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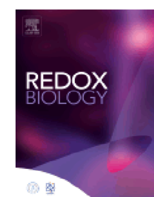

Review Article

Upregulation of the mitochondrial Lon Protease allows adaptation to acute oxidative stress but dysregulation is associated with chronic stress, disease, and aging ☆

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ABSTRACT

The elimination of oxidatively modified proteins is a crucial process in maintaining cellular homeostasis, especially during stress. Mitochondria are protein-dense, high traffic compartments, whose polypeptides are constantly exposed to superoxide, hydrogen peroxide, and other reactive species, generated by 'electron leakage' from the respiratory chain. The level of oxidative stress to mitochondrial proteins is not constant, but instead varies greatly with numerous metabolic and environmental factors. Oxidized mitochondrial proteins must be removed rapidly (by proteolytic degradation) or they will aggregate, cross-link, and cause toxicity. The Lon Protease is a key enzyme in the degradation of oxidized proteins within the mitochondrial matrix. Under conditions of acute stress Lon is highly inducible, possibly with the oxidant acting as the signal inducer, thereby providing increased protection. It seems that under chronic stress conditions, however, Lon levels actually decline. Lon levels also decline with age and with senescence, and senescent cells even lose the ability to induce Lon during acute stress. We propose that the regulation of Lon is biphasic, in that it is up-regulated during transient stress and down-regulated during chronic stress and aging, and we suggest that the loss of Lon responsiveness may be a significant factor in aging, and in age-related diseases.
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Contents

Introduction . . . 259
  Protein oxidation and proteolysis . . . 259
  Degradation of oxidized proteins within mitochondria . . . 259
Lon function and inducibility during stress . . . 260
  Significance of Lon induction during acute stress and disease . . . 260
  Decline of Lon inducibility in aging and during chronic stress . . . 261
Conclusions and future prospects . . . 262
References . . . 262

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AAA, ATPases associated with diverse cellular activities; Aco1, Aconitase 1; Ccp1, mitochondrial cytochrome-c peroxidase; CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate; Clp, caseinolytic protease; ClpP, core catalytic protease unit; COX, cytochrome c oxidase; COX4-1, cytochrome c oxidase subunit IV isoform 1; COX4-2, cytochrome c oxidase subunit IV isoform 2; ERAD, endoplasmic reticulum-associated degradation; Fe/S, iron/SULFUR; FRDA, Friedreich's ataxia; HAART, highly active antiretroviral therapy; HIF-1, hypoxia inducible factor-1; HsIVU, bacterial ATP-dependent protease; HSP104, heat shock protein 104; HSP60, heat shock protein 60; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MPPβ, mitochondrial processing peptidase beta subunit; Nfκb, nuclear factor kappa-light-chain-enhancer of activated B csells; NRF-2, nuclear factor (erythroid-derived 2)-like 2; Pim1, ATP-dependent Lon protease from yeast; Protease La, ATP-dependent protease; *PRSS15*, *LON* gene; Prx1, mitochondrial peroxiredoxin 1; SLLVY-AMC, *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; SOD, cytosolic superoxide dismutase; SOD2, mitochondrial superoxide dismutase 2; SPG13, hereditary spastic paraplegia; WI-38, human lung fibroblast; Yjl200c, mitochondrial aconitase isozyme

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Introduction

Protein oxidation and proteolysis

Many oxidants are highly reactive and can cause major damage to macromolecules. Side chains of amino acids are prone to oxidation, which not only impairs protein structure and function, but also creates exposed hydrophobic patches that will attract and bind to other exposed, oxidized proteins, creating toxic protein aggregates and cross-linked inclusion bodies that interfere with normal cellular function [1]. Therefore, the prompt removal of an oxidatively modified, irreparably damaged protein prevents protein aggregation and accumulation. As a result, cells of all organisms studied have evolved 'vigilant' enzymatic systems that rapidly bind oxidized proteins as soon as they are produced, and either repair, or degrade them before further damage occurs. There is substantial evidence suggesting that failure to degrade or repair damaged proteins contributes significantly to age-associated protein aggregation, and to neurodegenerative diseases [2,3].

