Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats

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Leeuwenburgh, Christiaan, Polly Hansen, Aviv Shaish, John O. Holloszy, and Jay W. Heinecke. Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R453–R461, 1998.—Many lines of evidence implicate oxidative damage in aging. Possible pathways include reactions that modify aromatic amino acid residues on proteins. α-Tyrosine is a stable marker for oxidation of protein-bound phenylalanine by hydroxyl radical, whereas 3-nitrotyrosine is a marker for oxidation of protein-bound tyrosine by reactive nitrogen species. To test the hypothesis that proteins damaged by hydroxyl radical and reactive nitrogen accumulate with aging, we used isotope dilution gas chromatography-mass spectrometry to measure levels of α-tirosine and 3-nitrotyrosine in heart, skeletal muscle, and liver from young adult (9 mo) and old (24 mo) female Long-Evans/Wistar hybrid rats. We also measured these markers in young adult and old rats that received antioxidant supplements (α-tocopherol, β-carotene, butylated hydroxytoluene, and ascorbic acid) from the age of 5 mo. We found that aging did not significantly increase levels of protein-bound α-tirosine or 3-nitrotyrosine in any of the tissues. Antioxidant supplementation had no effect on the levels of protein-bound α-tirosine and 3-nitrotyrosine in either young or old animals. These observations indicate that the α-tirosine and 3-nitrotyrosine do not increase significantly in heart, skeletal muscle, and liver in old rats, suggesting that proteins damaged by hydroxyl radical and reactive nitrogen species do not accumulate in these tissues with advancing age.

A WEAULT OF INDIRECT EVIDENCE implicate oxidative damage of cellular constituents in aging, as well as in the pathogenesis of the degenerative diseases of later years (1, 4, 16, 23, 27, 32, 33, 35, 38). Overexpression of superoxide dismutase and catalase, two enzymes that scavenge the reactive intermediates superoxide and hydrogen peroxide, respectively, increases the average and maximum life span of Drosophila (28). Moreover, caloric restriction prolongs the life span of rodents and invertebrates. In many of the studies on caloric restriction, the level of antioxidant defense mechanisms was increased and the level of oxidative damage was reduced (reviewed in Refs. 9 and 25). Collectively, these results suggest that reactive intermediates derived from oxidative metabolism may play a causal role in the aging process.

Reactive oxygen intermediates are potentially damaging to nucleic acids, lipids, and proteins (1, 4, 8, 9, 13, 16, 25, 27, 28, 32, 34). Several different metal-catalyzed oxidation systems convert certain amino acid residues to carbonyl derivatives (32). Protein-bound carbonyls are present at low levels in dermal fibroblasts isolated from young to middle-aged donors, but they are increased twofold in fibroblasts from people over the age of 60 years (27). Moreover, fibroblasts obtained from patients with diseases of accelerated aging have dramatically higher levels of protein carbonyls (27). These results suggest that levels of oxidized proteins may increase with age, and that metal-catalyzed oxidation reactions may be partly responsible for protein oxidation. Other factors, such as less efficient removal of oxidized proteins through proteolytic cleavage, may also promote the accumulation of protein carbonyls with aging (32, 34).

The mechanisms for oxidative cellular damage during aging poorly understood because the toxic intermediates are short-lived and difficult to detect directly. An alternative approach is to monitor stable end products of oxidative reactions. Potential oxidants are hydroxyl radical and species of similar reactivity such as the ferryl complex, which are generated by metal-catalyzed oxidation systems (32), glucose autoxidation (4), and mitochondrial aerobic metabolism (1). Hydroxyl radical converts phenylalanine to α-tirosine (Fig. 1; see Refs. 13–15). Another potential mechanism for protein oxidation involves nitric oxide, a long-lived radical that plays a critical role in cellular signaling and cytotoxic host defense mechanisms (26). Reactive nitrogen species derived from nitric oxide generate 3-nitrotyrosine in vitro (Fig. 1; Refs. 5 and 17).

