Isotope Dilution Mass Spectrometric Quantification of 3-Nitrotyrosine in Proteins and Tissues Is Facilitated by Reduction to 3-Aminotyrosine

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Oxidative damage by reactive nitrogen species has been implicated in the pathogenesis of atherosclerosis and other inflammatory diseases. The mechanisms of tissue damage are poorly understood, however, because the toxic intermediates are short-lived. Previous in vitro studies have suggested that 3-nitrotyrosine represents a specific marker of protein oxidation by reactive nitrogen species. The detection of this nitrated aromatic amino acid may thus serve as an indicator of tissue injury by nitrogen species in vivo. Here we describe a highly sensitive and specific analytical method for quantifying free and protein-bound 3-nitrotyrosine. The assay involves acid hydrolysis of proteins, isolation of 3-nitrotyrosine by ion exchange chromatography, and reduction of 3-nitrotyrosine to 3-aminotyrosine with dithionite. The reduced amino acid is then converted to its n-propyl, per-heptafluorobutyryl derivative and quantified by isotope dilution gas chromatography/negative-ion chemical ionization mass spectrometry. Attomole levels of 3-nitrotyrosine can be reproducibly measured in this manner. Quantifying 3-nitrotyrosine levels of tissues by stable isotope dilution gas chromatography/mass spectrometry should provide a powerful tool for exploring the impact of reactive nitrogen species on oxidative reactions in vivo. © 1998 Academic Press

Nitric oxide synthase converts arginine to nitric oxide (NO), 2 a nitrogen-centered free radical, in a variety of cells (1, 2). NO generated by constitutive isoforms of this enzyme plays a critical role in normal physiology by regulating vasomotor tone. The larger amounts of NO produced by inducible isoforms contribute to the cytocidal actions of inflammatory cells on microbial organisms and tumor cells. Under pathological conditions, however, reactive nitrogen species derived from NO may injure normal tissue (3–8). Indeed, immunohistochemical studies suggest that reactive nitrogen species contribute to a variety of pathological conditions, including atherosclerosis (9), acute respiratory distress syndrome (10), and ischemia–reperfusion injury (11, 12).

One pathway for generating reactive nitrogen species involves the reaction of NO with superoxide anion (O2−) to yield the oxidant ONOO−, peroxynitrite (13, 14).

NO + O2− → ONOO−

In vitro, ONOO− yields potent hydroxylating and nitrating species (4, 8, 15, 16).

Recent studies indicate that myeloperoxidase, a heme protein secreted by phagocytes, may provide another pathway for generating reactive nitrogen species (17, 18). At plasma concentrations of halide ion, this enzyme's major product is hypochlorous acid (HOCl) (19, 20), which reacts with nitrite (NO2−), a major decomposition product of NO, to yield a reactive intermediate similar to NO2Cl, nitryl chloride (17, 18).

Abbreviations used: NO, nitric oxide; HFB, heptafluorobutyryl; HFBA, heptafluorobutyric anhydride; BSA, bovine serum albumin; DTPA, diethylenetriaminopentaacetic acid; NICI, negative-ion chemical ionization; LDL, low-density lipoprotein; Na2S2O4, sodium dithionite.

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2 Abbreviations used: NO, nitric oxide; HFB, heptafluorobutyryl; HFBA, heptafluorobutyric anhydride; BSA, bovine serum albumin; DTPA, diethylenetriaminopentaacetic acid; NICI, negative-ion chemical ionization; LDL, low-density lipoprotein; Na2S2O4, sodium dithionite.
In the presence of hydrogen peroxide and a phenol, myeloperoxidase oxidizes nitrite to a nitrogen dioxide (NO₂) radical-like species (17, 18).

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCI} + \text{H}_2\text{O} \]
\[ \text{HOCI} + \text{NO}_2 \rightarrow \text{NO}_2\text{Cl} + \text{HO}^- \]

The mechanisms for cellular damage in vivo by reactive nitrogen species are poorly understood because the toxic intermediates are short-lived and difficult to measure directly. One approach to this problem is to detect stable end-products of these oxidizing reactions. Each of the three reactive nitrogen species—ONOO⁻, NO₂Cl, and NO₂—generates 3-nitrotyrosine when oxidizing tyrosine (4, 6, 15–18, 21, 22). In vitro studies demonstrate that 3-nitrotyrosine is a highly specific marker for protein oxidation by reactive nitrogen species (22). This has raised interest in the possibility that it might serve as a biomarker in proteins modified by reactive nitrogen species (4, 6, 15–18, 21, 22).

