Acrolein Impairs ATP Binding Cassette Transporter A1-dependent Cholesterol Export from Cells through Site-specific Modification of Apolipoprotein A-I*

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Acrolein is a highly reactive α,β-unaturated aldehyde, but the factors that control its reactions with nucleophilic groups on proteins remain poorly understood. Lipid peroxidation and threonine oxidation by myeloperoxidase are potential sources of acrolein during inflammation. Because both pathways are implicated in atherogenesis and high density lipoprotein (HDL) is anti-atherogenic, we investigated the possibility that acrolein might target the major protein of HDL, apolipoprotein A-I (apoA-I), for modification. Tandem mass spectrometric analysis demonstrated that lysine 226, located near the center of helix 10 in apoA-I, was the major site modified by acrolein. Importantly, this region plays a critical role in the cellular interactions and ability of apoA-I to transport lipid. Indeed, we found that conversion of Lys-226 to N-(3-formyl-3,4-dehydropiperidino)lysine by acrolein associated quantitatively with decreased cholesterol efflux from cells via the ATP-binding cassette transporter A1 pathway. In the crystal structure of truncated apoA-I, Glu-234 lies adjacent to Lys-226, suggesting that negatively charged residues might direct the modification of specific lysine residues in proteins. Finally, immunohistochemical studies with a monoclonal antibody revealed co-localization of apoA-I with acrolein adducts in human atherosclerotic lesions. Our observations suggest that acrolein might interfere with normal reverse cholesterol transport by HDL by modifying specific sites in apoA-I. Thus, acrolein might contribute to atherogenesis by impairing cholesterol removal from the artery wall.

Chemical damage to key proteins may contribute to the pathogenesis of many disorders, including diabetes, Alzheimer disease, and ischaemia-reperfusion injury and perhaps to the aging process itself (1). Reactive aldehydes are an important class of agents that covalently modify proteins, and a variety of enzymatic and nonenzymatic mechanisms generate them in the body (2). Acrolein is the most reactive α,β-unsaturated aldehyde, and it rapidly modifies biological nucleophiles (3). This potent electrophile may be produced in the body through lipid peroxidation or peroxidation of threonine by myeloperoxidase, and high levels are found in cigarette smoke (4–6). Lipid peroxidation, myeloperoxidase, renal disease, and cigarette smoking all strongly associate with an increased risk of vascular disease (2, 7, 8). Moreover, epitopes recognized by a monoclonal antibody specific for lysine adducts of acrolein have been detected in macrophages of atherosclerotic lesions (4), raising the possibility that acrolein is one important agent for damaging proteins in the artery wall. Acrolein may also be a uremic toxin, and carboxyl stress appears to increase in patients with chronic renal failure (9).

Like other α,β-unsaturated aldehydes, acrolein selectively reacts with the sulfhydryl group of cysteine, the imidazole group of histidine, and the e-amino group of lysine (10–12). Indirect evidence suggests that it might also modify arginine (13). In vitro studies have identified a number of acrolein adducts with proteins, including Nε-(3-methylpyridinium)lysine (MP-Lys),2 and Nε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) (Scheme 1) (4, 12). Moreover, modification of a single histidine residue by acrolein apparently decreases the catalytic activity of carbonic anhydrase II (14). However, the underlying mechanism is poorly understood, and remarkably, little is known about the factors that control the reaction of acrolein with nucleophilic groups in proteins.

High density lipoprotein (HDL), which decreases the risk of atherosclerotic disease (15–17), may be one physiologically important target of aldehyde compounds. Its protective effect has been attributed partly to the ability of its major protein, apolipoprotein A-I (apoA-I), to mobilize cholesterol from macrophages of the arterial wall to the liver, a process termed reverse cholesterol transport (18). Because this protein accounts for ~70% of HDL total protein content, structural alterations to apoA-I that inhibit HDL function might be pivotal to atherogenesis (19). Because HDL is the major carrier of lipid hydroperoxides in plasma (20), it may be constantly exposed to reactive aldehydes derived from decomposed lipid hydroperoxides. Pathways that oxidize HDL and thereby impair its function may, thus, be pivotal to the development of atherosclerosis (19–23). Indeed, there is strong evidence that myeloperoxidase oxidizes HDL in vivo (24–27), and this enzyme promotes lipid peroxidation and the generation of acrolein in vitro (5, 28, 29).

A variety of reactive aldehydes modify HDL and appear to cross-link its proteins in vitro (30). Modification is associated with loss of the ability of HDL to activate lecithin-cholesterol acyltransferase (30), a key enzyme in reverse cholesterol transport. However, it is unknown whether HDL in the human artery wall is vulnerable to modification by acrolein or other reactive aldehydes.

HDL apolipoproteins remove cellular cholesterol and phospholipids 2 The abbreviations used are: MP-Lys, Nε-(3-methylpyridinium)lysine; apoA-I, apolipoprotein A-I; BHK cells, baby hamster kidney cells; ESI, electrospray ionization; FDP-Lys, Nε-(3-formyl-3,4-dehydropiperidino)lysine; HDL, high density lipoprotein; LC, liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization—time-of-flight mass spectrometry; MS, mass spectrometry; m/z, mass-to-charge ratio; HPLC, high performance liquid chromatography; ABCA1, ATP-binding cassette transporter A1; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; DTPA, diethylenetriaminepentaaacetic acid.

