

Modifying Apolipoprotein A-I by Malondialdehyde, but Not by an Array of Other Reactive Carbonyls, Blocks Cholesterol Efflux by the ABCA1 Pathway*

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Dysfunctional high density lipoprotein (HDL) is implicated in the pathogenesis of cardiovascular disease, but the underlying pathways remain poorly understood. One potential mechanism involves covalent modification by reactive carbonyls of apolipoprotein A-I (apoA-I), the major HDL protein. We therefore determined whether carbonyls resulting from lipid peroxidation (malondialdehyde (MDA) and hydroxynonenal) or carbohydrate oxidation (glycolaldehyde, glyoxal, and methylglyoxal) covalently modify lipid-free apoA-I and inhibit its ability to promote cellular cholesterol efflux by the ABCA1 pathway. MDA markedly impaired the ABCA1 activity of apoA-I. In striking contrast, none of the other four carbonyls were effective. Liquid chromatography-electrospray ionization-tandem mass spectrometry of MDA-modified apoA-I revealed that Lys residues at specific sites had been modified. The chief adducts were MDA-Lys and a Lys-MDA-Lys cross-link. Lys residues in the C terminus of apoA-I were targeted for cross-linking in high yield, and this process may hinder the interaction of apoA-I with lipids and ABCA1, two key steps in reverse cholesterol transport. Moreover, levels of MDA-protein adducts were elevated in HDL isolated from human atherosclerotic lesions, suggesting that lipid peroxidation might render HDL dysfunctional *in vivo*. Taken together, our observations indicate that MDA damages apoA-I by a pathway that generates lysine adducts at specific sites on the protein. Such damage may facilitate the formation of macrophage foam cells by impairing cholesterol efflux by the ABCA1 pathway.

The cardioprotective effects of high density lipoprotein (HDL)⁴ are generally attributed to reverse cholesterol transport (1, 2). In this scenario, HDL removes excess cholesterol from artery wall macrophages and transports it back to the liver for excretion in bile. Apolipoprotein A-I (apoA-I), the major protein of HDL, plays a critical role in the first step of reverse cholesterol transport by enhancing the sterol efflux from macrophages that is accomplished by the ATP-binding cassette transporter A1 (ABCA1) (3, 4). The ligand for ABCA1 is lipid-free apoA-I produced by remodeling HDL (5, 6). Activation of ABCA1 depends critically on repeats 1 and 10 of apoA-I, which are located at the N- and C-terminal regions of the protein, respectively (7–9). ApoA-I also activates lecithin:cholesterol acyltransferase, which converts free cholesterol to cholesteryl ester, a subsequent step in the maturation of HDL particles (10–12).

It has been proposed that HDL loses its cardioprotective effects in humans with established coronary artery disease, although the underlying mechanisms are unclear (13). One potential pathway involves modification of apoA-I by reactive intermediates, perhaps in the artery wall. Indeed, oxidation of apoA-I by myeloperoxidase, a heme protein that generates an array of reactive oxygen and nitrogen species, severely impairs cholesterol efflux by the ABCA1 pathway and inhibits the ability of the protein to activate lecithin:cholesterol acyltransferase (14–20). However, little is known about the ability of other reactive species to impair the ABCA1 activity of apoA-I.

One mechanism for modifying proteins involves reactive carbonyls (21, 22), which have been implicated in the pathogenesis of atherosclerosis and diabetic vascular disease (23–25). They result from oxidation of carbohydrates or amino acids and peroxidation of lipids (21, 22, 26). Major carbonyl products of carbohydrate oxidation *in vivo* are thought to be glyoxal, methylglyoxal, and glycolaldehyde, which are precursors of advanced glycation end products. Lipid peroxidation yields a different spectrum of reactive carbonyl compounds, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acro-

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⁴ The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; DTPA, diethylenetriaminepentaacetic acid; ESI, electrospray ionization; HNE, 4-hydroxynonenal; LC, liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; MDA, malondialdehyde; MS, mass spectrometry; RLU, relative light units; BHK, baby hamster kidney.

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lein (24, 26), which have been termed advanced lipoxidation end products (21). Importantly, advanced lipoxidation and advanced glycation end product levels are elevated in diabetes, which greatly increases the risk for coronary artery disease (21, 27). Reactive carbonyls can also be produced by oxidation of hydroxyamino acids (28). For example, myeloperoxidase uses hydrogen peroxide produced by the NADPH oxidase of phagocytes to convert L-serine to glycolaldehyde, which in turn can react with proteins to form *N*-(carboxymethyl)lysine (28). This pathway may be physiologically relevant, because *N*-(carboxymethyl)lysine formation during acute inflammation is impaired in NADPH oxidase-deficient mice (29).

HDL may be constantly exposed to reactive carbonyls, because it is the major carrier of lipid hydroperoxides in plasma (30). Moreover, when plasma is oxidized *ex vivo*, apoA-I is a major target for covalent modification by lipid oxidation products (31). Reactive carbonyls are also known to impair HDL function *in vitro*. Thus, high concentrations of carbonyls modified apoA-I and inhibited lecithin:cholesterol acyltransferase activation by HDL, but the underlying mechanisms were not identified (32). When apoA-I incorporated into discoidal, reconstituted HDL was exposed to methylglyoxal, it lost its ability to activate lecithin:cholesterol acyltransferase (33). This loss was associated with modification of arginine, lysine, and tryptophan residues. We previously showed that acrolein (an α,β -unsaturated carbonyl derived from oxidized lipids and myeloperoxidase-oxidized threonine) modifies a single lysine residue in repeat 10 of apoA-I. That specific modification associated quantitatively with impaired cholesterol transport by ABCA1 (34).

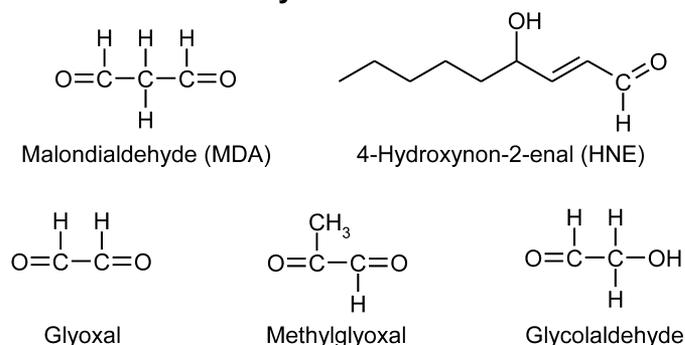
Neither the structures of the protein adducts nor the specific amino acid residues that MDA and other reactive carbonyls modify in apoA-I have been identified. In this study, we investigated the reactivity of carbonyls with apoA-I, and we determined whether these compounds altered the ability of the protein to promote cholesterol efflux by the ABCA1 pathway. We focused on five carbonyls implicated in the pathogenesis of cardiovascular disease (21, 23, 24, 35) as follows: MDA, HNE, glyoxal, methylglyoxal, and glycolaldehyde (Fig. 1A). We found that MDA selectively modifies Lys residues and dramatically impairs the ability of apoA-I to transport cellular cholesterol by the ABCA1 pathway. Moreover, we detected elevated levels of MDA adducts in HDL isolated from human atherosclerotic lesions, raising the possibility that MDA interferes with normal HDL cholesterol transport by modifying Lys residues in apoA-I in the artery wall.

EXPERIMENTAL PROCEDURES

Materials—All organic solvents were high performance liquid chromatography grade. 4-Hydroxynonenal (HNE) was purchased from Cayman Chemical (Ann Arbor, MI). Unless otherwise indicated, all other materials were purchased from Sigma.

Isolation of HDL and ApoA-I—Plasma was prepared from EDTA-anticoagulated blood collected from healthy adult subjects who had fasted overnight. HDL (density 1.125–1.210 g/ml) was isolated from plasma by sequential ultracentrifugation and depleted of apolipoproteins E and B100 by heparin-agarose chromatography (36). ApoA-I was purified from HDL

A. Reactive Carbonyls



B. Detected Carbonyl Adducts

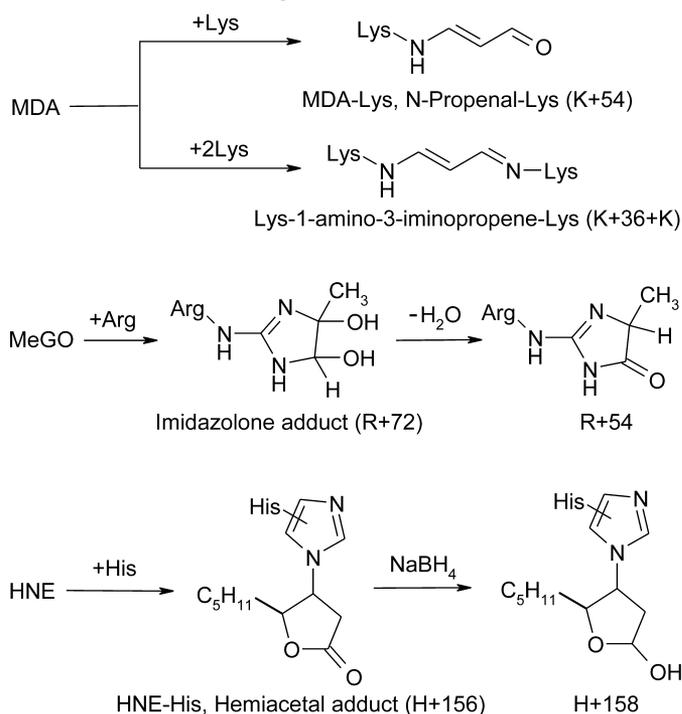


FIGURE 1. Structures of carbonyls and carbonyl adducts.

by ion-exchange chromatography (36). Protein concentration was determined using the Lowry assay (Bio-Rad), with albumin as the standard. The human studies committees at the University of Washington School of Medicine and University of Michigan approved all protocols involving human material.

