

Implications of Protein Degradation in Aging

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ABSTRACT: Aging is characterized by accumulation of potentially harmful altered proteins that could lead to gradual deterioration of cellular functions and eventually result in increased probability of death. Metabolic turnover of proteins thus plays an essential role in maintaining the life of an organism. In this article we summarize our current knowledge on age-related changes in protein turnover with special reference to degradation. Increase in half-life of proteins with advancing age is well documented. Qualitative rather than quantitative changes of proteasomes appear to be responsible for this change. Dietary restriction and moderate long-term exercise seem to restore higher proteasome activity and turnover rate of proteins in aged animals.

KEYWORDS: Proteasomes; Hepatocytes; Dietary restriction; Exercise; Ubiquitin

INTRODUCTION

Living organisms have evolved a variety of cellular and molecular mechanisms to cope with endogenous and exogenous stresses potentially harmful to life. Cells, if damaged seriously, are removed by apoptosis or necrosis and the damaged cells are then renewed by replication of remaining intact cells to establish a new steady state in an organism. Nonreplicating cells, such as neurons and cardiac muscle cells, or slowly replicating cells, as hepatocytes, are never or only slowly replaced by cell turnover.

Metabolic turnover of macromolecules, on the other hand, is an alternative means of escaping from the damaging consequences of possibly detrimental internal milieu or external environments. DNA with modified bases or base deletion can be repaired by a variety of repair enzymes,¹ without which death of cells or mutation might result. Peroxidized fatty acid esters in membrane phospholipids may also be repaired.² The oxidized phospholipids, if not repaired, can decrease membrane fluidity and/or give rise to various kinds of aldehydes that could modify adjacent proteins,³ thus deteriorating membrane functions such as signal transduction and membrane transport. Altered protein molecules would be degraded by proteolytic systems, and new intact

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molecules would be synthesized instead. Altered proteins do not only lose or lower cellular functions but also often gain toxicity if they accumulate, as in the aggregation of β -amyloid⁴ and paired helical filament⁵ in Alzheimer disease, α -synuclein in Parkinson's disease,⁶ or crystalline in cataract.⁷

Thus, the cellular and metabolic turnovers appear to be among the most fundamental survival strategies of an organism. Aging may therefore be characterized by a decrease in such life-maintenance systems, resulting in increased probability of death. It has been hypothesized that age-related decline of cellular functions is due to accumulation of harmful proteins or proteins with reduced or lost function, extreme cases being the serious age-related diseases mentioned above. We have investigated age-related changes of protein turnover with special reference to degradation and possible means to intervene in the aging process by preventing accumulation of altered proteins.

CHANGE IN DEGRADATION OF CELLULAR PROTEINS WITH AGE

Medvediev and Orgel independently presented an error catastrophe theory of aging that predicts catastrophic deterioration of cellular functions due to exponential increase of altered proteins or nucleic acids by expanding the rate of errors in replication, transcription, and/or translation.^{8,9} Although this theory has not gained experimental support so far (see, e.g., Refs. 10–12), an important realization has been advanced that one of the reasons errors do not increase is that error-containing molecules would be largely degraded, thus preventing their accumulation.^{13–15} Nevertheless, altered proteins do increase with age.^{16,17} Meanwhile, Sharma *et al.*¹⁸ and Reznick *et al.*¹⁹ convincingly demonstrated an age-related decrease in the turnover rate of pulse-labeled proteins in nematode and mouse, respectively. In addition, Lavie *et al.*²⁰ showed that prematurely terminated puromycinyl peptides are much more slowly degraded in the liver of old mice than in young counterparts. These findings suggested that degradation of normal and abnormal proteins is impaired in old animals.

Hepatocytes in primary culture isolated from young and old rodents provide a good model to investigate protein turnover as a function of age. They can easily be isolated and may be cultivated *in vitro* for the period necessary to study protein turnover without appreciable changes of protein metabolism.²¹ Proteins can be introduced into the cultured cells by the osmotic method involving enhanced formation of pinosomes containing the protein to be studied.²² We have reported that half-lives of proteins introduced into hepatocytes of old mice were extended significantly as compared with those in the cells from young animals (FIG. 1). Chicken lysozymes oxidatively modified *in vitro* were degraded as expected more rapidly than the unmodified counterpart in the cells of both young and old animals, the half-life again being longer in the latter cells (Takahashi *et al.*, in preparation).