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by a cholesterol-inducible active transport process mediated by a cell membrane protein called ATP-binding cassette transporter A1 (ABCA1) (18, 31). Lipid poor apoA-I promotes the efflux of cholesterol and phospholipids from macrophages exclusively by this pathway. The process appears to involve the amphipathic α-helical domains of apoA-I (32). In the current studies, we demonstrate that acrolein impairs the ability of apoA-I to promote cellular cholesterol efflux by the ABCA1 pathway. Moreover, we show that a single lysine (Lys-226) in helix 10 of apoA-I is major target of acrolein and that conversion of this residue to MP-Lys is quantitatively associated with inhibition of ABCA1 activity. Importantly, our immunohistochemical studies demonstrate co-localization of acrolein adducts and apoA-I in human atherosclerotic lesions. Thus, acrolein and perhaps other reactive aldehydes may interfere with normal HDL cholesterol transport by modifying apoA-I in the human artery wall. A preliminary account of this work was presented at the Eighth International Symposium on the Maillard reaction (33).

EXPERIMENTAL PROCEDURES

Materials

HPLC grade acetonitrile (CH₃CN) and trifluoroacetic acid were obtained from Fisher. Unless otherwise indicated, all other materials were purchased from Sigma.

Methods

Isolation of HDL and ApoA-I—Blood collected from healthy subjects who had fasted overnight was anticoagulated with EDTA. HDL (density 1.215–1.210 g/ml) was prepared from plasma by sequential ultracentrifugation and was depleted of apolipoproteins E and B100 by heparin-agarose chromatography (34). ApoA-I was purified to apparent homogeneity from HDL (34). Protein concentration was determined using the Lowry assay (Bio-Rad), with albumin as the standard. The Human Studies Committees at University of Washington School of Medicine approved all protocols involving human material.

Acrolein Modification—Reactions with isolated apoA-I (25 μM, 0.7 mg protein/ml), HDL (1 mg protein/ml), or synthetic peptides (100 μM) 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 acrolein and terminated by adding a 20-fold molar excess (relative to acrolein) of aminoguanidine. When indicated, reaction mixtures were reduced with a 50-fold molar excess (relative to protein or peptide) of sodium borohydride (NaBH₄).

Proteolytic Digestion—Native or acrolein-modified apoA-I or HDL was incubated overnight at 37 °C with a 20:1 ratio (w/w) of endoprotease Glu-C (from Staphylococcus aureus V8), sequencing grade modified trypsin (Promega, Madison, WI), or Glu-C/trypsin in 50 mM NH₄HCO₃ (pH 7.8). Digestion was halted by acidifying (pH 2–3) with trifluoroacetic acid.

Liquid Chromatography Electro spray Ionization Mass Spectrometry (LC-ESI-MS)—LC-ESI-MS analyses were performed in the positive ion mode with a Finnigan Mat LCQ ion trap instrument (San Jose, CA) coupled to a Waters 2690 HPLC system (Milford, MA) (35, 36). Synthetic peptides and peptide digests were separated on a reverse-phase column (Vydac C18 MS column, 25 × 2.1 mm inner diameter) at a flow rate of 0.2 ml/min using solvent A (0.2% formic acid in water) and solvent B (0.2% formic acid in 90% CH₃CN and 10% water). Solvent B was kept at 0% for 8 min, increased to 10% in 2 min, and then increased to 35% over 35 min for synthetic peptides or to 40% over 60 min for peptide digests. The electrospray needle was held at 4500 V. Nitrogen, the sheath gas, was set at 60 units. The collision gas was helium. The temperature of the heated capillary was 220 °C.

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). The matrices were α-cyano-4-hydroxy cinnamic acid for peptide digests and sinapinic acid for intact protein. The spectra of peptide digests were obtained in the positive reflectron 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.

SDS-PAGE and Western Blotting—Proteins were separated under reducing and denaturing conditions on 4–12% Bis-Tris polyacrylamide gels (NuPAGE, Invitrogen). Proteins were visualized with Coomassie Blue. For Western blotting, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). ApoA-I was detected with a goat polyclonal antibody to human apoA-I (1:10,000 dilution; Rockland Immunochemicals, Gilbertsville, PA) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution). Acrolein-modified apoA-I was detected with the monoclonal antibody mAb5F6 (1:5,000 dilution) (4) followed by horseradish peroxidase-conjugated mouse anti-goat IgG (1:10,000 dilution). Visualization of immunoreactive bands was performed with enhanced chemiluminescence.

Immunohistochemical Studies—Human coronary artery segments were obtained from hearts excised at the time of cardiac transplantation. They were fixed in neutral buffered formalin and embedded in paraffin as described previously (37). Atherosclerotic plaques were identified by morphological criteria (38) from 6-μm sections stained with Movat’s pentachrome stains. A mouse monoclonal antibody (mAb5F6) was used to identify acrolein-lysine adducts (1:500 dilution) (4), and a goat polyclonal antiserum was used to identify apoA-I (1:750 dilution) (37). Single-label immunohistochemistry was performed using previously described techniques (37), except that NovaRED (Vector Laboratories, Burlingame, CA), which yields a red reaction product, was used as the peroxidase substrate.

