Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein

Greg Pankhurst,* Xing Li Wang,† David E. Wilcken, † Georg Baernthaler,* Ute Panzenböck,* Mark Raftery,§ and Roland Stocker[1],*,**

The Heart Research Institute,* 145 Missenden Road, Camperdown NSW 2050, Australia; Prince of Wales Hospital,† Randwick NSW 2031, Australia; and Department of Pathology§ and Centre for Thrombosis and Vascular Research,** School of Medical Sciences, Faculty of Medicine, The University of New South Wales, UNSW Sydney NSW 2052, Australia

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 the endothelial cell nitric oxide synthase a/b genotype that is associated with increased coronary artery disease contain increased concentrations of apoA-I with two MetO residues. Our results show that during the early stages, oxidation of HDL gives rise to specifically oxidized forms of apoA-I and apoA-II, some of which may be useful markers of in vivo HDL oxidation, and hence potentially atherosclerosis.—Pankhurst, G., X. L. Wang, D. E. Wilcken, G. Baernthaler, U. Panzenböck, M. Raftery, and R. Stocker. **Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein.** ***J. Lipid Res.*** **2003.** 44: **349–355.**

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The oxidation of LDL is widely thought to be critical to atherogenesis (1). Oxidation of the LDL-associated antioxidants ubiquinol-10 (2) and α-tocopherol (3) represents the initial step in this process, concomitant with formation of lipid hydroperoxides (2, 4) that in turn may then lead to oxidation of apolipoprotein B-100 (apoB-100) (5). Oxidized LDL (oxLDL) is present in human atherosclerotic lesions (6), and its detection in circulation by immunological assays is used as a surrogate for, or marker of, atherosclerotic disease (7, 8). However, the term oxLDL refers to a mixture of modified lipoproteins that remain chemically uncharacterized (9). This limits the use of measurement of oxLDL as a diagnostic for cardiovascular disease(s). In addition, there are several caveats concerning the accuracy of these measurements, including the extent of LDL oxidation, reproducibility of the reference oxLDL, and a better characterization of the epitope recognized by the antibody used (10).

HDLs are also subject to oxidative modification in vivo, as 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., cholesterylester 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

Abbreviations: AAPH, 2,2'-azo-*bis*(2-amidinopropane) dihydrochloride; BHT, butylated hydroxy toluene; DTPA, diethylenetriamine pentaacetic acid; eNOS, endothelial nitric oxide synthase; MetO, Met sulfoxide; oxLDL, oxidized low-density lipoprotein.

[1] To whom correspondence should be addressed.
e-mail: r.stocker@unsw.edu.au

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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_2O_2 (19, 20), myeloperoxidase-derived oxidants (21–24), lipid hydroperoxides (25–27), and peroxyl radicals (26) and Cu^{2+} (26) that modify apoA-I and apo-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 (MetOs) (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 autoxidation or exposed to chemically controlled oxidation by alkyl peroxyl radicals.

EXPERIMENTAL PROCEDURES

Materials

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

Plasma oxidation and HDL isolation

For autoxidation 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-amidinopropane) dihydrochloride (AAPH) (Wako, Japan), a generator of aqueous peroxyl radicals, prior to incubation of the plasma samples at 37°C. Where indicated, diethylenetriamine pentaacetic acid (DTPA) or butylated hydroxy toluene (BHT) was also added prior to incubation.

HDL was isolated from plasma by density gradient ultracentrifugation using a Ultima-X Benchtop Centrifuge equipped with a TL100.4 rotor (Beckman Instruments) centrifuged for 3 h at 15°C and 100,000 rpm (29). The resulting HDL (comprising both HDL_2 and HDL_3) was aspirated and gel-filtered (PD-10 column, Pharmacia, Sweden) to remove low molecular weight compounds using 10 mM phospate buffer, pH 7.4, containing 100 mM DTPA.

