Oxidation of Methionine Residues to Methionine Sulfoxides Does Not Decrease Potential Antiatherogenic Properties of Apolipoprotein A-I*

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The initial stage of oxidation of high density lipoproteins (HDL) is accompanied by the lipid hydroperoxide-dependent, selective oxidation of two of the three Met residues of apolipoprotein A-I (apoA-I) to Met sulfoxides (Met(O)). Formation of such selectively oxidized apoA-I (i.e. apoA-I32) may affect the antiatherogenic properties of HDL, because it has been suggested that Met86 and Met112 are important for cholesterol efflux and Met148 is involved in the activation of lecithin:cholesterol acyl transferase (LCAT). We therefore determined which Met residues were oxidized in apoA-I32 and how such oxidation of apoA-I affects its secondary structure, the affinity for lipids, and its ability to remove lipids from human macrophages. We also assessed the capacity of discoidal reconstituted HDL containing apoA-I32 to act as substrate for LCAT, and the dissociation of apoA-I and apoA-I32 from reconstituted HDL. Met86 and Met112 were present as Met(O), as determined by amino acid sequencing and mass spectrometry of isolated peptides derived from apoA-I132. Selective oxidation did not alter the α-helicity of lipid-free and lipid-associated apoA-I as assessed by circular dichroism, and the affinity for LCAT was comparable for reconstituted HDL containing apoA-I or apoA-I32. Cholesteryl ester transfer protein mediated the dissociation of apoA-I more readily from reconstituted HDL containing apoA-I32 than unoxidized apoA-I. Also, compared with native apoA-I, apoA-I32 had a 2- to 3-fold greater affinity for lipid (as determined by the rate of clearance of multilamellar phospholipid vesicles) and its ability to cause efflux of [3H]cholesterol, [3H]phospholipid, and [14C]α-tocopherol from lipid-laden human monocyte-derived macrophages was significantly enhanced. By contrast, no difference was observed for cholesterol and α-tocopherol efflux to lipid-associated apolipoproteins. Together, these results suggest that selective oxidation of Met residues enhances rather than diminishes known antiatherogenic activities of apoA-I, consistent with the overall hypothesis that detoxification of lipid hydroperoxides by HDL is potentially antiatherogenic.

High density lipoproteins (HDL) are generally regarded as antiatherogenic, an activity commonly attributed to the removal of extrahepatic cholesterol by HDL particles (1, 2) and apolipoproteins, mainly apolipoprotein A-I (apoA-I), that dissociate from HDL (3, 4). In addition to promoting cholesterol efflux, HDL has also been proposed to be antiatherogenic by aiding the removal and detoxification of proatherogenic oxidized lipids (5–7). Thus, cholesteryl ester transfer protein (CE-TRP) transfers oxidized lipids from low density lipoproteins (LDL) to HDL (7), and HDL carries the majority of cholesteryl ester hydroperoxides (CE-OOH, the first and major products formed during lipoprotein oxidation) in human plasma (5). In addition, CE-OOH and cholesteryl ester hydroxides (CE-OH) in HDL, but not LDL, are removed rapidly via selective uptake by liver parenchymal cells in vitro (6) and by perfused liver in situ (8). This uptake is associated with cellular detoxification of CE-OH (6) and is more rapid than that of the corresponding nonoxidized cholesteryl esters (CE) (6, 8). Furthermore, CE-OH are also rapidly removed from HDL via hepatic clearance in vivo (9), and this is associated with biliary secretion of the CE-OH-derived cholesterol (9), indicating that oxidation of the fatty acid moiety of CE may aid the elimination of cholesterol from the body.

Once associated with HDL, CE-OH are reduced to the corresponding CE-OH (10) that no longer contribute to or enhance the oxidation of lipoproteins. This “antioxidant” activity is expressed by reconstituted HDL particles (rHDL) containing apoA-I only and lipid-free apoA-I (11, 12) and extends to phospholipid hydroperoxides (11–13). The reduction of lipid hydroperoxides, added to or formed in HDL exposed to radical oxidants, results in the formation of selectively oxidized apoA-I (i.e. apoA-I32) that contains two Met sulfoxide [Met(O)] residues as the sole modification (11, 12). However, it is not known which Met residues of apoA-I become oxidized and how formation of Met(O) affects the ability of apoA-I/HDL to mediate

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1 The abbreviations used are: HDL, high density lipoproteins; AAPH, 2,2′-azobis(2-aminopropane) hydrochloride; apoA-I, apolipoprotein A-I; apoA-I32, selectively oxidized apoA-I (32 mass units heavier than native apoA-I and containing two Met sulfoxide residues); CD, circular dichroism; CE, cholesteryl esters; CE-OH, cholesteryl ester hydroxides; CE-OOH, cholesteryl ester hydroperoxides; CETP, cholesteryl ester transfer protein; DMPC, dimyristoylphosphatidylcholine; drHDL, discoidal reconstituted HDL; rHDL, spherical reconstituted HDL; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; acLDL, acetylated low density lipoproteins; hMDM, human monocyte-derived macrophages; Met(O), methionine sulfoxide; PBS, phosphate-buffered saline; α-TOH, α-tocopherol; RP-HPLC, reverse-phase high pressure liquid chromatography; BSA, bovine serum albumin; ESI-MS, electrospray ionization mass spectrometry.
efflux of lipids from macrophages and to act as substrate for lecithin:cholesterol acyltransferase (LCAT). These questions are relevant, because in human atherosclerotic lesions HDL is oxidized to an extent comparable to that of LDL (14) and a decreased and enhanced ability to promote cellular cholesterol efflux has been reported for in vitro oxidized HDL (see e.g. Refs. 15–17). In the present study, we therefore examined the physicochemical characteristics and biological activities of apoA-I.

