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Oxidation of methionine residues affects the structure and stability of apolipoprotein A-I in reconstituted high density lipoprotein particles

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Abstract

To determine the effect of oxidative damage to lipid-bound apolipoprotein A-I (apo A-I) on its structure and stability that might be related to previously observed functional disorders of oxidized apo A-I in high density lipoproteins (HDL), we prepared homogeneous reconstituted HDL (rHDL) particles containing unoxidized apo A-I and its commonly occurring oxidized form (Met-112, 148 bis-sulfoxide). The size of the obtained discoidal rHDL particles ranged from 9.0 to 10.0 nm and did not depend upon the content of the oxidized protein. Using circular dichroism methods, no change in the secondary structure of lipid-bound oxidized apo A-I was found. Isothermal and thermal denaturation experiments showed a significant destabilization of the oxidized protein to denaturation by guanidine hydrochloride or heat. This effect was observed with and without co-reconstituted apolipoprotein A-II. Limited tryptic digestion indicated that the central region of oxidatively damaged apo A-I becomes exposed to proteolysis in the rHDL particles. Implications of these data for apolipoprotein function are discussed.

Keywords: Apolipoprotein A-I; High density lipoproteins; Reconstituted; Methionine oxidation; Protein structure; Thermal unfolding; Protein denaturation

Nomenclature

apo A-I	apolipoprotein A-I
apo A-I_{unox}	unoxidized apo A-I as found in serum
apo A-I_{ox}	oxidized apo A-I contained in serum or obtained from unoxidized apo A-I using hydrogen peroxide
apo A-II	apolipoprotein A-II

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apo A-II_{unox}	unoxidized apo A-II contained in serum
CD	circular dichroism
DHPC	1,2-diheptanoyl-*sn*-glycero-3-phosphocholine
DMS	dimethylsuberimidate
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
EM	electron microscopy
GdnHCl	guanidine hydrochloride
GGE	gradient gel electrophoresis
HDL	high density lipoproteins
rHDL	reconstituted HDL
HPLC	high performance liquid chromatography
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline consisting of 0.02 M phosphate, 0.15 M sodium chloride, pH 7.2
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
rHDL-1	rHDL containing only apo A-I_{unox}
rHDL-2	rHDL containing only apo A-I_{ox}
rHDL-3	rHDL containing apo A-I_{unox} and apo A-I_{ox} with a molar ratio of 1:1
rHDL-4	rHDL containing apo A-I_{unox}, apo A-I_{ox} and apo A-II_{unox} with a molar ratio of 3:3:1
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline containing 0.01 M Tris–HCl, 0.14 M NaCl, 0.25 mM EDTA-Na_2, 0.15 mM sodium azide, pH 7.4 or 8.0

1. Introduction

The risk for an atherosclerotic event is strongly and inversely related to levels of high density lipoproteins (HDL; Kwiterovich, 1998). Recently, there is growing evidence that oxidative modifications of HDL can reduce their ability to function in reverse cholesterol transport (Panzenboeck et al., 1997; Gesquiere et al., 1997; Rifici and Khachadurian, 1996; Nagano et al., 1991), one of the mechanisms through which HDL are believed to protect against the development of atherosclerosis. Oxidized HDL also lose their capacity to protect low density lipoproteins (LDL) against oxidation (Parthasarathy et al., 1990), and these oxidized LDL derivatives are known to initiate and promote the atherosclerotic process, leading to the development of coronary heart disease (Holvoet and Collen, 1998; Berliner et al., 1995). In addition, it has also been reported that oxidized HDL can directly inhibit endothelium-dependent vasoreactivity (Chin et al., 1992) and exert a cytotoxic effect on macrophages (Hurtado et al., 1996). The structural changes induced in HDL by oxidation and the potential consequences of oxidative modification on the protective actions of HDL in vivo have been reviewed (Francis, 2000). Nevertheless, it still remains unclear which constituents of oxidized HDL result in functional disorders of these lipoproteins, and by what mechanism.

Apolipoprotein A-I (apo A-I), the major protein constituent of plasma HDL, plays an important role in lipid transport and metabolism. It promotes cholesterol efflux (Rothblat et al., 1992; Chambenoit et al., 2001), acts as a cofactor for the lecithin–cholesterol acyltransferase (LCAT) enzyme (Jonas, 1991; Frank et al., 1998) and as a ligand that binds to the class B scavenger receptor SR-BI (Acton et al., 1996). Apo A-I shows endotoxin neutralization (Massamiri et al., 1997) and also protects against the cytotoxic effects of oxidized LDL (Suc et al., 1997). Two of the three methionine residues (Met-112 and 148)

in apo A-I are susceptible to oxidation, and readily form methionine sulfoxides after treatment with some chemical oxidizing agents in vitro (Von Eckardstein et al., 1991; Anantharamaiah et al., 1988; Sigalov and Stern, 1998). Oxidation of Met-112 and 148 has been observed also as a common oxidative modification of HDL protein moiety in vivo (Von Eckardstein et al., 1991; Anantharamaiah et al., 1988; Sigalov and Stern, 1998). In addition, there is evidence for direct reduction of cholesteryl ester hydroperoxides and phosphatidylcholine hydroperoxides (Garner et al., 1998a,b; Mashima et al., 1998) by apo A-I, with conversion of Met-112 and 148 to methionine sulfoxides. Both the oxidized and unoxidized forms of apo A-I occur in vivo, and in fresh plasma samples the ratio of oxidized to unoxidized apo A-I exhibits considerable variability between individuals (Von Eckardstein et al., 1991; Sigalov and Stern, 1998). The oxidized form of apo A-I is also contained in advanced human atherosclerotic plaques (Garner et al., 1998b). Methionine oxidation can alter the ability of the apolipoprotein to associate with lipid (Von Eckardstein et al., 1991; Anantharamaiah et al., 1988; Sigalov and Stern, 1998), and oxidized lipid-free apolipoprotein exhibits structural alterations as detected by circular dichroism (CD) spectroscopy and proteolytic susceptibility (Anantharamaiah et al., 1988; Sigalov and Stern, 1998; Roberts et al., 1997). Recently, we have shown that cellular cholesterol efflux mediated by HDL depends upon the ratio of unoxidized/oxidized apo A-I, and that the difference in the extent of efflux can reach 30% (Sigalov et al., 1997). Met-112 and 148 of apo A-I are located within the central region of the molecule which is involved in the activation of the cholesterol esterifying enzyme (LCAT; Frank et al., 1998; Sorci-Thomas et al., 1998; McManus et al., 2000), and there is also some evidence that oxidation of these residues can lead to impaired ability of reconstituted HDL (rHDL) particles to activate LCAT (Jonas et al., 1993). Considering these functionally important consequences of oxidative damage to apo A-I, the structural features and stability of oxidized protein in HDL particles need to be elucidated more fully.

The objective of this study was to analyze structural features of rHDL particles containing oxidized apo A-I, in the presence or absence of apolipoprotein A-II (apo A-II), in order to examine the effects of oxidative damage in the context of the HDL particle. We found that oxidation of Met-112 and 148 resulted in a significant destabilization of the lipid-bound protein to denaturation by guanidine hydrochloride (GdnHCl) or to thermal denaturation. We also used limited proteolytic digestion to show that the conformation or accessibility of the functionally important central region of apo A-I in rHDL particles is substantially altered by methionine oxidation.

