Elevated levels of protein-bound *p*-hydroxyphenylacetaldehyde, an amino-acid-derived aldehyde generated by myeloperoxidase, are present in human fatty streaks, intermediate lesions and advanced atherosclerotic lesions

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Reactive aldehydes might have a pivotal role in the pathogenesis of atherosclerosis by covalently modifying low-density lipoprotein (LDL). However, the identities of the aldehyde adducts that form on LDL *in vivo* are not yet clearly established. We previously demonstrated that the haem protein myeloperoxidase oxidizes proteins in the human artery wall. We also have shown that *p*-hydroxyphenylacetaldehyde (pHA), the aldehyde that forms when myeloperoxidase oxidizes L-tyrosine, covalently modifies the N^{ϵ}-lysine residues of proteins. The resulting Schiff base can be quantified as N^{ϵ}-[2-(p-hydroxyphenyl)ethyl]lysine (pHA-lysine) after reduction with $NaCNBH_3$. Here we demonstrate that pHA-lysine is a marker for LDL that has been modified by myeloperoxidase, and that water-soluble, but not lipid-soluble, antioxidants inhibit the modification of LDL protein. To determine whether myeloperoxidase-generated aldehydes might modify LDL *in vivo*, we used a combination of isotope-dilution GC-MS to quantify pHA-lysine in aortic tissues at various stages of lesion evolution. We also analysed LDL isolated from atherosclerotic aortic tissue. Comparison of normal and atherosclerotic aortic tissue demonstrated a significant elevation (more than 10-fold) of the reduced Schiff base adduct in fatty streaks, intermediate lesions and advanced lesions compared with normal aortic tissue. Moreover, the level of pHA-lysine in LDL recovered from atherosclerotic aortic intima was 200-fold that in plasma LDL of healthy donors. These results indicate that pHA-lysine, a specific covalent modification of LDL, is generated in human atherosclerotic vascular tissue. They also raise the possibility that reactive aldehydes generated by myeloperoxidase have a role in converting LDL into an atherogenic lipoprotein.

Key words: antioxidant, carbonyl, hypochlorous acid, oxidative stress, oxidized low-density lipoprotein.

INTRODUCTION

An elevated level of low-density lipoprotein (LDL), the major carrier of blood cholesterol, is an important risk factor for atherosclerotic vascular disease. Paradoxically, LDL fails to exert atherogenic effects *in vitro*. It therefore seems that LDL must be modified to trigger the pathological events of atherosclerosis. A wealth of human and animal studies suggests that one such mechanism involves oxidative reactions in the artery wall [1–3].

Reactive aldehydes generated during lipid peroxidation are one possible class of agents that modify LDL *in vivo*. Aldehydes react with nucleophilic targets, such as the free ϵ-amino groups of apolipoprotein B100 of LDL, to form stable adducts [4,5]. Chemical modification of apolipoprotein B100 with decomposition products of lipid peroxidation renders LDL a ligand for the macrophage scavenger receptors and promotes foam cell formation [6], a key early event in the atherosclerotic process [1–3]. Moreover, monoclonal antibodies generated against LDL that has been modified by a variety of low-molecular-mass aldehydes recognize similar epitopes in human and animal atherosclerotic lesions, as well as in LDL recovered from human atheroma [7]. Despite intense interest in modification of apolipoprotein B100 as a prerequisite for receptor-mediated uptake of LDL, the identities of the relevant aldehydes and the chemical structures of the resulting adducts generated *in vivo* are still unclear.

One potential pathway for LDL oxidation involves myeloperoxidase, an abundant haem protein secreted by activated phagocytes [8]. Catalytically active myeloperoxidase is present in human atherosclerotic lesions, where it co-localizes with lipid-laden macrophages [9]. The enzyme uses H_2O_2 generated by cells to oxidize Cl^- to the potent cytotoxic oxidant HOCl [10]:

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O$$

Exposure of LDL to reagent HOCl *in vitro* results in lipoprotein aggregation and conversion into a high-uptake form for macrophages [11]. Apolipoprotein B100-rich lipoproteins with similar properties have been isolated from atherosclerotic lesions [12–14]. Antibodies generated against proteins that have been modified by HOCl recognize epitopes in human atherosclerotic lesions and in LDL recovered from human aortas, suggesting that HOCl promotes oxidative reactions in the artery wall [15]. Moreover, mass spectrometric studies demonstrated that 3-chlorotyrosine, a specific product of HOCl, is significantly elevated in human atherosclerotic lesions and in LDL recovered from diseased artery wall [16]. In the presence of L-tyrosine, myeloperoxidase also generates tyrosyl radicals [17], which can peroxidize lipids in LDL [18] and form *o*,*o*′-dityrosine cross-links in proteins [19]. Dityrosine is markedly enriched in fatty streak lesions and in

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; LDL, low-density lipoprotein; NICI, negative-ion chemical ionization; PFP, pentafluoropropionyl; pHA, *p*-hydroxyphenylacetaldehyde; pHA-lysine, N^{ϵ}-[2-(p-hydroxyphenyl)ethyl]lysine.

