Oxidation of High Density Lipoproteins

I. FORMATION OF METHIONINE SULFOXIDE IN APOLIPROTEINS AI AND AII IS AN EARLY EVENT THAT ACCOMPANIES LIPID PEROXIDATION AND CAN BE ENHANCED BY \( \alpha \)-TOCOPHEROL

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The lipids of high density lipoproteins (HDL) are initially oxidized in preference to those in low density lipoprotein when human plasma is exposed to aqueous peroxyl radicals. In this work we report on the relative susceptibility of HDL protein and lipid to oxidation and on the role HDL's \( \alpha \)-tocopherol (\( \alpha \)-TOH) plays in modulating protein oxidation. Exposure of isolated HDL to either low fluxes of aqueous peroxyl radicals, \( \text{Cu}^{2+} \) ions, or soybean lipoxygenase resulted in the oxidation of apoAI and apoAII during the earliest stages of the reaction, i.e., after consumption of ubiquinol-10 and in the presence of \( \alpha \)-TOH. Hydro(pero)xides of cholesteryl esters and phospholipids initially accumulated together with specific oxidized forms of apoAI and apoAII, separated by high pressure liquid chromatography. The specific oxidized forms of apoAI were 16 and 32 mass units heavier than those of the native apolipoproteins and contained 1 and 2 methionine sulfoxide residues per protein, respectively. The third methionine residue in apoAI, as well as Trp residues, remained unoxidized during the earliest stages of HDL oxidation examined. Exposure of isolated apoAI to peroxyl radicals, \( \text{Cu}^{2+} \), or soybean lipoxygenase resulted in nonspecific (for peroxyl radicals) or no discernible protein oxidation (\( \text{Cu}^{2+} \) and soybean lipoxygenase). This indicated that the formation of the specific oxidized forms of apoAI observed with native HDL was not the result of direct reaction of these oxidants with the apolipoprotein. In vitro and in vivo enrichment of HDL with \( \alpha \)-TOH resulted in a dose-dependent increase in the extent of peroxyl radical-induced formation of HDL cholesteryl ester hydroperoxides (\( r = 0.96 \)) and cholesteryl ester hydroxides (\( r = 0.92 \)), as well as the loss of apoAI (\( r = 0.96 \)) and apoAII (\( r = 0.94 \)). \( \alpha \)-TOH enrichment also enhanced HDL lipid and protein oxidation induced by \( \text{Cu}^{2+} \) or soybean lipoxygenase. These results indicate that the earliest stages of HDL oxidation are accompanied by the oxidation of specific methionine residues in apoAI and apoAII and that in the absence of co-antioxidants, \( \alpha \)-TOH can promote this process.

Plasma levels of high density lipoprotein (HDL)\(^1\) cholesterol

\(^{1}\) The abbreviations used are: HDL, high density lipoproteins; AAPH, 2,2'-azo-bis(2-aminopropane) dihydrochloride; apoAI, apolipoprotein AI; apoAII, apolipoprotein AII; CE-OOH, cholesteryl ester hydroperoxides; CE-OH, cholesteryl ester hydroxides; \( \text{CoQ}_{10} \), ubiquinone-10; \( \text{CoQ}_{8} \), ubiquinol-10; LDL, low density lipoprotein; Met(O), methionine sulfoxide; PBS, phosphate-buffered saline; ROO\(^•\), peroxyl radical; SLO, soybean lipoxygenase; \( \alpha \)-TOH, \( \alpha \)-tocopherol; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; TMP, tocopherol-mediated peroxidation.

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of α-TOH is prevented by co-antioxidants that eliminate α-tocopherol radical (18), which otherwise propagates lipoprotein lipid peroxidation (14). It follows that α-TOH makes lipoproteins more reactive toward radical oxidants, and this can, depending on the conditions, lead to increased oxidation of lipoprotein lipids and, in principle, apolipoproteins.

