Activated human phagocytes employ the myeloperoxidase-H$_2$O$_2$-Cl$^-$ system to convert L-tyrosine to $p$-hydroxyphenylacetaldehyde (pHA). We have explored the possibility that pHA covalently reacts with proteins to form Schiff base adducts, which may play a role in modifying targets at sites of inflammation. Because Schiff bases are labile to acid hydrolysis, prior to analysis the adducts were rendered stable by reduction with NaCNBH$_3$. Purified pHA reacted with N$^\omega$-acetyllysine, an analog of protein lysine residues. The reduced reaction product was identified as N$^\omega$-acetyl-N$^\omega$-(2-($p$-hydroxyphenyl)ethyl)lysine by $^1$H NMR spectroscopy and mass spectrometry. The compound N$^\omega$-(2-($p$-hydroxyphenyl)-ethyl)lysine (pHA-lysine) was likewise identified in acid hydrolyzates of bovine serum albumin (BSA) that were first exposed to myeloperoxidase, H$_2$O$_2$, L-tyrosine, and Cl$^-$ and then reduced with NaCNBH$_3$. Other halides (F$^-$, Br$^-$, I$^-$) and the pseudohalide SCN$^-$ could not replace Cl$^-$ as a substrate in the myeloperoxidase-H$_2$O$_2$-L-tyrosine system. In the absence of the enzymatic system, pHA-lysine was detected in reduced reaction mixtures of BSA, L-tyrosine, and reagent HOCl. In contrast, pHA-lysine was undetectable when BSA was incubated with L-tyrosine and HOBr, peroxynitrite, hydroxyl radical, or a variety of other peroxidases, indicating that the aldehyde-protein adduct was selectively produced by HOCl. Human neutrophils activated in the presence of tyrosine also modified BSA lysine residues. pHA-lysine formation required L-tyrosine and cell activation; it was inhibited by peroxidase inhibitors and catalase, implicating myeloperoxidase and H$_2$O$_2$ in the reaction pathway. pHA-lysine was detected in inflamed human tissues that were reduced, hydrolyzed, and then analyzed by mass spectrometry, indicating that the reaction of pHA with proteins may be of physiological importance. These observations raise the possibility that the identification of pHA-lysine in tissues will pinpoint targets where phagocytes inflict oxidative damage in vivo.

Hypercholesterolemia and hyperglycemia are two important risk factors for atherosclerotic vascular disease (1, 2). A wealth of evidence indicates that low density lipoprotein (LDL), the major carrier of blood cholesterol, must be oxidatively modified to trigger the pathological events of atherosclerosis (3–7). Aldehydes derived from oxidized LDL lipids play a critical role in mediating many of these events. Diabetic vascular disease may similarly result from covalent modification of vascular wall and plasma proteins by glucose, which in its open chain form possesses an aldehyde moiety (8–10). Thus, reactive aldehydes derived from oxidized lipids and reducing sugars may be of central importance in atherogenesis.

Despite widespread interest in the potential importance of reactive aldehydes in the pathogenesis of disease (3–12), little is known regarding the nature of the covalent adducts formed between aldehydes and proteins in vivo. Most studies have relied on immunohistochemical methods to detect aldehyde-modified proteins (13–16), and the exact structure(s) of the cognate epitope(s) is unknown. Indeed, the only well characterized protein-bound aldehydes in vivo are those generated by the reaction of reducing sugars with amino groups, such as glucoselysine (17), fructoselysine (17), pentosidine (18), and N$^\omega$-(carboxymethyl)lysine (19).

One potential pathway for vascular injury involves myeloperoxidase, a heme protein secreted by phagocytes (7, 20–23). Myeloperoxidase uses H$_2$O$_2$ generated by phagocytes to produce diffusible cytotoxic oxidants (20, 21, 24–29). Active myeloperoxidase is a component of human atherosclerotic lesions, and the enzyme co-localizes with macrophages in transitional lesions (22). Immunohistochemical studies suggest that proteins modified by myeloperoxidase are present in atherosclerotic tissue (30). We have recently shown that 3-chlorotyrosine, a specific product of myeloperoxidase, is present at elevated levels in human atherosclerotic aorta and in LDL recovered from atherosclerotic aortic intima (23). Thus, myeloperoxidase may contribute to lipoprotein oxidation in the artery wall.

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This paper is dedicated to the memory of Professor Herman Esterbauer, a pioneer in the study of reactive aldehydes in biology.

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The abbreviations used are: LDL, low density lipoprotein; BSA, bovine serum albumin; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography; M$^*$, molecular anion; m/z, mass-to-charge ratio; GC/MS, gas chromatography/mass spectrometry; PBS, phosphate-buffered saline; PFP, pentfluoropropionyl; pHA, $p$-hydroxyphenylacetaldehyde; pHA-lysine, N$^\omega$-(2-($p$-hydroxyphenyl)ethyl)lysine; TOCSY, total correlation spectroscopy.
The best characterized product of myeloperoxidase is hypochlorous acid (HOCl), which is generated from chloride ion (Cl\(^-\)) in a two-electron oxidation reaction (Equation 1; Refs. 21, 24, and 25).

\[
\text{Cl}^- + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \quad \text{(Eq. 1)}
\]

HOCl bleaches heme proteins (31), converts amine to chloramines (32–34), inactivates sulfhydryl groups (35), and chlorinates unsaturated lipids (36–38). Another substrate for oxidation by myeloperoxidase is l-tyrosine, which undergoes a one-electron oxidation reaction to form tyrosyl radical (26, 27). Tyrosyl radical converts protein-bound tyrosyl residues to o,p\(^\prime\)-dityrosine and initiates lipid peroxidation, which may render LDL atherogenic (26, 27, 39, 40). Recent studies indicate that o,p\(^\prime\)-dityrosine is elevated in human atherosclerotic lesions, suggesting that oxidative damage within the human artery wall is mediated, in part, by tyrosyl radical (41).