Although oxidized sulfhydryl groups of cysteine/cystine and methionine residues can be directly repaired by various sulfhydryl reductase enzymes, the majority of amino acid oxidations within proteins cannot be repaired and must, instead, be removed by selective proteolytic degradation. In higher organisms, the majority of oxidatively damaged proteins are removed by the 20*S* proteasome and the immunoproteasome [4–6], which are found in most cellular compartments including the nucleus, cytoplasm, and the endoplasmic reticulum [7]. Proteasome is not, however, present in mitochondria, which are physically separated from the cytoplasm by a double membrane system. Mitochondria contain an electron transport chain, which transfers high energy electrons to a series of membrane protein complexes, before final acceptance by oxygen. Unfortunately, this process is not completely efficient and leakage of electrons occurs, generating superoxide and, through dismutation, hydrogen peroxide. Ultimately, even more reactive species, such as the hydroxyl radical, may be generated, causing damage to surrounding macromolecules [8]. Mitochondria are known to be one of the major sources of intracellular free radical production [9]. Management of oxidative damage inside mitochondria is, therefore, extremely important in the maintenance of cellular function and survival. Dysfunctional mitochondria and elevated production of oxidants has been associated with numerous diseases and with the aging process itself [10,11].

Within the mitochondrial matrix, there are multiple removal/repair systems for oxidized proteins. Reversible oxidative damage occurring to sulfur containing amino acids, such as cysteine, cystine, and methionine can be catalytically reduced by mitochondrial reductases, which can repair disulfide bridges or methionine sulfoxides [12,13]. Irreversible oxidative damage can form adducts of proteins with carbohydrates, and lipids, and may create bi-functional aldehydes and carbonyl groups, as well as intramolecular crosslinks [14]. Such damage can impair or completely inactivate enzymes and structural proteins which, if not rapidly removed, can aggregate, cross-link, and cause significant cellular toxicity. We have previously reported that such oxidized mitochondrial proteins are eliminated by the Lon Protease [15].

Degradation of oxidized proteins within mitochondria

The degradation of oxidized proteins in mitochondria is essential to maintain mitochondrial homeostasis [16]. There are 3 known proteases that have been shown to degrade damaged proteins in the mitochondria, all of which are ATP-stimulated. The AAA protease and the Clp-like protease are hetero-oligomeric complexes, while the Lon protease is homo-oligomeric. The AAA protease is localized to the mitochondrial inner membrane, while Clp and Lon are both found in the matrix. These proteases contribute to the degradation of short-lived, misfolded, or damaged proteins [17,18].

The Lon Protease is the most studied among the three, and has been shown to be the main protease for degradation of oxidized proteins [19]. Lon was initially shown to degrade stress response proteins and misfolded, missorted and nonassembled proteins in *Escherichia coli*, and then in yeast mitochondrial matrix [20–24]. After further analysis, Lon was considered to be part of the heat shock regulon of proteases in bacteria, along with HsIVU, and ClpP. Eventual deletion studies showed that Lon and HsIVU are required in the defense against carbonylation damage, however, deletion of ClpP had no effect and interestingly, Lon can fully compensate for the lack of ClpP [25]. Goldberg and colleagues found that the Protease La, the *E. coli* homolog for Lon, can degrade small hydrophobic peptides [26]. Indeed, the degradation of known Lon substrates, such as StAR [27] has been shown to occur via exposure of hydrophobic patches after protein oxidation.

The Lon protein consists of three domains, the substrate binding domain, the AAA Module, and the proteolytic domain [28]. ATP binds to the AAA module of the Lon complex resulting in a change in Lon conformation into a proteolytic active state. Oxidized proteins can give rise to exposed hydrophobic patches, which presumably binds to the substrate recognition sequence of Lon [29]. The degradation of folded proteins requires ATP mediated substrate unfolding [27]. An unstructured or denatured protein, does not require ATP hydrolysis, but its presence does maximize the rate of substrate degradation [30]. ATP binding activates the complex through a conformational change, and hydrolysis facilitates the translocation of the unfolded peptide into the proteolytic core for degradation [31]. Degradation of substrates is thought to occur in a processive manner in which translocation of the peptide chain into the proteolytic chamber of the Lon homo-oligomer occurs [27], from the amino to carboxy terminal, or vice versa.

