First Direct Evidence for Lipid/Protein Conjugation in Oxidized Human Low Density Lipoprotein*

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It has been postulated that lipids incorporated in atherosclerotic plaques are derived from the uptake of oxidized low density lipoprotein (LDL) by a macrophage-bound receptor. In vitro studies of LDL oxidation have established that reactive lipids are formed and that the exposure of native LDL to these products leads to modified protein with physical properties similar to oxidized LDL. Here we describe the application of highly specific tandem mass spectrometric techniques to the first characterization of lipid-modified LDL by demonstrating the addition of 4-hydroxy-2-nonenal to histidine residues of apolipoprotein B-100, following oxidation of LDL. The modified residues have been assigned to specific locations that have been previously shown to reside on the surface of the LDL particle.

The earliest recognized stage in the formation of atherosclerotic plaques is the formation of the arterial fatty streak resulting from the accumulation of foam cells beneath the endothelium. Most foam cells arise from monocyte-derived macrophages that have become embedded under the endothelium and have been loaded with cholesterol esters. Goldstein and co-workers (1) discovered a macrophage receptor which recognizes chemically modified LDL,1 but not native LDL. This “scavenger” receptor was found to take up chemically modified LDL at a high rate and in an apparently unregulated manner, suggesting it may be responsible for the accumulation of cholesterol and cholesterol esters by endothelial macrophages leading to their conversion to foam cells. It has since been established that oxidation (in vitro) converts LDL to a form recognized by the scavenger receptor (2). It has been suggested further that the key change to LDL resulting from exposure to oxidative conditions is the formation of lipid/protein conjugates (3). Studies of oxidized (in vitro) LDL have demonstrated the formation of reactive lipid products, including 4-hydroxy-2-nonenal (HNE) which was found to have the highest capacity for covalent modification of LDL among the peroxidation products studied (4). Lysine, cysteine, and histidine residues have been suggested as targets of HNE modification of protein (5–10). Material removed from aortic plaques of laboratory animals was recognized by antibodies raised against LDL exposed to HNE (11), suggesting an important role for HNE conjugates in the progression of atherosclerosis. Uchida et al. (12) reported the detection of HNE-modified histidine and lysine residues by amino acid analysis of oxidized LDL. Evidence conclusively locating lipid/protein conjugation in oxidized LDL has, however, hitherto been lacking. In a previous study with a model protein, apomyoglobin, we established that histidine residues were the principal sites of stable adduct formation with HNE (13).

ApoB-100, the primary protein constituent of low density lipoprotein, is one of the largest known monomeric proteins, consisting of 4536 amino acid residues and with a calculated molecular mass of 513 kDa. The primary sequence has been determined (14–16). Characterization of structural modifications of apoB-100 represents a formidable analytical problem. Proteolytic digestion of the protein generates peptides of a size amenable to characterization, but the complexity of the hydrolysate is such that isolation of individual constituents is extremely laborious (16). (For example, hydrolysis using trypsin should produce 497 peptides.) Furthermore, in structurally modified LDL, the complexity of the hydrolysate is further increased, and the modified peptides (incorporating nonconventional amino acid residues) are not amenable to complete structural characterization by conventional N-terminal sequence analysis.

We have therefore developed tandem mass spectrometric procedures to recognize and characterize peptides containing HNE-modified histidine residues of apoB-100. Precursor ion scanning allowed the selective detection of peptides containing HNE-modified histidine residues present in a complex mixture of other tryptic peptides. The ability to screen mixtures of components containing the targeted modifications allowed the use of a single simple HPLC fractionation step; isolation of individual components was unnecessary. The modified peptides, which were often present as minor components in a complex mixture, were then sequenced by product ion analysis without further purification.

EXPERIMENTAL PROCEDURES

40 μg of LDL was isolated according to standard procedures (17) and oxidized for 24 h at 37 °C in oxygen-saturated 50 mM phosphate buffer, pH 7.4, containing 5.5 μM CuSO 4. 10 ml of the 2 mg/ml LDL suspension obtained after oxidation was cooled to 0 °C and buffered to pH 7.3 with 1 M potassium phosphate. Reduction was performed by the addition of 0.1 g of NaBH 4, and the resulting precipitated protein was recovered by centrifugation. The precipitated LDL was delipidated by washing with CHCl 3:CH 3OH (2:1). The delipidated protein was resuspended in 8 ml of 0.1 M NH 4CO 3, 1 mM CaCl 2, pH 8.4, to which was added 0.32 mg of trypsin (Sigma). The suspension was stirred and maintained at 37 °C for 18 h. Portions (1 ml) of the digest solution were subjected to reverse-phase separation using an Analyst HPLC system (Perkin-Elmer) with UV detection at 217 nm. Separation was achieved using a Partisil C 18 2.1 × 150 mm column (Keystone Scientific) and gradient elution. HPLC fractions were collected over 1 min into 1.5 ml polypropylene tubes. The fractions from four HPLC analyses were combined and dried. The HPLC fractions were redissolved in 50 μl of H 2O:CH 3CN 1:1 with 0.1%
formic acid. The sample solutions were infused at 2 μl/min into a Quattro mass spectrometer, upgraded to Quattro II specifications (Micromass, Manchester, UK).

