Abstract  Atherosclerosis is a state of heightened oxidative stress. Oxidized LDL is present in atherosclerotic lesions and used as marker for coronary artery disease, although in human lesions lipids associated with HDL are as oxidized as those of LDL. Here we investigated specific changes occurring to apolipoprotein A-I (apoA-I) and apoA-II, as isolated HDL and human plasma undergo mild, chemically induced oxidation, or autoxidation. During such oxidation, Met residues in apoA-I and apoA-II become selectively and consecutively oxidized to their respective Met sulfoxide (MetO) forms that can be separated by HPLC. Placing plasma at −20°C prevents autoxidation, whereas metal chelators and butylated hydroxytoluene offer partial protection. Independent of the oxidation conditions, apoA-I and apoA-II (dimer) with two MetO residues accumulate as relatively stable oxidation products. Compared to controls, serum samples from subjects with atherosclerotic disease, include oxidized apolipoproteins in mildly oxidized high density lipoprotein, that could be present in blood of subjects with atherosclerotic disease. First, being substantially oxidized, HDLs or their component(s) over oxLDL as a potential marker of atherosclerotic disease. Second, whereas HDL lipids are at least as susceptible to oxidation as those of LDL (11). Also, lipids in HDL and LDL isolated from human atherosclerotic lesions are oxidized to a comparable extent (12) that increases with increasing severity of disease (13). These lesion lipoproteins retain normal concentrations of α-tocopherol (12, 14–16) and most of their major lipid oxidation products, i.e., cholesteryl ester hydro(pero)xides, accumulate in the presence of the vitamin (15). These findings suggest that oxidized lesion lipoproteins, including oxHDL, that could be present in blood of subjects with atherosclerotic disease, include early stage, oxidized lipoproteins.

Several considerations favor the potential use of oxHDLs or their component(s) over oxLDL as a potential marker of atherosclerotic disease. First, being substantially smaller and interacting less strongly with extracellular proteoglycans, vessel wall HDL is expected to re-enter the circulation more readily than LDL (17). Second, whereas
apoB-100 does not dissociate from LDL, HDL’s apolipoproteins dissociate readily and limited oxidation enhances this process in the case of apoA-I (18), further increasing the likelihood of its existence in circulation. Third, given their physical properties and smaller molecular size, oxidized forms of apoA-I and apoA-II are simpler to work with and to chemically characterize than apoB-100.

Compared with LDL, relatively little is known about how HDLs and their components become oxidized. Previous in vitro studies utilized different oxidants, including H$_2$O$_2$ (19, 20), myeloperoxidase-derived (21–24), lipid hydroperoxides (25–27), and peroxy radicals (26) and Cu$^{2+}$ (26) that modify apoA-I and apoA-II in different ways and to varying extent. In the case of apoA-I, HDL’s major apolipoprotein, a common feature of mild oxidation is that Met residues become oxidized to methionine sulfoxides (MetO) (19, 20, 24, 26, 28). Lipid hydroperoxides formed during HDL oxidation convert Met$^{112}$ and Met$^{86}$ of apoA-I to MetO (18, 26, 28). However, it remains unclear whether Met oxidation in HDL-associated apoA-I occurs in a stepwise or “none or two” manner, and how oxidation of HDL’s second most abundant apolipoprotein, apoA-II occurs. We therefore investigated the identity and rate of accumulation of oxidized apoA-I versus apoA-II formed in fresh human plasma undergoing autodissociation or exposed to chemically controlled oxidation by alkyl peroxy radicals.

**EXPERIMENTAL PROCEDURES**

**Materials**

All chemicals were obtained from Sigma (Australia) unless specified otherwise.

**Plasma oxidation and HDL isolation**

For autooxidation experiments, freshly isolated lithium heparin plasma was incubated at 37°C under air for the indicated period of time, then snap frozen at ~80°C to prevent further oxidation before analyses. Chemically controlled oxidation was achieved by the addition of 2,2’-azo-bis(2-aminopropane) dihydrochloride (AAPH) (Wako, Japan), a generator of aqueous peroxyl radicals, prior to incubation of the plasma samples at 37°C. Experiments were performed under basic conditions to prevent the oxidation of Met$^{86}$, and to chemically characterize apoA-I and apoA-II.

