Age, Vol. 20, 81-89, 1997

# AGE-ASSOCIATED, OXIDATIVELY MODIFIED PROTEINS: A CRITICAL EVALUATION

**Sataro Goto** and **Akihiro Nakamura**
Department of Biochemistry,
School of Pharmaceutical Sciences,
Toho University,
Miyama, Funabashi, 274 Chiba, Japan
Fax +81-474-72-1531 E-mail goto@phar.toho-u.ac.jp

## ABSTRACT

**Reactive oxygen species have been implicated in oxidative modifications of proteins, in many cases represented as carbonyls, which can lead to a variety of diseases and the age-associated decline of physiological functions. Considerable progress, as well as controversy, about oxidatively modified proteins and aging has unfolded in the last few years. In this article we critically evaluate changes in protein carbonyl content as a marker of the oxidative stress associated with age and other relevant issues on the degradation of oxidatively modified proteins.**

**A definitive conclusion on the age-related increase of protein carbonyls is currently viewed as having to await further confirmation using detailed analysis with new methodologies. Controversial methodological measurements and characterizations of protein carbonyls are discussed, emphasizing the merits of immunoblot analysis using two-dimensional gel electrophoresis. The degradation of oxidatively modified proteins has not yet been studied in depth in relation to their possible accumulation in old tissues. Recent efforts to establish a causal relation between the effect of oxidative stress on proteins and physiological declines with age are discussed briefly.**

## INTRODUCTION

In recent years molecular damage due to reactive oxygen species has been of increasing concern as a primary cause of aging and the mechanisms underlying a variety of age-associated pathologies including atherosclerosis, ischemia-reperfusion injuries of heart, brain, and lung, and neurodegenerative diseases. The story began as the free radical theory of aging originally proposed by Denham Harman in 1956; surviving as one of primary theories of aging for 40 years, it now appears to be gaining popularity (for reviews, see 1,2). In Harman's original theory, free radicals were assumed to primarily attack membrane lipids to form lipid peroxides, which can be harmful to cells and result in the impairment of physiological functions with advancing age. A modern version of the theory can be seen as the reactive oxygen hypothesis on aging, which might also include non-radical species.

Lipofuscin accumulating in postmitotic tissues with age has long been regarded as one of the major signs of lipid peroxidation. More recently, the pathological significance of oxidative modification of low density lipoproteins in atherosclerosis has been well documented. As a functional consequence of an age-related increase in the lipid peroxidation of organelle membranes, Yu and his collaborators have suggested that lipid peroxidation causes a decrease in membrane fluidity, thereby resulting in the functional declines of mitochondria and synaptic vesicles (3,4). It is noted, however, that molar ratios of lipid peroxides in unmodified membrane phospholipids appear rather low (i.e., on the order of $10^{-5}$ to $10^{-4}$) (5). Currently, because of methodological advances and increasing theoretical interests, studies on free radical-mediated attack on DNA and proteins have been initiated.

The extent of oxidative modifications of nuclear DNA bases in aged animals (on the order of magnitude of 1 to 2 in $10^5$ guanine nucleotides) appear too low to account for the general decline of cellular functions associated with aging (6,7). Obviously, however, an increase in the incidence of tumors with age could be explained by a single base modification in a cell. Implications of greater oxidative damage to mitochondrial DNA with age has been the focus of aging research (for review, see 8).

In contrast to oxidative damage to membrane lipids and nuclear DNA, accumulation of oxidized proteins can occur at higher rates (i.e., on the order of 10% of total cellular proteins on average) (9). This suggests a possibility of significant contributions by protein modifications to the widely occurring physiological decline associated with age. This review discusses some of the available results on protein oxidation related to aging, giving special reference to protein carbonyls.

More general views on oxidative stress and aging may be found in recent reviews (10,11).

### *Age-related Post-translational Modifications of Proteins*

Proteins undergo a variety of reversible or irreversible post-translational modifications that modulate their biological activities. Some modifications, however, are not physiological and can result in the loss or decrease of enzyme catalytic activity or in the deterioration of structural or regulatory proteins. Modifications such as racemization, deamidation, glycation, and oxidation are

reported to increase with age (12,13). Nonphysiological proteolytic cleavage of polypetide chains can also occur, as with the generation and deposition of β-amyloid in Alzheimer's patients and, to a lesser extent, in apparently healthy aged individuals (for recent review, see 14). Oxidation is believed to be theoretically one of the most important modifications relevant to aging because of its ubiquitous effect.