2. Materials and methods

2.1. Chemicals, enzymes and lipids

Dimethylsuberimidate (DMS), sodium cholate and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Chymotrypsin (TLCK treated, Type VII, EC 3.4.21.1, 55 U/mg) was purchased from Sigma Chemical Company. Trypsin (Sequencing grade, EC 3.4.21.4) was from Boehringer Mannheim GmbH (Mannheim, Germany). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2. Apolipoproteins

Apo A-I and apo A-II were isolated and purified from human serum as previously described (Sigalov et al., 1991). Unoxidized and oxidized apo A-I proteins as well as unoxidized apo A-II were isolated from the initial preparation by preparative high performance liquid chromatography (HPLC) as described (Sigalov and Stern, 1998). Protein composition of peaks in the HPLC profile was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% acrylamide) using the standard Laemmli system (Laemmli, 1970) as well as by analytical HPLC as previously described (Sigalov and Stern, 1998). The percentage of apo A-I_{ox} in eight apo A-I preparations obtained from pooled human sera (about 200 individual specimens in

each pool) using the same isolation technology has varied from 3 to 25%, providing further indirect evidence of considerable interindividual variability of the ratio oxidized/unoxidized apo A-I (Von Eckardstein et al., 1991). Both naturally occurring apo A-I_{ox}, and apo A-I_{ox} obtained from unoxidized protein by oxidation with hydrogen peroxide (see below), contain two methionine sulfoxide groups at Met-112 and 148 (Von Eckardstein et al., 1991), so in the text we use the term 'oxidized' to designate both these species. We use the terms 'unoxidized' to describe naturally occurring unoxidized apo A-I as found in serum (apo A-I_{unox}). Mass spectra of unoxidized and oxidized apo A-I, measured using a Voyager Elite STR mass spectrometer from PerSeptive Biosystems (Cambridge, MA), indicate that apo A-I_{ox} was 32 mass units greater than the initial apo A-I_{unox} as previously observed (Sigalov and Stern, 1998).

2.3. Chemical oxidation of apo A-I

Apo A-I_{ox} containing sulfoxides at Met-112 and 148, was obtained from the unoxidized protein by incubation at room temperature for 10 min in 3 M GdnHCl, pH 7.4 containing 0.9% hydrogen peroxide, and then was purified by preparative HPLC as previously described (Sigalov and Stern, 1998).

2.4. Preparation of reconstituted lipoproteins

The rHDL complexes were prepared by the sodium cholate dialysis method essentially as described (Sorci-Thomas et al., 1998) with an initial molar ratio of sodium cholate–POPC–cholesterol–apoA-I of 150:80:4:1. This method has been used previously to prepare rHDL with 2 or 3 apo A-I per particle and 9–11 nm diameter (Sorci-Thomas et al., 1998; Durbin and Jonas, 1997; Toledo et al., 2000; Davidson et al., 1995). Our characterization of rHDL particle size and composition (see below) was similar. All preparations were done in TBS, pH 7.4. Briefly, 65 μl of POPC in chloroform (~ 2.0 mg) and 5.0 μl of cholesterol in chloroform–ethanol, 1:1 (~ 50 μg) were mixed in a 1.5 ml polypropylene tube, dried in a stream of argon, and placed under vacuum for 30 min. Then, 100 μl of sodium cholate in TBS, pH 7.4, (about 2.1 mg) was added and vortexed. After incubation for 30 min at room temperature, 280 μl of a solution containing ~ 0.9 mg apo A-I_{unox}, A-I_{ox}, or a mixture of apo A-I_{unox} and A-I_{ox} (molar ratio of 1:1), or instead 1.1 mg of a mixture of apo A-I_{unox}–A-I_{ox}–A-II_{unox} (molar ratio of 1.5:1.5:1) were added. After additional incubation for 90 min at room temperature, cholate was removed by dialysis against 1 l of TBS, pH 7.4, for 1.5 h at room temperature and then against 3 l of the same buffer for 16 h at 4 °C. The obtained rHDL were then isolated on a calibrated Superdex 200 HR (10×300 mm^2) gel filtration column (Pharmacia) eluted at 0.4 ml/min with TBS, pH 7.4. Molecular weight of the rHDL particles was calculated from their retention times relative to gel filtration proteins standards supplied by Bio-Rad: thyroglobulin (670 000 Da), bovine gamma globulin (158 000 Da), chicken ovalbumin (44 000 Da), equine myoglobin (17 000 Da), and vitamin B-12 (1350 Da). The isolated rHDL samples were filtered through a 0.22 μm using Spin-X centrifuge tubes (Corning Costar Corporation, Cambridge, MA) and stored at 4 °C.

2.5. Characterization of reconstituted lipoproteins

Protein concentrations in the rHDL particles were measured using the Lowry method as modified by Markwell et al. (1978). Final protein compositions were determined in the prepared rHDL particles by analytical HPLC essentially as described above for lipid-free apolipoproteins, except that a solid GdnHCl was added to the analyzed rHDL samples to a final concentration of 6 M. Total cholesterol was determined enzymatically using a Boehringer-Mannheim kit and the manufacturer's suggested procedure. Phospholipids were determined by phosphorus assay (Van Veldhoven and Mannaerts, 1987). The number of apo A-I molecules per particle was determined by cross-linking performed by addition of one part DMS solution, 10 mg/ml in 1.0 M triethanolamine, pH 9.7, to ten parts rHDL solution, incubation for 2 h at room temperature (Swaney, 1986) and by determination of extent of

oligomer formation using SDS-PAGE (12.5% acrylamide). The gels were stained for protein with Coomassie Blue R250 and scanned with an Hewlett Packard ScanJet 3P. The obtained images were analyzed using a NIH Image 1.61 program (National Institutes of Health, Bethesda, MD) and a Scion Image 3b program (Scion Corporation, Frederick, MD).

The sizes and size distributions of the rHDL particles were estimated by both electron microscopy (EM) and nondenaturing gradient gel electrophoresis (GGE). The rHDL complexes (at a concentration of about 0.3 mg of protein/ml) were extensively dialyzed against 5 mM ammonium bicarbonate, mixed with the same volume of 2% phosphotungstate, pH 7.4, and were examined using a Phillips EM410 electron microscope on carbon-coated Formvar grids. Microphotographs were photographed at an instrument magnification of 63 000 and 110 000, and mean particle dimensions of 50 particles were determined from each negative. Nondenaturing GGE was performed on precast 4–20% gradient gels (Bio-Rad, Hercules, CA). Gel scanning and image analysis were performed as described above. Stokes' diameter and molecular weight of the rHDL particles were calculated from their mobility relative to proteins standards supplied by Pharmacia: thyroglobulin (17.0 nm, 669 000 Da), ferritin (12.2 nm, 440 000 Da), catalase (10.4 nm, 232 000 Da), lactate dehydrogenase (8.2 nm, 140 000 Da), and bovine serum albumin (7.1 nm, 67 000 Da). The protein and lipid compositions together with the apo A-I oligomer size determined by cross-linking and the particle molecular weight were used to estimate the particle molar composition.