Because α-tirosine and 3-nitrotyrosine are stable to acid hydrolysis and not normally present in proteins, they may serve as useful markers for oxidative damage. To test the hypothesis that proteins damaged by hydroxyl radical and reactive nitrogen species accumulate in tissues with age, we developed assays for oxidation products generated by each of these reaction pathways, using isotope dilution gas chromatography-mass spectrometry (GC/MS). In the present study, we quantified levels of α-tirosine and 3-nitrotyrosine in tissues of young adult and old rats and also assessed the effects of antioxidant therapy on protein oxidation.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise indicated, reagents were obtained from either Sigma Chemical (St. Louis, MO) or Aldrich Chemical (Milwaukee, WI). All organic solvents were high-performance liquid chromatography (HPLC) grade. Cambridge Isotope Laboratories (Andover, MA) supplied 13C-labeled amino acids for the preparation of internal standards. Isotopically labeled α-tirosine was synthesized using [13C6]-phenyl-
Protein isolation from animal tissues. Liver and muscle proteins used for in vitro oxidation experiments were isolated at 4°C. Freshly prepared rat tissue (100 mg wet wt) was homogenized in 10 ml of buffer A (0.1 mM DTPA, pH 7.4), freeze-thawed once, and centrifuged at 10,000 g for 10 min. The supernatant containing soluble tissue protein was dialyzed against H<sub>2</sub>O that was passed over a Chelex resin (Bio-Rad) column to remove free metal ions.

Protein oxidation by hydroxyl radical and peroxynitrite. Reactions (0.5 mg protein/ml) were performed at 37°C in buffer B (50 mM sodium phosphate, pH 7.4). Hydroxyl radical was generated using buffer B supplemented with 0.2 mM CuSO<sub>4</sub> and the indicated final concentration of H<sub>2</sub>O<sub>2</sub>. To inhibit residual catalase activity, 3-amino-1,2,4-triazole (0.1 mM) was included in reaction mixtures containing tissue protein. Reactions were terminated after 2 h by addition of 0.2 mM DTPA (pH 7.4), 300 mM catalase, and 0.1 mM butylated hydroxytoluene. Proteins were precipitated with ice-cold trichloroacetic acid (10% final concentration), acid hydrolyzed, and subjected to GC/MS analysis as described below.

Peroxynitrite was synthesized from 2-ethoxyethyl nitrite and H<sub>2</sub>O<sub>2</sub> (22) and stored at −80°C. Peroxynitrite was thawed immediately before use, and its concentration was determined spectrophotometrically (ε<sub>302</sub> = 1,670 M<sup>−1</sup>·cm<sup>−1</sup>; see Refs. 5 and 22). Reactions were initiated by addition of peroxynitrite and then terminated after a 5-min incubation at 37°C by acid precipitation of protein as described above. To determine whether peroxynitrite or reactive species derived from peroxynitrite were generating 3-nitrotyrosine, protein was added to the reaction mixture 2 min after addition of peroxynitrite. Decomposition of peroxynitrite under these conditions was confirmed by monitoring the change in absorbance at 302 nm of the reaction mixture.

Quantification of tissue vitamin levels. Liver α-tocopherol, β-carotene, and retinyl palmitate levels were determined using a C<sub>18</sub> reverse phase column and HPLC analysis (30). Results are expressed as nanomoles antioxidant per gram tissue (wet weight).

Determination of enzyme activity. Skeletal muscle homogenates (10%, wt/vol) were prepared in ice-cold 10 mM K<sub>2</sub>PO<sub>4</sub> (pH 7.2) and 2 mM EDTA using a glass Potter-Elvehjem homogenizer immersed in ice water. Enzyme activities were determined as previously described (3, 12, 20) under conditions where activity was a linear function of protein concentration.

Isolation of amino acids from tissue. Tissue was frozen in liquid N<sub>2</sub> and then pulverized under liquid N<sub>2</sub> using a stainless steel mortar and pestle. All subsequent procedures were carried out at 4°C. The tissue powder was suspended in buffer A and dialyzed for 2 h against H<sub>2</sub>O. Samples (~5 mg wet wt) were deproteinized by extraction with methanol (3 ml) and water-washed diethyl ether (10 ml) for 10 min. Protein precipitate was recovered by centrifugation at 500 g for 10 min. The resulting protein pellet was extracted once with water-washed diethyl ether and dried under N<sub>2</sub>. Samples (~1 mg protein) were dried under vacuum and immediately suspended in 0.5 ml 0.1 N HCl (Sequantial Grade, Pierce Chemical, Rockford, IL) containing 1% benzoic acid and 1% phenol (wt/vol). Isotopically labeled internal standards were added, and samples were hydrolyzed at 110°C for 24 h under N<sub>2</sub>.

Amino acids were isolated using a solid-phase C<sub>18</sub> column (3 ml; Supelclean SPE, Supelco, Bellefonte, PA). The column was conditioned with 2 ml of methanol, 6 ml of 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) containing 0.1 mM DTPA, and finally with 6 ml of 0.1% trifluoroacetic acid. Amino acid hydrolysate (~0.5 g).