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LDL recovered from human atheroma [20], suggesting that tyrosyl radical generated by myeloperoxidase [17,19] or other reaction pathways [21] promotes protein oxidation in the artery wall early in atherogenesis. Similarly, in the presence of nitrite, a major end product of nitric oxide metabolism, myeloperoxidase generates reactive nitrogen species; this promotes LDL lipid peroxidation and the nitration of apolipoprotein B100 tyrosine residues [22,23]. Immunohistochemical [24] and mass spectrometric [25] studies demonstrate that 3-nitrotyrosine levels are markedly higher in atherosclerotic lesions and in LDL recovered from human atheroma. Collectively, these results implicate myeloperoxidase as a physiological catalyst of LDL oxidation *in vivo*.

Myeloperoxidase oxidizes the amino acids commonly found in plasma and interstitial fluid to form a family of amphipathic, freely diffusable aldehydes [26–28]. The products could therefore covalently modify susceptible target molecules [29–31]. Indeed, the major tyrosine oxidation product of phagocytes, *p*-hydroxyphenylacetaldehyde (pHA) [26], forms a Schiff base with the ϵ-amino group of lysine residues in proteins [29] and the amino moieties of phosphatidylethanolamine and phosphatidylserine of red blood cells and LDL [31]. With the use of MS, HPLC and NMR, we identified the reduced protein adduct as N^{ϵ}-[2-(p-hydroxyphenyl)ethyl]lysine (pHA-lysine) [29].

In the present study we used a combination of GC and isotope-dilution MS to demonstrate that levels of pHA-lysine are markedly elevated in human aortic tissue at all stages of the atherosclerotic process and in LDL recovered from human aortic vascular lesions. These observations provide unambiguous, quantitative chemical evidence for a specific covalent modification of lysine residues of LDL *in vivo*. They also implicate the myeloperoxidase/H_2O_2/Cl^-/tyrosine system as one pathway for oxidative damage in the human artery wall.

EXPERIMENTAL

Materials

^2H_2O, L-[$^{13}C_6$]lysine and L-[ring labelled $^{13}C_6$]tyrosine were purchased from Cambridge Isotopes. HPLC solvents were purchased from Baxter. Chelex-100 resin and fatty-acid-free BSA were purchased from Boehringer-Mannheim. Sodium phosphate, ethyl acetate, H_2O_2, HBr and NaOCl were purchased from Fisher Chemical Company. Peroxynitrite ($ONOO^-$) was provided by Monsanto Corporation (St Louis, MO, U.S.A.). All other reagents were purchased from either Sigma Chemical Co. (St Louis, MO, U.S.A.) or the sources indicated.

General procedures

N^{α}-Acetyl-N^{ϵ}-[$^{13}C_6$]pHA-lysine was synthesized and isolated as described previously [29]. Buffers were treated with Chelex-100 resin to remove transition-metal ions. All glassware and buffers were demonstrated to be chlorine-demand free before use [32]. Myeloperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) was isolated (final A_{430}/A_{280} ratio 0.6) as described previously [17,33]. Enzyme concentration was determined spectrophotometrically (ϵ_{430} = 178 $mM^{-1}\cdot cm^{-1}$) [34]. Protein concentration was determined with the Markwell-modified Lowry protein assay [35] with BSA as standard. H_2O_2 concentration was determined spectrophotometrically (ϵ_{240} = 39.4 $M^{-1}\cdot cm^{-1}$) [36]. LDL (d = 1.019–1.063 g/ml) was isolated from non-fasted healthy male and female subjects by sequential density ultracentrifugation [37]. LDL oxidation by lipoxygenase was performed as described [38].

Tissue collection

Fresh surgical specimens of human aortic tissue were obtained from transplant donors and vascular surgery patients. Tissue was immediately rinsed in ice-cold normal saline, placed in buffer A [65 mM sodium phosphate (pH 7.4)/100 μM diethylenetriamine-penta-acetic acid (DTPA)/100 μM butylated hydroxytoluene] and stored at −80 °C until analysis. LDL was isolated from the intima of thoracic aortas obtained at necropsy within 10 h of death. Autopsy tissue was rinsed in ice-cold PBS supplemented with 100 μM DTPA and stored in buffer A at −80 °C until analysis.

