Human Atherosclerotic Intima and Blood of Patients with Established Coronary Artery Disease Contain High Density Lipoprotein Damaged by Reactive Nitrogen Species

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High density lipoprotein (HDL) is the major carrier of lipid hydroperoxides in plasma, but it is not yet established whether HDL proteins are damaged by reactive nitrogen species in the circulation or artery wall. One pathway that generates such species involves myeloperoxidase (MPO), a major constituent of artery wall macrophages. Another pathway involves peroxynitrite, a potent oxidant generated in the reaction of nitric oxide with superoxide. Both MPO and peroxynitrite produce 3-nitrotyrosine in vitro. To investigate the involvement of reactive nitrogen species in atherogenesis, we quantified 3-nitrotyrosine levels in HDL in vivo. The mean level of 3-nitrotyrosine in HDL isolated from human aortic atherosclerotic intima was 6-fold higher (619 ± 178 μmol/mol Tyr) than that in circulating HDL (104 ± 11 μmol/mol Tyr; p < 0.01). Immunohistochemical studies demonstrated striking colocalization of MPO with epitopes reactive with an antibody to 3-nitrotyrosine. However, there was no significant correlation between the levels of 3-chlorotyrosine, a specific product of MPO, and those of 3-nitrotyrosine in lesion HDL. We also detected 3-nitrotyrosine in circulating HDL, and linear regression analysis demonstrated a strong correlation between the levels of 3-chlorotyrosine and levels of 3-nitrotyrosine. These observations suggest that MPO promotes the formation of 3-chlorotyrosine and 3-nitrotyrosine in circulating HDL but that other pathways also produce 3-nitrotyrosine in atherosclerotic tissue. Levels of HDL isolated from plasma of patients with established coronary artery disease contained twice as much 3-nitrotyrosine as HDL from plasma of healthy subjects, suggesting that nitrated HDL might be a marker for clinically significant vascular disease. The detection of 3-nitrotyrosine in HDL raises the possibility that reactive nitrogen species derived from nitric oxide might promote atherogenesis. Thus, nitrated HDL might represent a previously unsuspected link between nitrative stress, atherosclerosis, and inflammation.

Elevated levels of low density lipoprotein (LDL) are a major risk factor for the development of atherosclerotic vascular disease, although a wealth of evidence suggests that LDL must be oxidatively modified to damage the artery wall (1, 2). In contrast, high density lipoprotein (HDL) protects the artery wall from atherosclerosis. This atheroprotective effect has been attributed in part to the ability of the major protein of HDL, apolipoprotein (apo) A-I, to mobilize cholesterol from arterial macrophages (3–5). However, HDL is also the major carrier of lipid hydroperoxides in plasma (6). Therefore, several other mechanisms, including the ability of HDL to inhibit LDL oxidation, reduce lipid hydroperoxides, and transport oxidized lipids to the liver for excretion, may also be cardioprotective (7–15). Pathways that oxidize HDL and thereby impair its function might thus be pivotal to the development of atherosclerosis (12, 14, 15). However, it is not yet known whether HDL in the circulation or human artery wall might also be targeted for oxidation.

We have described one pathway for LDL oxidation in humans (16). It involves hypochlorous acid and other reactive intermediates generated by myeloperoxidase, a heme protein secreted by phagocytes. High concentrations of enzymatically active myeloperoxidase have been found in human vascular lesions (16, 17), and characteristic protein and lipid oxidation products of the enzyme have been detected in LDL isolated from atherosclerotic tissue (18–20). Another oxidative pathway involves nitric oxide (nitrogen monoxide), which is generated by vascular wall cells (21). NO is a relatively stable free radical that is unable to oxidize LDL directly under physiological conditions (22, 23). However, it reacts rapidly with superoxide to form peroxynitrite (ONOO–) (24), a reactive nitrogen species that promotes peroxidation of the lipid moiety of LDL in vitro (25). Proteins also appear vulnerable to ONOO– because the oxidant reacts in vitro with tyrosine residues to yield the stable product 3-nitrotyrosine (26). LDL isolated from human atherosclerotic lesions contains much higher levels of 3-nitrotyrosine than does circulating LDL, as monitored by isotope dilution gas chromatography-mass spectrometry (GC/MS), a sensitive and specific method (27). These observations indicate that reactive nitrogen species oxidize LDL in the human artery wall.