In most cases, Lon does not recognize a specific consensus peptide sequence, it prefers hydrophobic sequences adjacent to the scissile bond [27,32,33]. Proteins with complex organization, such as those that harbor cofactors or prosthetic groups are more prone to Lon targeted degradation [34]. For example, Fe/S cluster proteins are generally highly susceptible to oxidation-dependent degradation [35]. However *E. coli* Lon can allosterically bind specific degron tags on substrate proteins, which modulate degradation rate. Different tags fused to the same protein can alter the speed and energetic efficiencies by 10-fold or more, according to physiological needs [36].

Protein damage has been demonstrated in *E. coli* after exposure to oxidants, and it has also been shown that such oxidatively damaged proteins are rapidly and selectively removed by proteolysis [37]. Pim1 is the yeast homolog of Lon, and strains lacking the gene have impaired ability to degrade mitochondrial matrix proteins [38] and maintain functional DNA [39,40]. We suspected that Lon might be the major protease for degradation of oxidized proteins in the mitochondria and, indeed, when purified Lon was incubated with native and oxidized aconitase, it preferentially degraded oxidized aconitase at a much higher rate than native aconitase [19].

A number of other Lon substrates have been identified. In *Saccharomyces cerevisiae* mitochondria, Pim1 degrades Ilv5, an acid

reductoisomerase that is prone to aggregation [41]. Under heat stress, Pim1 degrades ATPase, a subunit of the F_1F_0 ATPase, and the β subunit of MPPβ, a matrix processing peptidase [38]. With 2D PAGE and proteomics analysis, five metabolic enzymes were identified in Δpim1 yeasts, Ilv1, Ilv2, Lsc1, Lys4, and Yjl200c [34]. In another effort to globally screen for substrates of Pim1, a combination of proteomic techniques was used and a total of 19 protein substrates were identified, 14 of which were carbonylated proteins that accumulate in mutant Δpim1 cells. Seven of these had an oxidation index of greater than 1 and correspond to Pim1-specific oxidized protein substrates. The majority of the substrates are either mitochondrial metabolic enzymes or respiratory chain subunits [42]. The effects of oxidative stress on mitochondrial protein homeostasis were also tested using proteomic methods. Yeast cells were grown in media containing either menadione or H_2O_2, and protein profiles were analyzed by 2D PAGE and identified by mass spectrometry. In summary, enzymes containing oxidation sensitive prosthetic groups such as Ilv3, Lys4 and Aco1 were major targets for degradation under stress. Other antioxidant type enzymes, such as Ccp1, Yhb1, and Prx1 were also sensitive to Lon degradation. The idea that antioxidant enzymes would experience more oxidation-initiated degradation may initially seem counterintuitive. The very affinity of such enzymes for oxidized substrates, and their high rates of interaction with oxidants, however, presumably makes them highly susceptible to oxidative modification. Deletion studies of Pim1 cells were used to identify that Lon/Pim1 was the major protease in the degradation of oxidatively-modified proteins found in the matrix [43].

In bacteria, Lon activity can be inhibited naturally by T4 phage infection through the T4-encoded PinA protein, and occurs within a few minutes of infection. Certain inhibitors of the Proteasome can also inhibit Lon, suggesting similarities in the proteolytic mechanism [44], however, standard inhibitors of Lon are about 2000-fold more potent against the 20S proteasome [45]. Coumarinic derivatives do not inhibit the 20S proteasome, providing a better option for studying the physiological functions of Lon, but further tests are needed to determine their effects on other proteases, such as Clp. However, a recently developed therapeutic agent for lymphoma, the synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its derivative, CDDO-Me, specifically and selectively inhibit Lon proteolytic activity [46]. CDDO and CDDO-Me appear to act by forming covalent adducts with the proteolytic active site of Lon, thus rendering Lon irreversibly inactive [47]. Specific targeting of Lon was demonstrated by inability of the enzyme to degrade a fluorescently-labeled reporter substrate following incubation with CDDO. In contrast, no inhibitory effect was found with the 20S proteasome, as proteolytic activity was maintained following incubation of purified 20S proteasome with CDDO [46]. As more diseases are identified with underlying mitochondrial dysfunction, the potential of Lon suppression as a therapeutic intervention will spur increased interest to generate additional Lon specific inhibitors.