We have recently demonstrated that activated phagocytes also employ the myeloperoxidase-H\(_2\)O\(_2\)-Cl\(^-\) system to convert l-tyrosine to the amphipatic aldehyde, p-hydroxyphenylacet-aldehyde (pHA). At physiological concentrations of L-tyrosine to the amphipathic aldehyde, p-hydroxyphenylacet-aldehyde (pHA), is acid-stable and serves as a specific marker of valently modify proteins and identify the reduced Schiff base.

High Performance Liquid Chromatography—HPLC analysis of pHA was performed utilizing a C18 column (Beckman\textsuperscript{\textregistered} Murolon, 5-µm resin, 4.6 × 260 mm) equilibrated with solvent A (5% methanol, 0.1% trifluoroacetic acid, pH 2.5). Products were monitored by absorbance (A\(_{280}\)) and eluted at a flow rate of 1 ml/min with a nonlinear gradient generated with solvent B (90% methanol, 0.1% trifluoroacetic acid, pH 2.5) as follows: 0% solvent B over 0 min; isocratic elution at 35% solvent B for 20 min; 35–100% solvent B over 10 min. N\(^{-}\)Acetyl-pHA-lysine was isolated by reverse phase HPLC with the following gradient: 0–10% solvent B over 5 min; isocratic elution at 10% solvent B for 20 min; 10–100% solvent B over 10 min. Under these conditions, N\(^{-}\)acetyl-pHA-lysine is base-line resolved from pHA-lysine, free pHA, p-hydroxyphenylethanol and l-tyrosine.

Generation of pH-A-Lysine Adduct—Production of the protein-bound Schiff base was routinely quantified by reducing the products, subjecting them to acid hydrolysis, and then monitoring for the presence of the stable marker, pH-A-lysine (see below). Reactions were carried out under the conditions indicated in the figure legends. Schiff base adducts were reduced by addition of 10 mM NaCNBH\(_3\), and incubation for the indicated times at 37°C. Where indicated, 100 mM ammonium acetate, pH 7.2, was included in the reaction mixture during reduction to scavenge free pH-A.

Isolation of Membrane-associated and Soluble Proteins—Whole human blood anticoagulated with EDTA (5 mM) was diluted with phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.4; Sigma) supplemented with 0.1 mM DTPA and then modified with myeloperoxidase as indicated in the legend to Fig. 6. Following reduction with 10 mM NaCNBH\(_3\), in the presence of 100 mM ammonium acetate, the cells were pelleted by centrifugation at 5000 \(\times\) g for 15 min at 4°C, washed twice with PBS supplemented with 0.1 mM DTPA, and then homogenized on centrifugation at 100,000 \(\times\) g for 1 h at 4°C, delipidated with two sequential extractions with water-washed diethyl ether (1:1; v/v), and stored at -95°C.

Protein Hydrolysis—Pentafluoropropionyl (PFP) derivatives were generated by addition of [13C\(_6\)]pHA-lysine (300 nmol; a marker of protein content) and N\(^{-}\)acetyl-[13C\(_6\)]pHA-lysine (20 pmol) were added as internal standards. Following HBr hydrolysis and solid phase extraction on a C18 column, the content of pHA-lysine was determined by stable isotope dilution GC/MS as described below.

Preparation of N\(^{-}\)Acetyl-[13C\(_6\)]pHA-Lysine Internal Standard—\([13C\(_6\)]\)Tyrosine (2 mM) in 20 mM sodium phosphate, pH 7.0, was first converted to \([13C\(_6\)]\)pHA by addition of NaOCl as described above. N\(^{-}\)Acetyllysine (4 mM) was then added, the mixture was incubated at 37°C for 4 h, and the Schiff base was reduced by overnight incubation with 10 mM NaCNBH\(_3\). N\(^{-}\)Acetyl-[13C\(_6\)]pHA-lysine was isolated by reverse phase HPLC as described above.

Protein Hydrolysis—\(\sim 250 \mu\)g solutions were dried under vacuum in 2-ml glass reaction vials. Following the addition of \(12\) labeled internal standards and 0.5 ml of 6 N HBr supplemented with phenol (1%, w/v), samples were alternately evacuated and purged five times with argon gas. The argon-covered solution was hydrolyzed at 120°C for 24 h. The protein hydrolysate was diluted to 2.0 ml with 0.1% trifluoroacetic acid and applied to a C18 column (Supelpak LC-18, 5 ml, Supelco Co.) equilibrated with 0.1% trifluoroacetic acid. The column was washed with 2 ml of 0.1% trifluoroacetic acid, and pHA-lysine was recovered with 2 ml of 20% methanol in 0.1% trifluoroacetic acid.