Cultured endothelial cells, macrophages, and smooth muscle cells, all components of the atherosclerotic lesion, generate

* The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; DTPA, diethylenetriaminepentaacetic acid; GC/MS, gas chromatography-mass spectrometry; HDL, high density lipoprotein; HPLC, high pressure liquid chromatography.

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superoxide anion (28), suggesting that NO could form ONOO− or other reactive nitrogen intermediates in vascular tissue. The finding that antibodies to 3-nitrotyrosine recognize epitopes on all of these cell types in the artery wall is consistent with this proposal (29). Moreover, elevated levels of nitrosated plasma proteins associate with an increased risk of coronary artery disease, suggesting that oxidants derived from NO might modify circulating proteins or proteins that find their way into the bloodstream (30). Recent studies revealed that fibrinogen is one target for nitration in plasma (31). Also, exposing fibrinogen to nitrooxidants in vitro accelerated clot formation, suggesting that fibrinogen nitration in vivo might be prothrombotic (31). However, it is unclear whether NO and superoxide produced at physiologically plausible rates nitrate tyrosine efficiently in vitro (32, 33), leaving open the possibility that 3-nitrotyrosine has a different origin in vivo.

NO can also autoxidize to nitrite (NO2−), and plasma levels of NO2 rise markedly during acute and chronic inflammation (34). Because NO2− is a substrate for myeloperoxidase and other peroxidases, it might also be used to nitrate tyrosine in vivo (35, 36). Indeed, myeloperoxidase uses H2O2 and NO2− to generate reactive nitrogen species that nitrate free and protein-bound tyrosine residues and promote lipid peroxidation of LDL in vitro (37–40). These reactions might be physiologically relevant because tyrosine nitration is markedly impaired in a model of peritoneal inflammation in myeloperoxidase-deficient mice by a reaction pathway that appears to require NO2− or other intermediates derived from NO (41). In human atherosclerotic lesions, most cell-associated myeloperoxidase is found in and around macrophages (16). However, the enzyme has also been detected in endothelial cells (42), raising the possibility that reactive intermediates produced by peroxidases might generate the epitopes on macrophages and endothelial cells that are recognized by antibodies to 3-nitrotyrosine.

In the current study, we determined whether reactive nitrogen intermediates also oxidize HDL in vivo and in vitro. HDL isolated from atherosclerotic lesions and from plasma of humans with established coronary artery disease contained high levels of 3-nitrotyrosine as assessed by isotopic dilution GC/MS analysis. Our observations strongly support the hypothesis that reactive nitrogen intermediates oxidize HDL in vivo.

EXPERIMENTAL PROCEDURES

Materials

Myeloperoxidase (donor-hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was isolated by lectin affinity and size exclusion chromatography from human neutrophils (43, 44) and stored at −20 °C. Purified enzyme had an A405/A600 ratio of 0.8 and was apparently homogeneous on SDS-PAGE analysis; its concentration was determined spectrophotometrically (ε405 = 0.17 μM−1 cm−1) (40). Cambridge Isotope Laboratories (Andover, MA) supplied 13C-labeled amino acids. 3-Nitro13C3-tyrosine was synthesized using tetranitromethane under basic conditions (46), and its concentration was determined by comparison with authentic material during reverse phase HPLC (46). Sodium hypochlorite (NaOCl), trichloroacetic acid, and HPLC grade CH3CN and methanol were obtained from Fisher. All of the organic solvents were HPLC grade.

Methods

Isolation of HDL—Blood from coronary artery disease patients was collected from healthy adults and patients with clinically and angiographically documented coronary artery disease who had fasted overnight. HDL (d = 1.125–1.210 g/ml) was prepared from plasma by sequential ultracentrifugation (47). Isolated HDL was depleted of apo E and apo B100 by heparin-agarose chromatography (47). The Human Studies Committees at University of Washington School of Medicine and Wake Forest University School of Medicine approved all protocols involving human material.

