Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo

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Leeuwenburgh, Christiaan, Polly A. Hansen, John O. Holloszy, and Jay W. Heinecke. Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R128–R135, 1999.—Oxidative damage of proteins has been implicated in disease and aging. In vitro studies demonstrate that two unnatural amino acids, o,o'-dityrosine and o-tyrosine, are stable markers of protein oxidation. We have investigated the possibility that assaying these compounds in urine could provide a noninvasive way to determine levels of protein oxidation in vivo. Isotope dilution gas chromatography-mass spectrometry was used to quantify levels of o,o'-dityrosine and o-tyrosine in skeletal muscle and urine of aging rats subjected to two interventions: 1) dietary antioxidant supplementation and 2) exercise training. In both sedentary rats and exercise-trained rats, antioxidant therapy reduced levels of protein-bound o,o'-dityrosine in skeletal muscle. In contrast, antioxidant therapy or exercise training minimally affected o-tyrosine levels in this tissue. Levels of the oxidized amino acids in urine samples mirrored those of skeletal muscle proteins. Quantification of the levels of oxidized amino acids in urine may thus serve as a noninvasive measure of oxidative stress in vivo because they change in parallel with levels of protein-bound oxidized amino acids in skeletal muscle.

o,o'-dityrosine; ortho-tyrosine; antioxidants; exercise; protein oxidation

OXIDATIVE DAMAGE OF proteins, lipids, and nucleic acids has been implicated in diseases ranging from atherosclerosis to ischemia-reperfusion injury to cancer (1–3, 16, 32). Many lines of evidence also suggest that such damage plays a causal role in aging (1–3, 23, 28, 33, 34). One important target may be proteins (3, 33), which play fundamental roles as biological catalysts, gene regulators, and structural components of cells. One widely studied model of protein oxidation involves metal-catalyzed reactions that generate hydroxyl radical and other reactive species. These oxidants generate reactive carbonyls from certain amino acid residues (3, 23, 33). The discovery of elevated levels of protein carbonyls in many pathological states (3) and in tissues of old animals (3, 28, 33, 34) has implicated protein oxidation in the pathogenesis of disease and aging.

A major difficulty in evaluating the roles of oxidants in human disease has been the lack of precise measures of oxidative stress in vivo (7). Many of the currently available methods are nonspecific and prone to artifacts. A powerful approach to studying oxidative damage in vivo is the analysis of normal and diseased tissue for specific markers (9, 21, 26, 30, 31). Such markers have been identified as stable products of protein oxidation through in vitro studies. For example, the unnatural isomer o-tyrosine forms when hydroxyl radical oxidizes protein-bound phenylalanine residues (14, 15, 21). o,o'-Dityrosine appears when hydroxyl radical cross-links tyrosine residues (3, 5, 14, 21). A metal-catalyzed oxidation system therefore might produce both compounds in vivo. In contrast, only o,o'-dityrosine is generated when free or protein-bound tyrosine reacts with tyrosyl radical (11, 21), a reactive species that myeloperoxidase and other heme enzymes produce from H2O2 and tyrosine (10, 11). These findings suggest that the hydroxyl radical pathway might be distinguished from the tyrosyl radical pathway by determining the distribution of the two oxidized amino acids in tissue proteins (21). Because o,o'-dityrosine and o-tyrosine are stable to acid hydrolysis, they are potentially useful markers for protein oxidation in vivo (19, 21, 22, 30, 31).

Oxidized proteins have increased susceptibility to proteolytic degradation, resulting in the release of free oxidized amino acids (3, 29). Most of these products are modified compounds that cells are unlikely to use and that might be excreted in urine. To explore this possibility, we have used a highly sensitive and specific method [gas chromatography together with mass spectrometry (GC-MS)] to quantify levels of o,o'-dityrosine and o-tyrosine in rat skeletal muscle and urine. Skeletal muscle was studied because it represents one of the largest tissues in the rat and is affected by exercise training. We found that both antioxidant therapy and adaptation to exercise lowered o,o'-dityrosine levels in skeletal muscle and urine of aging rats. These results suggest that analyzing urine for oxidized amino acids might provide a noninvasive way to assess oxidative stress in vivo.

