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Mass Spectrometric Quantification of Markers for Protein Oxidation by Tyrosyl Radical, Copper, and Hydroxyl Radical in Low Density Lipoprotein Isolated from Human Atherosclerotic Plaques*

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Lipoprotein oxidation has been implicated in the pathogenesis of atherosclerosis. However, the physiologically relevant pathways mediating oxidative damage have not yet been identified. Three potential mechanisms are tyrosyl radical, hydroxyl radical, and redox active metal ions. Tyrosyl radical forms *o,o′*-dityrosine cross-links in proteins. The highly reactive hydroxyl radical oxidizes phenylalanine residues to *o*-tyrosine and *m*-tyrosine. Metal ions oxidize low density lipoprotein (LDL) by poorly understood pathways. To explore the involvement of tyrosyl radical, hydroxyl radical, and metal ions in atherosclerosis, we developed a highly sensitive and quantitative method for measuring levels of *o,o′*-dityrosine, *o*-tyrosine, and *m*-tyrosine in proteins, lipoproteins, and tissue, using stable isotope dilution gas chromatography-mass spectrometry. We showed that *o,o′*-dityrosine was selectively produced in LDL oxidized with tyrosyl radical. Both *o*-tyrosine and *o,o′*-dityrosine were major products when LDL was oxidized with hydroxyl radical. Only *o*-tyrosine was formed in LDL oxidized with copper. Similar profiles of oxidation products were observed in bovine serum albumin oxidized with the three different systems. Applying these findings to LDL isolated from human atherosclerotic lesions, we detected a 100-fold increase in *o,o′*-dityrosine levels compared to those in circulating LDL. In striking contrast, levels of *o*-tyrosine and *m*-tyrosine were not elevated in LDL isolated from atherosclerotic tissue. Analysis of fatty streaks revealed a similar pattern of oxidation products; compared with normal aortic tissue, there was a selective increase in *o,o′*-dityrosine with no change in *o*-tyrosine. The detection of a selective increase of *o,o′*-dityrosine in LDL isolated from vascular lesions is consistent with the hypothesis that oxidative damage in human atherosclerosis is mediated in part by tyrosyl radical. In contrast, these observations do not support a role for free metal ions as catalysts of LDL oxidation in the artery wall.

An elevated plasma level of low density lipoprotein (LDL)[1] is an important risk factor for the development of atherosclerotic vascular disease (1). Many lines of evidence suggest that LDL must be oxidatively modified before it can initiate atherosclerosis (2–7). LDL is oxidized *in vitro* by several different mechanisms, although the physiologically relevant pathways have not yet been identified (2–10). The most widely studied model involves free metal ions. LDL oxidation by cultured arterial cells requires micromolar concentrations of either iron or copper (11) and is inhibited by metal chelators (11–13). High concentrations of iron or copper catalyze LDL oxidation in the absence of cells (11, 13).

Metal-catalyzed oxidation systems represent another potential pathway for LDL oxidation. In these systems, redox active metals bound to proteins interact with hydrogen peroxide and a reducing agent to generate a hydroxyl radical-like intermediate (9). However, it is uncertain whether the metal ions required for these pathways are present *in vivo* because the body has intricate mechanisms for chelating metals and rendering them redox inactive (14).

We have described a mechanism for LDL oxidation that does not require free metal ions (15). The oxidation reaction involves tyrosyl radical generated by myeloperoxidase (16), a hemeprotein secreted by activated phagocytes (17, 18). Active myeloperoxidase has been detected in human atherosclerotic tissue (19), where it co-localizes with lipid-laden foam cells, the cellular hallmark of the early atherosclerotic lesions (2, 4, 7). Immunostaining of myeloperoxidase at different stages of lesion development (19) reveals patterns that are strikingly similar to those of protein-bound oxidation products in rabbit lesions (20). Moreover, oxidation products of the enzyme (21) have been detected by immunohistochemistry in vascular lesions (22). These observations raise the possibility that myeloperoxidase promotes LDL oxidation *in vivo*.

One strategy for determining whether free metal ions, metal-catalyzed oxidation systems, or tyrosyl radical oxidize proteins *in vivo* is to analyze normal and atherosclerotic vascular tissue for stable end products of these three pathways identified through *in vitro* studies. Hydroxyl radical generated by metal-catalyzed oxidation systems converts protein-bound phenylalanine residues to the unnatural isomers *o*-tyrosine and *m*-tyrosine (Fig. 1; Refs. 14 and 23–25). It also cross-links tyrosine residues into *o,o′*-dityrosine (23, 24). It has not been established whether LDL oxidation by free metal ions generates a similar pattern of oxidation products. With protein oxidation by tyrosyl radical, *o,o′*-dityrosine is a major product (Fig. 1; Refs. 26 and 27). These amino acid products are stable to acid hy-

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[1] The abbreviations used are: LDL, low density lipoprotein; BSA, bovine serum albumin; DTPA, diethylenetriamine pentaacetic acid; GC-MS, gas chromatography-mass spectrometry; *m/z*, mass-to-charge ratio.

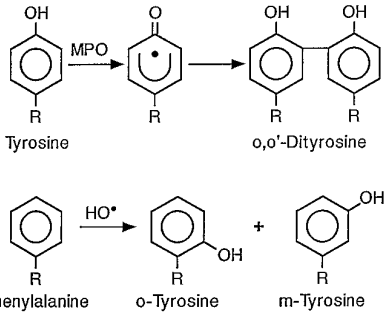

FIG. 1. **Reaction pathways for oxidation of protein-bound tyrosine and phenylalanine by myeloperoxidase and hydroxyl radical.** Tyrosyl radical generated by myeloperoxidase (*MPO*) leads to *o*,*o*′-dityrosine cross-linking. Metal ions catalyze the site-specific formation of hydroxyl radical (HO•), which abstracts a hydrogen atom from tyrosine and reacts with the aromatic ring of phenylalanine to generate predominantly *o*-tyrosine with smaller amounts of *m*-tyrosine and *o*,*o*′-dityrosine.

drolysis, making them potentially useful markers for protein oxidation *in vivo* (23, 26, 27).