Isolation of Lesion HDL—Atherosclerotic tissue was harvested at endarterectomy, snap frozen, and stored frozen at −80 °C until analysis. Lesions from a single individual (~0.5 g of wet weight) were minced with a scalpel and pulverized in a stainless steel mortar and pestle. All of the subsequent procedures were carried out at 4 °C. Powdered tissue was suspended in 2 ml of antioxidant buffer (138 mM NaCl, 2.7 mM KCl, 100 μM diethylenetriaminepentaaetic acid (DTPA), 100 μM butyldiol hydroxyl tolune, protease inhibitor mixture (Roche Applied Science), 10 μM sodium phosphate, pH 7.4) in a 2-ml centrifuge tube and rocked gently overnight. Tissue was removed by centrifugation, the supernatant was collected, and the pellet was extracted a second time with antioxidant buffer for 1 h. The pooled supernatants were centrifuged at 100,000 × g for 30 min, and the pellet and uppermost lipemic layer were discarded.

HDL was isolated from the tissue extract by sequential density ultracentrifugation (d = 1.063–1.210 g/ml) (47). DTPA and butyldihydroxytoluene (each 100 μM) were included in all solutions used for lipoprotein isolation. Lesion HDL was equilibrated with buffer A (0.1 mM DTPA, 100 mM sodium phosphate, pH 7.4) using a 100-kDa cut-off filter device (Millipore, Bredford, MA). Apo A-I in lesion HDL was detected by Western blotting using a rabbit IgG polyclonal antibody to human apo A-I (Calbiochem, La Jolla, CA) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence detection. Protein was determined using the Lowry assay, with albumin as the standard (Bio-Rad).

Isolation of Plasma Proteins—Red blood cells were pelleted from blood anticoagulated with EDTA by centrifugation at 2000 × g for 10 min. Plasma was collected and stored at 4 °C. Freshly thawed plasma was diluted 20:1 (v/v) with buffer B (0.1 mM DTPA, 50 mM sodium phosphate buffer, pH 7.4). The plasma proteins were precipitated with ice-cold trichloroacetic acid (10% v/v), collected by centrifugation, washed with 10% trichloroacetic acid, and delipidated twice with water/methanol/water-washed diethyl ether (1:3, v/v/v). HDL Oxidation in Vitro—Reactions were carried out in phosphate buffer (20 mM sodium phosphate, pH 7.4, 100 μM DTPA) supplemented with 1 mg/ml HDL protein, 50 mM myeloperoxidase, 250 μM H2O2, and 500 μM NO2−. The reactions were initiated by the addition of oxidant and terminated by adding 2.5 mM methionine and 200 mM catalase. Following the addition of NaOH to the reaction mixture (final pH >9), 3-nitrotyrosine formation was determined in a nitroblue tetrazolium assay with monitoring absorbance at 430 nm. Concentrations of HOCl and H2O2 were determined spectrophotometrically (εmax = 350 μM−1 cm−1) (48, 49).

