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Review

Implications of oxidative damage to proteins and DNA in aging and its intervention by caloric restriction and exercise

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Abstract

In this short review we describe implications of age-related changes of protein and DNA oxidation as a public mechanism of biological aging. Oxidatively modified protein and DNA have been demonstrated to increase with advancing age in rodents. Half-life of proteins is extended and DNA repair activity declines in old animals. Dietary restriction initiated late in life can shorten the half-life of proteins to levels of young animals, thus contributing to reduce level of altered proteins in old animals by the regimen. Regular exercise reduced oxidatively modified proteins in the brain with improved cognitive functions. It attenuated oxidative stress in the liver, i.e., ameliorating activation of nuclear factor κB, increasing reduced glutathione, and decreasing oxidized guanine base in nuclear and mitochondrial DNA. These findings suggest that regular exercise has systemic effects in reducing oxidative stress. Thus, life-styles such as diet and exercise may extend health span, by up-regulating overall anti-oxidant capacities that include proteins involved in protein turnover and DNA repair, resulting in reduction of damaged proteins and DNA that potentially promote physiological and pathological aging.

Keywords: Aging; Dietary restriction; DNA; Exercise; Oxidative stress; Protein

1. Introduction

One of the most serious current worldwide problems is social and economical as well as medical issues associated with ever increasing population of elderly people who are frail and/or sick and may therefore need extensive help and care by their families and societies. To cope with such problems, basic as well as translational researches of aging are highly needed from biological and medical perspectives. A major concern of industrialized societies is, therefore, to develop means to extend healthy lifespan or health span rather than simply prolong longevity. Reflecting such social trends, so called anti-aging medicine is getting popular not only among middle-aged and elderly people but also in clinicians as well as in industries that aim to enter in the big market. In this brief review we discuss the current status of studies on biological mechanism of aging with special emphasis on oxidative changes of proteins and DNA with age. Also discussed is possible intervention of aging by dietary restriction (DR) and regular exercise (RE) as viewed from age-related changes of proteins and protein metabolism in experimental animals.

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2. Molecular mechanisms of aging

2.1. Age-related alterations of proteins

Aging may be defined as a process of accumulation of damaged cellular constituents such as proteins, DNA, and membrane lipids that can result in the increased probability of death of an organism

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with gradual loss of homeostasis when exposed to internal and external stresses. A number of protective networks including antioxidants, antioxidant enzymes, chaperons, repair enzymes, and removal enzymes of damaged molecules are working in cells and tissues to maintain life against stresses. Nevertheless, increase in altered potentially harmful molecules is inevitable in the long run because protective systems themselves are subject to alterations and, therefore, the whole system is never perfect. Aging may be viewed as a deteriorative process of these systems that are active enough in young ages but become less and less active by time.

Of particular importance among these systems is protein turnover.[1] The rationale behind this idea is that even if, for example, DNA bases are oxidatively or otherwise modified, the cell would be able to restore normal functions if enzymes involved in the repair process would work efficiently and replace the damaged molecules by functionally active molecules via degradation and re-synthesis, i.e., metabolic turnover. Another possible way to replace damaged molecules is cell turnover or cell renewal that involves removal of cellular constituents as a whole. Here, we focus on metabolic turnover, particularly of proteins that can apply to both dividing and non-dividing cells such as neural and muscle cells that appear rate limiting of aging process of multicellular organisms.

Altered proteins are known to increase with age, most notable examples being accumulation of aggregated forms of proteins in neural tissues such as β-amyloid in Alzheimer's disease that is strongly implicated in its etiology[2] and α-synuclein in Parkinson's disease for which accumulation of the aggregated form is apparently responsible.[3] Less remarkable but more general altered forms of proteins can increase with age in any tissues of old animals.[4,5] Heat stability of an enzyme may decrease by slight chemical modifications of amino acid residues in proteins or conformational changes of proteins induced by unavoidable thermal perturbation. We have found that a greater percentage of enzymes is more heat-labile in various tissues of older rats and mice comparing with those of younger animals.[6,7] We and others have discovered the molecular activity of proteasome, a multicatalitic proteolytic enzyme, that is responsible for degradation of altered proteins is reduced with age as discussed in more detail in the later section.[8,9]