The aim of the present study was to compare the susceptibility of HDL's antioxidants, polar and neutral lipids, and apoAI and apoAII to oxidation, using controlled and low fluxes of radical oxidants favoring TMP. The results obtained show that following consumption of ubiquinol-10 (CoQ10H2), oxidation of HDL induced by ROO•, Cu2⁺, or soybean lipoygenase (SLO) resulted in the oxidation of apoAI and apoAII which occurred concomitantly with α-TOH consumption and lipid peroxidation. The oxidation of apoAI and apoAII was initially targeted toward specific Met residues in the early stages of the reaction. Supplementation of HDL with α-TOH resulted in a greater degree of both lipid and specific apolipoprotein oxidation, independent of the oxidant used. In the accompanying article (19) we investigate the mechanism of apoAI- and apoAII-Met oxidation and provide evidence for Met oxidation by HDL-associated lipid hydroperoxides.

EXPERIMENTAL PROCEDURES

Materials—2,2'-Azo-bis(2-aminopropane) dihydrochloride (AAPH) was purchased from Wako (Osaka, Japan); EDTA and SDS were from Sigma; potassium bromide and dimethyl sulfoxide (Me2SO) were from BDH (Poole, UK) and Merck (Kilsyth, Victoria, Australia), respectively. PD-10 Sephadex G-25 M columns were from Pharmacia Biotech Inc. (Uppsala, Sweden); guanidine hydrochloride (ultra pure) was from Life Technologies, Inc. (Paisley, UK), and trifuoracetic acid (Uppsala, Sweden); guanidine hydrochloride (ultra pure) was from Life Technologies, Inc. (Paisley, UK), and trifuoracetic acid (Uppsala, Sweden). Ethanol and tert-buty alcohol were from BDH and Rhône-Poulenc (Paris, France), respectively. All other organic solvents were from Mallinckrodt (Clayton, Australia). Buffers were prepared from the highest quality material available and using nanopure water (MODULAB).

Isolation of HDL and ApoAI—Human HDL was isolated rapidly from freshly obtained EDTA plasma using a two-step density gradient and ultracentrifugation in a TL 100.4 rotor (Beckman Instruments, Palo Alto, CA) (20). HDL was isolated directly by needle aspiration after 4 h centrifugation at 100,000 rpm. Immediately prior to use in experiments, low molecular weight compounds were removed by size exclusion chromatography (PD-10 column), supplemented with 1 mM EDTA. HDL protein concentrations were estimated using the bicinchoninic acid method (Sigma) with bovine serum albumin (Sigma) as a standard; the HDL particle concentration was calculated by cholesterol determination, assuming an average of 35 molecules of free cholesterol per HDL particle. ApoAI, isolated by a standard procedure (21) with minor modifications (22), typically consisting of 3–7 °C incubation of HDL for 5 h in the presence of 528 μM AAPH (1.75–4 mM) which produces ROO•, Cu2⁺, or soybean lipoygenase (SLO) resulted in the oxidation of apoAI and apoAII which occurred concomitantly with α-TOH consumption and lipid peroxidation. The oxidation of apoAI and apoAII was initially targeted toward specific Met residues in the early stages of the reaction. Supplementation of HDL with α-TOH resulted in a greater degree of both lipid and specific apolipoprotein oxidation, independent of the oxidant used. In the accompanying article (19) we investigate the mechanism of apoAI- and apoAII-Met oxidation and provide evidence for Met oxidation by HDL-associated lipid hydroperoxides.

