Acrolein Is a Product of Lipid Peroxidation Reaction

FORMATION OF FREE ACROLEIN AND ITS CONJUGATE WITH LYSINE RESIDUES IN OXIDIZED LOW DENSITY LIPOPROTEINS

Koji Uchida‡, Masamichi Kanematsu, Yasujiro Morimitsu, Toshihiko Osawa, Noriko Noguchi§, and Etsuo Niki§

From the Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya 464-8601 and the §Research Center for Advanced Science and Technology, The University of Tokyo, Komaba, Tokyo 153, Japan

Lipoprotein peroxidation, especially the modification of apolipoprotein B-100, has been implicated to play an important role in the pathogenesis of atherosclerosis. However, there have been few detailed insights into the chemical mechanism of derivatization of apolipoproteins during oxidation. In the present study, we provide evidence that the formation of the toxic pollutant acrolein (CH2═CH-CHO) and its conjugate with lysine residues is involved in the oxidative modification of human low density lipoprotein (LDL). Upon incubation with LDL, acrolein preferentially reacted with lysine residues. To determine the structure of acrolein-lysine adduct in protein, the reaction of acrolein with a lysine derivative was carried out. Employing N-ε-acetyllysine, we detected a single product, which was identified to be N-ε-(3-formyl-3,4-dehydropiperidino)lysine. The acido hydrolysis of the adduct led to the derivative that was detectable with amino acid analysis. It was revealed that, upon in vitro incubation of LDL with acrolein, the lysine residues that had disappeared were partially recovered by N-ε-(3-formyl-3,4-dehydropiperidino)lysine. In addition, we found that the same derivative was detected in the oxidatively modified LDL with Cu²⁺ and that the adduct formation was correlated with LDL peroxidation assessed by the consumption of α-tocopherol and cholesteryl ester and the concomitant formation of cholesteryl ester hydroperoxide. Enzyme-linked immunosorbent assay that measures free acrolein revealed that a considerable amount of acrolein was released from the Cu²⁺-oxidized LDL. Furthermore, metal-catalyzed oxidation of arachidonate was associated with the formation of acrolein, indicating that polyunsaturated fatty acids including arachidonate represent potential sources of acrolein generated during the peroxidation of LDL. These results indicate that acrolein is not just a pollutant but also a lipid peroxidation product that could be ubiquitously generated in biological systems.

Atherosclerosis is a complex vascular disorder that can lead to more serious conditions such as myocardial infarction. The events in atherosclerosis are monocyte migration from the blood stream, its differentiation into macrophage in situ, uptake of LDL by macrophage scavenger receptor, transformation of lipid-laden macrophage into foam cells, smooth muscle cell proliferation and transformation into foam cells, and thus the accumulation of foam cells leading to fatty streaks and subsequent plaque formation. Various lines of evidence indicate that an important part of the pathogenesis of atherosclerosis is the oxidative modification of plasma low density lipoprotein (LDL) (1−5). It has been proposed that LDL undergoes oxidative modification before it can give rise to foam cells, the key component of the progression of atherosclerosis. It has been believed that the oxidation of LDL in vivo can be reproduced by in vitro incubation of LDL with cultured cells such as endothelial cells (6−9), smooth muscle cells (7, 10), and macrophages (11) or by auto-oxidation catalyzed by cupric ion in the absence of cells (12). These cell-mediated or metal-catalyzed modifications convert LDL to a form that is recognized by macrophages far more readily than normal LDL (6, 13). The uptake of oxidized LDL leads to the formation of foam cells, the dominant cells in atherosclerotic lesions (4). During incubation of LDL with cells, the LDL molecule undergoes a large number of structural changes that alter its metabolism (1). Although a detailed mechanism for the oxidative modification of LDL has not been established, it is generally accepted that the primary generation of lipid hydroperoxide derivatives initiates a reaction cascade leading to rapid propagation and to amplification in the number of reactive oxygen species formed; this ultimately leads to extensive fragmentation of the fatty acid chains (14) and conversion of the LDL to a more atherogenic form (15).