Samples were evaporated to dryness under either anhydrous N\(_2\) or vacuum prior to derivatization. n-Propyl esters were prepared by the addition of 200 µl of 3.5 M HBr in n-propyl alcohol followed by heating at 65°C for 30 min. Propylated products were dried under N\(_2\), and pentafluoropropionyl (PFP) derivatives were generated by addition of 50 µl of pentafluoropropionyl acid anhydride (Pierce) in ethyl acetate (1:1; v/v) and heating at 65°C. Pentafluorobutyl derivatives were prepared by adding 50 µl of heptafluorobutyric acid anhydride/ethyl acetate (1:4, v/v) and heating for 30 min at 65°C.

Tissue Collection and Processing—Residual material from human tissues was frozen on临床 research. Tissue samples were mixed 1:1 (w/v) with reduction buffer (100 mM NaCNBH\(_3\), 100 mM ammonium acetate, 1 mM NaN\(_3\), 10 µg/ml catalase, 50 mM sodium phosphate, pH 7.4) and incubated at 37°C for 1 h. Preliminary experiments confirmed that no additional pHA-lysine was generated under these conditions when samples were supplemented with 20 nm myeloperoxidase and 100 µM H\(_2\)O\(_2\).
All procedures involving human tissue were approved by the Washington University Human Studies Committee.

Reduced tissue samples were delipidated employing a single phase extraction mixture of H₂O/methanol/water-washed diethyl ether (1:3:7, v/v/v) as described previously (20). The protein pellet was washed twice with 10% trichloroacetic acid at 0 °C under a fume hood and then subjected to acid hydrolysis, solid-phase extraction on a C18 minicolumn, and derivatization for GC/MS analysis.

Mass Spectrometric Analysis—Amino acids were quantified using stable isotope dilution GC/MS in the negative ion chemical ionization mode. Samples were analyzed with a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5988A mass spectrometer. The mass spectrometer was operated in the negative ion chemical ionization mode using selected ion monitoring. pHA-lysine was monitored using the base peak at m/z 706 (M−H2F)−, another major fragment ion at m/z 706 (M−H2F)−, and their corresponding isotopically labeled internal standard ions at m/z 732 and 712. L-Tyrosine was monitored using the base peak at m/z 367 (M−PF3)−, another major fragment ion at m/z 495 (M−HF)−, and their corresponding isotopically labeled internal standard ions at m/z 373 and 501. L-Lysine was monitored using the base peak at m/z 460 (M−HF)−, another major fragment ion at m/z 440 (M−2HF)−, and their corresponding isotopically labeled internal standard ions at m/z 466 and 446.

Quantification was based on an external calibration curve constructed with each authentic compound and its isotopically labeled internal standard. To ensure that no interfering ions co-eluted with the analyte, the ratio of ion currents of two characteristic ions of each compound and its internal standard were routinely monitored. All amino acids were base-line separated and co-eluted with 13C-labeled internal standards. The limit of detection (signal/noise >10) was <1 pmol for all compounds.

NMR Studies—Analyses were performed at 25 °C in D₂O/H₂O (1:9, v/v) with a Varian Unity-Plus 500 spectrometer (499.843 MHz for 1H) equipped with a Nalorac indirect detection probe. 1H chemical shifts were referenced to external sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d₄ (TSP) or D₂O. The amide proton of Nα-acetyl-pHA-lysine is not visible at neutral pH due to rapid exchange. To facilitate structural assignment Nα-acetyl-pHA-lysine was acidified with DCl (Cambridge Isotopes Inc.) until exchange of the amide proton was inhibited. For proton and total correlation spectroscopy (TOCSY) experiments, the intense water signal was attenuated by transmitter pre-irradiation. The proton NMR spectrum of Nα-acetyl-pHA-lysine was recorded at 25 °C from 64 transients under the following conditions: pre-acquisition delay = 2 s, acquisition time = 1.89 s (37,760 complex data points), pulse width = 7 μs (80° flip angle), and spectral width = 10,000 Hz. The free induction decay was processed with a line broadening apodization of 1.0 Hz. For TOCSY eight transients were collected for each of 200 t₂ domain increments. A 10-ms mixing period was employed resulting in cross peaks for only the strongest scalar couplings (geminal and vicinal). The acquisition time was 0.256 s in t₂ (2048 complex data points) and 0.050 s in t₁ (200 data points). TOCSY data was processed by the hypercomplex method with Gaussian weighting in both t₁ and t₂ dimensions. Digital signal processing was employed to suppress artifacts arising from the intense water resonance.