**HPLC analysis of HDL apolipoproteins**

Aliquots (typically 100 μl) of freshly isolated HDL (diluted 1:1, v/v, in HPLC-grade water) were applied to a 5 μm, 25 × 0.46 cm reverse phase C18 column (Vydac). The column was eluted at 0.5 ml/min and 50°C with an acetonitrile-water gradient containing 0.1% (v/v) TFA (Pierce) monitored at 214 nm. Following initial equilibration at 25% acetonitrile, the concentration was increased linearly to 45% over 5 min, and then to 55% over an additional 32 min. The acetonitrile content was then increased rapidly to 95% for 10 min and finally decreased to 25% for column re-equilibration.

**Characterization of HDL apolipoproteins by mass spectrometry**

Oxidized and non-oxidized HDL samples were subjected to HPLC, and the protein fractions collected, pooled, and lyophilized. Mass spectra were acquired using a single quadrupole mass spectrometer equipped with an electrospray ionization source (Platform, VG-Fisons Instruments). Samples (50 pmol, 10 μl) were injected into a moving solvent (10 μl/min) of acetonitrile-water (1:1, v/v) coupled directly to the ionization source. MALDI/peptide mass fingerprinting spectra of lyophilized proteins from HDL were determined after digestion with endoprotease AspN or trypsin (≈100 ng) in NH$_4$HCO$_3$ (25 μl, 20 mM, pH 8). After 14 h at 37°C, digests (1 μl) were analyzed directly after addition of matrix (DHB, 1 μl, 10 mg/ml) by MALDI over a mass range of m/z 500 to 7,000. Approximately 100 spectra were acquired in reflectron mode (Voyager STR, Perseptive Biosystems, Framingham, MA) with an accelerating voltage of 25,000 V. An extraction delay of 175 ns and spectra were calibrated externally using angiotensin I and insulin (oxy) B chain. Peptides were identified by comparison with theoretically determined peptide masses.

**RESULTS**

**Characterization of native and oxidized forms of apoA-I and apoA-II in HDL**

To assess the oxidation of HDL apolipoproteins in more detail, we first improved the HPLC method used previously (26), utilizing an initial acetonitrile concentration of 25% (v/v) that was increased to 45% over 5 min, followed by a slower gradient to 65% of 30 min. Addition of this initial rapid increase in acetonitrile concentration improved the separation of HDL-associated proteins (data not shown).

In native HDL, apoA-I and apoA-II were the two major proteins detected (Fig. 1A). ApoA-I eluted as a single species with a mass of 28081 Da as determined by electrospray mass spectrometry (predicted mass 28,078.7 Da). ApoA-II, which exists as a disulfide-linked homodimer, eluted as three distinct species with masses of 17,382,
17,255, and 17,123 Da, respectively. The largest of the three species corresponded to apoA-II homodimer with both N-terminal glutamine residues cyclized (predicted mass 17,381.8 Da), as described previously (32). Based on the mass changes, the two smaller species were assigned to apoA-II with one or both of the C-terminal glutamine residues removed (predicted masses 17,253.6 and 17,125.4 Da, respectively).

We then exposed HDL to mild controlled oxidation by exposure to the free radical generator AAPH (1 mM, 37°C). This resulted in the time-dependent consumption of endogenous α-tocopherol during which phospholipid- and cholesterylester hydroperoxides (11, 26) and their corresponding hydroxides accumulated (25, 28) (data not shown). Concomitant with these changes, oxidation of apolipoproteins occurred (26, 28), as characterized by decreasing amounts of native apoA-I and apoA-II and accumulation of new oxidized species (Fig. 1B). Three oxidized species of apoA-I were separated, two with an increase in mass of 16 Da (designated as apoA-I\_16), and the third with a mass increase of 32 (apoA-I\_32). MALDI-TOF analysis of these three species revealed that the mass increases were due to the oxidation of one or both Met residues 86 and 112 to MetO (Table 1). ApoA-I\_32 (MetO\_12, eluting at \~18.5 min in Fig. 1B) was the more prevalent of the two +16 species, perhaps suggesting increased exposure of this Met residue to the lipid hydroperoxides contained in the HDL particle. The two apoA-I\_16 species accumulated before apoA-I\_32 appeared. Thus, \~10-20% of the total apoA-I was converted to apoA-I\_16 species before apoA-I\_32 reached detectable levels (not shown).

All three apoA-II species behaved similarly with respect to oxidation, and as such will be referred to collectively throughout. ApoA-II was also converted into +16 and +32 forms by AAPH oxidation of HDL (Fig. 1B), as assessed by ESI-MS. These mass changes were the result of oxidation of one or both of the Met residues contained in the homodimer (Table 2). ApoA-II\_32 co-eluted with apoA-I, so that it was only detected under relatively harsh oxidizing conditions, i.e., when all native apoA-I was oxidized (data not shown).