*Oxidative Modification of Proteins with Age*

Mild oxidation, which mimics in vivo situations, can generate a number of different modifications of amino acid residues in proteins without cleavage of peptide bonds (15). One of the most easily oxidizable residues are cysteine residues, which undergo reversible oxidation/reduction. A decrease in the amount of protein sulfhydryl group has also been a marker of oxidative modifications of proteins in aging animals (16). More important, however, are modifications, mostly irreversible, by the catalytic action of transition metal ions such as $Fe^{+3}$ and $Cu^{+2}$ trapped in metal binding pockets of proteins (17,18) or indirect oxidative modifications with aldehydes derived from lipid peroxides (19). Site-specific, metal-catalyzed oxidation gives rise to carbonyl derivatives, predominantly proline, arginine, and lysine residues that are located at or near the metal binding sites (17,20). Other oxidative modifications reported to occur in metal-catalyzed oxidation of proteins in vitro or in vivo include, formation of *o*-tyrosine from phenylalanine, dityrosine from tyrosine (21,22), and 3,4-dihydroxyphenylalanine (DOPA) from tyrosine (23). Methionine can be oxidized to methionine sulfoxide (24), but it can be reduced back to methionine by peptide-methionine sulfoxide reductase, which is shown to be important in cellular protection against oxidative stress (25,26).

*Age-associated Changes in Protein Carbonyls*

Altered forms of various proteins, mostly enzymes, increase in aged animals (27). These age-related enzyme alterations often can be observed by a decrease in specific activity of purified enzymes, the presence of immunological cross-reactive substances, or an increase in thermal lability, which may be mimicked by metal-catalyzed oxidation in vitro (28-30, for review, see 31). Such findings prompted the speculation that oxidative modifications may occur to various cellular proteins during aging. Protein carbonyls can be measured by a spectrophotometric method using the carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH) (32-34). Oliver et al. were the first to report on the age-related increase of carbonyl contents in tissue proteins (28). They reported that proteins of fibroblasts isolated from normal individuals aged 9 to 80 show an age-dependent increase of carbonyls. Interestingly, these authors also noted that proteins of the cells from progeria and Werner's syndrome patients have higher levels of the carbonyls than the cells from normal individuals in the same age groups.

Subsequently, Starke-Reed and Oliver reported that protein carbonyls accumulate progressively with age in rat hepatocytes, increasing sharply between 20 and 26 months (35). In a more recent study, Sohal et al. found that the protein carbonyl content increases significantly with age from 9 to 23 months in the brain, heart, and kidney of mice (36). The same group reported that male Mongolian gerbils exhibit a significant increase of carbonyl levels in the brain and heart with age, but not in the kidney (37). The protein carbonyl content of houseflies in whole body homogenates also increase with age (38). Interestingly, the extent of protein oxidation was found to be positively correlated with the extent of physical activity of the flies, which is inversely correlated with the average life span. These authors provide support to the claim that protein carbonyl accumulation is closely related to the aging process.

In an attempt to confirm the important results of Starke-Reed and Oliver (35), we have determined the carbonyl content of proteins in various tissues from young (6-8 months) and old (28-34 months) male rats (Nakamura et al., in preparation). No significant difference in nmol of carbonyl per mg protein content was detected between the two age groups in the brain, lung, and heart, which exhibited average values between 2 to 2.3 nmole/mg. The liver showed slightly higher values of 3 to 3.5, but without a significant difference between the two groups. The kidney of old animals, however, contained 1.5 times more carbonyls than their young counterparts, having an average value of 4.1 nmole/mg protein. These findings are in general agreement with the results of Western blot (see below; Nakamura et al. in preparation).

Table 1 summarizes the age-related changes found in the carbonyl content of tissue proteins reported in the literature. One can notice a considerable variation in the data obtained in different laboratories from the same tissues of the same animal species. This issue is discussed in the next section.