Acrolein, an unpleasant and troublesome by-product of overheated organic matter, occurs as a ubiquitous pollutant in the environment, e.g. incomplete combustion of plastic materials, cigarette smoking, and overheating frying oils. Acrolein is also a metabolite formed in the biotransformation of allyl compounds and the widely used anticancer drug cyclophosphamide. Since it was identified as one of the “unnatural” components of tobacco smoke (16), a number of reports have appeared describing the damaging effects of acrolein on the tracheal ciliatory movement (17) and the pulmonary wall (18). Its high reactivity indeed makes acrolein a dangerous substance for the living cell. It has been shown that acrolein reduces the colony-forming efficiency of mammalian cells, forms cyclic adducts with nucleosides in vitro, and is a potent mutagen (19). Among all α,β-unsaturated aldehydes, acrolein is by far the strongest electrophile and, therefore, shows the highest reactivity with...
Acrolein as a Lipid Peroxidation Product

16059

nucleophiles, such as the sulphydril group of cysteine, imidazole group of histidine, and amino group of lysine (19).

To understand the mechanism of the oxidative modification of lipoproteins in vivo, we initiated studies on the identification of covalently modified amino acids generated during in vitro incubation of protein with lipid peroxidation products. In the present study, we identified a novel acrolein-lysine adduct, N \(^{-}\)-acetyl-3-dehydroepipiperidino)lysine (FDP-lysine) and found that the adduct is generated during in vitro peroxidation of human plasma lipoproteins. In addition, we demonstrate that the formation of FDP-lysine in the oxidized LDL is mediated by the free acrolein during the peroxidation. We provide evidence that free acrolein is indeed formed during the peroxidation of polyunsaturated fatty acids.

EXPERIMENTAL PROCEDURES

Materials—N \(^{-}\)-Acetyl-l-lysine, N \(^{-}\)-acetyl-l-histidine, acrolein, arachidonate, and bovine serum albumin were obtained from Sigma.

Reaction of Acrolein with N \(^{-}\)-Acetylated Protein—The reaction mixture (10 ml) contained acrolein (1–100 mM) and N \(^{-}\)-acetyl-l-histidine (100 mM) in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 24 h at 37 °C, the reaction mixture was analyzed by 1H-1H COSY experiments.

Amino Acid Analysis—Changes in the amino acid composition and the formation of acrolein-lysine adduct (FDP-lysine) were assessed by amino acid analysis. The authentic acrolein adducts (N \(^{-}\)-acetyl-lysine, N \(^{-}\)-acetyl-histidine, and N \(^{-}\)-acetyl-arachidonate) or the FDP-lysine residue generated in proteins was treated with NaBH4 prior to the acid hydrolysis. An aliquot (0.1 ml) of LDL incubated in the absence or presence of acrolein or Cu\(^{2+}\) was treated with 0.1 M NaBH4 (10 mM) in 0.1 M sodium phosphate buffer (pH 7.4) under atmospheric oxygen. Following washing with PBS containing 10% Tween 20 (PBS/Tween), 100 μl of the LDL or arachidonate solution was added to the wells. After incubation for 2 h at 37 °C followed by washing with Tween (0.1% each well was filled with 200 ml of Block Ace solution (100 mg/ml) for 30 min at 37 °C. The monoclonal antibody (mAb5F6) was then added to the wells at 100 μl/well of peroxidase-conjugated anti-mouse IgG anti-serum (1:4000) and incubated for 1 h at 37 °C. After washing, 100 μl/well of 1 mg/ml 3,3′-diaminobenzidine was added and incubated for several minutes at room temperature. The reaction was terminated by adding 2 μl sulfuric acid, and the absorbance at 490 nm was read on a micro-ELISA plate reader.

RESULTS

Identification of a Novel Acrolein-Lysine Adduct—The selectivity of the binding of acrolein to lysine residues was assessed by changes in the amino acid composition of acrolein-treated proteins. When LDL (0.5 mg/ml) was incubated with 0.5 mM acrolein for 24 h at 37 °C, about 40% of the lysine residues was lost, and no significant change in other amino acids was observed. The data suggested that the lysine residues of proteins represented primary targets for reaction with acrolein. Hence, in an effort to determine the structure of the acrolein-lysine adduct generated in the protein, the reaction of acrolein with N \(^{-}\)-acetylated lysine was carried out.