Intracellular protein degradation in eukaryotes occurs primarily by one of the two major proteolytic mechanisms, that is, the lysosomal or the proteasomal pathway. The proteasome has been implicated in selective degradation of oxidatively modified proteins²³ and the majority of normal cellular proteins.²⁴ The degradation of oxidatively modified and unmodified chicken lysozymes introduced into hepatocytes

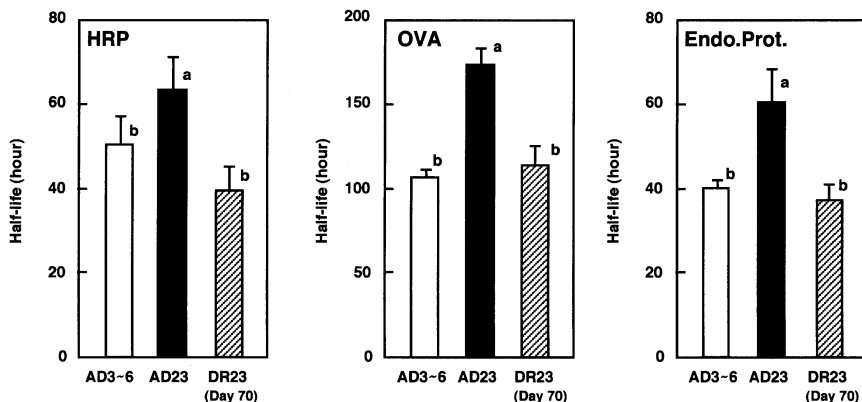


FIGURE 1. Effects of aging and dietary restriction initiated at old age on the half-lives of proteins in hepatocytes from mice (BDF1). HRP: horseradish peroxidase; OVA: ovalbumin; and Endo.Prot.: endogenous proteins. AD3-6: ad libitum-fed, 3–6 months old; AD23: ad libitum-fed, 23 months old; DR23: dietary restricted, 23 months old; mean \pm SD. (a) Significantly different from AD3-6 ($p < 0.05$); (b) significantly different from AD23 ($p < 0.05$).

from both young and old mice was inhibited by a proteasomal inhibitor, Z-leucyl-leucyl-norvalinal, suggesting that the proteasome is responsible for the age-related increase in the half-life of proteins (Kumiyama *et al.*, in preparation).

The proteasome is believed to exist *in vivo* in two different molecular forms, 20S and 26S proteasome, as detected on glycerol gradient centrifugation of cell extracts.²⁵ The 26S form consists of a 20S core proteasome and two 19S regulatory complexes. Proteins to be degraded by the 26S proteasome are marked with multiple ubiquitin molecules in steps requiring two or three kinds of enzymes. The polyubiquitinated proteins are recognized, deubiquitinated, and perhaps unfolded by a group of subunits called 19S regulatory complex in the 26S proteasome, and then the deubiquitinated proteins are degraded by the catalytic core of the 20S proteasome (Fig. 2). The 20S proteasome consists of two outer rings made up of seven different α -subunits each and two inner rings made up of seven different β -subunits each, some of which contain catalytic domains for peptide bond cleavage. Among at least five peptidase activities in the proteasomes, chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activities are often studied as proteasomal activity. Each of these peptidases catalyzes cleavage of a peptide bond at the carboxyl-terminal side of hydrophobic, basic, and acidic amino acid residues, respectively, giving rise to oligo peptides or amino acids from protein substrates. Inasmuch as the proteasome has been implicated in the degradation of oxidatively modified proteins,^{23,26} it was of interest to study age-related changes in them.