We have developed a quantitative assay for measuring tissue levels of *o*-tyrosine, *m*-tyrosine, and *o*,*o*′-dityrosine. It combines gas chromatography with stable isotope dilution mass spectrometry (GC-MS). Using this assay, we first investigated the relative yields of the three markers in LDL oxidized *in vitro* with free copper, hydroxyl radical, and tyrosyl radical. *o*-Tyrosine was a major oxidation product when either free copper or a hydroxyl radical system was used to oxidize LDL. In contrast, *o*,*o*′-dityrosine was selectively produced when tyrosyl radical was the oxidizing agent. A similar pattern of products was observed in bovine serum albumin (BSA) oxidized with the different systems. We then used the method to demonstrate that levels of *o*,*o*′-dityrosine, but not *o*-tyrosine or *m*-tyrosine, were elevated in LDL isolated from human atherosclerotic tissue. Collectively, these observations suggest that tyrosyl radical plays a role in oxidizing LDL in the artery wall.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, reagents were obtained from either Sigma or Aldrich Chemical Co. All organic solvents were high performance liquid chromatography grade. Cambridge Isotope Laboratories supplied ^{13}C-labeled amino acids. All buffers were passed over a Chelex-100 (Bio-Rad) column to remove transition metal ions.

Preparation and Isolation of Internal Standards—Isotopically labeled *o*-tyrosine and *m*-tyrosine were synthesized using [$^{13}C_6$]phenylalanine, copper, and H_2O_2 (23). *o*,*o*′-[$^{13}C_{12}$]Dityrosine was prepared with [$^{13}C_6$]tyrosine, horseradish peroxidase, and H_2O_2 (28). Concentrations of amino acids were determined by comparison with authentic standards using high performance liquid chromatography and monitoring of A_{276} (29). Compounds were analyzed at a flow rate of 1 ml/min using an Ultrasphere ODS column (250 × 4.6 mm, 5-μm diameter particles; Beckman) equilibrated with solvent A (5% methanol, 0.1% trifluoroacetic acid, pH 2.5) and eluted with solvent B (90% methanol, 0.1% trifluoroacetic acid, pH 2.5). The elution gradient was: 0–35% solvent B over 10 min; isocratic elution at 35% solvent B for 4 min; 35–100% solvent B over 12 min; isocratic elution at 100% solvent B for 5 min.

Isolation of LDL from Plasma—LDL was isolated rapidly using a two-step density gradient from plasma (EDTA, 1 mg/ml) prepared from normolipidemic, healthy subjects (30). LDL was collected by needle aspiration and subjected to size exclusion chromatography on a Bio-Rad 10 DG column equilibrated with Buffer A (50 mM sodium phosphate, pH 7.4).

Oxidation of BSA and LDL—All reactions were carried out at 37 °C in reaction mixture containing 1 mg of protein/ml of either BSA (fatty acid-free; Boehringer Mannheim) or LDL. Hydroxyl radical was generated by addition of 2 mM H_2O_2 to Buffer A supplemented with 0.1 mM $CuSO_4$ (23). Tyrosyl radical was generated by addition of 0.1 mM H_2O_2 to Buffer A supplemented with 0.1 mM diethylenetriamine pentaacetic acid (DTPA, pH 7.4), 0.2 mM L-tyrosine, and 20 nM myeloperoxidase (15, 16, 26). Reactions were terminated by the addition of 300 nM catalase, 100 μM butylated hydroxytoluene, and 200 μM DTPA, and proteins were immediately precipitated at 4 °C with ice-cold trichloroacetic acid (10%, v/v). The protein pellet was washed with 0.5 ml of 10% trichloroacetic acid and delipidated with 4 ml of methanol/water-washed diethyl ether (1:3, v/v). The protein residue was hydrolyzed as described below.

Collection of Human Arterial Tissue—Vascular tissue resected at surgery or autopsy was immediately placed in ice-cold antioxidant buffer (100 μM DTPA, 1 mM butylated hydroxytoluene, 1% (v/v) ethanol, 140 mM NaCl, 10 mM sodium phosphate, pH 7.4), and then frozen at −80 °C until analysis.

Isolation of LDL from Human Aorta—LDL was isolated from vascular lesions using a modified method of Steinbrecher and Lougheed (31). Isolated thoracic aortae were thawed in Buffer B (0.15 M NaCl, 10 mM sodium phosphate (pH 7.4), 0.3 mM EDTA, 100 μM DTPA, 50 μg/ml soybean trypsin inhibitor, 100 μM butylated hydroxytoluene, and 10 mM 3-aminotriazole). Fatty streaks and intermediate lesions were resected from aortic tissue (~9 g wet weight per aorta), frozen in liquid N_2, and pulverized under liquid N_2 with a stainless steel mortar and pestle. All subsequent procedures were carried out at 4 °C. Tissue powder was collected into 50-ml sterile conical tubes, Buffer B was added (5 ml/g tissue), and the tubes were rocked gently end-over-end overnight. Tissue powder was pelleted by centrifugation at 5000 × *g* for 15 min, the supernatant was subjected to centrifugation at 100,000 × *g* for 30 min, and the pellet and uppermost lipemic layer were discarded. LDL in the supernatant was isolated by sequential density ultracentrifugation (d = 1.02–1.07 g/ml; Ref. 32). A metal chelator (100 μM DTPA) and myeloperoxidase inhibitor (10 mM 3-aminotriazole; Refs. 15, 16, and 26) were included in all solutions used for lipoprotein isolation. LDL was extensively dialyzed against Buffer C (50 mM phosphate, 0.1 mM DTPA, pH 7.4) and then against Buffer D (0.1 mM DTPA, pH 7.4) under N_2 prior to analysis.

