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Oxidative Stress, Accumulation of Biological 'Garbage', and Aging

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ABSTRACT

Normal metabolism is associated with unavoidable mild oxidative stress resulting in biomolecular damage that cannot be totally repaired or removed by cellular degradative systems, including lysosomes, proteasomes, and cytosolic and mitochondrial proteases. Consequently, irreversibly damaged and functionally defective structures (biological 'garbage') accumulate within long-lived postmitotic cells, such as cardiac myocytes and neurons, leading to progressive loss of adaptability and increased probability of death and characterizing a process called aging, or senescence. Intralysosomal 'garbage' is represented by lipofuscin (age pigment), an undegradable autophagocytosed material, while extralysosomal 'garbage' involves oxidatively modified cytosolic proteins, altered biomembranes, defective mitochondria and other organelles. In aged postmitotic cells, heavily lipofuscin-loaded lysosomes perform poorly, resulting in the enhanced accumulation of defective mitochondria, which in turn produce more reactive oxygen species causing additional damage (the mitochondrial–lysosomal axis theory). Potential anti-aging strategies may involve not only overall reduction of oxidative stress, but also the use of intralysosomal iron chelators hampering Fenton-type chemistry as well as the stimulation of cellular degradative systems. *Antioxid. Redox Signal.* 8, 197–204.

AGE-RELATED BIOMOLECULAR DAMAGE IS A RESULT OF NORMAL METABOLISM

HALF A CENTURY AGO, Denham Harman postulated that reactive oxygen species (ROS), formed during normal oxygen metabolism, induce macromolecular damage, the accumulation of which accounts for progressive deleterious changes called aging, or senescence (37). This novel hypothesis (named the free radical theory of aging) has later been extensively supported by numerous *in vivo* and *in vitro* studies showing that age-related changes accelerate under the influence of oxidative stress, while various antioxidants slow aging (6, 8, 20).

Mitochondria account for the bulk of endogenously formed ROS (35, 38). An unavoidable respiratory electron leak results in the formation of superoxide anion radicals, $O_2^{\cdot -}$, which are toxic to mitochondrial enzymes such as aconitase, and also can reduce Fe^{3+} into Fe^{2+}, a catalyst of Fenton-type chemistry (see below). Although superoxide poorly crosses biological membranes, its uncharged protonated form, $HO_2^{\cdot}$, diffuses easily and is highly reactive (28). SOD2, manganese superoxide dismutase, reduces most mitochondrially produced superoxide into hydrogen peroxide, H_2O_2, while cytoplasmic superoxide (in particular formed by cytosolic oxidases) is reduced by copper-zinc superoxide dismutase, SOD1. Hydrogen peroxide, which readily diffuses throughout the cell, is eliminated by glutathione peroxidase and catalase (35). During Fenton reactions, hydrogen peroxide is partially converted into the extremely reactive hydroxyl radicals, $HO^{\cdot}$, which immediately attack surrounding macromolecules, including DNA, proteins and lipids. Hydroxyl radicals exert their pathogenic activity also indirectly, by reacting with unsaturated fatty acids under the formation of organic peroxides, which decompose to aldehydes. The latter are responsible for a number of toxic effects, including the formation of protein–protein aldehyde bridges (66).

Harman's theory, which recognizes endogenously produced ROS as the principal inducers of macromolecular

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damage, explains why age-related changes inevitably occur in any aerobic organism, especially within long-lived postmitotic cells (see below), even under absolutely favorable environmental conditions. The leading role of ROS in triggering aging does not, however, rule out the involvement of other factors that can damage biomolecules. These include advanced glycation end products (AGEs), forming as a result of reactions between sugars and amino groups of proteins and inducing protein-protein cross-linking and DNA mutations (13, 47), as well as the reactive metabolite S-adenosylmethionine, which alters the hydrogen-bonding ability of DNA bases through guanine methylation (41). In addition, macromolecules can undergo spontaneous nonoxidative modifications due to their inherent instability such as DNA strand breaks, depurination and deamination of DNA bases, isomerization, racemization, and deamidation of protein amino acid residues, or dephosphorylation of phosphoproteins (41). Theoretically, natural radioactivity may also contribute to aging, since hydroxyl radicals and other ROS form due to ionizing irradiation. In fact, the background radioactivity levels are too low to be of substantial importance in causing aging. In contradiction, very low doses of ionizing radiation (still higher than background) have been shown to increase the lifespan of mice (24).

