Acrolein Modification Impairs Key Functional Features of Rat Apolipoprotein E: Identification of Modified Sites by Mass Spectrometry

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Ⓢ *Supporting Information*

ABSTRACT: Apolipoprotein E (apoE), an antiatherogenic apolipoprotein, plays a significant role in the metabolism of lipoproteins. It lowers plasma lipid levels by acting as a ligand for the low-density lipoprotein receptor (LDLr) family of proteins, in addition to playing a role in promoting macrophage cholesterol efflux in atherosclerotic lesions. The objective of this study is to examine the effect of acrolein modification on the structure and function of rat apoE and to determine the sites and nature of modification by mass spectrometry. Acrolein is a highly reactive aldehyde, which is generated endogenously as one of the products of lipid peroxidation and is present in the environment in pollutants such as tobacco smoke and heated oils. In initial studies,

acrolein-modified apoE was identified by immunoprecipitation using an acrolein-lysine specific antibody in the plasma of 10-week old male rats that were exposed to filtered air (FA) or low doses of environmental tobacco smoke (ETS). While both groups displayed acrolein-modified apoE in the lipoprotein fraction, the ETS group had higher levels in the lipid-free fraction compared with the FA group. This observation provided the rationale to further investigate the effect of acrolein modification on rat apoE at a molecular level. Treatment of recombinant rat apoE with a 10-fold molar excess of acrolein resulted in (i) a significant decrease in lipid-binding and cholesterol efflux abilities, (ii) impairment in the LDLr- and heparin-binding capabilities, and (iii) significant alterations in the overall stability of the protein. The disruption in the functional abilities is attributed directly or indirectly to acrolein modification yielding an aldimine adduct at K149 and K155 (+38); a propanal adduct at K135 and K138 (+56); an N^ε-(3-methylpyridinium)lysine (MP-lysine) at K64, K67, and K254 (+76), and an N^ε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) derivative at position K68 (+94), as determined by matrix-assisted laser desorption/ionization—time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS). The loss of function may also be attributed to alterations in the overall fold of the protein as noted by changes in the guanidine HCl-induced unfolding pattern and to protein cross-linking. Overall, disruption of the structural and functional integrity of apoE by oxidative modification of essential lysine residues by acrolein is expected to affect its role in maintaining plasma cholesterol homeostasis and lead to dysregulation in lipid metabolism.
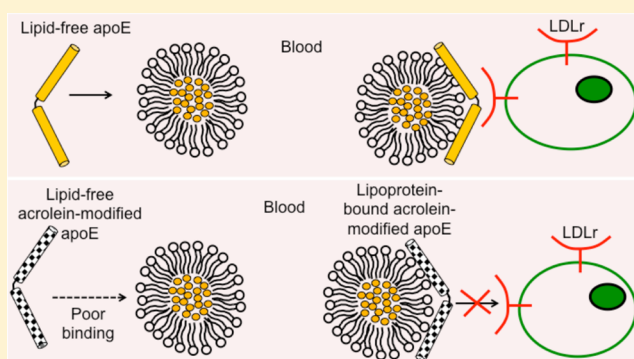
Apolipoprotein E (apoE) is a 34-kDa antiatherogenic protein that plays a crucial role in cardiovascular disease by regulating plasma cholesterol levels and lipoprotein metabolism.[1,2] By assisting in the transportation of very low-density lipoproteins (VLDL) and chylomicron remnants, apoE is able to remove excess cholesterol and triglyceride from the blood and into the liver for further processing. It mediates this role by acting as a ligand for cell surface localized lipoprotein receptors

such as the low density lipoprotein receptor (LDLr) and heparan sulfate proteoglycans (HSPG).[2,3] ApoE has the ability to exist in lipid-free and lipid (lipoprotein)-bound states, and undergoes a dramatic conformational change when going from one state to the other. Its ability to act as a ligand for the LDLr is elicited only in the lipid-bound state;[4,5] thus, its lipid-binding ability is a key functional prerequisite for apoE. ApoE also plays a role in promoting ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux from macrophages in atherosclerosis, a function that is typically mediated by apoAI under normal physiological conditions.[6]

The importance of apoE in lipoprotein metabolism is exemplified by studies with apoE-null mice, which display massive accumulation of cholesterol and triglyceride-rich lipoproteins in plasma and develop early and severe atherosclerotic lesions.[7,8] On the other hand, targeted overexpression of apoE in this mouse model lead to a marked resistance to diet-induced hypercholesterolemia and decreased plasma cholesterol levels.[9] In humans, apoE deficiency leads to type III hyperlipoproteinemia and premature development of atherosclerosis characterized by elevated plasma cholesterol levels and accumulation of VLDL.[10−12] These early studies established the key role of apoE in regulating plasma cholesterol and triglyceride levels, elevated levels of which are recognized as two of several risk factors for heart disease. The broad objective of this study is to investigate the role of oxidative stress modification of apoE and the potential molecular and physiological implications of the process with respect to lipoprotein metabolism.

Oxidative stress is recognized as a major factor in the onset of atherosclerosis and cardiovascular disease in humans,[13] with aging and life style (diet, smoking, exercise) playing significant roles.[14] Several lines of evidence implicate increased susceptibility of LDL to oxidative modification as potential factors.[15−17] Oxidative modification of LDL leads to its uptake by scavenger receptors located on macrophages, which eventually are converted to foam cells and deposited as fatty streaks in the vasculature, a signature feature of atherosclerotic plaques.[18] Both oxidized lipids[19−22] and oxidized proteins are believed to play crucial roles in atherogenesis, with oxidative damage to proteins such as apoB-100 likely increasing the atherogenicity of LDL.[23−26] However, little is known about the potential oxidative damage to apoE, a key protein component of chylomicrons, VLDL, and a subtype of HDL, and the contribution of oxidatively modified apoE to atherogenesis.

In this study, we follow modification of apoE by acrolein, one of the end products of lipid peroxidation generated endogenously in a process triggered by oxidative stress.[27] Acrolein is an α,β-unsaturated aldehyde (2-propenal, $CH_2=CH-CHO$) that is also present in the environment in pollutants such as tobacco smoke and heated oils. It is the strongest electrophile among all α,β-unsaturated aldehydes. Acrolein has been shown to play a significant role in oxidative modification of apoB-100 on LDL.[28] In this study, we note that endogenous generation of acrolein modifies apoE in rats; exposure of rats to environmental tobacco smoke (ETS) results in increased presence of acrolein-modified apoE in the lipoprotein-dissociated state. Further, direct exposure of purified recombinant rat apoE to acrolein caused impairment in LDLr-and HSPG-binding abilities and in lipid-binding and cholesterol efflux capabilities. We attributed the dysfunction of apoE to significant protein side chain modification of essential lysine residues mediated by acrolein, as evidenced by mass spectral analysis, and to overall alterations in the global protein fold.

EXPERIMENTAL PROCEDURES

Animal Exposures. The animal studies were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. They were in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the University of California, Davis, Institutional Review Board. Standardized research cigarettes were purchased from the University of Kentucky (Louisville, KY). Male Sprague−Dawley rats (10 weeks old) were exposed to aged and diluted side stream cigarette smoke (total suspended particulates (TSP) 0.97 ± 0.05 mg/m³) as a surrogate to ETS. Rats (12 per group) were exposed for 6 h/day for 3 consecutive days (body weight, 278 ± 9 g, mean ± SD); animals in the control group received filtered air (FA) under sham conditions (body weight, 276 ± 10 g, mean ± SD). Research cigarettes (3R4F) obtained from the University of Kentucky were maintained under humified and temperature-controlled conditions in a desiccator prior to use. Cigarettes were combusted using a TE10 smoking machine (Teague Enterprises, Woodland, CA) using a smoke puff volume setting of 35 mL of 2 s duration once per minute over a period of 8 min prior to the automatic replacement with a new cigarette in the smoking machine. Between smoke puffs, side stream cigarette smoke was collected and mixed with the mainstream puff volume, then passed through an aging and dilution chamber prior to further dilution and introduction into a whole body animal exposure system. Tobacco exposure conditions to aged and diluted smoke for the exposure period were as follows: relative humidity, 65% ± 1.7%; temperature, 22.8 ± 0.7 °C; TSP, 0.97 ± 0.05 mg/m³; nicotine, 0.25 ± 0.06 mg/m³; carbon monoxide, 3.6 ± 0.3 ppm. The conditions of exposure to ETS are highly representative of concentrations encountered in the home or other places where smoking occurs. It is known for people who smoke that they create a personal cloud of TSP approximating 2 mg/m³. Blood was withdrawn on the third smoke exposure day and centrifuged at 1000g for 15 min to obtain plasma.

Plasma Lipoprotein Isolation. Total lipoprotein fraction ($d < 1.21$ g/mL) was isolated from plasma by density gradient ultracentrifugation. Prior to use, the lipoprotein and lipid-free fractions (top and bottom fractions, respectively) were dialyzed against three changes of degassed 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl (phosphate buffered saline, PBS). Plasma apoE levels were determined by ELISA using mAb3H1. Plasma triglyceride and cholesterol levels were measured by enzymatic-end point reagent kits, according to the manufacturer's instructions, on a Gilford Impact500E autoanalyzer (Ciba-Corning Diagnostics Corp., Oberlin, OH).

