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Carbonylated proteins in aging and exercise: immunoblot approaches

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Abstract

Protein carbonyls were studied in aging and exercise by immunoblot followed by one- or two-dimensional polyacrylamide gel electrophoresis using antibodies against 2,4-dinitrophenylhydrazones. Proteins of rat kidneys exhibited significant age-related increase in the amount of carbonyl while those of the brain and liver did not. Major carbonylated proteins in the kidney included serum albumin. In nematodes in which protein carbonyls increased with age, one of the carbonylated proteins was identified as vitellogenin, an egg-yolk protein. A possible biological significance of this protein present in abundance even after egg-laying stages is discussed in terms of protection against oxidative stress. Exhaustive exercise induced significant increase in the carbonylation of selected but unidentified proteins in the lung. This oxidative stress might be caused by xanthine oxidase in this tissue and hypoxanthine derived from ATP-depleted muscles. Exercise at high altitude caused higher carbonylation of the skeletal muscle proteins, most notably a protein likely to be actin, than that at sea level but no significant difference was observed in lipid peroxidation. These studies emphasize the value of immunoblot analysis of tissue protein carbonyls in a variety of situations where oxidative stress is likely involved.

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1. Introduction

Active oxygen species generated in a variety of biological systems have been implicated in mechanisms of aging and age-associated pathologies such as arteriosclerosis, Alzheimer's and Parkinson's diseases, cardiac, hepatic and renal disorders (Stadtman, 1992; Ames et al., 1993; Martin et al., 1996). They also are able to induce damage to cells in tissue such as the muscle and lung during elevated energy metabolism in exercise (Reznick, 1998). Nucleic acids, proteins and membrane lipids are major targets of reactive oxygen species. Highly reactive hydroxyl radicals are thought to be generated in vivo by catalytic action of transition metals such as iron and copper that bind to appropriate sites of proteins and can modify nearby amino acid residues (Davies et al., 1987; Stadtman, 1990). Oxidative modifications of amino acid residues include derivatization of those such as proline, arginine, and lysine to carbonyl derivatives. Protein carbonyls can be determined spectrophotometrically by the reaction with a classical carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Recently we and others have developed immunoblot methods to study carbonylated proteins separated by one- or two-dimensional polyacrylamide gel electrophoresis (Nakamoto et al., 1994; Shacter et al., 1994; Nakamura and Goto, 1996). More quantitative and qualitative information may be obtained with this method regarding oxidative status of individual tissue proteins which can lead to deleterious consequences on cellular functions.

In this article we discuss oxidative stress in aging and exercise with special reference to our work on protein carbonyls.

2. Protein carbonyls in aging of rodents

Enzymes with altered activity are increased in tissues of senescent animals, some of which have been mimicked in vitro by metal-catalyzed oxidation, suggesting the involvement of active oxygen species in protein modifications in the aging process (Oliver et al., 1985; Takahashi and Goto, 1990). The amount of carbonyl per mg of protein is reported to increase progressively with age in rat hepatocytes (Starke-Reed and Oliver, 1989), mouse (Sohal et al., 1994b) and Mongolian gerbil tissues (Sohal et al., 1994a), houseflies (Sohal et al., 1993), and human skin fibroblasts (Oliver et al., 1987). These findings have added support to the claim that oxidative damage to proteins can explain the physiological decline of bodily functions with age. There is, however, controversy regarding this claim as discussed elsewhere (Goto and Nakamura, 1997).

We have determined the carbonyl content of proteins of various tissues of young (6–8 months) and old (28–34 months) male F344 rats by a conventional spectrophotometric method (Nakamura et al., submitted). No significant difference was

found between the two age groups in the brain, liver, lung or heart. Kidney proteins of old animals, however, contained approximately 1.5-fold more carbonyls in terms of nmol per mg protein. Our results on the liver and brain are not in agreement with the report that the protein carbonyl content in hepatocytes isolated from 26-month-old rats is significantly higher than that of younger (3–20-month-old) animals (Starke-Reed and Oliver, 1989). The reason for this apparent discrepancy is not clear, but it is possible that ischemia-reperfusion stress during the isolation of hepatocytes might influence the oxidation status of cellular proteins (Oliver et al., 1990; Caraceni et al., 1997), older cells possibly being more susceptible to the stress. The higher carbonyl content of the kidney proteins of old rats could be due to lesions often seen in that organ of aged rodents (Maeda et al., 1985).

To avoid complications due to possible problems such as incomplete removal of the free reagent before spectrophotometric measurement (Goto and Nakamura, 1997) we performed indirect immunoblot analysis for carbonyls using antibodies against DNPH in one- or two-dimensional gel electrophoresis of tissue proteins. Free DNPH which can remain in a sample for electrophoresis did not react with the antibodies even if it bound to transfer membranes. Results of one-dimensional immunoblot analysis were in good agreement with the results of our spectrophotometric measurement in that the signal intensity was significantly stronger only in the kidney of old animals compared with their young counterparts. In two-dimensional analysis it was noted that the pattern of protein staining and that of the immunological signal were markedly different from each other, indicating that specific cellular proteins are more susceptible to oxidative modifications than others and/or possibly contain carbonyl groups as essential moieties for their functions.