Lon function and inducibility during stress

Significance of Lon induction during acute stress and disease

In order to study the physiological relevance of Lon, a number of Lon silencing experiments were performed. Yeast cells lacking a functional *LON* gene contained a nonfunctional mitochondrial genome, were respiratory deficient, and lacked the ability to degrade damaged proteins, resulting in the accumulation of electron dense inclusions in their mitochondria [38]. In cultured human cells, Lon knockdown resulted in impaired mitochondrial function, accumulation of electron dense bodies in the mitochondria, damaged mitochondrial morphology resembling aged cells, and cell death occurring via apoptosis and necrosis [18].

The regulation of Lon is currently not well studied, and the transcription factors of Lon have not been well defined. It is likely that Lon is regulated by transcription factors that are associated with mitochondrial biogenesis and oxidative stress. Promoter characterization studies of the *LON* gene, *PRSS15*, identified putative binding sites for the transcription factors NRF-2, Nkx-2, and Nfκb, and Lyf-1, using computer analysis [48]. Deletion studies of NRF-2 and Nfκb suggests that Lon might be regulated by at least these two transcription factors [48].

Hypoxia-inducible factor 1 (HIF-1) is known as a master regulator for a battery of genes involved in oxygen homeostasis. Under hypoxic conditions, HIF-1 is activated due to a decline in hydroxylation. Fukuda et al. [49] suggest that, under hypoxia, the generation of free radicals actually increases due to inefficient electron transfer at complex I and complex III, causing elevated superoxide production, and increased overall H_2O_2 levels [49]. Analysis of the *LON* gene (*PRSS15*) promoter revealed three HIF-1 sites that can regulate Lon transcription. Cells exposed to hypoxic conditions displayed an induction of Lon (up to 5-fold) in a variety of cell and tissue types [49]. HIF-1 upregulates both Lon and a more stress resistant COX4-2, allowing for the degradation of COX4-1. The switch between COX proteins seems to be an adaptive response to optimize cytochrome c oxidase activity under hypoxic stress. These data are further corroborated by another group, when a different set of *in vitro* studies with hypoxia, ischemia, and ER stress resulted in the enhanced expression of *Lon* mRNA [50]. These authors agree that the enhanced Lon expression regulates the assembly and/or degradation of cytochrome c oxidase during stress.

Although the regulatory mechanism(*s*) governing Lon levels is/are still somewhat elusive, there is a growing body of evidence to show that Lon is modulated by stress. Indeed, we have explicitly proposed that Lon is a human stress protein [51]. In rhabdomyosarcoma cells, exposure to multiple independent stressors, including heat shock, serum starvation, and oxidative stress, all resulted in dramatic increases in Lon protein levels. This induction of Lon prevented the accumulation of carbonylated cellular proteins, thus resulting in improved cell survival and conserved mitochondrial function. Blockage of Lon induction with siRNA resulted in dramatically increased levels of carbonylated proteins, and the loss in mitochondrial function and cell survival [51]. A similar study in yeast showed that the expression of Lon/Pim1 is also induced in both heat stress and oxidative stress [43]. The induction of Lon has also been observed in situations which encourage mitochondrial biogenesis. In rats bearing the Zajdela hepatoma tumor, or hyperthyroid rats, the mRNA and protein, as well as the actual activity of Lon, were all enhanced. This induction of Lon was also correlated with the induction of mitochondrial biogenesis [52].

Frataxin is a mitochondrial protein that is involved with the regulation of iron-sulfur protein (Fe-S protein) activities. A loss of frataxin leads to the accumulation of iron, accumulation of oxidative damage, and reduced lifespan in FRDA models [53,54]. In a Friedreich Ataxia mouse model, in which frataxin has been deleted in striated muscles, an increase in ClpP and Lon mRNA, protein, and activity was observed in the isolated mitochondria of mice between 5 and 10 weeks of age [55]. The upregulation of Lon and ClpP was accompanied by a progressive loss of mitochondrial Fe-S proteins with no change in mRNA levels, suggesting degradation.