Although immunohistochemical studies with specific antibodies directed against 3-nitrotyrosine have yielded valuable information (9–12), their qualitative nature has made it difficult to determine the exact contribution of reactive nitrogen species to oxidative tissue damage. In contrast, methods that quantify specific long-lived products of such species would provide valuable tools for examining the roles of distinct oxidative pathways in the genesis of disease.

Here we describe a highly sensitive and specific isotope dilution gas chromatographic/mass spectrometric (GC/MS) assay for 3-nitrotyrosine. It involves reduction of 3-nitrotyrosine to 3-aminotyrosine, which then is converted to a fluoroacyl derivative that has excellent gas chromatographic and electron-capturing properties. GC/MS analysis can detect as little as 400 amol of this compound. We used this method to quantify both standard free 3-nitrotyrosine and protein-bound 3-nitrotyrosine in tissue after acid hydrolysis, isolation by ion-exchange chromatography, followed by reduction with dithionite.

**EXPERIMENTAL PROCEDURES**

**Materials**

3-Nitro-L-tyrosine, 3-aminol-L-tyrosine, sodium dithionite (sodium hydrosulfite; Na₂S₂O₄), and heptfluorobutyric anhydride (HFBA) were obtained from Sigma Chemical (St. Louis, MO). Cambridge Isotope Laboratories (Andover, MA) supplied ¹³C-labeled amino acids. 3-Nitro-[¹³C₆]tyrosine was synthesized from [¹³C₆]tyrosine with tetranitromethane (23) and its concentration was determined by HPLC following calibration with standard 3-nitrotyrosine (24).

**Methods**

Standard curve of free 3-nitrotyrosine and 3-nitro-[¹³C₆]tyrosine. A constant amount (500 pmol) of 3-nitro-[¹³C₆]tyrosine was added to a series of tubes containing varied amounts (0, 200, 500, 1000, 2000 pmol) of unlabeled 3-nitrotyrosine. The contents of each tube were then treated with Na₂S₂O₄ to reduce the 3-nitro group, and the products were extracted and treated sequentially with n-propanol/HCl and then with HFBA to form the n-propyl, tris-HFB derivative of 3-aminotyrosine.

Standard curve of bovine serum albumin standard containing 3-nitrotyrosine residues. Bovine serum albumin (BSA) was treated with tetranitromethane to yield a nitrated preparation containing 3-nitrotyrosine residues (23). To prepare a dilution curve of this material to demonstrate its 3-nitrotyrosine content, a series of tubes were prepared which contained a constant amount (200 pmol) of free 3-nitro-[¹³C₆]tyrosine internal standard, and varied amounts (0, 0.05, 0.075, 0.1, 0.2, 0.25, 0.375 mg) of the nitrated-BSA preparation. The contents of the tubes were then subjected to acid hydrolysis, anion exchange chromatography, reduction, derivatization, and GC/MS analysis as described below.

Preparation of tissue protein hydrolysates. Tissues were stored at −70°C until analysis. Thawed tissue was homogenized for 1 min at room temperature with 1 ml of 50 mM sodium phosphate, pH 7.4, 100 µM diethylenetriaminepentaacetic acid (DTPA) and 100 µM butylated hydroxytoluene using a Teckmar Tissumizer. Protein was precipitated with 1 ml of acetonitrile and collected by centrifugation at 2000g for 10 min. The protein pellet was washed twice with acetonitrile. One milliliter of 6 N HCl supplemented with 1% phenol (v/w), 100 pmol of [¹³C₆]nitrotyrosine, and 100 nmol of [¹³C₆]tyrosine was added to approximately 10 mg protein. Tissue proteins were hydrolyzed by heating at 110°C for 18–24 h. Samples were then concentrated to dryness under a stream of nitrogen.