Immunoprecipitation. Protein G-Sepharose (GE Healthcare, Pittsburgh, PA) was incubated with 0.1 μg of mAb5F6 (an antibody specific for acrolein-lysine adducts, obtained from Japan Institute for the Control of Aging, Fukuroi, Shizuoka, 437-0122 Japan), in PBS containing 0.1% Tween 20 for 1 h at 4 °C and washed four times with this buffer to remove unbound antibody. The total lipoprotein or lipid-free fraction (25 μg of protein) isolated from plasma of rats exposed to FA or ETS was then incubated with conjugated protein G-Sepharose for 1 h at 24 °C, followed by four washes as above to remove unbound proteins. The samples were treated with nonreducing SDS sample treatment buffer for Western blot analysis using horseradish peroxidase (HRP)-conjugated goat anti-human apoE antibody (BIODESIGN International, Saco, ME).

Acrolein Modification of Rat apoE. Recombinant rat apoE bearing a hexa-His tag was overexpressed, isolated, and purified using a HiTrap nickel-affinity column (HisTrap HP, GE Healthcare, Uppsala, Sweden) as described recently.[29] In initial experiments, purified apoE (5 mg) was incubated with acrolein (Alfa Aesar, Ward Hill, MA) (1:2.5 to 2000:1 molar ratio, acrolein/apoE) in PBS for 4 h at 37 °C. In control reactions, apoE was incubated as such, with no additives. Excess unreacted acrolein was removed by extensive dialysis against PBS for 48 h with three changes. Unmodified and acrolein-modified apoE were visualized by SDS-PAGE on a 4−20% acrylamide gradient gel (Invitrogen, Carlsbad, CA). In subsequent experiments, we focused on apoE modified with acrolein at 10:1 molar ratio (acrolein/apoE).

Western Blot. Western blot analysis of unmodified and acrolein-modified apoE (∼0.5 μg) was carried out using anti-apoE−HRP antibody (1:1000 dilution) or mAb5F6 (1:500 dilution)[30] followed by HRP-conjugated anti-mouse IgG (1:10000 dilution) (Chemicon) for detecting acrolein-lysine modifications (N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine)), using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Uppsala, Sweden).

Circular Dichroism (CD) Spectroscopy. The secondary structure of rat apoE was examined by CD spectroscopy on a Jasco J-810−150S spectropolarimeter at 24 °C.[31] Far-UV CD scans were recorded between 185 and 260 nm in 10 mM sodium phosphate buffer, pH 7.4, using protein concentrations of 0.2 mg/mL in a 0.1 cm path length cuvette. Far-UV CD profiles were the average of four independent scans recorded with a response time of 1 s and bandwidth of 1 nm. The molar ellipticity ($[\theta]$) in deg cm^2 dmol^{-1} at 222 nm was obtained using the equation

$$[\theta]_{222nm} = \mathrm{MRW}(\theta)/(10lc) \tag{1}$$

where MRW is the mean residue weight (obtained by dividing molecular weight by the number of residues) calculated to be 115.64, θ is the measured ellipticity in degrees at 222 nm, l is the cuvette path length (in cm), and c is the protein concentration (in g/mL). The percent α-helix content was calculated as described by others:[32]

$$\%\alpha\text{-helix} = \{(-[\theta]_{222} + 3000)/39000\} \times 100 \tag{2}$$

GdnHCl-Induced Unfolding. Unfolding of rat apoE was assessed by following changes in molar ellipticity at 222 nm as a function of increasing concentration of GdnHCl as described earlier.[33] The samples (0.2 mg/mL) were treated with 0−6 M GdnHCl in 10 mM sodium phosphate buffer, pH 7.4, for 18 h at 24 °C. The percent maximal change was calculated from the ellipticity values at 222 nm as

$$\{\theta \text{ at 0 M GdnHCl} - \theta \text{ at given [GdnHCl]}\}$$
$$/\{\theta \text{ at 0 M GdnHCl} - \theta \text{ at 6 M GdnHCl}\} \times 100 \tag{3}$$

Free energy of stabilization of unmodified and acrolein-modified apoE was calculated using the relationship:

$$\Delta G_{\mathrm{D}} = \Delta G_{\mathrm{D}}^{\mathrm{H_2O}} - \Delta nRT \ln(1 + ka) \tag{4}$$

where ΔG_{D} is the free energy change between 0 and 6 M GdnHCl, $\Delta G_{\mathrm{D}}^{\mathrm{H_2O}}$ is the free energy of denaturation in the absence of denaturant, Δn is the difference in the number of GdnHCl binding sites between the native and denatured states of protein, R is the gas constant, T is the temperature, k is the GdnHCl binding constant (0.8), and a is the molarity of GdnHCl. The free energy change, ΔG_{D}, was calculated as described previously.[34,35]

Fluorescence Spectroscopy. Steady state fluorescence analyses were performed on a PerkinElmer LS55B fluorometer at 24 °C. Fluorescence emission spectra of unmodified or acrolein-modified apoE (0.02 mg/mL) were recorded in PBS between 290 and 490 nm following excitation at 280 nm at a scan speed of 50 nm/min (5 nm excitation and emission slit widths); an average of 10 scans were recorded.

Lipid Binding Assay. The ability of unmodified and acrolein-modified apoE to bind lipids was determined as described previously.[29,36] The assay was performed in a PerkinElmer UV/vis spectrophotometer equipped with a Peltier controlled (PTP-6) cell holder. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids, Alabaster, AL) MLVs (500 μg) were preincubated at 24 °C in PBS in the cuvette followed by addition of 100 μg of apoE. The decrease in absorbance was measured at 325 nm. Data were normalized to initial absorbance at 325 nm prior to addition of protein. The time required for initial absorbance to decrease by 50% ($T_{1/2}$) and the rate constant (K, reciprocal of $T_{1/2}$) were determined.

Preparation of DMPC/apoE Complex. DMPC/apoE complexes were prepared as described earlier[37] using rat apoE or human apoE3(1−191). ApoE was incubated with DMPC vesicles (2.5:1 lipid/protein weight ratio or 125:1 molar ratio) at 24 °C for 16 h; lipid-bound protein was separated from the unbound protein by density gradient ultracentrifugation using a KBr gradient. About 10 fractions were collected, and protein assay was carried out on each fraction by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL) and phospholipid assay using the phospholipid assay kit (Wako Chemicals USA, Inc., Richmond, VA). Fractions containing both protein and lipid were pooled and concentrated. The lipoprotein complexes were characterized in terms of particle size and diameter and lipid and protein composition as described earlier.[38,39] The protein and lipid contents of the complexes were estimated using the *DC* protein (BioRad Laboratories, Hercules, CA) and phospholipid assay. Nondenaturing PAGE of the isolated lipoprotein complexes was carried out to evaluate the molecular mass and average particle size using Amersham high molecular weight standard (GE Healthcare, Uppsala, Sweden) on a 4−20% gradient gel for 24 h at 110 V and stained with Amido Black.

LDLr Binding Assay. To examine the ability of acrolein-modified apoE to bind to the LDLr, a coimmunoprecipitation (co-IP) assay was performed as described previously.[40] A construct bearing the LDLr ligand binding domains LA3−LA6 with a c-Myc epitope was employed. This construct represents the essential ligand binding elements of the extracellular soluble portion of the mature LDLr and is represented as sLDLr. DMPC/unmodified apoE or DMPC/acrolein-modified apoE (0, 1, and 10 μg of protein) was incubated with 10 μg of sLDLr in the presence of 2 mM Ca^{2+} in PBS for 1 h at 4 °C. This was followed by co-IP with an anti-c-Myc antibody-linked agarose (Sigma-Aldrich, St. Louis, MO) to capture the DMPC/apoE/sLDLr complexes. ApoE was detected by Western blot analysis using HRP-conjugated polyclonal apoE antibody. A replica experiment was conducted wherein an anti-c-Myc antibody (9E10) was utilized to identify the presence of LDLr in each reaction.

Heparin Binding Assay. A typical functional feature of apoE is its ability to interact with cell surface-localized HSPG in the blood vessels. *In vitro*, this is followed using the HiTrap heparin-Sepharose column (GE Healthcare, Uppsala, Sweden) appended to the ÄKTA FPLC system (Amersham Pharmacia Biotech,

Uppsala, Sweden) as described by us previously.[41,42] Unmodified or acrolein-modified apoE was dialyzed against 20 mM sodium phosphate buffer, pH 7.4, and injected onto the column at a flow rate of 1.0 mL/min. The bound protein was eluted with a salt gradient (0 to 1.0 M NaCl) and monitored at 280 nm. The salt concentration in the eluate was monitored by conductivity measurements.