Efflux of Cellular Cholesterol and Phospholipid—Baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 were...
radiolabeled with either $[^{3}H]$cholesterol or $[^{3}H]$choline. Expression of ABCA1 was induced by incubating cells for 20 h with Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin and 10 nM mifepristone. Efflux of $[^{3}H]$cholesterol or $[^{3}H]$choline-labeled phospholipids was measured after a 2-h incubation with Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin without or with apoA-I as described (39, 40). Cholesterol or phospholipid efflux mediated by apoA-I was calculated as the percentage of total $[^{3}H]$lipid (medium plus cell) released into the medium after subtracting the value obtained with Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin alone. BHK cells incubated with native or acrolein-modified apoA-I for up to 4 h showed no changes in morphology or in cell protein or cholesterol and phospholipid content per well.

RESULTS

FDP-Lys, MP-Lys, and $N_{\text{terminus}}^\text{propanalhistidine}$ Are the Major Products when Synthetic Peptides Are Exposed to Acrolein—In vitro studies have suggested that acrolein modifies amino groups, guanidine groups, sulfhydryl groups, and imidazole groups (3, 10, 13). To investigate the reactions with acrolein of amino acid side chains containing these nucleophiles, we synthesized four model peptides: Ac-GEYAHKY (HK), Ac-GEYAEEK (EK), Ac-GEYARKY (RK), and Ac-GEYAREY (RE). In peptide HK the imidazole and amino groups compete directly to react with acrolein. Peptides RE and RK were designed to determine the reactivity of Arg. Peptides EK and RK explored the impact of an adjacent positively or negatively charged group on the reactivity of Lys. The N-terminal group of each peptide was acetylated to prevent the primary amine from participating in the reactions. We did not explore the reactivity of thiol groups because apoA-I lacks cysteine residues. Each peptide was exposed to acrolein in phosphate buffer supplemented with DTPA (to chelate redox-active metal ions (41)) for 24 h at 37 °C. The reaction was terminated with aminoguanidine or with subsequent reduction with NaBH$_4$ and then analyzed by LC-ESI-MS.

We initially focused on peptide HK. After the peptide was exposed to acrolein and reduced with NaBH$_4$, LC-ESI-MS analysis detected several products (Fig. 1). One product exhibited a major ion at m/z 967.4, which was consistent with the addition of 58 atomic mass units to the protonated peptide [peptide + 58 atomic mass units + H$^+$]. MS/MS analysis of this product demonstrated b$_5$ and b$_6$ ions that had gained 58 atomic mass units (Fig. 1A), indicating that acrolein had converted His to His+58. In the absence of reduction the apparent mass of the His in the modified peptide increased by 56 atomic mass units, strongly suggesting that the adduct was $N_{\text{terminus}}^\text{propanalhistidine}$ (His+56) (4). Two other products exhibited major ions at m/z 1005.3 [peptide + 96 atomic mass units + H$^+$] and m/z 985.5 [peptide + 76 atomic mass units + H$^+$]. MS/MS analysis demonstrated that Lys was modified in these peptides and that its apparent mass had increased by 96 or 76 atomic mass units (Fig. 1, C and D; K+96 and K+76). In the absence of reduction, the mass of the Lys+96 adduct was shifted to Lys+94, but the Lys+76 adduct exhibited the same apparent M$_r$ in the presence or absence of reduction. These observations are consistent with previous studies demonstrating that acrolein reacts with Lys to form FDP-Lys (K+94) and MP-Lys (K+76) (4, 12).

We also used LC-ESI-MS and MS/MS to determine whether peptides EK, RE, and RK reacted with acrolein. FDP-Lys and MP-Lys were readily detectable in the peptides containing Lys. In contrast, we failed to detect adducts of Arg in peptides RE or RK (with or without reduction), which suggests that the guanidine group of these peptides had failed to react with the α,β-unsaturated aldehyde or that acrolein adducts of Arg were reversible under our experimental conditions.

We quantified the product yields of FDP-Lys and MP-Lys using reconstructed ion chromatograms of the precursor and product peptides. At a mole ratio of acrolein to peptide of 10:1 and an incubation time of 24 h, all the peptides gave a similar (25–42%) product yield of FDP-Lys (K+94) (Fig. 2C). In contrast, the product yields of MP-Lys varied over a 10-fold range (1–15%), suggesting that the local amino acid environment might strongly affect the reaction pathway. At relatively low mole ratios of aldehyde to peptide (<10:1), the product yield of the Lys adducts was linearly dependent on the concentration of acrolein (Fig. 2A). Acrolein initially reacted rapidly with the Lys residue in each peptide, but the reaction rate gradually decreased (Fig. 2B). Under all conditions, FDP-Lys was the major product in peptides EK and RK. MP-Lys was also a major product in peptide HK (Fig. 2), whereas MP-Lys was always a minor product in peptides EK and RK (data not shown).