Isolation of Lesion HDL—Atherosclerotic tissue was harvested at endarterectomy, snap-frozen, and stored frozen at -80°C until analysis. Lesions from a single individual (~ 0.5 g wet weight) were mixed with dry ice and pulverized in a stainless steel mortar and pestle. Lesion HDL was isolated by ultracentrifugation from extracts of tissue powder (14, 16), using buffers supplemented with $100\ \mu\text{M}$ diethylenetriaminepentaacetic acid (DTPA), $100\ \mu\text{M}$ butylated hydroxytoluene, and protease inhibitor mixture (Sigma). ApoA-I was detected by immunoblotting, using a rabbit IgG polyclonal antibody to human apoA-I (Calbiochem) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG. Detection was enhanced by chemiluminescence.

Preparation of Malondialdehyde (MDA)—MDA was prepared immediately before use by rapid acid hydrolysis of maloncarbonyl bis-(dimethylacetal) (37, 38). Briefly, 20 μl of 1 M HCl was mixed with 200 μl of maloncarbonyl bis-(dimethylacetal), and the mixture was incubated at room temperature for 45 min. The reaction mixture was diluted with 980 μl of 10 mM phosphate buffer (pH 7.4). After further dilution, the MDA concentration of the stock solution was determined by absorbance at 245 nm, using $\epsilon = 13,700 \text{ M}^{-1} \text{ cm}^{-1}$ (39).

Modification of ApoA-I by MDA and Other Carbonyls—Reactions of lipid-free apoA-I (5 μM , 0.14 mg of protein/ml) with MDA or other carbonyls (HNE, glyoxal, methylglyoxal, and glycolaldehyde) were carried out at 37 °C for 24 h in 50 mM sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. Reactions were initiated by adding MDA or other carbonyls and terminated by adding a 20-fold molar excess (relative to carbonyls) of aminoguanidine. When indicated, reaction mixtures were reduced with 10 mM NaBH₄.

Modification of HDL by MDA—Reactions with HDL (0.2 mg of protein/ml) were carried out at 37 °C for 24 h in 50 mM sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. Reactions were initiated by adding MDA and terminated by adding a 20-fold molar excess (relative to MDA) of aminoguanidine.

Efflux of Cellular Cholesterol—Baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 were radiolabeled with [³H]cholesterol. Expression of ABCA1 was induced by incubating the cells for 20 h with Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin (Dulbecco's modified Eagle's medium/bovine serum albumin) and 10 nM mifepristone. Efflux of [³H]cholesterol was measured after a 2-h incubation with Dulbecco's modified Eagle's medium/bovine serum albumin without or with apoA-I (17). Cholesterol efflux mediated by apoA-I was calculated as the percentage of total [³H]cholesterol (medium plus cell) released into the medium after the value obtained with Dulbecco's modified Eagle's medium/bovine serum albumin alone was subtracted. BHK cells incubated with native or carbonyl-modified apoA-I for up to 4 h showed no changes in morphology or in cell protein or cholesterol content per well.

Matrix-assisted Laser Desorption Ionization-Time-of-Flight-Mass Spectrometry (MALDI-TOF-MS)—MALDI-TOF-MS was performed using a Voyager-DE STR system equipped with delayed extraction (Applied Biosystems, Foster City, CA) (34). The matrices were α -cyano-4-hydroxycinnamic acid for peptide digests and sinapinic acid for intact proteins. Samples were desalted with a ZipTip pipette tip (ZipTip_{C18} or ZipTip_{C4} for peptides or proteins, respectively; Millipore Corp., Billerica, MA) before they were applied to the MALDI plate. Spectra of peptide digests were obtained in the positive reflector mode, using an accelerating voltage of 20 kV. The spectra of intact proteins were obtained in the positive linear mode, using an accelerating voltage of 25 kV.

Proteolytic Digestion of ApoA-I and LC-ESI-MS—Native or carbonyl-modified lipid-free apoA-I was digested with sequencing grade modified trypsin (Promega) or sequencing grade endoproteinase Glu-C (Roche Applied Science) and fractionated by LC as described previously (17, 34, 40). MS and

MS/MS analyses were performed in the positive ion mode (mass range 200–2,000 Da) with a Thermo-Finnigan LCQ Deca XP Plus instrument (40).

Quantifying ApoA-I Modifications with ¹⁵N-Labeled ApoA-I—Loss of precursor peptides and product yields of modified peptides were quantified by isotope dilution (41) with reconstructed ion chromatograms of precursor and product peptides, using ¹⁵N-labeled apoA-I as internal standard (18). An equal amount of ¹⁵N-labeled apoA-I was added to control or modified apoA-I prior to digestion. Loss of precursor peptide was calculated from the ratio of the peak area of precursor peptide of apoA-I from control or modified apoA-I to that of the corresponding ¹⁵N-labeled peptide from ¹⁵N-labeled apoA-I. Thus, precursor peptide loss (%) = $(1 - (\text{peak area of precursor ion from modified apoA-I} / \text{peak area of precursor ion from } ^{15}\text{N-apoA-I})) \times (\text{peak area of precursor ion from } ^{15}\text{N-apoA-I} / \text{peak area of precursor ion from control apoA-I}) \times 100$.

Product yields of modified peptides were calculated from the ratio of the peak area of product peptide of apoA-I relative to that of the corresponding ¹⁵N-labeled peptide from ¹⁵N-labeled apoA-I. Thus, product yield (%) = $100 \times (\text{peak area of product peptide in modified apoA-I} / \text{peak area of } ^{15}\text{N-labeled precursor peptide}) \times (\text{peak area of } ^{15}\text{N-labeled precursor peptide} / \text{peak area of precursor peptide from control apoA-I})$. When the product peptide was cross-linked, the product ion peak area was compared with the sum of the peak areas of all precursor peptides that formed the cross-linked product peptide. Thus, product yield (%) = $100 \times (\text{peak area of cross-linked product peptide in modified apoA-I} / \text{sum of peak area of } ^{15}\text{N-labeled precursor peptides}) \times (\text{sum of peak areas of } ^{15}\text{N-labeled precursor peptides} / \text{sum of peak areas of precursor peptides from control apoA-I})$. This method assumes that the precursor ions and product ions have similar MS response characteristics (40).

Immunochemical Detection of MDA Adducts—A chemiluminescence immunoassay, using monoclonal MDA2 that detects MDA-lysine adducts, was performed with modifications as described previously (42). In brief, 96-well Microfluor-2 White plates (Thermo Electron Corp.) were coated overnight at 4 °C with HDL (3 $\mu\text{g}/\text{ml}$ protein; isolated from plasma or atherosclerotic carotid lesions) in phosphate-buffered saline buffer supplemented with 0.27 mM EDTA and 20 μM butylated hydroxytoluene. The wells were then washed with TBS buffer (150 mM NaCl, 50 mM Tris base, 0.27 mM EDTA (pH 7.4)) containing 1% bovine serum albumin before biotinylated MDA2 (2 $\mu\text{g}/\text{ml}$) was added for 1 h at room temperature. After additional washings, bound MDA2 was detected by adding NeutrAvidin alkaline phosphatase (Pierce) and Lumi-Phos 530 (Lumigen, Inc.) and monitoring chemiluminescence (Dynex Luminometer, Thermo Electron Corp.). Each sample was assayed in triplicate. Results are expressed in relative light units/100 ms (RLU/ms). All samples were measured in a single assay.

Modeling MDA-modified ApoA-I—Energy minimization calculations (Accelrys Discovery Studio Client 2.5) used a hybrid model combining the conformational information for amino acids 1–180 (43) and amino acids 181–243 (44). To examine the effect of cross-linking on the apoA-I conformation, we imposed 2.5–5.6 Å distance constraints (5.6 Å is the estimated maximum length of the MDA cross-link) among the N ϵ atoms of Lys

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residues 182, 195, 206, 208, 226, and 239, and CHARMM forcefield was applied to the apoA-I model with a CFF partial charge forcefield (45) based on the distance constraints. To evaluate the effect of restrictions in mobility induced by cross-links, we performed two minimization protocols on our initial model as follows: the steepest descent method (maximum number of steps 500, root mean square gradient of 0.1, and energy change 0.0 kT/e) followed by the conjugate gradient method (maximum number of steps 500, root mean square gradient of 0.0001, and energy change 0.0 kT/e). After energy minimization modeling, we executed equilibration molecular dynamics with 1,000 steps of 0.001 time steps and a target temperature of 310 K. The final model was evaluated with the MODELER protein verification tool of Discovery Studio (45) to ensure that it was energetically more favorable than the initial conformation.