**Oxidation of HDL apolipoproteins in plasma**

To assess the rates at which the oxidized forms of apoA-I and apoA-II were formed, plasma was incubated at 37°C in the presence of AAPH, and the HDL isolated at various time points and then subjected to HPLC analysis. Oxidized forms of both apoA-I and apoA-II accumulated in a time- and AAPH-concentration-dependent manner. At low AAPH concentration, apoA-I\_16 and apoA-II\_16 accumulated before and at concentrations higher than apoA-I\_32 (Fig. 2A), whereas these differences became smaller at higher rates of peroxyl radical generation (Fig. 2B). Both forms of apoA-I\_16, i.e., MetO\_16 and MetO\_12, were formed at comparable rates and stages of oxidation, and are therefore presented together. Although it was impossible to accurately quantify apoA-I concentrations as it co-elutes with apoA-II\_32 (28), it was clear from the chromatograms that formation of the oxidized species was accompanied by a decrease in native apoA-I and apoA-II (data not shown).

Although AAPH is widely used as a model of controlled oxidative stress, we also wished to establish the identity of oxHDL apolipoproteins and their rate of formation dur-

**TABLE 1. ESI and MALDI-TOF analysis of native and selectively oxidized apoA-I and Met-containing peptides derived from them**

<table>
<thead>
<tr>
<th>Protein</th>
<th>ESI Mass</th>
<th>Mass Peptide Residues 73-88 (DNLEKETEGLRQE)</th>
<th>MSK</th>
<th>Mass Peptide Residues 108-116 (WQEFEMELRYI)</th>
<th>Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA-I</td>
<td>28,861</td>
<td>1906.9</td>
<td>1283.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA-I_16  (Met_86)</td>
<td>28,095</td>
<td>1926.2</td>
<td>1283.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA-I_16  (Met_112)</td>
<td>28,095</td>
<td>1907.1</td>
<td>1299.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA-I_32</td>
<td>28,114</td>
<td>1929.2</td>
<td>1299.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Native or mildly oxidized (1 mM AAPH, 6 h, 37°C) HDL, was subjected to RP-HPLC, and the apolipoproteins isolated and then subjected to ESI-MS as described under Experimental Procedures. Remaining samples were lyophilized, then digested with either trypsin or AspN, and the resultant peptide fragments subjected to MALDI-TOF. ApoA-I contains three Met residues of which Met\_86 and Met\_112 become oxidized during AAPH-induced oxidation of HDL. The results shown are typical of three separate analyses using different preparations of oxHDL.

**Fig. 1.** HPLC chromatograms of HDL apolipoproteins before and after oxidation induced by 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH). HDL prior to oxidation (A) or following oxidation by AAPH (1 mM) for 6 h at 37°C (B) was subjected to HPLC analysis as described under Experimental Procedures.
ing the autoxidation of whole plasma to validate the outcomes of the previous experiments. Plasma aliquots were filter-sterilized and incubated under air for up to 148 h at 37°C before HDL was isolated and analyzed by HPLC. After 24 h, both MetO\(^{36}\) and MetO\(^{112}\) were detected (Fig. 3), while apoA-I\(_{1,32}\) was not observed until after 72 h of incubation. After 120 h, the concentrations of apoA-I\(_{1,32}\) species detected were maximal. Subsequently, apoA-I\(_{1,32}\) (and apoA-II\(_{1,16}\)) concentrations declined, indicating that this form of oxidized apoA-I (apoA-II) represents a transient species that becomes progressively more oxidized. In contrast, apoA-I\(_{1,32}\) continued to accumulate at an enhanced rate, suggesting that it was derived from apoA-I\(_{1,16}\) and represented a more stable form of oxidized apoA-I. Under no circumstance did we observe apoA-I with all three Met residues oxidized, as judged by the lack of appearance of a novel, nonassigned peak eluting between the solvent front and nonoxidized apoA-I (not shown). Possibly the packing of apoA-I into the HDL particle does not result in Met\(^{148}\) being exposed to the lipid hydroperoxides, although it appears accessible to reagent hypochlorite (24).