RESULTS

pHA Forms a Schiff Base Adduct with the Free Amino Group of Nα-acetyllysine—We initially studied the reaction of pHA with Nα-acetyllysine, a model compound for free amino groups on proteins, to facilitate the isolation and characterization of products. Reactions were carried out at pH 7.4 and 37 °C in phosphate-buffered physiological salt solution. Reverse phase HPLC analysis of the complete reaction mixture after reduction with NaCNBH₃ revealed a single major product (Fig. 1; retention time 14.0 min). Formation of the compound required the presence of both pHA and Nα-acetyllysine (Fig. 1). In the absence of reduction, a compound with an identical retention time was observed; however, this product slowly decomposed under the acidic conditions employed for HPLC. The acid lability of the nonreduced compound suggested initial formation of a Schiff base between pHA and the Nα-amino group of Nα-acetyllysine. Reduction of the imine with NaCNBH₃ would then yield the acid-stable adduct (Scheme I). This hypothesis is strongly supported by structural analysis of the reduced form of the product (see below) which demonstrated that it was the Nα-acetyl derivative of Nα-(2-{p-hydroxyphenyl}ethyl)lysine (pHA-lysine).

To determine the structure of the compound, the reaction mixture was reduced with NaCNBH₃, and HPLC-purified material was derivatized and subjected to GC/MS analysis. A single major peak of material was apparent in the total ion chromatogram (Fig. 2, left panel). The negative ion chemical ionization mass spectrum of the n-propyl ester, per-PFP derivative of the compound (Fig. 2, right panel) was consistent with the proposed structure of pHA-lysine (Fig. 2, inset). The compound demonstrated a low abundance ion on selected ion monitoring at m/z 746, the anticipated m/z of the molecular ion (M⁺), that co-eluted with the major ions seen in the mass...
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The major product of the reaction between N\(^{-}\)acetyllysine and pH\(A\) (Fig. 1) was reduced with NaCNBH\(_\text{4}\) isolated by reverse phase HPLC, and its n-propyl ester, per-PFP derivative subjected to GC/MS as described under "Methods." Derivatized amino acids were eluted from a 15-m DB-5 capillary column (J & W Scientific; 0.35 mm inner diameter, 1.0-\(\mu\)m film thickness) with the following temperature gradient: 60–150 °C at 60 °C/min, then 150–250 °C at 10 °C/min. Left panel, total ion chromatogram of the derivatized product. Right panel, negative ion chemical ionization mass spectrometry of the derivatized product. The mass spectrum is consistent with the proposed structure of the n-propyl ester, per-PFP derivative of N\(^{-}\)acetyl-pHA-lysine.

2. Negative ion chemical ionization mass spectrum of the reduced Schiff base formed between pHA and N\(^{-}\)acetyllysine. The major product of the reaction between N\(^{-}\)acetyllysine and pH\(A\) (Fig. 1) was reduced with NaCNBH\(_\text{4}\) isolated by reverse phase HPLC, and its n-propyl ester, per-PFP derivative subjected to GC/MS as described under "Methods." Derivatized amino acids were eluted from a 15-m DB-5 capillary column (J & W Scientific; 0.35 mm inner diameter, 1.0-\(\mu\)m film thickness) with the following temperature gradient: 60–150 °C at 60 °C/min, then 150–250 °C at 10 °C/min. Left panel, total ion chromatogram of the derivatized product. Right panel, negative ion chemical ionization mass spectrometry of the derivatized product. The mass spectrum is consistent with the proposed structure of the n-propyl ester, per-PFP derivative of N\(^{-}\)acetyl-pHA-lysine.

\[ \text{pHA} \text{ Generated by the Myeloperoxidase-H}_\text{2} \text{O}_\text{2-Cl}^- \text{ System Covalently Modifies Lysine Residues of BSA—Preliminary experiments utilizing L-[14C]tyrosine demonstrated that BSA was covalently modified by an L-tyrosine-derived product in the presence of pHA and HOCl (or enzyme-bound hypochlorite; Refs. 51 and 52) as an intermediate in the formation of the reduced Schiff base (Fig. 3). Cross peaks permit the assignment of resonances of the reduced Schiff base (Fig. 3). Artifacts near 4.7 ppm in F1 and F2 arise from the intense water signal. With HOCl (or enzyme-bound hypochlorite; Refs. 51 and 52) as an intermediate in the formation of pHA and pHA-lysine.

pHA-Lysine Is a Specific Marker for Protein Modification by the Myeloperoxidase-H\(_\text{2}\text{O}_\text{2-Cl}^-\) Tyrosine System—To establish the specificity of pHA-lysine as a marker for protein modification by myeloperoxidase, we examined the ability of a variety of in vitro oxidation systems to generate the adduct on BSA in-
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| Condition | pHA-lysine nmol |
|-----------|----------------|
| Complete MPO system | 9.8 |
| Complete MPO system minus MPO | <0.1 |
| Complete MPO system plus Catalase | <0.1 |
| Complete MPO system plus NaCNBH$_3$ | <0.1 |
| Complete MPO system plus NaN$_3$ | <0.1 |
| Complete MPO system with other halides MPO + H$_2$O$_2$ + l-tyrosine | 15.4 |
| MPO + H$_2$O$_2$ + l-tyrosine + Br$^-$ | <0.1 |
| MPO + H$_2$O$_2$ + l-tyrosine + F$^-$ | <0.1 |
| MPO + H$_2$O$_2$ + l-tyrosine + I$^-$ | <0.1 |
| MPO + H$_2$O$_2$ + l-tyrosine + SCN$^-$ | <0.1 |
| Other oxidation systems CaSO$_4$ + H$_2$O$_2$ + l-tyrosine | <0.1 |
| ONOO$^-$ + l-tyrosine | <0.1 |

* The yield of pHA-lysine was 9.8% of H$_2$O$_2$ in the complete system and 15.4% of reagent HOCl.