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 HPLC grade. Cambridge Isotope Laboratories (Andover, MA) supplied 13C-labeled tyrosine and phenylalanine for the preparation of o-[13C6]tyrosine and o,o'-[13C6]dityrosine, respectively (10, 14). Concentration of 13C-labeled amino acids was determined by HPLC analysis (8).
Methods

Animals. Specific pathogen-free, female, colony-bred Long-Evans and/or Wistar hybrid rats were individually housed in stainless steel cages measuring $18 \times 36 \times 20$ cm in a temperature (18–22°C)- and light-controlled room with a 12:12-h light-dark cycle. Rats were maintained under pathogen-free conditions, provided with water ad libitum, and fed constant formula rodent diet containing 23% protein, 4.5% fat, 5.8% crude fiber, 7.3% ash, 4.5 ppm β-carotene, 15 IU/kg vitamin A, and 40 IU/kg vitamin E (laboratory rodent diet 5001; Harlan-Teklad, Madison, WI).

At 5 mo of age animals were randomly assigned either to continue the control diet or to begin the antioxidant-supplemented diet, which contained 0.5% ascorbic acid, 0.2% racemic α-tocopherol, 0.05% butylated hydroxytoluene, and 0.015% β-carotene. One-half of the rats in each group were assigned to sedentary or exercise subgroups. Rats in the exercise group were housed in cages equipped with stainless steel running wheels (11.2 cm circumference) and a revolution counter that monitored the number of miles run. Rats were studied when they were 24 mo of age. The Washington University School of Medicine animal studies committee approved all procedures.

Urine collection. Animals that had fasted from 6 PM were placed in stainless steel metabolic cages designed for urine collection; all subsequent procedures were performed under subdued light. Urine was collected from 12 AM until 9 PM in amber-colored glass vials containing 50 µl of 6% (wt/vol) phenol (an antioxidant and bactericidal agent) and 6 mM diethylenetriaminepentaacetic acid (DTPA, a metal chelator) and then stored at −80°C until analysis. Urine creatinine levels were measured using Sigma diagnostics kit 555-A.

Tissue collection. Animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt) injected intraperitoneally. The plantaris muscle, gastrocnemius, extensor digitorum, and the deep portion of the vastus lateralis muscle were quickly dissected out and rinsed with ice-cold saline (140 mM NaCl) to remove excess blood. Tissues were immediately placed in ice-cold antioxidant buffer [50 mM NaH2PO4, pH 7.4, 100 µM DTPA, 1 mM butylated hydroxytoluene, 1% (vol/vol) ethanol] and stored frozen at −80°C until analysis.

Tissue vitamin levels and enzyme activities. Levels of vitamin A were determined using a C18 reverse-phase column and HPLC analysis (27). Enzyme activities of cytosolic and mitochondrial superoxide dismutase, glutathione peroxidase, and catalase (12, 18) were measured in skeletal muscle homogenates (10%, wt/vol) prepared using a glass Potter-Elvehjem homogenizer and ice-cold 10 mM K2PO4 (pH 7.2) and 2 mM EDTA. Assays were performed under conditions where activity was a linear function of protein concentration.

Isolation of amino acids from urine. Urine (1 ml) was supplemented with 10% (vol/vol) trichloroacetic acid and centrifuged at 14,000 rpm for 5 min in an Eppendorf microfuge. Amino acids in the supernatant were isolated by solid-phase extraction on a C18 column (3 ml, Supelclean SPE; Supelco, Bellefonte, PA) using a vacuum manifold system (Supelco). The column was conditioned with 2 ml of methanol, 6 ml of 50 mM NaH2PO4 (pH 7.4) containing 0.1 mM DTPA, and finally with 6 ml of 0.1% trifluoroacetic acid. Then 0.4 ml of urine supplemented with 100 µl of trichloroacetic acid and 13C-labeled internal standards were added onto the column. The column was washed with 6 ml of 0.1% trifluoroacetic acid. Amino acids were eluted with 3 ml of 10% methanol and concentrated to dryness under vacuum for derivatization. Preliminary experiments demonstrated that the recovery of authentic o,o′-dityrosine and α-tyrosine subjected to this procedure was >90%.