Upon incubation of N-acetylated lysine (100 mM) with various concentrations of acrolein for 12 h at 37 °C, one product was detected exclusively even in the presence of a large excess of the lysine derivative (Fig. 1). After purification, the product was characterized by 1H-13C NMR and LC-MS along with 1H-1H COSY experiments. The connectivity from C-2 to C-6 of the product was established by analyzing the results of 1H-1H COSY experiments between the two acrolein-lysine structures in the product: δH 7.06, 6H, H-δ, 1.60 (2H, m, H-β), 1.71 (1H, m, H-β), 1.82 (3H, s, N-acetyl), 3.07 (2H, t, J = 8.3 Hz, H-ε), 4.14 (1H, dd, J = 9.0, 5.1 Hz, H-α), δC 21.5 (N-acetyl), 22.0 (C-γ), 23.0 (C-δ), 29.8 (C-β), 52.3 (C-α), 55.8 (C-ε), 174.2 (N-acetyl), 175.6 (COOH). Six signals (δC 23.3 t, 47.1 t, 48.4 t, 133.0 a, 148.8 d, and 193.6 d) remained in the 13C-NMR spectrum of the product, which seemed to originate from two molecules of acrolein in the reaction with N-acetyllysine. The 1H- and 13C-NMR spectra (in D\(_2\)O) of the product are shown in Fig. 1. Compared with the 1H- and 13C-NMR spectra between N-acetylated lysine and the product, the following signals were easily assigned for the presence of native N-acetylated lysine structure in the product: δH 1.25 (2H, m, H-γ), 1.60 (2H, m, H-δ), 1.60 (1H, m, H-β), 1.71 (1H, m, H-β), 1.82 (3H, s, N-acetyl), 3.07 (2H, t, J = 8.3 Hz, H-ε), 4.14 (1H, dd, J = 9.0, 5.1 Hz, H-α), δC 21.5 (N-acetyl), 22.0 (C-γ), 23.0 (C-δ), 29.8 (C-β), 52.3 (C-α), 55.8 (C-ε), 174.2 (N-acetyl), 175.6 (COOH). Six signals (δC 23.3 t, 47.1 t, 48.4 t, 133.0 a, 148.8 d, and 193.6 d) remained in the 13C-NMR spectrum of the product, which seemed to originate from two molecules of acrolein in the reaction with N-acetyllysine. The 1H- and 13C-NMR spectra of the product showed the presence of one methylene (δH 2.62/δC 23.3), two N-linked methylenes (δH 3.53, 3.96/δC 47.1 and δH 3.05, 3.48/δC 48.4), one olefinic methine (δH 7.06/δC 148.8), one formylmethylene (δH 9.21/δC 193.6), and one olefinic quaternary carbon (δC 133.0). The three signals (δH 7.06/δC 148.8, δH 9.21/δC 193.6, and δC 133.0) suggested the presence of conjugated double bond moiety (–CH=O–)). A partial structure, –CH₂–CH₂–CH=C(CH=O)–CH₂–, was constructed by analyzing the results of 1H-1H COSY experiments (Fig. 3). The connectivity from C-2 to C-6 of the product was

K. Uchida, M. Kanematsu, K. Sakaï, T. Matsuda, N. Hattori, Y. Mizuno, D. Suzuki, T. Miyata, N. Noguchi, E. Niki, and T. Osawa (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4882–4887.
Acrolein as a Lipid Peroxidation Product

Fig. 1. HPLC profiles of N\(^{-}\)-acetyl-lysine incubated with acrolein. The reaction mixture contained 100 mM N\(^{-}\)-acetyllysine and 1 mM (bottom), 10 mM (middle), or 100 mM (top) acrolein in 50 mM sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. HPLC was performed on a Develosil ODS-HG-5 column (8 × 250 mm) equilibrated in a solution of 5% methanol in 0.1% trifluoroacetic acid, flow rate being 2.5 ml/min. The elution profiles were monitored by absorbance at 227 nm. A major new peak was detected at about 17 min.

observed including the long-range coupling between C-2 and C-4 (indicated by bold lines and an arrow in Fig. 2). Finally, the six signals were assigned as follows: 6H 2.62 (2H, m, H-5), 3.05 (1H, dt, Ha-6), 3.48 (1H, dt, J = 12.5, 3.9 Hz, Hb-6), 3.53 (1H, d, J = 16.2 Hz, Ha-2), 3.96 (1H, d, J = 16.2 Hz, Hb-2), 7.06 (1H, br s, H-4), 9.21 (1H, s, 3-formyl); 8C 23.3 (C-5), 47.1 (C-2), 48.4 (C-6), 133.0 (C-3), 148.8 (C-4), and 193.6 (3-formyl). The LC-MS data for the product clearly showed the (M+H)\(^{+}\) peak at m/z 283 (Fig. 4). This was in agreement with the proposed structure (Fig. 5A). Based on these characteristics, it was determined that the product was a novel acrolein-lysine adduct, N\(^{-}\)-acetyl-FDP-lysine.