2.6. Circular dichroism

CD spectra were collected on solutions of 3.6 μM (0.1 mg/ml) lipid-free apo A-I proteins and of 1.8 μM (0.05 mg of protein/ml) rHDL particles in TBS, pH 7.4 with a 1 mm path-length quartz cuvette using an AVIV 62A DS spectropolarimeter (AVIV, Lakewood, NJ). Data were collected at 25 °C every nanometer from 190 to 260 nm with 1.0 s averaging per point and a 1 nm bandwidth. Spectra of at least six scans were signal averaged and baseline corrected by subtracting an averaged buffer spectrum. The spectra were normalized to molar residue ellipticity using a mean residue weight of 115.2 and 113.6 Da for human apo A-I and A-II, respectively. For comparison with previous results we calculated an apparent fractional percent α-helix content from the molar ellipticities at 222 nm by the method of Chen et al. ($[\phi]_{222} = -30\,300$, $f_H = 2340$, where f_H is the fraction of α-helical structure; Chen et al., 1972).

In experiments on temperature-induced unfolding the data were collected on solutions of 3.6 μM (0.1 mg/ml) lipid-free apo A-I and 1.8 μM (0.05 mg of protein/ml) rHDL particles in TBS, pH 7.4, with a 1 mm path-length quartz cuvette at 222 nm every 2–5 °C from 25 to 95 °C with 20.0 s averaging per point and a 1 nm bandwidth. T_m values for the broad transitions were estimated by curve-fitting using a seven-parameter equation (Zarutskie et al., 1999).

2.7. Isothermal denaturation studies

The effect of GdnHCl concentration on the structure of lipid-free and lipid-bound apo A-I was monitored by fluorescence emission of Trp as described previously (Tricerri et al., 1998). These measurements were made in a FluoroMax-2 spectrofluorimeter (SPEX Industries, Inc., Edison, NY) at 25 °C in 4×4 mm^2 cuvette. Emission spectra were taken by exciting at 285 nm with a resolution of 2 nm and by measuring the emission with a resolution of 4 nm. The molar GdnHCl concentrations (C) were determined from the solution refractive index (n) using the relationship: $C = 60.87n - 81.16$, as described (Kielley and Harrington, 1960). Aliquots of each lipid-free apo A-I protein (0.1 mg/ml) or rHDL sample (0.05 mg of protein/ml) were incubated with from 0 to 3.0 M GdnHCl (lipid-free proteins) or from 0 to 6.0 M GdnHCl (rHDL complexes) in TBS, pH 7.4, for 72 h at 4 °C. As shown previously, equilibrium is established during the 72-h incubation period (Sparks et al., 1992; Wald et al., 1990). Then, the Trp fluorescence spectra were taken as above described and the ratio of the fluorescence intensity at 353 nm to that at 333 nm was used to quantify the spectral shifts. Denaturation curves

were analyzed essentially as described (Sparks et al., 1992). Briefly, the following relationship between the free energy of denaturation (ΔG_D) and the GdnHCl activity (a) was used to estimate apo A-I conformational stability:

$$\Delta G_D^0 = \Delta G_D + \Delta n \times RT\ln(1 + Ka) \quad (1)$$

where $\Delta G_D{}^0$ is the standard free energy of denaturation (at zero denaturant concentration), R is gas constant (1.98 cal/degree mol), T is temperature (298 K), K is average association constant of GdnHCl and protein (0.6 M^{-1}), and Δn is the difference in the moles of the denaturant bound by the protein in the native and denatured states. The mean GdnHCl ionic activities (a) were calculated by the equation (Pace and Vanderburg, 1979):

$$a = 0.6761M + 0.1468M^2 + 0.02475M^3 + 0.001318M^4 \quad (2)$$

where M is the molar GdnHCl concentration. The equilibrium constants, K_D, were calculated from the 353 nm/333 nm fluorescence intensity ratios using the formula:

$$K_D = ([F]_N - [F])/([F] - [F]_D) \quad (3)$$

where $[F]$ is the observed 353 nm/333 nm fluorescence intensity ratio at a given GdnHCl activity and $[F]_N$ and $[F]_D$ are the 353 nm/333 nm fluorescence intensity ratios for the native and fully denatured forms of apo A-I measured in the absence or presence of 3 M GdnHCl (lipid-free proteins) or 6.0 M GdnHCl (rHDL complexes). Linear regression analysis was used to solve Eq. (1) and to determine $\Delta G_D{}^0$ and Δn. Stability of lipid-free and lipid-bound apo A-I was also determined by plotting the 353 nm/333 nm fluorescence intensity ratio vs the molar GdnHCl concentration and expressed as the concentration of the denaturant that reduced this ratio by 50% ($D_{1/2}$).

2.8. Limited proteolytic digestion with chymotrypsin and trypsin

Samples of lipid-free apo A-I or rHDL complexes (0.4 mg of protein/ml) in PBS (pH 7.2) were treated with chymotrypsin at 37 °C for 75 min using a protein–protease ratio (w/w) of 1000:1 (for lipid free apo A-I), or for 240 min using a ratio of 6:1 (for rHDL). Trypsin digestions were performed similarly, except in TBS (pH 7.4) for 120 min using a protein–protease ratio of 200:1 (w/w). Digested samples were analyzed by SDS-PAGE (15% acrylamide) and HPLC.

3. Results

3.1. Characterization of the size and composition of rHDL particles

We evaluated the ability of various apolipoprotein species, alone or in combination, to bind lipid and reconstitute into HDL-like particles. Cholate-solubilized POPC–cholesterol micelles were mixed with either A-I_{unox}, apo A-I_{ox}, a 1:1 mixture of unoxidized and oxidized species, or 1:1 mixture containing also a one-third molar equivalent of apo A-II, and detergent was removed by dialysis to form rHDL complexes (rHDL-1 through rHDL-4). In each case, characteristic discoidal particles with mean diameter 9–10 nm were formed (Table 1). As examined by EM, all obtained rHDL complexes had a similar ability to arrange in stacks (data not shown). No significant differences between different rHDL particles were found in their apparent particle size or shape. Protein and lipid molar composition of the reconstituted particles is indicated in Table 1. Chemical cross-linking of the protein molecules showed that rHDL-1, rHDL-2, and rHDL-3 complexes each contain three molecules of apo A-I per particle. The rHDL-4 complex after cross-linking exhibits a protein band at about 100 kDa that corresponds to three molecules of apo A-I and one molecule of apo A-II. These data are very similar to those calculated from the ratio between the total rHDL molecular weights determined by nondenaturing GGE (and by gel filtration chromatography) and the minimal molecular weights obtained from the rHDL molar composition.

Thus, oxidation of two of three methionine residues (Met-112 and 148) in apo A-I molecule does not lead to any significant differences between prepared rHDL complexes in their lipid and protein compositions as well as in their size or shape.

Table 1
Properties of rHDL particles

Complex	Particle composition POPC–Chol–Prot (mol:mol:mol)[a]	Protein composition (molar ratio)[b]	Particle diameter (EM; nm)[c]	Particle diameter (GGE; nm)[d]	α-Helix (%)[e]
apo A-I_{unox}					62 (4)
apo A-I_{ox}					42 (4)
rHDL-1	180 (16):5 (1):3	Only A-I_{unox}	10.2 (1.0)	9.4 (0.7)	78 (5)
rHDL-2	180 (14):3 (1):3	Only A-I_{ox}	10.3 (1.0)	9.6 (0.5)	81 (5)
rHDL-3	190 (19):5 (1):3	A-I_{unox}–A-I_{ox} (1:1)[f]	9.9 (1.0)	9.6 (0.6)	82 (5)
rHDL-4	150 (22):4 (1):3.5	A-I_{unox}–A-I_{ox}:A-II (3:3:1)[f]	10.3 (1.0)	9.6 (0.8)	78 (5)

[a] Composition determined as described (Section 2). Mean and S.D. (in parentheses) of three different preparations are given. The number of apolipoprotein molecules per particle was obtained by cross-linking with DMS (Swaney, 1986) and protein analysis by SDS-PAGE.
[b] Determined by the reversed-phase HPLC as described (Section 2).
[c] Mean and S.D. of 50 particles determined from negative staining EM.
[d] Mean and S.D. of three different preparations determined from nondenaturing GGE using reference globular proteins.
[e] Mean and S.D. of three different preparations determined from molar ellipticities at 222 nm.
[f] Average compositions that may reflect subpopulations with different molar composition.