DAMAGED BIOLOGICAL STRUCTURES ACCUMULATE BECAUSE THEIR REMOVAL IS NOT PERFECT

Biological organisms continuously recycle (turn over) their constituents, provided that damaged and obsolete structures are degraded into their basic building blocks and replaced with newly synthesized complex structures. Cells possess a number of recycling systems. The lysosomal compartment, represented by acidic vacuolar organelles containing a large number of hydrolytic enzymes, is perhaps the most important of them. In a process called autophagy (which includes macro-, micro- and chaperone-mediated autophagy), lysosomal enzymes degrade not only cytosolic biomolecules, such as proteins and lipids, but also (in macroautophagy) whole organelles, including mitochondria, endoplasmic reticulum, ribosomes and peroxisomes (25, 48). Importantly, worn-out lysosomes apparently can also be autophagocytosed, as suggested by the presence of lysosomal membrane components inside lysosomes (7, 61) and by the accumulation of altered lysosomes following inhibition of autophagy (68). A process called heterophagy stands for lysosomal degradation of initially extracellular material that enters cells through endocytosis. Proteins, mainly short-lived, are also decomposed by proteasomes (multicatalytic proteinase complexes), which are active in the cytoplasm, as well as in the nucleus (52, 88). In addition, many short-lived cytosolic proteins are cleaved by calpains, calcium dependent cysteine proteases (64). Mitochondria have their own proteolytic system. The matrix Lon and membrane-bound AAA proteases (2, 3) allow mitochondria to degrade some defective proteins without being autophagocytosed. Cellular degradative pathways are schematically presented in Figure 1.

Although most damaged biomolecules and organelles are thus successfully removed by degradation, some of them accumulate with age, suggesting an inherent insufficiency of the recycling mechanisms. Because these damaged structures are functionally effete or even completely worthless, yet occupy a certain part of cellular space and interfere with normal functions (see below), they are usually called biological 'garbage' or 'waste' (74). Biological 'waste' includes lipofuscin that is an undegradable autophagocytosed material (intralysosomal 'garbage') as well as extralysosomally located damaged macromolecules and organelles (Fig. 1).

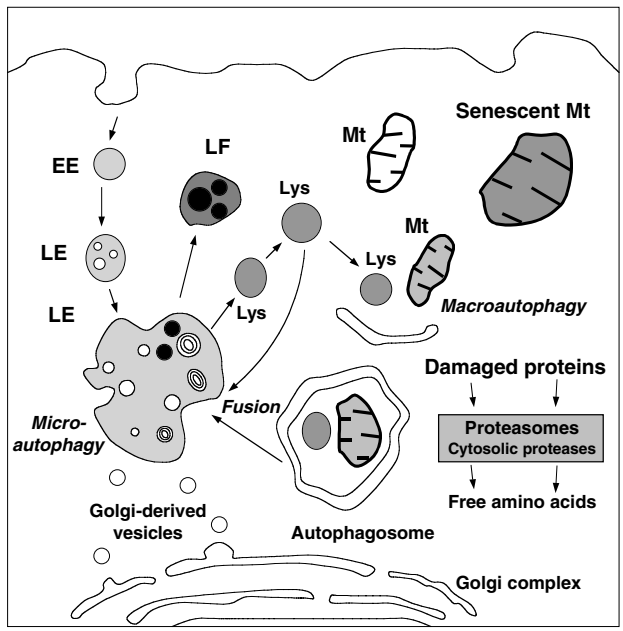

FIG. 1. Scheme showing major cellular degradative pathways and their relationship to aging. Autophagic and endocytic pathways converge in late endosomes, which are also called autophagolysosomes, pointing to their relation to autophagy. Late endosomes possess mannose phosphate receptors (MPR), that they receive from Golgi-derived vesicles carrying MPR-bound newly synthesized lysosomal enzymes. Mature lysosomes derive from late endosomes following a decrease of pH, enrichment with lytic enzymes, and loss of MPR. Cellular material to be degraded enters lysosomes through macroautophagy, microautophagy, and chaperone-mediated autophagy (not shown). Macroautophagy is associated with the formation of an isolating membrane and sequestration of material within an autophagosome and provides for recycling of many long-lived proteins, proteasomes, and all organelles, including mitochondria and lysosomes. During microautopagy, macromolecular compounds enter lysosomes through invagination of the lysosomal membrane. Nonlysosomal degradative systems include proteasomes, cytosolic proteases, and mitochondrial Lon and AAA proteases (not shown). Imperfect function of cellular degradative systems results in the accumulation of biological 'garbage' such as cytosolic damaged proteins (often forming undegradable aggregates), defective (senescent) mitochondria, and intralysosomal indigestible material (lipofuscin). EE, early endosome; LE, late endosomes; LF, lipofuscin-loaded lysosome; Lys, lysosomes; Mt, mitochondria. References are given in the text.