Cholesterol Efflux Activity. The ability of unmodified or acrolein-modified apoE to stimulate cholesterol efflux was assessed in J774 mouse macrophages (which do not synthesize apoE) as described previously.[43−45] Cells were plated onto 24-well culture plates and labeled with [^{3}H]cholesterol (1 μCi/mL) in RPMI-1640 with 1% fetal bovine serum (FBS) for 48 h. A cAMP analogue (cpt-cAMP) was added (18 h) to up-regulate ABCA1 expression. Cells were rinsed extensively and then exposed to unmodified or acrolein-modified apoE in serum-free RPMI-1640 medium at the indicated concentrations. In control experiments, unmodified or acrolein-modified human plasma-derived apoAI was used under identical conditions. The amount of [^{3}H]cholesterol appearing in the medium was expressed as a percentage of the radioactivity initially present in cells at time zero. Background release of [^{3}H]cholesterol to serum-free medium was subtracted from values obtained with added proteins.
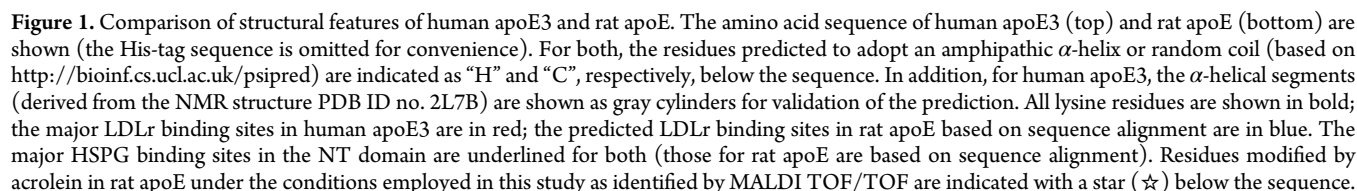
Mass Spectrometric Analysis. Purified recombinant rat apoE was incubated with acrolein (10-fold molar excess over apoE) at 37 °C for 4 h. For identification of acrolein-modified sites, all sample proteolytic digestions were performed with sequencing-grade trypsin (Promega, Madison, WI), AspN (from a mutant of *Pseudomonas fragi*, Sigma-Aldrich, St. Louis, MO), and GluC (from *Staphylococcus aureus* V8, Sigma-Aldrich, St. Louis, MO) or AspN + GluC in solution. Samples were incubated with a 50:1 ratio (protein/protease w/w) of GluC for 40 h at 37 °C in 100 mM ammonium bicarbonate, pH 8.0. Approximately 18 h prior to termination of GluC digestion, a 100:1 ratio (protein/protease w/w) of AspN was added to the mixture, and the incubation was continued to completion. The proteolytic digest was cleaned using C_{18} ZipTip pipette tips (Millipore Corporation, Billerica, MA) as per manufacturer's instructions. The digests of unmodified or acrolein-modified apoE were then subjected to matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI TOF/TOF MS). The samples were directly spotted on MALDI target plates, mixed with α-cyano-4-hydroxy cinnamic acid (CHCA) matrix (Protea Co, Morgantown, WV) in 50% acetonitrile (Millipore Corporation, Billerica, MA) and 0.1% trifluoroacetic acid (Fisher Scientific, Fair Lawn, NJ) and then analyzed on an AB 4800 mass analyzer (Applied Biosystems, South San Francisco, CA). Mass spectra were collected from 1000 laser shots per spot and tandem mass spectrometric (MS/MS) data were collected from 3000 laser shots in the 4800 mass analyzer. The peptides with signal-to-noise ratio above 15 at the MS mode were selected as the strongest peaks first for MS/MS experiments; a maximum of 20 MS/MS was allowed per spot. Mass calibration in MALDI TOF MS and MALDI TOF/TOF MS/MS was achieved through internal calibration (TOF/TOF Calibration Mixture, AB SCIEX). The spectra of digested peptides were acquired in the positive reflectron mode using an accelerating voltage of 20 kV. Raw data files obtained from the AB 4800 mass analyzer were processed using GPS Explorer, version 3.6 (Applied Biosystems, South San Francisco, CA) and then searched against Swiss-Prot protein sequence database downloaded from UniProt under the genus restriction of *Rattus* using the in-house licensed Mascot searching program (version 2.1.03). Note that the residue numbering matches that of the native sequence: the His-tag introduced for the purpose of isolation and purification is not numbered. The following parameters were specified: (i) enzyme, AspN+GluC; (ii) missed cleavage, 2; (iii) variable modification, methionine oxidation, acrolein112 (K), acrolein38 (K), acrolein56 (C), acrolein56 (H), acrolein56 (K), acrolein76 (K), and acrolein94 (K); (iv) peptide tolerance, 300 ppm; (v) MS/MS tolerance of 0.8 Da. The peptides containing acrolein-modified amino acids with $p < 0.05$ and peptide ion score and ion score confidence were further confirmed by manually checking the MS/MS spectra. Quantification of acrolein labeling was carried out using peak area-based label free quantitation method.[46] MS/MS spectra were further processed by Data Explorer software (version 4.9) (Applied Biosystems) with the noise removal method to reduce background noise and improve the signal-to-noise ratio.
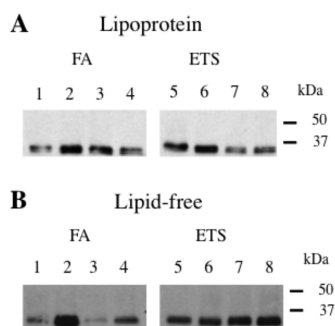
■ RESULTS

Rat apoE is a 34 kDa, 294 amino acid protein with 73.5% sequence similarity with human apoE3, Figure 1. It is rich in basic residues bearing 32 arginines and 11 lysines, many of which play a critical role in interacting with the LDLr family of proteins to facilitate receptor-mediated endocytosis and in maintaining the structural integrity of the protein. Lysine residues are considered particularly susceptible to oxidative modification; in initial studies, we determined whether plasma apoE is modified by acrolein *in vivo* as a result of natural cellular oxidation or due to environmental oxidative stress by exposing 10-week old male rats to FA or low dose of ETS. ELISA revealed that the total plasma apoE levels were slightly lower in ETS group compared with the FA group (0.034 ± 0.016 versus 0.049 ± 0.009 mg/mL for ETS and FA groups, respectively) ($P < 0.2$); when the lipid-free fraction alone was examined, the apoE level was found to be marginally higher in the ETS than in the FA group (0.025 ± 0.001 versus 0.019 ± 0.006 mg/mL for ETS and FA groups, respectively) ($P < 0.2$). The lipoprotein and lipid-free fractions of the ETS and FA-exposed groups were then evaluated for acrolein modification. IP analysis was performed in lipoprotein fractions pooled from three rats, Figure 2A. Acrolein modification of apoE was noted in both groups, with no significant differences in the extent of modification between the two. On the other hand, in the lipid-free fractions, Figure 2B, the ETS group displayed more acrolein-modified apoE compared with the FA group. An exception was one pooled fraction in the FA group (lane 2) that showed an intense acrolein-modified apoE band (the reason for this outlier is not known; it is possibly due to variations in endogenous lipid peroxidation, as discussed later). Examining individual rat plasma samples, we identified the possible contribution of one individual outlier that displays intense acrolein modification in the FA group (not shown).

In terms of the lipid peroxidation status of plasma (i.e., free and protein-bound Schiff base conjugates), there was no significant difference in the total malondialdehyde levels between the two groups (FA group = 16.5 ± 1.3 μM and ETS = 15.7 ± 1.6 μM). In addition, the susceptibility of LDL to Cu^{2+}-induced lipid peroxidation was evaluated. Lipid peroxidation was initiated by addition of 50 μM freshly prepared $CuSO_4$ to LDL from FA or ETS group maintained at 37 °C. For the two groups, the total diene concentration (169.7 ± 46.2 and 168.3 ± 57.8 nmol/mg LDL protein for FA and ETS groups, respectively) and the propagation rates, 2.8 ± 0.8 and 2.8 ± 0.9 nmol of dienes

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                    10        20        30        40        50        60
Human apoE3: KVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQEELLSSQVTQEL

NMR:

Prediction: CCCCCCCCCCCCCHHHHCCCCCCCCHHHHHHHHHHHHHHHHCHHHHHHHHHHCHHHHHH

                    70        80        90       100       110       120
            RALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRG

            HHHHHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCHH

                   130       140       150       160       170       180
            EVQAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAREGAERGLSAIRER

            HHHHHCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCHHHHHHH

                   190       200       210       220       230       240
            LGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVR

            HCHHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH

                   250       260       270       280       290       299
            AKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH

            HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCC


                     10        20        30        40        50        60
Rat apoE:    EGELEVTDQLPGQSDQPWEQALNRFWDYLRWVQTLSDQVQEELQSSQVTQELTVLMEDTM
Prediction:  CCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHHHHHHHHHHHHH
                                                                         *
                     70        80        90       100       110       120
             TEVKAYKKELEEQLGPVAEETRARLAKEVQAAQARLGADMEDLRNRLGQYRNEVNTMLGQ
             HHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHCHHHHHHHHCCHHHHHHHHHH
               *   **
                    130       140       150       160       170       180
             STEELRSRLSTHLRKMRKRLMRDADDLQKRLAVYKAGAQEGAERGVSAIRERLGPLVEQG
             CHHHHHHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHHHHHHCHHHHHHHHCCCHHHH
                         *   *          *    *
                    190       200       210       220       230       240
             RQRTANLGAGAAQPLRDRAQALSDRIRGRLEEVGNQARDRLEEVREQMEEVRSKMEEQTQ
             HHHHHHHHHHHCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
                                                                      *
                    250       260       270       280       294
             QIRLQAEIFQARIKGWFEPLVEDMQRQWANLMEKIQASVATNSIASTTVPLENQ
             HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCC
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Figure 1. Comparison of structural features of human apoE3 and rat apoE. The amino acid sequence of human apoE3 (top) and rat apoE (bottom) are shown (the His-tag sequence is omitted for convenience). For both, the residues predicted to adopt an amphipathic α-helix or random coil (based on http://bioinf.cs.ucl.ac.uk/psipred) are indicated as "H" and "C", respectively, below the sequence. In addition, for human apoE3, the α-helical segments (derived from the NMR structure PDB ID no. 2L7B) are shown as gray cylinders for validation of the prediction. All lysine residues are shown in bold; the major LDLr binding sites in human apoE3 are in red; the predicted LDLr binding sites in rat apoE based on sequence alignment are in blue. The major HSPG binding sites in the NT domain are underlined for both (those for rat apoE are based on sequence alignment). Residues modified by acrolein in rat apoE under the conditions employed in this study as identified by MALDI TOF/TOF are indicated with a star (☆) below the sequence.