On the other hand, in a preliminary study using bovine serum albumin, it became evident that the reaction with acrolein leads to a significant loss of histidine residues. To characterize an acrolein-histidine adduct, we carried out the reaction of N\(^{-}\)-acetylhistidine with acrolein and isolated the formylethylated histidine derivative (N\(^{-}\)-acetyl-N-formylethylhistidine), which was previously reported by Pocker and Janjic (24). It has been proposed that the adduct is generated from the nucleophilic attack of the imidazole nitrogen on the ethylenic bond of acrolein. The adduct is presumed to be a mixture of the isomeric forms of N\(^{-}\)- and N\(^{\prime}\)-substituted adducts of the imidazole ring (Fig. 5B and C).

Formation of FDP-lysine in the Acrolein-modified LDL—The fact that FDP-lysine was the most predominant form of acrolein upon reaction with the lysine derivative led us to assess the formation of this adduct in the in vitro modification of the protein. To assess the formation of FDP-lysine in proteins, we first attempted to detect the adduct in the hydrolyzed samples of authentic acrolein-N\(^{-}\)-acetyllysine adduct (N\(^{-}\)-acetyl-FDP-lysine). Upon acid hydrolysis followed by amino acid analysis of N\(^{-}\)-acetyl-FDP-lysine, a new peak that was distinguishable from other amino acids was detected. Whereas, it should be noted that even if the acrolein-N\(^{-}\)-acetylhistidine adduct (N\(^{-}\)-acetyl-N\(^{\prime}\)-formylethylhistidine) was reduced with NaBH\(_4\), acid hydrolysis of the adduct gave a single peak that was eluted at a retention time completely identical to that of histidine (data not shown). This observation suggested that the acid hydrolysis of N\(^{-}\)-acetyl-N-formylethylhistidine led to quantitative release of the histidyl moiety as free histidine.

To assess the formation of FDP-lysine in protein, the hydrolysate of LDL treated with acrolein was analyzed. As shown in Fig. 6A, incubation of LDL with acrolein (0–1 mM) for 2 h at 37 °C resulted in the loss of up to 110.3 lysine residues per mole of LDL (the total number of lysine residues per mole of LDL apoB obtained by amino acid analysis was 390 molecules). These losses were accompanied by the formation of FDP-lysine (12.1 mol/mol), accounting for about 11% of the lysine residues that had disappeared. Fig. 6B demonstrates the time-dependent formation of FDP-lysine in the LDL treated with 0.5 mM acrolein at 37 °C. The concentration reached about 7 molecules of the adduct per protein molecule after 1 h of incubation. This accounted for about 30% of the lysine residues that had disappeared.

Formation of FDP-lysine in the Oxidized LDL—Of particular importance is the observation that FDP-lysine is detected in the oxidized LDL (Fig. 7). Various lines of evidence have indicated that an important part of the pathogenesis of atherosclerosis is the oxidative modification of plasma LDL (1–5). In addition, it has been shown that oxidation of LDL generates a variety of reactive aldehyde products that covalently bind to the LDL apolipoproteins (25–27). Hence, we examined the kinetics of LDL peroxidation and the formation of FDP-lysine. When the oxidation of LDL was induced by 5 \(\mu\)M Cu\(^{2+}\), a small amount of \(\alpha\)-tocopherol remained after 2 h of incubation (Fig. 8A). Cholesteryl esters, which have polyunsaturated acid moieties, were consumed with time, and all of them disappeared by 12 h of incubation (Fig. 8B). The depletion of cholesteryl ester occurred in the following order: cholesteryl docosahexaenoate...
cholesteryl arachidonate (CE20:4) and cholesteryl linoleate (CE18:2). The consumption of free cholesterol was observed after substantially all of the cholesteryl esters having polyunsaturated acid moieties were depleted. The formation of cholesteryl linoleate hydroperoxide (CE18:2-OOH) increased and then decreased due to its decomposition. Because the formation and decomposition of cholesteryl arachidonate hydroperoxide (CE20:4-OOH) and cholesteryl docosahexaenoate hydroperoxide (CE22:6-OOH) proceeded faster than that of CE18:2-OOH, the accumulation of CE20:4-OOH and CE22:6-OOH was not observed at 2 h of incubation. When the oxidation of LDL was initiated at a slower rate, the formation and decomposition of both CE20:4-OOH and CE22:6-OOH was observed (data not shown). The lipid peroxidation in LDL was accompanied by a time-dependent increase in the formation of FDP-lysine (Fig. 8C). The formation of the FDP-lysine reached a plateau as soon as CE18:2 was depleted. After 12 h of incubation, approximately 4 molecules of the adduct per LDL molecule were formed. The amount corresponded to 3.4% of the lysine residues that disappeared during LDL peroxidation. During this period, at least 750 molecules of CE18:2 per LDL molecule disappeared and 85 molecules of CE18:2-OOH per LDL molecule were accumulated.