**TABLE I**

pHA-lysine is a specific product of the myeloperoxidase-H$_2$O$_2$-Cl$^-$-tyrosine system

BSA (1 mg/ml) and l-tyrosine (100 μM) were incubated at 37 °C for 1 h in sodium phosphate (20 mM, pH 7.0), DTPA (100 μM), NaCl (100 mM), and the indicated oxidation systems. Reaction products were reduced for 2 h at 37 °C with 10 mM NaCNBH$_3$. BSA was acid-precipitated, and the pellet was washed twice with 10% trichloroacetic acid at 0 °C. Protein was subjected to acid hydrolysis and the pHA-lysine content determined by stable isotope dilution GC/MS as described under "Methods." The complete MPO system consisted of myeloperoxidase (MPO) (40 nm), H$_2$O$_2$ (100 μM), and NaCl (100 mM). Where indicated, catalase (10 μg/ml), NaN$_3$ (1 mM), or NaCN (1 mM) were included, or lactoperoxidase (10 μg/ml) or horseradish peroxidase (10 μg/ml) were substituted for myeloperoxidase. HOCI, HOBr, and peroxynitrite (ONOO$^-$) were used at 100 μM. The sodium salts (100 mM) of Br$^-$, F$^-$, I$^-$, and SCN$^-$ were present in place of NaCl where indicated. BSA was exposed to CuSO$_4$ (100 μM) and H$_2$O$_2$ (2 mM) for 24 h at 37 °C in the absence of DTPA. Results are the mean of duplicate determinations. Similar results were observed in two independent experiments.

The complete MPO system plus catalase and NaCNBH$_3$ for 1 h at 37 °C, and modified BSA was then precipitated with 10% trichloroacetic acid at 0 °C. The pellet was washed twice by resuspension in 1% Tween 20 and precipitated with ice-cold 10% trichloroacetic acid. Residual detergent was extracted with diethyl ether, and the dried pellet was subjected to acid hydrolysis with HBr as described under "Methods." The amino acid hydrolysate was analyzed by reverse phase HPLC employing a linear gradient of 0–100% methanol in 0.1% trifluoroacetic acid, pH 2.5, over 20 min at 1 ml/ min. L-Tyrosine derived from BSA co-elutes with pHA-lysine under these conditions and contributes to the absorbance at 276 nm. Fractions were dried and analyzed by scintillation spectrometry. The identity of the major radioactive compound as pHA-lysine was confirmed by GC/MS analysis.

hydrolysate co-migrated with authentic pHA-lysine on reverse phase HPLC (Fig. 5). The identity of the radiolabeled compound as pHA-lysine was confirmed by GC/MS analysis. Amino acid analysis demonstrated that lysine was the major target for covalent modification by pHA. Incubation of BSA (1 mg/ml) with purified pHA (1 mM) in the presence of NaCNBH$_3$ resulted in the consumption of 34% of total l-lysine residues in the protein. Small but consistent losses of l-arginine (~6%) were also observed and may account for the late eluting product seen in Fig. 5.

Schiff bases are in equilibrium with their parent aldehyde and amino moieties. In the presence of NaCNBH$_3$, the formation of pHA-lysine is enhanced by the reduction of existing Schiff bases. To estimate the number of protein lysine residues modified in the absence of a reducing agent, we incubated BSA with pHA and then stabilized the Schiff base adduct by reduction with NaCNBH$_3$ in the presence of high concentrations of ammonium acetate to scavenge non-reacted pHA. Amino acid hydrolysis confirmed that lysine was a major target for covalent modification by pHA under these conditions (Table II). The loss of l-lysine residues was accompanied by the appearance of a product that co-eluted with L-glycine on amino acid analysis and that likely represents pHA-lysine.

**FIG. 5.** Reverse phase HPLC analysis of BSA reacted with radiolabeled pHA. BSA (1 mg/ml) was incubated at 37 °C for 2 h with HPLC-purified $[^{14}$C]pHA (1.0 mM) in PBS supplemented with DTPA (100 mM). Reaction products were reduced by incubation with 10 mM NaCNBH$_3$ for 1 h at 37 °C, and modified BSA was then precipitated with 10% trichloroacetic acid at 0 °C. The pellet was washed twice by resuspension in 1% Tween 20 and precipitated with ice-cold 10% trichloroacetic acid. Residual detergent was extracted with diethyl ether, and the dried pellet was subjected to acid hydrolysis with HBr as described under "Methods." The amino acid hydrolysate was analyzed by reverse phase HPLC employing a linear gradient of 0–100% methanol in 0.1% trifluoroacetic acid, pH 2.5, over 20 min at 1 ml/ min. L-Tyrosine derived from BSA co-elutes with pHA-lysine under these conditions and contributes to the absorbance at 276 nm. Fractions were dried and analyzed by scintillation spectrometry. The identity of the major radioactive compound as pHA-lysine was confirmed by GC/MS analysis.