### Table 1. Composition of antioxidant diet

<table>
<thead>
<tr>
<th>Component, g/kg diet</th>
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<tbody>
<tr>
<td>Constant Formula Rodent Diet</td>
<td>992</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5.0</td>
</tr>
<tr>
<td>Racemic α-tocopherol</td>
<td>2.0</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>0.50</td>
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<tr>
<td>β-Carotene</td>
<td>0.15</td>
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ml) supplemented with 10% trichloroacetic acid (vol/vol) was passed over the column. The column was washed with 6 ml of 0.1% trifluoroacetic acid. Amino acids were eluted with 2 ml of 25% methanol and dried under vacuum for derivatization. Preliminary studies using authentic standards demonstrated that >80% of amino acid oxidation products were recovered from the C18 column using this procedure.

Derivatization of amino acids. Amino acids were converted to N-propyl esters by addition of 200 µl HCl/n-propanol (1:3, vol/vol) and heating for 1 h at 65°C. After evaporation of excess reagent under N₂, heptafluorobutyryl anhydride/ethyl acetate (1:3, vol/vol) was added, and the samples were heated at 65°C for 15 min.

Mass spectrometric analysis. Amino acids were quantified using isotope dilution negative-ion chemical ionization GC/MS. Samples were dried under N₂, redissolved in 50 µl ethyl acetate, and 1 µl aliquots were then analyzed on a Hewlett-Packard 5890 Gas Chromatograph equipped with a 12-m DB-1 capillary column (0.20 mm ID, 0.33-µm film thickness, J & W Scientific) interfaced with a Hewlett-Packard 5988A Mass Spectrometer with extended mass range (11, 19, 21). Both the injection and detector temperature of the gas chromatograph were set at 250°C. Full scan mass spectra and selected ion monitoring were obtained with the J & W Scientific mass spectrometer interfaced with a Hewlett-Packard 5988A. The initial column temperature of 70°C was passed over the column. The column was washed with 6 ml of 1:100 split prior to mass analysis. The initial column temperature of 70°C was increased to 180°C at 60°C/min and then raised to 205°C at 4°C/min.

Mass spectrometric analysis. Amino acids were quantified using isotope dilution negative-ion chemical ionization GC/MS. Samples were dried under N₂, redissolved in 50 µl ethyl acetate, and 1 µl aliquots were then analyzed on a Hewlett-Packard 5890 Gas Chromatograph equipped with a 12-m DB-1 capillary column (0.20 mm ID, 0.33-µm film thickness, J & W Scientific) interfaced with a Hewlett-Packard 5988A Mass Spectrometer with extended mass range (11, 19, 21). Both the injection and detector temperature of the gas chromatograph were set at 250°C. Full scan mass spectra and selected ion monitoring were obtained with the mass spectrometer interfaced with a Hewlett-Packard 5988A Mass Spectrometer with extended mass range (11, 19, 21).

Preliminary studies using authentic standards demonstrated that >80% of amino acid oxidation products were recovered from the C18 column using this procedure. Quantification of oxidized amino acids. Quantification was based on an external calibration curve using each amino acid as a standard and the corresponding isotopically labeled amino acid as internal standard. The ratio of ion currents for each amino acid divided by that of the internal standard was a linear function of unlabeled amino acid for all ranges over which the amino acids were measured. Internal standards were present at a final ratio of 1–10 mol/mol relative to the naturally occurring amino acids. The limit of detection (signal/noise > 10) was < 1 pmol for all of the amino acids.

Statistical analysis. Results are presented as means ± SE. Differences between two groups were compared using an unpaired Student’s t-test. Multiple comparisons were performed using a two-way analysis of variance. A P value < 0.05 was considered significant.

RESULTS

Protein oxidation in vitro with hydroxyl radical and peroxynitrite. To evaluate the potential usefulness of o-tyrosine and 3-nitrotyrosine as markers for oxidation by hydroxyl radical and reactive nitrogen species in vitro, we investigated the product yield of these markers in proteins that had been oxidized in vitro. Bovine serum albumin (BSA) and soluble proteins isolated from muscle and liver were exposed to a hydroxyl radical-generating system (copper plus H₂O₂) or peroxynitrite. The levels of o-tyrosine and 3-nitrotyrosine in amino acid hydrolysates of the proteins were then determined using isotope dilution GC/MS analysis.