EXPERIMENTAL PROCEDURES

Isolation and Selective Oxidation of HDL—Isolated HDL was prepared from fresh plasma obtained from normolipidemic donors (18), and its protein content was determined by the bicinchoninic acid method (Sigma) using bovine serum albumin (BSA) (Sigma) as standard. HDL was oxidized in phosphate-buffered saline (PBS), pH 7.4, containing 1 mM EDTA by aerobic incubation at 37 °C for 19 h in the presence of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH, 80 μmol/liter and mg HDL protein), a generator of aqueous peroxyl radicals. After oxidation, AAPH was removed by gel filtration (11).

Isolation of Native and Selectively Oxidized ApoA-I—ApoA-I and apoA-I 32 were isolated by semipreparative reverse-phase high pressure liquid chromatography (RP-HPLC), as described in detail (11, 19). Peaks corresponding to apoA-I and apoA-I 32 were collected, placed immediately on dry ice, freeze-dried, redissolved in reconstitution buffer (3 mM guanidine HCl, 10 mM Tris, 0.01% EDTA, pH 8.2), and then dialyzed extensively against PBS (two buffer changes) and 0.5 mM Tris, pH 7.4 (three changes). Protein concentrations were determined by absorbance or by the bicinchoninic acid method described above, and the phospholipid content was determined using an enzymatic kit (Roche Molecular Biochemicals).

Preparation and Characterization of Discoidal Reconstituted HDL—Discoidal reconstituted HDL (drHDL A-I) or apoA-I 32 (drHDL A-I 32) were prepared by cholate dialysis (20) using the respective purified apolipoproteins. Compositional analyses were performed on a Cobas autoanalyzer (Roche Diagnostics). The Stokes’ diameter and surface charge of the particles were determined by nondenaturing gradient gel electrophoresis (23). Dissociation was determined using an enzymatic kit (Roche Molecular Biochemicals).

Preparation of Selectively Oxidized ApoA-I—Id-free apoA-I and apoA-I 32 were isolated by semi-preparative phase reverse-phase high pressure liquid chromatography (RP-HPLC), as described in detail (11, 19). The lipid affinity of apoA-I and apoA-I 32 was determined using the 32 phospholipid/cholesterol/apoA-I (i.e. 91/12/1 and 90/11/1 for apoA-I and apoA-I 32, respectively, with a stoichiometric variation of <5% between preparations), electrophoretic mobility, and particle size. Because a change in electrophoretic migration indicated a change in the secondary and/or tertiary structure of apoA-I (21, 22), the results indicate that introduction of two Met(O) does not cause a major conformational change of lipoprotein-associated apoA-I.

Apolipoprotein Dissociation Studies—Spherical reconstituted HDL (srHDL A-I) and srHDL A-I 32 were prepared from drHDL A-I and drHDL A-I 32, respectively, and purified by ultracentrifugation (23). Dissociation of apoA-I and apoA-I 32 from srHDL was assessed by incubating the particles with CETP and the phospholipid triglyceride emulsion Intrapal (20% triglyceride, Kalvitrin AB). Details of the incubations are in the legend to Fig. 1. Changes in srHDL size were determined by nondenaturing gel electrophoresis (25). Dissociation was determined by immunoblotting with anti-apoA-I polyclonal antibodies and visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech). A Sharp JX-610 high resolution scanner was used to scan Coomassie-stained gradient gels and immunoblots.

Apolipoprotein Self-association Studies—Size-exclusion HPLC of lipid-free apoA-I and apoA-I 32 was performed using a Phenomenex Biosep SEC-S2000 column (300 × 7.8 mm, Bio-Rad), using 0.05 M phosphate, 0.5 mM NaCl, pH 7.5, as the mobile phase at 0.5 ml/min and UV detection at 214 nm. Protein size was calculated using a gel filtration standard (Bio-Rad) for comparison. For cross-linking experiments, apoA-I or apoA-I 32 (25 μg/ml PBS) was preincubated for 30 min at 37 °C, followed by further incubation for 30 min at room temperature in the presence of a 100-fold molar excess of bis-sulfosuccinimidyl substrate (Pierce) and separation of the proteins on 8% Tris-glycine SDS-polyacrylamide gel electrophoresis. Bands were visualized by silver staining.

