

Reactive Nitrogen Intermediates Promote Low Density Lipoprotein Oxidation in Human Atherosclerotic Intima*

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Christiaan Leeuwenburgh‡, Medora M. Hardy§, Stanley L. Hazen¶, Peter Wagner‡, Shuji Oh-ishi‡, Urs P. Steinbrecher||, and Jay W. Heinecke‡***

From the Departments of ‡Medicine and of **Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110, §Searle Research, Monsanto Company, St. Louis, Missouri 63167, and the ||Department of Medicine, University of British Columbia, Vancouver, BC Canada V5Z 4E3

Oxidized low density lipoprotein (LDL) may be of central importance in triggering atherosclerosis. One potential pathway involves the production of nitric oxide (NO) by vascular wall endothelial cells and macrophages. NO reacts with superoxide to form peroxynitrite (ONOO⁻), a potent agent of LDL oxidation *in vitro*. ONOO⁻ nitrates the aromatic ring of free tyrosine to produce 3-nitrotyrosine, a stable product. To explore the role of reactive nitrogen species such as ONOO⁻ in the pathogenesis of vascular disease, we developed a highly sensitive and specific method involving gas chromatography and mass spectrometry to quantify 3-nitrotyrosine levels in proteins. *In vitro* studies demonstrated that 3-nitrotyrosine was a highly specific marker for LDL oxidized by ONOO⁻. LDL isolated from the plasma of healthy subjects had very low levels of 3-nitrotyrosine (9 ± 7 μmol/mol of tyrosine). In striking contrast, LDL isolated from aortic atherosclerotic intima had 90-fold higher levels (840 ± 140 μmol/mol of tyrosine). These observations strongly support the hypothesis that reactive nitrogen species such as ONOO⁻ form in the human artery wall and provide direct evidence for a specific reaction pathway that promotes LDL oxidation *in vivo*. The detection of 3-nitrotyrosine in LDL isolated from vascular lesions raises the possibility that NO, by virtue of its ability to form reactive nitrogen intermediates, may promote atherogenesis, counteracting the well-established anti-atherogenic effects of NO.

An elevated level of low density lipoprotein (LDL)¹ is a major

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¶ Howard Hughes Medical Institute Physician Postdoctoral Fellow.

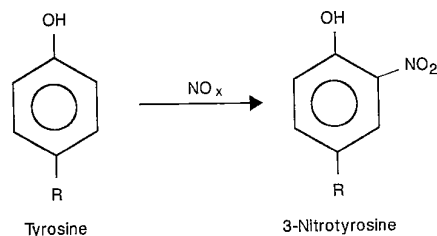
‡‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Division of Atherosclerosis, Nutrition and Lipid Research, Box 8046, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-6923; Fax: 314-362-0811; E-mail: heinecke@im.wustl.edu.

¹ The abbreviations used are: LDL, low density lipoprotein; BSA,

risk factor for premature atherosclerotic vascular disease. However, a wealth of evidence suggests that LDL must be oxidatively modified to damage the artery wall (1, 2). Pathways that oxidize lipid and protein may thus be pivotal to the development of atherosclerosis. LDL oxidation has been widely studied *in vitro*, but the mechanisms that promote oxidation within the artery wall remain poorly understood (2).

We have described one potential pathway for LDL oxidation that involves oxidants generated by myeloperoxidase, an enzyme secreted by phagocytes (3). Another pathway involves nitrogen monoxide (nitric oxide; NO) generated by vascular wall cells (4). NO is a relatively stable free radical that fails to oxidize LDL at physiological pH (5). However, NO reacts rapidly with superoxide to form peroxynitrite (ONOO⁻; Ref. 6), a reactive nitrogen species that promotes peroxidation of the lipid moiety of LDL *in vitro* (7). Cultured endothelial cells, macrophages and smooth muscle cells, all components of the atherosclerotic lesion, generate superoxide anion (2), suggesting that ONOO⁻ or other reactive nitrogen intermediates derived from NO could form in the artery wall.

In vitro studies demonstrate that ONOO⁻ spontaneously reacts with tyrosine residues to yield the stable product 3-nitrotyrosine (Scheme 1; Ref. 8). Macrophages and endothelial cells may play a role because antibodies for 3-nitrotyrosine detect epitopes in human atherosclerotic lesions that are associated predominantly with these two cell types (9). However, these studies did not quantify levels of 3-nitrotyrosine or evaluate the extent of LDL nitration.