**Peroxidation of Polyunsaturated Fatty Acids Generates Free Acrolein**—The observation that a significant amount of acrolein-derived lysine adduct (FDP-lysine) was detected in the oxidized LDL led us to examine whether LDL peroxidation is associated with the production of free acrolein. In the present experiments, to assess the formation of acrolein during LDL peroxidation, we developed the immunochemical procedure using the monoclonal antibody (mAb5F6) specific to FDP-lysine. Taking advantage of the fact that acrolein reacts with proteins and forms stable adducts with lysine and histidine residues, we developed a procedure that distinguishes free acrolein from acrolein covalently attached to the proteins. In this procedure, only free acrolein in the samples can react with the protein that has been coated on the immunoplate (Fig. 9A). The acrolein-derived epitopes generated in the coating protein are then detected by ELISA using the monoclonal antibody (mAb5F6) specific to FDP-lysine. It is thus expected that this method can specifically detect acrolein that is not conjugated to proteins or other molecules in the samples. As shown in Fig. 9B, it was clearly revealed that the incubation of LDL with Cu²⁺ led to a time-dependent formation of acrolein. To further examine the source of acrolein in LDL, we assessed whether the auto-oxidation of polyunsaturated fatty acids generates acrolein. As shown in Fig. 9C, the auto-oxidation of arachidonate with an iron/ascorbate-mediated free radical generating system was associated with a significant formation of acrolein; whereas, upon incubation of arachidonate with iron alone, acrolein was virtually undetectable. The result suggested that arachidonate represent a potential source of acrolein generated during peroxidation of LDL. In addition, we also observed that other polyunsaturated fatty acids, such as linoleate, cis-5,8,11,14-eicosapentaenoic acid, and cis-4,7,10,13,16,19-docosahexaenoic acid, generated acrolein during peroxidation (data not shown). Taken together, acrolein and its conjugate with protein represent general indicators of lipid peroxidation.

**DISCUSSION**

In the present study, we identified a novel acrolein-lysine adduct (FDP-lysine) and found that the adduct is indeed generated in the acrolein-treated and in the oxidized LDL. The formation of FDP-lysine can be reasonably explained by the following mechanism (Scheme I). Like other a,b-unsaturated aldehydes, acrolein should be susceptible to nucleophilic addition of the lysine amino group (I) at the double bond (C-3) to form a secondary amine derivative (II) with retention of the aldehyde group. This intermediate further reacts with another acrolein molecule via a Michael addition and generates an imine derivative (III and IV). After aldol condensation followed by dehydration, the reaction is completed to generate the FDP-lysine derivative (V). It is noteworthy that, even in the presence of a large excess of lysine molecules, the secondary amine derivative (II) is scarcely detected and, instead, FDP-lysine is exclusively formed (Fig. 1). Although the detailed mechanism...
FIG. 3. Two-dimensional total correlation spectrum of Nα-acetyl-FDP-lysine. The sample used for this spectrum was identical to that in Fig. 2. Cross-peaks permit the assignment of resonances of the partial structure, –CH2–CH2–CH=CH(CHO)–CH2–. The connectivity from C-2 to C-6 of the product was observed including the long range coupling between C-2 and C-4 (indicated by bold lines and an arrow).
remains unclear, it is postulated that the secondary amine derivative (II) is an unstable intermediate that preferentially reacts with acrolein, leading to the formation of FDP-lysine. Alternatively, it may not be unlikely that an acrolein dimer having free aldehyde groups reacts with the lysine amino group, generating the formation of a condensed ring product such as FDP-lysine. Acrolein can also react by a Michael addition mechanism with either or both nitrogens of the imidazole ring of histidine to generate \(N^p\)-formylethylhistidine (24, 28).