RESULTS AND DISCUSSION

The tryptic hydrolysate of apoB-100 derived from oxidized low density lipoprotein following reduction with NaBH4 was fractionated by reverse-phase HPLC. The prior reduction step was included to stabilize the product formed by Michael addition of HNE to histidine; experiments with model proteins have established that the conditions employed for tryptic digestion may lead to reversal of the initial addition reaction.2 The chromatogram was extremely complex (data not shown), as expected from the prediction of nearly 500 tryptic fragments of apoB-100. HPLC fractions (1 min) were collected and analyzed by conventional electrospray ionization MS and by tandem MS with scanning for precursors of m/z 268, where m/z 268 corresponds to the histidine immonium ion modified by the addition of an HNE moiety followed by a 2-hydrogen atom reduction (Structure I).

A previous study established that scanning for precursors of the HNE-modified histidine immonium ion is an effective procedure for the selective detection of peptides containing this modification (13). The conventional mass spectrum obtained from one fraction (37; Fig. 1A) included many abundant ions which were readily assigned to native tryptic peptides. In contrast, the spectrum of precursors of m/z 268 (Fig. 1B) contained only one ion at high relative abundance, m/z 658.3. The initial stage of interpretation consisted of comparing the m/z values of the ions in the precursor spectrum with those values calculated for the predicted tryptic peptides of native apoB-100 and apoB-100 where every histidine residue was replaced with a reduced HNE-adducted histidine residue. For tryptic peptides which contained more than one histidine residue, values were calculated with all possible combinations of native and adducted residues. The m/z values of the (M + nH)n+ ions with n = 1–5 were calculated for each peptide. The ion at m/z 658.3 in the precursor ion spectrum from fraction 37 corresponded to the predicted value for the (M + 2H)2+ ion from tryptic fragment T430 containing a single HNE-adducted histidine residue. The predicted value m/z for the (M + 3H)3+ ion was 439.2, and an ion was detected in the precursor ion spectrum at approximately the correct m/z value (439.8) for the (M + 3H)3+ ion but with very low signal-to-noise. The experimentally determined molecular mass for the adducted peptide was 1314.6 Da (using only the value of the (M + 2H)2+ ion) which was in precise agreement with the theoretical value for HNE-adducted T430. Product ion spectra were recorded for the ions of m/z 658.3 and 439.2 to confirm the presence of HNE-adducted T430. The product ion spectrum obtained from the triply protonated ion at m/z 439.2 (Fig. 1C) contained sufficient structural information to confirm the sequence of 8 of the 10 residues. The product ion spectrum obtained from the doubly protonated ion at m/z 658.3 (Fig. 1D) contained a complete set of C-terminal fragment ions which confirmed the entire amino acid sequence including the presence of an HNE-adducted histidine, third from the C terminus of T430, and corresponding to amino acid residue 3960 in apoB-100. Additional apoB-100 tryptic peptides containing HNE-adducted histidine residues were detected in other HPLC

2 M. S. Bolgar and S. J. Gaskell, unpublished results.
fractions. The detected HNE-adducted histidine residues were 21 or 2940 (where the ambiguity arises because the modified histidine residue was contained in a peptide which corresponded to two tryptic peptides with the same amino acid sequence but derived from different portions of apoB-100), 763, 916, 2074, 3281, 3948, and 3960. It is noteworthy that all of the modified residues identified so far are incorporated in portions of apoB-100 shown to reside on the surface of the LDL particle (16, 18). Thus, these modifications may significantly affect the interaction with macrophage-bound receptors.

In summary, we have conclusively demonstrated the addition of HNE to apoB-100 following oxidation of LDL, providing the first direct evidence for the significance of lipid/protein conjugation. The approach used here for the detection of HNE adduction is substantially more informative than methodology in which the protein is hydrolyzed to individual amino acids followed by an assay for HNE-adducted histidine. By identifying and mapping the tryptic peptides containing HNE-adducted histidine residues onto the apoB-100 sequence, a connectivity is established between the adducted residue and the protein of origin; this can be achieved without exhaustive purification of the protein. Furthermore, the assignment of modified residues to specific locations that have been previously shown to reside on the surface of the LDL particle has implications for receptor recognition of the oxidized protein.

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