*Methodological Problems in Carbonyl Determination*

Since Levine and his collaborators described in detail the procedure for determining the carbonyl content of tissue proteins by the DNPH method (32), and because investigators interested in oxidative stress began to realize the advantage of this method in evaluating the extent of stress, a number of reports have been published on the topic. The DNPH method has shown, however, to sometimes produce variable results, as seen in Table 1; and has recently been criticized by Cao and Cutler (39). One of the main points they cite is the effect of nucleic acid contamination in tissue extracts. They claim that a reliable measurement of protein carbonyls in tissue extracts cannot be achieved without removal of nucleic acids by streptomycin sulfate treatment. Precipitation of nucleic acids with this reagent before carbonyl measurement was previously recommended by Levine et al. (32), but this step seems often to have been omitted. In our experiments, RNA did not

**Table 1**: Age-related Protein Carbonyl Contents in Animal Tissues and Cells

| *Animal* | *Tissue (cell)* | *Age #* | *Carbonyl** | *Reference* |
|---|---|---|---|---|
| rat | hepatocyte | 3 m | 1.9±0.5 | 35 |
| | | 12 m | 2.4±0.1 | |
| | | 20 m | 2.7±0.8 | |
| | | 26 m | 5.9±0.3 | |
| rat | liver | 9 w | 1.8 | 71 |
| | | 15 w | 2~3 | |
| | | 60 m | 3.36 | |
| rat | brain: frontal cortex | 5 m | 2.22 | 73 |
| | | 24 m | 2.11 | |
| | hippocampus | 5 m | 1.27 | |
| | | 24 m | 1.51 | |
| rat | liver | 6 m | 0.75 | 39 |
| | | 12 m | 0.65 | |
| | | 24 m | 0.65 | |
| rat | plasma | 5 m | 1.5 | 74 |
| | | 18 m | 2.0 | |
| | | 31 m | 2.5 | |
| | splenocyte | 5 m1 | 1.8 | |
| | | 18 m | 2.4 | |
| | | 31 m | 3.8 | |
| rat | heart | 2 m | 3.4 | 75 |
| | | 20.5 m | 4.4 | |
| mouse | brain | 9 m | 2.6 | 36 |
| | | 17 m | 3.0 | |
| | | 23 m | 3.4 | |
| | heart | 9 m | 2.0 | |
| | | 17 m | 2.6 | |
| | | 23 m | 3.3 | |
| | kidney | 9 m | 3.4 | |
| | | 17 m | 4.0 | |
| | | 23 m | 6.3 | |
| mouse | brain | 23 m | 0.8 | 67 |
| mouse | brain: cortex | 22 m | 1.0 | 66 |
| | hippocampus | | 2.1 | |
| | striatum | | 1.8 | |
| | midbrain | | 1.5 | |
| | cerebellum | | 1.3 | |
| | hindbrain | | 1.5 | |
| gerbil | brain: cortex | 3 m | 4.3 | 60 |
| | | 15-18 m | 8.7 | |
| gerbil | brain: cortex | 3 m | 1.3 | 67 |
| | | 15 m | 1.5 | |
| | hippocampus | 3 m | 0.8 | |
| | | 15 m | 1.2 | |
| | striatum | 3 m | 1.1 | |
| | | 15 m | 1.2 | |
| | cerebellum | 3 m | 0.9 | |
| | | 15 m | 1.2 | |
| | midbrain | 3 m | 1.0 | |
| | | 15 m | 1.2 | |
| | hindbrain | 3 m | 1.0 | |
| | | 15 m | 1.1 | |
| | heart | 3 m | 0.6 | |
| | | 15 m | 1.2 | |
| house fly | whole body | 5~8 d | ~2 | 38 |
| | | 16 d | 4.5 | |
| fruit fly | whole body | 6 d | 4.2 | 72 |
| | | 21 d | 4.8 | |
| | | 35 d | 5.5 | |
| human | fibroblast: | | | 28 |
| | healthy | 13 y | 2.2 | |
| | | 60 y | 2.7 | |
| | | 80 y | 4.7 | |
| | Werner progeria | 28-60 y | 4.3~5.4 | |
| | | 2-13 y | 3.5~6 | |
| human | brain: frontal pole | | | 59 |
| | healthy | 28.8±5.7 y | 2.5 | |
| | | 69.9±3.1 y | 6.8 | |
| | Alzheimer | 74.3±2.3 y | 7.6 | |
| | occipital pole | | | |
| | healthy | 28.8±5.7 yr | 3.0 | |
| | | 69.9±3.1 y | 4.4 | |
| | Alzheimer | 74.3±2.3 y | 4.6 | |