formed/(min·mg of LDL protein), respectively (data not shown) were similar; however, the lag period for the ETS group was shorter ($\sim$12 min) than that for the FA group ($\sim$20 min). Further, there was no difference in the total cholesterol levels between the two groups (FA, 74.4 $\pm$ 3.7 mg/dL; ETS, 72.1 $\pm$ 1.2 mg/dL); the triglyceride levels were $\sim$25% higher in the ETS group compared with the FA group (115 $\pm$ 9.5 and 92 $\pm$ 17.2 mg/dL, respectively; $n = 12$, $P < 0.3$). Nonetheless,

the observation that plasma apoE can be oxidatively modified provided the rationale for us to propose that acrolein modification disrupts key functional features of apoE.

To test the molecular basis of this hypothesis, purified recombinant rat apoE was treated with PBS or increasing amounts acrolein in PBS (1:2.5 to 2000:1 molar ratio, acrolein/apoE), followed by extensive dialysis to remove unbound acrolein. SDS-PAGE analysis under reducing conditions, Figure 3A, indicated a

Figure 2. Effect of FA or ETS exposure on oxidative status of plasma apoE: detection of acrolein-modified apoE. The lipoprotein (A) and lipid-free fractions (B) (25 μg of protein) of rats exposed to FA or ETS were incubated with 5F6 conjugated to protein G-Sepharose to capture all acrolein-modified proteins, followed by Western blot using anti-apoE-HRP.
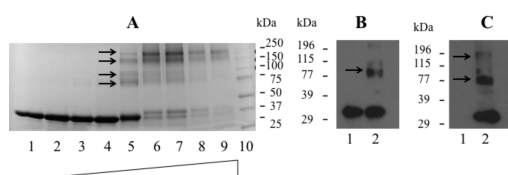
Figure 3. Acrolein modification of recombinant rat apoE. About 10 μg of apoE was treated with PBS (lane 1) or increasing amounts of acrolein at the ratios indicated below, electrophoresed on a 4–20% acrylamide gradient gel and stained with Amido Black (panel A). The lane assignments for the various acrolein/apoE molar ratios were as follows: lane 2, 1:2.5; lane 3, 1:1; lane 4, 2:1; lane 5, 20:1; lane 6, 200:1; lane 7, 400:1; lane 8, 1000:1; lane 9, 2000:1. Lane 10 shows the low molecular mass standards. Arrows draw attention to cross-linked species. Immunoblot analyses of 0.5 μg of unmodified (lane 1) and acrolein-modified (lane 2) apoE was carried out with anti-apoE–HRP (panel B) and 5F6 (panel C) antibody for apoE modified at 10:1 acrolein/apoE ratio.

variety of species depending on the molar excess of acrolein used: at 1:2.5, 1:1, and 2:1 ratios (lanes 2, 3, and 4, respectively), the major species were monomeric with minimal intermolecular cross-linking. At 20:1 ratio (lane 5), there was evidence of significantly modified monomeric species as seen by the slight shift in the 34 kDa band to lower mobility; in addition covalently cross-linked dimeric and some oligomeric species were noted (indicated by arrows). Between 200:1 and 2000:1 ratios (lanes 6–9), there was a progression toward formation of higher molecular weight species. In subsequent experiments, we used 10:1 ratio, the rationale being that there are 11 lysines in rat apoE and there would be ∼1 acrolein for each lysine. At this ratio, there is evidence of cross-linked dimer, as seen in the immunoblot using anti-apoE–HRP, Figure 3B. Acrolein modification was verified by Western blot using mAb5F6, Figure 3C, lane 2; the results confirm that acrolein modification of recombinant apoE generates epitopes that are recognized by 5F6, indicative of lysine modification. In addition, the presence of higher molecular weight bands is indicative of acrolein-facilitated intermolecular cross-linking via lysines (indicated with arrows). The presence of monomeric band in the immunoblot is indicative of lysine modification without intermolecular cross-linking. As expected, the 5F6 antibody does not recognize unmodified recombinant apoE as noted in control experiments, Figure 3C, lane 1.

Far-UV CD spectra of acrolein-modified apoE shows typical features of a highly helical protein, characterized by troughs at 208 and 222 nm, Figure 4A. The α-helical content was calculated
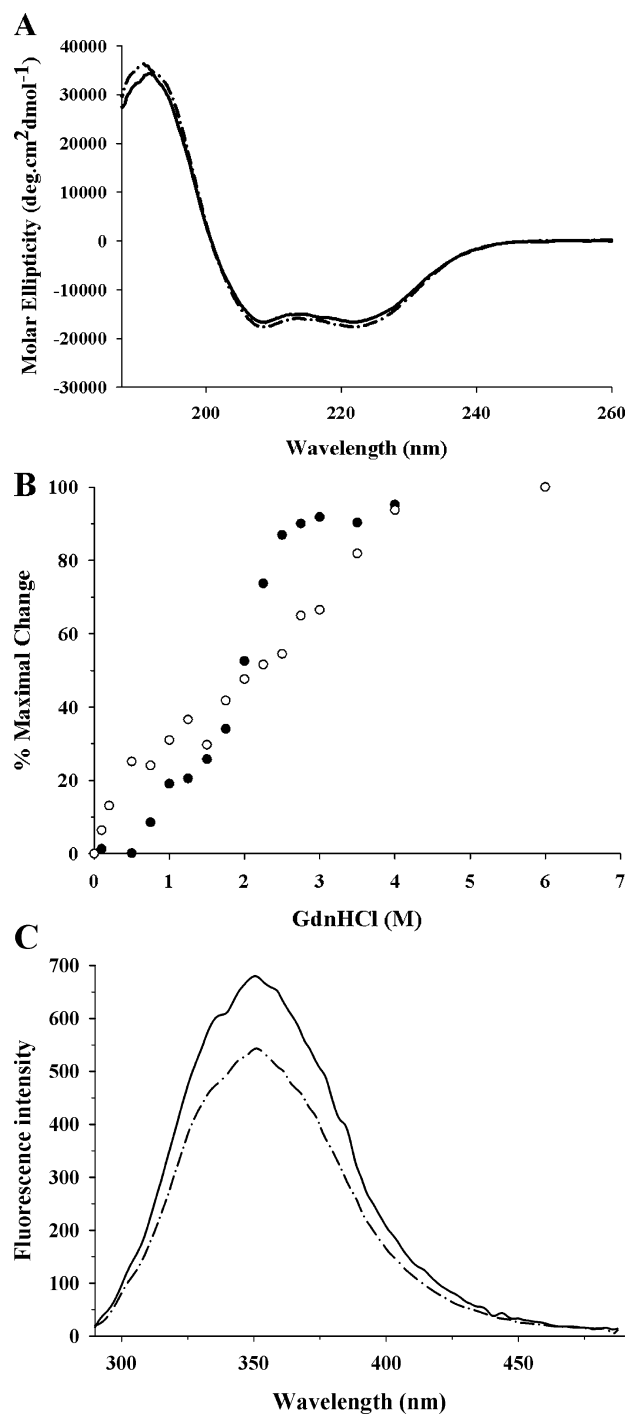
Figure 4. Effect of acrolein modification on secondary structure and tertiary fold of apoE. (A) Far-UV CD spectra. The spectra of unmodified (solid line) and acrolein-modified apoE (dashed line) (0.2 mg/mL) were recorded in 20 mM sodium phosphate buffer, pH 7.4. The scans were obtained from 185 to 260 nm using a 0.1 cm path length cuvette, scan speed of 20 nm/min, and response time of 1 s (average of three scans shown). (B) GdnHCl-induced unfolding. The % maximal change in ellipticity of 0.2 mg/mL unmodified (●) and acrolein-modified (○) apoE at 222 nm was plotted as a function of increasing GdnHCl concentration. The % maximal change was calculated using eq 3. (C) Fluorescence emission spectra. Fluorescence emission spectra of unmodified (solid line) and acrolein-modified apoE (dashed line) (0.02 mg/mL) were recorded in PBS. Emission scans were recorded between 290 and 490 nm following excitation at 280 nm (5 nm excitation and emission slit widths).

from the molar ellipticity values at 222 nm by eq 2 to be 46% ± 6%, which is very similar to that noted for unmodified apoE, 45% ± 5%, as reported recently.[29] GdnHCl-induced unfolding of acrolein-modified apoE was performed by following changes in the molar ellipticity at 222 nm as a function of varying GdnHCl concentration, Figure 4B. Unmodified apoE undergoes a two-phase unfolding process with a distinct plateau between the two phases, corresponding to unfolding of the C-terminal and N-terminal domains (midpoints of denaturation around 0.8 and 2.2 M GdnHCl, respectively). Each phase of the biphasic denaturation profile was treated as individual two-state denaturation equilibrium as described earlier.[34] The corresponding $\Delta G_D^{H_2O}$ values using eq 4 are 4.7 ± 1.7 and 9.9 ± 1.6 kcal/mol, respectively. In contrast, acrolein-modified apoE displayed a single-phase denaturation profile, with a midpoint of denaturation corresponding to 2.06 ± 0.3 M GdnHCl and a $\Delta G_D^{H_2O}$ of 1.5 ± 0.2 kcal/mol, suggesting the possibility of domain−domain interaction and a change in the overall tertiary fold. This is supported by a 25% decrease in intrinsic fluorescence emission at 350 nm (predominantly due to tryptophan), upon excitation at 280 nm, Figure 4C. Together these results are indicative of alterations in the tertiary fold, including intramolecular cross-linking between the N and C-terminal domains.