The idea of imperfect renewal of biological structures underlies a number of so-called error theories of aging. The somatic mutation (18) and error catastrophe (56) theories are probably the most important of them. Unlike the 'garbage' accumulation, or 'garbage' catastrophe theory (74), they point to the importance of synthetic errors in triggering aging. However, later studies have not found sufficient evidence in favor of the synthesis error theories, which failed to explain by themselves many manifestations of aging (45). Although errors of DNA and protein synthesis do occur, they would not accumulate and induce any serious disorders if the malformed biomolecules were reasonably well removed, but if the removal process is not perfect, the accumulation of damaged material ('garbage') is inevitable.

It should be pointed out that some synthetic errors may result in nonlethal mutations of nuclear or mitochondrial DNA, which in turn can accumulate. Malignant transformation is the most serious potential consequence of such errors, representing, however, an age-related pathology rather than the aging process itself.

AGE-RELATED 'GARBAGE' ACCUMULATION PRIMARILY OCCURS IN LONG-LIVED POSTMITOTIC CELLS

It has long been known that lipofuscin accumulation and other manifestations of cellular aging are primarily observed in cells that are commonly called 'postmitotic' or 'terminally differentiated', such as neurons, cardiac myocytes, and skeletal muscle fibers (67). In contrast, such changes are not typical of intestinal epithelium, epidermis, bone marrow, or other actively proliferating cell populations. Although mature enterocytes, keratinocytes, and blood cells are also postmitotic, they are frequently replaced as a result of differentiation of stem cells, which is associated with constant division and efficient dilution of biological 'garbage'. Over recent years, adult brains and hearts have also been shown to contain stem cells that can differentiate into mature neurons and myocytes (55, 92). However, the replacement of differentiated neurons and myocytes (if any) occurs very slowly, making their life long enough to accumulate considerable amounts of 'waste' material.

The anti-aging role of frequent cell replacement is evident in invertebrates. Hydra, a primitive Cnidarian animal, in which no long-lived cells are found, has been shown to escape aging under favorable experimental conditions (49), whereas adult forms of rotifers, nematodes, and insects, which are largely composed of long-lived postmitotic cells, exhibit rapid senescence (1, 29, 32). Similarly, cultured malignant cells with unlimited proliferation potential do not show any senescence-like changes, while growth-arrested normal cells undergo progressive alterations with age in the same way as postmitotic cardiac myocytes do (21, 76, 77).

The only type of age-related damage that can readily accumulate in proliferating cell populations is nonlethal mutations that have not been repaired or eliminated as a result of apoptosis. Such nuclear and mitochondrial DNA mutations may accumulate in proliferative tissues (72, 85), some resulting in the development of malignant neoplasms (22). These facts, however, do not exclude a fundamental role of the progressive alterations of long-lived postmitotic cells in aging (5, 39, 67).

INTRALYSOSOMAL 'GARBAGE': LIPOFUSCIN

Lipofuscin (age pigment) is an intralysosomal undegradable material, which is primarily composed of oxidatively modified protein and lipid degradation residues. It also contains some carbohydrates and traces of metals (up to 2%), including iron, copper, aluminum, zinc, calcium, and manganese (43, 78). Lipofuscin has a natural yellow-brownish color and displays a wide-spectrum autofluorescence. In the electron microscope, lipofuscin-loaded lysosomes usually appear as irregularly shaped osmiophilic granules of variable size and density.

Cultured cardiac myocytes and fibroblasts have not been shown to lose lipofuscin via exocytosis effectively (76, 77, 80). These *in vitro* observations seem to be challenged by a recent study, demonstrating that the removal of an eyestalk of a decapod crustacean may lead to a decrease of lipofuscin in the neurons of the contralateral eyestalk (30). This study, however, does not completely exclude a possible reduction in lipofuscin content due to initiation of neuronal stem cell division and differentiation in response to trauma.