# d: day; w: week; m: month; y: year; * nmol/mg protein (Note: values in the table were mostly calculated from the original data given as histograms or plots and therefore may not be precise)

influence the carbonyl assay to a significant extent at levels present in the postmitochondrial supernatants, but contamination of DNA did when a significant amount released from nuclei was present. Reznick and Packer minced tissues gently, rather than using a homogenizer to avoid breaking the nuclei and releasing DNA (34). The possible influence of DNA should be recognized and checked in a sample from each tissue preparation, because no method of homogenization is guaranteed against DNA released from damaged nuclei. It should also be pointed out that detergents in the homogenization buffer (34,36) and/or the sonication of tissues (28,59,60) can influence the quality of proteins extracted and/or the release of DNA from damaged nuclei.

Another possible problem often noticed in the DNPH method is the way to remove excess or loosely bound DNPH from reaction mixtures or derivatized proteins. The protein 2,4-dinitrophenylhydrozones formed are usually washed repeatedly in combination with centrifugation to recover them as precipitates. A part of the derivatized proteins tends to be lost during this procedure because the amount of proteins used is usually small (i.e., around 1 mg); hence, the spectrophotometric measurement of final solutions of the precipitates can give a value lower than it should be. One way to avoid this problem is to remove the excess reagent by chromatography and measure the carbonyl content and the protein concentration spectrophotometrically, as described by Levine et al. (33). Ayene and Fisher pointed to a significant intra-assay variability of the original method and used gel filtration to remove excess and loosely bound reagents (40). Another way to avert the problem of possible loss of proteins during washing is to measure the amount of protein in the final precipitates dissolved in acidic 6 M guanidine hydrochloride (34) or neutral 8.5 M urea (41). The latter solvent is suitable for the subsequent analysis of the derivatized proteins in Western blot, particularly analysis involving two-dimensional gel electrophoresis (see below). It should be mentioned here that the carbonyl contents of rat liver proteins reported by Cao and Cutler appear too low (0.06 nmol/mg, compared to most frequently reported values of 1 to 4 nmol/mg for rat tissues) in their recommended method using streptomycin sulfate (39). The low carbonyl content may be partly attributed to loss of proteins during repeated washing.

Regarding the origin of carbonyls in tissue proteins, a possibility exists that glycosylated moieties of glycoproteins, or oxidatively modified forms of them, may contribute to a portion of the reactive carbonyls in these proteins, although this has been ruled out in recent papers (41,42). Most researchers working on protein carbonyls seem to postulate that carbonyls are generated primarily by direct oxidation of amino acid residues. It should be noted, however, that reactive carbonyls in proteins can also be formed by the reaction with aldehydes derived from lipid peroxides and by glycation (41,43-45). When evaluating protein carbonyls in mixtures of tissue proteins, the possibility should also be

taken into account that some proteins such as copper-containing amine oxidases, which have reactive carbonyl groups in covalently bound cofactors, contribute to the overall carbonyl content (46).

In most of the carbonyl determinations of tissue or serum proteins, measurements were made of protein mixtures, so little information has been obtained on the kind of proteins carbonylated. It is interesting and important to characterize the changing patterns of individual carbonylated proteins with age. To achieve this, immunoblot analyses of proteins, separated in one- or two-dimensional gel electrophoresis have been developed (41,47-49). Shacter et al. studied oxidative modification of rat plasma proteins and glutamine synthetase by DNPH-derivatization, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot using a monoclonal antibody against 2,4-dinitrophenyl group (47). They found that individual proteins are carbonylated to different extents by the iron/ascorbate method, fibrinogen being much more susceptible to oxidative modifications (more than 28 nmol/mg) than albumin (6.7 nmol/mg ) or transferrin (10 nmol/mg). Male rat liver soluble proteins gave multiple immunological signals with the most prominent being carbonic anhydrase III, and was detected only in young adults. The carbonyl content of this androgen-dependent enzyme (12 to 15 nmol/mg) did not show significant age-related changes in animals between 2 and 18 months old (48). It is noted that no other major difference in the signal intensities was seen between ages 2 and 24 months. This observation is not in agreement with the previous finding on the protein carbonyls of hepatocytes from young and old rats using spectrophotometric measurements (35).