2.2. Generation of altered proteins

Enzymes or proteins with reduced molecular activity or heat-stability can be generated in theory by mutation, translational error or post-translational modifications. The rate of mutation, however, is too low to account for observed age-related increase in heat-labile enzymes or decline of molecular activity of enzymes that may lead to deterioration of biological functions with age.[10] The error frequency of gene expression has been shown not to change significantly with age, at least in translational fidelity, contrary to what error catastrophe theory of aging predicted.[11,12] Error catastrophe theory of aging, once a popular theory of aging, hypothesized that since fidelity of transfer of genetic information in replication and translation is not perfect, errors may increase gradually because the translational machinery by itself is made up of proteins that are subjected to errors. A number of investigations including our own studies have been conducted to verify the theory, reporting that it is not the case, however.[13,14] This is most probably because altered proteins are degraded and replaced by new proteins thus escaping accumulation of potentially harmful molecules, as discussed later.

Post-translational modifications of proteins reported to occur in aging include deamidation, racemization, glycation (glycoxidation), methylation, phosphorylation, and oxidation, as well as conformational changes with no apparent chemical changes.[15] Among these modifications, the oxidation is of particular interest because oxidative modifications of proteins can occur in any cells by reactive oxygen species (ROS) generated during oxygen metabolism most notably in mitochondria that consume more than 90% of oxygen that cells require. ROS is also generated in reactions catalyzed by oxidative and reductive enzymes as potentially harmful byproducts[16] or products that play essential physiological roles.[17]

We addressed whether ROS might be involved in age-related alteration of enzymes. Rat liver aminoacyl tRNA synthetases exposed to ROS generating mixtures *in vitro* exhibited heat-inactivation kinetics similar to the enzymes extracted from tissues of old animals, suggesting a possibility that ROS is involved in the alteration of the proteins.[18] Other investigators also reported that glucose-6-phosphate dehydrogenase oxidatively modified *in vitro* showed similar heat-lability to that found in old cells.[19] Aconitase, a mitochondrial enzyme, and glutamine synthetase, a cytoplasmic enzyme in the brain, are shown to be highly prone to oxidation *in vivo* and therefore have been used as a marker of oxidative stress.[20,21] It was suggested that enzymes having transition metal binding sites are readily inactivated or labilized by metal-catalyzed Fenton reaction. Tyrosyl tRNA synthetase appeared much more readily heat-labilized than leucyl tRNA synthetase, providing a potential example of differential sensitivity to ROS due to the difference in the metal binding properties.[18]

2.3. Detection of oxidatively modified proteins

Side chains of amino acid residues in proteins can be modified in many different ways by metal catalyzed oxidation with ROS at or near the metal binding sites. The modified amino acids include dityrosine from tyrosine, *o*- or *m*-tyrosine from phenylalanine, 2-oxohistidine from histidine, *N*-formylkynurenine from tryptophan, 4- or 5-hydroxyleucine from leucine, 3- or 4-hydroxyvaline from valine, methionine sulfoxide and methionine sulfone from methionine, cystine from cysteine, and carbonyl derivatives from lysine, arginine, proline, and so on.[15] Some of these modifications such as those of methionine and cyteine residues are reversible *in vivo* and therefore can be of physiological significance.[22] While most of the modifications are not easy to measure, carbonyl moieties that occur on many amino acid residues can easily be studied using a classical so called carbonyl reagent, 2,4-dinitrophenylhydrazine (DNPH) that specifically reacts with carbonyl groups. The products, 2,4-dinitrophenylhydrazones, are quantified with a spectrophotometer based on molar extinction coefficient so that molar content of the carbonyls per milligram of

proteins or per mole of a protein can be evaluated. Furthermore, since 2,4-dinitrophenyl moiety is highly immunogenic, monospecific antibodies can readily be produced. Thus, we have developed an immunological method to detect protein carbonyls in Western blot and immuno-histochemistry.[23,24] Levine et al.[25] also reported a similar method independently. Two dimensional separation of protein 2,4-dinitrophenylhydrazones have allowed us proteomic studies of carbonylated proteins.[24] Results of such studies have suggested that susceptibility to oxidation is highly variable among different proteins in aging.