One of the highly carbonylated proteins in the kidney of old animals was identified as serum albumin by amino acid sequencing of the isolated protein. Since the serum albumin present in the serum is only slightly carbonylated, extensive carbonylation of the counterpart found in the kidney might mean that oxidatively modified proteins tend to be trapped in the tissue or the modification may occur in situ due to local inflammatory reaction. Other possible mechanisms of protein carbonyls to be generated are glycation (Hayase et al., 1989; Nakamura and Goto, 1996; Liggins and Furth, 1997) and modification with aldehydes derived from lipid peroxides (Esterbauer et al., 1991; Uchida et al., 1995). A low percentage of plasma albumin is glycated in mature rats, of which the rate of excretion into urine tends to decrease in old animals (Bakala et al., 1995). Receptors for glycated albumin are reported to be present in the brush border membranes which appear to increase with age (Verbeke et al., 1996). In our immunohistochemical studies the carbonylated proteins were found throughout the renal structure but were more concentrated in renal tubules than in the glomerulus. The amount of total iron but not copper in the kidney was found to be increased with age, suggesting a possibility that iron is responsible for the generation of oxidatively damaged proteins in this tissue. The distribution of non-heme iron, however, did not match that of carbonylated proteins. It is therefore conceivable that proteins in this tissue are carbonylated by other mechanisms such as glycation and/or the reaction with aldehydes generated from lipid peroxides rather than by direct oxidation of amino acid residues (Kristal and Yu, 1992).

Immunoblot analysis of kidney proteins separated in two-dimensional polyacrylamide gel electrophoresis revealed prominent immunological signals in the young rats, but these were absent in old animals. The corresponding proteins are likely to be modified forms of α-2u-globulin, as judged from their molecular weight, approximate isoelectric point and abundance. α-2u-Globulin is synthesized in the liver androgen dependently and hence disappears in old animals in which the hormone ceases to be produced. The reason for carbonylation of this protein is not clear. The protein is normally excreted in urine as a major urinary protein but can accumulate in lysosomes of proximal tubules under pathological conditions (Bumett et al., 1989). It is therefore likely that modified forms of α-2u-globulin are trapped in the kidney of adult rats before or after carbonylation by the mechanisms mentioned above. This interpretation is consistent with the finding that α-2u-globulins in the kidney are more acidic than the isoforms found in the urine since carbonylated proteins should have lost amino moieties (Lane and Neuhaus, 1972). These findings further suggest that spectrophotometric measurement of the total protein carbonyl must be interpreted with caution since it does not necessarily reflect the carbonylation of proteins in general.

3. Carbonylated proteins in aging nematodes

The nematode *Caenorhabditis elegans* is an excellent model for the study of aging of multicellular eukaryotic organisms because of its short life-span, small number of cells in the body and availability of a variety of age-related mutants. A number of mutants have been isolated which exhibit either long or short life-spans (Friedman and Johnson, 1988; Ishii et al., 1990; Kenyon et al., 1993). The mean and maximum life-spans of the *age*-1 are over 50% longer than those of the wild-type animal N2 (Friedman and Johnson, 1988), while the *mev*-1 mutant has about a 30% shorter life-span than the wild type in a normal atmosphere (Ishii et al., 1990). The *age*-1 mutant exhibits elevated catalase and SOD activities (Larsen, 1993; Vanfleteren, 1993) while SOD activity of the *mev*-1 mutant is half that of the wild-type animal (Ishii et al., 1990). It is therefore conceivable that the level of anti-oxidative defense is responsible for the difference in the life-span of mutant and wild-type animals. In fact, Adachi et al. found an excellent correlation between the protein carbonyl content of whole body extracts as detected spectrophotometrically and survival percentage during the life of *age*-1, wild-type and *mev*-1 animals: i.e. the higher the rate of increase in carbonyl content, the shorter the life-span (Adachi et al., 1998). We confirmed these findings by Western blot and immunoblot after two-dimensional polyacrylamide gel electrophoresis of the proteins in the extract.

One of the abundant and highly carbonylated proteins with a molecular mass of around 110 000 Da in aged wild animals was identified as vitellogenin 6, a major egg yolk protein, by amino acid sequencing of two independent protease V8 fragments of the protein (Nakamura et al., in preparation). It is noted that fluorescent materials accumulate in the intestine and/or the body fluid of nematodes with age at a much higher rate in *mev*-1 mutants than in wild-type animals

(Hosokawa et al., 1994). These lipofuscin-like materials are assumed to be complex mixtures of reaction products of lipid peroxides and cross-linked proteins. Vitellogenins, a family of proteins stored in the yolk of developing oocytes, are synthesized as high-molecular weight precursors in the intestine, processed and secreted for egg formation. It is possible that carbonylated vitellogenins constitute the lipofuscin-like materials in the intestine or the body fluid of old nematodes. Biological significance of the presence of this protein in old nematodes is not clear since vitellogenins should be required only during younger egg-laying stages. It is interesting to note that vitellogenin can bind metals such as zinc, cadmium and iron (Richards and Steel, 1987; Heusden et al., 1991). This abundant secretory protein therefore may have a biological role in the body fluid in later life other than a component of egg yolk, such as protection against oxidative damage to other cellular components serving as a kind of sink for metals which catalyze reactions forming active oxygen species. In accordance with this idea Halliwell proposed that albumin, an abundant extracellular protein, may be an important antioxidant (Halliwell, 1988) and, in fact, albumin is capable of inhibiting lipid peroxidation by binding iron (Loban et al., 1997).