Immunohistochemical Studies—Hearts were excised at the time of cardiac transplantation in humans with cardiomyopathy (50). Coronary artery segments obtained from hearts were fixed in neutral buffered formalin and embedded in paraffin. Atherosclerotic plaques were identified by morphological criteria (50). Morphology was determined from 6-μm sections stained with Movat’s pentachrome stains. Macrophages, myeloperoxidase, 3-nitrotyrosine, and apo A-I were identified with monoclonal antibody HAM-56 (1:10 dilution; Dako Cytomation, Carpinteria, CA), rabbit polyclonal antiserum (1:300 dilution; Dako), immunofluorescence-purified rabbit polyclonal antibody (1:300 dilution; Upstate Biotechnology, Inc.), and goat polyclonal antiserum (1:750 dilution), respectively. Single-label immunohistochemistry used previously described techniques (50). Nova red (Vector Laboratories, Burlingame, CA), which yields a red reaction product, was used as the peroxidase substrate, and the cell nuclei were counterstained with hematoxylin. Protein Hydrolysis—HDL protein was precipitated with ice-cold trichloroacetic acid (10% v/v), collected by centrifugation, washed with 10% trichloroacetic acid, and delipidated twice with water/methanol/water-washed diethyl ether (1:3, v/v/v) (46). Isotopically labeled internal standards were added, and the samples were hydrolyzed at 110 °C for 16 h under argon with 6 M HCl and 500 μM sodium sulfonate, which was then quantified by reverse-phase HPLC (46). Acid hydrolysates were analyzed for 3-nitrotyrosine using the following ion-pairing reverse-phase HPLC conditions: 5 μm Hypersil ODS, pH 2.5, 0.1% acetonitrile and 0.1% phosphoric acid, 1 ml/min. 3-Nitrotyrosine was detected using negative ion chemical ionization GC/MS (52). The level of 3-nitrotyrosine was determined using the internal standard of 3-nitrotyrosine (M+–butyl(dimethyl)silyl)− and the ion of m/z 524 derived from 3-nitro[13C8]tyrosine. The level of 3-chlo-
rottyrosine was quantified using the ratio between the ion of m/z 489 derived from 3-chlorotyrosine ([M-H2O2-Cl-t-butyldimethylsilyl]+) and the ion of m/z 495 derived from 3-chloro[13C6]tyrosine. Potential artifact formation was monitored as the appearance of ions at m/z 528 (nitrination) or m/z 499 (chlorination) derived from L-[13C6]NO2-tyrosine added prior to sample work-up. Under these experimental conditions, artifact formation was <20% of 3-nitrotyrosine and <5% of 3-chlorotyrosine. l-Tyrosine is present at 10,000-fold higher levels than the oxidation products. Therefore the sample was diluted 1:100 and analyzed in a separate injection. l-Tyrosine and l-[13C6]tyrosine were quantified using the ions ([M-CO2-t-butyldimethylsilyl]+) at m/z 407 and m/z 413, respectively. Under these chromatography conditions, authentic compounds and isotopically labeled standards were base line-separated and exhibited retention times identical to those of analytes derived from tissue samples. The limit of detection (signal/noise > 10) was <100 attomol or <2 μmol/mol Tyr for all the amino acids. Results are normalized to protein content of Tyr, the precursor of 3-nitrotyrosine and 3-chlorotyrosine.

Statistical Analysis—Results represent the means ± S.E. The differences between two groups were compared using the Student’s t test. Correlations were determined using linear regression analysis for non-parametric data (Sigma Stat, SPSS). A p value of <0.05 was considered significant.

RESULTS

Myeloperoxidase Generates 3-Nitrotyrosine in HDL Protein under Physiologically Relevant in Vitro Conditions—To determine whether myeloperoxidase can nitrate tyrosine residues on HDL protein, we incubated the lipoprotein with the enzyme at neutral pH in phosphate buffer containing NO2 (500 μM) and H2O2 (250 μM). We monitored the formation of 3-nitrotyrosine spectroscopically by quantifying absorbance of the alkalinized reaction mixture at 430 nm.

3-Nitrotyrosine was readily detected in HDL exposed to the complete myeloperoxidase-H2O2-nitrite system. Nitration required each component of the reaction mixture: NO2, H2O2, and myeloperoxidase (Fig. 1A). The reaction depended on a NO2 concentration over a range of 0–1000 μM (Fig. 1B), was optimal at 250 μM NO2 (Fig. 1C), and was complete in 20 min (Fig. 1D). It was inhibited by the peroxide scavenger catalase (200 nM) (Fig. 1D) and the heme poison sodium azide (10 mM) (data not shown). These results indicate that myeloperoxidase nitrates HDL by a reaction that requires active enzyme, NO2, and H2O2.