Collection of blood samples

Cord blood samples were collected post-partum after normal full-term pregnancy and uncomplicated delivery from subjects recruited consecutively within the study period. We chose cord blood samples as they are less likely affected by environmental factors such as dietary vitamin supplements, as observed frequently in adult populations. The endothelial nitric oxide synthase (eNOS) intron 4 a/b polymorphism was determined from the cord blood DNA as described previously (30). Compared with the b/b genotype, carriers of the eNOSa allele who smoke have an increased risk for coronary artery stenosis and myocardial infarction (31). Smoking status of the mothers during pregnancy was also documented. Blood was centrifuged within 10 min of collection and serum samples stored at −70°C within a further 10 min. HDL was then isolated and analyzed immediately for oxidized apolipoproteins by the HPLC method described below.

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-oxHDL 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 finger printing spectra of lyophilized proteins from HPLC were determined after digestion with endoprotease AspN or trypsin (∼100 ng) in NH_4HCO_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 MADLI 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 (ox) 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,

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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_{+16} ($MetO^{112}$, 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).

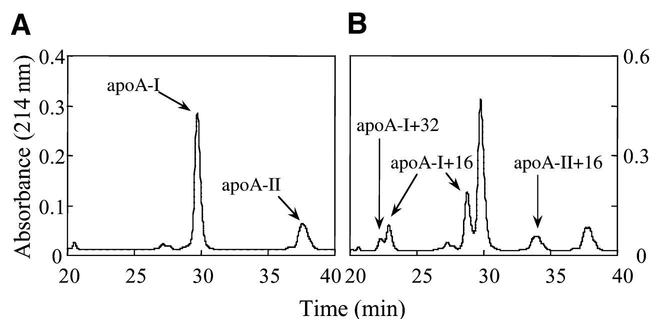

Fig. 1. HPLC chromatograms of HDL apolipoproteins before and after oxidation induced by 2,2'-azo-*bis*(2-amidinopropane) dihydrochloride (AAPH). HDL prior to oxidation (A) or following oxidation by AAPH (1 mM) for 6 h at 37°C (B) was subjected to HPLC analysis as described under Experimental Procedures.

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

Protein	ESI Mass	Mass Peptide Residues 73–88 (DNLEKETEGLRQE**M**SK)	Mass Peptide Residues 108–116 (WQEE**M**ELYR)
		Da	
apoA-I	28,081	1906.9	1283.4
apoA-I_{+16} (Met^{86})	28,098	1923.2	1283.6
apoA-I_{+16} (Met^{112})	28,095	1907.1	1299.5
apoA-I_{+32}	28,114	1923.2	1299.5

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.

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., $MetO^{86}$ and $MetO^{112}$, 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 2. ESI and MALDI-TOF analysis of native and selectively oxidized apoA-II and the Met-containing peptide derived from them

Protein	ESI Mass	Mass Peptide Residues 24–28 (DL**M**EK)
	Da	
apoA-II	17,252	635.3
apoA-II_{+16}	17,269	635.3, 651.3
apoA-II_{+32}	17,289	651.3

Native or mildly oxidized (1 mM AAPH, 6 h, 37°C) HDL were 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 trypsin, and the resultant peptide fragments subjected to MALDI-TOF. ApoA-II contains a single Met residue (Met^{26}) that becomes oxidized during AAPH-induced oxidation of HDL. The results shown are typical of three separate analyses using different preparations of oxHDL.

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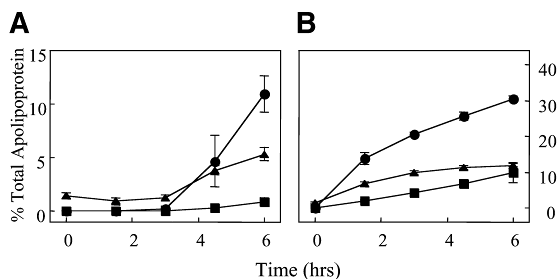

Fig. 2. Changes in HDL's apoA-I_{+16}, apoA-I_{+32}, and apoA-II_{+16} during the course of oxidation induced by AAPH in plasma. Plasma was exposed under air and at 37°C to (A) 10 mM AAPH or (B) 50 mM AAPH. At the time points indicated, HDL was isolated and apoA-I_{+16} (circle), apoA-I_{+32} (square), and apoA-II_{+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.