Both Membrane-associated and Soluble Proteins Are Modified by pHA—Previous studies revealed that ~90% of the pHA generated by activated neutrophils partitioned into the membrane fraction of the cells due to the amphiphilic nature of the aldehyde (28). We therefore determined whether myeloperoxidase-generated pHA preferentially modifies membrane-associated proteins. Whole blood was diluted with PBS and incubated with myeloperoxidase, H$_2$O$_2$, and physiological concentrations of l-tyrosine and Cl$^-$. After isolation of cells by centrifugation, the extent of pHA-lysine formation was determined in amino acid hydrolysates prepared from membrane-associated and sol-
H2O. The protein was then dried under vacuum, acid-hydrolyzed, and chromatography using a DG-10 column (Bio-Rad) equilibrated with incubation at 37 °C, modified protein was isolated by size exclusion reduce Schiff bases and scavenge free pHA, respectively). After a 2-h incubation at 37 °C, the protein was then dried under vacuum, acid-hydrolyzed, and subjected to amino acid analysis as described under "Methods." Cysteine, methionine, and tryptophan were not quantified. Values represent the mean of quadruplicate determinations and demonstrated less than 2% variability.

TABLE II

Amino acid composition of BSA modified by pHA

| Amino acid     | % native BSA |
|----------------|--------------|
| Alanine        | 100          |
| Arginine       | 101          |
| Aspartate/asparagine | 99      |
| Glutamate/glutamine | 100   |
| Glycine        | 145a         |
| Histidine      | 103          |
| Isoleucine     | 100          |
| Leucine        | 100          |
| Lysine         | 82           |
| Phenylalanine  | 101          |
| Proline        | 100          |
| Serine         | 101          |
| Threonine      | 97           |
| Tyrosine       | 98           |
| Valine         | 99           |

An unknown product co-eluted with glycine under these conditions.

Table. Substantial amounts of the adduct were generated on both membrane-associated and soluble proteins (Fig. 6, left panel). Selected ion monitoring GC/MS analysis demonstrated that the major ions expected for pHA-lysine co-eluted with those of synthetically prepared [13C6]pHA-lysine (Fig. 6, right panel). Generation of pHA-lysine required myeloperoxidase, H2O2, L-tyrosine, and protein. These results suggest that the amphipathic nature of pHA permits the aldehyde to react with cellular proteins in both lipid and aqueous environments. The relative enrichment of pHA-lysine in membrane-associated proteins may reflect high local concentrations of pHA or the location of the proteins at the interface between the intracellular milieu and extracellular space where free pHA is generated by myeloperoxidase.

**Human Neutrophils Form pHA-lysine Adducts on Model Proteins**—To determine whether activated human neutrophils similarly modified protein lysine residues with pHA, BSA was incubated with phorbol ester-activated human neutrophils in a balanced salt solution supplemented with plasma concentrations of L-tyrosine. BSA was then reduced with NaCNBH3, subjected to acid hydrolysis, and analyzed for the presence of pHA-lysine (Table III). Selected ion monitoring revealed the presence of multiple ions with the expected retention time and m/z of pHA-lysine. Moreover, the negative ion chemical ionization mass spectrum of the neutrophil-generated product was essentially identical to that of authentic pHA-lysine (compare Fig. 7 and Fig. 2, right panel).

**Covalent modification of lysine residues in BSA by activated human neutrophils**

Human neutrophils (1 × 106/ml) were incubated in medium A supplemented with BSA (1 mg/ml) and L-tyrosine (100 μM). Neutrophils were activated with phorbol ester (PMA) (200 nM) and maintained in suspension for 2 h at 37 °C by intermittent inversion (complete system). Neutrophils were removed by centrifugation, and Schiff bases in the supernatant were reduced by addition of NaCNBH3, subjected to acid hydrolysis, and the protein pellet was subjected to acid hydrolysis. The pHA-lysine content of the amino acid hydrolysate was determined by stable isotope dilution GC/MS. Results represent the mean of duplicate determinations. Similar results were observed in three independent experiments. SOD, superoxide dismutase.

| Condition      | pHA-lysine | pHA-lysine/BSA |
|----------------|------------|----------------|
| Complete system|            |                |
| Cells + L-tyrosine + BSA + PMA | 254 | 1.75 |
| Complete system minus Cells | <1 | <0.1 |
| L-tyrosine | <1 | <0.1 |
| PMA | <1 | <0.1 |
| Complete system plus SOD (10 μg/ml) | 466 | 3.22 |
| Catalase (200 μg/ml) | 7 | 0.05 |
| NaCNBH3 (10 mM) | 34 | 0.23 |
| NaCNBH3 (1 mM) | 12 | 0.08 |

Proteins were then subjected to amino acid analysis as described under “Methods.” Cysteine, methionine, and tryptophan were not quantified. Values represent the mean of quadruplicate determinations and demonstrated less than 2% variability.

**FIG. 6. Quantification of the pHA-lysine content of soluble and membrane-associated proteins exposed to the myeloperoxidase-H2O2-Cl–-tyrosine system.** Anticoagulated human blood (5 ml EDTA) was diluted with PBS containing 100 μM DTPA to a final concentration of 1 × 106 red blood cells/ml and then incubated for 1 h at 37 °C with 40 nM myeloperoxidase, 100 μM H2O2, 100 mM NaCl, and 100 μM L-tyrosine. Following reduction with 10 mM NaCNBH3, cells were pelleted, washed, and homogenized. Soluble and membrane-associated proteins were then isolated by ultracentrifugation as described under “Methods.” Left panel, the content of pHA-lysine in each protein fraction was determined by stable isotope dilution GC/MS as described under “Methods.” Right panel, detection of pHA-lysine in red blood cell proteins by selected ion monitoring. The base peak (m/z 726; M+–2HF) and another major fragment ion (m/z 706; M+–2HF) of pHA-lysine were monitored. * ions arising from [13C6]pHA-lysine internal standard.