Myeloperoxidase Generates 3-Nitrotyrosine by Directly Oxidizing NO2—It has been proposed that myeloperoxidase uses at least two distinct pathways to generate reactive nitrogen species (37). In the first pathway, the enzyme uses H2O2 and Cl to generate HOCI, which then reacts with NO2 to form nitryl chloride, a nitrating species. In the second pathway, myeloperoxidase uses a one-electron reaction to directly oxidize NO2 to nitric oxide radical, NO2. The radical might then oxidize tyrosine directly or might react with tyrosyl radical that myeloperoxidase also generates (38, 53).

To distinguish between these two pathways, we examined the effect of plasma concentrations of chloride ion (Cl−) or nitrite on nitration of HDL by the myeloperoxidase-H2O2-N02 system (Fig. 2). We also determined whether tauroine (2-aminoethanesulfonic acid), a potent scavenger of HOCI, inhibited nitration by the myeloperoxidase or HOCI-N02. The extent of HDL nitration by myeloperoxidase was independent of Cl− (Fig. 2A). Taurine also had no effect when myeloperoxidase nitrated HDL in the presence of Cl− (Fig. 2B). Moreover, we were unable to detect 3-nitrotyrosine in HDL exposed to HOCl-N02 (Fig. 2C). These observations indicate that HOCI produced by myeloperoxidase is not a major contributor to the nitration of HDL. Instead, the pathway likely involves direct oxidation of NO2 by compound 1 (a complex of myeloperoxidase and H2O2) and the reaction of the resulting NO2 with tyrosyl radical (54). It is noteworthy that myeloperoxidase preferentially oxidizes NO2 under these conditions, despite the presence of 200-fold greater levels of Cl−.

Myeloperoxidase Colocalizes with 3-Nitrotyrosine in Human Atherosclerotic Lesions—To determine whether apo A-I might be nitrated in vivo, we used immunohistochemical methods to study coronary arteries harvested from patients undergoing cardiac transplantation (n = 8). Apo A-I was rarely detected in nonatherosclerotic segments of these coronary arteries (data not shown). In contrast, the vast majority of lesions contained extracellular deposits of apo A-I (Fig. 3A), indicating that this protein is a characteristic component of atherosclerotic tissue (50).

Myeloperoxidase immunoreactivity was very prominent in intimal mononuclear cells. We detected such positive cells in all regions of atheroma, although immunoreactivity was especially evident in the subendothelial space, fibrous cap, and lipid core as well as near microvessels. We also detected extracellular myeloperoxidase immunoreactivity, both around macrophages...
Blood of Coronary Artery Disease Patients Has Nitrated HDL

**Figure 2. Effect of Cl⁻ or taurine on HDL nitration by the myeloperoxidase-H₂O₂-NO₂⁻ system or HOCl-NO₂⁻.** HDL was exposed for 60 min at 37 °C to myeloperoxidase in phosphate buffer supplemented with 250 μM H₂O₂, 500 μM NO₂⁻, and the indicated concentration of Cl⁻ (A) or the indicated concentration of taurine and 100 mM Cl⁻ (B). C. HDL was exposed for 60 min at 37 °C in phosphate buffer containing 500 μM NO₂⁻ and the indicated concentration of HOCl.

To establish which cells express myeloperoxidase, we immunostained atherosclerotic tissue with antibodies to myeloperoxidase and HAM-56, a specific marker for macrophages. These patterns were virtually identical, indicating that they were macrophages (Fig. 3D). Advanced plaques contained many cells that were positive for both myeloperoxidase and HAM-56, although some HAM-56-positive macrophages were negative for myeloperoxidase. These results indicate that human atherosclerotic lesions contain a major population of macrophages that express myeloperoxidase.

To determine whether reactive intermediates from myeloperoxidase might nitrate intimal proteins, we compared patterns of immunostaining for 3-nitrotyrosine and myeloperoxidase. These patterns were virtually identical (Fig. 3, B and D). Antibodies to both 3-nitrotyrosine and the enzyme reacted with material that associated closely with macrophages or was in the macrophages themselves. These observations raise the possibility that apo A-I is targeted for nitration in atherosclerotic intima. They also support the proposal that myeloperoxidase is an important pathway for generating 3-nitrotyrosine in the human artery wall.