Endoprotease Digest—ApoA-I and apoA-I 32 (50 μg in 200 μl) were digested at 37 °C for 20 h using endoprotease AspN (sequencing grade, Roche Molecular Biochemicals) in 400 μl of 100 mM ammonium bicarbonate, pH 8.0, at an enzyme-to-substrate ratio of 1:100 (w/v). The pH of each digest was then lowered to pH 2.0 with 1% trifluoroacetic acid, and the mixture was applied to a C18 RP-column (5 μm, 300 A, 4.6 × 250 mm, Separations Group, Hesperia). Peptides were eluted at 1 ml/min over 30 min using a gradient of 5–75% acetonitrile containing 0.1% trifluoroacetic acid. Peaks with major absorbance at 214 nm were collected, lyophilized, and then desalted by gel filtration (Bio-Rad) for comparison. For cross-linking experiments, apoA-I or apoA-I 32 were injected into a moving solvent (10 μl/min; acetonitrile/water (1:1, v/v)) and the mixture was applied to a C18 RP-column (5 μm, 300 A, 4.6 × 250 mm, Separations Group, Hesperia) containing 0.05% trifluoroacetic acid (50 μl) for mass determination.

Mass Spectrometry—Mass spectra were acquired using a single quadrupole mass spectrometer equipped with an electrospray ionization source (Platform, VG-Fisons Instruments). Samples (5–10 pmol, 10 μl) were injected into a moving solvent (10 μl/min; acetonitrile/water (1:1, v/v)) and the mixture was applied to a C18 RP-column (5 μm, 300 A, 4.6 × 250 mm, Separations Group, Hesperia) containing 0.05% trifluoroacetic acid (50 μl) for mass determination.

Biophysical Characterization—The secondary structure was analyzed by circular dichroism (CD). Spectra of apoA-I or apoA-I 32 (0.1 mg/ml 0.5 mM Tris, pH 7.4) and drHDL A-I or drHDL A-I 32 (0.16 mg of protein/ml of 1 mM PBS, pH 7.4) were recorded on a JASCO 720 CD spectropolarimeter at 25 °C using 1- and 0.1-mm path length cells, respectively. Acquisition parameters were: range = 184–260 nm; resolution = 0.5 nm; bandwidth = 1.0 nm; response time = 1 s; scan speed = 20 nm/min; and number of scans = 16. The mean residue ellipticity was calculated by [θ]αW = θαW × MRW/10 × c × l, where θαW is the observed raw data in millidegrees, MRW is the mean residue weight (i.e. 115.0 for apoA-I), c is the concentration, and l is the path length of the cell. The secondary structure was predicted by analyzing the CD spectra using the program “variable selection” (VARELSE). Spectra ofapoA-I 32 were also acquired in multichannel acquisition mode over the mass range 250–2000 Da in 10 s. Some spectra were recorded using a LC/MSD 1100 mass spectrometer (Hewlett Packard, Palo Alto, CA) employing similar conditions.

Automated N-terminal Sequencing—Peptides (typically 50–200 pmol) were N-terminally digested using an automated protein sequencer (model 470A, Applied Biosystems).

Kinetics of the LCAT Reaction—LCAT, prepared as described (23) and used for the experiments, esterified 340 nmol of CE/mg of LCAT/h under assay conditions. drHDL was labeled by placing [3H]cholesterol (48 C/mmol, Amersham Pharmacia Biotech) in a test tube, and the enzyme reaction was removed under N2. Ethanol buffered containing cholesterol were then added, and the mixture was incubated at 37 °C for 30 min before dilution with buffer and supplementation with β-mercaptoethanol (4 mM, final concentration) and fatty acid-free BSA (4 mg/ml). The final volume of the incubation mixtures was 135 μl, and the cholesterol concentration was varied from 0.1 to 5.0 μM. Following incubation at 37 °C for 30 min under N2, the LCAT reaction was initiated by adding 5 μl of a 1:8 (v/v) dilution of the LCAT preparation or buffer (control), and the mixture was incubated under N2 at 37 °C for 1 h. Following the reaction, radiolabeled CE were quantified by the digitonin precipitation method, exactly as described by Piran and Morin (26).

Cell Culture—Monocytes were isolated by counter-flow centrifugal elutriation (27) from buffy coats prepared from blood from normolipidemic volunteers. Cells were plated in 12-well tissue culture plates (Falcon) at a density of 1.5 × 106 cells per ml of RPMI medium containing 10% heat-inactivated human serum and left to adhere and differentiate into macrophages (hMDM) over the next 10 days. Subsequently, cells were washed and incubated in RPMI medium containing 10% heat-inactivated human serum-depleted in the presence of 100 μg/ml acetylated LDL (acLDL) for 48 h to generate lipid-laden cells, as described (28). For metabolic labeling of cellular cholesterol pools, acLDL was first labeled with [3H]cholesterol (51 C/mmol, Amersham Pharmacia Biotech) for 6 h (28), before [3H]cholesterol-acLDL was diluted in RPMI (100 μg/ml acLDL and 2 μCi/ml [3H]cholesterol), and then incubated with cells. The “labeling” medium was then replaced with RPMI medium without serum for 18 h to equilibrate labeled cholesterol among...
Characterization of Selectively Oxidized ApoA-I

Molecular mass, phospholipid content, and protein size of "lipid-free" apoA-I and apoA-I, were determined by ESI-MS, enzymatic assay and by size exclusion HPLC, respectively, as described under "Experimental Procedures." HPLC was performed using three separate preparations of apoA-I and apoA-I at 0.1, 0.5, or 1.0 mg/ml. Data shown represent means ± S.D. of triplicate or means of duplicate determinations.