**TABLE III**

Covalent modification of the lysine residues of BSA by activated human neutrophils

Human neutrophils (1 × 106/ml) were incubated in medium A supplemented with BSA (1 mg/ml) and L-tyrosine (100 μM). Neutrophils were activated with phorbol ester (PMA) (200 nM) and maintained in suspension for 2 h at 37 °C by intermittent inversion (complete system). Neutrophils were removed by centrifugation, and Schiff bases in the supernatant were reduced by addition of NaCNBH3, subjected to acid hydrolysis, and the protein pellet was subjected to acid hydrolysis. The pHA-lysine content of the amino acid hydrolysate was then determined by stable isotope dilution GC/MS. Results represent the mean of duplicate determinations. Similar results were observed in three independent experiments. SOD, superoxide dismutase.

| Condition | pHA-lysine | pHA-lysine/BSA |
|-----------|------------|----------------|
| Complete system |            |                |
| Cells + L-tyrosine + BSA + PMA | 254 | 1.75 |
| Complete system minus Cells | <1 | <0.1 |
| L-tyrosine | <1 | <0.1 |
| PMA | <1 | <0.1 |
| Complete system plus SOD (10 μg/ml) | 466 | 3.22 |
| Catalase (200 μg/ml) | 7 | 0.05 |
| NaCNBH3 (10 mM) | 34 | 0.23 |
| NaCNBH3 (1 mM) | 12 | 0.08 |

proteins to form a Schiff base.

**Neutrophils Activated in the Presence of Human Plasma Modify Protein Lysine Residues with pHA**—Human plasma possesses highly efficient antioxidant defense mechanisms (54,
protein modification by amino acid-derived aldehydes

**Fig. 7. Negative ion chemical ionization mass spectrum of pHA-lysine generated by activated human neutrophils.** Freshly harvested human neutrophils (1 x 10⁶/ml) were incubated in medium A supplemented with BSA (1 mg/ml) and L-tyrosine (100 μM). Cells were stimulated with phorbol myristate acetate (200 nM). Following a 2-h incubation at 37 °C, neutrophils were removed by centrifugation, and the supernatant was incubated with 10 mM NaCNBH₃ for 2 h at 37 °C. Protein was then precipitated with 10% trichloroacetic acid at 0 °C and washed three times with 10% trichloroacetic acid. ¹³C-Labeled internal standards were added, and the protein pellet was acid-hydrolyzed and subjected to GC/MS analysis as described under "Methods." Inset, proposed fragmentation pattern of the n-propyl ester, per PFP derivative of pHA-lysine. *, ions arising from [¹³C₆]pHA-lysine internal standard.

For example, oxidation of LDL by free metal ions is dramatically inhibited by the presence of <1% human plasma (55). To assess the potential physiological relevance of protein modification by neutrophil-generated pHA, we examined the effect of human plasma on pHA-lysine formation by phagocytes. Human neutrophils (4 x 10⁹/ml) were first incubated in medium supplemented with 200 μM L-tyrosine in the absence of human plasma. Following incubation at 37 °C for 4 h, products were reduced with NaCNBH₃ and the pHA-lysine content of total proteins determined by GC/MS analysis. Significant quantities of pHA-lysine were formed on endogenous proteins of phorbol ester-stimulated neutrophils (440 ± 110 nm pHA-lysine; mean ± S.E., n = 3) compared with resting neutrophils (<0.1 nm pHA-lysine; n = 3). Thus, phagocyte activation resulted in covalent modification of endogenous neutrophil proteins by pHA. The cell-mediated reaction was stimulated nearly 2-fold by superoxide dismutase and was inhibited by NaCN, NaN₃, or catalase. The presence of 20% human plasma markedly diminished adduct formation on total proteins in the reaction mixture; however, significant levels of pHA-lysine were still formed (6.4 ± 1.7 nm pHA-lysine; mean ± S.E., n = 3). Adduct formation required cell activation since minimal levels of pHA-lysine were generated in the absence of phorbol ester (0.11 ± 0.03 nm pHA-lysine; mean ± S.E., n = 3). Collectively, these results demonstrate that phagocyte activation results in covalent modification of proteins by the amino acid-derived aldehyde, pHA, through a peroxidase and H₂O₂-dependent mechanism. Furthermore, significant levels of the adduct were still formed in the presence of human plasma. Thus, formation of the Schiff base adduct might occur in vivo, particularly within a protected environment where local antioxidant defense mechanisms might become compromised.