Anion exchange chromatographic isolation of amino acids from tissue hydrolysates. Dried protein hydrolysate was reconstituted with 2 ml of 50 mM ammonium acetate (pH 7.0) and applied to an anion exchange column (0.5 × 1 cm; AG1-X8 resin; Bio-Rad Laboratories). The column was washed three times with 2 ml of H₂O. Under these conditions, 3-nitrotyrosine and other acidic, negatively charged amino acids bind to the resin, but neutral and basic amino acids do not. Because the pKₐ of the phenolic hydroxyl group of 3-nitrotyrosine is approximately 7 and therefore negatively charged at neutral pH, this modified amino acid
can be separated from non-acidic amino acids by anion exchange chromatography (Fig. 6). The 3-nitrotyrosine and other acidic amino acids were then eluted from the column with 2 ml of 1 M acetic acid.

Reduction of 3-nitrotyrosine to 3-aminotyrosine. The solution containing eluted amino acids was then concentrated to dryness under N2, and the residue was dissolved in 0.1 ml of 50 mM ammonium acetate (pH 7.0). Free 3-nitrotyrosine was then reduced to 3-aminotyrosine by the addition of 10 μl of a 10 mM Na2S2O4 solution freshly prepared in the ammonium acetate buffer (25). Control experiments confirmed that reduction was quantitative and complete within seconds under these conditions (25).

Derivatization of amino acids. Amino acids were converted to n-propyl carboxylic acid ester derivatives by heating for 1 h at 65°C with 1-propanol:concentrated HCl (3:1, v/v). The solution was then concentrated to dryness under N2, and 50 μl of a solution of heptafluorobutyric anhydride:ethyl acetate (1:3) was added. After vortex-mixing, samples were heated (5 min, 65°C) to form heptafluorobutyryl derivatives of the α-amino, aromatic amino, and phenolic hydroxyl groups. An aliquot (2 μl) of the resultant solution was then injected into the GC/MS system.

Gas chromatography/mass spectrometry. Unless otherwise noted, samples were injected into the gas chromatograph with a 30:1 split. The injector temperature was 240°C, and the transfer lines were maintained at 250°C. The gas chromatograph was a Hewlett-Packard 5890 equipped with a DB-1 column (12 m, 0.2 mm i.d., 0.33 μm methyl silicone film coating; P. J. Corbert, St. Louis, MO) or a DB-17 column (30 m, 0.25 mm i.d., 0.25 μm phenyl methyl silicone film coating; P. J. Corbert). The GC oven temperature was maintained at 150°C for the first 2 min after injection and was then increased at a rate of 20°C/min to final temperature of 270°C. All spectra were acquired on a Hewlett-Packard 5988A mass spectrometer (Palo Alto, CA) operated in the negative-ion chemical ionization mode. Emission current was set at 300 μA. The electron energy was 240 eV, and the source temperature was 240°C, and the transfer lines were maintained at 150°C. The source pressure was maintained at 0.6 Torr with methane as moderating gas.

**RESULTS**

Negative-ion chemical ionization (NICI) mass spectrometry is ~200 times more sensitive than electron impact mass spectrometry for quantifying trace levels of organic compounds (26, 27). NICI is most sensitive when used with target analytes that contain an electron-capturing moiety (e.g., fluorine).

Derivatization with heptafluorobutyric anhydride (HFBA) generates compounds whose high volatility and ability to capture electrons make them suitable targets for NICI GC/MS. HFBA reacts with several functional groups, including aliphatic or aromatic hydroxyl groups and primary or secondary amines. It derivatizes n-propyl tyrosine, forming a compound that yields a readily detectable NICI GC/MS signal at picomole levels (28). We therefore determined whether various HFBA derivatives of 3-nitrotyrosine could be quantified through NICI GC/MS.