Isolation of amino acids from skeletal muscle. Vastus lateralis muscle was pulverized in liquid N2, dialyzed versus 0.1 mM DTPA (pH 7), and delipidated with methanol/water-washed diethyl ether as previously described (22). Samples (~1 mg protein) were concentrated to dryness under vacuum and immediately suspended in 0.5 ml of 6 N HCl (sequential grade; Pierce Chemical, Rockford, IL) containing 1% benzoic acid and 1% phenol (wt/vol). 13C-labeled internal standards were added, and samples were hydrolyzed at 110°C for 24 h under N2. Amino acids were isolated from the acid hydrolysate (14, 25) by solid-phase extraction on a C18 column (3 ml, Supelclean SPE) using a vacuum manifold system (Supelco) and concentrated to dryness under vacuum for derivatization.

Derivatization of amino acids. Amino acids were converted to their n-propyl carbonyl acid esters by the addition of 200 µl of HCl/n-propanol (1:3, vol/vol) and heating for 1 h at 65°C. After concentration to dryness under N2, 50 µl of heptfluoro butyric anhydride/ethyl acetate were added, and the samples were heated at 65°C for 15 min.

Mass spectrometric analysis. Mass spectrometric analyses were performed in the negative-ion electron capture mode with methane as the reagent gas using 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, Folsom, CA) interfaced with a Hewlett-Packard 5988A mass spectrometer with extended mass range. When amino acids were isolated from urine by solid-phase extraction on a reverse-phase C18 column, derivatized with n-propanol and heptfluorobutyric anhydride, compounds were detected that exhibited major ions and retention times identical to those of the n-propyl, heptfluorobutyric anhydride derivatives of α-o,o′-dityrosine and α-tyrosine (11, 21, 22). The identities of the compounds were confirmed by comparing them with both heptfluorobutyryl and pentafluoropropionyl derivatives of each oxidized amino acid (21).

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

RESULTS

There were no significant differences in body weight between either the sedentary rats (control diet 433 ± 68 g, antioxidant diet 414 ± 62 g) or the exercise-trained rats (control diet 416 ± 62 g, antioxidant diet 395 ± 37 g) on the two different diets at 24 mo of age. The rats on the control diet exercised to the same extent as those on the diet supplemented with antioxidants, but both groups ran significantly fewer miles as they aged (data not shown).

Levels of α-tocopherol, β-carotene, and retinyl ester (a metabolic product of β-carotene) were all increased significantly in the liver of rats on the antioxidant-supplemented diet (Table 1): α-tocopherol levels were five times higher than in rats on the control diet, β-carotene increased to a high level with antioxidant feeding but was undetectable in the control animals, and retinol palmitate also increased significantly. Plasma levels of ascorbic acid increased twofold in rats on a similar antioxidant diet in a longevity study (13). These results indicate that the antioxidant supplements used in this study effectively increase liver levels of α-tocopherol, β-carotene, and retinol palmitate and plasma levels of ascorbic acid.

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Antioxidant Therapy Lowers Level of o,o'-Dityrosine in Skeletal Muscle

Antioxidant supplementation reduced levels of protein-bound o,o'-dityrosine in skeletal muscle in both the sedentary and exercise-trained rats (Fig. 1A, P < 0.05 by ANOVA). In rats on the control diet the level tended to be lower in exercise-trained animals than in sedentary animals, but this difference was not statistically significant. In contrast, neither antioxidant supplementation nor exercise training altered levels of protein-bound o-tyrosine in skeletal muscle (Fig. 1B). These results indicate that supplementing the diet with antioxidants reduces the basal level of o,o'-dityrosine but not o-tyrosine in skeletal muscle of sedentary and exercise-trained rats.