There are good reasons to believe that the conversion of autophagocytosed material into undegradable substances primarily occurs in lysosomes. The presence of redox active iron, free diffusion of hydrogen peroxide through the lysosomal membrane, and low pH promote the formation of hydroxyl radicals via Fenton reactions (see above). This causes massive radical damage to autophagocytosed biomolecules (many of which are membrane-derived proteins and lipids) and ensuing cross-linking of macromolecules, making them resistant to degradation by lysosomal enzymes (15). This hypothesis is strongly supported by the fact that the combination of oxidative stress and lysosomal protease inhibition (which delays degradation of autophagocytosed macromolecules and thus allows more time for their oxidation) dramatically accelerates lipofuscin formation in cultured cells (76).

Lipofuscin can originate from different types of auto- or heterophagocytosed material. In many cells, especially in highly aerobic ones, such as cardiac myocytes and neurons, efficiently autophagocytosed mitochondria constitute the bulk of intralysosomal undegradable material. The mitochondrial origin of lipofuscin components is evidenced by the presence of abundant ATP synthase subunit c in pigment-loaded lysosomes. As suggested by the experiments on growth-arrested fibroblasts, specific components of lysosomes, such as membranes and proteins, also may be sources of lipofuscin (68). In professional scavenger cells with active phagocytosis, such as macrophages, micro- and macroglial cells, and retinal pigment epithelial cells, a large portion of lipofuscin contents derives from heterophagocytosed extracellular material (17, 42, 62, 87).

Although lipofuscin has long been considered an innocent hallmark of aging, there is accumulating evidence of its

harmful properties. Most importantly, is has been found to interfere with autophagy (80), thus decreasing intracellular turnover. In addition, probably due to high content of iron, lipofuscin increases the susceptibility of cells to oxidant-induced injury (75). Lipofuscin-loaded retinal pigment epithelial cells show decreased capacity to phagocytose rod outer segments (69) and are increasingly sensitive to light (86), perhaps explaining the increased risk of macular degeneration in the elderly.

Interestingly, the rate of lipofuscin accumulation is inversely related to the lifespan, rapid in short-lived species, and slow in long-lived ones when identical types of postmitotic cells are compared (53), telling us that lipofuscin accumulation, indeed, most probably has deleterious effects on cellular function.

EXTRALYSOSOMAL CELLULAR 'GARBAGE'

Senescent cells are characterized by alterations of virtually all their compartments. Damaged cellular structures beyond lysosomes include, in particular, oxidatively modified cytosolic biomolecules (46, 66), altered biomembranes (9), and proteasomes (31), and defective organelles including ribosomes, peroxisomes, endoplasmic reticulum, and, most importantly, mitochondria (74, 79). Mitochondria are considered particularly affected by age due to the facts that they are the main sites of ROS generation (20), and that mitochondrial DNA is highly susceptible to oxidative damage (in comparison with nuclear DNA) (57). Senescent mitochondria show morphological abnormalities such as enlargement, swelling, loss of cristae, and destruction of inner membranes (71, 81). As a consequence, ATP production declines (89).

Oxidatively damaged proteins are prone to form partially unfolded or misfolded tertiary structures, which readily aggregate forming so-called aggresomes (34, 65). Protein aggregates often have a typical morphology and are chemically well characterized. For instance, alpha-synuclein aggregates form Lewy bodies within dopaminergic neurons of substantia nigra (10), while aggregates of the hyperphosphorylated protein tau compose neurofibrillary tangles and argyrophilic grains within perikarya and processes of brain neurons, respectively (36, 84). Corpora amylacea represent glycoproteinaceous inclusions that preferentially accumulate in astrocytes (60). The accumulation of intracellular protein aggregates may stay behind following cell death, resulting in the formation of extracellular aggregates. This is probably the case for senile plaques composed of amyloid beta-protein, which initially forms within neurons (33). Normally, the amount of aggresomes gradually increases with age, although particular types of protein aggregates accumulate dramatically in certain pathologies, such as alpha-synuclein aggregates in Parkinson and Lewy body diseases and neurofibrillary tangles and beta-amyloid in Alzheimer disease (83).

It should be pointed out that the increase of neuromelanin within aging dopaminergic neurons apparently differs by nature from alpha-synuclein accumulation. Melanin granules are lysosome-related acidic organelles, which are believed to bind toxic agents, such as oxygen-derived radicals and transition metals, thus protecting aging cells (54, 93). Unlike normal aging, Parkinson disease is associated with decreased content of neuromelanin, apparently due to degeneration of dopaminergic neurons (90).