Statistical Analysis—Unless indicated otherwise, results represent means and S.D. of triplicate determinations and are representative of at least two independent experiments.

RESULTS

Modification of ApoA-I by MDA, but Not by Four Other Reactive Carbonyls, Impairs Cholesterol Efflux by the ABCA1 Pathway—To determine whether any of the five reactive carbonyls affect cellular lipid metabolism, we measured the ability of apoA-I that had been modified by MDA, HNE, glyoxal, methylglyoxal, or glycolaldehyde to promote cholesterol efflux from cultured cells expressing high levels of ABCA1. Reactions with carbonyls were carried out at neutral pH in phosphate buffer supplemented with DTPA to chelate redox-active metal ions (46). When ABCA1-transfected BHK cells were incubated with 3 $\mu\text{g}/\text{ml}$ apoA-I that had been exposed for 24 h at 37 °C to a 50:1 ratio of carbonyl (mol/mol), the apolipoprotein that had been incubated with MDA was markedly less able to promote cholesterol efflux (Fig. 2A). In striking contrast, apoA-I incubated with the same molar ratio of each of the other carbonyls did not lose cholesterol efflux activity (Fig. 2A).

Exposure to increasing concentrations of MDA progressively and dramatically deprived apoA-I of its ability to promote cholesterol efflux (Fig. 2B). A 20:1 molar ratio of MDA reduced activity by ~50%, and the effect was time-dependent. At a 50:1 molar ratio, the loss was ~25% at 2 h and ~50% at 5 h (Fig. 2C), suggesting that the carbonyl reacts rapidly with apoA-I. Both the apparent K_m and V_{max} values for cholesterol efflux changed as apoA-I was exposed to increasing concentrations of MDA (Fig. 2D). These results demonstrate that MDA significantly reduces the ability of apoA-I to promote cholesterol efflux by the ABCA1 pathway. In contrast, modification by HNE, glyoxal, methylglyoxal, or glycolaldehyde had little effect.

ApoA-I Exhibits Increased Molecular Weight When Modified by Carbonyls—All of the carbonyls we investigated have two electrophilic centers (Fig. 1A). Therefore, they have the potential to form mono-adducts as well as intra- and intermolecular cross-links. To better understand the potential reactions of apoA-I, we exposed the protein to up to a 100:1 molar ratio of carbonyl and analyzed the intact protein with MALDI-TOF-MS. Using this approach, we observed a progressive decrease in the ion intensity of material with the mass-to-charge ratio (m/z) of native apoA-I (data not shown). However, we were unable to

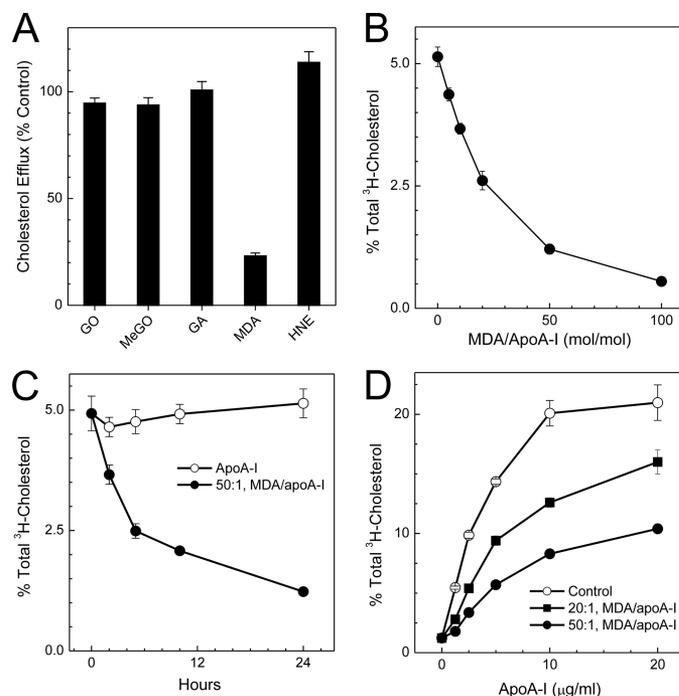


FIGURE 2. Impact of carbonyl modification on ability of apoA-I to promote cholesterol efflux by ABCA1. ApoA-I (5 μM) was incubated with 250 μM glyoxal, methylglyoxal, glycolaldehyde, MDA, or HNE (A), or with the indicated concentrations of MDA for 24 h (B and D), or with 250 μM MDA for the indicated times (C) at 37 °C in 50 mM sodium phosphate buffer (pH 7.4) containing 100 μM DTPA. Reactions were initiated by adding carbonyl and terminated by adding a 20-fold molar excess (relative to carbonyl) of aminoguanidine. A–C, [³H]cholesterol-labeled ABCA1-transfected BHK cells were incubated for 2 h with 3 $\mu\text{g}/\text{ml}$ of control (0 μM carbonyls) or carbonyl-modified apoA-I. D, [³H]cholesterol-labeled ABCA1-transfected BHK cells were incubated with the indicated concentrations of untreated or MDA-treated apoA-I (20:1 or 50:1, mol/mol, MDA/protein) for 2 h. At the end of the incubation, [³H]cholesterol efflux to the acceptor apolipoprotein was measured. HNE was added to apoA-I in ethanol. Control experiments showed that the same final concentration of ethanol (~1 μl per 200 μl) increased the efflux activity of native apoA-I by ~10%. Results represent two independent experiments.

detect an increase in ions with the anticipated m/z of multimers of native apoA-I, even though the carbonyls we investigated had two electrophilic centers. On the other hand, MALDI-TOF-MS showed that the molecular weight of apoA-I increased steadily as the molar ratio of MDA rose from 0 to 100 (Fig. 3, *solid squares*). At a 50:1 molar ratio, the increase was ~350 atomic mass units. These results indicate that MDA covalently modifies apoA-I and progressively increases the molecular weight of the protein. HNE and methylglyoxal also significantly increased the molecular weight of apoA-I (Fig. 3, *solid circles* and *empty triangles*, respectively), indicating that they covalently modify apoA-I. At a 50:1 molar ratio, the increase was ~700 atomic mass units with HNE and ~350 atomic mass units with methylglyoxal. In contrast, glyoxal and glycolaldehyde had little effect on the apparent molecular weight of apoA-I, indicating that they fail to yield adducts of apoA-I that are detectable under our experimental conditions. However, it is important to note that certain glycolaldehyde adduction reactions are reversible (47).

Based on these observations and the molecular weights of the carbonyl adducts we detected in apoA-I (Fig. 1B; see below), we estimate that a 50:1 molar ratio of carbonyl yielded ~7–8

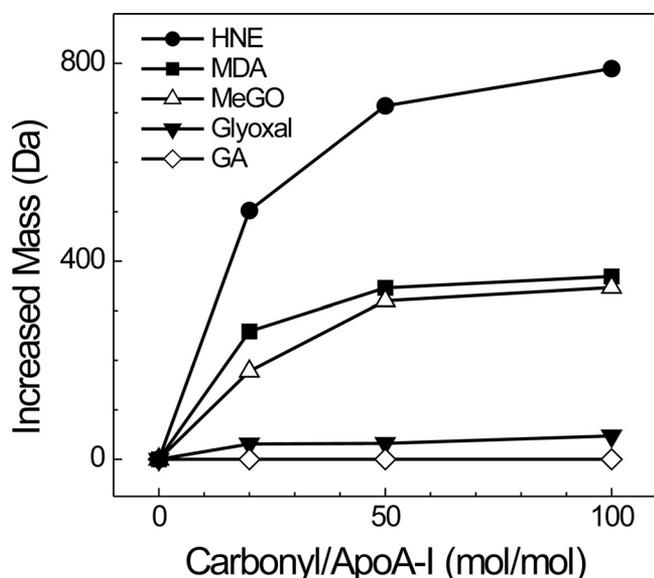


FIGURE 3. MALDI-TOF-MS of carbonyl-modified apoA-I. ApoA-I was exposed to the indicated concentrations of carbonyl for 24 h, as described in the legend to Fig. 2. After the reaction was terminated with aminoguanidine and reaction products reduced by NaBH_4 , intact proteins were desalted and analyzed by MALDI-TOF-MS. The masses of the intact native or carbonyl-modified proteins were determined, using myoglobin and bovine serum albumin as the internal standards.

MDA, 4–5 HNE, and 5–6 methylglyoxal adducts, respectively, per mol of apoA-I.

Peptides Containing Lys Residues Are Lost in High Yield When Lipid-free ApoA-I Is Exposed to MDA—ApoA-I contains 21 Lys, 16 Arg, and 5 His residues that could potentially react with carbonyls. To determine which residues are actually modified, we exposed the lipid-free protein to MDA, digested the modified protein with trypsin or Glu-C, and used LC-ESI-MS/MS to analyze the resulting peptide mixture. Because apoA-I is rich in Lys and Arg residues, a tryptic digest yields several small peptides that are poorly retained on a reverse-phase column. To ensure complete coverage and to unambiguously identify the site at which each peptide is modified, we also used the proteolytic enzyme Glu-C, which cleaves peptide bonds C-terminal to glutamic acid in ammonium bicarbonate buffer (40). Used in concert, peptides from tryptic and Glu-C digests span the entire sequence of apoA-I.