SCHEME 1. Nitration of tyrosine by reactive nitrogen species (NO_x).

To explore the role of reactive nitrogen species in oxidative damage *in vivo*, we developed a quantitative assay for measuring levels of 3-nitrotyrosine. The method combines gas chromatography with stable isotope dilution mass spectrometry (GC-MS). Using this assay, we first investigated the relative yields of protein-bound oxidation products in bovine serum albumin (BSA) and LDL that were oxidized with ONOO⁻ *in vitro*. In both cases, the major product was 3-nitrotyrosine. Other widely studied oxidation systems failed to generate significant levels of 3-nitrotyrosine in LDL. These observations indicate that 3-nitrotyrosine is a specific marker for oxidative damage by reactive nitrogen intermediates. We then demonstrated that 3-nitrotyrosine levels are highly elevated in LDL isolated from human atherosclerotic tissue. These observations implicate reactive nitrogen species as one pathway for LDL oxidation in the artery wall.

bovine serum albumin; *m/z*, mass-to-charge ratio; ONOO⁻, peroxynitrite; NO, nitrogen monoxide; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials—Cambridge Isotope Laboratories (Andover, MA) supplied ^{13}C -labeled amino acids. 3-Nitro- $^{13}\text{C}_6$ tyrosine was synthesized using tetranitromethane (10), and its concentration was determined by reverse phase HPLC analysis by comparison with authentic material (11). All organic solvents were HPLC grade. Autopsy material was supplied by the Division of Surgical Pathology, Washington University School of Medicine. Vascular tissue resected within 10 h of death was immediately placed in ice-cold antioxidant buffer (100 μM diethylenetriamine pentaacetic acid (DTPA), 1 mM butylated hydroxytoluene, 1% (v/v) ethanol, 10 mM 3-aminotriazole, 50 mM NaH_2PO_4 , pH 7.4), and then frozen at -80°C until analysis.

Protein Oxidation in Vitro—Reactions were carried out at 37°C in Buffer A (50 mM phosphate, 0.1 mM DTPA, pH 7.4) supplemented with 1 mg protein/ml LDL or BSA (fatty acid-free; Boehringer Mannheim). ONOO^- was synthesized from 2-ethoxyethyl nitrite and H_2O_2 (12) and stored at -80°C . ONOO^- was thawed just prior to the experiment, and its concentration was determined spectrophotometrically ($\epsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1}$; Refs. 6 and 8). Reactions were initiated by the addition of ONOO^- . Samples were incubated for 5 min at 37°C .

Isolation of LDL—LDL ($d = 1.02\text{--}1.07 \text{ g/ml}$) was isolated by sequential density ultracentrifugation from plasma (1 mg/ml EDTA) prepared from normolipidemic, healthy volunteers (13). LDL was extensively dialyzed against Buffer A (50 mM phosphate, 0.1 mM DTPA, pH 7.4) at 4°C prior to experiments.

Lesion LDL was isolated from thoracic aortae that were thawed in Buffer C (0.15 M NaCl, 10 mM sodium phosphate (pH 7.4), 0.3 mM EDTA, 100 μM DTPA, 50 $\mu\text{g/ml}$ soybean trypsin inhibitor, 100 μM butylated hydroxytoluene, and 10 mM 3-aminotriazole). Fatty streaks and intermediate lesions from a single individual were resected from aortic tissue ($\sim 9 \text{ g}$ 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 C was added (5 ml/g tissue), and the tubes were rocked gently overnight. Tissue was removed by centrifugation at $5000 \times g$ for 15 min, the supernatant was subjected to centrifugation at $100,000 \times g$ for 30 min, and the pellet and uppermost lipemic layer were discarded. LDL in the remaining supernatant was isolated by sequential density ultracentrifugation ($d = 1.02\text{--}1.07 \text{ g/ml}$; Ref. 13). A metal chelator (100 μM DTPA) and NO synthase inhibitor (10 mM 3-aminotriazole; Ref. 14) were included in all solutions used for lipoprotein isolation. Lesion LDL was extensively dialyzed at 4°C under N_2 against Buffer A (50 mM phosphate, 0.1 mM DTPA, pH 7.4) and then against 0.1 mM DTPA (pH 7.4) prior to analysis.