Extralysosomal damaged cellular structures can potentially be autophagocytosed and, unless degraded by lysosomal enzymes, form lipofuscin pigment. Some of them, however, probably escape autophagy and may remain within senescent postmitotic cells forever, such as various aggresomes and extremely large (so-called 'giant') mitochondria. As suggested by the study on cultured neonatal rat cardiac myocytes, enlarged mitochondria are autophagocytosed less efficiently than those of small sizes (81). De Grey postulated that mitochondria with defective DNA may be less targeted for autophagy compared to normal ones because, due to deficient respiration, their membranes are less affected by ROS (26). A number of observations suggest that in some cases mitochondria with mutated DNA may accumulate within aging cells as a result of enhanced replication (23, 44).

THE MITOCHONDRIAL–LYSOSOMAL AXIS THEORY OF AGING

Lipofuscin progressively accumulates within the lysosomal compartment, which, from a physiological point of view, may be considered as a box receiving enzymes by fusion with Golgi-derived vesicles (formerly, primary lysosomes) and substrates to be degraded by fusion with endosomes and autophagosomes. In each moment, however, the compartment is divided into a number of vacuoles with diameters usually not exceeding 1–3 μ*M*, showing fusion and fission and exchange of material (14).

Lipofuscin occupies increasingly larger portions of individual lysosomes, often not allowing them to contain any other type of material. The number of such lysosomes (earlier named residual bodies) within long-lived postmitotic cells increases with age. Golgi vesicles carrying newly produced lytic enzymes to the lysosomal compartment fuse with any kind of lysosomes having the right type of surrounding 10 nm thick membranes. The result being that also completely lipofuscin-occupied lysosomes receive lytic enzymes by fusion with Golgi-derived vesicles. In this case, since lipofuscin is undegradable, the lysosomal enzymes cannot perform any useful activity and, consequently, they are lost as if they were exocytosed. When lysosomal enzymes are worn-out they themselves need to be degraded.

Consequently, in senescent postmitotic cells, large numbers of lysosomes contain nothing but lipofuscin and lytic enzymes. As the number of lipofuscin-loaded lysosomes increases, more and more newly produced lysosomal enzymes would relocate from the Golgi area to lipofuscin-loaded lysosomes rather than to late endosomes or autophagosomes where they would be able to perform useful activities.

The aged cell may compensate for this 'drainage' of lysosomal enzymes from useful purposes by increasing the synthesis of lytic enzymes, but finally the production of such enzymes cannot any longer be increased, and from then on there will be a growing deficiency of lysosomal degradation capacity resulting in prolonged half-life of structures that normally are

turned-over by autophagy. The more extensive the formation of lipofuscin-loaded lysosomes, the larger the loss of lysosomal enzymes from normal activity and the longer the half-life of structures that are normally degraded by autophagy.

Since mitochondria are turned-over by autophagy, depression of this activity will result in the accumulation of aged mitochondria with abnormal functions, such as production of less ATP and more ROS than is normally the case (20, 57, 79). When the number of defective mitochondria exceeds a certain proportion, we believe the cell would be short of ATP and oxidative stress would grow abnormally intense. Such cells would be unable to respond to increased demands, being able to perform basic tasks only, and thus inflexible in a way that is a general characteristic of aging. Experimental support of the mitochondrial–lysosomal axis theory is provided by a recent observation on cultured neonatal rat cardiac myocytes showing that the rate of lipofuscin accumulation by single cells positively correlates with both oxidative stress and mitochondrial damage (82).

A decreased lysosomal degradative capacity due to lipofuscin overload would also promote the accumulation of extralysosomal protein aggregates (see above). These aggregates, in particular, beta-amyloid, are known to act as pro-oxidants by inducing ROS generation in the presence of transition metals such as iron and copper (19, 91). An increased ROS formation, in turn, would intensify lipofuscinogenesis and protein aggregation, as well as mitochondrial damage, suggesting the enhancement of the mitochondrial–lysosomal axis related mechanisms in cells with extensive formation of protein aggregates.