We have recently developed an immunoblot analysis of tissue carbonylated proteins separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in which DNPH-derivatized proteins were dissolved in 8.5 M urea rather than using the conventional, 6 M guanidine chloride method before first-dimensional electrophoresis (41). Most important, this method should enable us to examine the extent of carbonylation of individual proteins. In fact, patterns of immunological signals are significantly different from those obtained from protein staining to detect individual proteins, as marked by arrows in Figure 1. Notably, the prominent signals seen in the kidney and brain are likely albumin and tubulins, respectively, as judged from their molecular weights and isoelectric points, as Figure 1 demonstrates. Such proteins can constitute significant portions of total carbonylated proteins. It may be possible therefore that the observed changes in protein carbonyl content shown in Table 1 are due to changes in specific proteins such as these. Thus, two-dimensional analysis can provide more specific information about the oxidative status of tissue proteins than the conventional spectrophotometric method. Another advantage of the immunoblot method is that one can avoid most of the complications associated with the spectrophotometric method, such as the effect of free DNPH and the possible contamination of DNA as discussed above.

*Proteolysis of Oxidized Proteins in Aging*

Half-lives of proteins are extended in older animals (50,51). Accumulation of intracellular oxidized proteins, if any, may be at least partly a result of the imbalance between the generation and degradation of such proteins, if damage is not repaired. Curiously, however, relatively little attention has been given to the degradation of altered proteins in aging, despite its potential importance in the accumulation of such proteins with age (51). Earlier, Lavie et al. demonstrated that puromycinyl peptides, as a model of altered proteins, degrade much more slowly in old mice (52). We have recently shown that oxidatively modified chicken lysozymes introduced into mouse hepatocytes in primary culture exhibit a shorter half-life than the unoxidized lysozymes. The half-life of oxidized lysozymes in the cells from old mice (22 to 29 months old) was 25 to 40% longer than that of their young (5 to 10 months old) counterparts (51, Nakano et al. in preparation). Thus, altered or oxidized proteins were shown to degrade less efficiently in cells of aged animals.

Different kinds of cellular proteases in in vitro experiments have been suggested to be responsible for the degradation of oxidatively or otherwise modified proteins in vivo (53-55). Recently, Grune et al. demonstrated that protein degradation at cultured cells exposed to oxidative stresses was largely abolished by prolonged treatment with an antisense oligodeoxyribonucleotide of an essential proteasome subunit, strongly suggesting an important role for ATP- and ubiquitin-independent 20S proteasome in the selective degradation of oxidatively modified proteins in cells (56,57). We have recently shown that a proteasome inhibitor, leucyl-leucyl-norvalinal, markedly inhibits degradation of oxidatively modified chicken lysozymes introduced into mouse hepatocytes in a primary culture from young donors. Remarkably, the inhibition was marginal in the cells from aged animals (Nakano et al., in preparation). We and others have shown that a so-called insulin degrading enzyme can also degrade oxidized proteins preferentially at least in vitro (55, Kurochkin and Goto, submitted). This enzyme has been recently implicated in the degradation of oxidized proteins in peroxisomes where oxidative damage is likely to occur (58, Imanaka et al. submitted). Thus, a possible involvement of proteases, other than proteasomes in the degradation of oxidized proteins in vivo, should also be taken into account.