3.2. Secondary structure of apo A-I in rHDL

As shown in Fig. 1, the CD spectra of all four rHDL preparations exhibit pronounced double minima at 208 and 222 nm and a maximum about 192–195 nm, which are typical of proteins with a high degree of α-helical structure. In contrast, the spectrum of the lipid-free apo A-I_{ox} was considerably less intense than that of the unoxidized protein form (data not shown), indicating a reduction of apo A-I α-helical content upon methionine oxidation, consistent with our previous report (Sigalov and Stern, 1998). After lipid binding, the fractional helical content increased substantially from ~60% (apo A-I_{unox}) and ~40% (apo A-I_{ox}) to about 80% in each of the rHDL complexes (Table 1). The low α-helical content value estimated for the lipid-free apo A-I_{ox} is likely due to a partially unfolded structure of the oxidized protein. No significant differences were observed between the CD spectra of the four rHDL complexes (Fig. 1). The very high estimated helical content of apo A-I on all rHDL complexes is supported by the recently published crystal structure of a lipid-free apo Δ(1–43) A-I fragment (Borhani et al., 1997), reported to be in the lipid-bound form (Rogers et al., 1997), which shows the protein in a ring-shaped continuous helix. The observed high helical content of apo A-I together with the observation of three protein molecules per rHDL particle are consistent with

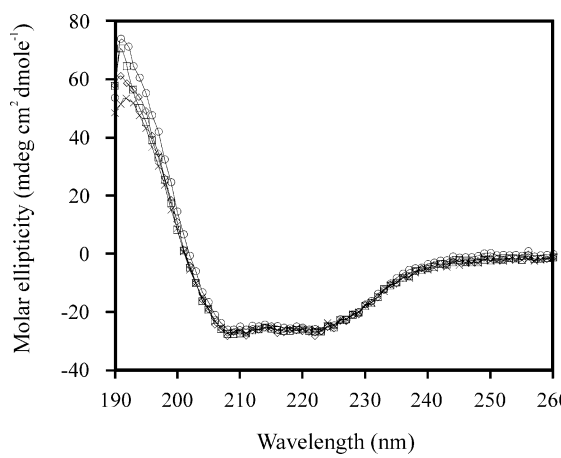

Fig. 1. Circular dichroic spectra of apo A-I on different rHDL particles. The spectra of lipid-associated apo A-I—(○) rHDL-1, (□) rHDL-2, (◇) rHDL-3 and (×) rHDL-4—were recorded from 190 to 260 nm at 25 °C using a 1 mm pathlength quartz cuvette on AVIV 62A DS spectropolarimeter. Samples were analyzed at a protein concentration of 1.8 μM (0.05 mg of protein/ml) rHDL particles in TBS, pH 7.4, and spectra of at least six scans were signal averaged and baseline corrected by subtracting an averaged buffer spectrum.

Table 2
Thermodynamic parameters of lipid-free and lipid-associated apo A-I

Complex	$D_{1/2}$ (M GdnHCl)[a]	$\Delta G_D{}^0$ (kcal/mol of apolipoprotein)[b]	Δn (mol GdnHCl/mol of apolipoprotein)[c]	T_m (°C)[d]
apo A-I_{unox}	1.0 (0.1)	4.7 (0.3)	29 (4)	64 (3)
apo A-I_{ox}	0.4 (0.1)[f]	1.4 (0.1)[f]	15 (3)[f]	–
rHDL-1	4.1 (0.3)[f]	3.7 (0.3)[f]	7 (1)[f]	82 (3)[f]
rHDL-2	3.3 (0.3)[f,g]	3.1 (0.3)[f,g]	7 (1)[f]	71 (3)[f,g]
rHDL-3	3.8 (0.3)[f]	3.0 (0.3)[f,g]	6 (1)[f]	74 (3)[f,g]
rHDL-4[e]	3.7 (0.3)[f]	3.2 (0.3)[f,g]	7 (1)[f]	74 (3)[f,g]

[a] Midpoints of GdnHCl denaturation. Mean and S.D. (in parentheses) of three different measurements are given.
[b] Free energy of denaturation at zero GdnHCl concentration (± S.D.).
[c] The number of the GdnHCl moles bound during denaturation (± S.D.).
[d] Midpoints of thermal denaturation (± S.D.).
[e] Mean number of apolipoprotein moles calculated from the data of reversed-phase HPLC.
[f] $P<0.05$, comparison vs apo A-I_{unox}.
[g] $P<0.05$, comparison vs rHDL-1.

the molecular belt model LL5/5 proposed for discoidal HDL particles (Segrest et al., 1999).

Thus, the secondary structure of oxidized apo A-I molecules in rHDL complexes remains similar to that of the unoxidized protein in contrast to the substantially altered secondary structure of the oxidatively damaged lipid-free protein.

3.3. Isothermal denaturation of lipid-free and lipid-associated apo A-I

The denaturation of apo A-I has been shown previously to be a reversible process with a midpoint of denaturation ($D_{1/2}$) of $\approx$1.0 M GdnHCl for lipid-free apo A-I and $\approx$3–4 M GdnHCl for lipid-bound apo A-I (Jonas et al., 1993; Sorci-Thomas et al., 1998; Sparks et al., 1992; Wald et al., 1990). We obtained similar values for lipid-free apo A-I_{unox} (0.9–1.0 M) and for all four rHDL complexes (3.3–4.1 M; Table 2 and Fig. 2). In contrast, the $D_{1/2}$ for lipid-free apo A-I_{ox} (0.4 M) was substantially lower than for lipid-free apo A-I_{unox}, indicating significantly reduced structural stability. The apo A-I_{ox} denaturation curve is broadened and shifted to lower denaturant concentration, such that even in the absence of denaturant apo A-I_{ox} is partially unfolded. Significantly reduced stability of the oxidized protein is observed also in the reconstituted lipoprotein particle. The rHDL-1 complex, carrying only apo A-I_{unox}, exhibits half-maximal denaturation at 4.1 M (Table 2), as compared to the other apo A-I_{ox}-carrying rHDL complexes, which exhibit half-