We also quantified $N_{\text{terminus}}^\text{propanalhistidine}$ (His+56) after exposing peptide HK to acrolein (10:1, mol/mol) for 24 h (Fig. 2). Approximately
50% of the His residue was converted to Nε-propanalhistidine, indicating that the yield of His+56 was similar to that of Lys+96 (FDP-Lys) plus Lys+76 (MP-Lys). It should be noted that we also observed peptides with adducts of both His and Lys (H+56, K+94, and H+56, K+76) when we exposed peptide HK to acrolein. This suggests that modifying one of these amino acids does not prevent the side chain of the other from reacting. Collectively, these observations indicate that Lys and His are major targets of acrolein in the synthetic peptides HK, EK, and RK. Moreover, high yields of FDP-Lys were produced in all the Lys-containing peptides, whereas the yield of MP-Lys depended on the sequence of each peptide. In contrast, we were unable to detect stable adducts of Arg after we incubated RK or ER for variable times with a wide range of acrolein concentrations.

MP-Lys, but Not FDP-Lys or Nε-propanalhistidine, Is the Major Product When ApoA-I Reacts with Acrolein—The 10 amphipathic helical domains in apoA-I, the major protein in HDL, are thought to play essential roles in lipid binding, lipoprotein stability, and reverse cholesterol transport (42–44). Our peptide studies suggested that acrolein reacts with residues that contain primary amines and imidazoles, and apoA-I contains 21 Lys and 5 His residues (Fig. 3). To determine which of these amino acids acrolein can modify, we exposed apoA-I to the α,β-unsaturated aldehyde at neutral pH in phosphate buffer containing a metal chelator for 24 h at 37 °C. After terminating the reaction with aminoguanidine, a proteolytic digest of the protein was analyzed by LC-ESI-MS, LC-ESI-MS/MS, and MALDI-TOF-MS. Because apoA-I is rich in Lys and Arg residues, a tryptic digest yields many small peptides that are poorly retained on a reverse-phase column. ApoA-I digested with Glu-C (which cleaves peptide bonds C-terminal to glutamic acid in ammonium bicarbonate buffer (45)) produces fewer, larger peptides, but several contain multiple Lys residues. To ensure complete coverage and to unambiguously identify the site at which each peptide is modified, we therefore used Glu-C, trypsin, or both Glu-C and trypsin to digest apoA-I and acrolein-modified apoA-I.

LC-ESI-MS analysis of a Glu-C digest of native apoA-I detected 11 peptides containing 17 of the protein’s 21 Lys residues. We did not detect peptides containing Lys-133, Lys-195, and Lys-206 or Lys-208. When we exposed apoA-I to a 20:1 mole ratio of acrolein (aldehyde/protein), all 11 peptides that contained Lys gained 76 atomic mass units (TABLE ONE). Fig. 4 shows the MS/MS analysis of SFKVSFLSALEE, which contains Lys-226, in native and acrolein-exposed apoA-I. In the acrolein-modified protein, every b ion from b3 to b11 of the peptide had gained 76 atomic mass units (TABLE ONE), suggesting that Lys-226 had been converted to MP-Lys. MS analysis demonstrated that all of the Lys-containing peptides in acrolein-modified apoA-I had gained 76 atomic mass units (TABLE ONE), suggesting that Lys had been converted to MP-Lys in all 11 peptides.

MALDI-TOF-MS analysis of a Glu-C digest of native and acrolein-modified apoA-I detected 8 of the same peptides detected by LC-ESI-MS as well as 3 additional Lys-containing peptides: Lys-133, Lys-195, and (Lys-206, Lys-208) (data not shown). Fig. 5A shows the MALDI-TOF-MS spectrum of peptide SFKVSFLSALEE (peptide + H⁺; m/z 1356.6) in the native protein. When apoA-I was incubated with acrolein, a major peak of material at m/z 1432.6 was detected in the peptide digest (Fig. 5B), consistent with the formation of SF(K+76)VSFLSALEE. Note that this ion was not detected in control apoA-I (Fig. 5A). A major ion (m/z 1372.6) derived from the Glu-C digest peptide containing His-162 was also present in this region of the full scan mass spectrum (Fig. 5A). However, we did not detect any prod-
Site-specific Modification of ApoA-I by Acrolein

**TABLE ONE**

Detection of acrolein-modified lysine residues in a Glu-C digest of apoA-I using LC-ESI-MS

| Position | Sequence | Predicted m/z | Observed m/z | Modification |
|----------|----------|---------------|--------------|--------------|
| 1–13     | DEPPQSWDVRKK* | 1568.7 | 1644.6, 822.8, 549.1 | K7+ +76 |
| 14–34    | LATVYVDVLK*DSGRDVVSQFE | 2404.2 | 1240.7, 827.7 | K7+ +76 |
| 35–62    | GSAKLKGWQLNLK*LLDNWDSVTSTFSK*LE | 3120.7 | 1066.6, 800.5, 640.7 | M +76 |
| 71–78    | FWDNLKE*K | 1080.5 | 1156.5, 579.0 | K7+ +76 |
| 86–92    | MSK*KDLEE | 851.4 | 927.4, 464.3 | K7+ +76 |
| 93–111   | VK*AK*VQPYLDFQK*K*LQWR | 2379.2 | 1228.5, 819.4, 492.0 | M +76 |
| 114–125  | LRYOK*KVEPLRAE | 1501.8 | 1577.7, 789.7, 527.0, 395.5 | K7+ +76 |
| 140–147  | K*PLGGE | 872.5 | 948.6, 474.9 | K7+ +76 |
| 180–191  | ALK*ENGGARLAE | 1228.7 | 1305.4, 653.4, 436.2 | K182 +76 |
| 224–235  | SFK*VSFLSALEE | 1356.7 | 1432.6, 717.0 | K226 +76 |
| 236–243  | YTK*K*LNTQ | 995.6 | 1071.7, 536.6 | M +76 |