| Parameter determined | ApoA-I | ApoA-I, |
|----------------------|--------|--------|
| Mass (Da)            | 28,079.1 ± 1 | 28,112.5 ± 2 |
| Phospholipid (mol/mol) | ≤0.07 | ≤0.10 |

### Results

#### Characterization of ApoA-I and ApoA-I,32

To assess HDL protein oxidation and to isolate pure native apoA-I and apoA-I,32, we adapted the HPLC method previously described (11, 12, 19). In native HDL, apoA-I and apoA-II were the major proteins and oxidized forms of apoA-I or apoA-II were not detected. Oxidation of the same HDL with AAPH (as described under "Experimental Procedures") decreased the content of apoA-I and apoA-II with the concomitant formation of new peaks. The two apoA-I-derived peaks have been shown previously to correspond to modified apoA-I containing one (referred to as apoA-I,16) and two (apoA-I,32) Met(O) instead of Met (11, 12, 19).

#### Characterization of Lipid-free apoA-I and apoA-I,32

Molecular mass, phospholipid content, and protein size of "lipid-free" apoA-I and apoA-I,32 were determined by ESI-MS, enzymatic assay and by size exclusion HPLC, respectively, as described under "Experimental Procedures." HPLC was performed using three separate preparations of apoA-I and apoA-I,32 at 0.1, 0.5, or 1.0 mg/ml. Data shown represent means ± S.D. of triplicate or means of duplicate determinations.
The ability of apoA-I to promote cellular lipid efflux, we preincubated hMDM for 48 h with medium containing Met(O)-containing oxidized peptides 4' and 7' in B as indicated by arrows.

Fig. 3, the kinetics of liposome clearance were similar, although the time required for the initial relative turbidity \( \left( \frac{A_0 - A_n}{A_n} \right) \) to decrease to half (i.e. \( \frac{1}{2} \)) was significantly shorter for apoA-I \( 32 \) than apoA-I. Thus, the rate constant, \( k_{1/2} \) (i.e. \( \frac{1}{2} \)) (25), was 0.7 ± 0.4 and 1.6 ± 0.8 min \(^{-1} \) for apoA-I and apoA-I \( 32 \), respectively (mean ± S.D., \( n = 9 ; p = 0.0003 \)). The resulting ratio of \( k_{1/2} \) for apoA-I \( 32 \) was 2.4 ± 0.5, indicating that apoA-I \( 32 \) converted multilamellar liposomes to small unilamellar vesicles two to three times faster than did apoA-I. This suggests that the introduction of the sulfoxide moieties increased the ability of lipid-free apoA-I to interact with phospholipids.

**Cellular Studies—**ApoA-I promotes the efflux of cholesterol from peripheral cells (3), and α-helices containing Met(112) are thought to be important for this process (40, 41). To test whether selective oxidation of apoA-I altered cholesterol efflux, we preincubated hMDM for 48 h with medium containing [\(^3\)H]cholesterol and [\(^3\)H]cholesterol and then overnight with medium without supplements (see “Experimental Procedures”). During such lipid loading and subsequent equilibration, cells acquired 6.6% of the radioactivity added (Table III). Our findings are consistent with Met(112) not being oxidized in apoA-I \( 32 \) (Table II).

Because the ability to associate with lipids is a feature determining the ability of apoA-I to promote cellular lipid efflux, we next examined the rate of clearance of multilamellar DMPC liposomes by lipid-free apoA-I and apoA-I \( 32 \). As can be seen in
apoA-I at all time points (p < 0.001). After 6 h, 11.3 ± 1.4% and 7.0 ± 1.2% of the cellular \(^{3}H\)cholesterol was released into the medium for apoA-I and apoA-I \(_{\text{32}}\) as described under “Experimental Procedures.” Michaelis-Menten kinetic parameters were determined in three separate experiments, each using a different preparation of drHDL. V\(_{\text{app}}\) is given in nanomoles of CE/ml of LCAT/h, and \(k_{\text{cat}}\) represents the efficiency of the enzyme-substrate reaction (\(k_{\text{cat}} = V_{\text{app}}/K_{\text{m}}\)). The correlation coefficient for the respective Lineweaver-Burk plots is represented by \(r\).

a Secondary structures (%): H, \(\alpha\)-helix; A, antiparallel \(\beta\)-sheet; P, parallel \(\beta\)-sheet; T, \(\beta\)-turn; O, “other” structures.

b Data shown represent means ± S.D. of three separate protein preparations.

c Significantly different from corresponding value for apoA-I (p \(\leq\) 0.02).