To determine which apoA-I residues MDA modifies, we used isotope dilution to monitor loss of precursor peptides. This approach provides a global, unbiased view of protein damage (18). Peptide loss was quantified using ^{15}N -labeled apoA-I as internal standard. Loss of precursor peptide was calculated using the ratio of the peak area of precursor peptide of apoA-I from control or modified apoA-I to that of the corresponding ^{15}N -labeled peptide from ^{15}N -labeled apoA-I.

We focused on Lys residues first, because previous studies identified them as major targets of MDA (24, 48). When apoA-I was exposed to a 20-fold molar ratio of MDA for 24 h, isotope dilution revealed that multiple peptides containing one or more Lys residues were lost in high yield. This observation suggests that the amino side chain of the residue was a major target for MDA (Fig. 4A). We detected <50% of three native peptides containing Lys residues as follows: (i) Lys²²⁶; (ii) Lys⁹⁴, Lys⁹⁶,

Lys¹⁰⁶, and Lys¹⁰⁷; and (iii) and Lys¹² and the free N-terminal amino group. In contrast, there was little loss of other Lys-containing peptides (e.g. Lys⁷⁷ or Lys⁸⁸), suggesting that MDA does not modify certain Lys residues in apoA-I (Fig. 4A).

MDA Converts Free Amino Groups in ApoA-I to the Propenal Adduct (K+54) but Not to the Dihydropyridine Adduct—*In vitro* studies have identified a number of adducts between MDA and Lys residues (Fig. 1B), including *N*-propenal-lysine (K+54) and dihydropyridine (DHP)-lysine (K+134 (48)). To determine which adducts form in apoA-I, we exposed the protein to MDA, digested it, and analyzed the resulting peptides by LC-ESI-MS. MS/MS detected K+54 in multiple peptides, strongly suggesting that *N*-propenal-Lys is a major adduct when MDA modifies Lys. In contrast, we failed to detect DHP-Lys when we exposed apoA-I to a wide range of MDA concentrations.

To confirm that K+54 was generated when apoA-I was exposed to MDA, we applied LC-ESI-MS/MS to Glu-C or trypsin digests. Fig. 5A shows a spectrum from a representative Glu-C peptide of apoA-I: LYRQKVEPLRAE, where K was Lys¹¹⁸ in the native protein (peptide + H⁺; m/z 1501.8). When apoA-I was incubated with MDA and digested, we detected a major product peptide at m/z 1555.8. This was consistent with the formation of LYRQ(K+54)VEPLRAE. This ion was not detectable in control apoA-I. In the MDA-treated protein, the b_5 – b_7 ions had gained 54 atomic mass units, but the y_5 and y_6 ions were unmodified (Fig. 5B), strongly suggesting that Lys¹¹⁸ had been converted to *N*-propenal-Lys. Because trypsin fails to cleave peptide bonds C-terminal to a modified Lys residue, we used the missing cleavages in tryptic digests to confirm our observations. Both tryptic and Glu-C digestion also identified the *N*-propenal adduct of the N-terminal amino group of apoA-I. Thus, MS analysis of Glu-C and tryptic digests demonstrated that Lys residues that contained an ϵ -amino group as well as the N-terminal α -amino group of apoA-I could form the *N*-propenal adduct when lipid-free apoA-I was exposed MDA.

MDA Converts a Subset of Residues to N-Propenal-Lys in High Yield—To determine whether MDA targets specific Lys residues in apoA-I, we exposed the protein to a 20:1 molar ratio of the carbonyl for 24 h and used isotope dilution LC-ESI-MS to quantify the product yields of modified Lys residues. The estimated product yield for K+54 is shown in Fig. 4B. Lys¹¹⁸, Lys¹³³, and Lys¹⁹⁵, as well as the N-terminal amino group, were selectively converted to the *N*-propenal adduct in relatively high yields. It is noteworthy that of the two precursor peptides lost in highest yield (Fig. 4A), one contained Lys²²⁶ and the other contained Lys⁹⁴, Lys⁹⁶, Lys¹⁰⁶, and Lys¹⁰⁷. However, those two peptides were converted to *N*-propenal-Lys in relatively low yield (compare Fig. 4, A with B). These observations suggest that K+54 accounts for only a subset of the peptides that are lost in high yield when apoA-I is exposed to MDA.

MDA Forms Lys-1-Amino-3-iminopropene-Lys Cross-links (Lys-MDA-Lys, K+36+K) in ApoA-I—The *N*-propenal-Lys adduct contains a carbonyl group that could cross-link with a second Lys residue to form the Lys-MDA-Lys adduct (Fig. 1B). Indeed, when apoA-I was exposed to low molar ratios of MDA, LC-ESI-MS/MS identified K+36+K, the Lys-1-amino-3-iminopropene-Lys (Lys-MDA-Lys) cross-link in which two Lys residues have gained 36 atomic mass units (39). For example,

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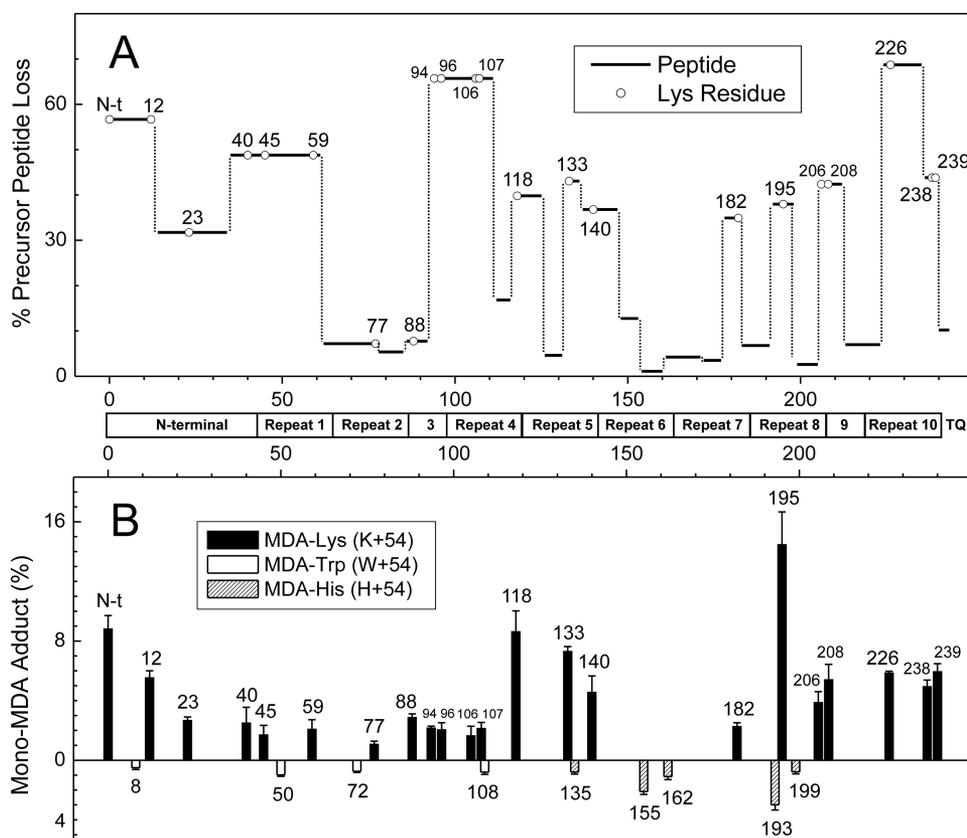


FIGURE 4. Precursor loss and product yields of *N*-propenal-Lys (K+54), *N*-propenal-Trp (W+54), and *N*-propenal-His (H+54) in MDA-modified apoA-I. ApoA-I was exposed to a 20-fold molar ratio of MDA, as described in the legend to Fig. 2. A mixture of 5 μ g of modified or control apoA-I and 2.5 μ g of 15 N-labeled apoA-I was digested with trypsin or Glu-C. A, following LC-ESI-MS/MS analysis, the ratio of precursor peptides derived from modified or control apoA-I relative to 15 N-labeled peptides derived from 15 N-labeled apoA-I was determined from extracted ion chromatograms. Precursor peptide loss was calculated as described under "Experimental Procedures." B, product yields of K+54, W+54, and H+54 were calculated from the ratio of peak area of product peptide from modified apoA-I to that of the corresponding 15 N-labeled peptide from 15 N-labeled apoA-I, as described under "Experimental Procedures." The *N*-propenal adducts were identified by LC-ESI-MS/MS analysis. *N-t*, N-terminal amino group. Results represent three independent experiments.

peptide KAKPALE (m/z 756.5), containing Lys²⁰⁶ and Lys²⁰⁸, was readily detected in Glu-C digests of lipid-free apoA-I. After apoA-I was exposed to MDA, the intensity of this precursor peptide fell significantly, although two new peaks of material appeared of m/z 810.5 and 792.5. This observation is consistent with the formation of K+54 and K+36+K. In the product peptide of m/z 810.5, MS/MS indicated that Lys²⁰⁶ or Lys²⁰⁸ was converted to K+54, although in the product peptide of m/z 792.5, Lys²⁰⁶ and Lys²⁰⁸ formed the K+36+K cross-link (Fig. 5D). In the peptide containing K+36+K, the b_3 to b_5 ions gained 36 atomic mass units, whereas the y_1 to y_4 ions were unchanged (compare Fig. 5, C and D). These findings are consistent with the conversion of Lys²⁰⁶ and Lys²⁰⁸ to Lys-MDA-Lys.