Preparation of Tissue—Vascular tissue was thawed at room temperature. Regions of normal and atherosclerotic aortic tissue were defined morphologically using the criteria of the Pathobiological Determinants of Atherosclerosis in Youth Study (33). Resected arterial tissue was frozen in liquid N_2 and pulverized using a stainless steel mortar and pestle. Tissue powder (~60 mg wet weight) was suspended in 1 ml of Buffer D, dialyzed (2 h) *versus* Buffer D at 4 °C, and then delipidated by incubation (10 min) with 13 ml of methanol/water-washed diethyl ether (3:10, v/v) on ice. The sample was centrifuged at 5000 × *g* for 10 min, the supernatant was removed, and the fluffy protein powder was again delipidated with 10 ml of water-washed diethyl ether.

Protein Hydrolysis and Isolation of Amino Acids—The protein or tissue residue was dried under N_2, ^{13}C-labeled internal standards were added, and the sample was then hydrolyzed at 110 °C for 24 h in 0.5 ml of 6 N HCl (Sequenal grade, Pierce Chemical) supplemented with 1% benzoic acid and 1% phenol. The samples were supplemented with 10% trichloroacetic acid (v/v) and passed over a reverse phase column (3 ml, LC-18 SPE Tube, Supelco Inc. Bellefonte, PA), which was previously washed with 12 ml of 50 mM $NaHPO_4$ and 100 μM DTPA (pH 7.4) followed by 12 ml of 0.1% trifluoroacetic acid. Amino acids were eluted with 25% methanol (2 ml) and dried under vacuum for derivatization. Preliminary studies using authentic standards demonstrated that the amino acids were stable to acid hydrolysis and that >80% of tyrosine oxidation products were recovered from the C-18 column using this procedure.

Derivatization of Amino Acids—Amino acids were converted to carboxylic acid esters by the addition of 200 μl of HCl/*n*-propyl alcohol (1:3, v/v) and heating for 1 h at 65 °C. Excess reagent was evaporated under N_2. To prepare the heptafluorobutyryl derivatives of the amino acids, 50 μl of heptafluorobutyric anhydride/ethyl acetate (1:3, v/v) was added, and the samples were heated at 65 °C for 10 min. To prepare the pentafluoropropionyl derivatives of the amino acids, 50 μl of pentafluoropropionic anhydride/ethyl acetate (1:4, v/v) was added, and the samples were heated at 65 °C for 30 min.

Mass Spectrometric Analysis—Amino acids were quantified by stable isotope dilution GC-MS. Derivatized samples were dried under N_2 and redissolved in 50 μl of 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 inside diameter, 0.33-μ film thickness, J & W Scientific) interfaced with a Hewlett Packard 5988A Mass Spectrometer with extended mass range (26, 34). The injector and ion source temperature were set at 250 °C and 150 °C, respectively. Full scan mass spectra and selected ion monitoring were obtained with both the *n*-propyl heptafluorobutyryl and the *n*-propyl pentafluoropropionyl derivatives of both authentic and isotopically labeled amino acids in the negative-ion chemical ionization mode with methane as the reagent gas.

For phenylalanine and *p*-tyrosine, an aliquot of derivatized amino acid was diluted 1:100 (v/v) with ethyl acetate. A 1-μl sample was injected into the gas chromatograph with a 1:100 split prior to mass analysis. The initial column temperature of 120 °C was maintained for 1 min and then increased to 220 °C at 10 °C/min. The mass spectrum of the *n*-propyl heptafluorobutyryl derivative of phenylalanine included a small molecular ion (M^-) at mass-to-charge (*m/z*) 403 and a prominent ion at *m/z* 383 ($M^- - HF$). Phenylalanine was quantified using the *m/z* 383 ion. The mass spectrum of the *n*-propyl heptafluorobutyryl derivative of *p*-tyrosine revealed prominent ions at *m/z* 595 ($M^- - HF$) and 417 ($M^- - CF_3$ $(CF_2)_2CHO$). The *m/z* 417 ion was used to quantify tyrosine.

Tyrosine and phenylalanine oxidation products were analyzed without dilution and injected in the splitless mode. The initial column temperature was 150 °C: it was increased to 300 °C at 40 °C/min. The mass spectrum of the *n*-propyl heptafluorobutyryl derivative of *o,o′*-dityrosine included a small molecular ion at *m/z* 1228 (M^-) and prominent ions at *m/z* 1208 ($M^- - HF$) and 1030 ($M^- - CF_3$ $(CF_2)_2CHO$). The *m/z* 1208 ion was used to quantify *o,o′*-dityrosine levels. The mass spectrum of the *n*-propyl heptafluorobutyryl derivative of *o*-tyrosine exhibited prominent ions at *m/z* 595 ($M^- - HF$) and 417 ($M^- - CF_3$ $(CF_2)_2CHO$). The *m/z* 595 ion was used for quantification. The mass spectrum of the *n*-propyl heptafluorobutyryl derivative of *m*-tyrosine exhibited prominent ions at *m/z* 595 ($M^- - HF$) and 417 ($M^- - CF_3$ $(CF_2)_2CHO$). The *m/z* 417 ion was used for quantification.

The generation of oxidized amino acids by the analytical procedure was investigated in preliminary experiments. The increase in the level of *o*-tyrosine in phenylalanine subjected to acid hydrolysis was less than 10 μmol of oxidation product/mol of precursor amino acid, which is less than 5% of the value found in tissue. Under these conditions, there was no detectable conversion of tyrosine to *o,o′*-dityrosine. The level of oxidation products in model proteins (BSA, RNase, IgG) subjected to acid hydrolysis, derivatization, and GC-MS analysis was variable. For *o*-tyrosine it ranged from 100 to 1,100 μmol/mol, and for *o,o′*-dityrosine it ranged from undetectable (less than 5 μmol/mol) to 60 μmol/mol. The level of oxidation products in different model proteins remained consistent during repeated analyses, strongly suggesting that the oxidized amino acids were present endogenously. These observations indicate that the analytical procedure itself was not a source of protein oxidation.

To determine whether post-mortem changes were likely to be contributing to protein oxidation, human aortic tissue obtained at either surgery or autopsy was subjected to acid hydrolysis, derivatization, and negative-ion chemical ionization GC-MS analysis. There were no significant differences in the levels of *o*-tyrosine and *o,o′*-dityrosine in the surgical and autopsy tissue, suggesting that post-mortem changes are unlikely to result in significant alterations in the levels of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine in human vascular tissue.