Mass Spectrometric Analysis—BSA and LDL were precipitated at 4°C with ice-cold trichloroacetic acid (10% v/v). LDL lipids were extracted with methanol/water-washed diethyl ether and water-washed diethyl ether.² Protein residue ($\sim 0.5 \text{ mg}$) 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 (Sequal grade, Pierce) supplemented with 1% benzoic acid and 1% phenol. Amino acids were isolated from the acid hydrolysate using a C18 column.² Authentic 3-nitrotyrosine was stable to acid hydrolysis under these conditions, and recovery of the amino acid from the C18 column was $>80\%$. The *N*-propylheptafluorobutyl derivatives of the amino acids were prepared,² dried under N_2 , and redissolved in 50 μl of ethyl acetate, and 1- μl aliquots were analyzed on a Hewlett Packard 5890 Gas Chromatograph equipped with a 12-m DB-1 capillary column (0.20 mm inside diameter, 0.33- μm film thickness, J & W Scientific) interfaced with a Hewlett Packard 5988A Mass Spectrometer equipped with extended mass range. Both the injection and detector temperature of the gas chromatograph were set at 250°C . Full scan mass spectra and selected ion monitoring were obtained in the negative ion chemical ionization mode with methane as the reagent gas. The limit of sensitivity for all of the amino acids was $<1 \text{ nmol}$ (signal to noise > 10).

The mass spectrum of the *N*-propylheptafluorobutyl derivative of 3-nitrotyrosine included prominent ions at m/z 464 (M^-) and m/z 444 ($\text{M}^- \text{HF}$). The base ion (m/z 464) and its ^{13}C -labeled internal standard ion (m/z 470) were used for quantification. A 1:10 split injection was used for analysis of 3-nitrotyrosine; the initial column temperature of 70°C was increased to 180°C at $60^\circ\text{C}/\text{min}$ and then raised to 205°C at $4^\circ\text{C}/\text{min}$. *p*-Tyrosine, *o,o'*-dityrosine, phenylalanine, and *o*-tyrosine were subjected to GC-MS analysis as described.²

² Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) *J. Biol. Chem.* **272**, in press.

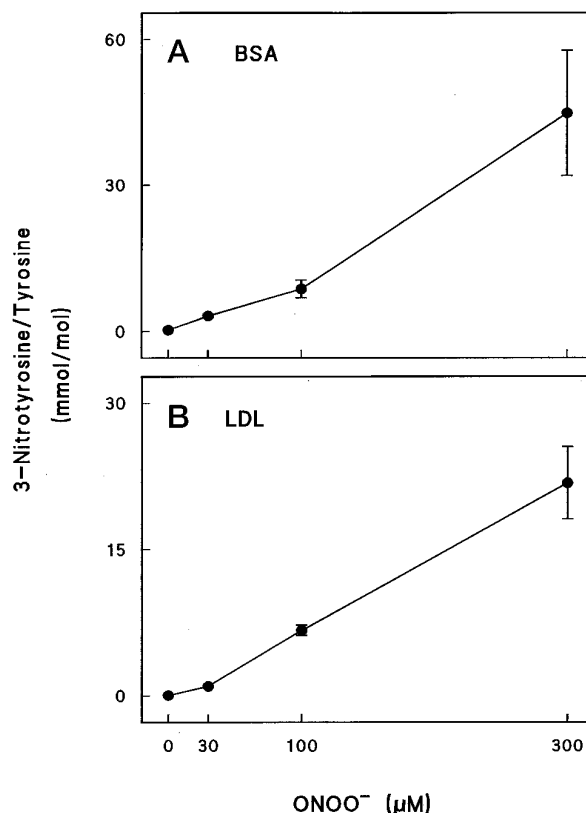


FIG. 1. Product yield of 3-nitrotyrosine in BSA (A) and LDL (B) oxidized *in vitro* with ONOO^- . BSA or LDL was exposed for 5 min at 37°C to the indicated final concentration of ONOO^- in Buffer A. 3-Nitrotyrosine content of the protein was then determined using stable isotope dilution GC-MS as described under "Experimental Procedures." The level of 3-nitrotyrosine is normalized to the content of the precursor amino acid L-tyrosine determined using ^{13}C -labeled internal standard.

