Effects of Protein Oxidation on the Structure and Stability of Model Discoidal High-Density Lipoproteins[†]

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ABSTRACT: High-density lipoproteins (HDLs) prevent atherosclerosis by removing cholesterol from macrophages and by providing antioxidants for low-density lipoproteins. Oxidation of HDLs affects their functions via the complex mechanisms that involve multiple protein and lipid modifications. To differentiate between the roles of oxidative modifications in HDL proteins and lipids, we analyzed the effects of selective protein oxidation by hypochlorite (HOCl) on the structure, stability, and remodeling of discoidal HDLs reconstituted from human apolipoproteins (A-I, A-II, or C-I) and phosphatidylcholines. Gel electrophoresis and electron microscopy revealed that, at ambient temperatures, protein oxidation in discoidal complexes promotes their remodeling into larger and smaller particles. Thermal denaturation monitored by far-UV circular dichroism and light scattering in melting and kinetic experiments shows that protein oxidation destabilizes discoidal lipoproteins and accelerates protein unfolding, dissociation, and lipoprotein fusion. This is likely due to the reduced affinity of the protein for lipid resulting from oxidation of Met and aromatic residues in the lipid-binding faces of amphipathic α-helices and to apolipoprotein cross-linking into dimers and trimers on the particle surface. We conclude that protein oxidation destabilizes HDL disk assembly and accelerates its remodeling and fusion. This result, which is not limited to model discoidal but also extends to plasma spherical HDL, helps explain the complex effects of oxidation on plasma lipoproteins.

High-density lipoproteins (HDLs)[1] remove excess cholesterol from the body and thereby prevent atherosclerosis. Plasma HDLs form a heterogeneous population of particles differing in their protein and lipid composition, shape, size, and functional properties. Nascent discoidal HDLs are comprised of a cholesterol-containing phospholipid bilayer and apolipoproteins wrapped around the particle perimeter in a belt-like amphipathic α-helical conformation (*1*). Mature spherical HDLs contain proteins, phospholipids, and free (unesterified) cholesterol (FC) in their surface and apolar lipids (mainly cholesterol esters) in the core (*2*).

Apolipoprotein A-I (apoA-I, 28 kDa) is a key structural and functional component of HDL that comprises ~70% of the total HDL protein content. Plasma levels of HDL and apoA-I are inversely correlated to the risk of atherosclerosis (*3*). HDLs protect against atherosclerosis by removing excess cholesterol from arterial macrophages and other peripheral cells via the reverse cholesterol transport (RCT) pathway (*2*). Other cardioprotective mechanisms have been proposed to involve the antioxidant and anti-inflammatory properties of HDL and its constituent proteins, including apoA-I (*4–10*). ApoA-I also plays key roles at various stages of RCT. It promotes efflux of cell cholesterol and phospholipids to HDL via the interactions with ATP-binding cassette transporter A1 (*11*). It provides a cofactor for lecithin:cholesterol acyltransferase (LCAT) that esterifies cholesterol and converts nascent discoidal to mature spherical HDL (*2*). It binds to the hepatic scavenger receptor and mediates selective uptake of HDL cholesterol esters by the liver (*12*).

Oxidative stress is thought to be among the key initiating factors in atherogenesis. According to the oxidation modification hypothesis, retention of oxidized low-density lipoproteins (oxLDLs) in the arterial wall triggers a cascade of pro-atherogenic events culminating in foam cell formation, a hallmark of early atherosclerosis (*13*). In contrast to oxLDL, functional consequences of HDL oxidation remain unclear (*14–16*). Some studies report that oxidation impairs the ability of HDL to promote cellular cholesterol efflux (*14, 17*), while others suggest that oxidized HDL may actually enhance cholesterol efflux (*18, 19*). This apparent controversy may result from the differences in the oxidizing agents and their products as well as in the extent of oxidation used in different studies. In fact, Pirillo and colleagues reported that mild oxidation by copper improves the ability of HDL to accept cell cholesterol, while extensive oxidation inhibits this ability (*20*). Our recent study is consistent with this report and shows

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[1] Abbreviations: RCT, reverse cholesterol transport; HDL, high-density lipoprotein; rHDL, reconstituted HDL; LDL, low-density lipoprotein; oxLDL, oxidized LDL; (oxA-I):DMPC, complexes reconstituted using oxidized apoA-I; ox(A-I:DMPC), complexes that were reconstituted using intact apoA-I and then oxidized; apo, apolipoprotein; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; FC, free cholesterol; MetO, methionine sulfoxide (S=O double bond); PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; T-jump, temperature jump; EM, electron microscopy.