Antioxidant Therapy and Adaptation to Exercise Lower Level of o,o'-Dityrosine in Urine

Isotope dilution GC-MS was used to quantify levels of o,o'-dityrosine and o-tyrosine in urine samples from the four groups of animals. Selected ion monitoring in the electron capture mode demonstrated that the negative ions derived from the amino acids coeluted with ions derived from authentic 13C-labeled internal standards (Fig. 2). To correct for individual differences in glomerular filtration, levels of oxidized amino acids were normalized to levels of urinary creatinine. We found that urine from the sedentary rats that received antioxidant supplements contained only about one-half as much o,o'-dityrosine as urine from sedentary rats on the control diet (Fig. 3A, P < 0.05). When exercise-trained control animals were compared with the sedentary control animals, there was about a 50% reduction in o,o'-dityrosine levels (Fig. 3A, P < 0.005). o,o'-Dityrosine levels in the urine of exercise-trained rats were not affected by antioxidant supplementation. As in the tissue samples, the o-tyrosine content of urine was similar regardless of the diet or exercise treatment group (Fig. 3B).

Aging Does Not Result in Skeletal Muscle Atrophy

Aging might result in muscle wasting that was prevented by exercise training or antioxidant supplementation. It would be inappropriate to normalize oxidized amino acids to urine levels of creatinine in animals suffering from muscle atrophy. However, there were no differences in the weights of the gastrocnemius or extensor digitorum longus muscles in 9- and 24-mo-old animals (Table 2). Antioxidant dietary supplementation also failed to affect the weight of either muscle (Table 2). These results indicate that muscle wasting is not likely to account for the differences in urine levels of o,o'-dityrosine in the different groups of animals.

Altered Renal Excretion of Amino Acids Does Not Account for Effect of Antioxidant Supplementation or Exercise Training on Levels of Oxidized Amino Acids in Urine

To determine whether exercise or antioxidants might alter the rate at which the kidneys excrete either

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Table 1. Liver content of vitamins in control and antioxidant-fed animals

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Control Diet</th>
<th>Antioxidant Diet</th>
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<tbody>
<tr>
<td>α-Tocopherol</td>
<td>15 ± 2</td>
<td>74 ± 12*</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>&lt;0.1</td>
<td>2.2 ± 0.9†</td>
</tr>
<tr>
<td>Retinol palmitate</td>
<td>630 ± 70</td>
<td>1,450 ± 140*</td>
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Values are means ± SE in nmol/g wet wt; n = 6 animals/group. Antioxidant content of right liver lobe of 24-mo-old sedentary rats fed the control and antioxidant-supplemented diets was determined by reverse-phase HPLC analysis as described under Methods. *P < 0.05, †P < 0.005.

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Fig. 1. Protein-bound o,o'-dityrosine (A) and o-tyrosine (B) in skeletal muscle of rats. At 5 mo of age female animals were continued on control diet or fed control diet supplemented with antioxidants (ascorbic acid, α-tocopherol, butylated hydroxytoluene, and β-carotene). One-half the animals in each group had access to a running wheel for exercise. Rats were killed at 24 mo of age, and levels of oxidized amino acids in acid hydrolysates of skeletal muscle were quantified by isotope dilution gas chromatography-mass spectrometry (GC-MS). Tissue contents of o,o'-dityrosine and o-tyrosine are normalized to content of precursor amino acids tyrosine and phenylalanine, respectively. Results represent means ± SE (n = 6/group). *P < 0.05 compared with animals fed control diet.
creatinine or the precursor amino acids of \textit{o,o'-dityrosine} and \textit{o-tyrosine}, the amounts of creatinine and of tyrosine and phenylalanine in urine were quantified. Creatinine levels were lower in the animals on the antioxidant diet (Table 3, \textit{P}, 0.05) than in the animals on the control diet. The ratio of tyrosine to creatinine in the urine was similar in all groups (Table 3), suggesting that differences in amino acid excretion were not responsible for the differences in \textit{o,o'-dityrosine} levels observed in the urine samples.