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 $MetO^{86}$ and $MetO^{112}$ were detected (**Fig. 3**), while apoA-I_{+32} was not observed until after 72 h of incubation. After 120 h, the concentrations of apoA-I_{+16} species detected were maximal. Subsequently, apoA-I_{+16} (and apoA-II_{+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_{+32} continued to accumulate at an enhanced rate, suggesting that it was derived from apoA-I_{+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).

Very similar trends were observed for apoA-II and its oxidized forms, though the aforementioned difficulties associated with the accurate quantification of apoA-II_{+32} levels precluded detailed analysis and quantification.

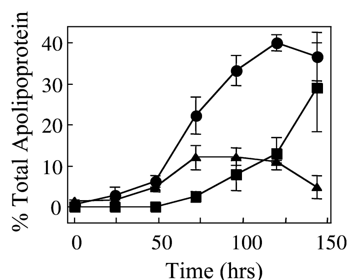

Fig. 3. Changes in HDL's apoA-I_{+16}, apoA-I_{+32}, and apoA-II_{+16} during the course of autoxidation in plasma. Plasma was incubated under air and at 37°C, and at the time points indicated, the HDL was isolated and apoA-I_{+16} (circle), apoA-I_{+32} (square), and apoA-II_{+16} (triangle) analyzed as described under Experimental Procedures. Data are shown as percent of total apolipoprotein peak area, and represent means ± SE of three separate experiments.

Inhibition of autoxidation

Their unambiguous separation and identification suggests that the specifically oxidized species of apoA-I and apoA-II may be useful markers of in vivo oxHDL and/or oxidative stress/damage to lipid and protein. We therefore investigated simple means to overcome ex vivo formation of these modified apolipoprotein species. Therefore, the transition metal ion chelator DTPA and the lipid-soluble antioxidant BHT were examined for their ability to inhibit apolipoprotein autoxidation, as was the impact of cooling or freezing of the plasma sample. BHT inhibits lipoprotein lipid peroxidation in the presence of vitamin E (33).

After 48 h of incubation of freshly obtained, untreated plasma under air at 37°C, 6% of the apoA-I and 5.3% of the apoA-II were found in their respective oxidized forms (normalized to 100% in **Table 3**). Both DTPA and BHT inhibited the oxidation of both proteins (Table 3), and when used in combination, their inhibitory effects were additive. However, in no case was complete inhibition of the oxidation process observed. Addition of methionine (50 mM), ascorbate (1 mM), EDTA (1 mM), or azide (1 mM) also failed to inhibit autoxidation (data not shown). In contrast, maintaining the plasma sample at 4°C greatly decreased, and storage at −20 or −80°C completely prevented, formation of any of the oxidized forms of apoA-I and apoA-II as compared with time 0 (Table 3).

Presence of oxidized apolipoproteins in vivo

Having established conditions that allowed collection and storage of samples without significant autoxidation of apoA-I and apoA-II to take place, we applied the modified HPLC method to groups of serum samples with or with-

TABLE 3. Effect of incubation time, temperature, and different inhibitor(s) on the formation of specifically oxidized apoA-I and apoA-II during the autoxidation of freshly obtained human plasma

Time	Temperature	Inhibitor	ApoA-I Oxidation	ApoA-II Oxidation
hours	*°C*		%	
0	37	—	0 ± 0	24.7 ± 4.1
48	37	—	100 ± 0	100 ± 0
48	37	DTPA (100 μM)	84.8 ± 2.9[a]	94.6 ± 4.0
48	37	BHT (100 μM)	65.3 ± 0.7[a]	76.8 ± 1.9[a]
48	37	DTPA (100 μM) + BHT (100 μM)	59.9 ± 2.2[a]	68.2 ± 2.3[a]
48	4	—	15.8 ± 10.4	30.5 ± 4.9
48	−20	—	3.0 ± 3.0	26.4 ± 2.8
48	−80	—	3.0 ± 3.0	26.7 ± 2.3