Tissue processing and LDL isolation from human atherosclerotic intima

Aortic tissue was thawed in buffer A supplemented with 100 mM $NaBH_3CN$. All subsequent steps were performed at 4 °C unless indicated otherwise. Atherosclerotic lesions were classified morphologically with the use of the criteria of the Pathobiological Determinants of Atherosclerosis in Youth Study [39]. Aortic intima was dissected from the medium, frozen in liquid nitrogen and pulverized under liquid nitrogen with a stainless steel mortar and pestle. Tissue powder (approx. 20 mg wet weight) was suspended in 5 ml of buffer A supplemented with 20 mM $NaBH_3CN$, 5 mM EGTA and 50 μM PMSF. The mixture was maintained in suspension with constant agitation at 37 °C for 1 h with additional $NaBH_3CN$ (20 mM increments) added from a fresh stock (200 mM $NaBH_3CN$ in water) every 10 min. After reduction, the mixture was delipidated twice with water/methanol/water-washed diethyl ether (1:3:7, by vol.). With each delipidation step, the single-phase extraction mixture was vortex-mixed and incubated for 30 min at 0 °C; the protein precipitate was recovered by centrifugation (5000 ***g*** for 10 min). The protein pellet was suspended in and dialysed against 50 mM sodium phosphate (pH 7)/100 μM DTPA, then dialysed against water supplemented with 100 μM DTPA.

Several control experiments were performed to evaluate whether the formation of protein-bound pHA-lysine was taking place *ex vivo*. First, homogenates of normal and atherosclerotic aortic intima were incubated under air in buffer A supplemented with [$^{13}C_6$]tyrosine overnight at 25 °C. Under these conditions, no detectable formation of [$^{13}C_6$]pHA-lysine occurred, as monitored by isotope-dilution GC-MS. Secondly, there was no significant change in the pHA-lysine content of either normal or atherosclerotic aortic tissue that had been incubated for 10 h in buffer A at room temperature before analysis. Thirdly, the levels of pHA-lysine were comparable in aortic tissue obtained at autopsy and fresh at the time of vascular surgery. Lastly, there was no significant increase in the content of pHA-lysine in aortic intima supplemented with 40 nM myeloperoxidase and incubated overnight under air in buffer A at 25 °C. These results indicate that post-mortem changes were unlikely to be contributing to protein modification by pHA.

Each preparation of lesion LDL was isolated from tissue harvested from a single donor. Lesion LDL was isolated by sequential density ultracentrifugation (d = 1.019–1.063 g/ml) from fatty streaks and intermediate lesions prepared from human thoracic aortic tissue essentially as described [32]. Freshly prepared $NaBH_3CN$ (20 mM) was included in all solutions used for lipoprotein isolation and in the buffers used for lipoprotein extraction from intima. Additional $NaBH_3CN$ (20 mM final concentration) was added to intimal tissue powder suspended in buffer after 1, 2, 3 and 4 h of incubation. High-resolution size-exclusion chromatography (sequential Superose 6 and 12

columns; Pharmacia LKB) of soluble lesion LDL preparations prepared by this method demonstrated elution profiles of immunoreactive apolipoprotein B100 and total cholesterol with an apparent molecular mass identical with that of LDL isolated from plasma. Western blotting of lesion LDL with a rabbit antibody (G. Schonfeld, Washington University School of Medicine, St Louis, MO, U.S.A.) monospecific for apolipoprotein B100 showed immunoreactive material that co-migrated with intact apolipoprotein B100 on SDS/PAGE, as well as both higher-molecular-mass and lower-molecular-mass forms of the protein [16,20,25]. Similar properties have been reported for lesion LDL isolated by other investigators [12–14].

Protein hydrolysis

Protein (approx. 250 μg) solution was dried under vacuum in a 2 ml glass reaction vial [40]. Known quantities of $^{13}C_6$-labelled internal standards and 0.5 ml HBr (6 M) supplemented with phenol (1 %, w/v) were then added to the vial. Samples were alternately evacuated and purged with argon gas five times. The argon-covered solution was hydrolysed at 120 °C for 24 h. The protein hydrolysate was diluted to 2 ml with 0.1 % (v/v) trifluoracetic acid and applied to a C_{18} mini-column (Supelclean, 3 ml; Supelco Co.) equilibrated with 0.1 % (v/v) trifluoroacetic acid. After a 2 ml wash with 0.1 % (v/v) trifluoroacetic acid, pHA-lysine was recovered with 2 ml of 20 % (v/v) methanol in 0.1 % (v/v) trifluoroacetic acid. Previous studies have shown that the adduct is stable to acid hydrolysis and recovered in more than 90 % yield during solid-phase extraction [29].