2.4. Degradation of proteins

The steady state level of proteins in cells is dependent on both synthesis and degradation, i.e., metabolic turnover. Accumulation of altered proteins in tissues with age can be due to either a higher rate of oxidation and other types of modifications or a lower rate of degradation of the modified proteins, or both. We and others have found that half-lives of proteins in tissues and cells are significantly extended with age.[26−28] Half-lives of proteins in hepatocytes isolated from old mice were longer than in those of young counterparts by 50%−95%, suggesting a cause of age-related accumulation of altered proteins being due at least partly to decrease in protein degradation. So far, little attempt has been made to learn the degradation of altered proteins *in vivo* except a report by Lavie et al.[29] who demonstrated that puromycinyl peptides as a model of altered proteins are degraded much more slowly in livers of older mice. To investigate half-life of oxidized proteins we introduced oxidatively modified lysozyme into hepatocytes from young and old mice. Half-life of the modified lysozyme was much shorter than that of its unoxidized counterpart. The finding is consistent with reports that denatured proteins are more readily recognized by proteases, consistent with findings *in vitro* by other investigators.[30] Oxidatively modified lysozyme was degraded significantly more slowly in the cells of older mice than in those of young animals. Thus, the lower protein degradation appears to play a role in the increased steady state level of oxidized proteins in old animal tissues. Consistent with this finding, the proteasome activity of the liver of old animals was found significantly lower than that of the younger counterpart.[9] The proteasome is a multicatalytic protease that degrades altered or regulatory proteins with at least three different substrate specificities with respect to amino acid residues and exists in two forms, i.e., 20 S and 26 S forms. The 26 S proteasome is responsible for the degradation of ubiquitinated proteins while the 20 S proteasome is involved in the degradation of non-ubiquitinated proteins. Both forms of the enzyme consist of the same seven different α subunits and seven different β subunits with additional regulatory subunits in the 26 S proteasome. Our finding showed that activities of both forms of the proteasome are equally reduced with age. What is interesting here is that the amount of proteasome subunits was not reduced but rather tended to increase with age as determined by Western blot for the subunits.[28] This means that the molecular activity of the proteasome might be reduced in aged animals, suggesting that the proteasome by itself is altered with age, leading to reduced protein degradation. In fact, Ishii et al.[31] reported that proteasome itself can be modified by oxidative modification in cells and also *in vitro*, resulting in decreased enzyme activity to degrade substrates.

3. Intervention of aging

3.1. DR initiated late in life in rodents

DR or caloric restriction (CR) is the only robust means to delay age-related decline in physiological functions and onset of age-associated diseases, extending mean and maximum lifespans to a significant extent (30%−40%) in mice and rats.[32] Some of the anti-aging effects of DR/CR can be observed also in animals such as nematodes, fruit flies, spiders, and water fleas.[33] In majority of rodent studies DR/CR is initiated early in life, i.e., soon after weaning or at early stages of growth. In view of the wide range of beneficial effects and positive effects in many animal species of DR/CR to apparently retard aging, it is likely to influence fundamental or "public" mechanism(s) of aging such as increased resistance against oxidative stress.[34] In rodents, DR/CR initiated much later in life was also demonstrated to have beneficial effects: reduction of tumor incidence resulting in the extension of lifespan, albeit less remarkable compared with earlier-onset regimens.[35−37] Many theories have been proposed to explain the mechanism of the anti-aging effect of DR/CR, including attenuation of oxidative stress,[38] lowering of energy metabolism,[39] hormesis effect due to mild stress of elevated plasma glucocorticoid concentration,[40] reduced glycation due to lower plasma glucose level[41] or increased physical activities due to searching for food,[42] *etc*.

Regardless of the primary mechanism(s) of the anti-aging action of DR/CR, it was hypothesized that effects of DR on proteins and protein metabolism may be important since proteins are involved in all life maintenance processes as discussed in the previous section. We have, therefore, studied proteins and protein metabolism in middle-aged or older animals subjected to DR.[36] DR initiated at an old age was able to reduce age-related increase in the percentage of heat-labile enzymes in different tissues within 2 months to the level of young animals.[43] It was hypothesized that protein turnover is decreased in the old, leading to the increase in altered proteins, and that DR might reverse this process by increasing degradation of damaged proteins. In fact, prolonged half-lives of proteins in hepatocytes of old mice was shortened to that of young animals by DR.[44] Half-life of the degradation of oxidatively modified lysozymes introduced into the hepatocytes was also extended in old cells,[28] although effect of DR remained to be seen in the modified proteins.