Quantification of Oxidized Amino Acids—The base peak ion of each amino acid was used for quantification. To ensure that interfering ions were not co-eluting with the analyte, the ratio of ion currents of the two most abundant ions of each amino acid were monitored in all analyses. Under these chromatographic conditions, authentic compounds and isotopically labeled standards were baseline separated and exhibited retention times identical to those of analytes derived from tissue samples.

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. The limit of detection (signal/noise > 10) was ≤ 1 pmol for all of the amino acids. The coefficients of variation for the analyses of tyrosine and phenylalanine in different LDL preparations were 0.8% and 4.8%, respectively.

RESULTS

To investigate the relative yields of the amino acid markers after protein oxidation by hydroxyl radical, metal ions, and tyrosyl radical, BSA and LDL were exposed to either a copper-H_2O_2 oxidation system (hydroxyl radical), free copper (metal ion), or a myeloperoxidase-H_2O_2-tyrosine system (tyrosyl radical). We selected BSA for our initial studies because albumin is abundant in plasma and has been widely investigated as a target for oxidative damage *in vitro*. To determine whether lipid components might affect the pattern of oxidation products, we performed similar experiments with LDL. LDL was rapidly isolated using a procedure that generates very low levels of endogenous lipid hydroperoxides (30). The presence of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine was determined using stable isotope dilution GC-MS analysis.

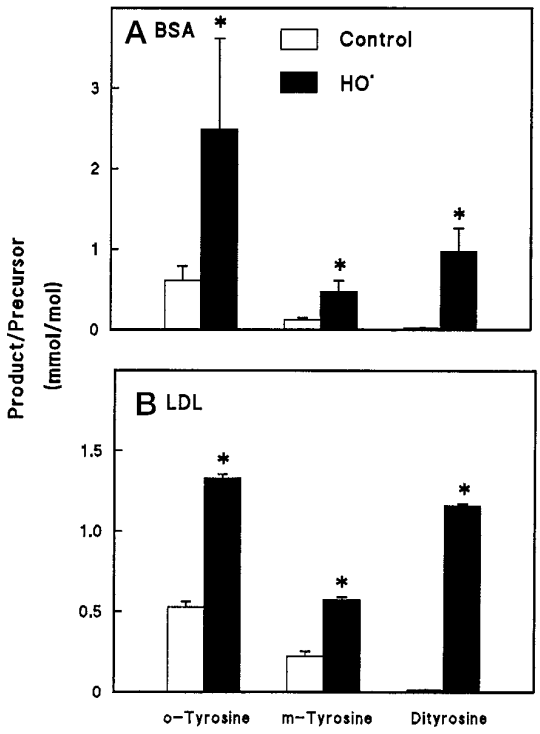

FIG. 2. **Phenylalanine and tyrosine oxidation products in BSA (*A*) and LDL (*B*) exposed to hydroxyl radical (HO·).** BSA and LDL were incubated for 2 h at 37 °C in Buffer A (50 mM sodium phosphate, pH 7.4) supplemented with 0.1 mM $CuSO_4$ and 2 mM H_2O_2. Reactions were initiated by the addition of H_2O_2 and terminated by the addition of catalase, butylated hydroxytoluene, and DTPA. BSA and LDL were acid-precipitated and LDL was delipidated. Proteins were subjected to acid hydrolysis, the amino acids were isolated from the hydrolysate by solid phase extraction on a C-18 column, the *n*-propyl heptafluorobutyryl derivatives of the amino acids were prepared, and the formation of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine was determined using stable isotope dilution negative-ion chemical ionization GC-MS as described under "Experimental Procedures." *o,o′*-Dityrosine and unnatural tyrosine isomer contents of proteins are normalized to the content of the precursor amino acids *p*-tyrosine and phenylalanine, respectively, determined using ^{13}C-labeled internal standards. Values are the mean of duplicate determinations. Similar results were observed in three independent experiments. *, $p < 0.05$ compared with control using the paired *t* test.

Detection of o-Tyrosine, m-Tyrosine, and o,o′-Dityrosine in BSA and LDL Oxidized by Hydroxyl Radical—Hydroxyl radical was generated using a system containing 0.1 mM copper and 2 mM H_2O_2. The accumulation of *o*-tyrosine in BSA oxidized by hydroxyl radical was initially linear and then reached a plateau after 2 h (data not shown). *o*-Tyrosine was the major product when BSA was oxidized by hydroxyl radical, and smaller amounts of *m*-tyrosine and *o,o′*-dityrosine were also formed (Fig. 2*A*). When LDL was oxidized by hydroxyl radical under the same experimental conditions, the major products were *o*-tyrosine and *o,o′*-dityrosine, with smaller amounts of *m*-tyrosine (Fig. 2*B*). These results indicate that *o*-tyrosine is a major product of protein oxidation by hydroxyl radical.

Detection of o-Tyrosine, m-Tyrosine, and o,o′-Dityrosine in BSA and LDL Oxidized by Copper—Copper is a potent catalyst for LDL oxidation *in vitro* (11, 13), but the yield of aromatic oxidation products in the modified lipoprotein has not been reported. We therefore monitored the formation of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine in LDL exposed to copper. To