Samples were evaporated to dryness under either anhydrous nitrogen or vacuum before derivatization. n-Propyl esters were prepared by the addition of 200 μl 3.5 M HBr in propan-1-ol followed by heating at 65 °C for 60 min [40,41]. Propylated products were dried under nitrogen and pentafluoropropionyl (PFP) derivatives were generated by the addition of excess pentafluoropropionic acid anhydride (Pierce Chemical Co.) in ethyl acetate (1:3, v/v) and heating for 60 min at 65 °C. Heptafluorobutyryl derivatives of n-propyl ester-derivatized products were prepared by the addition of 50 μl of heptafluorobutyric acid anhydride/ethyl acetate (1:4, v/v) and heating for 60 min at 65 °C [40,41].

Mass spectrometric analysis

Derivatized amino acids were analysed on a Hewlett Packard 5890 gas chromatograph interfaced with a Hewlett Packard 5988A mass spectrometer equipped with an extended mass range [40]. Chromatographic separations were performed with a 30 m DB-17 capillary column (J & W Scientific; 0.25 mm internal diam., 0.25 μm film thickness) with helium as the carrier gas. An aliquot of derivatized sample was diluted 1:50 (v/v) with ethyl acetate and then 1 μl was analysed with a 1:50 split before mass analysis. The injector, transfer line and source temperatures were set at 250, 250 and 130 °C respectively.

Amino acids were quantified as their n-propyl, per-PFP derivative with selected ion monitoring in the electron-capture negative-ion chemical ionization (NICI) mode [40]. pHA-lysine was monitored by using the base ion at m/z 726 $(M–HF)^-$, another major fragment ion at m/z 598 $(M–PFP–H)^-$ and their corresponding isotopically labelled internal standard ions at m/z 732 and m/z 604. L-Tyrosine was monitored by using the base ion at m/z 367 $(M–PFP)^-$, another major fragment ion at m/z 495 $(M–HF)^-$ and their corresponding isotopically labelled internal standard ions at m/z 373 and m/z 501. L-Lysine was monitored by using the base ion at m/z 460 $(M–HF)^-$, another major fragment ion at m/z 440 $(M–2HF)^-$ and their corresponding isotopically labelled internal standard ions at m/z 466 and m/z 446.

Quantification was based on an external calibration curve constructed with each authentic compound and its isotopically labelled internal standard [40]. To ensure that interfering ions were not co-eluting with the analyte, the ratio of ion currents of two characteristic ions of each compound and its internal standard were monitored. All amino acids were baseline separated and co-eluted with ^{13}C-labelled internal standards. The limit of detection (signal-to-noise ratio more than 10:1) was less than 1 pmol for all compounds.

Statistical analysis

The statistical significance of differences between groups were assessed with the unpaired Student's t test.

RESULTS

Our previous studies demonstrated that pHA reacts with the ϵ-amino group of lysine residues in model proteins to yield a Schiff base, pHA-lysine. To determine whether the protein component of LDL would undergo this reaction, we exposed LDL to the myeloperoxidase/H_2O_2 system in sodium phosphate buffer supplemented with the metal chelator DTPA, and plasma concentrations of both Cl^- ion and L-tyrosine (100 mM and 100 μM respectively). After incubation for 2 h at 37 °C, we reduced the lipoprotein with $NaBH_3CN$, delipidated the product, hydrolysed apolipoprotein B100 with acid and determined the content of pHA-lysine by isotope-dilution GC-MS. In the presence of the complete myeloperoxidase system, pHA-lysine was readily detectable in the acid hydrolysate (Table 1). Its production required myeloperoxidase, H_2O_2, chloride and tyrosine and was blocked by the peroxide scavenger catalase. The haem poisoned azide, and cyanide also inhibited pHA-lysine formation (results not shown). These results indicate that myeloperoxidase generates pHA-lysine adducts on LDL by a reaction requiring H_2O_2, Cl^- and L-tyrosine.