Three and half months of DR initiated at 26.5 months of age up-regulated the proteasome activity significantly in rat livers.[28] The amount of the proteasome as detected by Western blot did not differ between DR/CR and freely fed animals. It is, therefore, conceivable that DR/CR promotes turnover of the proteasome itself, replacing the impaired enzymes in the old tissues by newly synthesized intact molecules, and thus

leading to more efficient degradation of altered proteins. It thus appears that DR/CR initiated late in life can promote removal of potentially harmful or useless altered proteins accumulated in old animals.

3.2. Regular physical exercise

While regular exercise is believed good for health, it is often claimed that exercise may induce oxidative stress because of an excessive oxygen uptake that can result in elevated generation of ROS in mitochondria and enzymatic systems such as reactions catalyzed by xanthine oxidase in purine catabolism and nicotinamide adenine dinucleotide phosphate (NAD(P)H) dehydrogenase in inflammatory processes accompanied by increased consumption of ATP and muscle damage in unaccustomed exercise. As a result of massive generation of ROS, proteins, nucleic acids, and membrane phospholipids could be more oxidatively modified in animals subjected to exercise than in sedentary situation, potentially leading to detrimental consequences. In fact, a bout of exhaustive exercise increased protein oxidation in the skeletal muscle, liver and lung of sedentary animals unprepared to the increased oxidative stress.[45–47] We hypothesized that moderate regular exercise can be beneficial in aged animals by upregulating the protective capabilities particularly in repair and replacement of oxidatively or otherwise damaged molecules. We have tested this hypothesis in tissues of middle-aged or older rats using two different protocols of regular exercise.

3.3. Regular swimming exercise and oxidative modification of proteins in rat brain

Swimming exercise is an experimental paradigm to study physiological, biochemical and other changes associated with forced physical activities in rodents. We have studied effects of swimming exercise on cognitive function and oxidative modification of the brain proteins in rats.[48] The animals were subjected to 60–90 min of swimming exercise per day, 5 days per week. Exercised animals showed improved cognitive functions in passive and active avoidance tests in both age groups after 9 weeks of the regular exercise. These functional changes were accompanied by decrease in protein carbonyl, a marker of oxidative stress, of the brain. The activity of proteasome that can degrade altered proteins was up-regulated, suggesting that the increased proteasome activity is responsible for the decrease in the oxidatively modified proteins. These findings are consistent with the report that age-related decline of cognitive function and the increase in protein carbonyls in the brain of gerbils was reversed by the treatment with a spin trap compound, N-tert-butyl-α-phenylnitrone (PBN) with concomitant increase in proteasome activity[49] (but see Floyd et al.[50] for an alternative mechanism of PBN action). Since the moderate regular exercise and the PBN treatment up-regulated the activity of proteasome that is responsible for the degradation of oxidatively or otherwise modified proteins, the beneficial effects of the regular exercise and the PBN treatment are likely brought about by increased degradation of such proteins by the enzyme. Upregulation of proteasome activity by exercise has been observed also in the skeletal muscle.[51] As to potential beneficial effects on brain functions, long-term physical activity has been reported to increase neurogenesis in the brain.[52,53] It appears possible that a mechanism behind this response involves increased generation of ROS by exercise since division of neural precursor cells *in vitro* is inhibited by α-lipoic acid, a potent antioxidant.[54] Remarkably, physical exercise reduced β-amyloid deposition in the brain of transgenic model mice for Alzheimer's disease by possibly upregulating the activity of the enzyme that can degrade β-amyloid,[55,56] suggesting that regular exercise may reduce the risk of dimentia in human. In fact, epidemiological studies have shown that even regular practice of walking can reduce risk of Alzheimer's disease and other types of dementias.[57] Thus, regular exercise appear to have beneficial effects on tissues other than the skeletal and cardiac muscles on which the exercise effect are likely to influence. We therefore studied effects of regular exercise on the liver.