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that mild oxidation by copper or hypochlorite destabilizes mature human HDL and accelerates heat-induced protein dissociation and lipoprotein fusion, while extensive oxidation inhibits these reactions (*21*). To clearly understand these oxidation-induced changes in functions and remodeling of plasma HDL, one has to differentiate between the complex effects of multiple protein and lipid modifications that occur concomitantly at various stages of oxidation. To do so, we use discoidal reconstituted HDLs (rHDLs) of controlled composition, such as complexes of human apoA-I with dimyristoylphosphatidylcholine (DMPC), as simple experimental models for dissecting the effects of protein oxidative modifications by hypochlorite on HDL structure and stability.

Hypochlorite is a product of myeloperoxidase (MPO), a heme protein expressed by macrophages in atherosclerotic lesions that is thought to be an important in vivo mediator of lipoprotein oxidation (*15, 22, 23*). The active form of MPO and the proteins modified by MPO-generated HOCl have been found in human atherosclerotic lesions, supporting the notion that MPO mediates HDL oxidation in the artery wall (*24, 25*). Physiological concentrations of HOCl have not been accurately determined, although some in vivo-based reports suggest that, at sites of acute inflammation, the molar ratio of oxidant to HDL may be as high as 30:1 (ref *26* and references therein). At these and lower HOCl levels and near-neutral pH, oxidative modifications in HDL are mainly limited to apolipoproteins (*26, 27*). We use this property of HOCl to test the effects of apolipoprotein oxidation on the structure and stability of model discoidal HDL.

Dose-dependent HOCl-induced modifications in the major HDL proteins, apoA-I and apoA-II, have been well-documented by using limited proteolysis, mass spectrometry, and liquid chromatography; the general consensus is that oxidation of Met and Cys occurs first, followed by modifications of Lys and aromatic residues and, eventually, protein cross-linking into homo- or heterodimers and trimers (*24, 26–30*). Specifically, limited proteolysis and mass spectrometry studies of apoA-I in solution or on HDL suggest that, at a 5:1 HOCl:protein molar ratio, Met 86, Met 112, and Met 148 are converted to methionine sulfoxides (MetO); at a 10:1 ratio, Phe 37 and Phe 71 are the primary targets of oxidation, and at a 25:1 ratio, Tyr 192 is chlorinated (*25, 28*). In addition, progressive oxidation of Trp residues in apoA-I has been detected by fluorescence (*31*). Oxidative modifications in these and other hydrophobic residues, which are located in the apolar faces of the amphipathic apolipoprotein α-helices, are expected to impair the lipid binding affinity of apoA-I and affect its functions. In fact, oxidation of Met 112 and Met 148 to MetO reportedly impairs the ability of apoA-I to activate LCAT (*29*); it also leads to partial unfolding and destabilization of the α-helices in lipid-free and in lipid-bound apoA-I and apoA-II, as well as to reduced protein affinity for lipid that is attributed to the reduced hydrophobicity of the Met-containing apolar helical faces (*29, 30, 32*). Oxidative cross-linking of apoA-I and apoA-II in HDL, which occurs via the tyrosyl radicals, reportedly alters apoA-I conformation, leading to increased solvent exposure of the protein α-helices (*33, 34*) and to the enhanced ability of HDL to promote efflux of cholesterol from fibroblasts (*35*). Here, we analyze the effects of these oxidative modifications on thermal stability and remodeling of discoidal rHDLs.

Earlier, we established a kinetic mechanism of lipoprotein stabilization and showed that thermal or chemical denaturation of model discoidal and plasma spherical HDLs leads to protein unfolding and dissociation and to particle fusion (*36–40*). Importantly, this denaturation reaction resembles metabolic HDL remodeling and fusion by plasma factors such as LCAT (*41, 42*). Thus, our in vitro studies of HDL denaturation provide a useful experimental approach for understanding lipoprotein remodeling in vivo. Here, we use this approach to analyze the effects of protein oxidation on the structure, stability, and remodeling of discoidal rHDLs. The results complement our recent oxidation studies of plasma spherical HDL (*21*) and help dissect the effects of protein and lipid oxidation on HDL remodeling and functions.

MATERIALS AND METHODS

Materials. Diethylenetriaminepentaacetic acid (DTPA, >98% pure) and sodium hypochlorite (NaOCl, <5% chlorine) were from Across Organic. Lipids, including DMPC, dipalmitoyl-PC (DPPC), palmitoyloleoyl-PC (POPC), and FC, were from Avanti Polar Lipids (≥95% pure). All chemicals were of the highest-purity analytical grade.

Protein Preparation and Characterization. ApoA-I and apoA-II (an S−S-linked dimer of two 77-amino acid monomers and a second major HDL protein) were isolated and purified from human plasma HDL and refolded as described (*38*). Human apoC-I (a minor HDL protein of 57 amino acids that is a structural and functional prototype of larger exchangeable apolipoproteins) was custom-synthesized and purified to ≥97% purity at 21st Century Biochemicals as described (*36*).