Table 1 Effect of water-soluble and lipid-soluble antioxidants on pHA-lysine formation during LDL oxidation by the myeloperoxidase/H_2O_2/Cl^-/tyrosine system

LDL (512 μg/ml protein) was incubated with a mixture containing myeloperoxidase (40 nM), H_2O_2 (100 μM), NaCl (100 mM) and L-tyrosine (100 μM) at 37 °C for 2 h in 20 mM sodium phosphate (pH 7)/100 μM DTPA (complete MPO system) or in the additional presence of the indicated antioxidants (100 μM). At the end of the incubation, reaction products were reduced with 100 mM $NaBH_3CN$ in the presence of 100 mM ammonium acetate for 2 h at 37 °C. LDL was precipitated with 10% (w/v) trichloroacetic acid at 0 °C in a fume hood. Protein was then delipidated and subjected to acid hydrolysis; the pHA-lysine content was determined by isotope-dilution GC–MS. α-Tocopherol, β-carotene and probucol were added to LDL from concentrated ethanolic stocks [final concentration of ethanol less than 0.5% (v/v)] and incubated at 37 °C for 1 h under nitrogen before use. Results are means of duplicate determinations and are representative of the results found in two independent experiments.

	pHA-Lysine	
Addition to LDL	(nM)	(% of complete MPO system)
None	< 2	< 7
Complete MPO system	28	100
Complete MPO system plus		
Ascorbate	< 2	< 7
Uric acid	16	57
β-Carotene	29	104
Probucol	29	104
α-Tocopherol	32	114

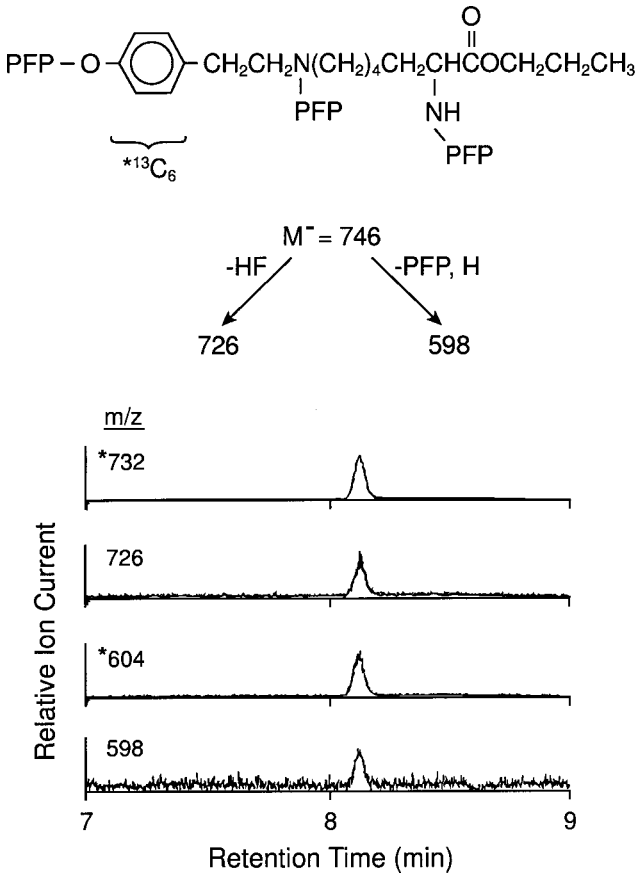

Figure 1 Electron-capture NICI–MS detection of pHA-lysine in human vascular tissue

Fatty streak intima from a fresh surgical specimen of human aorta was rapidly dissected, pulverized in liquid nitrogen and then reduced with $NaBH_3CN$. After delipidation, $^{13}C_6$-labelled internal standards were added, the protein was hydrolysed with acid, and amino acids were isolated from the acid hydrolysate by chromatography on a C_{18} mini-column. The n-propyl per-PFP derivatives of the amino acids were subjected to electron-capture NICI–GC-MS analysis with selected ion monitoring. Note that the retention times of ions were identical with the corresponding ions derived from $^{13}C_6$-labelled pHA-lysine internal standard.

pHA-lysine is a marker for the modification of LDL by the myeloperoxidase/H_2O_2/chloride/tyrosine system

At plasma levels of halides, Cl^- is the major substrate of myeloperoxidase; however, myeloperoxidase can also use H_2O_2 to convert Br^- and SCN^- ions into their respective hypohalous acids. To determine whether these oxidants could generate pHA-lysine, we exposed LDL to HOCl, HOBr or the myeloperoxidase/H_2O_2 system supplemented with various halides. A physiological concentration of L-tyrosine (100 μM) was included in all of the reaction mixtures. Significant levels of pHA-lysine were formed when LDL plus L-tyrosine were exposed to either the complete myeloperoxidase/H_2O_2/chloride system (29 nM) or reagent HOCl (23 nM). In contrast, no pHA-lysine was formed with HOBr, the major oxidant formed by eosinophil peroxidase [42]. pHA-lysine was also undetectable (< 0.1 nM) when LDL was incubated with myeloperoxidase, H_2O_2 and other halides (Br^-, I^- or F^-) or with the pseudohalide SCN^-. These results suggest that a chlorinating oxidant such as HOCl is an intermediate in the reaction that generates pHA-lysine. They also are consistent with our previous studies identifying the α-monochloramine of tyrosine as an intermediate in the myeloperoxidase-dependent formation of pHA [27,28].