### TABLE II

Apolipoproteins isolated from RP-HPLC were digested with endoprotease AspN. The resulting peptides were then separated by RP-HPLC (see Fig. 2), collected, lyophilized, and subjected to ESI-MS as described under “Experimental Procedures.” Peptides shown are numbered according to the peak labels shown in Fig. 2.

| Peptide peak | Residues | Sequence\(^{a}\) | ApoA-I | ApoA-I \(_{\text{32}}\) |
|--------------|----------|-----------------|--------|-----------------|
| 1 | 150–156 | | 823.9 | 824.4 |
| 2 | 234–243 | | 1253.4 | 1253.8 |
| 3 | 157–167 | | 1243.4 | 1244.0 |
| 4, 4’ | 73–88\(^d\) | DNLEKETGLRQEMSK | 1907.1 | 1907.3 |
| 5 | 89–101 | EGRQKHELQEKLPGL6ER | 1531.8 | 1532.1 |
| 6 | 128–149\(^d\) | EGARQKLHELQEKLPGL6ER | 2578.9 | 2579.7 |
| 7, 7’ | 102–127\(^d\) | DDGPQQKQEMELRQKVEPLRAELQ | 3337.8 | 3338.1 |
| 8 | 25–47 | | 2223.5 | 2224.1 |
| 9 | 223–233 | | 1227.4 | 1227.8 |
| 10 | 51–72 | | 2555.8 | 2556.6 |

\(^{a}\) Amino acid sequence of the Met-containing peptides as determined by automated N-terminal sequencing (see “Experimental Procedures”). M in bold indicates Met residues identified to be modified in apoA-I \(_{\text{32}}\)-derived peptides.

### TABLE III

Analysis of the secondary structure was performed using the “variable selection” procedure described under “Experimental Procedures.”

| CD spectra were recorded on a Jasco spectrometer between 184 and 260 nm as described under “Experimental Procedures.” |
| --- |
| ApoA-I\(^b\) | 52.0 ± 2.5 | 3.6 ± 2.8 | 4.3 ± 1.1 | 16.0 ± 1.7 | 213 ± 0.5 | 98 ± 1.0 |
| ApoA-I \(_{\text{32}}\)\(^b\) | 52.6 ± 2.5 | 2.6 ± 3.0 | 1.7 ± 1.5 | 20.7 ± 1.5 | 22.0 ± 1.0 | 19 ± 3.0 |
| drHDL \(_{\text{A-I}}\) | 69.0 | 1.0 | 5.0 | 13.0 | 12.0 | 100 |
| drHDL \(_{\text{A-I}}\) \(_{\text{32}}\) | 68.0 | 1.0 | 4.0 | 13.0 | 14.0 | 100 |

### TABLE IV

Lipid-associated apoA-I and apoA-I \(_{\text{32}}\) have a comparable ability to activate LCAT

LCAT activation was examined using drHDL containing apoA-I or apoA-I \(_{\text{32}}\) as described under “Experimental Procedures.” Michaelis-Menten kinetic parameters were determined in three separate experiments, each using a different preparation of drHDL. V\(_{\text{app}}\) is given in nanomoles of CE/ml of LCAT/h, and \(k_{\text{cat}}\) represents the efficiency of the enzyme-substrate reaction (\(k_{\text{cat}} = V_{\text{app}}/K_{\text{m}}\)). The correlation coefficient for the respective Lineweaver-Burk plots is represented by \(r\).

| Apparent \(K_{\text{m}}\) (M) | \(V_{\text{max}}\) | \(k_{\text{cat}}\) | \(r\) |
| --- | --- | --- | --- |
| drHDL \(_{\text{A-I}}\) | 3.5 ± 0.9 x 10\(^{-7}\) | 29.8 ± 13.2 | 8.3 ± 2.0 x 10\(^{-7}\) | 0.965 |
| drHDL \(_{\text{A-I}}\) \(_{\text{32}}\) | 2.4 ± 0.8 x 10\(^{-7}\) | 21.1 ± 8.5 | 8.5 ± 0.8 x 10\(^{-7}\) | 0.988 |

apoA-I at all time points (p < 0.001). After 6 h, 11.3 ± 1.4% and 7.0 ± 1.2% of the cellular \(^{3}H\)cholesterol was released into the medium for apoA-I \(_{\text{32}}\) and apoA-I, respectively (Fig. 4), whereas only 2.6 ± 0.4% of the cellular radioactivity was released in controls (medium alone) (p < 0.05). All of the effluxed \(^{3}H\)radioactivity was associated with unesterified cholesterol, as determined by HPLC with UV \(_{210}\) nm and on-line radiometric detection (not shown). After 24-h incubation 2.3- and 2.8-fold more \(^{3}H\)cholesterol effluxed in the presence of apoA-I and apoA-I \(_{\text{32}}\), respectively, compared with control incubations; cholesterol efflux remained significantly greater for apoA-I \(_{\text{32}}\) than apoA-I but only by a factor of 1.2 (Fig. 4). Thus, the enhanced removal of cholesterol by lipid-free apoA-I \(_{\text{32}}\) is observed primarily during early stages of incubation of the apolipoprotein with the cells, consistent with the enhanced lipid affinity of apoA-I \(_{\text{32}}\) versus apoA-I (Fig. 3).

We next examined the concentration dependence of \(^{3}H\)cholesterol efflux from lipid-laden hMDM by lipid-free apoA-I and apoA-I \(_{\text{32}}\). \(^{3}H\)Cholesterol efflux increased dose dependently and was more pronounced for apoA-I \(_{\text{32}}\) than for apoA-I, independent of the incubation time used (Fig. 5). The enhanced efflux by apoA-I \(_{\text{32}}\) seemed more apparent at low protein concentration, suggesting that dissociation of even a small proportion of apoA-I \(_{\text{32}}\) from oxidized HDL may enhance cholesterol efflux compared with apoA-I dissociated from unoxidized HDL.