Fig. 1. Consumption of antioxidants and formation of lipid hydroperoxides in isolated HDL oxidized by AAPH. HDL was oxidized by AAPH (4 mM) at 37 °C in PBS supplemented with 1 mM EDTA. At the time points indicated, lipids and lipophilic antioxidants were analyzed as described under "Experimental Procedures." A shows depletion of CoQ10H2 (●) and α-TOH (●) and accumulation of CoQ10H2 (○). B shows formation of CE-OH (●), CE-OOH (△), and phosphatidylcholine hydroperoxides (○). Data shown are means ± S.E. of four separate experiments.
HDL Lipid and Protein Oxidation and Modulation by α-Tocopherol

FIG. 2. HPLC chromatograms of HDL apolipoproteins during oxidation initiated by AAPH. HDL was oxidized by AAPH (4 mM) at 37 °C. At the time points indicated below, samples were taken and apolipoproteins analyzed as described under “Experimental Procedures.” The major forms of apoAI and apoAII are labeled. The molecular masses of the various forms of apoAI, determined by MS, are given in parentheses; values are means from three experiments. The arrows indicate the appearance of oxidized forms of apoAI, designated apoAI-16 (see arrow a) and apoAI-32 (see arrow b). A through F represent samples taken at 0 (i.e. directly after AAPH addition) and 2–6 h of incubation, respectively. Data are from one experiment representative of four. Note the difference in scale between the y ordinates of A and B and C–F.

increased to 1.0 ml/min, and the content of acetonitrile increased to 90% over 5 min, and finally decreased to 40% over 10 min.

Characterization of Oxidized ApoAI by Mass Spectrometry—AAPH-oxidized HDL was subjected to HPLC, and the fractions of oxidized and unoxidized apoAI were collected, pooled, and analyzed by electrospray ionization MS using a single quadrupole mass spectrometer equipped with an electrospray ionization source (Platform, VG-Fisons Instruments, Manchester, UK). Samples (10 μl) were injected into a moving solvent (10 μl/min; H2O:acetonitrile 1:1 v/v, 0.05% trifluoroacetic acid) coupled directly to the ionization source via a fused silica capillary interface (50 μm x 40 cm). The source temperature was 50 °C, and N2 was used as the nebulizer gas. Sample droplets were ionized at a positive potential of ~3 kV, transferred to the mass spectrometer with a cone voltage of 60 V, and the peak width at half-height of 1 Da. Spectra were scanned over the mass range of 700 to 1800 Da in 5 s and calibrated with horse heart myoglobin (Sigma).

Amino Acid Analysis of ApoAI—Fractions of unoxidized and oxidized apoAI, collected as described above for the MS analysis, were dried under reduced pressure before 100 μl of a 50 mM CNBr solution in 100% acetonitrile, and 400 μl of formic acid were added. The mixture was top gassed with N2, sealed, and incubated in the dark for 18 h at 22 °C. H2O (5 volumes) was then added, the sample dried under reduced pressure, hydrolyzed in gaseous 6 M HCl containing 1.0% phenol (v/v), 0.01% mercaptoacetic acid (v/v), and analyzed for amino acids after derivatization with o-phthaldialdehyde (26). Trp loss was estimated by serial UV-210 nm and fluorescence (Ex280 nm/Em350 nm) monitoring of apoAI following HPLC separation and calculated from the UV/fluorescence ratio. Loss of endogenous fluorescence was also used as an index of Trp oxidation in intact human HDL (27). For this, aliquots (400 μl) of oxidizing HDL were added to 500 μl of PBS containing 1% (v/v) SDS, and the fluorescence was measured (Hitachi F-4010 fluorescence spectrophotometer) with Ex280 nm/Em350 nm.