O-Tyrosine accumulated in all three types of proteins incubated with the hydroxyl radical-generating system (Fig. 2). The increase in level of o-tyrosine was dependent on the concentration of H₂O₂ in the reaction mixture (Fig. 2). Copper alone had little influence on o-tyrosine formation. The relative yield of o-tyrosine varied with the different types of protein; it was highest in BSA and lowest in muscle protein. The lower yield of o-tyrosine in muscle and liver may reflect differences in metal ion binding or tissue antioxidant defenses against oxidative damage. Since the muscle and liver proteins were extensively dialyzed prior to oxidation, it is unlikely that enzymes requiring low-molecular-weight cofactors, such as glutathione peroxidase, were affecting the level of oxidation. In contrast, enzymes such as superoxide dismutase may have retained activity and influenced the absolute level of protein oxidation.

Amino acid analysis of BSA exposed to the copper-H₂O₂ system demonstrated major losses in the content of histidine, tyrosine, and phenylalanine. Proline and glycine were partially destroyed by hydroxyl radical (Table 2). There was no significant change in the levels of the other amino acids measured. It is important to note that o-tyrosine formation accounted for only a small percentage of the loss of phenylalanine in BSA exposed to copper and H₂O₂. Thus o-tyrosine is an excellent marker for protein damage in vitro, but it is not the major product of phenylalanine oxidation by hydroxyl radical. Collectively, these observations indi-
cate that o-tyrosine should be useful as a marker for 
hydroxyl radical-mediated damage in vivo.

BSA, skeletal muscle, and liver contained very low 
levels of protein-bound 3-nitrotyrosine. There was a 
dramatic concentration-dependent increase in 3-nitro-
tyrosine content after peroxynitrite exposure (Fig. 3).
The product yield of 3-nitrotyrosine was greater in liver 
than in muscle or BSA. When BSA was added 2 min 
after addition of peroxynitrite, there was minimal 
nitration of the protein, indicating that peroxynitrite or 
a short-lived species derived from peroxynitrite medi-
ates nitration of the aromatic ring of protein-bound 
tyrosine. Because peroxynitrite also hydroxylates 
aromatic compounds (5, 19), we determined whether 
o-tyrosine was formed in proteins exposed to peroxyni-
trite. The product yield of o-tyrosine in BSA exposed to 
peroxynitrite was <5% that of 3-nitrotyrosine. In vitro 
studies of model proteins have demonstrated that 
3-nitrotyrosine is a specific marker for protein oxida-
tion by reactive nitrogen species (17, 19). Taken to-
gether, these results indicate that 3-nitrotyrosine should 
serve as an excellent marker for proteins oxidized by 
reactive nitrogen in vivo.

Antioxidant intervention in animals. Female Long-
Evans/Wistar hybrid rats were randomly assigned to 
either the control or antioxidant diet at 5 mo of age, and 
the diets were continued until the animals were killed 
at 9 and 24 mo of age. Antioxidant-treated animals 
received the control diet supplemented with both a 
water-soluble vitamin (ascorbic acid) and lipid-soluble 
vitamins (α-tocopherol, β-carotene) and a synthetic 
lipid-soluble antioxidant (butylated hydroxytoluene) 
(Table 1). Approximately one-half of the rats fed the two 
different diets had died by 24 mo of age, indicating that 
the animals were reaching the end of their life spans. 
There were no significant differences in the body weights 
of either the young rats (9 mo of age; control diet, 350 ± 
14 g; antioxidant diet, 353 ± 14 g) or the old rats (24 mo 
of age; control diet, 433 ± 28 g; antioxidant diet, 416 ± 
25 g) fed the two different diets. The treatment groups 
did not differ significantly with respect to heart and 
quadriceps weight (data not shown).

Liver levels of the antioxidants. To determine the 
effectiveness of the antioxidant regimen at increasing 
tissue levels of the lipid-soluble antioxidants, we mea-
ured the α-tocopherol, β-carotene, and retinyl ester 
concentrations in the livers of the animals. There were
significant increases in the levels of all these antioxidants in the livers of 24-mo-old rats fed the antioxidant-supplemented diet. α-Tocopherol levels increased fivefold (74 nmol/g; \( P < 0.05 \)) above control levels. Moreover, β-carotene, which was undetectable in the control animals, increased to a high level (2.2 nmol/g) with antioxidant feeding. We also measured retinyl palmitate levels because the rat intestinal epithelium rapidly cleaves β-carotene to retinoic acid, retinal, and other products. There was a significant twofold increase in liver retinyl palmitate content in the animals fed β-carotene and the other antioxidants (1,450 nmol/g; \( P < 0.05 \)).