MDA Cross-links Both Juxtaposed and Distant Lys Residues in ApoA-I Primary Sequence—The estimated distance between the two Ne atoms of Lys-MDA-Lys is ~ 5.6 Å. If the lysine side chain (~ 7.6 Å) is included, the total distance of Lys-MDA-Lys cross-linking is ~ 20.8 Å, suggesting that Lys residues lying 0–4 amino acids apart in the primary sequence of apoA-I might be the primary targets of MDA. To test this proposal, we used LC-ESI-MS/MS to identify the sites of K+36+K in Glu-C and tryptic digests of MDA-treated apoA-I. This approach identi-

fied all four potential cross-links in adjacent Lys residues: Lys⁹⁴-MDA-Lys⁹⁶, Lys¹⁰⁶-MDA-Lys¹⁰⁷, Lys²⁰⁶-MDA-Lys²⁰⁸, and Lys²³⁸-MDA-Lys²³⁹ (Fig. 6B, bottom panel).

We also identified 27 cross-links between more distant Lys residues in lipid-free apoA-I and between the free N-terminal amino group and Lys¹² (Fig. 6A). It is noteworthy that the C terminus of apoA-I readily formed K+36+K adducts between residues that were far apart in the primary sequence. For example, most of the peptide containing Lys²²⁶ was lost from apoA-I exposed to MDA (Fig. 4A), and we identified cross-links between that residue and Lys¹¹⁸, Lys¹³³, Lys¹⁸², Lys¹⁹⁵, and Lys²⁰⁶ (Fig. 6A). Those peaks of material exhibited high ion currents, suggesting that Lys-MDA-Lys might be an important product when MDA modifies apoA-I.

Lys-MDA-Lys Cross-link Is a Major Product When ApoA-I Is Exposed to MDA—To determine the relative yields of K+36+K, we exposed apoA-I to a 50:1 molar ratio of MDA, digested the modified protein with Glu-C or trypsin, and used reconstructed ion chromatograms with isotope dilution to quantify the ion current of each product peptide.

All four pairs of closely situated Lys residues (Lys⁹⁴ and Lys⁹⁶, Lys¹⁰⁶ and Lys¹⁰⁷, Lys²⁰⁶ and Lys²⁰⁸, Lys²³⁸ and Lys²³⁹) formed K+36+K in high yield (Fig. 6B, bottom panel). However, certain Lys residues that were widely separated in the primary sequence of apoA-I also produced a high yield of Lys-MDA-Lys. Thus, Lys¹³³ and Lys¹⁴⁰, Lys¹¹⁸ and Lys¹⁴⁰, and Lys¹⁹⁵ and Lys²⁰⁶ were cross-linked to about the same extent as juxtaposed Lys residues (Fig. 6B, top panel).

We next compared the product yields of *N*-propenal adducts of Lys (K+54) and amino-3-iminopropene cross-links of Lys (K+36+K) in apoA-I incubated with different concentrations of MDA for different times. We found that when the concentration of MDA or the incubation time was increased, the product yield of K+36+K for Lys²⁰⁶ and Lys²⁰⁸ was much greater than that of the individual K+54 adducts (data not show). These observations strongly suggest that Lys-MDA-Lys is the major product when MDA modifies apoA-I.

Lys Residues in the C Terminus of ApoA-I Form Multiple K+36+K Adducts—Several individual Lys residues formed cross-links with multiple Lys residues. Thus, Lys¹³³ linked to five Lys residues, Lys¹⁸² to five Lys residues, Lys¹⁹⁵ to seven Lys residues, and Lys²²⁶ to five Lys residues (Fig. 6A). Importantly, most of those Lys residues lie in the C-terminal domain of apoA-I.

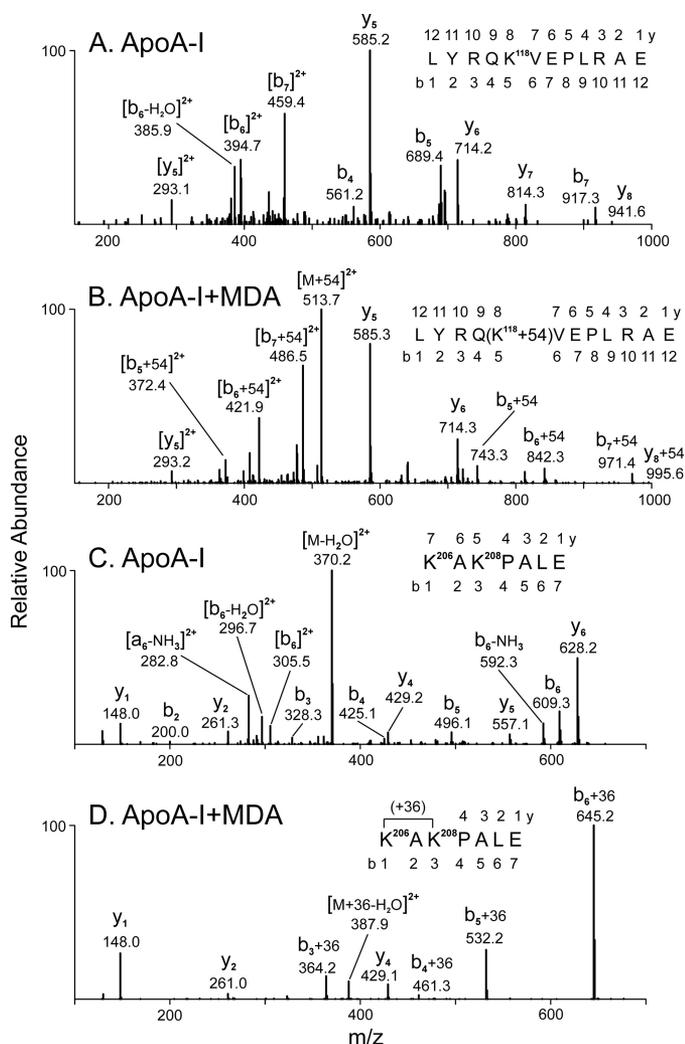


FIGURE 5. MS/MS analysis of N-propenal-Lys (K+54) and Lys-MDA-Lys (K+36+K) in MDA-modified apoA-I. ApoA-I was incubated in buffer alone (A and C) or exposed to a 20-fold molar excess of MDA (B and D), as described in the legend to Fig. 2. A Glu-C peptide digest was analyzed by LC-ESI-MS/MS, as described under "Experimental Procedures." A, MS/MS spectrum of [LYRQKVEPLRAE + H]⁺ (*m/z* 1501.8) where K is Lys¹¹⁸. B, MS/MS spectrum of [LYRQ(K+54)VEPLRAE + H]⁺ (*m/z* 1555.8), where K is Lys¹¹⁸. C, MS/MS spectrum of [KAKPALE + H]⁺, where the first K is Lys²⁰⁶ and the second K is Lys²⁰⁸ (*m/z* 756.5). D, MS/MS spectrum of [KAK(K+36+K)PALE + H]⁺, where the first K is Lys²⁰⁶ and the second K is Lys²⁰⁸ (*m/z* 792.5, Lys²⁰⁶+Lys²⁰⁸+36).

Lys Residues in Repeats 9 and 10 of ApoA-I Are Converted to K+54 and K+36+K in High Yield—By using apoA-I exposed to three different concentrations of MDA, we identified the 10 most reactive and 8 least reactive Lys residues in apoA-I (Table 1). Six of the 10 most reactive Lys residues were in repeats 8–10 at the C terminus (Lys¹⁹⁵, Lys²⁰⁶, Lys²⁰⁸, Lys²²⁶, Lys²³⁸, and Lys²³⁹), whereas all 8 of the least reactive were located at the N terminus (Lys²³, Lys⁴⁰, Lys⁴⁵, Lys⁵⁹, Lys⁷⁷, Lys⁸⁸, Lys¹⁰⁶, and Lys¹⁰⁷). Importantly, repeats 9 and 10 of the C-terminal domain are needed to promote sterol efflux by ABCA1 (7–9). Our observations suggest that MDA adduction and cross-linking of Lys residues in the C terminus might be particularly detrimental to the ability of apoA-I to transport cholesterol by ABCA1.