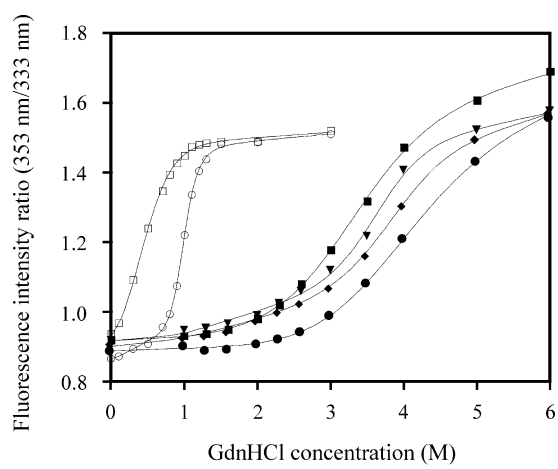

Fig. 2. Denaturation by GdnHCl of lipid-free apo A-I and rHDL complexes. Aliquots of (○) unoxidized and (□) oxidized lipid-free apo A-I proteins or prepared (●) rHDL-1, (■) rHDL-2, (◆) rHDL-3 and (▼) rHDL-4 complexes were incubated at 4 °C with 0–6 M GdnHCl in 10 mM TBS, pH 7.4 for 72 h. Fluorescence intensities at 353 and 333 nm were measured at 25 °C using a 4×4 mm^2 cuvette on a FluoroMax-2 spectrofluorimeter with sample protein concentrations between 0.05 and 0.1 mg of protein/ml. Emission spectra were taken by exciting at 285 nm with a resolution of 2 nm and by measuring the emission with a resolution of 4 nm. The ratio of fluorescence intensity at 353 nm to that at 333 nm is plotted against the GdnHCl molar concentration.

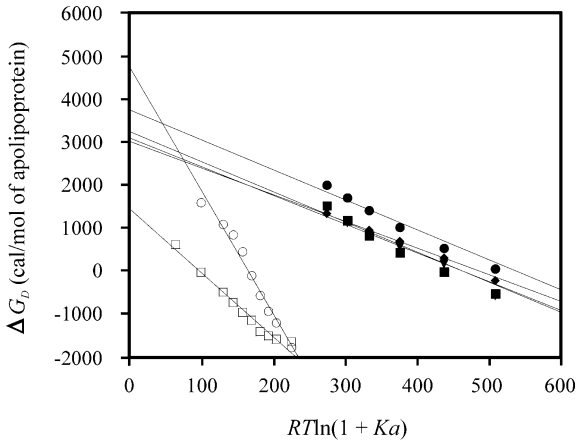

Fig. 3. The free energy of denaturation of lipid-free apo A-I and rHDL complexes as a function of the ionic activity of GdnHCl. The free energy values of denaturation (calculated from change in the ratio of fluorescence intensity at 353 nm to that at 333 nm, Fig. 2) are fitted using linear regression equations and plotted against $RT\ln(1 + Ka)$ for (○) unoxidized and (□) oxidized lipid-free apo A-I proteins or prepared (●) rHDL-1, (■) rHDL-2, (◆) rHDL-3 and (▼) rHDL-4 complexes. The standard free energy of denaturation ($\Delta G_D{}^0$) and the number of the bound GdnHCl moles (Δn) were computed from the intercepts on the vertical axis and the slopes of the regression lines, respectively, as described (Sparks et al., 1992).

maximal denaturation at lower concentrations of GdnHCl (3.3–3.8 M). The free energy of denaturation and the number of molecules of GdnHCl bound during denaturation (Δn) were calculated for each apo A-I and rHDL species (Table 2), by regression analysis of free energies plotted against $RT\ln(1 + Ka)$ for the transition region (Fig. 3; Section 2 and Sparks et al., 1992). The estimated $\Delta G_D{}^0$ value for lipid-free A-I_{unox} (4.7 kcal/mol) is close to those derived from previous thermal (Gursky and Atkinson, 1996) and GdnHCl isothermal (Edelstein and Scanu, 1980; Reijngoud and Phillips, 1982) denaturation experiments. The conformational stability of lipid-free apo A-I is substantially decreased by oxidation, and exhibits $\Delta G_D{}^0 \sim 1.4$ kcal/mol. Notably, the difference in the lipid-bound state is much smaller but still remains significant (3.1 and 3.7 kcal/mol for rHDL-2 and rHDL-1 complexes, respectively). No substantial differences in conformational stability of apo A-I were observed among the apo A-I_{ox}-carrying rHDL complexes, including rHDL-4 which also contains apo A-II. The number of denaturant molecules bound during the unfolding transition was also affected by apolipoprotein oxidation. Denaturation of lipid-free apo A-I_{unox} involves the binding of twice as much GdnHCl (Δn) as does the denaturation of the oxidized protein, consistent with partial unfolding of apo A-$I_{ox.}$ All of the rHDL denaturations are characterized by similar values of Δn, which are several fold smaller than for the lipid-free protein (Table 2). The large differences between Δn for lipid-free and lipid-bound apolipoproteins underscores the observation (Sparks et al., 1992) that isothermal $D_{1/2}$ values may not directly reflect the protein conformational stability but also different interactions with the denaturant. Thus, the oxidative damage to apo A-I in rHDL complexes can induce a significant destabilization of the protein to denaturation by GdnHCl.

3.4. Thermal denaturation of lipid-free and lipid-associated apo A-I

Thermal denaturation curves for rHDL complexes were obtained using CD spectroscopy (Fig. 4). The stability of apo A-I to thermal denatura-

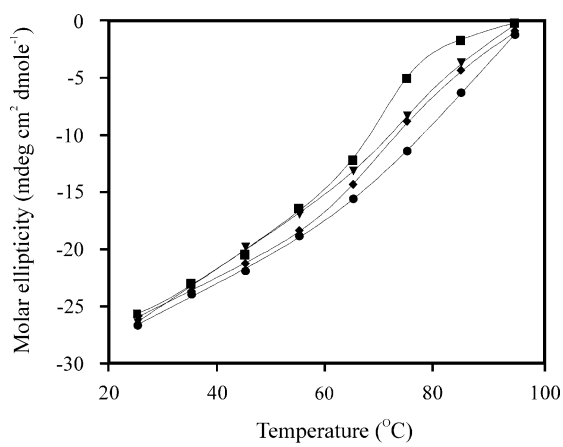

Fig. 4. Temperature-induced unfolding of rHDL complexes. The CD data were collected at 222 nm every 2–5 °C from 25 to 95 °C on solutions of 1.8 μM (0.05 mg of protein/ml) prepared (●) rHDL-1, (■) rHDL-2, (◆) rHDL-3 and (▼) rHDL-4 complexes (B) in TBS, pH 7.4, with a 1 mm pathlength quartz cuvette on AVIV 62A DS spectropolarimeter.

tion is enhanced in lipid-bound state, and rHDL complexes exhibit denaturation midpoints in the range of 71–82 °C, with T_m value for rHDL-1 particles significantly higher than those for rHDL-2, rHDL-3 and rHDL-4 complexes (Table 2). For comparison, the melting range for lipid-free apo A-I_{unox} is about 40–80 °C (data not shown), with a midpoint (T_m) at 64 °C (Table 2), which is similar to prior reports (Sparks et al., 1992; Gursky and Atkinson, 1996) and to our previous data (Sigalov and Stern, 1998). In contrast, lipid-free apo A-I_{ox} does not exhibit any cooperative unfolding transition (data not shown), suggestive of a largely unfolded structure, as it has been also found in our previous studies (Sigalov and Stern, 1998). Thus, the thermal denaturation data also indicate that oxidation of two methionine residues in apo A-I molecule results in a significant destabilization of this protein in rHDL complexes.