In a parallel longevity study, there was a twofold increase in the plasma level of ascorbic acid in rats fed the antioxidant diet (J. O. Holloszy, unpublished observations). These results indicate that the antioxidant supplementation used in this study effectively increased liver levels of α-tocopherol, β-carotene, and retinyl palmitate, and plasma levels of ascorbic acid.

Mass spectrometric analysis of tissue oxidation products. When amino acids from acid hydrolysates of rat liver, heart, and skeletal muscle were isolated and derivatized with heptafluorobutyric anhydride and then analyzed by GC/MS in the negative-ion chemical ionization mode, we detected compounds that exhibited major ions and retention times identical to those of α-tyrosine and 3-nitrotyrosine. Selected ion monitoring demonstrated that the ions derived from the amino acids coeluted with ions derived from authentic \(^{13}\)C-labeled internal standards. Identity of the compounds was confirmed by comparison with authentic standards using both heptafluorobutyryl and pentfluoropropionyl derivatives of each oxidized amino acid (11, 19, 21). These results indicate that acid hydrolysates of these tissue proteins contain detectable levels of oxidized amino acids.

Protein oxidation in heart. The levels of α-tyrosine and 3-nitrotyrosine in heart muscle did not increase significantly with age (Fig. 4, A and B). Antioxidant supplementation resulted in no significant change in the levels of either α-tyrosine or 3-nitrotyrosine in cardiac tissue (Fig. 4, A and B). These results suggest that aging does not have major effects on the levels of protein-bound α-tyrosine and 3-nitrotyrosine in the heart. Moreover, dietary antioxidant supplementation did not alter the level of protein oxidation.

Protein oxidation in skeletal muscle. As observed with cardiac tissue, there were no alterations in levels of protein-bound α-tyrosine and 3-nitrotyrosine (Fig. 5, A and B) in skeletal muscle as a result of aging. In addition, antioxidant supplementation had no significant effect on skeletal muscle levels of α-tyrosine and 3-nitrotyrosine in both the young and old rats (Fig. 5, A and B).

Protein oxidation in liver. Neither dietary supplementation with antioxidants nor aging had a significant effect on the levels of protein-bound α-tyrosine in liver (Fig. 6, A and B). Control and antioxidant-supplemented old rats tended to have higher levels of 3-nitrotyrosine in the liver than did the young rats, but this difference was not statistically significant (Fig. 6B; \( P = 0.12 \)). Antioxidant supplementation had no effect on 3-nitrotyrosine levels in either group of rats.

Antioxidant and metabolic enzyme activities in skeletal muscle. Activities of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), the mitochondrial enzyme citrate synthase, and the glycolytic enzyme lactate dehydrogenase were determined in skeletal muscle of 9-mo- and 24-mo-old rats.
fed the control or antioxidant-supplemented diet. Antioxidant supplementation did not alter the activities of any of the enzymes significantly. There were also no differences in enzyme activities found between the
young and the old rats, except for glutathione peroxidase, which exhibited a small decrease of 8% in the old animals. This finding is consistent with previous observations that catalytically inactive enzymes fail to accumulate in this tissue with aging (18).

DISCUSSION

The role of oxidative stress in aging has been extensively investigated; however, the molecular basis for protein oxidation has not been established. In the current studies, we have focused on hydroxyl radical and reactive nitrogen species as potential pathways for oxidative damage. We selected o-tyrosine and 3-nitrotyrosine as markers for oxidation by these pathways because they are unnatural amino acids and therefore should represent posttranslational modifications of proteins. The results of our study using isotope dilution GC/MS to determine tissue levels of o-tyrosine and 3-nitrotyrosine suggest that proteins oxidized by hydroxyl radical and reactive nitrogen species do not accumulate in cardiac muscle, skeletal muscle, and liver of aging rats. Thus other pathways are likely to account for any age-related increase in protein oxidation in these tissues. It is important to note that our markers of oxidation might fail to increase in proteins damaged in vivo by other reactive intermediates.

A second important observation is that dietary antioxidant supplementation with a combination of a water-soluble antioxidant (ascorbic acid) and lipid-soluble antioxidants (α-tocopherol, butylated hydroxytoluene, and β-carotene) failed to significantly affect levels of o-tyrosine and 3-nitrotyrosine in all tissues examined. These results demonstrate that antioxidant intervention with this combination of agents was unable to reduce the levels of o-tyrosine and 3-nitrotyrosine, at least in liver, skeletal muscle, and heart.