Suitably Juxtaposed, Positively Charged Amino Acids May Help MDA Modify Lys—To determine whether the local amino acid sequence of apoA-I might direct Lys modification by

MDA, we noted the 8 residues flanking each of the most and least reactive Lys residues (Table 1). Of the most reactive Lys residues, 60% (6 of 10 residues) had a positively charged amino acid located 2 residues toward the N terminus (−2 position), whereas 0% (0 of 8 residues) of the least reactive Lys residues exhibited this feature. We also observed that 60% (5 of 8) of poorly reactive Lys residues had a negatively charged residue 3 amino acids toward the C terminus (+3 position), whereas only 20% (2 of 10) of the highly reactive ones exhibited that feature. Furthermore, the 4 most reactive Lys residues all had a positively charged residue at the −2 position, whereas the 3 least reactive Lys residues all had a negatively charged residue at the +3 position. Our observations suggest that, in apoA-I, nearby positively charged amino acids help MDA modify Lys, whereas negatively charged amino acids might inhibit that reaction.

Energy Minimization Model of C Terminus of ApoA-I That Has Been Cross-linked by MDA—We used molecular dynamics energy minimization together with previous structural models (43, 44) to determine how K+36+K cross-links in repeats 9 and 10 affect the structure of lipid-free apoA-I. When the cross-links that we observed between residues Lys¹⁸², Lys¹⁹⁵, Lys²⁰⁶, Lys²⁰⁸, Lys²²⁶, and Lys²³⁹ (Fig. 7A) were incorporated into apoA-I with 2.5–5.6 Å distance constraints between the Lys Ne atoms, the C terminus of apoA-I became highly condensed (Fig. 7B). The overall structure of the cross-linked C terminus can be represented schematically as a hexagonal shape (Fig. 7, C and D). These observations, which represent an array of potential conformers of the various cross-linked forms of apoA-I, suggest that intramolecular cross-linking of the C terminus limits conformational adaptability as well as the ability of the domain to interact with ABCA1.

HDL Isolated from Human Atherosclerotic Lesions Is Enriched in Proteins That Have Been Modified by MDA—To quantitatively assess whether MDA modifies HDL *in vivo*, we isolated HDL by sequential density gradient ultracentrifugation from human plasma and carotid atherosclerotic tissue recovered at surgery. To prevent artifactual oxidation of lipoproteins, we used buffers containing high concentrations of DTPA (a metal chelator) and butylated hydroxytoluene (a lipid-soluble antioxidant). Immunoblotting with a monospecific rabbit antibody demonstrated that apoA-I accounted for >50% of the protein in lesion HDL.

We used the monoclonal antibody MDA2, which detects MDA-lysine adducts on a wide variety of modified proteins (42), to quantify MDA-HDL. We first showed that HDL exposed to MDA under the conditions used for our *in vitro* studies yielded material that was immunoreactive with MDA2 (Fig. 8A). Using MDA2 in an immunoassay, we found that the level of MDA-modified proteins in HDL isolated from the atherosclerotic lesions (407 ± 167 RLU/100 ms; *n* = 10) was 3.6-fold higher than in circulating HDL (113 ± 63 RLU/100 ms; *n* = 5; *p* < 0.0005) (Fig. 8B). These observations provide strong evidence that HDL can be modified by MDA in the human artery wall.

HNE Modifies ApoA-I His Residues in High Yield—His and Lys residues are the major targets for 4-hydroxynonenal *in vitro* (24, 49). To determine whether they are targeted in apoA-I, we exposed the protein to a 50:1 molar ratio of HNE and analyzed

Modification of ApoA-I by Carbonyls

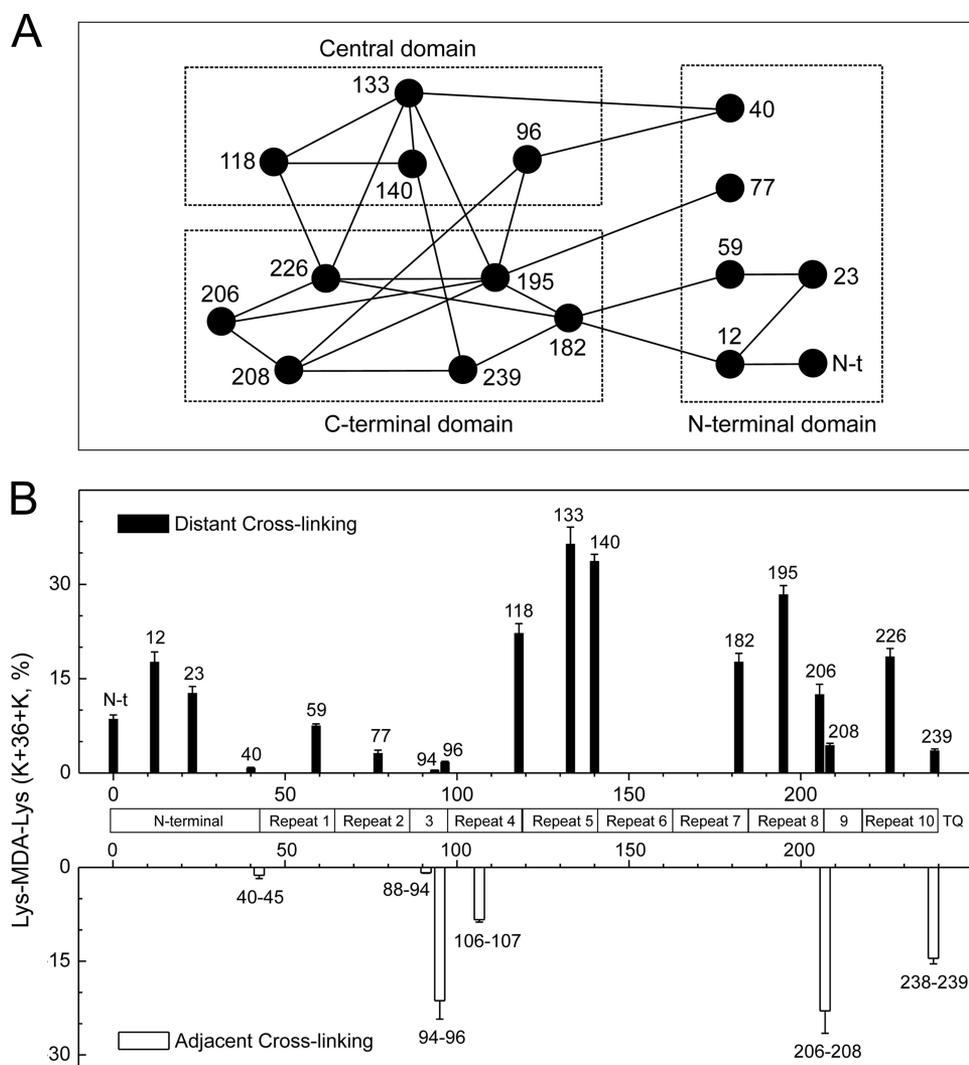


FIGURE 6. Product yield of Lys-MDA-Lys (K+36+K) and cross-linking map between Lys residues in MDA-modified apoA-I. ApoA-I was exposed to a 50-fold molar ratio of MDA, as described in the legend to Fig. 2. A mixture of control or modified apoA-I (5 μ g) and 15 N-labeled apoA-I (2.5 μ g) was digested with trypsin or Glu-C. Following LC-ESI-MS/MS analysis, the product yields of Lys-MDA-Lys (K+36+K) between Lys residues were calculated from the ratio of peak area of product peptide from modified apoA-I to that of the corresponding 15 N-labeled peptide from 15 N-labeled apoA-I, as described under "Experimental Procedures." All cross-linked adducts were confirmed by LC-ESI-MS/MS analysis. A, distant cross-linking map between Lys residues in MDA-modified apoA-I; B, product yield of adjacent cross-linking (bottom panel) and product yield of distant cross-linking (top panel).

a proteolytic digest by LC-ESI-MS/MS. All five peptides that contained His were lost in near-quantitative yield. MS/MS analysis revealed that the His residue in each peptide gained 158 or 156 atomic mass units without NaBH_4 reduction, suggesting that His was converted to the hemiacetal adduct (Fig. 1B) (49). In contrast, we were unable to detect HNE-Lys or HNE-Arg after exposing apoA-I to HNE.

We determined the product yield of HNE-His with reconstructed ion chromatograms of product and precursor peptides from apoA-I exposed to a 50:1 molar ratio of HNE (Fig. 9, purple bars). This concentration of HNE does not affect the ability of the protein to promote cholesterol efflux by the ABCA1 pathway. All five His residues were modified in near-quantitative yield (\sim 90%). This observation strongly suggests that the His residues of apoA-I are the major target of HNE and that HNE adducts of His have little impact on the ability of the protein to promote cholesterol efflux by the ABCA1 pathway.

Methylglyoxal Modifies Arg Residues in ApoA-I—Arg residues are the major target for methylglyoxal *in vitro* (50). LC-ESI-MS analysis of a Glu-C digest of native apoA-I demonstrated that the 18 Arg residues of the protein were distributed among 12 peptides. When we exposed apoA-I to a 50:1 molar ratio of methylglyoxal, we detected all 12 peptides containing R+54 and/or R+72. MS/MS analysis confirmed that the Arg residues in those peptides had gained 54 or 72 atomic mass units, suggesting the formation of imidazolone adducts (Fig. 1B) (50). We determined the product yields of methylglyoxal-Arg adducts with reconstructed ion chromatograms of product and precursor peptides. Arg⁶¹ was the major target of methylglyoxal in apoA-I. When the protein was exposed to a 50:1 molar ratio of the carbonyl, the product yield of imidazolone adducts involving Arg⁶¹ was \sim 80% (Fig. 9, yellow bars). However, modifying apoA-I with a 50:1 molar ratio of methylglyoxal failed to affect the ability of the protein to promote cellular cholesterol efflux by ABCA1.