It was reported that the activity of alkaline protease of rat hepatocytes for oxidatively modified proteins declines progressively with age, resulting in an 80% decrease between 2- and 24-months-of-age (35). The alkaline protease apparently was postulated as an enzyme(s) preferential to oxidatively modified proteins, though not verified relevant to the role of the enzyme(s) in aging in either the paper or subsequent publications

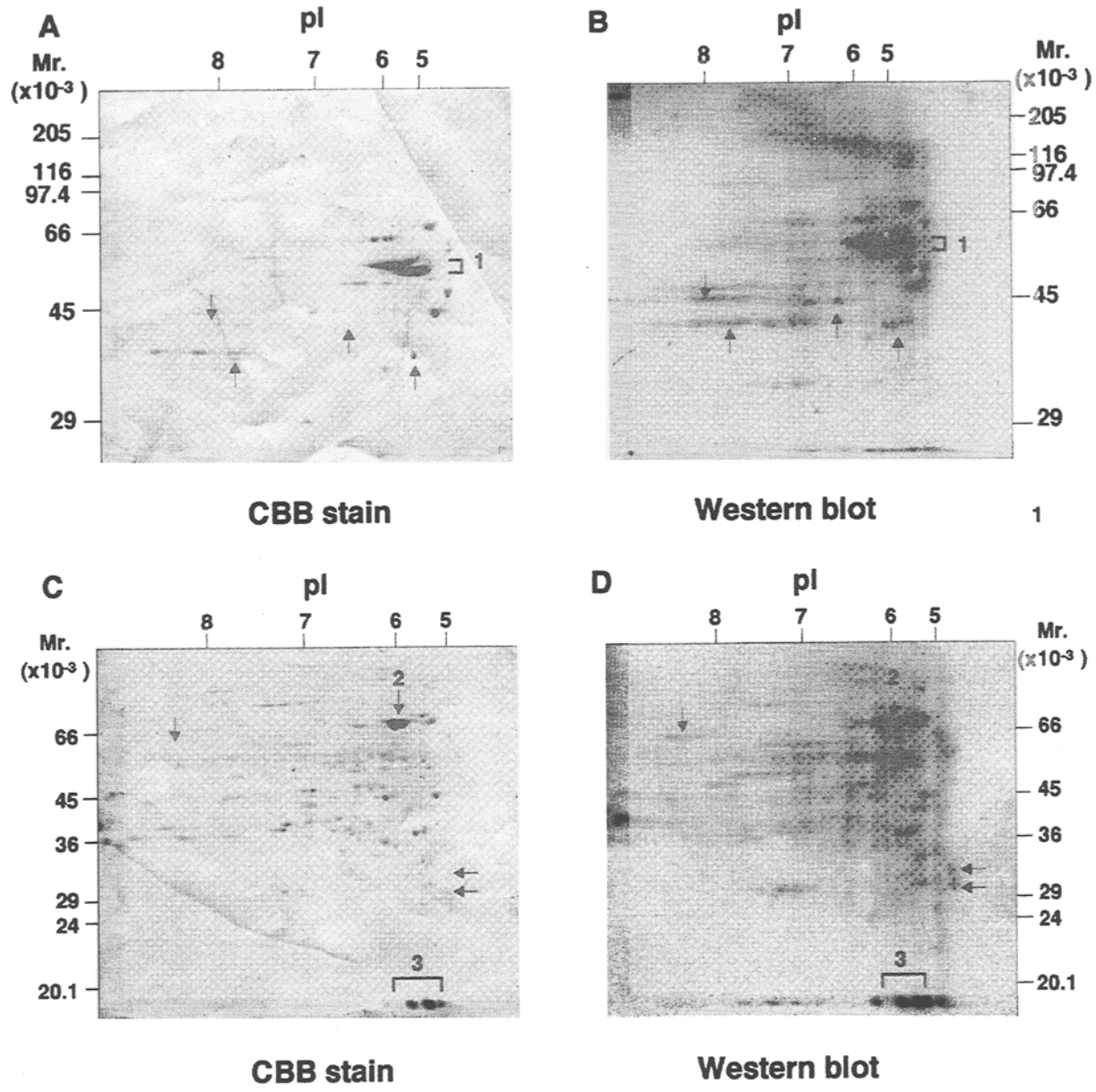


**Figure 1:** Two-dimensional immunoblot analysis of carbonylated proteins of rat brain and kidney
(A), (B): brain; (C), (D): kidney. The sheets were immunostained for the 2,4-dinitrophenyl group (B, D) and then stained with Coomassie brilliant blue (A,C) (41). Major signals were tentatively assigned as α,β-tubulins (1), albumin (2) and α2u-globulin, (3) from molecular weights, isoelectric points and relative abundance. Note that arrows indicate marked difference between the protein staining and immunological signals.