We converted the carboxyl group of 3-nitrotyrosine to the n-propyl ester and reacted the resulting compound with HFBA. Under mild conditions, nitrotyrosine’s α-amino group but not its phenolic hydroxyl group became fluoroacylated (22). The structure and NICI mass spectrum of n-propyl, HFB-nitrotyrosine are illustrated in Fig. 1. The molecular anion (m/z 464) is abundant, and an ion (m/z 444) reflecting neutral loss of HF from the molecular anion is also observed. Although this mono-HFB derivative is detectable at femtomole levels, its unprotected phenolic hydroxyl group results in a broad GC signal (Fig 3). Repeated injections of this derivative also reduce capillary GC column lifetime.

Under harsh reaction conditions, HFBA fluoracylates the phenolic hydroxyl group as well as the α-amino group of n-propyl nitrotyrosine. Although this bis-HFB derivative behaves more favorably on GC, its NICI mass spectrum contains no structurally informative ions at high abundance, and more than 90% of the total ion current derives from heptafluorobutyrate (m/z 213) (not shown). We also examined several other fluoroacylating reagents, but none of the resulting 3-ni-
trotyrosine derivatives were suitable for GC/MS analysis.

We attributed this difficulty to the presence of the nitro group, whose relative polarity could contribute to poor GC behavior and whose bulk could sterically hinder the reaction of the phenolic hydroxyl on the adjacent ring carbon. Moreover, the group is strongly electron-withdrawing and therefore increases the acidity of the phenolic hydroxyl. This could reduce the stability of the reaction product of HFBA and the phenolic hydroxyl by increasing its susceptibility to hydrolysis.

Attempting to circumvent these difficulties, we reduced the 3-nitro group to an aromatic amine, which is less bulky, does not withdraw electrons, and might itself be susceptible to fluoracylation. We found that reagent Na2S2O4 readily reduced 3-nitrotyrosine to 3-aminotyrosine (25), which we then converted to the n-propyl carboxylic acid ester. The latter reacted with HFBA to yield a tris-derivative in which the fluoracyl moiety protected the α-amino, aromatic amino, and phenolic hydroxyl groups. The structure and NICI mass spectrum of this derivative are illustrated in Fig. 2. Although the molecular anion (m/z 826) is of low abundance, ions reflecting neutral losses of HF (m/z 806), two HF molecules (m/z 786), HF plus CO2 (m/z 762), and C3F7CHO (m/z 628) were observed. High-resolution and tandem mass spectrometric experiments confirmed that the ion at m/z 762 arises from consecutive losses of HF and CO2 (Hsu et al., unpublished).

Figure 3 illustrates the greater sensitivity of the tris-HFB, n-propyl ester derivative of 3-aminotyrosine relative to the mono-HFB, n-propyl ester of 3-nitrotyrosine for GC/MS analyses. In this experiment, we simultaneously injected 500 fmol of each derivative (with a 20:1 split ratio) into the GC/MS system and monitored selected ions of the derivatives of authentic 3-nitrotyrosine (m/z 464) and 3-aminotyrosine (m/z 762). The derivative of 3-aminotyrosine exhibited a shorter GC retention time (255 s vs 297 s), a narrower GC peak width (2.8 s vs 4.8 s), a higher peak amplitude (7 × 10^4 vs 0.4 × 10^4 relative ion abundance) and a
higher signal to noise ratio than the derivative of 3-nitrotyrosine. As shown in Fig. 4, we could detect 400 amol of the tris-HFB, n-propyl ester of 3-aminotyrosine with a signal to noise ratio exceeding 10 on GC/MS analysis with selected ion monitoring in the NICI mode.

To determine whether we could use the 3-aminotyrosine derivative to develop a sensitive, structurally specific isotope dilution GC/MS method for quantifying 3-nitrotyrosine, we prepared a standard curve using 3-nitrotyrosine labeled with a heavy isotope. We then added a constant amount (500 pmol) of the 3-nitro-[13C6]tyrosine internal standard to a series of tubes containing known amounts (0, 200, 500, 1000, 2000 pmol) of unlabeled 3-nitrotyrosine. After treating the contents of each tube with Na2S2O4 to reduce the nitro group, the products were extracted and treated sequentially with n-propanol/HCl and then HFBA to form the n-propyl, tris-HFB derivative of 3-aminotyrosine. These derivatives were then analyzed by NICI GC/MS with selected monitoring of ions arising from the target analyte (m/z 762) and the internal standard (m/z 768). Peaks at the appropriate GC retention time were integrated, and the ratio for the peak integral reflecting the target analyte divided by that for the internal standard is plotted as a function of the amount of unlabeled 3-nitrotyrosine initially added.