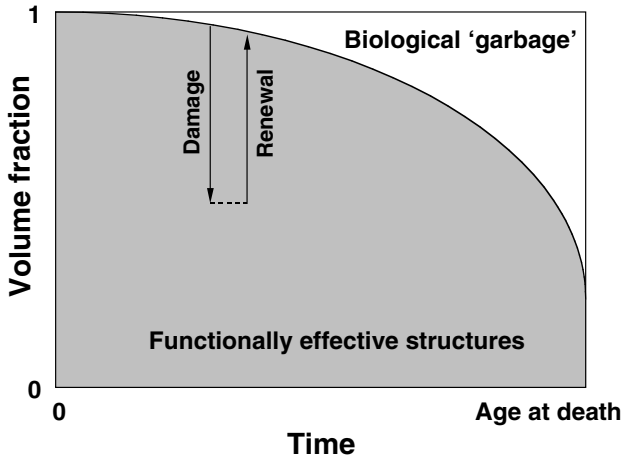

FIG. 2. Scheme illustrating failure of a biological system as a result of age-related 'garbage' accumulation. *Arrows* represent single acts of damage and renewal of biological structures. It is assumed that the renewal is not perfect (as indicated by a *shorter 'renewal arrow'* compared to the '*damage arrow'*) because damaged structures (biological 'garbage') are not completely removed. Consequently, biological systems progressively lose functionally effective structures and accumulate waste products. This deleterious process apparently accelerates towards the end of life, as explained in the text (the mitochondrial–lysosomal axis theory of aging). When the proportion of effective structures decreases below a critical level, the biological system dies.

The mitochondrial–lysosomal axis theory of aging thus suggests that decreased adaptability of senescent postmitotic cells is a function of intralysosomal accumulation of lipofuscin that creates a situation where lack of lysosomal enzymes make normal autophagic turnover impossible. The result is the accumulation of defective structures, especially mitochondria, which will disturb metabolism of the senescent postmitotic cell in several ways. Lack of ATP would be an important feature. Since lipofuscin is rich in iron, increased cellular oxidative stress may also result in lysosomal rupture due to intralysosomal Fenton-type reactions and in apoptotic cell death if lysosomal destabilization exceeds a certain value (16). Figure 2 schematically illustrates the progressive cellular dysfunction and death as a result of biological 'garbage' accumulation.

PREVENTION OF BIOLOGICAL 'GARBAGE' ACCUMULATION AS POTENTIAL ANTI-AGING INTERVENTION

It is clear from the preceding discussion that prevention of biological 'waste' products accumulation may become an important anti-aging strategy. So far, many efforts have been made to slow aging by decreasing free radical damage (8, 40). This approach still remains important, and new effective interventions reducing ROS-mediated injury are in demand. Considering the role of intralysosomal oxidative stress in lipofuscinogenesis, it would be attractive to develop antioxidants and iron chelators with lysosomotropic properties. Recently, alpha-lipoic acid plus, an agent suppressing Fenton-type chemistry by binding intralysosomal iron, has been shown to protect cultured cells from oxidant-induced injury (58). An intriguing question is whether this drug could also suppress lipofuscin formation, known to be dependent on iron-catalyzed intralysosomal generation of ROS (15). In support of such a possibility, a potent iron chelator, desferrioxamine, was previously shown to inhibit lipofuscin accumulation within cultured cardiac myocytes (50). Unlike true lysosomotropic agents, which are usually weak bases of a relatively low molecular weight, desferrioxamine is a large molecule that is internalized through endocytosis and thus enters the lysosomal compartment. Desferrioxamine is effective when used in relatively high doses (around 1 m*M*), shows cumulative effect and, therefore, appears too toxic for practical applications by causing cellular iron starvation (73). The use of lysosomotropic iron chelators such as alpha-lipoic acid plus, which appeared up to 5000 times as effective as desferrioxamine in preventing oxidant-induced lysosomal damage (58), might thus become a challenging approach for reducing manifestations of aging and age-related diseases.

Stimulation of degradative processes represents another possible way preventing biological 'garbage' accumulation. Such interventions would not remove already formed 'waste' products that are resistant to cellular digestive enzymes. Yet, this approach might accelerate catabolism, thus preventing further oxidation of biomolecules and their conversion into indigestible products, such as lipofuscin. Although no drugs with such specific activities has been suggested, the idea is supported by the facts that calorie-restricted animals, known

to have an extended lifespan (11, 12), as well as long-lived *daf-2 C. elegans* mutants (51), show enhanced autophagy. Both calorie restricted animals and *daf-2* mutants also show reduced ROS production (4, 59, 63, 70), which apparently works in tandem with stimulation of autophagy. It should be pointed out that the reduction of oxidative stress to some extent may occur secondary to the enhancement of autophagic degradation, which removes defective mitochondria producing increased amounts of ROS, and prevents the accumulation lipofuscin, being not only a product of oxidation but also a pro-oxidant (see above). A more radical approach to the reduction of biological 'garbage' accumulation might involve transfection of aging cells with bacterial or fungal genes coding for enzymes called xenohydrolases, which are capable to degrade lipofuscin (27).

ABBREVIATIONS

mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

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