Lipoprotein Reconstitution. DMPC complexes with apolipoproteins were obtained by adding a protein stock solution to a DMPC suspension (protein:lipid ratio 1:4 w/w) followed by overnight incubation at 24 °C. Discoidal complexes of apoA-I with DPPC, POPC, or PC and FC were prepared by sodium cholate dialysis (*43*). For DPPC and POPC complexes, PC:A-I:cholate molar ratios of 80:1:80 were used; complexes containing FC were prepared using PC:FC:A-I initial molar ratios of 100:50:1. Lipoprotein formation was confirmed by negative staining electron microscopy (EM) and nondenaturing polyacrylamide gel electrophoresis (PAGE).

Protein Oxidation by Hypochlorite. To oxidize apoA-I to various stages, 1 mg/mL protein (lipid-free or in complex with lipid) in standard buffer [10 mM sodium phosphate and 100 μM DTPA (pH 7.5)] was incubated for 12 h at 24 °C with NaOCl using oxidant:protein molar ratios from 5:1 to 50:1 (*44*). The concentration of HOCl was determined spectrophotometrically, using an extinction coefficient ϵ_{290} of 350 M^{-1} cm^{-1} (*45*). The reaction was terminated with 2.5 mM Met; the samples were dialyzed overnight at 4 °C against standard buffer prior to use. Complexes reconstituted from intact apoA-I and DMPC that were oxidized to various stages by HOCl [termed ox(A-I:DMPC) throughout this work] were compared with similar complexes that were reconstituted from oxidized apoA-I and DMPC [termed (oxA-I):DMPC]. The absence of oxidative lipid modifications in ox(A-I:DMPC) was confirmed by thin-layer chromatography as described (*46*).

Gel Electrophoresis. SDS−PAGE [12% (w/v) gel] was performed under nonreducing conditions in a Mini Protean II chamber (Bio-Rad) for 2 h at 120 V; 10 μg protein aliquots were applied per lane. The gel was stained with Imperial protein stain (Pierce).

Nondenaturing PAGE was performed using self-made 4–15% gradient gels. Protein samples (10 μg) were run for 2 h at 120 V. The gel was stained with Imperial protein stain. The particle diameters were determined from comparison with the high-molecular weight standard (GE Healthcare).

Circular Dichroism Spectroscopy. Far-UV CD and 90° light scattering data were collected using an AVIV 215 spectropolarimeter with thermoelectric temperature control as described (*38, 47*). In temperature-jump (T-jump) experiments, the sample temperature was rapidly increased at time zero from 25 °C to a constant value ranging from 75 to 95 °C and the time course of the protein unfolding was monitored at 222 nm. The helical content was calculated from the measured molar residue ellipticity [Θ_{222}] as described (*36*).

Electron Microscopy. Protein–lipid complexes were visualized by negative staining EM under low-dose conditions in a CM12 transmission electron microscope (Philips Electron Optics) as described (*36, 38*). The disk diameters were determined from these micrographs using 200–350 particles.

Fluorescence Spectroscopy. Trp emission spectra were recorded at 25 °C with a Fluoromax-2 spectrofluorimeter from apoA-I solutions of 50 μg/mL concentration in standard buffer. The excitation wavelength was 280 nm; the spectra were recorded from 310 to 500 nm with 5 nm excitation and emission slit widths.

DMPC Clearance. DMPC multilamellar vesicles were prepared by thin film evaporation; a DMPC solution in methanol and chloroform (2:1, v/v) was evaporated under nitrogen, and the lipid film was hydrated with the standard buffer. Clearance of DMPC multilamellar vesicles by normal or oxidized apoA-I was monitored at 24 °C by turbidity at 325 nm using a Varian Cary-300 UV−vis absorption spectrophotometer equipped with thermoelectric temperature control as described (*40*).

All experiments in this study were repeated three to five times to ensure reproducibility.

RESULTS

First, we analyzed A-I:DMPC complexes at various stages of oxidation corresponding to oxidant:protein ratios from 5:1 to 50:1. Protein cross-linking was assessed by SDS−PAGE (Figure 1). Intact apoA-I ran as a single band corresponding to monomeric protein (~28 kDa, lane 0 in Figure 1). Oxidation of lipid-free (lanes 1′ and 2′) and DMPC-bound apoA-I (lanes 1 and 2) produced two additional bands near 50 and 100 kDa reflecting oxidation-induced dimer and trimer formation that was confirmed by SDS−PAGE (*44*). Increasing oxidant:protein ratios to 25:1 and beyond resulted in near-complete conversion of monomeric to cross-linked protein on discoidal particles (lanes 3 and 4); lipid-free apoA-I (data not shown) undergoes extensive intramolecular cross-linking under these conditions (*44*). At lower oxidant:protein ratios (≤10:1) that encompass the range of physiologically relevant conditions (*26*), lipid-bound apoA-I and lipid-free apoA-I show a comparable population distribution