**HDL Isolated from Human Atherosclerotic Lesions Contains 3-Nitrotyrosine**—To determine whether reactive nitrogen species damage lipoproteins in vivo, we quantified 3-nitrotyrosine in lesion HDL. We isolated the HDL by sequential ultracentrifugation from atherosclerotic tissue that was freshly harvested from patients undergoing carotid endarterectomy. To prevent artifactual oxidation of lipoproteins, we used buffers containing high concentrations of DTPA (a metal chelator) and butylated hydroxytoluene (a lipid soluble antioxidant). Western blotting with a monospecific rabbit antibody confirmed that lesion HDL contained a high concentration of apo A-I and a range of apparently larger immunoreactive proteins (Fig. 4A). Quantitative Western blotting demonstrated that apo A-I accounted for >50% of the protein in the HDL.

To quantify 3-nitrotyrosine, isolated HDL was delipidated and hydrolyzed, and the amino acids in the hydrolysate were isolated by solid phase extraction on a C18 column. The reisolated amino acids were derivatized and analyzed by GCMS with selected ion monitoring in the negative ion chemical ionization mode. The derivatized amino acids isolated from lesion HDL contained a compound that exhibited the major ion identical to that of authentic 3-nitrotyrosine (Fig. 4C). Selected ion monitoring showed that this ion (Fig. 4B) co-eluted with the ion derived from 13C-labeled internal standard (3-nitro[13C₆]tyrosine). In contrast, there was little evidence for 3-nitrotyrosine formation during sample work-up and analysis (3-nitro[13C₆]₉[15N]tyrosine; Fig. 4B). These results indicate that 3-nitrotyrosine is present in HDL isolated from human atherosclerotic lesions and that it is not an artifact of sample preparation.

**HDL Isolated from Human Atherosclerotic Lesions Is Enriched in 3-Nitrotyrosine**—To assess quantitatively the contribution of nitration to the oxidation of artery wall lipoproteins, we isolated HDL from plasma of healthy humans and from human atherosclerotic aortic tissue. HDL was delipidated and hydrolyzed, the resulting amino acids were isolated and derivatized, and the derivatized amino acids were quantified with isotope dilution GC/MS with selected ion monitoring (Fig. 5A). The concentration of 3-nitrotyrosine in HDL isolated from the atherosclerotic lesions was six times higher (619 ± 178 μmol/mol Tyr; n=10) than that in circulating HDL (104 ± 39 μmol/mol Tyr; n=17; p<0.01). These observations provide strong evidence that HDL is one target for damage by reactive nitrogen intermediates in the human artery wall.

**HDL Modified by Reactive Nitrogen Species Circulates in the Blood of Humans with Established Coronary Artery Disease**—To determine whether nitrated HDL also circulates in blood, we used isotope dilution GC/MS to quantify 3-nitrotyrosine levels in HDL isolated by sequential ultracentrifugation from the blood of healthy humans (8 males, ages 33–63) and humans with established atherosclerosis (7 males and 2 females, ages 33–67). The subjects with atherosclerosis had lesions documented by clinical symptoms and coronary angiography. The healthy subjects were normolipidemic with no known history of coronary artery disease.

Circulating HDL isolated from patients with established atherosclerosis contained a 2-fold higher concentration of 3-nitrotyrosine (136 ± 11 μmol/mol Tyr; n=9) than circulating HDL (68 ± 7 μmol/mol Tyr; n=8) isolated from the healthy humans (Fig. 5B; p<0.01). In a second analysis, we compared the levels of 3-nitrotyrosine in HDL and total plasma proteins isolated from the blood of the same individuals (Fig. 6). The mean level of 3-nitrotyrosine in HDL isolated from plasma of healthy subjects (n=5) was 7-fold higher (57 ± 10 μmol/mol Tyr) than that in total plasma proteins (8 ± 6 μmol/mol Tyr; p=0.01). These observations provide strong evidence that human blood contains HDL that is nitrated much more extensively than total plasma proteins and that 3-nitrotyrosine levels in circulating HDL are higher in humans with clinically established coronary artery disease than in healthy humans.