As noted above, the electron-withdrawing properties of the nitro group increase the acidity of the phenolic hydroxyl group of 3-nitrotyrosine. The resulting net negative charge at neutral pH makes 3-nitrotyrosine adsorb to AG1-X8 anion exchange resin. Other acidic amino acids (Glu and Asp) also adsorb to the resin under these conditions, but neutral or positively charged ones do not and elute from the column. The 3-nitrotyrosine-enriched solution can then be washed from the column with acid. When we added 3-nitro-[13C6]tyrosine to a sample of tissue protein hydroly-

![FIG. 4. Detection of 3-nitrotyrosine by negative-ion chemical ionization–mass chromatography–mass spectrometry is sensitive to the attomole range. The n-propyl, per-HFB derivative of 400 amol of 3-nitrotyrosine was analyzed in the selected ion monitoring mode with NICI GC/MS. The chromatogram was obtained with monitoring of m/z 762 (M−–HF−–CO2).](image)

![FIG. 5. Standard curve for isotope dilution GC/MS measurement of 3-nitrotyrosine as its reduced derivatized product. A constant amount (500 pmol) of the 3-nitro-[13C6]tyrosine internal standard was then added to a series of tubes containing varied but known (0, 200, 500, 1000, 2000 pmol) of unlabeled 3-nitrotyrosine. The contents of each tube were then treated with Na2S2O4 to reduce the 3-nitro group, and the products were extracted and treated sequentially with n-propanol/HCl and then with HFBA to form the n-propyl, tris-HFB derivative of 3-aminotyrosine. These derivatives were then analyzed by NICI GC/MS with selected monitoring of ions arising from the target analyte (m/z 762) and the internal standard (m/z 768). Ion current peaks at the appropriate GC retention time were then integrated, and the ratio for the peak integral reflecting the target analyte divided by that for the internal standard is plotted as a function of the amount of unlabeled 3-nitrotyrosine initially added.](image)
sate, it adsorbed to an AG1-X8 resin and was quantitatively desorbed with 1 M acetic acid, as determined by quantitative GC/MS analysis of the eluate's 3-nitrotyrosine content. We also monitored some naturally occurring amino acids in this experiment. For example, 99% of L-tyrosine eluted in the application solution, and only 1% eluted with 3-nitrotyrosine in the acid solution (Fig. 6). Because tyrosine and [13C6]tyrosine behave identically on ion exchange chromatography, the tyrosine in this fraction may be used to estimate the protein content of the tissue sample. In other experiments, we determined that 3-aminotyrosine does not bind to the resin (data not shown).

In control experiments, we showed that 3-nitrotyrosine is stable to acid (6 N HCl, 110°C) hydrolysis in the absence or presence of a variety of reducing agents (Table 1), as assessed quantitatively by HPLC. Detectable amounts of 3-aminotyrosine did not form from 3-nitrotyrosine under these conditions. These observations suggest that 3-nitrotyrosine is not reduced to 3-aminotyrosine by biologically occurring reducing agents and that it might be detectable in acid hydrolysates of proteins.

To examine this possibility, we treated bovine serum albumin with tetranitromethane (23) to nitrate this standard protein. We then added varying amounts of the product to a series of tubes, each containing a fixed amount (100 pmol) of 3-nitro-[13C6]tyrosine as an internal standard. After acid hydrolysis, we isolated 3-nitrotyrosine by anion exchange chromatography and reduced it with Na2S2O4. The resulting 3-aminotyrosine was then converted to the n-propyl ester, tris-HFB derivative and analyzed by GC/MS in the NICI mode with selected monitoring of ions from both the target analyte (m/z 762) and internal standard (m/z 768). As illustrated in Fig. 7, the peak area ratio of the ion current peak at m/z 762 divided by that at m/z 768 at the GC retention time appropriate for derivatized 3-aminotyrosine was a linear function of the amount of the nitrated BSA initially added to the reaction vessel over at least one order of magnitude.