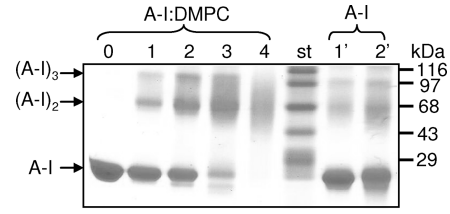

FIGURE 1: Effects of apoA-I oxidation to various stages of protein cross-linking analyzed by SDS−PAGE. Lipid-free apoA-I (lanes 0, 1′, and 2′) or A-I:DMPC disks (lanes 1–4) containing 1 mg/mL protein in standard buffer [10 mM sodium phosphate and 100 μM DTPA (pH 7.5)] were incubated with various concentrations of HOCl as described in Materials and Methods. Lane numbers correspond to oxidant:protein molar ratios of 0:1 (0), 5:1 (1 and 1′), 10:1 (2 and 2′), 25:1 (3), and 50:1 (4). The gel was stained with Imperial protein stain.

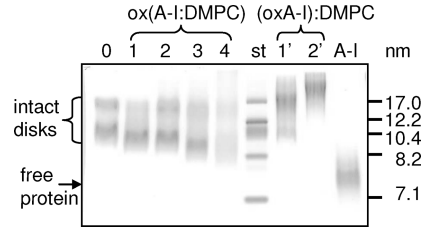

FIGURE 2: Effects of protein oxidation on the size of apoA-I:DMPC complexes at 25 °C analyzed by nondenaturing PAGE. Ten microliters of a 1 mg/mL apoA-I solution was applied to each lane; at this concentration, lipid-free apoA-I aggregates and runs at 7–8 nm [lane A-I (*1*)]. Lanes 0–4 show discoidal complexes reconstituted from intact apoA-I and DMPC that were oxidized using oxidant:protein molar ratios of 0:1 (0), 5:1 (1), 10:1 (2), 25:1 (3), and 50:1 (4). Lanes 1′ and 2′ show complexes reconstituted from oxidized apoA-I and DMPC; the oxidant:protein ratio is 5:1 (1′) or 10:1 (2′). Molecular size standards (st) in nanometers are indicated.

of monomeric, dimeric, and trimeric species (lanes 1 and 2 and lanes 1′ and 2′ compared); therefore, we used these relatively low ratios in our analysis of (oxA-I):DMPC complexes and their comparison with ox(A-I:DMPC) disks.

Effects of protein oxidation on the particle size at 25 °C were analyzed by native PAGE (Figure 2). Intact disks formed two distinct populations 11 and 13 nm in size (Figure 2, lane 0). Oxidation of apoA-I:DMPC disks progressively increased their heterogeneity, leading to formation of smaller and larger particles (Figure 2, lanes 1–4). Complexes reconstituted from oxA-I and DMPC appeared to be larger than intact or ox(A-I:DMPC) disks and exhibited an increase in the particle size with an increase in the degree of protein oxidation (Figure 2, lanes 1′ and 2′).

EM analysis confirmed these results (Figure 3). Electron micrographs of intact apoA-I:DMPC complexes showed disks with an average diameter $\langle d \rangle = 11$ nm (Figure 3A). Oxidation of these disks using oxidant:protein ratios of ≥10:1 led to formation of smaller and larger ox(A-I:DMPC) particles with a broad diameter distribution from 9 to 22 nm (Figure 3C−E). Complexes reconstituted from oxidized apoA-I and DMPC, (oxA-I):DMPC, were larger than intact or ox(A-I:DMPC) disks, and the average diameter of these complexes was 22 nm (Figure 3F,G). Thus, the results of native PAGE and EM studies consistently show that oxidation of apoA-I in discoidal particles leads to remodeling of disks into larger and smaller particles, while oxidation of lipid-free A-I prior to lipoprotein reconstitution leads to formation of only larger particles.