Starke-Reed and Oliver²⁷ were perhaps the first to show that the activity of alkaline protease in the liver of rat (strain not described) declines with age. The major alkaline protease was apparently believed to be an enzyme, later called proteasome. They found a dramatic decline of the activity between 16 and 26 months of age using soluble liver proteins oxidatively modified by metal catalyzed oxidation. This observation was confirmed by Agarwal and Sohal²⁸ who reported an age-related decline

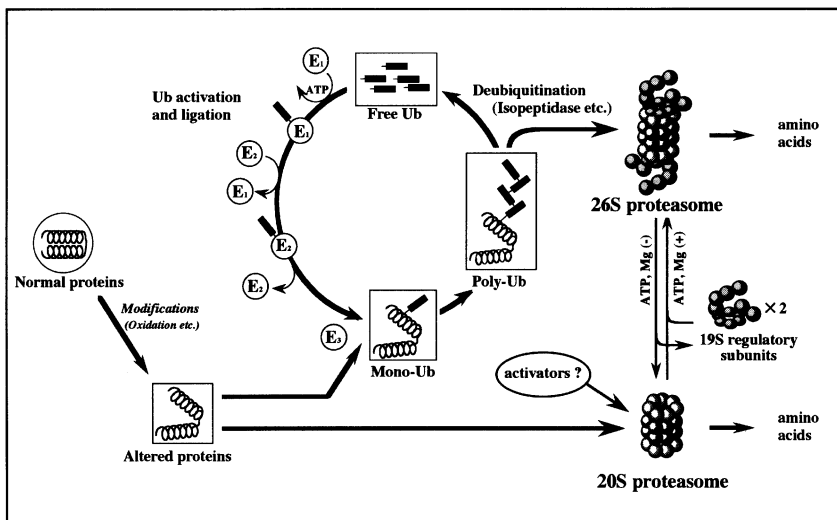


FIGURE 2. 20S and 26S proteasome pathways of altered protein degradation. Ub: ubiquitin.

of alkaline protease activity in the liver of SD rats using X-ray irradiated bovine serum albumin as a substrate. No significant change of the activity, however, was observed in the brain or heart among animals of 3, 13, and 23 months of age. Carney *et al.*²⁹ also found significant decrease in alkaline protease activity in the brain of old gerbils. Cao and Cutler³⁰ critically examined alkaline protease activity in the brain of aging male F344 rats using oxidized glutamine synthetase (GS) or oxidized brain cortex protein as substrate. They found no change of the enzyme with age, making a conclusion that the reported age-dependent change in alkaline protease activity remains to be confirmed. Sahakian *et al.*³¹ also found that the multicatalytic protease activity in liver homogenates of F344 rats at 8, 14, and 26 months of age did not change with age significantly when oxidatively modified bulk liver soluble proteins or oxidatively modified GS were used as substrates. The same group of investigators also reported that specific activity of the PGPH activity in purified multicatalytic proteinase declined by 50% with age when young (8 months old) and old (24 months old) rats were compared.³² In accordance with the finding of age-related decrease in PGPH activity, Shibatani *et al.*³³ reported that the activity in F344 rat liver supernatant (100,000 × g) decreased by 40% with age (7 vs. 26 months of age). More recently, Keller *et al.*³⁴ described that chymotrypsin-like activity of proteasome decreases with age (3, 12, 24, and 28 months old) in the homogenates of heart, lung, kidney, and liver as well as some portions of the brain of male F344 rats. In all of the investigations cited above, tissue homogenates or the supernatant was used as enzyme sources. It is, therefore, likely that the 20S and 26S proteasome activities together or the 20S proteasome activity alone was measured, depending on what substrate was used and under what assay conditions the activity was determined. This is because the two forms of the proteasome appear to have distinct functions, the 26S

form being involved in ATP-dependent degradation of ubiquitinated proteins and the 20S form degrading proteins ATP and ubiquitin independently. The 20S form does not appear to be simply an artificially dissociated form of the 26S proteasome (Ref. 35, see FIG. 2). We therefore studied changes in the two forms of the proteasome with age.