(59,60). The reliability of the assay for measuring the alkaline protease activity was challenged, and the reported age-related decline of the activity failed confirmation in a recent publication (61). It should also be pointed out that the description of the properties of oxidized liver soluble proteins used as substrates do not confirm the findings in the previous paper (35). In fact, Sahakian et al. were unable to confirm the age-related decline of alkaline protease activity of rat liver homogenates or hepatocyte extracts, using either oxidatively modified glutamine synthetase or oxidized liver homogenates as substrates (62). Nor did they find any substantial age-related difference in the activity of the purified liver multicatalytic protease, which is likely involved in the degradation of oxidatively modified proteins. The same group of investigators recently reported that the liver multicatalytic proteinase purified from old rats exhibits approximately 50% of the peptidylglutamyl peptide hydrolase activity that their young conterparts do, but without a reduction in other peptide hydrolase activities (63).

These findings, however, do not necessarily mean that the total activity of the enzyme or the specific activities in the homogenates decline with age, because the activity of the enzyme in the original supernatant used for the purification was even greater in the older animals. Another complication in evaluating the results is low recovery of the enzyme (i.e., about 1 to 2%, as calculated from the data). Shibatani and Ward reported similar age-related changes of peptide hydrolyzing acitivities in proteasomes using high-speed supernatants of rat livers (64). Agarwal and Sohal (65) showed that total alkaline protease activity with X-ray-oxidized BSA as a substrate declines linearly with age by about 50% in rat livers between 3 and 23 months old, essentially confirming the previous results (35), and 20% in hearts between 13 and 23 months old, but almost no decline in the brain. Thus, results reported so far prohibit us from drawing definitive conclusions on age-related change of proteases activity that is likely involved in the degradation of oxidatively modified proteins.

To avoid a possible bias due to extensive purification or complications by use of homogenates, which should contain various proteases, we have examined the proteasome activity of rat brains by centrifuging the crude extracts on glycerol gradients to separate 20S and 26S proteasomes from other proteolytic activities by virtue of their much heavier molecular weights than the rest of the cellular proteases. Protease activities evaluated using three kinds of fluorogenic peptide as substrates were lower in the cerebral cortex of older animals but higher in the brain stem (Makita et al., in preparation). Thus, the age-related change of proteasome activities is not uniform for different substrates and tissues. Further experiments are required to establish a possible relation between protease activities and accumulation of oxidatively modified proteins.

*Future Perspectives*

In spite of increasing interest and progress in the research of oxidative modifications of proteins in aging, some major points remain to be established. First, although considerable efforts have been made to clarify the causal relation between protein oxidation and functional deterioration of cells and tissues with age, evidence supporting this idea is just now emerging (60,66). Carney et al. showed that the increase in protein carbonyl content in the brain cortex and memory loss in aged gerbils occur in parallel and that these changes are reversed by administration of the spin-trapping compound, N-*tert*-butyl-alfa-phenylnitrone (PBN) (60). So far, to our knowledge, two reports attempt to confirm this important effect of PBN; one provides corroborative results in the gerbil brain but not in the gerbil heart or the mouse brain cortex (67), and the other report failed to do so in rat livers (39). Foster et al. found that age-related changes in learning/memory and walking skills are linearly related with protein carbonyl contents in the corresponding brain regions (i.e., the higher the carbonyl content, the lower the success score) (66). These exciting findings are expected to be confirmed by other investigators. Also, it will be interesting to identify oxidatively damaged proteins in the brain that are responsible for the decline of these functions using Western blot for carbonylated proteins on 2-D PAGE, followed by amino acid sequencing.

Second, so far, only limited information is available on the chemical entities of oxidized groups, particularly carbonyls of tissue proteins. Moreover, the available data are mostly those for proteins modified in vitro by metal-catalyzed oxidation or radiation (68-70). In view of the findings that carbonyls can also be introduce into proteins via reaction with aldehydes or glycation, in addition to possible direct oxidation of amino acid residues, structures of carbonyls in proteins obtained from tissues also should be identified to gain better insight into the possible implications of protein oxidation in aging.

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