In every case only peptides consistent with the formation of K +76 were detected. A full scan mass spectrum containing three modified peptides is shown in Fig. 5D. Each peptide had gained 76 atomic mass units, suggesting that Lys-96, Lys-106, and Lys-107 had been converted to MP-Lys.

In a Glu-C digest of apoA-I exposed to acrolein, we detected 4 peptides containing 2, 3, or 4 Lys residues (peptides 35–62, 93–111, 206–212, and 236–243) that had each gained 76 atomic mass units. At low mol ratios of acrolein (<20:1, acrolein/protein) we were unable to detect any modified peptide that exhibited a 2-, 3-, or 4-fold increase of 76 atomic mass units. When apoA-I was exposed to a high molar ratio of the aldehyde (≥50:1) and then digested with Glu-C, peptides containing multiple modified Lys residues (K +76) were readily detected (data not shown). Under all conditions, acrolein preferentially modified certain residues in peptides containing multiple Lys (e.g. Lys-59, Lys-208, and Lys-238) (data not shown). We took advantage of the inability of trypsin to cleave adjacent to modified Lys residues as well as digestion with both Glu-C and trypsin to confirm these results. These observations indicate that only one Lys residue in peptides derived from apoA-I that contain multiple reactive residues is modified at low concentrations of acrolein.

It is important to note that we failed to detect FDP-Lys or Nm-propanalhistidine when we exposed apoA-I to acrolein, even though these compounds were major products when we incubated the synthetic peptides with acrolein. Instead, we detected only Lys +76. Analysis of peptides containing multiple Lys residues indicated that acrolein modifies only a single Lys. This strongly suggests that acrolein reacts with Lys residues in apoA-I to selectively yield MP-Lys, which becomes the major product of amino acid modification under these conditions. Moreover, our results suggest that specific Lys are targeted for modification.

**FIGURE 4.** LC-ESI-MS/MS identification of Lys-226 in a Glu-C digest of acrolein-modified apoA-I.

As described in the legend to TABLE ONE. Peptide digests were analyzed with LC-ESI-MS/MS. In every case only peptides consistent with the formation of K +76 were detected. A full scan mass spectrum containing three modified peptides is shown in Fig. 5D. Each peptide had gained 76 atomic mass units, suggesting that Lys-96, Lys-106, and Lys-107 had been converted to MP-Lys.
single Lys in each detected peptide was modified and that the extent of modification varied for each residue. The product yield for each Lys residue in these peptides was, thus, lower than the overall product yield for the peptide. This approach demonstrated that Lys-226 was the major residue modified by acrolein (Fig. 6A) and that MP-Lys was the major product.

We used LC-ESI-MS to characterize the reaction conditions for the modification of Lys residues 226 and 94 of apoA-I by acrolein. Because all peptide 94* in a Glu-C digest of apoA-I contains multiple Lys residues, we estimated the product yield for Lys-94 by dividing the overall product yield by 3. At low molar ratios of aldehyde to apoA-I (20:1), the product yield of K+76 was linearly dependent on the concentration of acrolein for both residues 226 and 94 (Fig. 7A). Acrolein initially reacted rapidly with each Lys residue, but the reaction rate gradually decreased (Fig. 7B). Under all conditions MP-Lys was the only product we detected; the ratio of modified Lys-226 to Lys-94 was approximately constant, and Lys-226 was always the major target for modification.

Lys-226 Is the Major Target of Acrolein in Lipid-associated ApoA-I—We determined whether lipidation affects the reaction of acrolein with apoA-I by incubating either lipid-free apoA-I or HDL with the reactive aldehyde (20:1, acrolein/protein, mol/mol) and identifying the modifiable sites and product yields for Lys residues in apoA-I (Fig. 6B). Because apoA-I contains
Site-specific Modification of ApoA-I by Acrolein

21 Lys residues and 243 amino acid residues, the mole ratio of acrolein to Lys was ~1:1 and that of acrolein to total amino acids was ~1:10. In both lipid-free and lipid-associated apoA-I, ~30% of Lys-226 was converted to MP-Lys. The product yields of MP-Lys for most of the Lys residues in apoA-I were similar for lipid-free and lipid-associated protein. However, Lys-182 was more reactive when apoA-I was incorporated into HDL than when it was lipid-free. The reverse was true for Lys-12 and Lys-118. These observations indicate that Lys-226 is the major target of acrolein in both lipid-free apoA-I and HDL-associated apoA-I and, therefore, that lipidation has little effect on the protein’s ability to react with acrolein.