To determine whether acrolein modification alters the functional abilities of apoE, the following assays were performed: (i) the LDLr binding ability of acrolein-modified apoE was followed using sLDLr/LA3-LA6/Myc. Following incubation of DMPC/apoE (unmodified or acrolein-modified) with sLDLr/LA3-LA6/Myc, the receptor-bound complexes were captured by co-IP with anti-c-Myc-agarose and detected by anti-apoE−HRP antibody, Figure 5 (top panel) or anti-Myc antibody (bottom
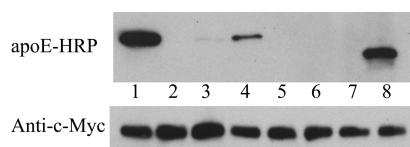
Figure 5. Effect of acrolein modification on LDLr binding ability of apoE. DMPC/apoE complexes were incubated with 10 μg of sLDLr in PBS and 2 mM CaCl₂ for 1 h at 4 °C, followed by co-IP with anti-c-Myc antibody-linked agarose to capture the DMPC/apoE/sLDLr complexes. LDLr-bound apoE was detected by Western blot using anti-apoE-HRP antibody (top panel). A duplicate analysis was carried out in parallel with anti-c-Myc antibody (bottom panel) to confirm the presence of similar levels of LDLr in each reaction mixture. The lane assignments were as follows: Lane 1: Western blot control in both panels (0.5 μg apoE in *top panel* and 10 μg sLDLr/LA3-LA6/Myc in *bottom panel*); lanes 2, 3, and 4: 0, 1, and 10 μg protein, respectively, of DMPC/unmodified apoE; lanes 5, 6, and 7: 0, 1, and 10 μg protein, respectively, of DMPC/acrolein-modified apoE. Lane 8 contains DMPC/human apoE3/(1−191) (10 μg), which served as an additional control to support the robustness of the assay.

panel). Lipid-free apoE (0.5 μg) and sLDLr/LA3-LA6/Myc (10 μg) were loaded in lane 1, top and bottom panels, respectively, as immunoblot controls. DMPC/unmodified apoE elicits the ability to bind the sLDLr/LA3-LA6/Myc in a concentration-dependent manner in the presence of Ca²⁺ (0, 1, and 10 μg of apoE, lanes 2, 3, and 4, respectively). The binding ability was abolished when Ca²⁺ was omitted and when 2 mM EDTA was included to chelate residual Ca²⁺ in the incubation mixture (data not shown), indicative of the specificity of interaction of apoE with sLDLr and the requirement of Ca²⁺ for maintaining the structural and functional integrity of the latter. In contrast,

DMPC/acrolein-modified rat apoE was unable to interact with the sLDLr (0, 1, and 10 μg of apoE, lanes 5, 6, and 7, respectively). The lipid-bound form of human apoE3/(1−191) (10 μg) bearing the LDLr binding segment was used as a positive control, lane 8.

(ii) The heparin binding ability of acrolein-modified apoE was assessed in comparison with that of unmodified protein, Figure 6.
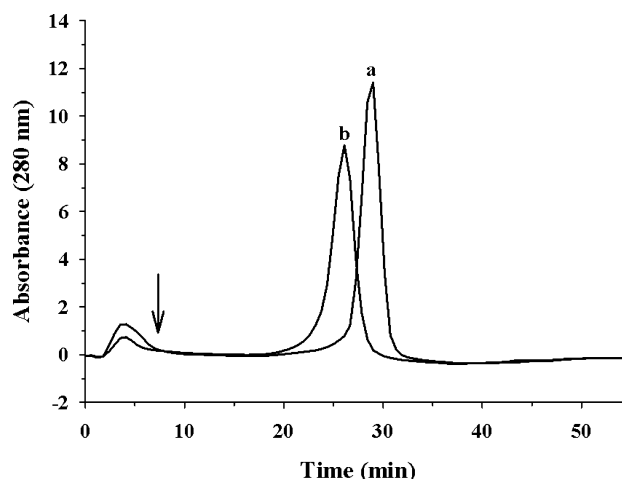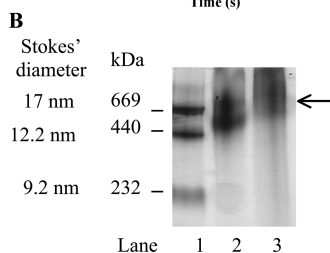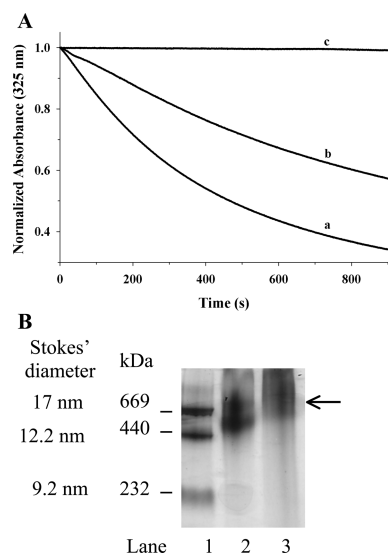

Figure 6. Heparin binding ability of unmodified and acrolein-modified apoE. Unmodified (a) or acrolein-modified (b) apoE (100 μg) was loaded onto a heparin-Sepharose column in 20 mM sodium phosphate, pH 7.4, in an ÄKTA FPLC system. The flow rate was maintained at 1 mL/min. A salt gradient of 0−1.0 M NaCl was used to elute the bound protein; the elution of the protein was monitored at 280 nm. The arrow represents the start of the gradient.

Unmodified apoE binds to heparin-Sepharose, requiring 0.49 ± 0.01 M NaCl to elute the bound protein. Acrolein-modified apoE also binds to heparin-Sepharose; however, its binding is relatively weaker, requiring 0.39 ± 0.04 M to be eluted.

(iii) The ability of acrolein-modified apoE to interact with lipids was next evaluated in comparison with that of unmodified apoE. The rationale was to see whether the increase in modified apoE in the lipid-free bottom fraction from plasma of ETS-exposed rats was due to decreased lipid-binding ability. Typically, lipid binding of an apolipoprotein is assessed by determining its ability to transform DMPC MLVs to discoidal bilayer complexes at 24 °C, the gel−crystalline transition temperature of DMPC. Conversion of the large vesicles (∼200 nm diameter) to the smaller lipid/protein complexes (diameter ∼20 nm) is accompanied by a decrease in turbidity that can be followed as changes in absorbance at 325 nm, especially in the initial stages of the assay. Figure 7A shows changes in absorbance obtained when DMPC MLVs (500 μg of lipid) were treated with unmodified (curve a) or acrolein-modified apoE (curve b) (100 μg of protein) or PBS (curve c). The absorbance of DMPC vesicles remained mostly unchanged in absence of protein. It decreased rapidly upon addition of unmodified apoE ($T_{1/2}$ = 8.8 ± 1.0 min and rate constant, K = 0.12 ± 0.01 min⁻¹), as reported earlier. On the other hand, the $T_{1/2}$ and K were significantly altered in acrolein-modified apoE, 22 ± 1.7 min and 0.045 ± 0.003 min⁻¹, respectively. Upon prolonged incubation, DMPC/acrolein-modified apoE complex formation occurs eventually. The lipoprotein complexes formed after overnight incubation were isolated by density gradient ultracentrifugation revealing more heterogeneous particles that are slightly larger in size, ∼17 nm

Figure 7. Lipid-binding ability of unmodified and acrolein-modified apoE. (A) Transformation of DMPC MLVs to smaller lipid/protein complexes by apoE. About 500 μg of DMPC MLVs was treated with 100 μg of unmodified (curve a) or acrolein-modified (curve b) apoE or PBS (curve c), and the change in absorbance at 325 nm was followed as a function of time at 24 °C. The data are normalized to absorbance at 0 min. Representative curves from three different experiments are shown. (B) Nondenaturing PAGE analysis of DMPC/apoE complexes prepared with unmodified or acrolein-modified apoE. About 50 μg of DMPC/apoE complexes were loaded on a 4−20% acrylamide gradient gel and electrophoresed in 10 mM Tris-glycine, pH 8.4, for 24 h at 110 V and stained with Amido Black. Lane 1, high molecular mass standard; lane 2, DMPC/unmodified-apoE; lane 3, DMPC/acrolein-modified apoE complex (arrow draws attention to the major band). The molecular mass and average particle sizes were calculated from a calibration curve using the following standards and their corresponding molecular masses and Stokes diameters: thyroglobulin (669 kDa, 17 nm), ferritin (440 kDa, 12.2 nm), and catalase (232 kDa, 9.2 nm).

diameter, and mass, ∼670 kDa, compared with that noted for unmodified apoE (∼15 nm and ∼630 kDa, respectively) as seen by nondenaturing PAGE, Figure 7B.