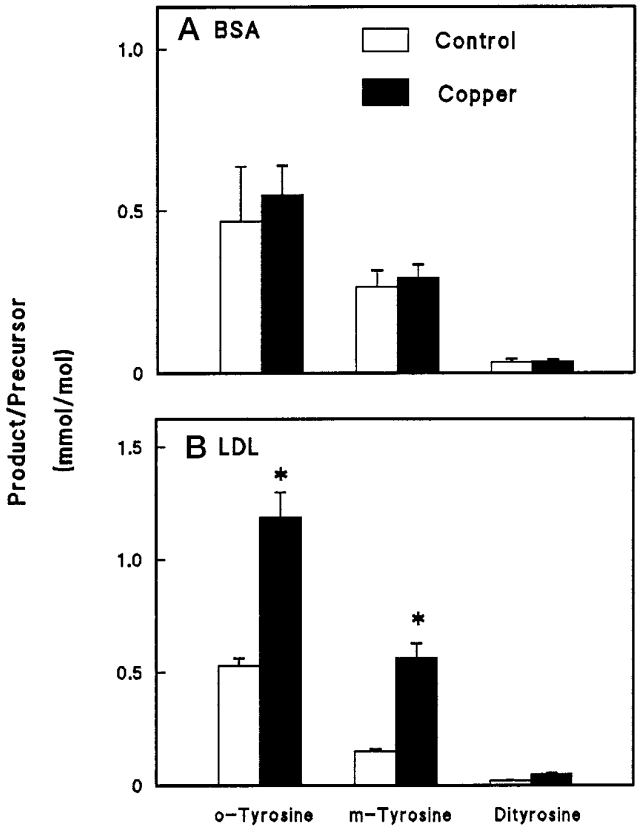

FIG. 3. **Phenylalanine and tyrosine oxidation products in BSA (*A*) and LDL (*B*) exposed to copper.** BSA (1 mg/ml) and LDL (1 mg of protein/ml) were incubated at 37 °C in Buffer A supplemented with 0.1 mM $CuSO_4$ for 2 h and 24 h, respectively. Reactions were initiated by the addition of $CuSO_4$ and terminated by the addition of catalase, butylated hydroxytoluene, and DTPA. Protein-bound oxidation products were determined using stable isotope dilution GC-MS as described in the legend to Fig. 2. *, $p < 0.05$ compared with control using the paired *t* test.

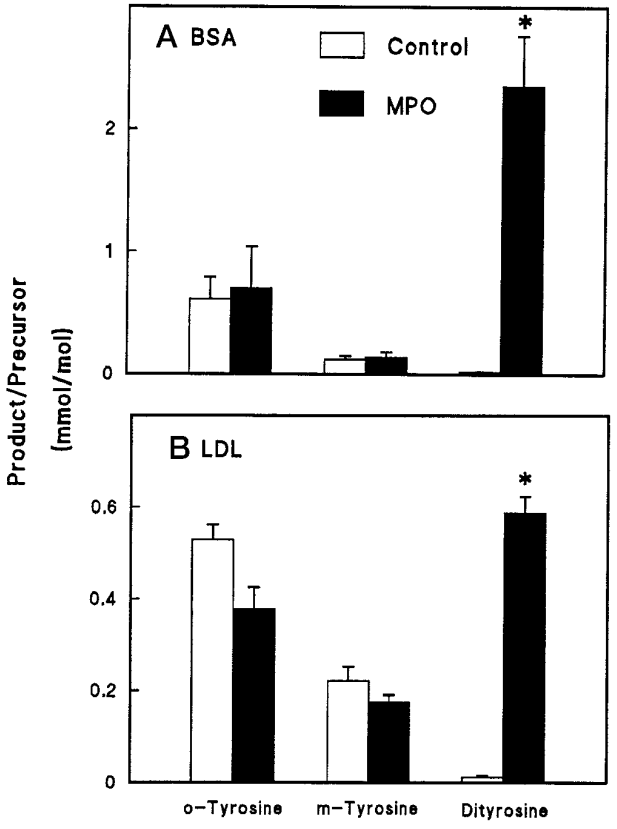

FIG. 4. **Phenylalanine and tyrosine oxidation products in BSA (*A*) and LDL (*B*) exposed to tyrosyl radical generated by myeloperoxidase (*MPO*).** BSA and LDL were incubated for 2 h at 37 °C in Buffer A supplemented with 0.1 mM H_2O_2, 0.1 mM DTPA, 0.2 mM L-tyrosine, and 20 nM myeloperoxidase. Reactions were initiated by the addition of H_2O_2 and terminated by the addition of catalase, butylated hydroxytoluene, and DTPA. Protein-bound oxidation products were determined using stable isotope dilution GC-MS as described in the legend to Fig. 2. *, $p < 0.05$ compared with control using the paired *t* test.

explore the effect of the lipid environment of LDL on its susceptibility to oxidation, we also determined the product yield of the oxidation products in BSA. The levels of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine were unaffected by exposing BSA to high concentrations of copper alone (Fig. 3*A*). The oxidation of tyrosine and phenylalanine residues in BSA by the hydroxyl radical generating system thus requires both H_2O_2 and copper under these experimental conditions (compare Figs. 2*A* and 3*A*).

In contrast to BSA, both *o*-tyrosine and *m*-tyrosine increased in LDL exposed to copper (Fig. 3*B*). However, there was little change in the level of *o,o′*-dityrosine in LDL incubated with copper alone (Fig. 3*B*). When LDL was exposed to copper, the kinetics of protein oxidation (monitored as *o*-tyrosine formation) and lipid oxidation (monitored as thiobarbituric acid reacting substances; Ref. 32) were similar. These results suggest that alkoxyl and/or peroxyl radicals formed during metal-catalyzed lipid peroxidation play a role in the hydroxylation of protein-bound phenylalanine. In contrast, reactive intermediates generated during copper-catalyzed LDL oxidation apparently fail to promote *o,o′*-dityrosine formation.

Detection of o-Tyrosine, m-Tyrosine, and o,o′-Dityrosine in BSA and LDL Oxidized by Tyrosyl Radical—Oxidation of BSA by myeloperoxidase-generated tyrosyl radical was initially rapid and then leveled off after 0.5 h as monitored by *o,o′*-dityrosine formation (data not shown). The progress curve for protein dityrosine cross-linking by myeloperoxidase was similar to that previously reported for the synthesis of free *o,o′*-dityrosine (16) and protein tyrosylation (26). *o,o′*-Dityrosine was the major product when BSA was exposed to tyrosyl radical, with little increase in the levels of either *o*-tyrosine or *m*-tyrosine (Fig. 4*A*). *o,o′*-Dityrosine also was the only product formed in LDL exposed to tyrosyl radical, with no significant change in the levels of either *o*-tyrosine or *m*-tyrosine (Fig. 4*B*). These results indicate that *o,o′*-dityrosine is a major product of protein oxidation by tyrosyl radical generated by myeloperoxidase.