Modification of ApoA-I with Acrolein Generates Complexes with Higher Apparent Molecular Masses—Two reactive moieties of acrolein, an aldehyde group and an unsaturated bond, have the potential to cross-link to nucleophilic groups on two different proteins. To investigate this possibility, we subjected acrolein-modified apoA-I or acrolein-modified HDL to gel electrophoresis under denaturing and reducing conditions (Fig. 8). Multiple protein bands with apparent molecular masses of ~60, 90, 120, and 180 \( \times 10^3 \) daltons were apparent in Coomassie Blue-stained gels of lipid-free apoA-I. HDL yielded a different pattern, with bands with apparent molecular masses of ~38, 60, 68, 76, and 90 \( \times 10^3 \) daltons. Formation of the more slowly migrating material was dependent upon incubation time and the molar ratio of aldehyde to protein. For HDL, bands with apparent molecular mass values consistent with those of apoA-I-apoA-II (AI-AII) multimers (~38, 60, 68, 76 kDa) were also observed (Fig. 8). When the molar ratio of acrolein to apoA-I was increased to 100, native apoA-I almost disappeared from the gel (data not shown), perhaps because the modified protein failed to react with Coomassie Blue or because all of the native protein had formed complexes with higher \( M_r \) values.

Using MALDI-TOF-MS to analyze apoA-I that had been exposed to increasing concentrations of acrolein, we observed a decrease in the ion intensity of material with the \( m/z \) of the native protein. However, we were unable to detect an increase in intensity of ions that exhibited the \( m/z \) of multimers of native apoA-I (data not shown). On the other hand, the molecular mass of acrolein-modified apoA-I increased steadily from ~28 to ~30 kDa as the concentration of acrolein in the reaction mixture rose from 0 to a 100-fold molar excess relative to that of apoA-I (data not shown). These results confirm that acrolein covalently modifies apoA-I and progressively increases the apparent \( M_r \) of the protein. The failure of MS to detect ions with the molecular mass of apoA-I multimers suggests that the slowly migrating species observed by SDS-PAGE in acrolein-modified apoA-I might represent noncovalent complexes. Alternatively, variability in the sites at which acrolein cross-links apoA-I could make such complexes difficult to detect by MS analysis.

Modification of ApoA-I by Acrolein Impairs Cholesterol and Phospholipid Efflux by the ABCA1 Pathway—To investigate the potential effects of acrolein on cellular lipid metabolism, we assessed the ability of acrolein-modified apoA-I to promote cholesterol and phospholipid efflux from cultured cells. When ABCA1-transfected BHK cells were incubated with 5 µg/ml of apoA-I that had been exposed to increasing concentrations of acrolein, the apolipoprotein progressively lost its ability to promote cholesterol and phospholipid efflux (Fig. 9A). When apoA-I was incubated with a 20:1 or 50:1 molar ratio of acrolein, the modified apolipoprotein lost about 30 or 60% of this ability. Fig. 9B shows the cholesterol-removing ability of apoA-I when increasing amounts of untreated or acrolein-modified apoA-I were incubated with ABCA1-transfected BHK cells. The results demonstrate that modification by acrolein reduces the ability of apoA-I to promote cholesterol and phospholipid efflux by the ABCA1 pathway.

To determine whether modification of Lys-226 might account for this loss of function, we examined the relationship between the disappearance of the peptide containing this residue and the loss of ABCA1 activity (Fig. 10). ApoA-I exposed to varying concentrations of acrolein was incubated with ABCA1-transfected BHK cells. In parallel experiments, apoA-I or modified apoA-I was digested with Glu-C, and modification of Lys residues was quantified using reconstructed ion chromatograms and LC-ESI-MS analysis. As the molar ratio of acrolein to apoA-I increased from 1 to 50, both the ion current of the native peptide and the ability of acrolein-modified apoA-I to promote cholesterol efflux steadily decreased (Fig. 10A). Loss of the precursor peptide containing Lys-226 associated strongly with a decrease in the ability of acrolein-modified apoA-I to promote cholesterol efflux (Fig. 10A). Generation of the peptide containing MP-Lys-226 increased in parallel with the loss of cholesterol removal by acrolein-modified apoA-I (Fig. 10B).

In contrast to the peptide containing MP-Lys-226, the production of modified peptides containing other Lys residues had little association
with inhibition of cholesterol removal by acrolein-modified apoA-I (data not shown). Our observations, therefore, suggest that selective modification of Lys-226 by acrolein may impair the ability of apoA-I to promote cholesterol and phospholipid efflux by the ABCA1 pathway.

**ApoA-I Co-localizes with Acrolein-lysine Adducts in Human Atherosclerotic Lesions—**To determine whether acrolein might modify apoA-I in vivo, we harvested coronary arteries from patients undergoing cardiac transplantation and then immunostained the tissue with antibodies specific for apoA-I and acrolein-modified proteins (4). It is important to note that the monoclonal antibody used to detect acrolein adducts reacts specifically with MP-Lys (12), the major product detected in acrolein-modified apoA-I. As we described previously (37), most of the epitopes that reacted with the antibody to apoA-I were located in proteoglycan-rich areas of atherosclerotic intima that contained little cellular material (Fig. 11, A and B). Most acrolein adducts were also extracellular, and they localized to the same proteoglycan-rich areas that reacted with the antibody to apoA-I (Fig. 11C). Cellular regions adjacent to endothelium showed much less immunostaining for apoA-I, and we were unable to detect apoA-I in normal intima (data not shown). These observations imply that apoA-I is an important target for acrolein in the artery wall. We also detected adducts for acrolein in macrophage-rich areas, confirming previous reports that acrolein-modified proteins are abundant both around and within macrophages (4).