To correct for differences in renal excretion of amino acids, the urine content of \textit{o,o'-dityrosine} was normalized to that of its precursor amino acid tyrosine. The level of \textit{o,o'-dityrosine} in urine was significantly lower in the sedentary animals on the antioxidant diet (Fig. 4A, \textit{P}, 0.04) than in the sedentary animals on the control diet. When exercise-trained control animals were compared with the sedentary control animals, there was about a 70\% reduction in urinary \textit{o,o'-dityrosine} levels (Fig. 4A, \textit{P}, 0.005). In contrast, there

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**Fig. 2.** Detection of \textit{o,o'-dityrosine} (A) and \textit{o-tyrosine} (B) in urine of rats by selected ion monitoring negative-ion electron capture GC-MS analysis. Note coelution of major ion expected for \textit{o,o'-dityrosine} (m/z 1,208) and \textit{o-tyrosine} (m/z 595) with that of authentic \textit{13C}-labeled \textit{o,o'-dityrosine} (m/z 1,220) and \textit{o-tyrosine} (m/z 601), respectively.

**Fig. 3.** Levels of \textit{o,o'-dityrosine} (A) and \textit{o-tyrosine} (B) in urine of rats. At 5 mo of age animals were assigned to antioxidant-supplemented diet and exercise training as described in legend to Fig. 1. When animals were 24 mo of age urine was collected during an overnight fast as described under Methods. Levels of oxidized amino acids in urine were quantified by isotope dilution GC-MS. \textit{o,o'-Dityrosine} and \textit{o-tyrosine} are normalized to creatinine content of urine. Results represent means ± SE (\textit{n} = 6/group). *\textit{P} < 0.05 antioxidant vs. control group. +\textit{P} < 0.005 compared with sedentary control animals.
These results further support the conclusion that anti-

Table 2. Muscle weight of animals at 9 and 24 mo of age

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Diet</th>
<th>Sedentary</th>
<th>Exercise</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Antioxidant</td>
<td>Control</td>
</tr>
<tr>
<td>Gastrocnemius, g</td>
<td>9 mo</td>
<td>1.54±0.16</td>
<td>1.66±0.15</td>
</tr>
<tr>
<td></td>
<td>24 mo</td>
<td>1.71±0.24</td>
<td>1.74±0.29</td>
</tr>
<tr>
<td>Extensor digitorum, mg</td>
<td>9 mo</td>
<td>140±5</td>
<td>155±16</td>
</tr>
<tr>
<td></td>
<td>24 mo</td>
<td>152±13</td>
<td>150±24</td>
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Values are means ± SD; n = 4–5/group (9 mo) or 6–10/group (24 mo).

were no differences among the four groups when the o-tyrosine content of urine was normalized to that of its precursor amino acid phenylalanine (Fig. 4B).

Urine levels of o,o'-dityrosine and o-tyrosine normalized to creatinine correlated with urine levels of o,o'-dityrosine and o-tyrosine normalized to tyrosine and phenylalanine (r = 0.80 and r = 0.47, respectively). These results further support the conclusion that antioxidant supplementation lowers the level of o,o'-dityrosine in urine while not affecting o-tyrosine levels.

Exercise Increases Activity of Antioxidant Enzymes in Skeletal Muscle

The lower o,o'-dityrosine levels in skeletal muscle of exercise-trained animals might result from increased antioxidant defenses. To evaluate this possibility, the activities of antioxidant enzymes (mitochondrial and cytosolic superoxide dismutase, catalase, and glutathione peroxidase) were determined using skeletal muscle samples (Table 4).

The activities of two major antioxidant enzymes, mitochondrial superoxide dismutase and cytosolic glutathione peroxidase, were significantly higher (P < 0.05) in exercise-trained animals than in sedentary animals (Table 4). In contrast, antioxidant supplementation did not significantly alter the activities of these enzymes. There was no significant change in the activities of catalase and cytosolic superoxide dismutase in the exercise-trained animals. These results indicate that increased activities of the antioxidant enzymes glutathione peroxidase and mitochondrial superoxide dismutase might account in part for the decline in protein oxidation in exercise-trained animals.