Stimulation of Lon activity has also been associated with increased carbonyl content *in vivo* in a cardiac ischemia/reperfusion model and in a yeast frataxin homolog-deficient strain. Cardiac ischemia/reperfusion is associated with mitochondrial free radical production, where reperfusion injury causes oxidative damage to proteins. Levels of oxidized aconitase, an Fe-S protein, was measured after reperfusion in hearts of rats, and the activity of Lon was increased while the levels of oxidatively modified proteins

decreased [56]. Interestingly, frataxin and aconitase interact with one another to reverse the inhibition of aconitase during oxidative stress. Bulteau et al. [57] propose that if the damage incurred during oxidative stress is mild and reversible, frataxin might act as a chaperone and promote re-assembly. On the other hand, when such stress results in irreversible inactivation and 4Fe-S cluster disassembly, degradation of such proteins would take place through the Lon protease [57]. These data suggest that Lon induction might be a regulated event to help reduce irreparable damage to the mitochondria.

Troglitazone is an insulin sensitizing, antidiabetic drug that was withdrawn from the market due to risks of drug induced liver injury. Specifically, troglitazone causes mitochondrial membrane depolarization [58,59], and increased intramitochondrial oxidant stress [60]. A study with $SOD^{+/-}$ mice, which are sensitized to mitochondrial oxidant stress when treated with a hepatotropic drug challenge, showed an induction of adaptive response proteins, including heat shock proteins, catalase, and the Lon protease [61]. Treatment of animals after 2 weeks induced these adaptive response proteins, during the first phase, and was able to prevent the carbonylation of mitochondrial proteins. Prolonged treatment for 4 weeks, however, resulted in a second phase, where distinct proteome expression changes occur that indicated irreparable oxidant damage, and increased protein carbonyls [61].

One of the side effects of HIV patients treated with HAART type drugs, which are nucleosidic inhibitors (NRTIs), is lipodystrophy associated with mitochondrial toxicity [62–64]. In a global screen of mRNA levels in HIV-positive patients with lipodystrophy, the Lon protease was expressed at significantly higher levels. Patients with NRTIs also showed a decrease in mitochondrial DNA. Analysis of Lon upregulation in the model cell line SW872, showed that it is not the decrease in mitochondrial DNA *per se* that induced Lon, rather, it was the NRTI induced production of superoxide and hydrogen peroxide that resulted in an approximate 4-fold increase in Lon mRNA and protein[65].

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is a disease most frequently associated with a point mutation with an $A > G$ transition on the mitochondrial $tRNA^{LEU(UUR)}$ gene [66,67]. In an immortalized B cell line, harboring the A3243G mutation in 10% of the mitochondrial DNA, in other words, a heteroplasmic state, Lon protease protein levels were increased by 2.5-fold [68]. The upregulation of Lon protein is associated with increased Lon activity, as measured by degradation of the peptide SLLVY-AMC, and aconitase activity. MELAS cells however, displayed impaired transmembrane potential compared to normal cells [68]. This contradicts previous data that show that downregulation of Lon results in loss of transmembrane potential. It is likely that during such disease conditions, a decoupling between Lon induction and transmembrane potential occurs, or perhaps the increased Lon is not effective in maintaining mitochondrial homeostasis under chronic stress conditions.

Decline of Lon inducibility in aging and during chronic stress

Under physiologic concentrations, endogenous O_2 and H_2O_2 help to maintain homeostasis. Adaptive responses caused by an acute stress seem to be an effective coping mechanism in cellular tolerance and survival. Mild doses of an oxidant such as hydrogen peroxide can induce a repertoire of adaptive response proteins, one of which is Lon, which will temporarily equip cells with increased defenses against high levels of stress. However, when oxidants (including H_2O_2) are produced in great excess for prolonged periods of time, they cause chronic oxidative stress, with serious adverse effects. In a proteomic study of acute versus chronic oxidative stress on peripheral blood mononuclear cells, it was observed that cells treated with an acute dose of H_2O_2 induced classical indicators for stress such as chaperones, redox regulating enzymes, and DNA repair proteins, to minimize O_2 and H_2O_2 production. On the other hand, cells displaying chronically higher levels of oxidation also exhibit proteomic alterations which appear to be related to inflammation and apoptosis regulation [69].