In addition, the formation of this adduct has been suggested in the carbonic anhydrase II treated with acrolein (24); however, a definitive method to detect the \(N^p\)-alkylated histidine in proteins has not yet been established. Acrolein shows the greatest reactivity with thiols (19). Indeed, the exposure of cells to acrolein leads first, in a concentration-dependent manner, to a loss of cellular glutathione. It has been suggested that the reaction of acrolein with thiols generates thioether adducts via a Michael addition mechanism; however, as far as we know, the adduct has not been isolated and chemically characterized. Hence, we attempted to prepare an acrolein-thiol adduct using \(N\)-acycysteine but could not isolate the adduct, despite the formation of a single product. This might be attributed to the reversal of the addition reaction during purification.

By means of the amino acid analysis technique, we detected FDP-lysine in the acrolein-modified protein. The stability of FDP-lysine in the acrolein-modified protein. The stability of FDP-lysine in the acrolein-modified protein. The stability of FDP-lysine against acid hydrolysis suggests the involvement of an enal-dienol tautomerism (Scheme II). This study also provided evidence for generation of the adduct in the oxidatively modified LDL. Because FDP-lysine is an end result of lipid peroxidation, the adduct may therefore serve as a useful marker for assessing the role of lipid peroxidation in the pathogenesis of numerous diseases. However, it should be noted that the considerable loss of lysine residues upon reaction with acrolein is attributed only in part to the formation of FDP-lysine. As can be seen in Fig. 6B, the measured 7 mol of
FDP-lysine in the LDL treated with 0.5 mM acrolein for 1 h corresponds to only 30% of the lysine residues lost. The incomplete recovery of FDP-lysine probably reflects the formation of Schiff base-type acrolein-lysine adducts and the occurrence of secondary reactions in which the aldehyde moiety of the primary Schiff base derivatives further reacts with another lysine amino group, leading to intra- and intermolecular cross-linked derivatives. These secondary reactions are likely responsible for the acrolein-provoked polymerization of protein. In fact, it has been reported that the treatment of adult human hemoglobin with acrolein leads to a heterogeneous mixture of modified hemoglobin molecules including cross-linked dimers (29).

During the lipid peroxidation process, the decomposition of lipid hydroperoxides leads to the generation of many compounds as reactive intermediates. In turn, these can bind to the lysine residues of proteins, generating relatively stable end products. Oxidative modification of LDL is also associated with the formation of a large number of lipid peroxidation products that could covalently bind to LDL apoB; however, there have been few detailed insights into the chemical mechanism of the derivatization of apoB in the oxidized LDL. It has been shown that a highly reactive aldehyde, such as 4-hydroxy-2-nonenal (HNE), generated during the oxidative modification of LDL forms stable Michael addition-type products with histidine and lysine residues of LDL apoB (25, 27, 30, 31). Immunochemical analysis of HNE protein adducts has demonstrated that HNE-derived epitopes are indeed present in the LDL oxidatively modified by Cu²⁺ or cultured endothelial cells (25) and in human atherosclerotic lesions (31). We have recently reported that the N-2-propenal-type MDA-lysine adduct, N²(2-propenal)lysine, represents one of the major constituents of oxidized LDL (32). N²-(Carboxymethyl)lysine, the advanced glycation end product formed on the protein by combined non-enzymatic glycation and oxidation (glycoxidation) reactions, has also been reported to be a product of LDL peroxidation (26). A mechanism has been proposed in which the metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein leads to the formation of glyoxal, an intermediate formed during lipid peroxidations, reacting with lysine residues to generate N²-(carboxymethyl)lysine (26). In addition to these adducts, the chemical nature of oxidized LDL was elucidated, in part, by

![Fig. 7](image-url) Detection of FDP-lysine by amino acid analysis. The authentic Nα-acetyl-FDP-lysine (lower) and oxidized LDL with 5 μM Cu²⁺ for 24 h (upper) were analyzed by amino acid analysis followed by acid hydrolysis.