The concentrations of oxidant and redox catalyst were very different in the hydroxyl radical and tyrosyl radical systems. There was 20 times as much H_2O_2 in the hydroxyl radical system as in the tyrosyl radical system (2 mM *versus* 0.1 mM), and there was 5,000 times as much copper as myeloperoxidase (100 μM *versus* 20 nM). Despite the marked differences in concentrations of redox active components in the reaction mixtures, the relative yields of *o*-tyrosine (per mol of phenylalanine) and *o,o′*-dityrosine (per mol of tyrosine) were similar when BSA was exposed either to hydroxyl radical or to tyrosyl radical. These observations imply that under our experimental conditions myeloperoxidase-generated tyrosyl radical is much more efficient than metal-catalyzed hydroxyl radical at oxidizing protein-bound aromatic amino acids.

Detection of o-Tyrosine, m-Tyrosine, and o,o′-Dityrosine in LDL Isolated from Human Atherosclerotic Lesions—To determine whether free metal ions, hydroxyl radical, or tyrosyl radical damage lipoproteins *in vivo*, we looked for the amino acid markers in LDL isolated from human artery wall. LDL (*d* =

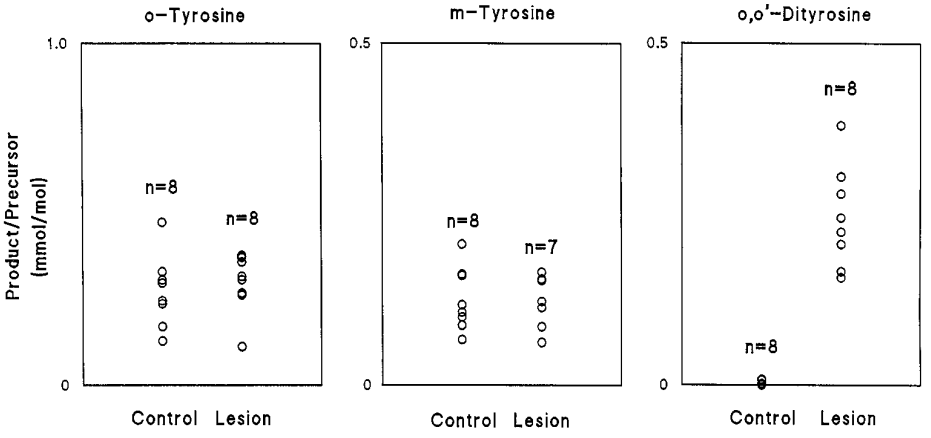

FIG. 5. **Levels of protein-bound *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine in LDL isolated from plasma (*Control*) and in LDL isolated from human atherosclerotic tissue (*Lesion*).** Levels of amino acids in protein hydrolysates were quantified by selected ion monitoring stable isotope dilution GC-MS analysis as described under "Experimental Procedures." *o,o′*-Dityrosine and unnatural tyrosine isomer contents of proteins are normalized to the content of the precursor amino acids *p*-tyrosine and phenylalanine, respectively, determined using ^{13}C-labeled internal standards.

1.02 to 1.07 g/ml) was prepared by sequential ultracentrifugation from aortic atherosclerotic tissue obtained at autopsy (31, 32). The buffers used for tissue processing contained high concentrations of DTPA (a metal chelator), butylated hydroxytoluene (a lipid soluble antioxidant), and 3-aminotriazole (a peroxidase inhibitor) to prevent artifactual oxidation of lipoproteins. Western blotting with a rabbit antibody monospecific for human apolipoprotein B100 (35), the major protein of circulating LDL, confirmed that intact apolipoprotein B100 was present in lesion LDL. As previously noted by other investigators (5, 31), aggregated and a range of lower molecular weight forms of immunoreactive proteins also were present in LDL isolated from lesions.

Compared with circulating LDL isolated from plasma, there was a striking 100-fold increase in *o,o′*-dityrosine levels in LDL isolated from atherosclerotic lesions (Fig. 5). In contrast, the levels of *o*-tyrosine and *m*-tyrosine in circulating LDL and lesion LDL were similar. The distribution of oxidation products in LDL isolated from aortic tissue was thus similar to that found in LDL oxidized by tyrosyl radical.

Detection of o-Tyrosine, m-Tyrosine, and o,o′-Dityrosine in Human Vascular Tissue—To determine whether metal ions, hydroxyl radical, or tyrosyl radical damage proteins *in vivo*, we looked for the amino acid markers in human atherosclerotic tissue. Amino acids isolated from acid hydrolysates of advanced lesions were treated with *n*-propyl alcohol and pentafluoropropionyl 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 *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine. Selected ion monitoring showed that the ions derived from the amino acids co-eluted with the ions derived from ^{13}C-labeled internal standards, as shown in Fig. 6 for *o,o′*-dityrosine. The identity of each compound was confirmed by comparison with authentic standards using both heptafluorobutyryl and pentafluoropriopionyl derivatives of each oxidized amino acid. These results indicate that *o,o′*-dityrosine, *o*-tyrosine, and *m*-tyrosine are present in acid hydrolysates of proteins from human atherosclerotic lesions.

Phenylalanine and Tyrosine Oxidation Products at Different Stages of Atherosclerosis in Human Aortic Tissue—To determine the levels of oxidative damage to proteins at different stages of atherosclerosis, we analyzed autopsy samples of human aortic tissue. The abundance of this material allowed us to identify areas of normal tissue and areas at different stages of atherosclerotic disease from each individual donor. Tissue was prepared and analyzed by stable isotope dilution negative-ion chemical ionization GC-MS as described above. Four independent analyses of tissue obtained from 16 different individuals were performed, and each donor provided all four types of aorta, for a total of 64 analyses.