To determine whether proteins from native mammalian tissue might contain 3-nitrotyrosine residues, we precipitated the proteins in rat heart homogenate with acetonitrile. We then added 3-nitro-[13C6]tyrosine and

![FIG. 6. Recovery of 3-nitrotyrosine and other amino acids subjected to anion exchange chromatography. A mixture of 3-aminotyrosine, 3-nitrotyrosine, leucine, isoleucine, and tyrosine (each 100 μM) in 50 mM ammonium acetate was subjected to anion exchange chromatography on Bio-Rad AG1-X8 resin as described under Experimental Procedures. Recovery of amino acids was monitored in the column flowthrough, after two washes of the column with H2O, and following elution of the column with 1 M acetic acid. The amino acid content of each eluant fraction was determined by GC/MS analysis after suitable derivatization (22, 28).](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>3-Nitrotyrosine (% recovery)</th>
</tr>
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<tbody>
<tr>
<td>No addition</td>
<td>99%</td>
</tr>
<tr>
<td>Glutathione</td>
<td>97%</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>95%</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>98%</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>96%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>100%</td>
</tr>
<tr>
<td>Methionine</td>
<td>96%</td>
</tr>
</tbody>
</table>

Note. 3-Nitrotyrosine (100 μM) was incubated at 37°C for 24 h either alone (no addition) or with 1 mM of each of the indicated compounds. Reaction products were then dried under vacuum, hydrolyzed in 6 N HCl at 120°C for 24 h, and quantified by reverse-phase HPLC as described under Experimental Procedures. Results represent the mean of duplicate determinations.
We previously developed an isotope dilution GC/MS method for quantitating 3-nitrotyrosine that uses 3-nitro-[13C6]tyrosine as an internal standard and forms a fluoroacyl derivative (22). The derivative is strongly electron-capturing and permits the use of NICI mass spectrometry, which is approximately 200 times more sensitive than electron impact mass spectrometry for many analytes (26, 27). This method detects femtomole levels of 3-nitrotyrosine and has been successfully applied to 3-nitrotyrosine from biological sources (22). As with many compounds containing a nitro group or other polar moiety, however, the GC signal tended to be poorly resolved. This may occur because the nitro group stereoelectronically destabilizes the phenolic hydroxyl, which is not fluoroacylated in the chemical derivatization process.

In the study described here, we improved the assay by first reducing the nitro group to an amine. This permitted facile fluoroacylation of the phenolic hydroxyl, the aromatic amino group, and α-amino group of the compound. The resulting derivative was substantially more volatile than the monofluoroacylated derivative of 3-nitrotyrosine, and it exhibited excellent chromatographic properties on a GC column. Moreover, it yielded a greater signal on NICI analysis, reflecting the excellent electron capturing properties of its higher fluoroacyl content. Using this approach, we successfully measured the 3-nitrotyrosine content of acid hydrolysates of a standard protein and native tissue proteins. To facilitate these measurements, we developed an anion exchange chromatographic method for isolating 3-nitrotyrosine that exploits the nitro group’s effect on the acid-base properties of the phenolic hydroxyl. This reduces chemical noise by separating the modified amino acid from other components in biological material.

GC/MS analysis is likely to be an improvement over immunochemical methods for detecting 3-nitrotyrosine in biological tissues because immunochemical methods are only semi-quantitative and their results may be confounded by cross-reacting but structurally distinct molecules. The NICI GC/MS method also offers advantages over existing chromatographic approaches to quantifying 3-nitrotyrosine; these include an HPLC method using tandem UV and an electrochemical method that detects a reduced, derivatized product from 3-nitrotyrosine (21). While these methods have excellent sensitivity and are more specific than immunochemistry, they provide no structural information about the analyte. In contrast, GC/MS provides specific structural information, reducing the potential for confusion with extraneous compounds that coelute with the target analyte during chromatographic separation. In addition, GC/MS analyses permit the use of an internal standard which, apart from its heavy isotope, is structurally identical to the target analyte and therefore behaves identically during extraction, processing, and chromatographic analyses. Including this standard corrects for analyte loss during extraction, processing, and chromatographic analyses. Including this standard corrects for analyte loss during extraction, processing, and chromatographic analyses.
We intend to apply the new method to our continued studies of atherosclerosis because it appears that low-density lipoprotein (LDL), the major carrier of blood cholesterol, must be oxidized to trigger pathological events (29, 30). Immunological evidence has aroused considerable interest in the possibility that levels of 3-nitrotyrosine in tissues and cells might reflect oxidative injury by activated nitrogen species. Our recent work demonstrates the utility of isotope dilution mass spectrometric analysis for exploring the roles of oxidative damage in disease (22). We used GC/MS to demonstrate that nitrotyrosine levels are much higher in LDL isolated from atherosclerotic tissue than in circulating LDL (22). This observation indicates that reactive nitrogen intermediates represent one pathway for LDL oxidation in the artery wall.