To establish the specificity of pHA-lysine as a marker for myeloperoxidase-mediated protein modification, we examined the ability of a variety of oxidation systems *in vitro* to generate the adduct. pHA-lysine was undetectable in LDL exposed to L-tyrosine and Cl^- plus a wide variety of other oxidation systems: a hydroxyl-radical-generating system (copper plus H_2O_2) [21], redox-active free metals (iron or copper), a low molecular mass metal chelate (Cu/EDTA), haemin, other peroxidases (lactoperoxidase plus H_2O_2, or horseradish peroxidase plus H_2O_2), a reducing sugar (glucose), a lipid hydroperoxide-generating system (lipoxygenase plus phospholipase A_2) [38] and a reactive nitrogen species ($ONOO^-$). All of these systems produced the expected levels of lipid oxidation products (thiobarbituric reacting substances assay [43] and lipid hydroperoxides [18]) but failed to generate pHA. Collectively, these results suggest that pHA-lysine is a specific marker for modification of LDL by the myeloperoxidase/H_2O_2/chloride/tyrosine system.

pHA-lysine is present in surgical specimens of human atherosclerotic tissue

To determine whether the myeloperoxidase-generated adduct was present in vascular tissue, we obtained fresh aortic specimens during vascular surgery and during the harvesting of organs for transplantation. We then characterized the extent of aortic lesions, isolated intimal tissue and subjected it to delipidation, hydrolysis and derivatization. Electron-capture NICI–GC-MS analysis of the n-propyl per-PFP derivatives of the amino acids revealed a compound that exhibited major ions and a retention time identical with those of authentic pHA-lysine (Figure 1). Ions that co-chromatographed with ions derived from isotopically labelled pHA-lysine were observed at m/z 726 $(M–HF)^-$ and m/z 598 $(M–CF_3CF_2CHO)^-$ (Figure 1). Low-abundance ions with m/z 706 $(M–2HF)^-$ and m/z 746, the anticipated m/z of the molecular anion (M^-), were also observed (results not shown). These results suggest strongly that pHA-lysine is present in atherosclerotic lesions.

To confirm that pHA-lysine was the compound detected in acid hydrolysates of atherosclerotic tissue, we prepared the n-propyl perheptafluorobutyryl derivatives of amino acids and subjected them to electron-capture NICI–GC-MS analysis. Selected ion monitoring demonstrated major ions at m/z 876 $(M–HF)^-$, m/z 856 $(M–2HF)^-$ and m/z 698 $(M–CF_3CF_2CF_2CHO)^-$ that co-chromatographed with those derived from their corresponding isotopically labelled internal standard (results not shown). These results confirm that pHA-lysine is present in amino acid hydrolysates prepared from human atherosclerotic tissue.

pHA-lysine content of aortic intima varies with the stage of atherosclerotic disease

To determine whether levels of pHA-lysine are higher in atherosclerotic tissue than in normal aortic tissue, we used isotope-dilution GC-MS to quantify the amount of the adduct in human aortic intima at various stages of lesion evolution (Figure 2). Whereas there was a low level of pHA-lysine in normal aortic intima [43 ± 19 (mean $\pm$ S.E.M.) μmol of pHA-lysine/mol of lysine], there was a 15-fold higher content in fatty streaks (600 ± 100 μmol of pHA-lysine/mol lysine). There also was a similar elevation in intermediate lesions (650 ± 70 μmol pHA-lysine/mol of lysine), although advanced atherosclerotic lesions exhibited only 7-fold as much pHA-lysine (320 ± 85 μmol of pHA-lysine/mol of lysine) as normal aortic intima (Figure 2).