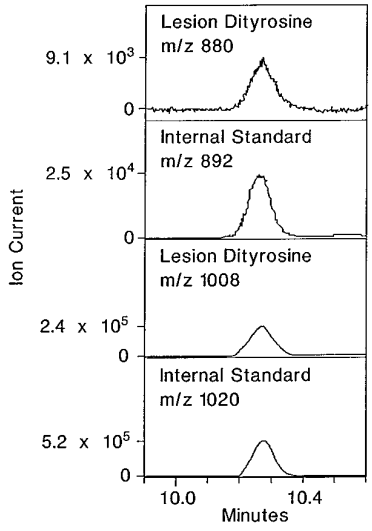

FIG. 6. **Detection of the *n*-propyl pentafluoropropionyl derivative of *o,o′*-dityrosine in human atherosclerotic tissue by selected ion-monitoring negative-ion chemical ionization GC-MS.** Human aortic tissue obtained at autopsy was pulverized in liquid N_2, dialyzed to remove low molecular weight components, delipidated, and subjected to acid hydrolysis. After addition of *o,o′*-[$^{13}C_{12}$]dityrosine as internal standard, the amino acids were isolated by solid phase extraction on a C-18 column, the *n*-propyl pentafluoropropionyl derivatives of the amino acids were prepared, and the derivatized amino acids were subjected to negative-ion chemical ionization GC-MS analysis as described under "Experimental Procedures." The predicted molecular weight of the *o,o′*-dityrosine derivative is 1008. The loss of HF (20 units) and CF_3CF_2CHO (148 units) from the molecular ion (M^-) of *m/z* 1028 would yield ions with *m/z* values of 1008 and 880, respectively. Note co-elution of the major ions expected for *o,o′*-dityrosine (*m/z* 880 and 1008) with those of *o,o′*-[$^{13}C_{12}$]dityrosine (*m/z* 892 and 1020).

There was a striking 11-fold elevation in the level of protein-bound *o,o′*-dityrosine in fatty streaks compared with normal aorta (Fig. 7). The content of protein-bound *o,o′*-dityrosine also was elevated in advanced lesions, being 6 times greater than in normal tissue. In contrast, levels of *o*-tyrosine and *m*-tyrosine were not significantly different at any stage of the atherosclerotic process, although there was a trend toward higher levels of both markers in advanced atherosclerotic lesions (Fig. 7). The pattern of elevation of oxidation products was identical in each analysis, with marked increases in fatty streaks and advanced lesions observed in all four independent experiments. These results indicate that aortic tissue levels of protein-bound *o,o′*-dityrosine, but not *o*-tyrosine or *m*-tyrosine, increase in fatty streaks and advanced lesions obtained through autopsy from humans.

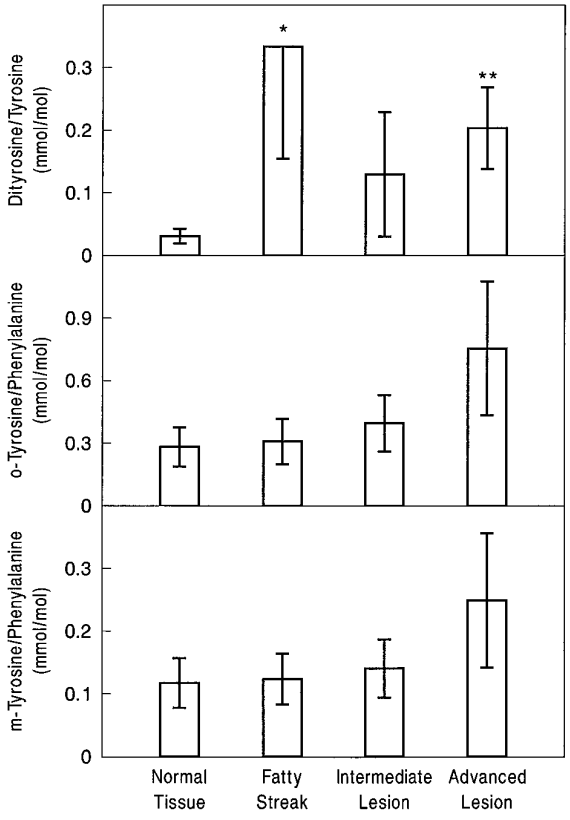

FIG. 7. **Levels of protein-bound *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine at different stages of atherosclerosis in human aortic tissue obtained at autopsy.** Normal aortic tissue and atherosclerotic lesions ($n = 64$) were classified using the criteria of the Pathobiological Determinants of Atherosclerosis in Youth Study (33). Each donor ($n = 16$) provided all four types of tissue. Tissue levels of amino acids were quantified by selected ion-monitoring stable isotope dilution negative-ion chemical ionization GC-MS as described under "Experimental Procedures." *o,o′*-Dityrosine and unnatural tyrosine isomers contents of tissue are normalized to the protein content of the precursor amino acids p-tyrosine and phenylalanine, respectively. Results represent the mean ± S.E. of four individual experiments; the same pattern of elevations in oxidation products was observed in each analysis. *, $p < 0.05$ and **, $p < 0.01$, respectively, compared to normal arterial tissue using the Mann-Whitney test.

TABLE I

Patterns of tyrosine and phenylalanine oxidation products formed in BSA and LDL exposed to hydroxyl radical, copper and tyrosyl radical in vitro, and in LDL isolated from human atherosclerotic tissue

	Oxidized amino acid[a]		
	o-Tyrosine	*m*-Tyrosine	*o,o′*-Dityrosine
Hydroxyl radical			
BSA	↑	↑	↑
LDL	↑	↑	↑
Copper			
BSA	–	–	–
LDL	↑	↑	–
Tyrosyl radical			
BSA	–	–	↑
LDL	–	–	↑
Lesion LDL	–	–	↑

[a] ↑, increased; –, no change.

DISCUSSION

The long-term goal of our research is to identify the molecular mechanisms of oxidative damage in human vascular disease. In the current studies we have focused on three pathways, free metal ions, hydroxyl radical, and tyrosyl radical, that potentially play a role in LDL oxidation and the pathogenesis of atherosclerosis. We selected *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine as markers for oxidation because they are unnatural amino acids and therefore should represent post-translational modifications of proteins.