Lon mRNA levels were originally shown by Lee et al., in 1999, to be approximately 4-fold lower in the muscle tissues of aged mice than in young controls. This apparent down-regulation was completely prevented by caloric restriction [70]. Subsequently, we reported that Lon protein levels and Lon activity were similarly depressed in the muscles of aged normal mice, and were even lower in muscles from (mangano) superoxide dismutase heterozygotes [15]. These results were then tested in human cells. WI-38 lung fibroblasts cells undergo a finite number of doublings in culture, eventually leading to cellular senescence. The cells can be classified by population doubling number, as early, middle, and late passage cells. Carbonyl protein damage increased dramatically in late passage cells compared to early passage cells [71]. Concurrently, the level of Lon from isolated mitochondria stayed the same in senescent cells, but was significantly decreased when measured in whole cells. Lon levels declined sharply in senescent cells and Lon inducibility, after an acute H_2O_2 treatment, was abolished [72]. Importantly, the induction of Lon in early passage cells was associated with a reduction in background protein carbonylation, while in middle passage cells, Lon induction was sluggish and was associated with delayed carbonyl reduction. Of, perhaps, greatest interest was the finding that Lon was not inducible at all in senescent cells, and that the lack of Lon induction was accompanied by ever increasing levels of carbonyl-containing proteins that were never removed/degraded [72].

In mice, the level of protein carbonylation increases dramatically with age. Protein carbonyl accumulation is even greater in heterozygous mitochondrial $SOD2^{+/-}$ knockout mice, and reaches significant levels even in young animals [15]. This effect is largely attributed to the decreased ability of $SOD2^{+/-}$ mice to neutralize superoxide, making them a model for chronic oxidative stress. The levels of Lon were also measured in these mice, and there seems to be an inverse relationship between protein oxidation levels and Lon [15]. Taken together, these data suggest that exposure to age-associated, chronic oxidative stress, down-regulates Lon, and the protection against protein damage is lost.

The age-associated modulation of Lon levels and activity is tissue specific. The Lon activity of old rats was decreased by 2.5-fold in the liver, however, the protein level remained the same in the mitochondrial matrix, suggesting Lon inactivation [73]. In contrast, Lon activity remained the same in old rat hearts but the protein level was increased by 5-fold, suggesting compensation for lost activity [74]. A more detailed review on the effects of aging and oxidative stress on Lon has been published by Ugarte et al [28].

A decline of Lon and ClpP protein levels has been reported in cells from a patient with Hereditary Spastic Paraplegia (SPG13). These cells encode a mutant HSP60 protein, caused by a single point mutation that decreases the efficiency of mitochondrial protein folding normally provided by this chaperone. The authors suggest that a compensation mechanism in these cells down-regulates the expression of ClpP and Lon protease, in order to prevent the rapid degradation of misfolded peptides, allowing more folding attempts instead of premature degradation. Unfortunately, these patients suffer from gradual axon degradation in specific motor neurons, and is believed to be caused by the accumulation of misfolded proteins due to insufficient clearance of proteins by proteases [75].

On the contrary, a study in the fungus *Podospora Anserina* reported that constitutive upregulation of Lon results in lifespan extension

without any adverse effects on respiration, growth, or fertility [76]. These fungi exhibit increased ATP-stimulated protease activity (resulting in higher resistance against oxidative stress), lower levels of oxidized and glycodized proteins, and a reduction in the production of H_2O_2, [76].

The budding yeast, *S. cerevisiae* has only the Lon homolog, Pim1, to degrade oxidized mitochondrial proteins [77]. Senescent yeast show a marked decline in Pim1 proteolytic activity, coupled with accumulation of electron-dense inclusion bodies and increased carbonyl content [78]. Strains of *pim1*Δ exhibit reduced lifespan and a parallel increase of oxidized and ubiquitinylated cytosolic proteins which, as previously demonstrated [3,7], are potent inhibitors of the 20S proteasome [78]. Interestingly, proteasome activity is nearly fully restored, in *pim1*Δ strains that overexpress Hsp104, a cytosolic chaperone protein, indicating that a lack of Pim1 can contribute to elevated cytosolic protein aggregates [78]. The dual role of Pim1/Lon may be critical for protein maintenance and mitochondrial protein degradation to prevent impairment of the 20S proteasome [78]. This observation from yeast highlights the interdependency between cytosolic and mitochondrial repair systems, with the loss of mitochondrial Pim1 also leading to a loss of cytosolic 20S proteasome activity [78].