A key question is the specificity of the oxidized amino acids we used as markers for in vivo damage mediated by hydroxyl radical or reactive nitrogen species. In vitro studies using metal-catalyzed oxidation systems (Refs. 13, 15, 21; Fig. 2), photooxidation (14), and ionizing radiation (13) indicate that o-tyrosine levels increase dramatically in proteins oxidized by hydroxyl radical. The failure to demonstrate an increase in o-tyrosine thus argues against the notion that the steady-state level of hydroxyl radical-mediated protein oxidation increases during aging.

Metal ions bind to phenylalanine and tyrosine with high affinity via p-cation interactions (7), and these amino acids are major targets for oxidation by hydroxyl radical in proteins (Ref. 13 and Table 2), suggesting that redox-active free metal ions are likely to interact with aromatic amino acids in vivo. We observed large increases in o-tyrosine in BSA, as well as muscle and liver proteins exposed to copper-H₂O₂ in vitro. Moreover, protein oxidation by hydroxyl radical generated by ionizing radiation results in large increases of o-tyrosine (13). These observations further support the notion that o-tyrosine is an excellent marker for oxidation of proteins by hydroxyl radical in vivo.

Nitric oxide, a long-lived radical generated by many cell types (26), may represent another pathway for protein oxidation in vivo. Nitric oxide reacts with molecular oxygen to generate poorly characterized reactive nitrogen intermediates that nitrate tyrosine in
Protein oxidation, antioxidants, and aging

Indirect evidence suggests that oxidized proteins accumulate with aging in tissues (1, 4, 16, 23, 27, 32, 33, 38). It should be noted that most of these studies used nonspecific methods to quantify protein oxidation and that the chemical nature of the oxidation products is uncertain. In the only previous study that used mass spectrometry to monitor specific amino acid oxidation, the chemical nature of the oxidation products was nonspecific methods to quantify protein oxidation (33, 38). It should be noted that most of these studies accumulate with aging in tissues (1, 4, 16, 23, 27, 32, 33, 38). It should be noted that most of these studies did not accumulate with aging in these tissues. Interventions using ascorbic acid, α-tocopherol, butylated hydroxytoluene, and β-carotene had no significant effect on the level of o-tyrosoine and 3-nitrotyrosoine in these tissues. This observation suggests that dietary supplementation with a combination of water and lipid-soluble antioxidants had little effect on the rate of formation and/or degradation of proteins oxidized by hydroxyl radical and reactive nitrogen species vivo, as monitored by the levels of o-tyrosoine and 3-nitrotyrosoine.

Perspectives

Oxidative modification alters the function of proteins and is thought to play an important role in the decline of cellular function during aging. However, the pathways that promote protein oxidation in the body are poorly understood, in part because it is very difficult to detect the highly reactive intermediates that induce protein oxidation. Hydroxyl radical and reactive nitrogen species are potent oxidants that damage proteins in the test tube, forming compounds that are stable and thus easy to measure. In this study, very specific and sensitive analytic methods were used to measure the levels of certain of these "fingerprints" of oxidative damage in tissue proteins of aging rats. We found that there was very little change in the levels of the compounds with age. This was surprising because previous studies using nonspecific measures had suggested that proteins damaged by hydroxyl radical and reactive nitrogen accumulate in the tissues of old animals. Our results suggest that aging rats show very little change in the level of proteins oxidized by hydroxyl radical and reactive nitrogen. It should be noted that we measured only a limited number of markers of oxidation and that other compounds and proteins oxidized by different pathways might accumulate with aging. It is also important to mention that certain specific proteins might undergo a much larger increase in oxidative damage. Because we measured the average level of protein oxidation in tissue, we might have missed detecting a marked increase in protein oxidation in a few selected proteins.

We thank Drs. L. Sage, S. Hazen, and J. Turk for critical reading of the manuscript and N. Holmberg, M. Chen, S. Simcox, D. Van Hoornbeek, D. Mueller, L. Frazier, and M. Kennedy for technical assistance.

Gas chromatography-mass spectrometry experiments were performed at Washington University School of Medicine Mass Spectrometry Resource (RR00954). This research was supported by National Institute on Aging research Grants AG-00425G, AG-15013, and AG-12293, and Claude D. Pepper OAIc Grant AG-13629. C. Leeuwenburgh is a recipient of the National Service Research Award from the National Institute on Aging. J. W. Heinecke is an Established Investigator of the American Heart Association.

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Received 26 June 1997; accepted in final form 17 October 1997.

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