3.5. Limited proteolytic digestion

To study the effect of oxidative damage on the proteolytic stability of apo A-I in rHDL particles, and to obtain some information on the region(s) of apo A-I that may be more exposed to proteolysis after oxidation of two methionine residues, limited proteolytic digestion of rHDL complexes was performed (Fig. 5). After 2 h digestion there are no digestion fragments visible for rHDL-1 (Lane 1) while three major protein fragments with molecular weights of about 22, 18 and 14 kDa are observed for all other rHDL complexes (Lanes 2–4). Densitometric scans indicated that about 50, 25 and 25% of the total apo A-I had been digested in the rHDL-2, rHDL-3 and rHDL-4 complexes, respectively. HPLC analysis provided similar results (not shown). Thus, the amount of the digested apo A-I protein is strongly related to the apo A-I_{ox} content in rHDL particles. Furthermore, about 40% of the digested apo A-I appears as a 14 kDa fragment approximately half the size of the 28 kDa intact apo A-I, indicating a primary cleavage site in the central region of the molecule. An interesting analogy is observed between these tryptic digestion data and recently published results (Durbin and Jonas, 1997) on the tryptic digestion of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine–rHDL complexes containing only apo A-I or both apo A-I and A-II. These authors have shown that in the presence of apo A-II a newly accessible tryptic site was observed near the middle of the apo A-I molecule, with about 40% of the digested apo A-I from these hybrid particles observed as a 14 kDa fragment (Durbin and Jonas, 1997). Limited proteolysis of the rHDL complexes with chymotrypsin did not reveal any significant differences in their digestion patterns.

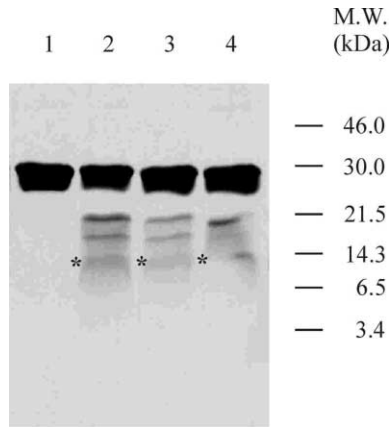

Fig. 5. SDS-PAGE analysis of the trypsin digestion products of rHDL complexes. The rHDL complexes were treated by trypsin at room temperature for 2 h. (Lane 1) rHDL-1 containing only apo A-I_{unox}. (Lane 2) rHDL-2 containing only apo A-I_{ox}. (Lane 3) rHDL-3 containing apo A-I_{unox} and apo A-I_{ox} with a molar ratio of 1:1. (Lane 4) rHDL-4 containing apo A-I_{unox}, apo A-I_{ox} and apo A-II_{unox} with a molar ratio of 3:3:1. The positions and molecular weights of the protein standards are indicated on the right of the gel. The 14 kDa fragment is identified with an asterisk.

Thus, the oxidative damage to the apo A-I molecule leads to the appearance of an accessible tryptic site in the central region of the lipid-bound apo A-I molecule.

4. Discussion

In this study we have investigated the functionally important effects of oxidative damage to apo A-I in well-defined reconstituted discoidal lipoprotein particles. The naturally occurring oxidation of two of three methionine residues (Met-112 and 148) in apo A-I resulted in significant destabi-

lization of this protein in discoidal rHDL particles. The structure of the central region of oxidized lipid-bound apo A-I was substantially altered due to this oxidative damage and became accessible to proteolysis. Because these experiments were performed with discoidal rHDL particles including those that contain apo A-II, these findings are especially relevant to native nascent HDL, but they also should apply to mature HDL (Brouillette and Anantharamaiah, 1995) including those containing only apo A-I (LpAI) or both apo A-I and apo A-II (LpAI/AII).

There is now growing evidence that not only apo A-I in HDL particles but also lipid-free apo A-I plays an important role in promoting cellular cholesterol efflux—the first step in reverse cholesterol transport (Yokoyama, 1998; Asztalos et al., 1997; Gillotte et al., 1999). In addition, the apo A-I in plasma has been also shown to alternate between association with HDL and a lipid-free state (Liang et al., 1995). Moreover, since methionine sulfoxides in apo A-I molecule can be formed in vivo by direct reduction of naturally occurring cholesteryl ester hydroperoxides (Garner et al., 1998a,b) and/or phosphatidylcholine hydroperoxides (Mashima et al., 1998), as well as by oxidation by HOCl formed in vivo by the myeloperoxidase/H_2O_2/Cl^- system (Bergt et al., 1999, 2000), it is apparent that the functionally important alterations of the structure and stability of apo A-I in HDL particles caused by an oxidative damage may be of a physiological importance. The current study shows that oxidation of two methionine residues in lipid-bound apo A-I significantly decreases its thermodynamic stability (Table 2; Figs. 2 and 3). Due to destabilization the oxidized apo A-I might become more readily stripped from HDL. Therefore, this destabilized protein could provide a pool of lipid-free oxidized apo A-I with potentially altered functional properties that in turn could also form new nascent HDL particles containing increased amount of oxidized protein.

We observed increased susceptibility of oxidized apo A-I to proteolysis even after incorporation into rHDL particles. Recently, it has been shown that a naturally occurring apo A-I variant (apo A-I_{FIN}, L159A) observed in individuals with isolated familial hypoalphalipoproteinemia, is prone to proteolysis in vivo, and that there is a direct correlation between the extent of apo A-I_{FIN} proteolysis and the decrease in apo A-I and HDL-cholesterol in vivo (McManus et al., 2001). Mild trypsinization of HDL is also known to effectively abolish apolipoprotein-mediated cholesterol efflux in vitro (Mendez and Oram, 1997). Thus, the oxidation-induced changes in the accessibility of the apo A-I central region to proteolysis also may interfere with HDL-mediated efflux in vivo.

Several studies have highlighted the importance of the central region of apo A-I molecule in lipid binding (residues 100–121; McManus et al., 2000), in the efflux of intracellular and plasma membrane cholesterol (residues 140–150; Sviridov et al., 1996), in LCAT activation and HDL maturation (residues 143–164; Sorci-Thomas et al., 1998; Frank et al., 1998; McManus et al., 2000). Furthermore, a naturally occurring apo A-I variant (apo A-$I_{Seattle}$) with a deletion in the central region (Δ146–160) is associated in vivo with an atherogenic lipoprotein profile characterized by a deficiency in HDL related to an impaired ability of apoA-$I_{Seattle}$ to react with LCAT (Lindholm et al., 1998). These studies suggest that if oxidation of Met-112 and 148 residues in the lipid-bound apo A-I molecule leads to conformational changes, it might result in functional disorders of HDL. Indeed, the oxidation-induced alterations of apo A-I in rHDL observed in this study could explain our previously observed results that efflux of cellular cholesterol is decreased progressively as the ratio of oxidized to unoxidized apo A-I in HDL particles is increased (Sigalov et al., 1997). In line with these findings it has been recently reported that HOCl modification of rHDL particles led to decreased cholesterol efflux from macrophages at physiologically occurring HOCl concentrations, and that the degree of cholesterol efflux decreasing was dependent on the degree of HDL oxidative modification (Bergt et al., 1999). In addition, the observed decreased reactivity of rHDL particles containing oxidized apo A-I towards LCAT (Jonas et al., 1993) and the inhibition of LCAT in the reaction with rHDL particles in the presence of phosphatidylcholine hydroper-

oxides (Davit-Spraul et al., 1999), could be also linked to the oxidation-induced structural alterations.