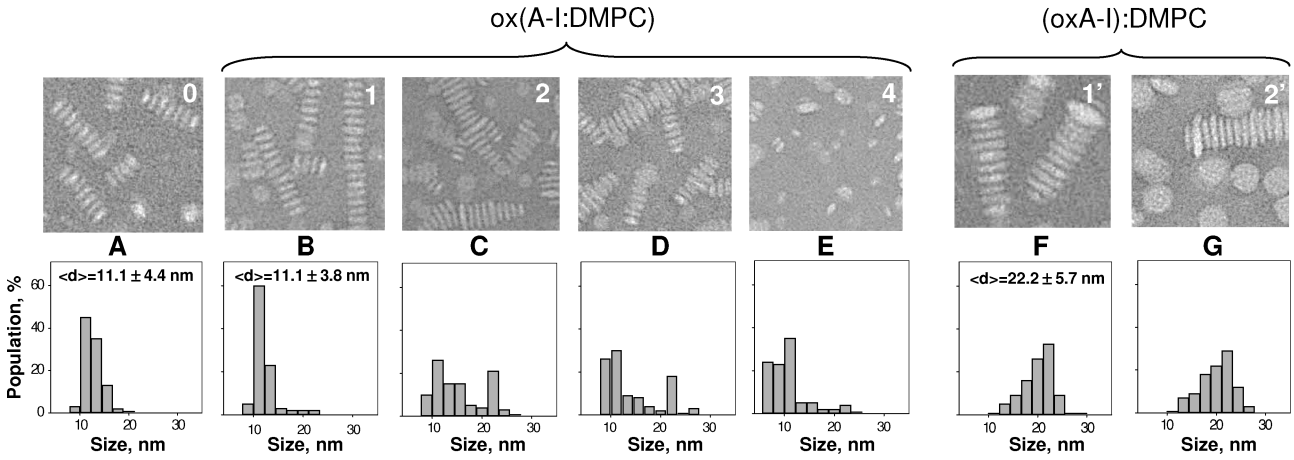

FIGURE 3: Effects of protein oxidation on the size and morphology of A-I:DMPC complexes analyzed by negative staining EM. The top row shows electron micrographs and the bottom row the corresponding particle size distributions. Numbers in panels A−E correspond to those in Figure 2 and show intact A-I:DMPC disks (0) and disks that were treated with oxidant:protein ratios of 5:1 (1), 10:1 (2), 25:1 (3), and 50:1 (4). Panels F and G show disks reconstituted from oxidized apoA-I and DMPC; the HOCl:A-I ratios were 5:1 (1′) and 10:1 (2′). Average particle diameters with standard deviations are indicated in selected panels.

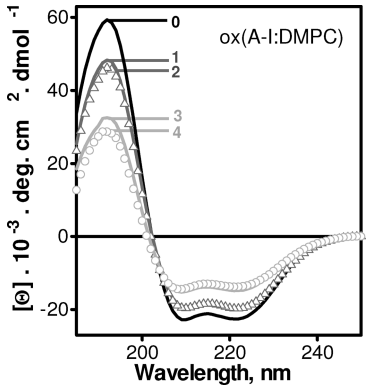

FIGURE 4: Effect of oxidation on the secondary structure of apoA-I in DMPC complexes at 22 °C. Far-UV CD spectra were recorded from the ox(A-I:DMPC) disks (20 μg/mL protein) that were incubated with HOCl using oxidant:protein ratios of 0:1 (0), 5:1 (1), 10:1 (2), 25:1 (3), and 50:1 (4).

Oxidation-induced secondary structural changes in apoA-I were analyzed by CD spectroscopy. Far-UV CD spectra in Figure 4 suggest that intact disks have 60% α-helical content that is reduced to 50% at oxidant:protein molar ratios of 5:1 and 10:1 and to 40% α-helix at oxidant:protein ratios of 25:1 and 50:1. Similarly, lipid-free protein exhibited up to 20% loss in its helical content under these oxidative conditions (CD data not shown). These results are consistent with the earlier studies reporting partial α-helical unfolding in apoA-I upon oxidation (*28*).

Oxidation-induced remodeling of A-I:DMPC disks at ambient temperatures (Figure 3), together with their reduced α-helical content (Figure 4), suggests that protein oxidation destabilizes the disk assembly. To test this notion, we analyzed thermal denaturation of A-I:DMPC disks that were oxidized to various stages. Intact and ox(A-I:DMPC) complexes were heated and cooled from 10 to 98 °C at a rate of 11 °C/h, and thermal denaturation and reconstitution were monitored at 222 nm by CD and by 90° light scattering for changes in α-helical structure and in particle size, respectively (*35*). The results show that oxidation leads to progressive low-temperature shifts in the apparent temperature of disk denaturation, from $T_m = 82$ °C in intact disks to 60 °C in ox(A-I:DMPC) disks that were modified using a 50:1 oxidant:protein ratio (Figure 5A,B). Thus, the CD and light scattering melting data confirm that oxidation destabilizes A-I:DMPC disks and promotes their fusion.

To test whether this notion applies to (oxA-I):DMPC complexes, we compared the melting data recorded for these complexes by CD (Figure 5C) and light scattering (not shown) with similar data recorded for ox(A-I:DMPC) disks. This comparison was made using 5:1 and 10:1 oxidant:protein ratios that produce similar cross-linking patterns in lipid-bound and lipid-free protein (Figure 1, lanes 1 and 1′ and lanes 2 and 2′). The results show that the melting data of ox(A-I:DMPC) and (oxA-I):DMPC complexes largely overlap (Figure 5C). Thus, despite large difference in the diameters of these complexes observed at ambient temperatures (panels B and C of Figure 3 and panels E and F of Figure 3), their apparent thermal stability is similar and is significantly lower than that of intact disks.