The association of phospholipids with apoA-I yields distinct lipoprotein particles that are better acceptors of cellular cholesterol than is lipid-free apoA-I (3). Consistent with this, more \(^{3}H\)cholesterol was released from hMDM to drHDL \(_{\text{A-I}}\) and drHDL \(_{\text{A-I}}\) \(_{\text{32}}\) than the corresponding lipid-free apolipoproteins in short and long-term incubations (compare Figs. 4 and 6). After 6 h of incubation, 16.1 ± 2.1% (for drHDL \(_{\text{A-I}}\)) and 16.7 ± 1.6% (for drHDL \(_{\text{A-I}}\) \(_{\text{32}}\)) of the cellular \(^{3}H\)cholesterol was present in the medium. However, drHDL \(_{\text{A-I}}\) and drHDL \(_{\text{A-I}}\) \(_{\text{32}}\) were equally effective (Fig. 6), in contrast to the lipid-free apolipoproteins. Thus, once associated with substantial amounts of phospholipids and cholesterol, apoA-I and apoA-I \(_{\text{32}}\) have similar cholesterol efflux activity.
Cholesterol loading enhances the apolipoprotein-mediated efflux of cholesterol and phospholipids, and apolipoprotein-mediated cholesterol efflux appears to be critically dependent on the initial removal or microsolvilization of membrane phospholipids (42). Fig. 7 shows that, similar to cholesterol, the efflux of [3H]phospholipids from lipid-laden hMDM was greater for lipid-free apoA-I32 than for apoA-I.

In addition to cholesterol and phospholipids, α-TOD is another important constituent of all cell membranes. We therefore assessed whether apoA-I32 is also more effective than apoA-I in removing this lipid from [14C]-labeled hMDM. Incubation of such cells with lipid-free apolipoprotein A-I resulted in a time-dependent efflux of [14C]-α-TOD. The extent of this efflux was greater for apoA-I32 than apoA-I (Fig. 8). As α-TOD and cholesterol efflux experiments were performed under comparable conditions (see “Experimental Procedures”), the relative extents of release of the two lipids were compared. Cells acquired 2.65 ± 0.02 × 10^5 cpm/mg of cell protein (mean ± S.D., n = 3) or 4.9% of the [14C]-α-TOD added. 4.4 ± 0.5% and 5.6 ± 0.6% of cell [14C]-α-TOD was released after 3 h, compared with 4.5 ± 0.5% and 6.1 ± 0.8% of cellular cholesterol for apoA-I and apoA-I32, respectively. Thus, apolipoprotein A-I removes these two lipids with comparable efficacy from hMDM, although apoA-I32 is superior in doing this compared with apoA-I. Similar to the efflux of [3H]cholesterol, lipid-associated apoA-I32 and apoA-I had comparable efflux-promoting activity for [14C]-α-tocopherol (not shown).

**DISCUSSION**

Oxidized LDL is now generally thought to contribute to atherogenesis, because it can cause lipid accumulation in macrophages and has a number of other potentially proatherogenic properties (43). There is convincing evidence for the presence of oxidized LDL in human atherosclerotic lesions (see e.g. Ref. 44), and this has recently been extended to HDL (14). Therefore, studying the properties of oxidized HDL may be relevant to atherogenesis. The present study characterized and compared known properties of in vitro selectively oxidized (i.e. apoA-I32) with native, nonoxidized apoA-I. Compared with native apoA-I, lipid-free apoA-I32 had a greater affinity for lipids and showed an enhanced ability to promote lipid efflux from lipid-laden human macrophages. This increased activity was no longer seen with lipid-associatated apoA-I32 (drHDL32). Selective oxidation was not associated with a decrease in the ability of lipid-associated apolipoprotein to activate LCAT, and compared with apoA-I, apoA-I32 dissociated more readily from srHDL in the presence of CETP. Together our findings suggest that selective and limited oxidation does not decrease, but rather increases, the potential antiatherogenic properties of lipid-free apoA-I.

HPLC-purified apoA-I32 was used throughout the present study. Compared with native apoA-I, apoA-I32 contains two Met(O) instead of Met residues (11, 19). The presence of Met(O) was confirmed indirectly by coelution from the HPLC column of apoA-I32 used in the present study with modified forms of apoA-I produced during AAPH-induced oxidation of HDL (not shown) and the increased mass (Table I). Therefore, the enhanced ability of apoA-I32 to associate with phospholipids (Fig. 3) and to cause lipid efflux from lipid-laden hMDM (Figs. 4, 7, and 8) can be ascribed to the introduction of two sulfoxide groups. Previous mass spectrometric studies of proteolytic fragments of H2O2-oxidized apoA-I indicated that the two sulfoxide