Other Procedures—All buffers were passed over a Chelex 100 column (Bio-Rad) to remove transition metal ions. Myeloperoxidase was isolated as described (11). Protein was measured using the Markwell-modified Lowry procedure (16) with BSA as the standard. Results are presented as means \pm S.E.

RESULTS

ONOO⁻ Generates 3-Nitrotyrosine When It Oxidizes BSA and LDL—After exposing BSA to ONOO^- , we used stable isotope dilution GC-MS analysis to quantify the level of protein-bound 3-nitrotyrosine. Native BSA contained very low levels of 3-nitrotyrosine, but there was a dramatic, concentration-dependent increase after ONOO^- exposure (Fig. 1A). When BSA was added to the reaction mixture 2 min after ONOO^- , however, its 3-nitrotyrosine content barely increased. These results indicate that ONOO^- or short-lived intermediates derived from ONOO^- mediated nitration of protein tyrosyl residues.

ONOO^- also behaves like a hydroxyl radical in that it oxidizes the amino acids phenylalanine and tyrosine to the unnatural compounds *o*-tyrosine and *o,o'*-dityrosine (6, 17, 18). To determine whether these products were formed in proteins oxidized by ONOO^- , we quantified *o*-tyrosine and *o,o'*-dityrosine levels in BSA incubated with 0.3 mM ONOO^- . The levels of *o*-tyrosine and *o,o'*-dityrosine increased in oxidized BSA; however, the yield of the modified amino acids was $<5\%$ of the level of 3-nitrotyrosine. Nitration and hydroxylation rates of free amino acids are strongly influenced by H^+ (8, 17, 18). However, the pH of the reaction mixture measured immediately after addition of ONOO^- was not significantly altered. These results indicate that 3-nitrotyrosine is the major product

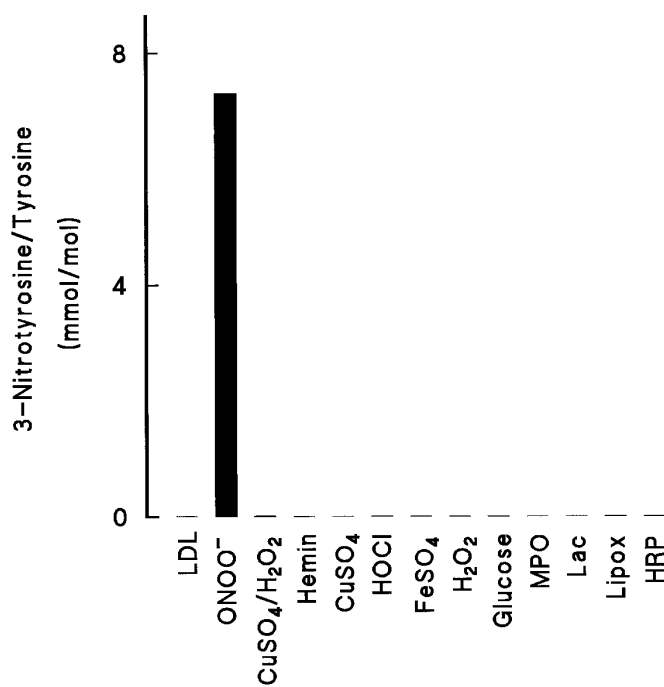


FIG. 2. Formation of 3-nitrotyrosine in LDL exposed to different oxidation systems. LDL (0.5 mg of protein/ml) was incubated at 37 °C in 50 mM phosphate buffer (pH 7.4), 100 mM NaCl alone (LDL), or with the indicated oxidation system. LDL was incubated with 100 μ M ONOO⁻ for 5 min. LDL was exposed to either HOCl alone (100 μ M) or myeloperoxidase (MPO; 21 nM), horseradish peroxidase (HRP; 10 μ g/ml), and lactoperoxidase (Lac; 10 μ g/ml) in buffer supplemented with 100 μ M H₂O₂ for 60 min. All other reactions were carried out for 24 h. Final concentrations of other oxidants were: CuSO₄, 10 μ M; CuSO₄ and H₂O₂, 100 μ M and 2 mM, respectively; FeSO₄, 10 μ M; hemin, 10 μ M; and glucose, 200 mM. LDL oxidation by lipoxygenase (Lipox) was performed in 50 mM borate (pH 9), 150 mM NaCl supplemented with phospholipase A₂ as described (15). At the end of the incubation, the 3-nitrotyrosine content of LDL was determined by stable isotope dilution GC-MS analysis.

when ONOO⁻ oxidizes BSA tyrosyl residues at physiological pH.