In summary, the HDL particles containing oxidized apo A-I might have physiological role and clinical significance different from undamaged ones. A possible atherogenic role of oxidized HDL partially resulted from some loss of their cholesterol effluxing capacity and from inactivation of LCAT is now widely discussed (Bonnefont-Rousselot et al., 1999). Considering the results presented herein, the atherogenic role of oxidized HDL may be linked not only with the HDL lipid peroxidation but also and perhaps, even to a greater extent, with the oxidative damage to the major protein constituent of HDL—apo A-I. Thus, there might be a possible diagnostic value in determination of the content of oxidized apo A-I, or the ratio of oxidized to unoxidized apo A-I, in human serum.

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References

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., Krieger, M., 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271, 518–520.

Anantharamaiah, G.M., Hughes, T.A., Iqbal, M., Gawish, A., Neame, P.J., Medley, M.F., Segrest, J.P., 1988. Effect of oxidation on the properties of apolipoproteins A-I and A-II. J. Lipid Res. 29, 309–318.

Asztalos, B., Zhang, W., Roheim, P.S., Wong, L., 1997. Role of free apolipoprotein A-I in cholesterol efflux. Formation of pre-alpha-migrating high-density lipoprotein particles. Arterioscler. Thromb. Vasc. Biol. 17, 1630–1636.

Bergt, C., Oettl, K., Keller, W., Andreae, F., Leis, H.J., Malle, E., Sattler, W., 2000. Reagent or myeloperoxidase-generated hypochlorite affects discrete regions in lipid-free and lipid-associated human apolipoprotein A-I. Biochem. J. 346 (Pt. 2), 345–354 I prone.

Bergt, C., Reicher, H., Malle, E., Sattler, W., 1999. Hypochlorite modification of high density lipoprotein: effects on cholesterol efflux from J774 macrophages. Fed. Eur. Biochem. Soc. Lett. 452, 295–300.

Berliner, J.A., Navab, M., Fogelman, A.M., Frank, J.S., Demer, L.L., Edwards, P.A., Watson, A.D., Lusis, A.J., 1995. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 91, 2488–2496.

Bonnefont-Rousselot, D., Therond, P., Beaudeux, J.L., Peynet, J., Legrand, A., Delattre, J., 1999. High density lipoproteins (HDL) and the oxidative hypothesis of atherosclerosis. Clin. Chem. Lab. Med. 37, 939–948.

Borhani, D.W., Rogers, D.P., Engler, J.A., Brouillette, C.G., 1997. Crystal structure of truncated human apolipoprotein A-I suggests a lipid-bound conformation. Proc. Natl. Acad. Sci. USA 94, 12291–12296.

Brouillette, C.G., Anantharamaiah, G.M., 1995. Structural models of human apolipoprotein A-I. Biochim. Biophys. Acta 1256, 103–129.

Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., Chimini, G., 2001. Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. J. Biol. Chem. 276, 9955–9960.

Chen, Y.H., Yang, J.T., Martinez, H.M., 1972. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. Biochemistry 11, 4120–4131.

Chin, J.H., Azhar, S., Hoffman, B.B., 1992. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. J. Clin. Invest. 89, 10–18.

Davidson, W.S., Gillotte, K.L., Lund-Katz, S., Johnson, W.J., Rothblat, G.H., Phillips, M.C., 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. J. Biol. Chem. 270, 5882–5890.

Davit-Spraul, A., Therond, P., Leroy, A., Palmade-Rieunier, F., Rousset, C., Moatti, N., Legrand, A., 1999. Inhibition of lecithin cholesterol acyltransferase by phosphatidylcholine hydroperoxides. Fed. Eur. Biochem. Soc. Lett. 447, 106–110.

Durbin, D.M., Jonas, A., 1997. The effect of apolipoprotein A-II on the structure and function of apolipoprotein A-I in a homogeneous reconstituted high density lipoprotein particle. J. Biol. Chem. 272, 31333–31339.

Edelstein, C., Scanu, A.M., 1980. Effect of guanidine hydrochloride on the hydrodynamic and thermodynamic properties of human apolipoprotein A-I in solution. J. Biol. Chem. 255, 5747–5754.

Francis, G.A., 2000. High density lipoprotein oxidation: in vitro susceptibility and potential in vivo consequences. Biochim. Biophys. Acta 1483, 217–235.

Frank, P.G., N'Guyen, D., Franklin, V., Neville, T., Desforges, M., Rassart, E., Sparks, D.L., Marcel, Y.L., 1998. Importance of central alpha-helices of human apolipoprotein A-I in the maturation of high-density lipoproteins. Biochemistry 37, 13902–13909.

Garner, B., Witting, P.K., Waldeck, A.R., Christison, J.K., Raftery, M., Stocker, R., 1998a. Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. J. Biol. Chem. 273, 6080–6087.

Garner, B., Waldeck, A.R., Witting, P.K., Rye, K., Stocker, R., 1998b. Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. J. Biol. Chem. 273, 6088–6095.

Gesquiere, L., Loreau, N., Blache, D., 1997. Impaired cellular cholesterol efflux by oxysterol-enriched high density lipoproteins. Free Radic. Biol. Med. 23, 541–547.

Gillotte, K.L., Zaiou, M., Lund-Katz, S., Anantharamaiah, G.M., Holvoet, P., Dhoest, A., Palgunachari, M.N., Segrest, J.P., Weisgraber, K.H., Rothblat, G.H., Phillips, M.C., 1999. Apolipoprotein-mediated plasma membrane microsolubilization. Role of lipid affinity and membrane penetration in the efflux of cellular cholesterol and phospholipid. J. Biol. Chem. 274, 2021–2028.

Gursky, O., Atkinson, D., 1996. Thermal unfolding of human high-density apolipoprotein A-1: implications for a lipid-free molten globular state. Proc. Natl. Acad. Sci. USA 93, 2991–2995.

Holvoet, P., Collen, D., 1998. Oxidation of low density lipoproteins in the pathogenesis of atherosclerosis. Atherosclerosis 137 (Suppl.), S33–S38.

Hurtado, I., Fiol, C., Gracia, V., Caldu, P., 1996. In vitro oxidised HDL exerts a cytotoxic effect on macrophages. Atherosclerosis 125, 39–46.

Jonas, A., 1991. Lecithin–cholesterol acyltransferase in the metabolism of high-density lipoproteins. Biochim. Biophys. Acta 1084, 205–220.

Jonas, A., von Eckardstein, A., Churgay, L., Mantulin, W.W., Assmann, G., 1993. Structural and functional properties of natural and chemical variants of apolipoprotein A-I. Biochim. Biophys. Acta 1166, 202–210.

Kielley, W.W., Harrington, W.F., 1960. A model for the myosin molecule. Biochim. Biophys. Acta 41, 401–421.

Kwiterovich, P.O. Jr., 1998. The antiatherogenic role of high-density lipoprotein cholesterol. Am. J. Cardiol. 82 (9A), 13Q–21Q.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680–685.

Liang, H.Q., Rye, K.A., Barter, P.J., 1995. Cycling of apolipoprotein A-I between lipid-associated and lipid-free pools. Biochim. Biophys. Acta 1257, 31–37.

Lindholm, E.M., Bielicki, J.K., Curtiss, L.K., Rubin, E.M., Forte, T.M., 1998. Deletion of amino acids Glu146→Arg160 in human apolipoprotein A-I (ApoA-$I_{Seattle}$) alters lecithin:cholesterol acyltransferase activity and recruitment of cell phospholipid. Biochemistry 37, 4863–4868.