![Fig. 8](image-url) Kinetics of LDL peroxidation and the formation of FDP-lysine. LDL (0.5 mg protein/ml) was incubated with 5 μM Cu²⁺ at 37 °C. A, the consumption of α-tocopherol (●), free cholesterol (○), and cholesteryl esters (CE18:2, ○; CE20:4, △; CE22:6, □) was followed. The formation of CE18:2-OOH (●) measured by an HPLC-UV was plotted. [Toc]/[Toc]₀ shows the ratio of the remaining fraction of α-tocopherol to that of time 0. B, the consumption of cholesteryl linoleate (18:2, ○), arachidonate (20:4, △), and docosahexaenoate (22:6, ◻), and free cholesterol (□) is shown by a fraction of the remaining substrate. C, FDP-lysine (■), generated in the oxidized LDL, was measured by amino acid analysis.
detecting the acrolein-lysine adduct in the Cu\(^{2+}\)-oxidized LDL. Our present findings add FDP-lysine to a growing list of protein-bound lipid peroxidation products.

To verify the presence of protein-bound acrolein \textit{in vivo}, we have recently raised the monoclonal antibody (mAb5F6) against acrolein-modified protein and have shown that FDP-lysine constitutes an epitope of the antibody.\(^2\) Furthermore, immunohistochemical analysis of atherosclerotic lesions from a human aorta revealed that FDP-lysine recognized by the antibody indeed constitutes the lesions, in which intense positivity is associated primarily with macrophage-derived foam cells. The result is consistent with the view (1) that protein alterations, including oxidative modification, predispose LDL to clearance by scavenger receptors of macrophages, intracellular deposits of lipoprotein-derived cholesterol, and the formation of foam cells. Acrolein may therefore play an important role in the formation of arterial foam cells and contribute to the development of atherosclerosis, while additional studies will be needed to establish a direct connection between the acrolein modification of proteins and their atherogenic properties.

Regarding the quantification of free acrolein in biological samples, several methods, such as HPLC and gas-liquid chromatography, might be available. However, most of them are based on derivatizations of acrolein followed by chromatographic analysis. In these methods, there has been a general problem in the determination of free acrolein, which arises from the variation in the amount of acrolein detected with the assay method employed. A principal problem in determining acrolein in biological samples is the possibility of acrolein being formed during the derivatization procedures. As previously discussed, acrolein can form adducts with the amino acid residues of proteins, while acrolein may in part be weakly bound, for example, to an amino group of lysine residues or bound in the form of a Schiff’s base to a protein. They can be easily split off by weak acids or heating. Such artificial formation of acrolein can be avoided in our new method. Using this procedure, the results of the current study provide the first description of acrolein as a physiologically important aldehyde that could be ubiquitously generated under oxidative stress. The observations (Fig. 9) that free acrolein was detected during LDL per-
Acrolein as a Lipid Peroxidation Product

oxidation and auto-oxidation of arachidonate indicated that polyunsaturated fatty acids might represent potential sources of acrolein. This was also supported by the result (Fig. 8) that the formation of FDP-lysine during LDL peroxidation reached a plateau after all cholesteryl ester polyunsaturated acid esters were oxidized; however, the mechanism for the acrolein production during lipid peroxidation has not yet been experimentally resolved. It has also been shown that in vitro oxidation of free hydroxy-amino acids with myeloperoxidase in the presence of H₂O₂ and chloride ion also generates acrolein via the formation of 2-hydroxypropanal (33). The fact that (i) myeloperoxidase is expressed in human atherosclerotic lesions (34) and (ii) myeloperoxidase can generate reactive species and catalyze lipoprotein oxidation in vitro (35) suggests that acrolein generated from a myeloperoxidase-catalyzed lipid peroxidation plays a role in converting LDL into an atherogenic particle.

In summary, we describe the identification of a novel lysine adduct (FDP-lysine) with acrolein. We also demonstrate that the adduct is indeed formed in the acrolein-modified LDL or Cu²⁺-oxidized LDL. Because lipid peroxidation and subsequent modification of proteins are known to be involved in widespread biological processes, FDP-lysine may be a useful biological marker in the studies of a number of biological systems. These findings also indicate that acrolein is not just a pollutant but also a lipid peroxidation product. Additional studies should provide insight into the biological significance of acrolein as the lipid peroxidation product that could be ubiquitously generated in biological systems.