To explore the possibility that a lipid environment affects the susceptibility of tyrosine to nitration, we isolated LDL from plasma and exposed it to the concentrations of ONOO⁻ described above. Native LDL contained very low levels of 3-nitrotyrosine, but 3-nitrotyrosine increased dramatically when LDL was exposed to ONOO⁻ (Fig. 1B). The product yield of *o*-tyrosine and *o,o'*-dityrosine in LDL oxidized by ONOO⁻ was <1% of the yield of 3-nitrotyrosine. These results confirm that 3-nitrotyrosine is a major product of protein oxidation by ONOO⁻. They also suggest that nitration of protein tyrosine residues is favored over hydroxylation and hydrogen atom abstraction by this reactive nitrogen intermediate.

The 3-nitrotyrosine content of LDL depended on ONOO⁻ concentration (Fig. 1B), and adding LDL 2 min after ONOO⁻ drastically reduced the extent of protein nitration. At equal concentrations of ONOO⁻, the yield of 3-nitrotyrosine (expressed as millimoles of nitrotyrosine per mol of tyrosine) in LDL was only half that in BSA, perhaps because its lipids (or lipid-soluble antioxidants) compete with the apolipoprotein for ONOO⁻ (19) or because its tyrosine residues are less accessible to the short-lived nitrating intermediate.

3-Nitrotyrosine Is a Specific Marker for LDL Oxidized by Reactive Nitrogen Species—To evaluate the specificity of 3-nitrotyrosine as a marker for protein damage by reactive nitrogen species, we examined a variety of *in vitro* oxidation systems for their ability to generate 3-nitrotyrosine in apolipoprotein B100, the major protein of LDL.

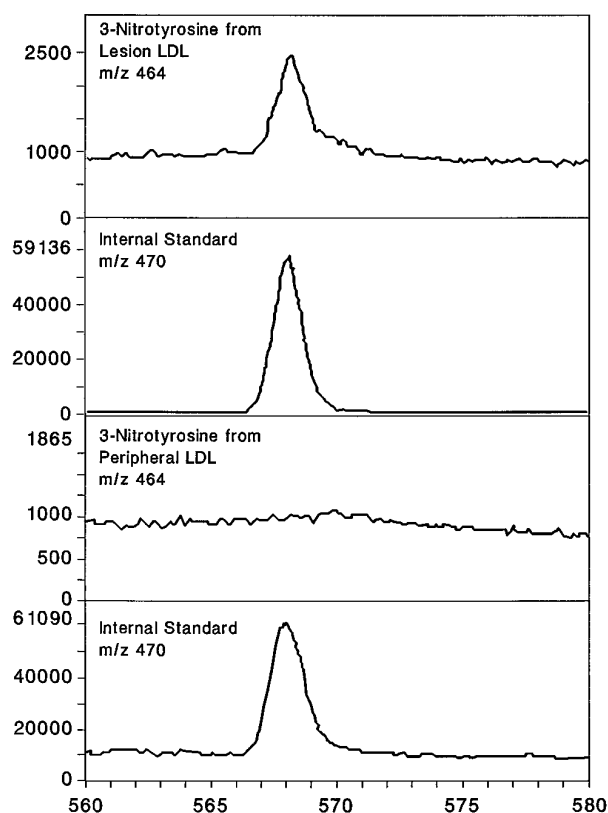


FIG. 3. Detection of 3-nitrotyrosine in LDL isolated from human atherosclerotic lesions by selected ion monitoring negative ion chemical ionization GC-MS analysis. Note co-elution of the major ion expected for 3-nitrotyrosine with that of authentic ¹³C-labeled 3-nitrotyrosine.