RESULTS

Oxidation of HDL Lipids and Antioxidants—To define the temporal relationship between the consumption of HDL’s CoQ10H2 and α-TOH and the accumulation of oxidized lipids, isolated HDL was subjected to a constant low flux of ROO’ at 37 °C. The HDL particle concentration in these experiments was 14 ± 4 μM (mean ± SD, n = 4). Prior to oxidation, HDL contained α-TOH and total coenzyme Q10 at 0.56 ± 0.10 and 0.012 ± 0.005 molecules/particle, consistent with previous observations (7). Approximately 50% of HDL’s coenzyme Q10 was present as CoQ10H2 (Fig. 1), indicating that relatively little adventitious oxidative damage to HDL had occurred during its isolation. Upon initiation of oxidation, HDL’s CoQ10H2 was oxidized to CoQ10 within 30 min (Fig. 1), and this was followed by a gradual, linear loss of CoQ10. α-TOH was consumed in a time-dependent manner from the onset of oxidation and was below the limit of detection after 3–4 h incubation (Fig. 1). Fig. 1 also shows the kinetics of formation of phosphatidylcholine hydroperoxides, CE-OOH and CE-OH in AAPH-oxidizing HDL. The concomitant formation of CE-OOH and CE-OH in AAPH-oxidizing HDL were also used as an index of Trp oxidation in intact human HDL (27).
Amino acid content of apoAI in native and AAPH-oxidized HDL

| Amino acid | apoAI | apoAI-16 | apoAI-32 |
|------------|-------|----------|----------|
| Ala<sup>a</sup> | 100   | 100      | 100      |
| Arg        | 100 ± 3 | 94 ± 2  | 103 ± 2  |
| Asx        | 100 ± 2 | 97 ± 2  | 99 ± 3   |
| Glx        | 100 ± 1 | 103 ± 8 | 112 ± 7  |
| Gly        | 100 ± 3 | 101 ± 2 | 111 ± 4  |
| His        | 100 ± 3 | 100 ± 12| 100 ± 6  |
| Leu        | 100 ± 2 | 98 ± 2  | 99 ± 1   |
| Lys        | 100 ± 8 | 108 ± 3 | 94 ± 5   |
| Met<sup>b</sup> | 100 ± 5 | 61 ± 9  | 34 ± 6   |
| Met(O)<sup>c</sup> | 10 ± 1 | 49 ± 5  | 100 ± 5  |
| Phe        | 100 ± 1 | 109 ± 4 | 109 ± 4  |
| Ser        | 100 ± 4 | 104 ± 3 | 107 ± 8  |
| Thr        | 100 ± 1 | 108 ± 2 | 104 ± 4  |
| Tyr        | 100 ± 1 | 110 ± 5 | 104 ± 1  |
| Val        | 100 ± 2 | 109 ± 4 | 102 ± 4  |
| (Trp)<sup>d</sup> | 100 ± 8 | 97 ± 8  | 90 ± 10  |

<sup>a</sup> Samples were standardized to their content of Ala.

<sup>b</sup> Following reaction with CNBr, Met residues are converted to homoserine and its lactone. Therefore, the data for Met is based on the levels of homoserine detected.

<sup>c</sup> CNBr does not react with Met(O) which is reduced to Met during acid hydrolysis, and the data for Met(O) are based on the levels of Met detected.

<sup>d</sup> Trp not stable under the conditions of acid hydrolysis, was determined using serial UV 214 nm absorbance and fluorescence detection.

**Oxidation and Characterization of HDL Apolipoproteins**—To compare HDL lipid versus protein oxidation, we adapted an HPLC method that separates native apoAI and apoAI from oxidized forms containing specifically oxidized Met residues (24). Fig. 2A shows a representative chromatogram of apolipoproteins in native HDL; apoAI and apoAII were the major apolipoproteins identified, in agreement with previous observations (8). Oxidized forms of apoAI and apoAII were not detected in freshly isolated HDL. Apolipoproteins were eluted before apoAI (Fig. 2; see also Refs. 8 and 29). The mass of apoAI in native HDL was 28,079.5 ± 1.1 Da (mean ± SD, n = 3), in agreement with that predicted from its amino acid sequence (i.e. 28,078.7).