Glycolaldehyde Fails to Yield Detectable Adducts in ApoA-I—Glycolaldehyde converts Lys residues to N^ϵ -(carboxymethyl)lysine (21, 51) and glycolaldehyde-pyridine *in vitro* (52). To identify the major targets in apoA-I, we exposed the protein to a 50:1 molar ratio of glycolaldehyde, reduced the protein with NaBH_4 , and analyzed a proteolytic

digest with LC-ESI-MS/MS. However, we were unable to detect any major products. These results are consistent with our MALDI-TOF-MS analysis, which failed to detect an increase in molecular weight after apoA-I was exposed to glycolaldehyde. Our observations suggest that glycolaldehyde does not generate a high yield of stable adducts in apoA-I, even though we readily detected glycolaldehyde-Arg adducts when we studied model peptides.

DISCUSSION

Conversion of HDL to a dysfunctional form has been proposed to impair the cardioprotective properties of the lipoprotein, but the underlying mechanisms remain poorly understood (13, 53). However, many lines of evidence implicate reactive carbonyls in the pathogenesis of atherosclerotic and diabetic vascular disease (21, 27, 54, 55). Therefore, we determined whether pathophysiologically relevant carbonyls, MDA, HNE,

TABLE 1

Analysis of amino acid sequences flanking the Lys residues most reactive and least reactive with MDA in apoA-I

ApoA-I (5 μ M) was exposed to 10, 100, or 250 μ M MDA, as described in the legend to Fig. 2. Product yields were calculated by isotope dilutions using 15 N-labeled apoA-I. Results represent two independent experiments.

Most Reactive	-4	-3	-2	-1	K	1	2	3	4
1	G	A	R	Q	K133	L	H	E	L
2	E	Y	H	A	K195	A	T	E	H
3	L	Y	R	Q	K118	V	E	P	L
4	S	E	K	A	K208	P	A	L	E
5	E	Y	T	K	K239	L	N	T	Q
6	L	E	S	F	K226	V	S	F	L
7	T	L	S	E	K206	A	K	P	A
8	E	E	Y	T	K238	K	L	N	T
9	W	D	R	V	K112	D	L	A	T
10	E	V	K	A	K96	V	Q	P	Y
Least Reactive	-4	-3	-2	-1	K	1	2	3	4
1	Q	L	N	L	K45	L	L	D	N
2	D	N	L	E	K77	E	T	E	G
3	Q	E	M	S	K88	D	L	E	E
4	S	A	L	G	K40	Q	L	N	L
5	D	D	F	Q	K106	K	W	Q	E
6	D	F	Q	K	K107	W	Q	E	E
7	V	D	V	L	K23	D	S	G	R
8	S	T	F	S	K59	L	R	E	Q

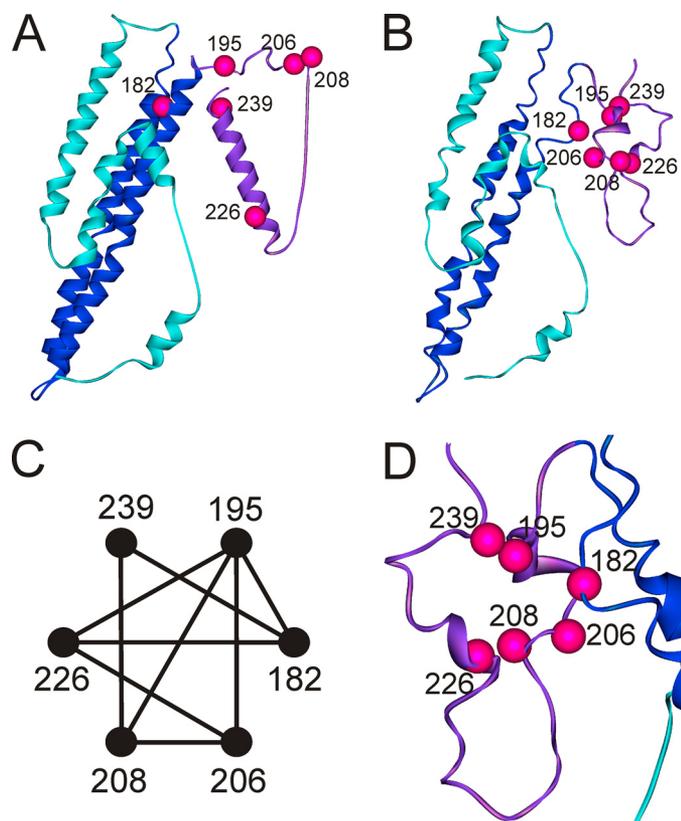


FIGURE 7. Hexagon model of MDA-modified C terminus of apoA-I. A, location of Lys residues modified in high yield by MDA in the C-terminal region of lipid-free apoA-I. B, energy minimization model of repeats 7–10 of apoA-I cross-linked by MDA. C, hexagon model of cross-links between C-terminal lysine residues in MDA-modified apoA-I. D, close-up view of C-terminal in the energy minimization model of apoA-I cross-linked by MDA.

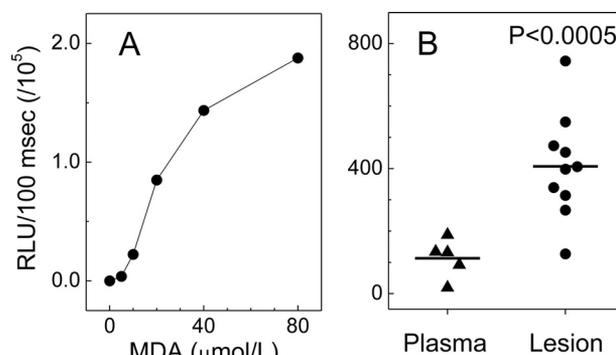


FIGURE 8. Quantification of MDA-modified proteins in HDL isolated from plasma and atherosclerotic tissue of humans. A, HDL (from healthy plasma, 0.2 mg of protein/ml) was exposed to indicated concentrations of MDA as described in the legend to Fig. 2. B, plasma was obtained from healthy humans. Human atherosclerotic tissue was obtained at surgery from subjects undergoing carotid endarterectomy. HDL was isolated from plasma and tissue by ultracentrifugation, and equal amounts of HDL protein were added to each well, and then MDA-modified proteins were quantified by enzyme-linked immunosorbent assay using the MDA2 monoclonal antibody (RLU/ms = relative light units/ms). Each value shown is the average of triplicate measurements as described under "Experimental Procedures."

glyoxal, methylglyoxal, and glycolaldehyde, might impair a key aspect of cardioprotective effects of apoA-I, *i.e.* cholesterol removal by the ABCA1 pathway. Remarkably, we found that MDA, unlike the other carbonyls we tested, potentially damages this critical step in reverse cholesterol transport. These observations may be biologically important because we showed that HDL isolated from human atherosclerotic lesions had been modified by MDA.

The nature of selective vulnerability of apoA-I to MDA is a key question. Three lines of evidence strongly support the proposal that MDA modifies the ϵ -amino group of Lys residues in the C terminus of apoA-I and that the resulting Lys-MDA adducts impair cholesterol efflux by the ABCA1 pathway. First, quantitative analysis of proteolytic digests of apoA-I by isotope dilution MS revealed that certain Lys residues were converted in high yield to the MDA-Lys or Lys-MDA-Lys adduct. Six of the 10 most reactive Lys residues resided in repeats 7–10 of the C terminus of apoA-I (Fig. 9), suggesting that modification of this region might be an important mechanism for impairing ABCA1 activity. Second, HNE failed to affect the ability of apoA-I to promote cholesterol efflux even though it modified all five His residues of the central domain of apoA-I in high yield (Fig. 9). Glyoxal and methylglyoxal, which likewise failed to affect the ABCA1 activity of apoA-I, modified residues in the N terminus of the protein and the central region of the protein but not its C terminus (Fig. 9). Third, the C terminus of apoA-I was the major site for cross-linking by MDA (Fig. 9). A model based on molecular dynamics energy minimization suggested that cross-linking caused the C terminus of apoA-I to become highly condensed, which should markedly affect its conformation and conformational adaptability. Importantly, studies of deletion mutants of apoA-I suggest that repeat 10 of the C terminus of the protein helps activate ABCA1 (7, 9). Taken together, these observations support the proposal that modification of the C terminus of apoA-I by MDA deprives the protein of its ability to promote cholesterol efflux by the ABCA1 pathway.