Significant levels of the nitrated amino acid were present in LDL exposed to ONOO⁻ (Fig. 2). In contrast, there was little change in the 3-nitrotyrosine content of LDL oxidized by copper, iron, a hydroxyl radical generating system (H₂O₂ plus copper), myeloperoxidase, lactoperoxidase, horseradish peroxidase, glucose, or lipoxygenase (Fig. 2). All of the systems oxidized LDL as monitored by the appearance of lipid oxidation products (thiobarbituric reacting substances assay and lipid hydroperoxides; Refs. 13 and 20). Collectively, these results demonstrate that 3-nitrotyrosine is a highly specific marker for LDL oxidation by reactive nitrogen species.

3-Nitrotyrosine Is Present at Greatly Elevated Levels in LDL Isolated from Human Vascular Lesions—To assess the possible role of reactive nitrogen intermediates such as ONOO⁻ in oxidizing lipoproteins *in vivo*, we isolated LDL (*d* = 1.02–1.07 g/ml) from human atherosclerotic tissue recovered at autopsy and then determined its 3-nitrotyrosine content. 3-Aminotriazole, an inhibitor of NO synthase (14) and myeloperoxidase (11), was included during tissue processing and lipoprotein isolation. Lesion LDL subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting analysis with a rabbit polyclonal antibody monospecific for human apolipoprotein B100 (21) demonstrated a protein with the predicted molecular mass of apolipoprotein B100. Forms of immunoreactive apolipoprotein B100 with lower molecular mass were also present, as previously reported by other investigators (1, 2).

LDL isolated from human atherosclerotic lesions was delipidated, hydrolyzed, and derivatized, and the derivatized amino acids were subjected to GC-MS analysis in the negative ion chemical ionization mode. We detected a compound in the amino acid hydrolysate that exhibited major ions and retention times identical to those of authentic 3-nitrotyrosine. Selected

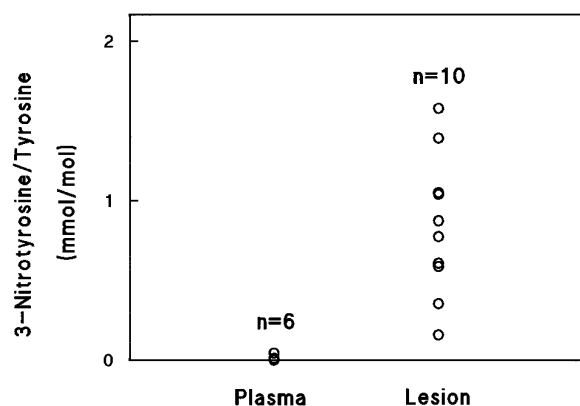


FIG. 4. Levels of protein-bound 3-nitrotyrosine in LDL isolated from plasma (*Plasma*) and human atherosclerotic tissue (*Lesion*). The 3-nitrotyrosine content of LDL was determined using stable isotope dilution GC-MS analysis as described under "Experimental Procedures."

ion monitoring showed that the ions derived from the amino acid co-eluted with those derived from 3-nitro[$^{13}\text{C}_6$]tyrosine (Fig. 3). The identity of 3-nitrotyrosine was confirmed further by comparison with an authentic standard using both heptafluorobutyl and pentafluoropropionyl derivatives. These results indicate that 3-nitrotyrosine is present in LDL isolated from human atherosclerotic lesions.

To determine whether protein nitration may contribute to the oxidation of artery wall lipoproteins, we isolated LDL from plasma and from human atherosclerotic aortic tissue. We then delipidated and hydrolyzed the proteins and subjected the derivatized amino acid hydrolysates to stable isotope dilution GC-MS analysis (Fig. 4). There was a striking 90-fold increase in the level of protein-bound 3-nitrotyrosine in lesion LDL ($840 \pm 140 \mu\text{mol/mol}$ of tyrosine; $n = 10$) compared with circulating LDL ($9 \pm 7 \mu\text{mol/mol}$ of tyrosine; $n = 6$).

DISCUSSION

In this study, we examined the potential role of reactive nitrogen species in LDL oxidation. *In vitro* studies demonstrated that 3-nitrotyrosine was a highly specific marker for LDL oxidized by ONOO^- , a reactive nitrogen species. Analysis of LDL isolated from human atherosclerotic lesions revealed a remarkable 90-fold elevation of the level of 3-nitrotyrosine compared with that in circulating LDL. These observations strongly support the hypothesis that reactive nitrogen intermediates derived from NO contribute to LDL oxidation in the artery wall.