3.4. Regular treadmill exercise and oxidative stress in aging rat liver

Middle-aged (18-month-old, roughly equivalent to 45–50 years of age in human) and old (28-month-old, similarly 70–75 in human) male rats were subjected to regular treadmill training (4 times a week, 60–90 min per day) in to study oxidative status in the liver. In both age groups maximal oxygen uptake increased by about 40% after 8 weeks of RE,[58] showing that ability to respond to the oxygen requirement is well retained in the old animals. ROS level measured with a fluorescent probe was significantly higher in the liver extracts of old sedentary animals than in middle-aged counterparts. The RE attenuated the age-associated increase. The redox status evaluated by glutathione level showed more than two-fold increase of the reduced form (GSH) with decreased level of oxidized form (GSSG) in both middle-aged and older exercised animals. It thus appears that the cellular milieu is shifted to anti-oxidative state, suggesting an increase in preventive capacity by the exercise regimen even at old ages. We investigated the activity of nuclear factor κB (NF-κB), an important redox sensitive transcription factor, that regulates various inflammatory and immune responses.[59] Binding activity of NF-κB of nuclear extracts to deoxy-oligonucleotide with the responsive element increased with age as expected for the increased oxidative stress mentioned above. The binding was reduced after the regular exercise, suggesting that the regimen may attenuate or reverse inflammatory processes in the aged animals that are exacerbated, and thus reduces risk of many age-associated diseases such as atherosclerosis and cancer.

Glucocorticoids (GC) have anti-inflammatory activities and are used to treat inflammation in chronic diseases such as asthma and rheumatoid arthritis. GC inhibits gene expression of pro-inflammatory cytokines including various interleukins such as IL-1 and IL-6 and tumor necrosis factor α (TNF-α) as well as enzymes or receptors responsible for inflammatory processes such as inducible nitric oxide synthase (iNOS) and cyclo-xygenase-2 (COX-2). GC receptor (GR) is a transcription factor that influences directly or indirectly gene expression of

inflammation-related proteins. We found that binding activity of GR to the responsive DNA element is significantly decreased in the liver of aged animals, but 8 weeks of regular exercise was able to reverse the change.[60] No significant difference in the amount of GR protein was detected between young adult and older animals, suggesting, therefore, that the quality rather than the quantity of GR is altered with age. Serum level of GC was significantly higher in the exercised older animals than in the sedentary counterparts. In view of the anti-inflammatory activities of GC, these observations also support the view that RE may have a beneficial effect in reducing inflammation. It is interesting to note that GR can directly interact with NF-κB.[61] It is likely, therefore, that transcription factors GR and NF-κB synergistically downregulate the expression of inflammation related genes.

Interestingly, proteins in cardiac muscles of rats subjected to regular swimming exercise for 9 weeks were more resistant to oxidative challenge of intraperitoneal injection of H_2O_2.[62] The exercise preconditioning increases proteasome and DT-diaphorase (NAD(P)H quinone oxidoreductase) activities, thereby, apparently reducing increased carbonyl modification of proteins by the challenge. RE is reported to increase anti-oxidant enzyme activities in the skeletal muscle[63,64] and the liver of rats.[65] Taken together, these results support the view that RE upregulates protection against oxidative stress.

4. Perspective

Thus, RE can apparently improve age-related functional decline and delay onset of age-related diseases by attenuating potentially harmful oxidative damage and suppressing inflammatory processes even in old ages. We suggest that RE can induce adaptive response against oxidative and perhaps other stresses by moderately increasing ROS in many tissues[66,67] even in old animals. Others have reported similar findings.[68] Responses induced by exercise may be a form of hormesis in that while excessive generation of ROS by exhaustive exercise is harmful to an unprepared organism, moderate generation of ROS by modest RE may induce adaptive response to be able to cope with future stronger stresses. In support for the idea that ROS is a basis of beneficial outcomes of RE, Ristow et al.[69] have reported possible involvement of ROS in health promoting effects of exercise in human. They found that vitamins C and E supplements abolished beneficial effects of RE, i.e., upregulation of glutathione peroxidase mRNA, serum adiponectin and glucose uptake capacities of tissues. It is thus suggested that anti-oxidant activities of these vitamins are responsible for the reduced beneficial effects of RE. It should be mentioned in this regard that contradictory results are reported in a rat study.[70]

We have emphasized that beneficial outcomes of regular exercise in old ages are likely brought about in part by mild oxidative stress induced by the physical activities.[67,71,72]

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