It is noteworthy that myeloperoxidase, a heme enzyme secreted by phagocytes, is present in human atherosclerotic lesions (31). This enzyme generates 3-nitrotyrosine in vitro (17, 18). It also is the only human enzyme known to generate hypochlorous acid, HOCl, at plasma concentrations of halide (19, 20). When HOCl reacts with tyrosine, it forms 3-chlorotyrosine, making the latter a specific marker of protein oxidation by the myeloperoxidase system (32, 33). Using isotope dilution GC/MS, we detected elevated levels of 3-chlorotyrosine, 3-nitrotyrosine, and several other myeloperoxidase products in atherosclerotic lesions (28, 34). These findings raise the possibility that myeloperoxidase is a catalyst for nitration and chlorination reactions in vivo.

Reactive nitrogen species also have been implicated in a wide range of other inflammatory diseases, ranging from ischemia–reperfusion injury to arthritis to cancer and muscle cachexia (1–15). The availability of a sensitive and specific isotope dilution GC/MS method

![Graph](image)

**FIG. 8.** Demonstration of 3-nitrotyrosine in rat heart by GC/MS. Proteins in samples of rat heart were hydrolyzed to their constituent amino acids after the addition of internal standard as described under Experimental Procedures. Native 3-nitrotyrosine and the internal standard were then recovered from the hydrolysate by anion exchange chromatography and reduced to 3-aminotyrosine, which was then converted to the n-propyl, perheptafluorobutryl derivative and analyzed by NICI GC/MS. Qualitative identification of the native analyte was achieved by demonstrating coelution of two ions rising from it at the appropriate GC retention time (A). These ions are m/z 762 (M–HF–CO) and m/z 806 (M–HF). Quantitation was achieved (B) by comparing the relative intensities of the ions reflecting elimination of HF and CO from the native analyte (m/z 762) and from the internal standard (m/z 768).

**TABLE 2**

<table>
<thead>
<tr>
<th>Rat Heart</th>
<th>3-Nitrotyrosine (µmol/mol)</th>
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<tbody>
<tr>
<td>Animal 1</td>
<td>178</td>
</tr>
<tr>
<td>Animal 2</td>
<td>155</td>
</tr>
<tr>
<td>Animal 3</td>
<td>97</td>
</tr>
<tr>
<td>Animal 4</td>
<td>197</td>
</tr>
<tr>
<td>Animal 5</td>
<td>144</td>
</tr>
<tr>
<td>Animal 6</td>
<td>156</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>154 (31)</td>
</tr>
</tbody>
</table>

Note. Amino acid hydrolysates was prepared from rat heart as described under Experimental Procedures. Samples were then processed for mass spectrometric analysis and 3-aminotyrosine was quantified by monitoring its ions at m/z 762 and its corresponding isotopically enriched counterpart at m/z 768 as described in the legend to Fig. 8. Levels of 3-nitrotyrosine are normalized to protein content of the precursor amino acid tyrosine, determined using [13C6]tyrosine as internal standard (22, 28).
for precisely measuring 3-nitrotyrosine should help identify the specific reaction pathways that generate this oxidant in vivo. Such studies may have important implications for the potential of oxidative reactions to modify proteins in pathophysiologic environments. They also may suggest interventions to interrupt these harmful events.

ACKNOWLEDGMENTS

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