Markwell, M.A., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87, 206–210.

Mashima, R., Yamamoto, Y., Yoshimura, S., 1998. Reduction of phosphatidylcholine hydroperoxide by apolipoprotein A-I: purification of the hydroperoxide-reducing proteins from human blood plasma. J. Lipid Res. 39, 1133–1140.

Massamiri, T., Tobias, P.S., Curtiss, L.K., 1997. Structural determinants for the interaction of lipopolysaccharide binding protein with purified high density lipoproteins: role of apolipoprotein A-I. J. Lipid Res. 38, 516–525.

McManus, D.C., Scott, B.R., Frank, P.G., Franklin, V., Schultz, J.R., Marcel, Y.L., 2000. Distinct central amphipathic alpha-helices in apolipoprotein A-I contribute to the in vivo maturation of high density lipoprotein by either activating lecithin–cholesterol acyltransferase or binding lipids. J. Biol. Chem. 275, 5043–5051.

McManus, D.C., Scott, B.R., Franklin, V., Sparks, D.L., Marcel, Y.L., 2001. Proteolytic degradation and impaired secretion of an apolipoprotein A-I mutant associated with dominantly inherited hypoalphalipoproteinemia. J. Biol. Chem. 276, 21292–21302.

Mendez, A.J., Oram, J.F., 1997. Limited proteolysis of high density lipoprotein abolishes its interaction with cell-surface binding sites that promote cholesterol efflux. Biochim. Biophys. Acta Lipids Lipid Metab. 1346, 285–299.

Nagano, Y., Arai, H., Kita, T., 1991. High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proc. Natl. Acad. Sci. USA 88, 6457–6461.

Pace, C.N., Vanderburg, K.E., 1979. Determining globular protein stability: guanidine hydrochloride denaturation of myoglobin. Biochemistry 18, 288–292.

Panzenboeck, U., Raitmayer, S., Reicher, H., Lindner, H., Glatter, O., Malle, E., Sattler, W., 1997. Effects of reagent and enzymatically generated hypochlorite on physicochemical and metabolic properties of high density lipoproteins. J. Biol. Chem. 272, 29711–29720.

Parthasarathy, S., Barnett, J., Fong, L.G., 1990. High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. Biochim. Biophys. Acta 1044, 275–283.

Reijngoud, D.-J., Phillips, M.C., 1982. Mechanism of dissociation of human apolipoprotein A-I from complexes with dimyristoylphosphatidylcholine as studied by guanidine hydrochloride denaturation. Biochemistry 21, 2969–2976.

Rifici, V.A., Khachadurian, A.K., 1996. Oxidation of high density lipoproteins: characterization and effects on cholesterol efflux from J774 macrophages. Biochim. Biophys. Acta 1299, 87–94.

Roberts, L.M., Ray, M.J., Shin, T.-W., Hayden, E., Reader, M.M., Brouillette, C.G., 1997. Structural analysis of

apolipoprotein A-I: limited proteolysis of methionine-reduced and -oxidized lipid-free and lipid-bound human apo A-I. Biochemistry 36, 7615–7624.

Rogers, D.P., Brouillette, C.G., Engler, J.A., Tendian, S.W., Roberts, L., Mishra, V.K., Anantharamaiah, G.M., Lund-Katz, S., Phillips, M.C., Ray, M.J., 1997. Truncation of the amino terminus of human apolipoprotein A-I substantially alters only the lipid-free conformation. Biochemistry 36, 288–300.

Rothblat, G.H., Mahlberg, F.H., Johnson, W.J., Phillips, M.C., 1992. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. J. Lipid Res. 33, 1091–1097.

Segrest, J.P., Jones, M.K., Klon, A.E., Sheldahl, C.J., Hellinger, M., De Loof, H., Harvey, S.C., 1999. A detailed molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein. J. Biol. Chem. 274, 31755–31758.

Sigalov, A., Alexandrovich, O., Strizevskaya, E., 1991. Large-scale isolation and purification of human apolipoproteins A-I and A-II. J. Chromatogr. 537, 464–468.

Sigalov, A.B., Petrichenko, I.E., Kolpakova, G.V., 1997. The ratio of non-oxidized/oxidized forms of apolipoprotein A-I can affect cholesterol efflux from human skin fibroblasts mediated by high density lipoprotein. Eur. J. Clin. Chem. Clin. Biochem. 35, 395–396.

Sigalov, A.B., Stern, L.J., 1998. Enzymatic repair of oxidative damage to human apolipoprotein A-I. Fed. Eur. Biochem. Soc. Lett. 433, 196–200.

Sorci-Thomas, M.G., Curtiss, L., Parks, J.S., Thomas, J., Kearns, M.W., Landrum, M., 1998. The hydrophobic face orientation of apolipoprotein A-I amphipathic helix domain 143–164 regulates lecithin:cholesterol acyltransferase activation. J. Biol. Chem. 273, 11776–11782.

Sparks, D.L., Lund-Katz, S., Phillips, M.C., 1992. The charge and structural stability of apolipoprotein A-I in discoidal and spherical recombinant high density lipoprotein particles. J. Biol. Chem. 267, 25839–25847.

Suc, I., Escargueil-Blanc, I., Troly, M., Salvayre, R., Negre-Salvayre, A., 1997. HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL. Arterioscler. Thromb. Vasc. Biol. 17, 2158–2166.

Sviridov, D., Pyle, L., Fidge, N., 1996. Identification of a sequence of apolipoprotein A-I associated with the efflux of intracellular cholesterol to human serum and apolipoprotein A-I containing particles. Biochemistry 35, 189–196.

Swaney, J.B., 1986. Use of cross-linking reagents to study lipoprotein structure. Meth. Enzymol. 128, 613–626.

Toledo, J.D., Tricerri, M.A., Corsico, B., Garda, H.A., 2000. Cholesterol flux between lipid vesicles and apolipoprotein AI discs of variable size and composition. Arch. Biochem. Biophys. 380, 63–70.

Tricerri, A., Corsico, B., Toledo, J.D., Garda, H.A., Brenner, R.R., 1998. Conformation of apolipoprotein AI in reconstituted lipoprotein particles and particle–membrane interaction: effect of cholesterol. Biochim. Biophys. Acta 1391, 67–78.

Van Veldhoven, P.P., Mannaerts, G.P., 1987. Inorganic and organic phosphate measurements in the nanomolar range. Anal. Biochem. 161, 45–48.

Von Eckardstein, A., Walter, M., Holz, H., Benninghoven, A., Assmann, G., 1991. Site-specific methionine sulfoxide formation is the structural basis of chromatographic heterogeneity of apolipoproteins A-I, C-II, and C-III. J. Lipid Res. 32, 1465–1476.

Wald, J.H., Krul, E.S., Jonas, A., 1990. Structure of apolipoprotein A-I in three homogeneous, reconstituted high density lipoprotein particles. J. Biol. Chem. 265, 20037–20043.

Yokoyama, S., 1998. Apolipoprotein-mediated cellular cholesterol efflux. Biochim. Biophys. Acta 1392, 1–15.

Zarutskie, J.A., Sato, A.K., Rushe, M.M., Chan, I.C., Lomakin, A., Benedek, G.B., Stern, L.J., 1999. A conformational change in the human major histocompatibility complex protein HLA-DR1 induced by peptide binding. Biochemistry 38, 5878–5887.