**DISCUSSION**

Acrolein, an $\alpha,\beta$-unsaturated aldehyde, is generated when lipids are peroxidized and myeloperoxidase oxidizes threonine, but the factors that control its reactivity with nucleophilic groups in proteins remain poorly understood. Because HDL helps prevent vascular disease (15–17) and lipid oxidation products and myeloperoxidase have been implicated in atherogenesis (46, 47), we investigated the reactivity of acrolein with apoA-I, the major protein in HDL. Our studies of model peptides showed that acrolein reacts with Lys and His residues to yield MP-Lys, FDP-Lys, and Nim-propanalhistidine (4, 12). However, we did not detect any stable adducts of acrolein with Arg (13). FDP-Lys and Nim-propanalhistidine were the major products when we exposed synthetic
peptides to acrolein. In striking contrast, the only modified amino acid we detected after exposing apoA-I to acrolein was MP-Lys, a pyridinium compound resulting from the reaction of two acrolein molecules with the amino group of Lys (12). Remarkably, Lys-226 was the single major target of acrolein in apoA-I. Conversion of Lys-226 to MP-Lys associated strongly with impairment in apoA-I ability to promote the efflux of cholesterol and phospholipid from cells by the ABCA1 pathway. Immunohistochemical studies with a monoclonal antibody that reacts specifically with MP-Lys found that the adduct co-localized with apoA-I in human atherosclerotic lesions. Taken together, our observations suggest that acrolein might make a critical contribution to atherosclerosis by impairing cholesterol removal from artery wall cells, perhaps by promoting site-specific modification of Lys-226 in apoA-I.

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. Our results indicate that Lys-226 in helix 10 is the major site for modification by acrolein in both lipid-free and lipid-associated apoA-I. Thus, the local environment around Lys residues in proteins is likely to have a major impact on the reaction of the amino group with acrolein in vivo, where low concentrations of acrolein are likely to be generated over prolonged periods.

A key question, then, becomes the mechanisms that direct the site-specific reaction of Lys-226 with acrolein. One important factor may be...
negatively charged amino acids, which could promote electrophilic attack on the side chain of Lys residues by promoting deprotonation of the ε-amino group, thereby lowering its $pK_a$ and increasing its nucleophilicity. MP-Lys is derived from the reaction of 2 acrolein molecules with the ε-amino group of Lys, and it is possible that negatively charged amino acids promote the reaction of the initial acrolein adduct with a second molecule of acrolein or the cyclization reaction necessary for formation of the methypyridinium group. In the crystal structure of an N-terminal-truncated form of apoA-I (48), Lys-226 forms a salt bridge with Glu-234, suggesting that this interaction might promote the reaction of Lys with acrolein. However, other factors may also contribute. In the amphipathic α-helices that dominate the apoA-I secondary structure (49), amino acids separated by two or three other residues lie adjacent on the same face of a helix. It is noteworthy that, except for Lys-118 and Lys-206, every Lys residue in the proposed α-helical domains of apoA-I lies either two or three residues away from a negatively charged (Glu, Asp) amino acid side chain. Thus, virtually every Lys residue in an amphipathic α-helical domain resides in a $\text{KXXX(E/D), (E/D)XXK}$, $\text{KXXX(E/D), or (E/D)XXX}$ motif. This reflects the important role of salt bridges in maintaining the α-helical structure of apoA-I (48, 49).

The unique reactivity of Lys-226 may, thus, partly reflect its interaction with two negatively charged side chains of two amino acids: Glu-223, located 2 residues away in the linear sequence, and Glu-234, which forms a salt bridge with the residue in the crystal structure of truncated apoA-I. Previous studies have suggested that acrolein reacts with His, Cys, Arg, and Lys residues in proteins (6, 10, 13). We used model peptides containing His, Lys, and/or Arg to identify the targets of acrolein. We found that peptides containing His and Lys, but not Arg, were targeted for modification. The major products were $\text{N^\text{im}-propanalhistidine}$ (His+56), FDP-Lys (Lys+94), and MP-Lys (Lys+76). Although apoA-I contains five His residues, we failed to detect $\text{N^\text{im}-propanalhistidine}$. This might reflect the formation of a relatively unstable adduct (6), although we readily detected $\text{N^\text{im}-propanalhistidine}$ in the model peptide. High levels of FDP-Lys formed in all the synthetic peptides, whereas the yield of MP-Lys was generally lower. However, we detected only MP-Lys when we modified apoA-I with acrolein, although other investigators have identified FDP-Lys in acrolein-modified proteins (6).

Our observation that MP-Lys is the only detectable adduct in acrolein-modified apoA-I further supports the proposal that α-helical structure and the local amino acid environment are important for controlling acrolein reactivity with proteins.