The proteasomal peptidase activities in the liver extracts of young (8- to 10-month-old), middle-aged (15- to 18-month-old), and old (25- to 28-month-old) male F344 rats were studied using fluorogenic peptide substrates for chymotrypsin-like, trypsin-like, and PGPH activities. The $100,000 \times g$ supernatant of the extracts in 5% glycerol was fractionated on glycerol gradient (15–35%) centrifugation to separate 20S and 26S.³⁶ Glycerol is believed to preserve the physiological state of the proteasomes in cells.²⁵ The trypsin-like activity in the sum of 20S and 26S fractions decreased significantly by 17% when the level of the old group was compared with that of the young. The chymotrypsin-like activity in the absence of the activator sodium dodecyl sulfate (SDS) declined significantly by 30%, but age-related change was abolished in the presence of SDS under which condition much higher activity was attained. The age-related percentage decrease in the total PGPH activity of 20S and 26S regions in the presence of SDS was most notable, being 60% (old vs. young) (FIG. 3b). Differences between the 20S and 26S forms in age-related changes of the activities were not significant (FIG. 3a), indicating that both ubiquitin-dependent and -independent proteasomal protein degradation are equally impaired in old animals. Remarkably, the relative amount of an α -subunit (C2) of the proteasomes per milligram tissue protein did not decrease appreciably with age, as shown by Western blot analysis.³⁶ Shibatani *et al.*³³ also noted that the amount of 20S proteasome did not change with age, as detected by immunoblot analysis of $100,000 \times g$ supernatant

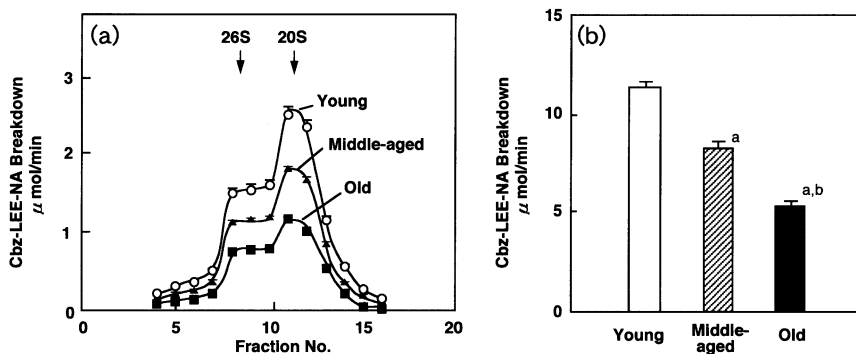


FIGURE 3. Age-related changes in the activity of peptidylglutamyl-peptide hydrolyzing (PDPH) activities of proteasomes (26S and 20S) in the livers of rats (F344). (a) Distribution of proteasomal activities on the glycerol gradient centrifugation. (b) The PGPH activity in each fraction of the gradient shown in FIG. 3a was totaled for individual animals. Young: 8 to 10 months old; middle-aged: 15 to 18 months old; old: 25 to 28 months old. Values are mean \pm SE ($n = 6$). (a) Significantly different from young ($p < 0.05$); (b) significantly different from middle-aged ($p < 0.05$).³⁶

proteins of the liver of male F344 rats 7, 16, and 26 months of age. These findings suggest that the quality rather than quantity of proteasome subunits is decreased with age. It is possible that the proteasomes are themselves modified oxidatively either directly or by aldehydes derived from peroxidized lipids.^{31,34} We recently found in male BDF1 mouse liver that although the PGPH activity tended to decrease with age, though not significantly, a significant 50% increase in the amount of the proteasome subunit was found in old animals, again suggesting that the quality of the proteasome decreases with age (Takenouchi *et al.* in preparation). Thus, an age-related decline in the quality of proteasomes could be at least part of the cause of the accumulation of altered proteins.