To further test this notion and to determine the effect of oxidation on the kinetics of lipoprotein denaturation, we monitored the rate of α-helical unfolding at 222 nm in T-jump experiments. Lipoprotein samples were rapidly heated from 25 °C to high temperature, and the protein unfolding was monitored by CD at 222 nm as a function of time. Figure 6 illustrates the results of such kinetic experiments at 80 °C. Kinetic T-jump data at this and other temperatures clearly show progressive acceleration of protein unfolding upon oxidation, with the slowest unfolding observed in intact disks and fastest in ox(A-I:DMPC) disks that were oxidized using a 50:1 HOCl:protein ratio (lines 0–4 in Figure 6). Furthermore, at similar oxidant:protein ratios of 5:1 and 10:1, the kinetic CD data recorded for (oxA-I):DMPC and ox(A-I:DMPC) complexes fully overlapped, confirming their identical stability (lines 1 and 1′ in Figure 6). Although T-jump data of intact A-I:DMPC disks were well-approximated by single exponentials (*38*), similar data of oxidized complexes could not be fitted with exponential functions, thereby precluding quantitative Arrhenius analysis of the effects of oxidation on the kinetic lipoprotein stability. Nevertheless, our CD and light scattering melting and kinetic data (Figures 5 and 6) clearly show that protein oxidation progressively destabilizes lipoproteins and accelerates protein unfolding and lipoprotein remodeling and fusion.

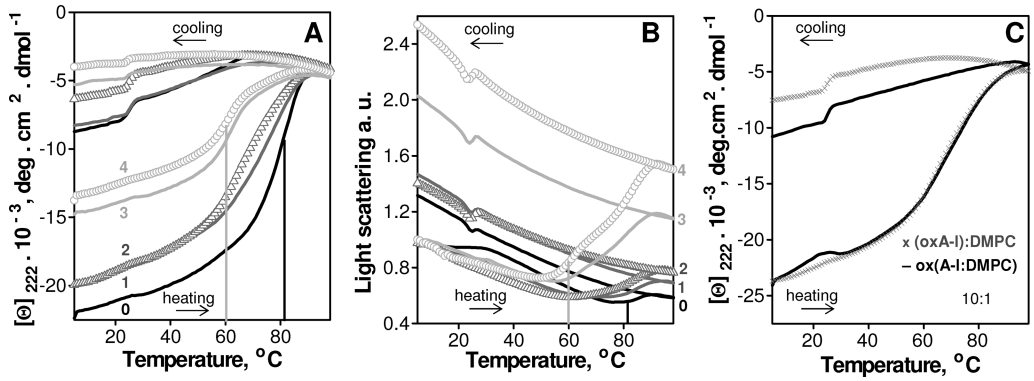

FIGURE 5: Effects of oxidation on thermal denaturation of A-I:DMPC disks. Disk samples, which were prepared as described in the legend of Figure 4, were heated and cooled from 10 to 98 °C at a constant rate of 11 °C/h, and the CD (A) and light scattering (B) melting data were recorded simultaneously at 222 nm to monitor changes in protein helical content and in particle size, respectively; the negative slopes in the light scattering data result from an optical artifact in the CD instrument and from the temperature dependence of the refractive index (*47*). The heating curves of nonoxidized disks (0, black lines in panels A and B) show a transition centered at 82 °C that corresponds to protein unfolding and concomitant lipoprotein fusion (*38*). The apparent transition temperature decreases upon oxidation; the heating curves of highly oxidized disks (4, light gray) show a similar transition centered at 60 °C followed by an additional fusion transition observed by light scattering at higher temperatures (B). Panel C shows CD melting data recorded at 222 nm at a scan rate of 11 °C/h of ox(A-I:DMPC) complexes (which were reconstituted from intact apoA-I and then oxidized) and (oxA-I):DMPC complexes (which were reconstituted from oxidized apoA-I); the oxidant:protein ratio was 10:1.

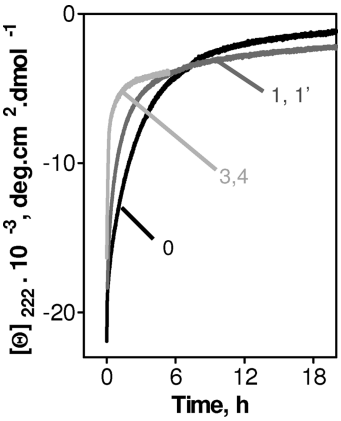

FIGURE 6: Effects of oxidation on the protein unfolding kinetics in DMPC complexes. Intact A-I:DMPC (0), ox(A-I:DMPC) (1–4), and ox(A-I):DMPC complexes (1′ and 2′), which were treated using HOCl:A-I molar ratios of 5:1 (1 and 1′), 10:1 (2 and 2′), 25:1 (3), and 50:1 (4), were subjected to T-jumps from 25 to 80 °C. The time course of the protein unfolding was monitored by CD at 222 nm. The data traces 2 and 2′ (not shown) and 1 and 1′ partially overlap.