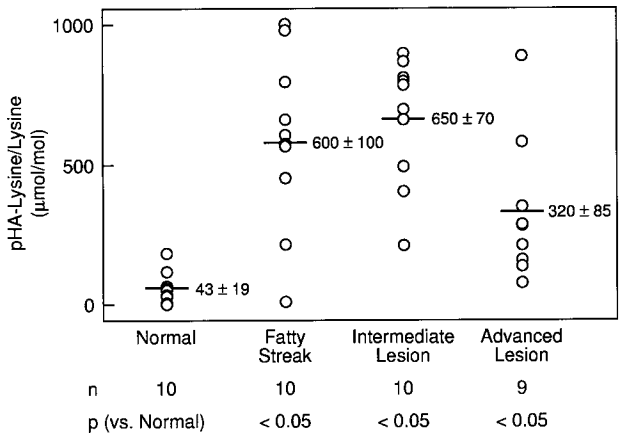

Figure 2 Quantification of pHA-lysine content of normal and atherosclerotic human aortic tissue harvested at surgery by isotope-dilution GC-MS

The content of pHA-lysine in intima from various stages of lesion evolution from human thoracic aortic tissue obtained at surgery was determined by isotope-dilution electron-capture NICI–GC-MS analysis. Each value (mean ± S.E.M.) is the result obtained with tissue harvested from a single donor.

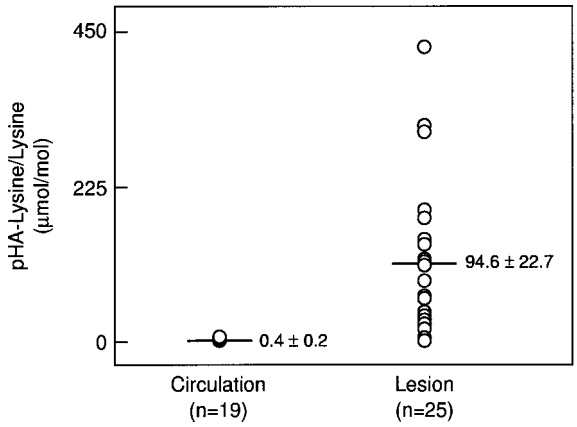

Figure 3 Quantification of pHA-lysine in LDL isolated from human atherosclerotic lesions and from plasma by isotope-dilution GC-MS

LDL was isolated by sequential ultracentrifugation from plasma and atherosclerotic aortas respectively. The pHA-lysine content of n-propyl per-PFP derivatives of amino acid hydrolysates prepared from the lipoproteins was determined by isotope-dilution electron-capture NICI–GC-MS analysis. Each value (mean ± S.E.M.) is the result obtained with LDL isolated from tissue harvested from a single donor.

Collectively, these results suggest that aldehydes produced by the myeloperoxidase system might modify proteins during all stages of the atherosclerotic process, and that such modifications might be particularly prevalent early in lesion development.

pHA-lysine levels are markedly elevated in LDL isolated from human atherosclerotic lesions

To determine whether LDL is modified by myeloperoxidase in the artery wall, we isolated the lipoprotein from atherosclerotic aortas and measured its pHA-lysine content by isotope-dilution NICI–GC-MS. Levels of the adduct were 200-fold higher than those observed in circulating LDL (lesion LDL, 95 ± 23 μmol of pHA-lysine/mol of lysine; circulation LDL, 0.4 ± 0.2 μmol of pHA-lysine/mol of lysine) (Figure 3). This finding indicates that pHA, a reactive aldehyde generated by myeloperoxidase, can modify LDL in the human artery wall.

Water-soluble but not lipid-soluble antioxidants inhibit pHA-lysine formation and LDL modification by the myeloperoxidase/H_2O_2/Cl^-/tyrosine system

The ability of antioxidants to inhibit the formation of pHA-lysine was quantified by monitoring the extent of adduct formation in reactions containing LDL, L-tyrosine, individual antioxidants and the myeloperoxidase/H_2O_2/Cl^- system (Table 1). All incubations contained equimolar amounts of antioxidant and H_2O_2 and were initiated by the addition of peroxide. pHA-lysine formation was potently inhibited by ascorbate, which reacts rapidly with HOCl [44]. Uric acid, another abundant aqueous antioxidant in plasma and interstitial fluid, partly attenuated pHA-lysine formation. Recent studies indicate that uric acid also scavenges HOCl (J. P. Henderson and J. W. Heinecke, unpublished work). In contrast, none of the lipid-soluble antioxidants examined were effective. These results are consistent with the idea that both the generation of pHA and the subsequent formation of a Schiff base by nucleophilic attack of the N^{ϵ}-amino group of protein lysine residues occurs in an aqueous compartment.

DISCUSSION

A wealth of evidence suggests that the oxidation of LDL is involved in the pathogenesis of atherosclerosis [1–3]. Aldehydes derived from oxidized LDL lipids have been proposed to have a critical role in mediating many of these events [1–7]. Similarly, diabetic vascular disease might result from the covalent modification of vascular wall and plasma proteins by glucose and other carbonyl compounds [45]. However, despite widespread interest in the potential importance of reactive aldehydes in the pathogenesis of vascular disease, little is known about the nature of the covalent adducts that form between aldehydes and proteins in human atheroma.