**pHA-Lysine Is Present in Human Inflammatory Fluids**—To investigate whether pHA might react with proteins in vivo, we searched for the presence of pHA-lysine in a variety of inflammatory fluids. Human tissue samples were obtained from an infected site (a pilonidal cyst), sterile pus (a culture-negative peripancreatic abscess), and from an noninfectious inflammatory condition (arthrocentesis fluid from an acutely inflamed gouty knee). Samples from these sites were chosen because they should possess high numbers of activated phagocytes and were likely sites of pHA formation. Immediately following tissue collection, an aliquot of residual specimen was removed, supplemented with inhibitors of the myeloperoxidase-H₂O₂-Cl⁻ system (azide and catalase), incubated with NaCNBH₃ in the presence of excess ammonium acetate to scavenge free aldehyde, and then prepared for GC/MS analysis. As shown in Fig. 8 for abscess fluid, pHA-lysine was readily detected by selected ion monitoring in amino acid hydrolysates prepared from each of the tissues. These results demonstrate that pHA-modified proteins are present in vivo.

**Discussion**

Covalent modification of proteins by reactive aldehydes is thought to play a critical role in vascular disease and inflammation (3–19). Our results indicate that pHA, a major product of L-tyrosine oxidation by phagocytes, reacts with the free amino group of lysine residues on proteins to form a Schiff base (Scheme I). The structure of the reduced adduct was established as pHA-lysine by mass spectrometric analysis and high resolution NMR spectroscopy. pHA-lysine was the major adduct detected in amino acid hydrolysates prepared from model proteins that were first exposed to pHA and then reduced with NaCNBH₃ in the presence of ammonium acetate. This finding raises the possibility that the Schiff base adduct of pHA is a significant product of phagocyte activation. Indeed, we have identified pHA-lysine in acid hydrolysates prepared from inflamed human tissues that were treated with NaCNBH₃ and ammonium acetate, strongly suggesting that pHA forms a Schiff base with proteins in vivo.

pHA-lysine was detected in amino acid hydrolysates of model proteins exposed to either synthetically prepared pHA or to l-tyrosine oxidized by either HOCI, the myeloperoxidase-H₂O₂-Cl⁻ system, or activated human phagocytes. These results implicate free pHA generation by myeloperoxidase as a critical step in the reaction pathway (28). Consistent with this proposal, addition of the H₂O₂ scavenger catalase, and the peroxidase inhibitors azide or cyanide, inhibited pHA-lysine gener-
of other halides were unable to replace Cl\(^-\) in reactions of the reactive intermediate derived from HOCl. In contrast, a variety of phagocyte-generated aldehydes in a variety of inflammatory disease states.

The irreversible modification of proteins by lipid- and glucose-derived aldehydes has been implicated in the pathogenesis of diseases ranging from atherosclerosis to ischemia-reperfusion injury to diabetic vascular disease (3—19). The reversible covalent modification of proteins by Schiff base formation has received less attention. Schiff base formation by aromatic aldehydes has been proposed to modulate cytokine production in vitro (56), with potent effects on the immune system including antigen-induced T-cell proliferation, suppression of viral replication, and inhibition of solid tumor growth. One of the aldehydes employed in these pharmacological studies was pHA, which substantially enhanced T-cell proliferation in vitro and in vivo (56). These observations raise the possibility that phagocytes represent a physiological pathway for the production of aromatic aldehydes that regulate the immune system.

We have suggested that activation of phagocytes exposed to plasma concentrations of free amino acids and Cl\(^-\) might result in the generation of a family of low molecular weight, freely diffusible aldehydes (28, 57). These selectively reactive amphipathic aldehydes could then covalently modify susceptible target molecules. The detection of pHA-lysine in proteins at sites of inflammation is consistent with this hypothesis. Mass spectrometric studies quantifying structurally defined aldehydes and their adducts in human and animal tissues should further our understanding of the precise biochemical mechanisms underlying oxidative damage in vitro, with important implications for the role of activated phagocytes in the pathogenesis of disease.

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FIG. 8. Detection of pHA-lysine in human inflammatory tissue by selected ion monitoring: Residual fluid from an intra-abdominal abscess was mixed (1:1, v/v) with sodium phosphate buffer containing catalase, azide, NaCNBH\(_3\), and ammonium acetate and incubated for 1 h at 37 °C as described under “Methods.” Reduced tissue was then delipidated, washed with 10% trichloroacetic acid, and the protein residue subjected to acid hydrolysis as described under “Methods.” Amino acids were isolated using a C18 solid phase extraction column and subjected to GC/MS. The n-propyl-per FPP derivative of amino acids were eluted from a 30-m DB-17 capillary column (J & W Scientific; 0.25 mm inner diameter, 0.25-μm film thickness) with the following temperature gradient: 175 °C for 3 min, then 175–270 °C at 40 °C/min. Ions consistent with the n-propyl-per FFP derivative of pHA-lysine were observed at m/z 746 (M\(^+\)), m/z 726 (M – HF\(^-\)), m/z 706 (M – 2HF\(^-\)), m/z 682 (M – HF – CO\(_3\)^-), m/z 598 (M – CF\(_2\)CF\(_2\)CHO\(^-\)), and m/z 479 (M – CF\(_2\)CF\(_2\)COO-C\(_6\)H\(_4\)-CH\(_2\)CH\(_2\))\(^-\). The retention times of the ions were identical to the corresponding ions derived from \(^{13}\)C\(_6\)pHA-lysine internal standard (data not shown).