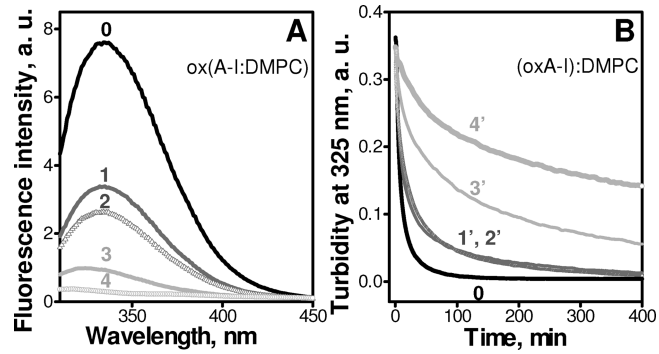

FIGURE 7: Effects of protein oxidation on the Trp modifications in apoA-I:DMPC disks and on the kinetics of DMPC clearance. (A) Trp emission spectra of normal A-I:DMPC disks (0) or of similar disks that were oxidized by using HOCl:A-I molar ratio of 5:1 (1), 10:1 (2), 25:1 (3) or 50:1 (4). (B) Time course of DMPC clearance. DMPC multilamellar vesicles in standard buffer were incubated at 24 °C. The clearance was triggered at time zero by adding the protein; the final concentrations were 20 μg/mL apoA-I and 80 μg/mL DMPC. ApoA-I was nonoxidized (0) or oxidized by using a HOCl:apoA-I molar ratio of 5:1 (1′), 10:1 (2′), 25:1 (3′), or 50:1 (4′).

Fluorescence spectra of lipid-free apoA-I and of A-I:DMPC complexes showed a gradual reduction in the intensity of the Trp emission peak centered at 330 nm with an oxidant:protein ratio increasing from 5:1 to 50:1 (Figure 7A), which is characteristic of Trp oxidation (*31*). Furthermore, turbidity measurements showed a gradual reduction in the rate of DMPC clearance by apoA-I with an increased level of protein oxidation (Figure 7B). These results are consistent with earlier reports (*29, 32*) and with our stability studies (Figures 4 and 5) and indicate that oxidation of apoA-I progressively reduces its affinity for lipid. This may result from oxidative modifications of hydrophobic residues (Met, Trp, Tyr, Phe, etc.) located in the lipid-binding helical faces, which reduce the lipophilicity of these helices. In addition, apolipoprotein cross-linking, which reportedly increases solvent exposure of the α-helices (*33, 34*) and is accompanied by partial α-helical unfolding on the disks (Figure 4), may also contribute to the reduced lipophilicity.

To test whether oxidation-induced lipoprotein destabilization is limited to A-I:DMPC disks or is a general property of discoidal HDLs, we analyzed the effects of oxidation on the thermal stability of complexes reconstituted from various proteins and lipids. In addition to apoA-I, we used homologous exchangeable apolipoproteins apoA-II (*38, 39*) and apoC-I; these proteins contain a single Met in the apolar helical face that can form MetO, as well as several Tyr residues that can be cross-linked by HOCl. In addition to DMPC (14:0, 14:0), we reconstituted disks with longer chain PCs that were fully saturated or monounsaturated, such as dipalmitoyl-PC (16:0, 16:0) or palmitoyl:oleoyl:PC (16:0, 18:1); such PCs better approximate the lipid composition of plasma HDL. Furthermore, we reconstituted A-I:DMPC:FC disks containing 10% FC, oxidized them to various stages with HOCl, and assessed the ensuing changes in their thermal stability by CD. The CD melting data in Figure 8 illustrate gradual destabilization of A-I:DMPC:FC complexes with an

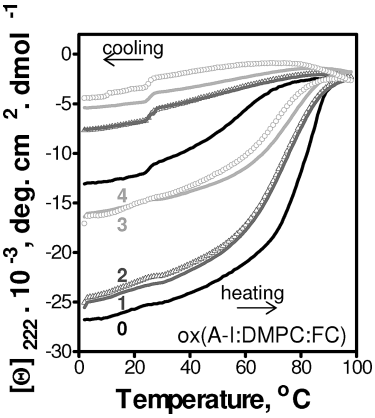

FIGURE 8: Effect of oxidation on the thermal denaturation of A-I:DMPC−FC disks. Disk samples containing 10 % cholesterol were prepared as described in Materials and Methods. The disks were heated and cooled from 10 to 98 °C at a constant rate of 11 °C/h. The protein unfolding was monitored by CD at 222 nm. Numbers correspond to those in Figures 4 and 5 and show intact disks (0) and disks that were oxidized using a HOCl:apoA-I molar ratio of 5:1 (1), 10:1 (2), 25:1 (3), or 50:1 (4).

increased degree of protein oxidation. These and other results consistently show that, similar to A-I:DMPC disks, selective protein oxidation in other discoidal rHDLs gradually destabilizes these rHDLs and accelerates protein unfolding and dissociation, as well as lipoprotein remodeling and fusion.