Fig. 3. Changes in HDL’s apoA-I\(_{1,16}\), apoA-I\(_{1,32}\), and apoA-II\(_{1,16}\) during the course of autoxidation in plasma. Plasma was incubated under air and at 37°C, and at the time points indicated, the HDL was isolated and apoA-I\(_{1,16}\) (circle), apoA-I\(_{1,32}\) (square), and apoA-II\(_{1,16}\) (triangle) analyzed as described under Experimental Procedures. Data are shown as percent of total apolipoprotein peak area, and represent means ± SE of three separate experiments.
out increased risk for coronary artery disease according to their eNOS genotypes and exposure to cigarette smoking. We observed a significant correlation between eNOS genotype and percentage of HDL’s apoA-I present as apoA-I \(_{1+32}\) (Fig. 4). HDL isolated from serum of the eNOS\(_a/b\) genotype (16.6 ± 6.5%, \(n = 4\)) had five times higher apoA-I \(_{1+32}\) than that of serum obtained from subjects with the common eNOS\(_b/b\) genotype (3.3 ± 0.9%, \(n = 8\)) (\(P = 0.016\)). Similarly, HDL isolated from serum with exposure to tobacco smoking (14.1 ± 4.8, \(n = 5\)) also had a higher proportion of apoA-I being present as apoA-I \(_{1+32}\) than HDL from serum of nonexposed sera (5.5 ± 1.9%, \(n = 12\)) (Fig. 4), although this difference did not reach statistical significance (\(P = 0.064\)).

**DISCUSSION**

Atherosclerosis is now generally considered a state of elevated oxidative stress, associated with the presence of oxidized molecules in the diseased vessel wall, including oxidized LDL and HDL. The present study shows that the two major apolipoproteins of HDL, apoA-I and apoA-II, become specifically oxidized as the isolated lipoprotein or human plasma undergoes an oxidative challenge. During the early stages, when \(\alpha\)-tocopherol remains present, apoA-I \(_{1+16}\) (MetO\(_{86}\) or MetO\(_{112}\)) and apoA-II \(_{1+16}\) are formed and accumulate initially as specific oxidation products, whereas apoA-I \(_{1+32}\) accumulates comparatively later and appears to be a more stable product. Therefore, oxidation of Met residues in apoA-I (and apoA-II) to MetO is a step-wise, early process during the oxidation of isolated HDL and plasma. We also detected increased concentrations of apoA-I \(_{1+32}\) in serum of subjects with increased risk of coronary artery disease, consistent with some of the specifically modified apolipoprotein(s) being useful as novel measures of in vivo lipoprotein oxidation, and possible atherosclerosis.

During atherogenesis, different oxidants contribute to different extents and at different stages to the modification of lipoproteins in the vessel wall (34). Previous in vitro studies on the oxidation of HDL and its apolipoproteins also utilized different oxidants that modify apoA-I and apoA-II in different ways and to varying extents. For example, high molar ratios of hypochlorite to HDL results in gross changes to its apolipoproteins and oxidation of amino acids in addition to Met (23), whereas at low molar oxidant-to-lipoprotein (up to 6:1), oxidation is limited to MetO formation (24). Given this complexity we decided for the present study to (1) use oxidizing conditions that cause lipid peroxidation, and (2) to limit the extent of HDL/plasma oxidation to a stage where \(\alpha\)-tocopherol remains present. Both decisions are based on our present knowledge of lipoprotein oxidation in the context of atherogenesis. First, HDL lipid peroxidation occurs in human lesions (12). Second, lipid peroxidation is a feature common to most oxidants, including all one-electron (1e) oxidants and conditions that give rise to 1e oxidation reactions (4, 35), including exposure to hypochlorite (36), and therefore is likely of general relevance. Third, HDL is the major carrier of cholesterylester hydroperoxides in plasma (11), so that the conditions employed here where apolipoprotein oxidation is a secondary process mediated by lipoprotein-associated lipid hydroperoxides (28) are biologically relevant. Finally, most oxidized lipids present in human lesions are formed in the presence of \(\alpha\)-tocopherol (15), so that carrying out in vitro oxidation under the continued presence of the vitamin is likely meaningful from a pathophysiological point of view. Showing that identical oxidized species of apoA-I and apoA-II are formed under the three experimental conditions validates our choices.

A key feature of the present and previous studies is that mild oxidation of HDL results in the formation of specific defects.
forms of oxidized apoA-I and apoA-II, the chemical modification of which is defined unambiguously. In the case of apoA-I, modification of Met residues is a step-wise process, with a single Met residue (Met^{112} or Met^{86}) oxidized first, followed by the second Met residue; the third Met residue (Met^{148}) does not become oxidized under the conditions employed here. The situation is comparable for apoA-II, with a consecutive conversion of the two Met^{26} residues in the apoA-II homodimer to MetO^{26}. Therefore, the formation of apoA-I_{1+16} and apoA-II_{1+16} precedes that of apoA-I_{3+32} and apoA-II_{3+32}, and hence reflects a comparatively earlier stage of HDL oxidation.