Acrolein and 4-hydroxynonenal, another α,β-unsaturated aldehyde produced by lipid oxidation, have been proposed to cross-link HDL proteins (30, 50). Using SDS-PAGE, we confirmed that proteins with higher apparent $M_r$ values appeared when apoA-I or HDL was exposed to acrolein. However, MALDI-TOF-MS failed to detect major ions with $m/z$ ratios consistent with the formation of multimers of apoA-I. Thus, the more slowly migrating protein bands observed by SDS-PAGE might represent noncovalent complexes of acrolein-modified apoA-I (and apoA-I and apoA-II in HDL). Alternatively, molecular heterogeneity in the sites of cross-linking may have made it difficult to detect high $M_r$ complexes by MS analysis. In future studies, it will be important to investigate the structural basis for the apparent increase in $M_r$ that is seen when apoA-I is exposed to acrolein.

Two lines of evidence suggest that the acrolein reaction with apoA-I could be physiologically relevant. First, the apoA-I ability to promote cholesterol and phospholipid efflux from cells by the ABCA1 pathway became progressively impaired by increasing concentrations of acrolein. Importantly, this impairment associated quantitatively only with loss of precursor peptides containing Lys-226. Moreover, there was a strong linear correlation between the appearance of MP-Lys-226 and decreased lipid efflux from cells. We cannot exclude the possibility that other modifications of apoA-I contributed to impaired cholesterol efflux, but our observations suggest that modification of Lys-226 is particularly important. Consistent with this proposal, deletion of helix 10 and/or helices 9 plus 10 of apoA-I greatly diminishes ABCA1-dependent cholesterol efflux (51, 52). Moreover, a synthetic peptide that mimics the 9/10 helix of apoA-I can mediate high affinity cholesterol efflux via ABCA1 (53). These investigators have suggested that a specific structural element possessing a linear array of acidic residues spanning two apoA-I amphipathic α-helices is required to mediate cholesterol efflux and stabilize ABCA1. It is noteworthy that modification of Lys-226 could disrupt this linear array, perhaps accounting for the impairment in cholesterol transport that we observed when we modified apoA-I with acrolein.

Second, we found evidence that acrolein modifies apoA-I in the human artery wall. Histochemical studies with a monoclonal antibody specific for acrolein-lysine adducts (mAb5F6 (4, 12)) demonstrated intense immunoreactivity with epitopes in human atherosclerotic intima. Most of these epitopes were extracellular in regions of the lesions that were relatively depleted of cells, and they co-localized extensively with epitopes recognized by an antibody specific for apoA-I. Thus, apoA-I appears to be a major target for acrolein in arterial tissue. Previous studies have demonstrated that binding of the acrolein-modified protein to mAb5F6 is inhibited much more potently by MP-Lys than by FDP-Lys, suggesting that the antibody is specific to MP-Lys (12). MP-Lys was the only product that we detected in acrolein-modified apoA-I and HDL, and acrolein-modified apoA-I reacted strongly with mAb5F6 in both Western blots and enzyme-linked immunosorbent assays (data not shown). These observations indicate that MP-Lys is likely the epitope recognized by mAb5F6. The co-localization of acrolein adducts with apoA-I suggests that apoA-I is targeted and modified by acrolein in atherosclerotic intima.

Although acrolein is highly reactive, it is long-lived compared with most oxidizing intermediates. Lipid peroxidation and threonine oxidation by myeloperoxidase are potential sources of this aldehyde in the artery wall, suggesting that acrolein might initially be generated in macrophage-rich regions and then diffuse long distances to react with proteins. It is important to note that HOCl-modified proteins and acrolein adducts produce similar patterns of immunostaining in human atherosclerotic intima (25, 26, 54). Collectively, these observations suggest that acrolein modifies specific proteins in the human artery wall and that apoA-I might be a major target.

We also found strong evidence for co-localization of acrolein adducts and apoA-I in macrophage-rich regions of atherosclerotic intima, confirming previous reports (4). Our observations suggest the following model. Lipid peroxidation and amino acid oxidation by myeloperoxidase in the artery wall generate acrolein (5, 28). The aldehyde compound then reacts with apoA-I. Modification of Lys residues impairs apoA-I ability to remove cholesterol from lipid-laden macrophages, contributing to the formation of atherosclerotic lesions. Because phagocytes store NADPH oxidase and myeloperoxidase in their plasma membrane and secretory compartments, respectively, oxidation is likely to be restricted in space by local changes in oxidant concentrations. However, acrolein is a relatively long-lived molecule that might diffuse some distance before reacting with proteins (3). It is important to note that apoA-I promotes cholesterol efflux from cells by interacting with ABCA1 at the plasma membrane of macrophages. Our findings suggest that acrolein production by phagocytes is a physiological mechanism for
modifying apoA-I and inhibiting HDL function, thereby promoting atherogenesis.

In conclusion, our studies establish that acrolein interferes with normal HDL cholesterol transport by the ABCA1 pathway, perhaps by site-specific modification of Lys-226 in apoA-I. This interference might play a critical role in atherogenesis by impairing cholesterol removal from artery wall cells. Our observations raise the possibility that modification of apoA-I by acrolein promotes foam cell formation and the development of atherosclerosis.

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