![Figure 4](image_url) Enhanced efflux of [3H]cholesterol from hMDM to lipid-free apoA-I32 versus apoA-I. Cells were isolated by elutriation, matured, and then loaded with acLDL in the presence of [3H]cholesterol and subjected to efflux in triplicates as described under “Experimental Procedures.” Cells were extracted at t = 0 min for lipid analysis by HPLC with UV 210 nm and on-line radiometric detection. Total cellular cholesterol mass and radioactivity were 153 ± 34 nmol/mg cell protein and 1.1 ± 0.1 × 10^6 cpm/mg cell protein, respectively (mean ± S.D., n = 3 experiments). After equilibration, cells were washed and subjected to efflux by incubation with RPMI only (control, ●) or apoA-I32 (▲). At times indicated, aliquots of the medium were removed and centrifuged (to remove any cells) and the radioactivity was determined in the resulting supernatant. At 6 h, cells were lysed and cell-associated radioactivity and protein were determined. Separate cultures were used for the 24-h time point, and cell lysates were prepared as for the 6-h time point. Data shown are means ± S.E. for nine cultures obtained in three separate experiments (0–6 h) and means ± S.E. for 12 cultures obtained in four separate experiments (24 h). The lines for apoA-I and apoA-I32 are statistically different (p < 0.0001, two-way ANOVA). The inset shows early time points as means ± S.D. from a single experiment representative of two, performed in triplicate (p < 0.0001 for two-way ANOVA for apoA-I versus apoA-I32).

![Figure 5](image_url) Dose-dependent efflux of [3H]cholesterol from hMDM to lipid-free apoA-I32 and apoA-I. hMDM were loaded with acLDL, and the cellular cholesterol pools were labeled as described under “Experimental Procedures.” After equilibration, cells were washed and subjected to efflux by incubation with RPMI supplemented with the indicated concentration of apoA-I (●) or apoA-I32 (▲). At time points of 1 (A), 3 (B), or 5 h (C), aliquots of the medium were removed and centrifuged, and the radioactivity was determined in the resulting supernatant. After 5 h, cells were lysed to determine cell-associated radioactivity and protein. Data shown represent means ± S.D. of a single experiment performed in triplicate. The lines for apoA-I and apoA-I32 are statistically different for A, B, and C (p < 0.0001, two-way ANOVA).
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moieties were located at Met^{112} and Met^{148} (19). In contrast, the two sulfoxide moieties in apoA-I_{32} formed during the oxidation of HDL with aqueous peroxyl radicals (this study) are located at Met^{86} and Met^{112}, as demonstrated unambiguously by amino acid sequencing and mass determination of the proteolytic fragments of apoA-I_{32} (Table II). This suggests that different Met residues may become oxidized depending on the prevailing oxidizing conditions. In preliminary experiments, we observed that H_2O_2 also caused the conversion of Met^{86} and Met^{112} to the corresponding Met(O), independent of whether lipid-free or lipid-associated apoA-I is used. Possible reasons for the apparent discrepancies between these findings and those reported earlier by others (19) are presently being investigated. In any case, the results obtained with reagent H_2O_2 contrasts those obtained with peroxyl radicals and other conditions giving rise to one electron oxidants, where lipid association, i.e. the formation of lipid hydroperoxides, is required for the selective oxidation of apoA-I, as we have demonstrated previously (11, 12). Our observations obtained with apoA-I_{32}

isolated from AAPH-oxidized, lipid hydroperoxide-containing HDL may be of general relevance, because radical oxidants generally induce the formation of CE-OOH (11, 12). Also, as CE-OOH are detected in HDL of human plasma (6) and atherosclerotic lesions (14), our findings may be relevant in vivo.

The mechanism responsible for the enhanced ability of isolated apoA-I_{32} to associate with liposomal and cellular lipids is not known at present. The fact that enhanced efflux is seen with three different classes of lipids (phospholipids, cholesterol, and α-TOH) suggests that it relates to a general characteristic of the modified apolipoprotein. Amphiphilic helices of apoA-I are responsible for its association with lipids. Because there was no significant difference in the α-helical content of apoA-I_{32} and apoA-I (Table III), an oxidation-induced change in this secondary structure per se is not likely to explain the present findings. The hydrophobic residues Met^{86} and Met^{112} are each located in the nonpolar face adjacent to the polar face at the surface of amphipathic helices 2 and 4 of apoA-I, respectively (Fig. 9) (45). Jonas et al. (45) suggested that introduction of the polar sulfoxide moiety at the boundary between the polar and nonpolar faces changes the ratio of charged to neutral surface area. Such change decreases the hydrophobic moment and hence could alter the lipid affinity of the corresponding helices of the apolipoprotein (Fig. 9) (45). Met^{148} represents a hydrophobic residue in one of the apoA-I canonical "modified" heptad repeats, and this modified heptad repeat is particularly rich in aromatic residues and has a high hydrophobic moment (45, 46). The interruption of a canonical heptad repeat has been proposed to rotate the orientation of the hydrophobic residues that follow (46). Therefore, introduction of a polar sulfoxide could reorient the direction of the hydrophobic face following Met^{112}, and this could conceivably alter the lipid affinity. In any case, the fact that apoA-I_{32} and its oxidized peptides (Fig. 2) elute before apoA-I and the respective nonoxidized peptides, respectively, on RP-HPLC, clearly indicates a substantial change in overall polarity of repeats 2 and 4 as the sole result of conversion of the Met residues into Met(O).

A striking feature of the present study is that the observed increased ability of apoA-I_{32} to cause lipid efflux from cells was lost upon reconstitution of the apolipoprotein into discoidal particles. It is well established that the conformation of apoA-I is altered upon association with phospholipids (46). These polar

FIG. 6. Efflux of [3H]cholesterol from hMDM to dHDL containing apoA-I or apoA-I_{32}. The experimental conditions were as described in the legend to Fig. 4, except that cells were washed and incubated in RPMI in the absence (control, ○) or presence of 25 μg of protein/ml of dHDL-A-I (●) or dHDL-A-I_{32} (▲). Data shown represent means ± S.D. of one experiment performed in triplicate, representative of two independent experiments.