DISCUSSION

In the current studies we used isotope dilution GC-MS to simultaneously quantify levels of o,o'-dityrosine and o-tyrosine in skeletal muscle and urine. This allowed us to examine the effects of supplemental dietary antioxidants and adaptation to exercise, two interventions designed to alter levels of protein oxidation products in vivo. o,o'-Dityrosine and o-tyrosine were monitored for three reasons. First, levels of these two markers should represent oxidative modifications of proteins that occur posttranslationally (5, 10, 11, 14, 15, 21). Second, in vitro studies indicate that proteins damaged by hydroxyl radical and tyrosyl radical exhibit distinct patterns of these products (11, 14, 21). Third, we suspected that the oxidatively modified amino acids might be excreted in urine instead of being recycled for protein synthesis.

Our results indicate that skeletal muscle levels of o,o'-dityrosine, a specific marker of oxidation, are significantly lower if sedentary or exercise-trained rats receive antioxidants. Importantly, levels of o,o'-dityrosine in urine mirrored those in tissue of animals subjected to the different interventions. In contrast, there was little difference in either skeletal muscle or urine levels of o-tyrosine among any of the groups of animals. These results suggest that levels of these compounds in urine reflect the overall steady-state level of oxidized amino acids in tissues. This finding raises the possibility that quantifying the levels of oxidized amino acids in urine could provide a noninvasive method for determining oxidative stress in vivo.

The results led us to propose a working model for the formation and excretion of oxidized amino acids. Oxidants derived from a variety of pathways, including enzymatic reactions and mitochondrial respiration, modify amino acid residues of proteins (1–3, 11, 16, 17, 32). Aromatic amino acids are likely to be vulnerable because they are susceptible to oxidation (6, 14, 15, 19, 21). The oxidized proteins then are targeted for degradation or broken down during tissue remodeling (3, 29). The resulting free oxidized amino acids are released from cells, filtered out of blood by the kidneys, and excreted in urine.

A potential advantage of measuring oxidation products in urine is that their levels may provide an integrated assessment of the rate of endogenous oxidative stress. Another advantage is the relatively high concentration of oxidized amino acids in urine, which facilitates their measurement by GC-MS. A potential disadvantage is that localized oxidative stress, as in atherosclerotic lesions in the artery wall (9, 21, 32), may not contribute to the overall excretion of oxidized amino acids. Moreover, the fate of oxidized amino acids in the body is unknown, and it is possible that a portion of these products is metabolized into other substances or reincorporated into proteins. Indeed, levels of o-tyrosine in skeletal muscle were ~40-fold higher than those of o,o'-dityrosine, which suggests that this amino

Table 3. Levels of amino acids and creatinine in urine

<table>
<thead>
<tr>
<th>Diet</th>
<th>Sedentary</th>
<th>Exercise</th>
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<tbody>
<tr>
<td>Control</td>
<td>Antioxidant</td>
<td>Control</td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>5.1±0.4</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Tyrosine-creatinine, mmol/mol</td>
<td>5.9±1.0</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>Phenylalanine-creatinine, mmol/mol</td>
<td>9.9±2.1</td>
<td>7.6±0.8</td>
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Values are means ± SE; n = 6 24-mo-old rats/group. *P < 0.05 compared with rats on control diet.
acid may be formed by other mechanisms. One possibility is that the diet contains o-tyrosine, which is taken up by the intestines and then misincorporated into proteins. It is also possible that oxidative stress increases the excretion of oxidized amino acids in the urine. In future experiments it will be important to evaluate the turnover rates of oxidized model proteins as well as the metabolic fates of oxidized amino acids.

Our observation that tissue and urine levels of o,o'-dityrosine, but not o-tyrosine, are selectively altered by antioxidant intervention and exercise training support the notion that tyrosyl radical may be one pathway for protein oxidation in vivo. We previously have reported that o,o'-dityrosine levels in cardiac and skeletal muscle of mice increase with aging (22). In contrast, o-tyrosine levels of rats do not increase with age in either tissue (19). These results suggest that hydroxyl radical may not be an important agent of aging-related protein damage in these tissues, at least as monitored by o-tyrosine levels.