Consistent with this hypothesis, ubiquitinated proteins accumulate with age in various tissues, including different regions of the brain of mouse (FIG. 4).³⁷ It is interesting that proteins that contain errors in translation or that are misfolded during translation were shown to be polyubiquitinated and rapidly degraded by the proteasome, and the ubiquitinated proteins therefore accumulate if the proteasome activity is inhibited.³⁸ It is possible that the age-associated increase in ubiquitinated proteins is partly a result of abortive translation in addition to being due to well-documented nonphysiological posttranslational modifications. Regarding the accumulation of ubiquitinated proteins, it should be pointed out that their deposition in neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, may be causally related to the deterioration of the ubiquitin/proteasomal system of protein degradation.^{39,40}

DIETARY INTERVENTION FOR DECREASED PROTEIN TURNOVER IN AGED ANIMALS

Dietary restriction (DR) or caloric restriction initiated soon after weaning or even in middle age is perhaps the only reliable means of retarding aging in a variety of organisms.^{41,42} Because the majority of age-related changes are attenuated by this regimen, it is suggested that general mechanisms that are likely to be the basis of aging might be involved in the beneficial effects of DR. In the present context Ward and his collaborators reported that proteasomal PGPH activity that declines with age in ad libitum-fed F344 rats was maintained at higher levels by DR throughout life.³³ Interestingly, the age-related increase rather than decrease in chymotrypsin-like activity was abolished by DR. The significance of this change is unclear. Vittorini *et al.*⁴³ reported that proteasomal chymotrypsin-like and trypsin-like activities in the liver extract (23,000 × *g* supernatant) of SD rats fed every other day decreased significantly from those of ad libitum-fed animals at the age of 2, 24, and 27 months. On the other hand the PGPH activity that declined significantly between 24 and 27 months of age was not affected by the regimen. Thus, available data on the effect of life-long DR on proteasome activity are controversial.

Of particular interest is the mechanism of beneficial effects of DR initiated relatively later in life on the extension of life or a healthy life span because such intervention might provide a biological basis for human application. We have previously shown that DR initiated at old ages and continued for two months in BDF1 mice resulted in significant reduction of altered proteins and half-life of proteins, including

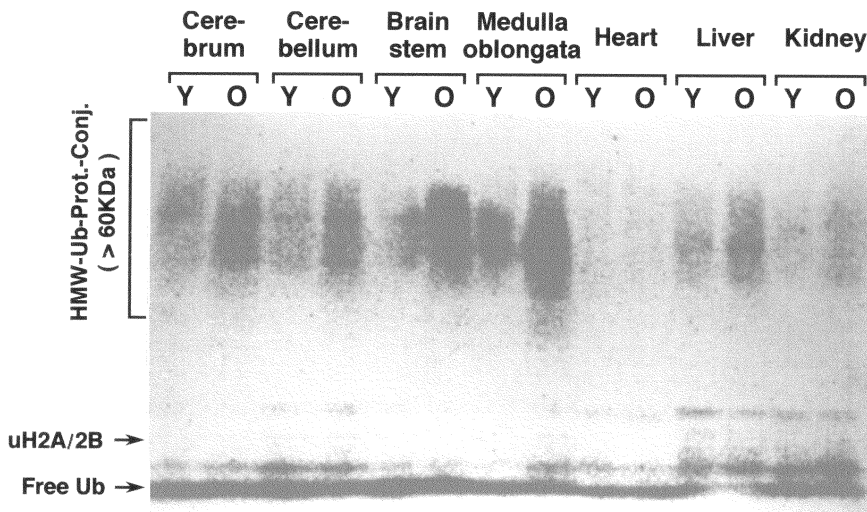


FIGURE 4. Accumulation of high-molecular-weight (HMW) ubiquitin conjugates in BDF1 mouse tissues with age. Y: young (3 to 6 months); O: old (32 to 36 months old). (Reproduced with permission from Ohtsuka *et al.*³⁷)

oxidatively modified ones^{44, 45} (Takahashi *et al.*, in preparation). These findings, and in view of protein turnover being involved in the general process of life, suggest that an increase in protein turnover by DR late in life may be part of the mechanism of its beneficial effects in prolonging life.