We first performed *in vitro* studies to establish the product yields of *o*-tyrosine, *m*-tyrosine, and *o,o′*-dityrosine in LDL and BSA exposed to the different oxidation systems. LDL oxidized by copper exhibited large increases in *o*-tyrosine and *m*-tyrosine. In contrast, there was little change in the level of *o,o′*-dityrosine in copper-oxidized LDL. There was no evidence of protein oxidation, as monitored by the amino acid oxidation products, in BSA exposed to high concentrations of copper alone. For both LDL and BSA, *o*-tyrosine was a major end product when the oxidant was hydroxyl radical-generated by a metal-catalyzed oxidation system. The level of *o,o′*-dityrosine in proteins exposed to hydroxyl radical was variable but was always accompanied by a striking increase in *o*-tyrosine. Similar results were reported by Huggins *et al.* (23) for RNase and lysozyme damaged with hydroxyl radical generated by metal ions and ionizing radiation. In contrast to copper and hydroxyl radical, there was a selective increase in the level of *o,o′*-dityrosine in BSA and LDL oxidized by tyrosyl radical generated by myeloperoxidase, with no significant increase in the amount of either *o*-tyrosine or *m*-tyrosine. Collectively, these results indicate that proteins damaged *in vitro* by free metal ions, hydroxyl radical, and tyrosyl radical exhibit distinct patterns of phenylalanine and tyrosine oxidation products (Table I).

We next determined the levels of the oxidation products in LDL isolated from human vascular tissue resected at autopsy. There was a remarkable 100-fold increase in *o,o′*-dityrosine levels compared with circulating LDL. In striking contrast, the levels of *o*-tyrosine and *m*-tyrosine in lesion LDL and circulating LDL were similar. The pattern of a selective increase in *o,o′*-dityrosine levels without significant change in *o*-tyrosine levels was remarkably similar to that observed in both LDL and BSA oxidized by tyrosyl radical (Table I). Furthermore, the selective increase in *o,o′*-dityrosine levels with little change in either *o*-tyrosine or *m*-tyrosine was different from that observed with LDL oxidized with either copper or the hydroxyl radical system. LDL exposed to either of the metal ion-dependent systems exhibited large increases in *o*-tyrosine with a variable increase in *o,o′*-dityrosine. These observations suggest that tyrosyl radical, but not free copper, may play a role in oxidizing LDL *in vivo*.

Finally, we determined the levels of the oxidation products in human vascular tissue resected at autopsy. Compared with normal aortic tissue, there was an 11-fold elevation of *o,o′*-dityrosine in fatty streaks, the earliest lesion of atherosclerosis. Protein-bound *o,o′*-dityrosine was elevated 6-fold in advanced atherosclerotic lesions. There was no significant increase in *o*-tyrosine or *m*-tyrosine in aortic tissue at any stage of the atherosclerotic process. The selective increase in *o,o′*-dityrosine levels without significant change in *o*-tyrosine levels in atherosclerotic aortic tissue is consistent with the hypothesis that tyrosyl radical represents one pathway for protein oxidation in the artery wall (Table I).

Because *o,o′*-dityrosine levels in aortic tissue were highest in fatty streaks, and also were elevated in advanced lesions, tyrosyl radical may contribute both to the initiation and progression of vascular disease. The trend toward higher levels of protein-bound *o,o′*-dityrosine in intermediate aortic lesions did not achieve statistical significance, perhaps reflecting large biological variations or a real decrease in tissue *o,o′*-dityrosine levels. The decline in *o,o′*-dityrosine levels seen in intermediate lesions may result in part from degradation of oxidized proteins during vascular remodeling. Indeed, *o,o′*-dityrosine has been proposed to target oxidized proteins for proteolytic breakdown (36). The variations in *o,o′*-dityrosine levels at different stages

of the atherosclerotic process may also reflect variations in the degree or mechanism of oxidative stress, as well as differences in the protein composition of normal and atherosclerotic tissue.

Previous studies have shown that tissue homogenates prepared from atherosclerotic lesions contain detectable levels of catalytically active metal ions (37, 38), suggesting that free metal ions or low molecular weight chelates of metal ions may promote LDL oxidation *in vivo*. However, it is difficult to exclude the possibility that homogenization of the tissue artifactually generated the redox active components observed in these experiments. Neither study reported the level of catalytically active metal ions in normal aortic tissue subjected to the same procedure. Because levels of *o*-tyrosine were similar in circulating LDL and lesion LDL, and because LDL oxidation by copper yielded large amounts of *o*-tyrosine with no change in *o*,*o*′-dityrosine, our results suggest that free metal ions are unlikely to promote LDL oxidation in the artery wall (Table I). Indeed, premature atherosclerosis is not a prominent feature of genetic disorders that cause iron and copper to accumulate in tissue and plasma (39, 40). It is possible, however, that endogenous *o*-tyrosine masked a metal ion-dependent increase in protein oxidation. There was a trend toward higher levels of *o*-tyrosine and *m*-tyrosine in advanced atherosclerotic lesions, suggesting that metal ion-catalyzed oxidation reactions may be important late in the disease process when cellular dissolution might promote the release of redox active metal ions.

The detection of a selective increase in *o*,*o*′-dityrosine in lesion LDL and atherosclerotic tissue, together with the distinct patterns of phenylalanine and tyrosine oxidation products formed in BSA and LDL oxidized *in vitro*, suggests that tyrosyl radical may play a role in lipoprotein oxidation *in vivo*. A potential pathway involves myeloperoxidase, a well-characterized source of tyrosyl radical (15, 16, 26). Active myeloperoxidase is present in human atherosclerotic tissue (19), and oxidation products of the enzyme have been detected by immunohistochemistry in vascular lesions (21, 22), suggesting that tyrosyl radical generated by myeloperoxidase may represent one pathway for LDL oxidation *in vivo*. In contrast, the failure to detect an increase of *o*-tyrosine in lesion LDL and atherosclerotic tissue suggests that free redox active metal ions are unlikely to serve as a catalyst for LDL oxidation in the artery wall.

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