Freshly obtained human plasma was incubated under air in the absence and presence of the inhibitor(s) indicated before HDL was isolated and their apolipoproteins separated by HPLC as described in Experimental Procedures. Results are shown as percent of total apolipoprotein peak area detected, normalized to 100% for the 48 h/37°C samples, and represent means ± SE of three separate incubations of a plasma sample obtained from a single donor. The 100%-values for oxidized apoA-I and apoA-II varied for the three experiments, ranging from 4.4–9.4% and 4.4–5.5%, respectively. Data were analyzed using a paired Student's *t*-test.

[a] $P < 0.05$.

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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_{+32} (**Fig. 4**). HDL isolated from serum of the eNOSa/b genotype (16.6 ± 6.5%, n = 4) had five times higher apoA-I_{+32} than that of serum obtained from subjects with the common eNOSb/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_{+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 α-tocopherol remains present, apoA-I_{+16} ($MetO^{86}$ or $MetO^{112}$) and apoA-II_{+16} are formed and accumulate initially as specific oxidation products, whereas apoA-I_{+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_{+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 α-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 (1*e*) oxidants and conditions that give rise to 1*e*-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 α-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

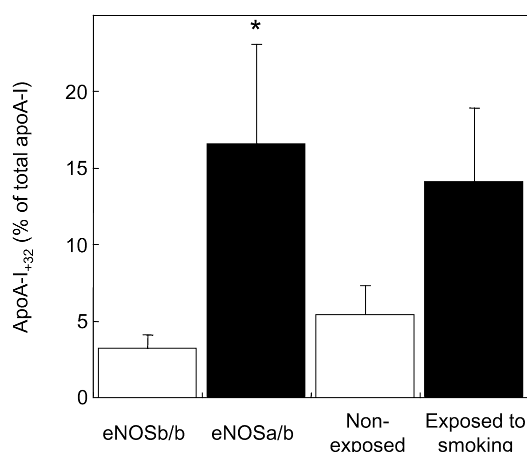

Fig. 4. Relationship between apoA-I_{+32} and eNOS genotypes and exposure to cigarette smoking. The concentration of apoA-I_{+32}, measured as described under Experimental Procedures, was determined in serum of cord blood in subjects of eNOSa/b (n = 4) and eNOSb/b genotype (n = 8), and in cord blood samples without (n = 12) and with exposure to cigarette smoking during pregnancy (n = 5). Both eNOSa allele carriers and cigarette smokers are associated with increased risk for vascular diseases. Results are expressed as a percentage of total apoA-I concentration and represent mean ± SD.

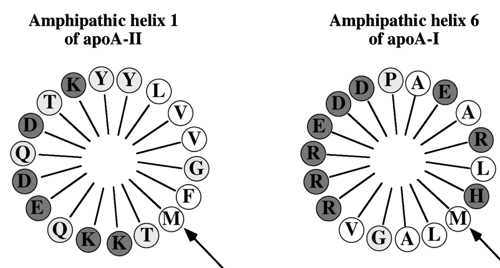

Fig. 5. Helical wheel diagrams of the putative amphipatic helices 1 and 6 of apoA-II and apoA-I, respectively. The models were constructed using the program PSAAM of Dr. A. Crofts, University of Illinois, and the rules of Chou and Fasman as previously described by Jonas et al. (40). Helical sequences begin with the Gln placed at the 0° position and proceed with the successive amino acid residue placed clockwise at 100° from the former. Empty, light gray, and dark gray filled circles represent hydrophobic, uncharged polar, and charged residues respectively. The position of the Met is marked with an arrow.

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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_{+16} and apoA-II_{+16} precedes that of apoA-I_{+32} and apoA-II_{+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*e*-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_{+16}/A-II_{+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_{+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_{+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_{+16} may offer sensitivity, the comparatively more stable nature of apoA-I_{+32} may make this form of oxidized apoA-I_{+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 nonpolar 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 nonpolar 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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