(iv) Subsequently, we determined whether acrolein modification alters the ability of apoE to promote cholesterol and phospholipid efflux from macrophages in comparison with unmodified and acrolein-modified apoAI, Figure 8, panel A. Unmodified rat apoE (10 μg/mL) stimulates a robust cholesterol efflux from macrophages (∼80% compared with that by human apoAI) in the presence of cAMP and lower levels in the absence of cAMP. Upon acrolein modification (50:1 acrolein/apoE, m/m), its ability to promote efflux decreased significantly to ∼5%. This trend was similar to that noted for apoAI, with acrolein modification leading to a dramatic decrease in cholesterol efflux ability, an observation also noted by others.[47] The ability to promote efflux was dose dependent in the range studied (0.1−30 μg/mL) for unmodified apoAI and apoE, with maximal effect noted at ∼10 μg/mL, Figure 8, Panel B; throughout the concentration range studied, acrolein-modified apoE (and apoAI) was still not effective, displaying only 5−10% efficiency as unmodified protein. The decreased cholesterol efflux capability of acrolein-modified apoE appears to correlate with the extent of modification, Figure 8, panel C, with 10:1 and 50:1 ratios (acrolein,apoE, m/m ratio) eliciting 67% and 36%, respectively, of unmodified apoE.

To determine whether the loss of function is due to modification of specific sites by acrolein, we carried out mass
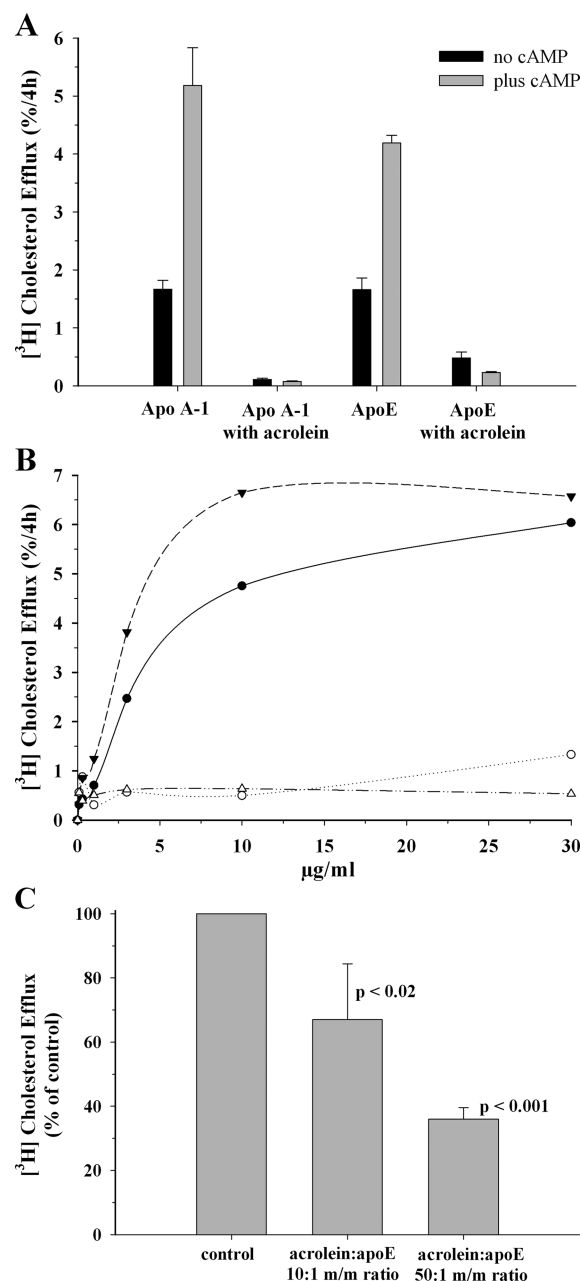
Figure 8. Effect of acrolein modification on cholesterol efflux capability of apoE. J774 macrophages were labeled with [³H]cholesterol (1 μCi/mL) in RPMI-1640 with 1% FBS for 48 h. A cAMP analogue was added to up-regulate ABCA1 expression for a period of 18 h, followed by exposure to 10 μg/mL (panel A) unmodified or acrolein-modified human apoAI or rat apoE (50:1 acrolein/protein, m/m ratio) in serum-free RPMI-1640 medium. The amount of [³H]cholesterol appearing in the medium after 4 h was expressed as a percentage of the radioactivity initially present in cells at time zero. A parallel experiment with no added cAMP was conducted to assess the efflux not mediated by ABCA1. Background release of [³H]cholesterol to serum-free medium was subtracted from values obtained with added proteins. Panel B shows dose-dependent (0.1−30 μg/mL) efflux for unmodified (●) or acrolein-modified rat apoE (○) and unmodified (▼) or acrolein-modified (△) human apoAI. All other conditions are as described above. Panel C shows correlation between extent of acrolein modification and efflux capability of apoE. Under conditions as described under panel A, efflux was measured with 3 μg/mL unmodified or acrolein-modified (10:1 and 50:1, acrolein/apoE, m/m ratio) apoE. Values are expressed as % of control unmodified apoE, mean ± SD.

Table 1. MALDI TOF/TOF Analysis of Peptides Obtained from AspN + GluC Digestion of Acrolein Modified Rat apoE[a]

position	sequence	predicted (m/z)	observed (m/z)	modification
63−69	VKAY**K**[67]KE	865.5	941.9	K[67]+76
58−69	DT**M**[60]TEV**K**[64]AY**K**[67]**K**[68]E	1442.7	1704.5	M[60]+16, K[64]+76, K[67]+76, K[68]+94
125−146	LRSRLSTHLR**K**[135]M**RK**[138]RLMRDADD	2754.2	2866.2	K[135]+56, K[138]+56
145−160	DDLQ**K**[149]RLAVY**K**[155]AGAQE	1804.9	1880.8	K[149]+38, K[155]+38
248−262	IFQARI**K**[254]GWFEPLVE[b]	1833.0	1908.7	K[254]+76

[a]Unmodified or acrolein modified apoE (20 μg) was treated with AspN + GluC and subjected to MALDI TOF/TOF analysis following cleanup using C_{18} ZipTip pipette tips as described under Experimental Procedures. The five major peptides with modified residues are shown. Data are shown only for acrolein-modified apoE. The modified residues are shown in bold; their positions are indicated in superscript. The observed m/z values were obtained by processing the raw data using the GPS Explorer, version 3.6 (Applied Biosystems, South San Francisco, CA), and then searched against Swiss-Prot protein sequence database. The mass increase at defined sites is shown in the last column. [b]Peptide 248−262 showed modification (+76 Da) even in absence of added acrolein, possibly due to air oxidation during sample handling. However, the modification at this site was ~36-fold higher upon exogenous addition of acrolein as inferred from quantification based on peak area.
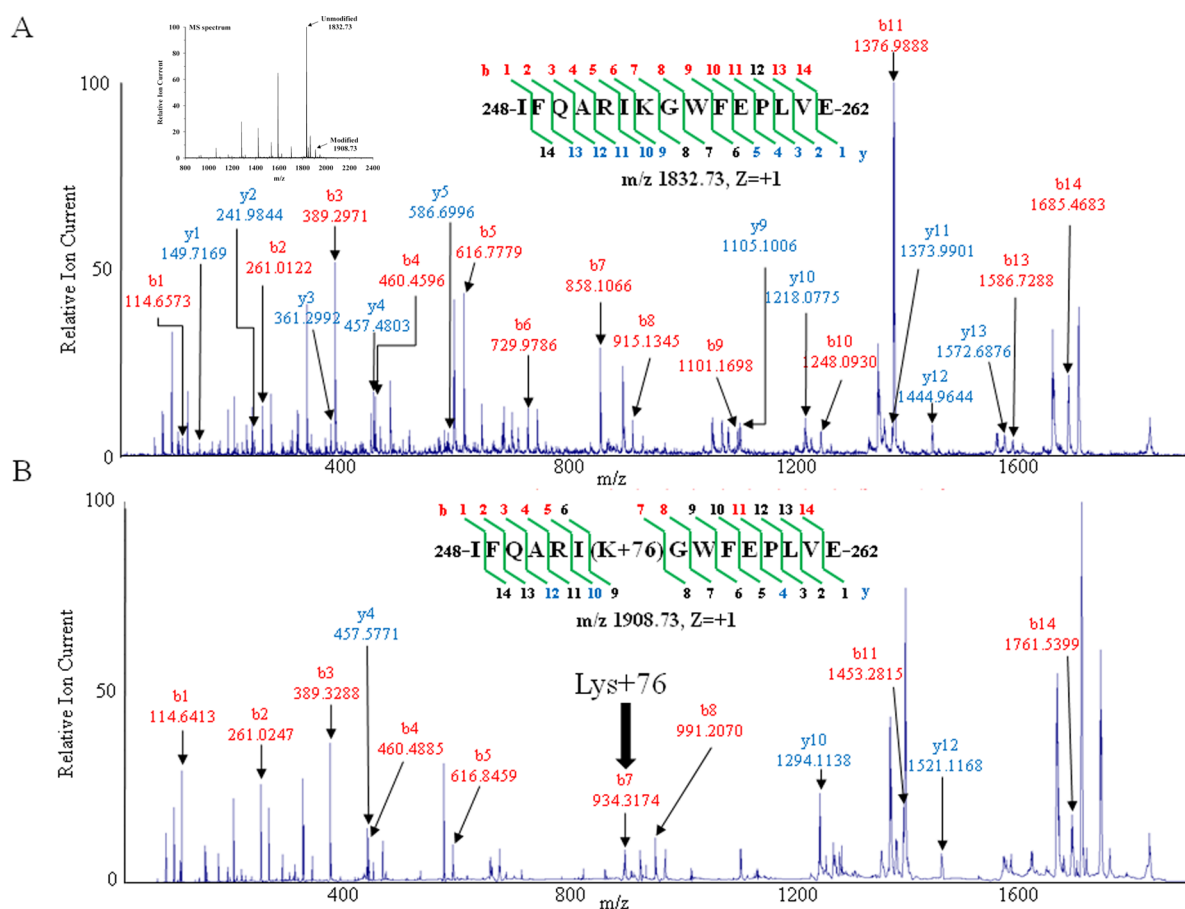
Figure 10. MALDI TOF/TOF identification of Lys254 in the AspN + GluC digestion of unmodified and acrolein-modified apoE. MS/MS analysis of peptide 248−262 obtained from AspN + GluC digestion of unmodified and acrolein-modified. (A) [IFQARIK[248]GWFEPLVE]$^+$ (m/z 1832.73, z = +1); (B) [IFQARIK[248]+76GWFEPLVE]$^+$ (m/z 1908.73, z = +1). Bold arrow draws attention to b7 ion from acrolein-modified apoE with 76 Da mass increase. Inset in panel A shows the mass spectrum for each parent ion for unmodified and acrolein-modified apoE peptide.
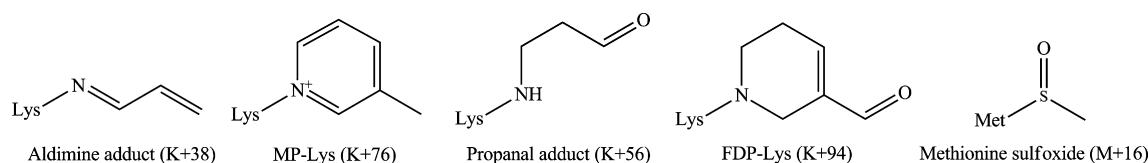
acrolein-exposed samples. Lastly, peptide 58−69 revealed an increase in mass units by 16 at Met60 that is attributed to oxidative modification of the −S-CH$_3$ group to methionine sulfoxide.