**Levels of 3-Nitrotyrosine Correlate Strongly with Those of 3-Chlorotyrosine in Circulating HDL but Not Lesion HDL**—To
determine whether myeloperoxidase might promote protein ni-
tration in vivo, we assessed the relationship between 3-chloro-
tyrosine, a marker of protein oxidation that is generated only by
myeloperoxidase at plasma concentrations of halide ion, and
levels of 3-nitrotyrosine in both circulating and lesion HDL (Fig.
7). Linear regression analysis demonstrated a strong correlation
between levels of 3-chlorotyrosine and levels of 3-nitrotyrosine
(\(r^2 = 0.51; p < 0.01\) in plasma HDL. In contrast, there was no
significant correlation (\(r^2 = 0.18; p = 0.10\)) between levels of
3-chlorotyrosine and those of 3-nitrotyrosine in lesion HDL.

These observations strongly support the hypothesis that my-
eloperoxidase promotes the formation of 3-chlorotyrosine and
3-nitrotyrosine in circulating HDL but suggest that other path-
ways also produce 3-nitrotyrosine in atherosclerotic tissue.

**DISCUSSION**

NO produced by endothelial cells regulates vasomotor tone
and inhibits smooth muscle cell proliferation and leukocyte
adhesion (21). The larger amounts produced by macrophages
help kill microbes and tumor cells. Under pathological condi-

**FIG. 3.** Immunohistochemical colocalization of apo A-I and proteins modified by reactive nitrogen species in human atherosclerotic plaque. Photomicrographs of neighboring sections of human coronary arteries harvested at cardiac transplant. Atherosclerotic plaque was immunostained for apo A-I (A), proteins containing 3-nitrotyrosine (B), macrophages (C), and myeloperoxidase (D). Positive immunohistochemical staining is indicated by a red immunoreaction product. Original magnification, 100× (hematoxylin counterstain).

**FIG. 4.** Detection by mass spectrometry of 3-nitrotyrosine in HDL isolated from plasma and atherosclerotic human aortic tissue harvested at surgery. Human atherosclerotic tissue was obtained from subjects undergoing carotid endarterectomy. Atherosclerotic lesions were frozen in dry ice and pulverized. Powdered tissue was suspended overnight in buffer (containing antioxidants and metal chelators) at 4 °C. HDL was isolated from the suspension by sequential ultracentrifugation. \(^{13}\)C-Labeled internal standards were added, and the protein was hydrolyzed with acid. A, Western blot analysis of HDL isolated from lesions and plasma with an antibody specific for apo A-I. Lanes 1–3, plasma HDL, 0.5, 0.1, and 0.05 \(\mu g\) of protein. Lane 4, lesion HDL, 1 \(\mu g\) of protein. Arrow, monomeric apo A-I. B, amino acids derived from HDL were isolated on a C18 solid phase column, derivatized, and analyzed by isotope dilution negative ion electron capture GC/MS with selected monitoring of ions of \(m/z\) 518 (for 3-nitrotyrosine), \(m/z\) 524 (for 3-nitro\(^{13}\)C\(_6\)tyrosine), or \(m/z\) 528 (for 3-nitro\(^{13}\)C\(_6\),\(^{15}\)N\)tyrosine derived from \(L\)-\(^{13}\)C\(_9\),\(^{15}\)N\)tyrosine). C, full scan mass spectrum of the \(t\)-butyl dimethylsilyl derivative of authentic 3-nitrotyrosine. Note that the major fragment ion has an \(m/z\) of 518.
Blood of Coronary Artery Disease Patients Has Nitrated HDL

In the current studies, we demonstrate that HDL is oxidized in vivo. Remarkably, we observed evidence that human blood contains HDL that is nitrated much more extensively than total plasma proteins. Thus, HDL can apparently be nitrated in vivo.