REFERENCES

1. Steinberg, D., Parthasarathy, S., Carew, T. E., Khon, J. C., and Witztum, J. L. (1989) N. Engl. J. Med. 320, 915–924
2. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) J. Clin. Invest. 84, 1086–1095
3. Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., and Steinberg, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1372–1376
4. Rosenfeld, M. E., Palinski, W., Yla-Herttuala, S., Butler, S., and Witztum, J. L. (1990) Arteriosclerosis 10, 336–349
5. Steinberg, D. (1997) J. Biol. Chem. 272, 29963–29966
6. Henriksen, T. E., Mahoney, E. M., and Steinberg, D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6499–6503
7. Morel, D. W., DeCorleto, P. E., and Chisolm, G. M. (1984) Arteriosclerosis 4, 357–364
8. Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3883–3887
9. Steinbrecher, U. P., Witztum, J. L., Parthasarathy, S., and Steinberg, D. (1987) Arteriosclerosis 7, 135–143
10. Heinecke, J. W., Baker, L., Rosen, H., and Chait, A. (1986) J. Clin. Invest. 77, 757–761
11. Cathcart, M. K., Morel, D. W., and Chisolm, G. M. (1985) J. Leukocyte Biol. 38, 341–350
12. Lenz, M. W., Hughes, H., Mitchell, J. R., Via, D. P., Guyton, J. R., Taylor, A. A., Gotto, A. M., Jr., and Smith, C. (1990) J. Lipid Res. 31, 1043–1050
13. Henriksen, T. E., Mahoney, E. M., and Steinberg, D. (1983) Arteriosclerosis 3, 149–159
14. Esterbauer, H., Gebicki, J., Puhl, H., and Jurgen, G. (1992) Free Radical Biol. Med. 13, 341–390
15. Quinn, M. T., Parthasarathy, S., Feng, L. G., and Steinberg, D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2995–2998
16. Johnston, R. A. W., and Plimmer, J. R. (1959) Chem. Rev. 59, 885–936
17. Kensler, C. J., and Battista, S. P. (1963) N. Engl. J. Med. 269, 1161–1166
18. Izard, C., and Liberman, C. (1978) Mutat. Res. 47, 115–138
19. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Free Radical Biol. Med. 11, 81–128
20. Ramos, P., Giese, S. P., Schuster, B., and Esterbauer, H. (1995) J. Lipid Res. 36, 2113–2128
21. Jessup, W., Mohr, D., Giese, S. P., Dean, R. T., and Stocker, R. (1992) Biochim. Biophys. Acta 1180, 73–82
22. Noguchi, N., Sakai, H., Kato, T., Tsuchiya, J., Yamamoto, Y., Niki, E., Horikoshi, H., and Kodama, T. (1996) Atherosclerosis 125, 227–234
23. Uchida, K., Osawa, T., Hiai, H., and Toyokuni, S. (1995) Biochem. Biophys. Res. Commun. 212, 1068–1073
24. Pocker, Y., and Janjie, N. (1988) J. Biol. Chem. 263, 6169–6176
25. Uchida, K., Toyokuni, S., Nishikawa, K., Kawakishi, S., Oda, H., Hiai, H., and Stadtman, E. R. (1991) Biochemistry 30, 12487–12494
26. Fu, M. X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W., and Thorpe, S. R. (1996) J. Biol. Chem. 271, 9982–9986
27. Requena, J. R., Fu, M. X., Ahmed, M. U., Jenkins, A. J., Lyons, T. J., Baynes, J. W., and Thorpe, S. R. (1997) Biochem. J. 322, 317–325
28. Gan, J. C., and Ansari, G. A. (1987) Res. Commun. Pathol. Pharmacol. 35, 419–422
29. Hoberman, H. D., and San George, R. C. (1988) J. Biochem. Toxicol. 3, 105–119
30. Uchida, K., Osawa, T., Hiai, H., and Toyokuni, S. (1994b) Biochem. Biophys. Res. Commun. 212, 1068–1073
31. Uchida, K., Itakura, K., Kawakishi, S., Hiai, H., Toyokuni, S., and Stadtman, E. R. (1995) Arch. Biochem. Biophys. 324, 241–248
32. Uchida, K., Sakai, K., Itakura, K., Osawa, T., and Toyokuni, S. (1997) Arch. Biochem. Biophys. 346, 45–52
33. Anderson, M. M., Hazen, S. L., Hau, F. F., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 424–432
34. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) J. Clin. Invest. 93, 437–444
35. Savenkova, M. I., Mueller, D. M., and Heinecke, J. W. (1994) J. Biol. Chem. 269, 20394–20400