4. Exercise and protein carbonylation

Moderate long-term physical exercise retards aging and extends the average life-span of experimental animals (Holloszy, 1993). In humans, several studies have suggested that lifelong regular exercise can reduce the incidence of diseases such as cardiovascular diseases and cancer, thereby prolonging life (Sarna et al., 1993; Blair et al., 1995). Mechanisms of such beneficial effects of physical exercise, however, are not well understood. Aerobic exercise is accompanied by an increase in the generation of active oxygen species in the skeletal muscle which can result in tissue damage (Davies et al., 1982). However, it is likely that adaptive response such as upregulation of antioxidant enzyme activities and other protective mechanisms can be induced by regular and moderate exercise, and in this way general protective activity is acquired to cope with stronger stress which might be encountered later (Booth et al., 1998).

A single bout in rats of exhaustive running or endurance training for 12 weeks has been shown to induce significant increase in protein oxidation of the skeletal muscles (Reznick et al., 1992; Witt et al., 1992). Supplementation of antioxidants attenuated the increase in protein oxidation due to a single bout of exercise, indicating that free radicals are involved in the process. We have shown that exhaustive running of rats induces a significant 40% increase of protein carbonyls in the lung, as determined by quantitation of Western blot signals (Radak et al., 1998). One possible scenario which could lead to oxidative stress in the lung in this situation is as follows. Under anaerobic conditions due to exhaustive exercise a large amount of ADP would be formed upon ATP depletion in the skeletal muscle. ATP would then be regenerated at the expense of two molecules of ADP by the catalytic action of adenylate kinase, AMP being formed as a by-product which is

metabolized to adenosine and then to hypoxanthine by adenosine deaminase. Hypoxanthine would diffuse out from the muscle into the circulating blood and thus be provided as a substrate for xanthine oxidase located on the surface of endothelial cells of the lung or in circulation, generating active oxygen species during the enzyme reaction. In support of this interpretation, it has been shown that oxidative stress due to acute exhaustive exercise was attenuated by the injection of long-lived derivatized SOD in spite of a marked increase in xanthine oxidase activity in the plasma (Radak et al., 1995). It would be of interest to see if a xanthine oxidase inhibitor, such as allopurinol, could attenuate the protein oxidation as in the case of ischemia/perfusion lung injury (Zhao et al., 1997). An alternative or additional possibility is the increased generation of active oxygen species due to high oxygen tension with extensive aeration in the tissue. It is likely that active oxygen species generated from activated alveolar macrophages with anaerobic running to exhaustion are involved in the protein oxidation as in inflammation (Kinnula et al., 1992).

Under reduced partial oxygen pressure at high altitude the generation of active oxygen species is assumed to decrease so that oxidative damage to biomolecules would be attenuated. Mn–SOD activity of the skeletal muscle is found decreased after long-term high-altitude training in rats, the situation apparently not being beneficial in protecting against oxidative stress (Radak et al., 1994). We have studied how such a situation affects the oxidation status of lipids and proteins in the muscles of animals trained at high altitude. Proteins of quadricept muscles of rats trained for 4 weeks at 4000 m altitude oxygen pressure exhibited a significantly higher extent of carbonylation than counterparts of untrained or trained animals at sea level (Radak et al., 1997). Reduced manganese–SOD might be partly responsible for higher oxidative stress in the animals trained at high altitude. Proteins which showed a marked increase in signal intensity in the Western blot appeared to be actins as judged by their molecular weight and abundance. We have observed that actins and myosin heavy chain in the cells of human arteries and veins are also highly carbonylated. These observations indicate that contractile proteins are susceptible to oxidation. Interestingly, contrary to the increased protein oxidation, the extent of lipid peroxidation as measured by thiobarbituric acid reactivity and the amount of lipid peroxides did not differ between animals exposed to sea level and high-altitude conditions. The apparent discrepancy between the oxidation status of lipids and proteins raises a question as to the mechanism of oxidative damage to these molecules. Protein carbonyls are often thought to be generated by direct oxidation of amino acid residues. Theoretically there are other possibilities which could account for carbonyls in proteins, including Michael addition of aldehydes to proteins such as malonaldehyde and 4-hydroxynonenal which are formed as degradation products of lipid hydroperoxides. However, increased oxidation of skeletal muscle proteins appears to be independent of lipid peroxidation in high-altitude training.

In conclusion, Western blot analysis of carbonylated proteins appears to provide better information than spectrophotometric measurement on the oxidative status of proteins in cells and tissues in aging and in diverse situations of exercise and pathologies.

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