FIG. 7. Efflux of phospholipids from hMDM to lipid-free apoA-I and apoA-I_{32}. Matured hMDMs were loaded with acLDL, washed, and labeled with [3H]methyl choline and then washed four times with RPMI and equilibrated for 1 h in RPMI only prior to efflux, as described under "Experimental Procedures." This procedure resulted in 97.8 ± 19.1 nmol of phospholipid or 9.9 ± 1.0 × 10^{6} cpm/mg of cell protein (means ± S.D.), corresponding to a specific activity of 10,0514 cpnm/mmol of phospholipid. Monolayers were incubated for up to 3 h with efflux media without (○) or with 25 μg/ml apoA-I (●) or apoA-I_{32} (▲). Extraction of phospholipids from the media and cells and determination of radioactivity were performed as described under "Experimental Procedures." Data shown are means ± S.E. for seven cultures obtained in two separate experiments. The lines for apoA-I and apoA-I_{32} are statistically different (p < 0.02, two-way ANOVA).

FIG. 8. Efflux of α-TOH from hMDM to lipid-free apoA-I or apoA-I_{32}. The experimental conditions were as described in the legend to Fig. 4, except that cells were preincubated with 0.4 μCi/ml all-rac-[14C]α-TOH during loading with acLDL. After equilibration, cellular α-TOH levels were 2.9 ± 0.5 nmol or 1.41 ± 0.01 × 10^{5} cpm/mg of cell protein (means ± S.D.; n = 3), corresponding to a specific activity of 50,130 cpnm/mmol of α-TOH. Monolayers were incubated for up to 3 h with efflux media without (○) or with 25 μg/ml apoA-I (●) or apoA-I_{32} (▲). Data shown are means ± S.E. for nine cultures obtained in three separate experiments. The lines for apoA-I and apoA-I_{32} are statistically different (p = 0.0012, two-way ANOVA).

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lipid efflux promoting activity of the apoA-I/apoA-II heterodimer, has an enhanced ability to promote cholesterol efflux from fibroblasts and to inhibit accumulation of LDL-derived cholesterol mass by fibroblasts (50). Our studies and those of Francis et al. (50) thus establish a precedent that selective oxidation of HDL’s apolipoproteins can promote rather than inhibit cellular lipid efflux. Interestingly, however, although the association with lipids increased the lipid efflux promoting activity of the apoA-I/apoA-II heterodimer (50), the difference in this activity between apoA-I and apoA-I observed here was lost upon reconstitution of the protein with lipid. This distinguishes our observations from those of Francis and coworkers, although both report a promotion of lipid efflux by oxidized apoA-I/HDL.

The physiological relevance of the present findings is presently unclear. At least two parameters need to be considered, namely, the occurrence of HDL oxidation leading to the formation of human apoA-I and the presence of lipid-free apoA-I/apoA-I in vivo. As indicated earlier, the contents of CE-OH and CE-OH associated with human lesion HDL are comparable to those in LDL (14), and CE-OH themselves or conditions that lead to their formation, including tyrosyl radicals, convert apoA-I to apoA-I (11, 12). Thus, it is conceivable that formation of Met(O) in apoA-I takes place in lesions. Indeed, preliminary results indicate that a substantial proportion of apoA-I present in HDL isolated from human lesions coelutes on HPLC with apoA-I. There is also evidence that apoA-I dissociates from HDL (4). The existence of lipid-free apoA-I in vivo has been indicated both in plasma and in interstitial fluid (51). Most apoA-I secreted by cells and pre-β-I HDL, thought to represent a major cholesterol acceptor in vivo (52), is associated with substantial amounts of phospholipids. This is not surprising, given the high affinity of apoA-I for phospholipids and the ready availability of phospholipids in vivo. However, this does not exclude the possibility that lipid-free apoA-I exists in vivo. K values for the reaction of lipid-free apoA-I with cells have been reported to be on the order of 0.2–1 × 10⁻⁷ M, i.e. only around 1/500 of its concentration in plasma (51). Therefore, only one of several hundred apoA-I molecules is necessary to be free to carry out cellular cholesterol and phospholipid efflux at Vmax (51). Indeed, upon removal of core lipids, lipid-free apoA-I may dissociate from the surface of HDL (3) and be transferred to cell surface sites to generate pre-β-I HDL with cellular lipid at a high rate (51). This reaction has been suggested to be relevant to physiological conditions (51). Our finding, i.e. apoA-I dissociates more readily from reconstituted HDL than apoA-I in the presence of CETP (Fig. 1), supports the notion that this process is relevant for apoA-I present on oxidized HDL in vivo. If a similar process were to occur in the intima of a developing lesion where HDL oxidation takes place (14), lipid-free apoA-I may be present and contribute to cholesterol efflux. Future studies are needed to investigate this possibility.

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Oxidation of Methionine Residues to Methionine Sulfoxides Does Not Decrease Potential Antiatherogenic Properties of Apolipoprotein A-I

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