DISCUSSION

The results reported here show that oxidation of the exchangeable apolipoproteins by HOCl progressively destabilizes discoidal rHDLs of various compositions and accelerates protein dissociation and lipoprotein remodeling and fusion (Figures 5, 6, and 8). This is consistent with the earlier reports of destabilization of α-helices in lipid-free and lipid-bound apoA-I and apoA-II upon oxidation with hydrogen peroxide (*29, 32*). This destabilization was attributed to the reduced affinity of protein for lipid upon MetO formation in the apolar lipid-binding faces of the amphipathic apolipoprotein α-helices (*29*). Such MetO formation occurs at early stages of oxidation and is followed by modifications of other protein groups, such as aromatic residues and Lys, and their cross-linking (*24–31*). Correlation of rHDL destabilization (Figures 5 and 6) with partial α-helical unfolding (Figure 4), Trp oxidation (Figure 7A), protein cross-linking (Figure 1), and decelerated lipid clearance (Figure 7B) suggests that not only MetO formation but also HOCl-induced modifications of other aromatic groups located in the lipid-binding faces of the protein α-helices, as well as protein cross-linking, may contribute to the reduced affinity of protein for lipid and to rHDL destabilization. In fact, cross-linking of lipid-bound apoA-I reduces its helical content (Figure 4) and reportedly increases solvent exposure of specific protein α-helices (*16, 33, 34*), which may weaken protein–lipid interactions and promote protein dissociation and lipoprotein remodeling and fusion.

Our results reveal that oxidative protein modifications in apoA-I:DMPC disks lead to remodeling of rHDL into larger and smaller particles. Such a remodeling was observed at or above a 10:1 oxidant:protein ratio (Figure 3C−E), which probably is well in excess of the normal HOCl levels and hence is unlikely to occur in vivo. Remodeling of spherical lipoproteins into larger and smaller particles has been well-documented. For example, conversion of plasma HDL into larger and smaller particles occurs upon remodeling by plasma factors, such as phospholipid transfer protein (*48*), probably due to imbalance between the core and surface of spherical particles. Another example is heat-induced conversion of very low-density lipoproteins into larger and smaller spherical particles, resulting in transient formation of small HDL-like lipoproteins (*49*). To the best of our knowledge, the study presented here provides the first report of remodeling of discoidal lipoproteins into larger and smaller particles. Larger particles are probably fusion products of intact-size disks; such fusion may result from conformational changes in oxidized apoA-I, leading to increased exposure of the lipid surface in discoidal complexes. The mechanism of dissociation of smaller particles is not entirely clear. Formation of these particles is concomitant with the disappearance of the monomeric apoA-I at oxidation stages 2–4 (lanes 2–4 in Figure 1 and panels C−E in Figure 3), suggesting that the conformation of apoA-I that is altered upon cross-linking or other oxidative modifications favors dissociation of such small particles.

Since apolipoprotein dissociation and lipoprotein fusion occur not only during denaturation of discoidal rHDLs but also during denaturation and metabolic remodeling of plasma spherical HDLs, analysis of the effects of oxidative modifications on these reactions may provide the molecular basis for understanding functional consequences of HDL oxidation. Interestingly, in contrast to discoidal rHDLs that are progressively destabilized upon oxidation (Figures 5 and 6), plasma spherical HDLs are destabilized only at early oxidation stages corresponding to a low HOCl:protein ratio of up to 10:1 but exhibit increased resistance to protein dissociation and particle remodeling at higher oxidant levels (*21*). We hypothesized that initial destabilization of plasma HDL results from oxidative modifications to its major proteins, apoA-I and apoA-II, while the reversal of this effect upon further oxidation is due to modifications of core and surface lipids (*21*).

The results of this work strongly support this hypothesis. They show that, regardless of the initial protein state (lipid-free or in discoidal rHDL), mild oxidation of apoA-I leads to protein cross-linking into dimers and trimers (Figure 1) and to destabilization of the rHDL assembly (Figures 5, 6, and 8). This suggests strongly that cross-linking of apoA-I into dimers and trimers that occurs at early oxidation stages in plasma spherical HDL is also destabilizing. We propose that such moderate destabilization of plasma HDLs benefits their functions, since it facilitates cholesterol efflux to mildly oxidized HDLs, promotes apolipoprotein exchange among lipoproteins, and accelerates HDL remodeling by plasma factors during RCT (*20, 21*). The results reported here show that this functional advantage results, at least in part, from modifications to HDL proteins that occur at early stages of oxidation, such as oxidation of Met and aromatic groups in the apolar helical faces, that destabilize HDL assembly and promote HDL remodeling.

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