In our immunohistochemical studies of atherosclerotic lesions, myeloperoxidase colocalized with epitopes recognized by antibodies to 3-nitrotyrosine, suggesting that it was an important source of reactive nitrogen species in the artery wall. However, there was no significant correlation between levels of 3-nitrotyrosine and 3-chlorotyrosine, a specific product of myeloperoxidase (51), in HDL isolated from atherosclerotic lesions, suggesting that pathways independent of myeloperoxidase also nitrate HDL in the artery wall. Alternatively, macrophage scavenger receptors might bind and internalize chlorinated HDL and nitrated HDL at different rates, altering their relative concentrations in lesion HDL (57). It is also possible that chlorinated HDL and nitrated HDL are extracted with different efficiencies from vascular tissue. Collectively, our observations provide compelling evidence that HDL is a specific target for nitration in the human artery wall and suggest that myeloperoxidase is involved in one reaction pathway. Thus, nitrated HDL may represent a previously unsuspected biochemical link between inflammation, nitrosative stress, and atherogenesis.

We also found that circulating HDL was nitrated on tyrosine residues. Importantly, the HDL content of 3-nitrotyrosine was twice as high in humans with established coronary artery disease as in healthy subjects, suggesting that levels of nitrotyrosine might serve as a marker for clinically active coronary artery disease. We also observed a strong relationship between levels of 3-nitrotyrosine and 3-chlorotyrosine in plasma HDL, suggesting that myeloperoxidase is the major pathway for nitrating HDL that appears in the circulation.

To determine how myeloperoxidase might generate 3-nitrotyrosine in HDL, we examined two pathways that produce reactive nitrogen intermediates from NO$_2$ (37, 38, 53). In one pathway, myeloperoxidase first uses H$_2$O$_2$ to convert Cl$^-$ to

![Fig. 5. Mass spectrometric quantification of 3-nitrotyrosine in HDL isolated from plasma and human atherosclerotic lesions.](Image)

![Fig. 6. Mass spectrometric quantification of 3-nitrotyrosine in plasma proteins and in HDL isolated from plasma.](Image)

![Fig. 7. Association between 3-nitrotyrosine and 3-chlorotyrosine levels in HDL isolated from human atherosclerotic lesions and plasma.](Image)
HOCI, which then reacts with NO2 to yield NO2Cl, a nitrating and chlorinating intermediate (37, 58). Sulfur-containing compounds that scavenge HOCI should inhibit this pathway. The other pathway, which should not require Cl−, involves direct one-electron oxidation of NO2 to NO3 by compound I of myeloperoxidase. We found that nitration of HDL by the myeloperoxidase-H2O2-NO2 system was not dependent upon Cl− and also was insensitive to taurine, a potent scavenger of HOCI. Moreover, myeloperoxidase should preferentially oxidize NO2 because the oxidation potential of NO2 and Cl− are −0.99 and −1.36 V (58, 59), respectively. These observations suggest that, under our experimental conditions, a NO2/H2O2 oxidant is involved.

Myeloperoxidase is likely to use NO2− as a physiological substrate when it generates reactive nitrogen species. We previously showed that myeloperoxidase-deficient mice had a markedly lower level of free 3-nitrotyrosine than wild-type mice after intraperitoneal infection with bacteria (41). In contrast, the two strains had comparable levels of the nitrated amino acid when peritoneal inflammation was induced by cecal ligation and puncture. Although both models of intra-abdominal inflammation produced an intense neutrophil response and a marked increase in the level of 3-chlorotyrosine, they differed in one important respect: levels of NO2 and NO3 were 20-fold higher in mice infected intraperitoneally with bacteria than in mice subjected to cecal ligation and puncture (41). These results indicate that myeloperoxidase in vivo generates oxidants that can nitrate tyrosine. They also suggest that the enzyme produces these oxidants only when levels of NO2 and NO3 increase substantially.

Collectively, our observations indicate that reactive nitrogen species oxidize HDL in the human artery wall. Nitrated HDL also circulates in blood, and our preliminary studies suggest that humans suffering from clinically significant atherosclerosis contain elevated levels of the oxidized lipoprotein in their plasma. The detection of 3-nitrotyrosine in HDL isolated from arterial samples of Washington).