A key question is whether NO generation by cells of the artery wall promotes or inhibits the development of atherosclerotic plaque. Cultured endothelial cells, macrophages, and smooth muscle cells generate superoxide (2), which may rapidly react with NO to form ONOO^- (6). Moreover, ONOO^- peroxidizes the lipid moieties of LDL *in vitro*, converting the lipoprotein to a form that is recognized by the macrophage scavenger receptor (7). Unregulated uptake of such modified lipoprotein may play a role in cholesterol accumulation by macrophages, a critical early step in atherogenesis (1, 2). In

keeping with this hypothesis, our detection of elevated levels of 3-nitrotyrosine in LDL isolated from atherosclerotic lesions suggests that reactive nitrogen intermediates derived from NO may indeed promote atherosclerotic vascular disease.

In contrast, other studies have shown that NO inhibits LDL oxidation by cultured macrophages (2, 5) and that NO may act as a chain-breaking antioxidant during lipid peroxidation (19). NO also may exert other anti-atherogenic effects *in vivo*, including inhibition of platelet aggregation and suppression of smooth muscle cell proliferation (4). Studies in hypercholesterolemic rabbits suggest that NO inhibits fatty streak formation, the cellular hallmark of the early atherosclerotic lesion (22). These results suggest that NO is anti-atherogenic in this animal model.

These apparently conflicting findings could be explained if the relatively stable free radical NO exerts potent anti-atherogenic effects while reactive intermediates derived from NO damage artery wall targets. The availability of superoxide may be critical in controlling this balance. Superoxide would both remove anti-atherogenic NO and produce pro-atherogenic ONOO^- . Thus, an imbalance in the pro- and anti-atherogenic effects of NO may be one important factor in the pathogenesis of atherosclerotic vascular disease.

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REFERENCES

- Witztum, J. L., and Steinberg, D. (1991) *J. Clin. Invest.* **88**, 1785–1792
- Berliner, J. A., and Heinecke, J. W. (1996) *Free Radical Biol. & Med.* **20**, 707–727
- Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) *J. Clin. Invest.* **94**, 437–444
- Moncada, S., and Higgs, A. (1993) *N. Engl. J. Med.* **329**, 2002–2012
- Jessup, W., Mohr, D., Giesig, S. P., Dean, R. T., and Stocker, R. (1992) *Biochim. Biophys. Acta* **1180**, 73–82
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1620–1624
- Graham, A., Hogg, N., Kalyanaram, B., O'Leary, V., Darley-Usmar, and Moncada, S. (1993) *FEBS Lett.* **330**, 181–185
- Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) *Methods Enzymol.* **233**, 229–240
- Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M.-A., Tarpey, M. M., and White, R. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 81–88
- Solkolovsky, M., Riordan, J. F., and Vallee, B. L. (1966) *Biochemistry* **5**, 3582–3589
- Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1996) *J. Biol. Chem.* **271**, 1861–1867
- Leis, J. R., Pena, M. E., and Rios, A. (1993) *J. Chem. Soc. Chem. Commun.* **16**, 1298–1299
- Heinecke, J. W., Suzuki, L., Rosen, H., and Chait, A. (1987) *J. Biol. Chem.* **262**, 10098–10103
- Buchmuller-Rouiller, Y., Schneider, P., Betz-Corradin, S., Smith, J., and Mauel, J. (1992) *Biochem. Biophys. Res. Commun.* **183**, 150–155
- Rankin, S. M., Parthasarathy, S., and Steinberg, D. (1991) *J. Lipid. Res.* **32**, 449–456
- Markwell, M. A. R., Haas, S. M., Breber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210
- Van der Vliet, A., O'Neil, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994) *FEBS Lett.* **339**, 89–92
- Ischiropoulos, H., and Al-Mehdi, A. B. (1995) *FEBS Lett.* **363**, 279–282
- Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaram, B., and Freeman, B. A. (1995) *Arch. Biochem. Biophys.* **324**, 15–25
- Savenkova, M. I., Mueller, D. M., and Heinecke, J. W. (1994) *J. Biol. Chem.* **269**, 20394–20400
- Krull, E. S., Tang, J., Keller, T. S., Clouse, R. E., and Schonfeld, G. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1069–1076
- Cooke, J. P., and Tsao, P. S. (1994) *Arterioscler. Thromb.* **14**, 653–655