Overall, there were four different types of acrolein-modified lysines: an aldimine adduct (+38) at Lys149 and Lys155; a propanal adduct (+56) at Lys135 and Lys138; an N^ε-(3-methylpyridinium)-lysine (MP-lysine) (+76) at Lys64, Lys67, and Lys254, and FDP-lysine (+94) at Lys68, Figure 11 and Table 1. The mass increase of 38 Da is believed to correspond to a Schiff base adduct between the aldehyde group of acrolein and the ε-amino group of lysine; this, in turn, reacts with a second acrolein via a Michael addition eventually

cyclizing to MP-lysine, a stable product registering a mass increase of 76 Da, via an imine intermediate.[50] The mass increase of 56 Da arises when acrolein undergoes nucleophilic addition at the double bond leading to the Michael addition-type adduct; the increase of 94 Da possibly arises from reaction of a lysine with two acrolein molecules via Michael addition followed by condensation and dehydration reactions.[28,30,51,52]

■ DISCUSSION

Several lines of evidence indicate a correlation between lipoprotein oxidation and atherogenicity of a lipoprotein particle,

Aldimine adduct (K+38)   MP-Lys (K+76)   Propanal adduct (K+56)   FDP-Lys (K+94)   Methionine sulfoxide (M+16)

Figure 11. Chemical structures of acrolein adducts identified in acrolein-modified rat apoE by MALDI TOF. The numbers in parentheses indicate the observed increases in mass units under the conditions employed in this study.

with oxidized LDL (containing apoB-100 as the major protein and generally lacking apoE) playing a significant role in the etiology of atherosclerosis.[53,54] We propose that oxidative modification of apoE located on other lipoproteins may be an additional mechanism for predisposing subjects toward an unfavorable plasma lipid (pro-atherogenic) profile. Acrolein is one of the major pro-oxidants generated *in situ* and is also one of the predominant environmental pollutants. Lysine and histidines are likely targets for acrolein modification.[30,51,52] Our aim was to determine whether plasma apoE is subjected to modification by acrolein from either of these sources. To address this issue, initial studies were carried out to determine the effect of exposure of rats to FA or ETS on plasma apoE.

Despite the marginal differences in the triglyceride levels and the LDL susceptibility to oxidation, the FA and ETS exposure studies offered three major findings: (i) sufficient acrolein is generated *in situ* to cause modification of plasma proteins as noted in acrolein modification of apoE in rats exposed to FA, (ii) ETS exposure leads to further acrolein modification of apoE as noted in its increased presence in the lipid-free fraction during ultracentrifugation, and (iii) acrolein modification appears to alter the lipoprotein binding property of apoE. These observations warranted further investigations to determine the molecular basis of the effect of acrolein modification on apoE at the structural and functional level.

As an exchangeable apolipoprotein, apoE can exist in a lipid-free or lipid (lipoprotein)-associated state. In the lipid-associated but not in the lipid-free state, apoE serves as a ligand for the LDLr. Rat apoE bears an overall sequence identity of 73.5% with human apoE3 and 73.9% with apoE4 and is expected to adopt a similar fold and three-dimensional structure as human apoE, based on the biphasic GdnHCl-induced unfolding pattern that we observed recently.[29] It is a highly helical protein that displays two midpoints of denaturation corresponding to 0.8 and 2.2 M GdnHCl, suggesting the presence of two independently folded domains as noted by others for human apoE3.[5,32] In apoE3, they correspond to unfolding of the C-terminal (CT) and N-terminal (NT) domains, respectively. The NT domain (residues 1−191) is a four-helix bundle bearing the LDLr binding sites, while the CT domain (residues 201−299) bears high-affinity lipid-binding sites. The CT domain also mediates apoE self-association leading to tetramers by helix−helix interactions in the lipid-free state. In humans, apoE is polymorphic, existing in 3 major isoforms (apoE2, apoE3 and apoE4) with significant differences between the isoforms in determining the predisposition of individuals towards hyperlipidemia and Alzheimer's disease: in apoE3 (allelic frequency ∼78%), considered the normal anti-atherogenic isoform, positions 112 and 158 have a Cys and Arg, respectively. In apoE2 (allelic frequency ∼7%), these positions bear a Cys: individuals bearing this isoform are predisposed to developing heart disease and hyperlipidemia. In apoE4 (allelic frequency ∼15%) both positions bear an Arg; individuals bearing apoE4 are at a high risk for developing Alzheimer's disease and heart disease. Although rat apoE bears an Arg at position 112 like human apoE4,

it likely resembles apoE3 from a functional standpoint.[5,57] From its sequence similarity and secondary structural predictions, it is expected to adopt a fold similar to apoE3. The lysine residues that are considered essential for LDLr binding are conserved in rat apoE. Of the 11 lysine residues, 8 are located toward the N-terminal part of rat apoE between residues 64 and 155 and 3 toward the C-terminal part. Thus, oxidative modification of lysine residues can be expected to have a significant impact on the ability of apoE to regulate plasma lipid homeostasis.

Acrolein modification generated epitopes on rat apoE that are recognized by mAb5F6, the antiacrolein-lysine antibody. SDS-PAGE analysis reveals the presence of monomers, cross-linked dimers, and oligomeric species in acrolein-modified apoE. Increasing the amount of acrolein resulted in increased extent of modification, with covalently cross-linked species appearing when there is at least one acrolein per lysine in apoE, Figure 3. The 34-kDa band in the immunoblot could also represent intramolecular cross-linking, as suggested by GdnHCl-induced unfolding pattern of acrolein-modified apoE. The distinct biphasic pattern of unfolding that represents unfolding of the two domains seen in unmodified apoE is lost upon acrolein modification, Figure 4B. Acrolein-mediated covalent cross-linking between lysines in the two domains would prevent independent unfolding and potentially alter the overall tertiary fold as seen in decreased intrinsic fluorescence emission, Figure 4C, without any significant change in the secondary structure, Figure 4A.