Our more recent results for F344 rat liver suggest that DR by feeding every other day initiated at an old age (26.5 months old) and continued for 3.5 months restored higher PGPH activity, comparable to the level of young animals (10 months old), with no apparent change in the amount of proteasome (Kumiyama *et al.*, in preparation). This finding suggests that proteasome complexes with reduced activity, possibly due to some kind of modification, are replaced by intact ones during DR. It is noted that such “rejuvenation” can be achieved even in the latest half of life (the average life span of male F344 rats being 29 months in our animal facility).

EFFECT OF EXERCISE ON THE PROTEOLYTIC SYSTEM IN AGING ANIMALS

Regular moderate exercise is believed to be beneficial for health, reducing age-related disorders and eventually extending functional life or health span and life span.^{46, 47} The biochemical basis of this well-documented phenomenon is not, however, well understood.⁴⁸ Paradoxically, physical exercise increases the generation of reactive oxygen species that are potentially harmful to proteins and other macromolecules in cells. It is true that a single bout of exercise does indeed damage these mac-

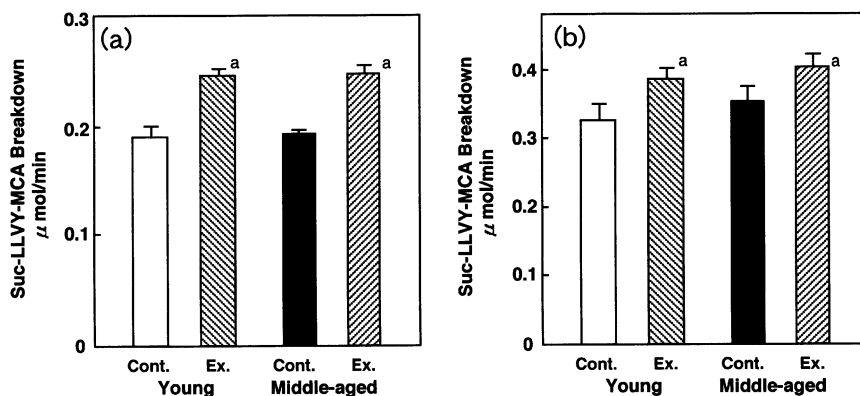


FIGURE 5. Effect of exercise on the activity of chymotrypsin-like activity of proteasomes (26S and 20S) in the (a) skeletal muscle and (b) brain of rats (Wistar). Cont.: control, Ex.: exercised. Young: 4 weeks old; middle-aged: 14 months old. Values are mean \pm SE (n = 6). a: significantly different from the control groups ($p < 0.05$).^{52, 53}

romolecules in laboratory rodents.^{49–51} This is perhaps not unexpected because sedentary laboratory rodents are probably not prepared for the massive oxidative stress caused by acute exercise. It is, however, conceivable that moderate long-term exercise induces higher protection mechanisms against oxidative damage due to slightly but significantly elevated generation of reactive oxygen species. To test this hypothesis we conducted a moderate swimming exercise of 5 days a week for 9 weeks on young (4-week-old) and middle-aged (14-month-old) male Wistar rats.⁵² Although the amount of protein carbonyls in the skeletal muscle was not changed significantly by the exercise, the activities of proteasomal chymotrypsin-like and trypsin-like peptidases increased significantly (FIG. 5a). Remarkably, the same exercise training improved cognitive functions assessed by active and passive avoidance tests of the rats with concomitant decrease in protein carbonyl and increase in proteasomal peptidase activities in the brain (FIG. 5b).⁵³ It is tempting to speculate that the decrease in protein carbonyls is involved in the improved learning and memory of rats by the exercise, and the enhanced proteasomal activity could have a role in the reduction of these carbonyls. Our findings are in accordance with previous reports on negative correlation between the extent of carbonylation of brain proteins and cognitive function, and the positive correlation between alkaline protease activity of the brain and cognitive function in aging animals.^{29,54} It is thus conceivable that, in addition to an increase in the activity of antioxidant enzymes known to occur in the brain as a result of regular physical exercise, upregulation of proteasomes to promote protein turnover may play a significant role in improved cognitive functions by reducing damaged proteins in the brain. It would be interesting to know whether or not improvement of cognitive functions can be attained by moderate regular exercise in older animals, such as those used in our DR experiments described above.

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