A number of factors complicate this simple interpretation. First, it is important to note that proteins oxidized by hydroxyl radical in vitro also exhibit a variable increase in o,o'-dityrosine and that tyrosine oxidation occurs with greater efficiency than phenylalanine hydroxylation (6, 14, 19, 21). Second, tyrosine is a major target for oxidation by hydroxyl radical (19). Indeed, aromatic amino acids bind metal ions with high affinity in vitro (4), raising the possibility that tyrosine might be selectively oxidized by site-specifically generated hydroxyl radical. Third, tissue levels of o,o'-dityrosine were significantly lower than those of o-tyrosine, making it easier to detect increases in o,o'-dityrosine levels. Therefore we cannot exclude a possible role for hydroxyl radical and free metal ions in o,o'-dityrosine generation in vivo.

An important advance has been the recent demonstration that a series of prostaglandin F2-like compounds, the F2 isoprostanes, are generated when arachidonic acid is oxidized in vivo (24, 25). Measurement of plasma levels of the F2 isoprostanes has implicated lipid peroxidation as one pathway for oxidative stress in humans. However, many different oxidants initiate lipid peroxidation in vitro (1–3, 7, 16, 17, 21), and lipid peroxidation may continue after the initial insult, suggesting that F2 isoprostanes are unlikely to provide insights into the specific reaction mechanisms that promote oxidative damage in tissue. Other potential limitations of the F2 isoprostanes as markers of oxidative stress are their low abundance, the complex work-up required for analysis of biological material, and generation of certain isomers by enzymatic reactions (24, 25).

In contrast to peroxidation of polyunsaturated lipids, certain amino acid oxidation products may be more specific markers of the cause of oxidative damage. For example, 3-nitrotyrosine or 3-chlorotyrosine are produced when reactive nitrogen or hypochlorous acid

![Fig. 4. Levels of o,o'-dityrosine (A) and o-tyrosine (B) in urine of rats. o,o'-Dityrosine and o-tyrosine are normalized to tyrosine and phenylalanine content of urine, respectively. Levels of oxidized amino acids in urine were quantified by isotope dilution GC-MS. Results represent means ± SE (n = 6/group). *P < 0.05 antioxidant vs. control group. †P < 0.005 compared with sedentary animals.](https://example.com/fig4.png)

<table>
<thead>
<tr>
<th>Table 4. Activities of antioxidant enzymes in skeletal muscle</th>
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<tr>
<td><strong>Enzyme</strong></td>
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<tr>
<td>Superoxide dismutase, U/g wet wt</td>
</tr>
<tr>
<td>Cytosolic</td>
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<tr>
<td>Mitochondrial</td>
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<tr>
<td>Glutathione peroxidase, µmol·min⁻¹·g wet wt</td>
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<tr>
<td>Catalase</td>
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Values are means ± SE; n = 6 24-mo-old rats/group. Enzymatic activities were determined in homogenates of skeletal muscle. *P < 0.05 compared with control rats.
oxidize proteins (9, 17, 20). They do not appear, however, when a wide variety of other oxidation systems, including hydroxyl radical, metal ions, and glycoxidation, are the oxidizing agents (9, 20). These observations suggest that measuring 3-nitrotyrosine and 3-chloro-tyrosine in urine might provide sensitive and specific measures of oxidative damage by reactive nitrogen species or activated phagocytes in vivo. In future studies it will be important to quantify urine levels of other oxidized amino acids that are selectively generated by specific reaction pathways.

A major problem in evaluating the role of oxidative stress in human disease has been the difficulty in determining which doses and combinations of antioxidants best prevent tissue damage. Our observation that antioxidant therapy lowers o,o'-dityrosine levels in the skeletal muscle and urine of rats raises the possibility that measuring amino acid oxidation products in urine samples could provide a noninvasive way to monitor the effectiveness of antioxidant therapy in humans. This approach also may be useful for evaluating the relative contributions of different reaction pathways to oxidative stress in various pathological conditions and aging.

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