The relationship between the 20S proteasome and Lon, found in senescent yeast, can be taken as an indication that multiple cellular redundancies may exist to limit the accumulation of oxidative damage. The age-related loss of Pim1 may trigger release of damaged mitochondrial proteins into the cytoplasm, eliciting a response similar to endoplasmic reticulum-associated degradation (ERAD) [79]. Inhibition of the 20S proteasome may, in turn, induce mitophagy in an attempt to limit further damage from the mitochondria [80]. A similar (probably mechanistically related) phenomenon is also witnessed in ischemic myocardial mitochondria [81]. Loss of mitochondrial proteolytic activity, due to oxidative damage during ischemia/reperfusion injury, potentially forces the cell to rely upon secondary cytoplasmic mechanisms (i.e. proteasome) to remove damaged mitochondrial proteins [81]. The 20S proteasome has been shown to independently degrade ubiquitin-free mitochondrial proteins and is tightly modulated following oxidative stress [81]. This indicates that the 20S proteasome may help to regulate mitochondrial function by acting in an alternate pathway to remove damaged mitochondrial proteins. Further work will be necessary to determine if the interdependency between the 20S proteasome and Lon discovered in yeast also operates in senescent mammalian cells. It will also be extremely important to determine the potential interplay between Lon and proteasome, and autophagic pathways for the removal of oxidized protein aggregates.

Conclusions and future prospects

Proteins have unique three-dimensional structures that are essential for their specific functions. Aerobic cells are constantly exposed to various oxidizing species that can change the native structure of proteins. The exposure of hydrophobic stretches of peptides increases the probability of irregular protein interactions and aggregation. Aggregates of oxidized proteins readily form covalent cross-links, which prevent their dissociation and can inhibit or preclude proteolytic removal. The accumulation of such cross-linked protein aggregates, and the inability of cells to remove such large clusters, poses a perpetual toxic process [82], that may be a major contributor to the aging process, and numerous age-related degenerative diseases.

An estimated 700 mitochondrial proteins make up the yeast mitochondrial proteome [83] and mammalian cells must contain an equal or greater number. Such a large number of proteins require the coordination of biosynthesis, complex assembly, and degradation systems, in order to maintain proper mitochondrial function, especially during stress. Interestingly, the Lon protease is a multi-faceted protein that performs several functions. In addition to its proteolytic properties, Lon exhibits a chaperone function [84] and it can also bind to promoter sequences in the mitochondrial DNA, as well as genomically important proteins such as mitochondrial polymerase γ, twinkle helicase, mitochondrial transcription factor B, mitochondrial transcription factor A, and single strand breaks in mitochondrial DNA [85–87]. These multiple functions further poise Lon as a "juggler" of responsibilities, involving the quality control of proteins, and the maintenance of vital mitochondrial functions.

The ability of cells to cope with stress through adaptive responses that induce the synthesis of key stress proteins, such as Lon, proteasome, heat shock proteins, chaperones, and antioxidants such as superoxide dismutase, is an effective mechanism ensuring temporary cellular survival, that has been widely conserved throughout evolution [4–6,51,72,88–90]. As cells age however, protective pathways are not as efficient, which can cause cumulative damage that will hamper cellular competence. For instance, oxidant-induced unfolding of proteins is an irreversible modification that may serve as a signal to target such damaged proteins to the Lon degradation pathway [91]. A prompt response by Lon would seem to be crucial in order to prevent aggregation of such damaged proteins. The Lon protease itself, is susceptible to inactivation by oxidants such as ONOO- [92]. Therefore, the presence of oxidants can both regulate or "de-regulate" protective actions by the cell. We suggest that during chronic stress conditions, there is a bias towards de-regulation, and a decline of Lon can further accelerate this deteriorative process. In a study measuring protein modification levels in four fractions of cells, it was shown that 44% of the protein modifications were found in mitochondria, 28% in the cytosol, 11% in the endoplasmic reticulum, and 8% in the cytoskeleton [93]. Therefore, a decline in Lon levels and activity, whether due to normal aging processes or chronic stress, might be a major factor in the age-related state of mitochondrial health. It now seems likely that Lon is a key element in stress protection and 'healthy' aging, which raises the intriguing possibility that preservation of Lon levels and activity throughout life (by means yet to be determined) might be a viable intervention to improve (or increase?) human life.

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