Modification of apoA-I by Carbonyls

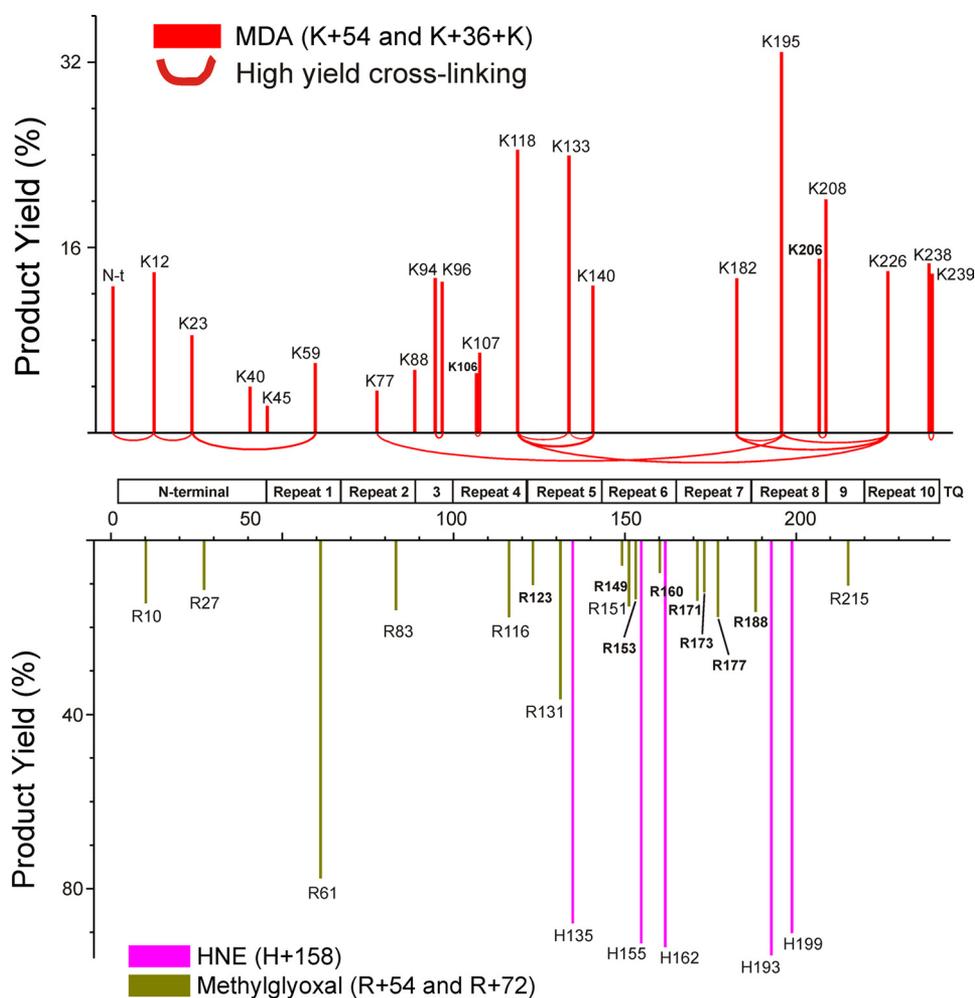


FIGURE 9. Summary of amino acid residues modified by MDA, HNE, or methylglyoxal in lipid-free apoA-I. The product yields of MDA-Lys (K+54) and Lys-MDA-Lys (K+36+K) (red), HNE-His (H+158, purple), or methylglyoxal-Arg (R+54 and R+72, yellow) were determined by isotope dilution MS. High yield cross-linking between lysine residues by MDA are indicated (red arc).

The C-terminal region of lipid-free apoA-I contains a stretch of random coil (residues 188–208) and a stretch of β -strand (residues 209–220) (44, 56–58). The random coil is thought to contribute to the energy requirements of lipid-free to lipid-bound conformational transition, and the β -strand stabilizes the soluble lipid-free conformation of apoA-I (44, 59). When the protein binds lipid, however, residues 188–220 undergo a transition to an α -helical structure (44). This conformational transition has been proposed to account for most of the free energy required to transform apoA-I from its lipid-free to lipid-bound states (44, 59). Molecular dynamics energy minimization modeling, based on the Lys-MDA-Lys adducts we observed in repeats 7–10, suggested that cross-linking limited the conformational adaptability of the C terminus of lipid-free apoA-I. Cross-linking would therefore minimize the enthalpic contribution of this region to lipid binding.

Collectively, our observations suggest that MDA alters the ability of apoA-I to promote sterol efflux by impairing two key steps in the ABCA1 pathway (60). First, MDA-Lys adducts alter the structure and conformation of repeat 10, preventing productive interactions with ABCA1 (61). Second, Lys-MDA-Lys cross-links limit the conformational adaptability of the C ter-

minus, reducing the amount of free energy available for the lipid-free to lipid-bound transition of apoA-I.

Modification of apoA-I could be physiologically relevant because MDA is one of the most abundant carbonyls generated through lipid peroxidation (24). Indeed, immunochemical analyses of HDL with MDA2, a monoclonal antibody that binds with high affinity to a variety of MDA-modified proteins (42), demonstrated that HDL isolated from atherosclerotic tissue contained a 3.6-fold higher concentration of MDA than HDL from the plasma of apparently healthy humans. Moreover, MDA concentration appears to increase in diseases, such as diabetes mellitus, that increase the risk of cardiovascular disease (54). Importantly, MDA adducts have also been detected in low density lipoproteins isolated from atherosclerotic lesions (62), and modification by MDA of Lys residues in apoB-100 of low density lipoprotein is implicated in macrophage foam cell formation (35, 63), a critical step in atherogenesis.

These observations suggest that modification of Lys residues by MDA generated during lipid peroxidation could promote the formation of macrophage foam cells by two distinct pathways as follows: (i)

conversion of low density lipoprotein into a ligand for scavenger receptors that admit cholesterol into macrophages; and (ii) modification of lipid-free apoA-I derived from HDL in a manner that impairs cholesterol efflux by the ABCA1 pathway.

Most studies of protein modification have focused on the vulnerability of individual amino acid side chains. Remarkably little is known about the influence of nearby residues, although it is clear that reactive intermediates can modify specific amino acid residues in proteins (19, 34, 64, 65). When we exposed apoA-I to low concentrations of MDA, isotope dilution MS revealed that certain Lys residues were modified in high yield, whereas others were almost completely resistant. Thus, MDA modifies Lys residues only at specific sites in lipid-free apoA-I. Analysis of nearby amino acids in the apoA-I sequence demonstrated that a positively charged amino acid located two residues away (toward the N terminus of the protein) from a Lys residue strongly associated with increased reactivity with MDA. In contrast, a negatively charged amino acid residue located three amino acids toward the C terminus associated with decreased modification by MDA. It is important to note that most of apoA-I has an α -helical structure and that amino acids two and three residues apart in a primary α -helical

sequence are closely located in three-dimensional space. Our observations suggest that positively charged amino acids that are appropriately juxtaposed to Lys residues in apoA-I may favor the formation of MDA-Lys adducts. Interestingly, positively charged surfaces in albumin were recently proposed to direct site-specific modification of Lys residues by MDA (66).

HNE is perhaps the best studied product of lipid peroxidation (24). It can react with imidazoles (His), thiols (Cys), or free amino (Lys) groups of proteins to form stable Michael adducts with hemiacetal structures (39, 67). We readily detected His adducts after we exposed apoA-I to HNE. Quantitative analysis by isotope dilution MS revealed that all five His residues in apoA-I were modified in high yield (~90%) when the protein was exposed to a 50:1 molar ratio of HNE. Remarkably, this concentration of HNE had no effect on the ability of apoA-I to promote cholesterol efflux by the ABCA1 pathway, likely because all five His residues are located in the central region of the protein, which does not appear to play a major role in activating the transporter.

Glyoxal and methylglyoxal can be generated by carbohydrate, lipid, and amino acid oxidation, and elevated levels of these reactive carbonyls are thought to play important roles in the accelerated vascular disease observed in diabetic humans (55). However, apoA-I exposed to either carbonyl was still fully competent to promote cholesterol efflux by the ABCA1 pathway. These observations are consistent with the fact that only one Arg residue, Arg²¹⁵, resides in repeats 9 and 10 of apoA-I. Importantly, this Arg was only minimally modified by glyoxal or methylglyoxal.

Glycolaldehyde is produced by carbohydrate oxidation (68) and by activated human neutrophils, which use myeloperoxidase to make it from L-serine (28, 29). However, we were unable to detect any modified amino acid residues in apoA-I exposed to glycolaldehyde. Our observations suggest that glycolaldehyde has little impact on the ability of apoA-I to promote cholesterol efflux by ABCA1, but it is unclear whether the carbonyl yields stable adducts in apoA-I under our experimental conditions.

In conclusion, we demonstrated that of five reactive carbonyls studied only MDA selectively and dramatically impaired the ability of apoA-I to transport cholesterol. The mechanism likely involves modification of Lys residues, especially cross-linking in repeats 9 and 10 in the C terminus. Our observations raise the possibility that modification of apoA-I by MDA generates dysfunctional HDL that can no longer remove cholesterol from macrophages. In future studies, it will be of interest to determine whether reactive carbonyls impair other key steps in reverse cholesterol transport, such as lecithin:cholesterol acyltransferase activation, that depend on apoA-I.

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