Two major physiological functions of apoE are LDLr- and HSPG-binding activities,[58,59] both involving basic residues. Both functions appear to be impaired with acrolein modification. The LDLr is a multidomain receptor that plays a crucial role in removing atherogenic neutral lipid containing lipoproteins from the circulation. The ligand binding domain of the LDLr is accommodated within the seven adjacent modular LDL-A repeats LA1−LA7,[60] of which LA5 is required for high-affinity binding of apoE containing lipoprotein particles.[40,61] A single Ca^{2+} ion contributes to the structural integrity of each module by coordinating with four conserved acidic residues. In the case of the high affinity LDLr interaction with human apoE3, basic residues located between 130 and 150 in helix 4 of the NT domain and Arg172 have been shown to be essential for binding with the LDLr ligand binding modules.[62,63] Pioneering studies by Weisgraber and colleagues have demonstrated that amino acid substitution or *in vitro* chemical modification of the lysine and arginine residues in apoE leads to a dramatic loss in LDLr binding ability.[5,64] The binding involves numerous electrostatic interactions that govern specificity between the basic residues on apoE and the acidic residues surrounding the Ca^{2+} in the receptor; in addition, the interface also involves a tryptophan and a stacking of histidine,[65,66] while the helix curvature that is conferred by the lipid or lipoprotein surface also likely plays an essential role in the binding interaction. Together these and other factors such as creation of a multivalent ligand induced by lipid binding[40,67] contribute to the high affinity binding of

lipid-associated apoE predominantly via residues TEELRVR-LASHLRKLRKRLLR. The sequence identity in this segment between rat and human apoE is ~81% (compared with overall identity of 73.5%), Figure 1, with all the basic residues being conserved (TEELRSRLSTHLRKMRKRLMR), signifying that these residues play a critical role in the binding interaction in rat apoE as well. Loss of LDLr binding ability upon acrolein modification, Figure 5, is indicative of direct modification of one or more of these lysine residues or changes in the overall fold of the protein or both. Mass spectral data provide direct evidence for modification of Lys135 and Lys138, Table 1, and Supporting Information, Figure S1, panel C, leading to a propanal adduct (+56 Da) on both these lysines, Figure 11.

In the case of heparin interaction, acrolein modification of Lys135 and Lys138 appears to be responsible for a significant decrease in the binding ability of rat apoE. Interaction with cell surface HSPG is a major factor in the physiological role of apoE in the hepatic clearance of lipoprotein remnants. This is mediated through the LDLr and LDLr-related protein (LRP) coupled to HSPG pathway. Together, these uptake pathways play a significant role in clearing fasting and postprandial remnant clearance.[68] ApoE initially interacts with HSPG, followed by the transfer of the lipoprotein particle to LRP. Defective binding to HSPG can therefore potentially lead to accumulation of the pro-atherogenic remnant particles.[68] The details of the interaction between human apoE and heparin have emerged from site-directed mutagenesis,[69] NMR, and surface plasmon resonance studies[69,70] and by monoclonal antibodies.[71] ApoE/heparin interaction possibly occurs as a two-step process, the first step involving a fast association with electrostatic interactions and the second step involving hydrophobic interactions.[69] In these studies, heparin, a highly sulfated version of HSPG that is isolated from bovine intestinal mucosa, was used as an *in vitro* model for studying cell surface HSPG. They point to the importance of Arg142, Lys143, Arg145, Lys146, and Arg147 in the NT domain of human apoE isoforms in high-affinity binding.[70] They are available for interaction in both lipid-free and lipoprotein-associated apoE. These residues are directly involved in ionic interaction with the negatively charged sulfated groups of heparin. In human apoE3, Lys143 and Lys146 appear to have unusually low pK_a (9.5 and 9.2, respectively), which was attributed to the strong positive electrostatic potential in their microenvironment.[72,73] Based on sequence alignment, the corresponding heparin-binding sites in rat apoE are Lys135 and Lys138 in the NT domain,[29] both of which are modified by acrolein. It has been suggested that the position of lysine in an amphipathic α-helix within salt-bridge forming distance such as a (E/D)XXK, KXX(E/D), (E/D)XXXK, or KXXX(E/D) motif may alter its nucleophilicity and thereby enhance reactivity with acrolein as noted for apoAI[47] and an HDL peptide mimetic.[74] However, in contrast to apoAI where most of the lysine is in this configuration, only 3 out of 11 lysine are within salt-bridge forming distance in rat apoE, making the enhanced nucleophilicity a less likely explanation for the altered reactivity to acrolein. Regardless of the nature of the chemical interaction, the modification significantly weakens the overall ionic interaction of rat apoE with the sulfated moieties of heparin.

It is possible that modified rat apoE retains its ability to bind heparin via sites in the CT domain. In human form, Lys233 appears to bear high affinity for heparin only in the isolated CT domain.[69] The corresponding site in rat apoE is an arginine (Arg225), which does not appear to bear any modification. Thus, Arg225 in the CT domain is a likely site for interaction of

acrolein-modified rat apoE with heparin, which may explain the weak interaction. Interestingly, we observed in a prior study that acrolein modification of *isolated* NT domain of human apoE3 practically abolished its heparin binding ability.[42] Nevertheless, the physiological implication of weak HSPG binding of acrolein-modified apoE is poor clearance of remnant lipoprotein particles in oxidative stress conditions such as aging and exposure to tobacco smoke and other environment pollution. It can be envisaged that acrolein can cause significant oxidative modification of the lysine residues as a result of ETS exposure, thereby disrupting its interaction with HSPG and the LDLr.

Acrolein also significantly impairs the lipid-binding ability of apoE as seen in the 60% decrease in the rate of conversion of DMPC vesicles to discoidal particles. This has important physiological implications, because lipid binding is an essential prerequisite that confers increased LDLr binding affinity as noted above. This observation derives support from *in vivo* studies, which revealed an increased presence of acrolein-modified apoE in the lipid-free fraction in the ETS group compared with the FA group. It is likely that oxidative modification of apoE weakened its lipid association and triggered its dissociation from the lipoprotein particle. Previous studies from our laboratory indicate that acrolein modification of the isolated N-terminal domain of human apoE3 also displayed a significant loss in its lipid binding ability.[42] The alternative possibility that apoE was oxidatively modified after its dissociation from the lipoprotein particle or that oxidative modification of lipids weakened the lipid-binding affinity of apoE cannot be excluded at this point.

Prior investigations established the critical role of human apoE isoforms in reverse cholesterol transport and in promoting ABCA1-mediated cholesterol efflux from cholesterol-laden macrophages, particularly in atherosclerotic lesions.[6,75−77] The ability of rat apoE to promote ABCA1-dependent cholesterol efflux noted in the present study is similar to that reported for human apoE3[43] with J774 mouse macrophages and with HeLa cells transfected with ABCA1 cDNA. This is consistent with previous reports showing that apolipoproteins are good acceptors of lipids from cells expressing ABCA1.[78−82] Although the molecular details of the process are not clear, the CT domain of apoAI and apoE appears to be critical for mediating efficient cholesterol efflux. Structural features of the α-helix such as hydrophobicity and amphipathic nature, rather than specific residues, are important determinants that govern cellular cholesterol efflux abilities of apolipoproteins.

In the present study, we show that acrolein modification significantly impairs the ability of rat apoE to promote cholesterol efflux. The site modified in the CT domain is Lys254, which may be a contributory factor toward decreasing ABCA1-mediated cholesterol efflux. Although there were no significant changes in the α-helical content following acrolein modification, it is possible that the lysine modifications disrupt intrahelical ionic interactions with glutamate residues at i, $i + 3$, or $i + 4$ position and destabilize the overall helix stability. GdnHCl-induced denaturation data indicate that the biphasic mode of unfolding is lost in acrolein-modified apoE, suggesting interdomain (intra- or intermolecular) cross-linking. This would alter the helix curvature and explain the impairment of cholesterol efflux. Other studies show that interaction of apoAI with acrolein resulted in modification of Lys226 located in helix 10 in the C-terminal domain, which disrupted its ability to promote ABCA1-mediated cholesterol efflux in baby hamster kidney cells.[47]

Taken together with our *in vitro* biochemical analysis and other researchers' reports, we predict that apoE is a potential *in vivo* target for interaction with acrolein in tobacco smoke. Oxidation of protein appears to represent the final stage of LDL oxidation process,[83] when the aldehydes react with side chain amino groups. It has been shown that acrolein preferentially modified lysine residues of LDL *in vitro*. Immunohistochemical studies from other laboratories show the presence of acrolein-modified proteins in atherosclerotic lesions from human aorta.[30,84] In the present study, we demonstrate that acrolein-modified apoE is found to a similar extent in the total lipoprotein fractions in both groups, while the ETS group had a greater extent of modified apoE in the lipid-free fraction. It is possible that acrolein present in the tobacco smoke either directly modified apoE or that other prooxidants triggered formation of acrolein in lipoprotein particles, eventually modifying apoE.

It is anticipated that other apolipoproteins may be potentially oxidized by ETS exposure as well, an aspect that requires further attention. The implication of our observations is that in addition to smokers, people exposed to second hand smoke and individuals routinely exposed to environmental pollutants may be susceptible to developing heart disease due to the oxidative damage to apoE and other apolipoproteins. Further studies are required to determine the global effects of age- and ETS-induced oxidative stress on the plasma lipoproteome.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MALDI TOF/TOF identification of M60, K64, K67, K68, K135, K138, K149, and K155 in AspN + GluC digested acrolein-modified apoE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ApoE, apolipoprotein E; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ETS, environmental tobacco smoke; FA, filtered air; FDP-lysine, N^ϵ-(3-formyl-3,4-dehydropiperidino)-lysine; HSPG, heparan sulfate proteoglycan; LDL, low density lipoprotein; LDLr, low density lipoprotein receptor; MALDI TOF, matrix-assisted laser desorption/ionization−time of flight; MLV, multilamellar vesicle; MP-lysine, N^ϵ-(3-methylpyridinium)-lysine; PBS, phosphate buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM sodium chloride); VLDL, very low density lipoprotein

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