The present study suggests that the identified specific forms of oxidized apoA-I and apoA-II may serve as in vivo markers of HDL oxidation, although several aspects need to be considered. First, their presence reflects lipid peroxidation and protein oxidation, as both lipid hydroperoxides and 2-oxidants generate the MetO identified. This is potentially useful as at present oxidation markers are limited to either lipid or protein oxidation. Second, their presence likely reflects HDL oxidation (although one cannot rule out the possibility that apoA-I becomes oxidized in lipid-free form and then associates with HDL). This distinguishes specifically oxidized apoA-I/II from general markers of lipid oxidation, such as F_{2}-isoprostanes that, e.g., do not distinguish cellular from lipoprotein lipid oxidation. Third, the methodology described here to measure oxidized apoA-I/II is relatively simple, and can be applied to sample numbers commonly employed in basic research. Fourth, the slow rate at which apoA-I_{1+16}/A-II_{1+16} are formed during autoxidation and the results in Table 3 suggest that short periods of plasma storage at room temperature are unlikely to affect and storage at −20°C preserves the oxidation state of apolipoproteins in HDL.

To establish the usefulness of the specific oxidized forms of apoA-I/II as markers for in vivo HDL oxidation, several aspects require further investigation. Foremost, it will be important to firmly establish the presence of specific oxidized forms of apoA-I in biological samples. To this end, our preliminary experiments reported here show that the circulating concentration of apoA-I_{3+32} is increased in subjects with increased risk for coronary artery disease, such as in carrier of the eNOSa/b genotype and exposure to cigarette smoking (Fig. 4). We also observed the content of apoA-I_{1+32} in human aortic lesions increasing relative to native apoA-I with increasing severity of disease (U. Panzenböck and R. Stocker, unpublished observation). It will be important to extend these studies to subjects with proven coronary artery disease to test whether circulating concentrations of any of the specifically oxidized forms of apoA-I and/or apoA-II correlate with, and hence can be used as a surrogate for, atherosclerosis in individuals. It will also be interesting to investigate the metabolism of specific oxidized forms of apoA-I. For example, peptide MetO reductase can reduce MetO residues in lipid-associated oxidized apoA-I (37), although the enzyme is present only inside cells and would be expected to reduce only one stereoisomer of MetO. In addition, it remains to be established which of the different oxidized apolipoprotein species characterized in the present study is the best marker of HDL oxidation. While detection of apoA-I/II_{1+16} may offer sensitivity, the comparatively more stable nature of apoA-I_{3+32} may make this form of oxidized apoA-I_{3+32} a more suitable marker, consistent with the present findings with samples from eNOSa/b genotype and smoking exposure. Finally, the present HPLC-based method has limitations with regards to its potential application to large numbers of clinical samples, as it is time consuming and hence costly.

We pointed out previously (18) that the “oxidation-sensitive” Met^{86} and Met^{112} of apoA-I are located in the non-polar face adjacent to the polar face at the surface of amphipathic helices. Similarly, the redox-sensitive Met^{26} of apoA-II is also found at the interface between the non-polar and polar surface faces of the first amphipathic helix of apoA-II (Fig. 5). These are the sites where the hydroperoxide moiety of lipoprotein lipid hydroperoxides are expected to be located (38) so that oxidation of Met residues may be facile. Met^{148} (Fig. 5) may be comparatively more resistant to oxidation as it is located opposite a cluster of three positively-charged arginine residues (Arg^{149}, Arg^{153}, and Arg^{160}) on the hydrophobic interface of helix 6 of apoA-I (39), and thus likely buried in the lipoprotein particle. In any case, the introduction of the polar sulfoxide moiety at these sites alters the properties of the apolipoprotein affected (18). This is also reflected in the striking change in retention time of the modified apolipoprotein on RP-HPLC, consistent with the idea that the specific oxidations reported here introduce new epitopes on the proteins. If so, it would raise the possibility of establishing an antibody-based assay for the detection of specifically oxHDL with high sensitivity. We are presently investigating these possibilities.

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REFERENCES


This page contains a list of references from a scientific journal article. It includes authors, titles, and publication details for various studies related to atherosclerosis and related conditions. The references cover a range of topics, including oxidative stress, lipoprotein modification, and the role of antioxidants in the prevention and treatment of atherosclerotic disease. The citations are from reputable sources such as the Journal of Clinical Investigation and the American Journal of Pathology, among others. The list is presented in a standard academic format, with proper citation details for each reference.