We have suggested that the myeloperoxidase-catalysed oxidation of common amino acids might be an alternative pathway for generating reactive aldehydes at sites of inflammation [26–31]. Our detection by GC-MS of pHA-lysine in LDL recovered from atherosclerotic intima is consistent with this hypothesis. Studies *in vitro* demonstrating that pHA-lysine is generated by HOCl, but not by a wide range of other hypohalous acids and oxidation systems, strengthen this conclusion. Because HOCl is a specific product of myeloperoxidase at plasma concentrations of halides, our results further implicate the enzyme in oxidative reactions in the artery wall. pHA-lysine was present at elevated levels in fatty streaks, intermediate lesions and advanced atherosclerotic lesions. These observations demonstrate that the covalent modification of apolipoprotein B100 by pHA is one mechanism for modifying LDL *in vivo* and that the reaction is taking place at all stages of the atherosclerotic process.

A key question is whether the covalent modification of LDL within the artery wall by myeloperoxidase-generated pHA is important in the development of vascular disease. We recently demonstrated that macrophages take up and degrade pHA-modified very-low-density lipoprotein more readily than native very-low-density lipoprotein [46]. Preliminary studies indicate that pHA generated by myeloperoxidase also converts LDL into a form that is rapidly degraded by human macrophages (J. W. Heinecke, S. L. Hazen and A. Chait, unpublished work). Our detection of elevated levels of pHA-lysine in LDL recovered from the artery wall suggests that myeloperoxidase-mediated modification of lipoproteins might help to transform macro-

phages into foam cells *in vivo*. It remains to be determined whether LDL modification by pHA is a pathological event or is a protective mechanism that facilitates the clearance of excess or potentially toxic LDL from the interstitial space.

Atherosclerosis exhibits many features of a chronic inflammatory condition [3]. Baseline plasma concentrations of C-reactive protein, an acute-phase reactant that serves as a marker for underlying systemic inflammation, independently predicts the risk of future myocardial infarction and stroke [47]. This and other findings implicate inflammatory cells such as monocytes, macrophages and lymphocytes in the development of atherosclerotic vascular disease [1–3]. Monocytes and subpopulations of tissue macrophages that normally participate in the immune response and host defences synthesize myeloperoxidase. Our detection of pHA-lysine in aortic intima from fatty streaks and more advanced lesions is consistent with continuing inflammation in atherogenesis. It also suggests that reactive aldehydes generated by myeloperoxidase might be a mechanism for mediating potent biological effects at different stages of atherosclerosis.

The oxidation hypothesis of atherosclerosis has focused considerable interest on the therapeutic potential of antioxidants [1–3]. Despite the promising results of epidemiological studies, results from the limited number of large prospective clinical trials have been mixed [48]. However, the pathways that promote LDL oxidation in the human artery wall are only recently becoming clear [49]. It remains to be determined whether these oxidation reactions are pathogenic and whether nutritional antioxidants such as vitamin E block the reactions *in vivo*. Our results *in vitro* indicate that antioxidants in the aqueous compartment, rather than lipid-soluble antioxidants such as vitamin E, inhibit pHA-lysine formation. Because both vitamin C and vitamin E are present in atherosclerotic tissue [50], the myeloperoxidase-dependent generation of pHA-lysine might occur within a protected environment *in vivo* in which antioxidant defences are compromised. One such environment might be the phagolysosome, where high concentrations of oxidants are generated. Indeed, antibodies against oxidized LDL react strongly with epitopes located in lysosome-like structures of macrophages [7].

The detection of elevated levels of chlorotyrosine in LDL isolated from atherosclerotic tissue is strong evidence that myeloperoxidase promotes the oxidative modification of lipoproteins *in vivo* [16]. The demonstration that 3-nitrotyrosine, *o*,*o*′-dityrosine and pHA-modified phosphatidylethanolamine are also increased in lesion LDL further supports a role for myeloperoxidase in contributing to the oxidative modification of diseased vascular tissues because these products can be formed by myeloperoxidase [20,25,31]. The present results identify yet another mechanism by which the myeloperoxidase system might modify LDL and arterial wall proteins during the evolution of atherosclerosis: the conversion of free amino acids into aldehydes that covalently modify amino groups of proteins. The biological consequences of pHA-lysine formation *in vivo* remain to be established. The many links between lipoprotein oxidation and myeloperoxidase-generated intermediates suggest that myeloperoxidase might influence the development of human vascular disease by a variety of mechanisms.

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