As oxidation progressed the content of HDL’s apoAI and apoAII decreased time-dependently. Representative chromatograms are shown for AAPH-induced oxidation (Figs. 2, B–F, and 3A), with qualitatively similar results being obtained with Cu<sup>2+</sup> or SLO as alternative oxidants (data not shown). Concomitant with the loss of unoxidized apoAI and apoAII, new peaks were detected (Fig. 2, B–F). The peak eluting before apoAI and apoAII has been designated as apoAI<sub>16</sub> and is known to contain one of the two Met<sub>32</sub> residues in apoAI dimer as Met sulfoxide (Met(O)) (8). In addition, a peak eluting with a retention time of 0.35 relative to apoAI increased in a time-dependent fashion (Fig. 2, B and F). The fraction corresponding to this peak was collected, and the mass of the compound was determined to be 28,111.9 ± 0.6 (mean ± S.D., n = 3), i.e. 32 mass units greater than that of unoxidized apoAI. This oxidized form of apoAI will be referred to as apoAI<sub>16</sub>. Formation of apoAI<sub>16</sub> is consistent with a previous study on proteolytic peptides derived from oxidized apoAI which suggested that the compound contained two (Met<sub>112</sub> and Met<sub>148</sub>) of the three Met residues as Met(O) (24).

In addition to apoAI<sub>16</sub> and apoAI<sub>32</sub>, oxidation of HDL with AAPH consistently resulted in the formation of a further product eluting close to apoAI (relative retention time of 0.97) (Fig. 2, B and F). During the early stages of oxidation this compound appeared as a leading shoulder on the apoAI peak (see arrow in Fig. 2B). As oxidation progressed, the compound became partially resolved from apoAI. By using a more shallow acetonitrile gradient (see “Experimental Procedures”) better separation was obtained, and a relatively pure preparation of this form of apoAI was collected. Upon re-chromatography of the collected fraction, a single peak was observed (not shown), the molecular mass of which was 28,095.9 ± 1.8 Da (mean ± S.D., n = 3), i.e. 16 Da greater than that of native apoAI; the compound was assigned apoAI<sub>16</sub>. The increased mass of 16 Da suggested introduction of one additional atom of oxygen, and the slight decrease in hydrophobicity was consistent with one of the three Met residues of apoAI being converted to Met(O). Amino acid analysis confirmed that approximately ~33% of the Met residues in apoAI<sub>16</sub> were depleted, whereas Met(O) levels were ~50% of those found in apoAI<sub>32</sub>, which contained 2 Met(O) (Table I). Amino acids other than Met were not oxidized in apoAI<sub>16</sub> (Table I), consistent with the molecular mass obtained. From this we conclude that apoAI<sub>16</sub> is a previously unrecognized oxidized product of apoAI, formed during the earliest stages of HDL oxidation.

Fig. 3 shows the time-dependent changes in the levels of apoAI, apoAII, and their oxidized forms apoAI<sub>16</sub>, apoAI<sub>32</sub>, and apoAI<sub>16</sub> during AAPH-induced oxidation of HDL. Protein oxidation was clearly detected after 1 h of incubation, i.e. after complete consumption of CoQ<sub>10</sub>H<sub>2</sub>, yet in the presence of α-TOPH (cf. Figs. 1 and 3). By 2 h, ~35 and 40% of apoAI and apoAII, respectively, were oxidized (Fig. 3), although α-TOPH...
that some Trp remained unoxidized. Portion of the grossly oxidized Trp products were also fluorescent orogenous fluorescence of

\[ \text{conductance} \]

of Isolated ApoAI—To assess whether the above described changes to apoAI (and apoAII) were due to direct interaction of the apolipoproteins with the oxidation-initiating species, we

\[ \text{oxidation of HDL devoid of} \]

first exposed isolated, lipid-free apoAI to AAPH. This resulted in a general broadening of the apoAI peak on HPLC chromatography without selective formation of apoAI\textsubscript{16} and apoAI\textsubscript{32} (Fig. 6), consistent with AAPH oxidizing several different amino acids in proteins (31), in addition to giving rise to Met(O) (32). Exposure of isolated apoAI to Cu\textsuperscript{2+} or SLO at 37 °C for up to 48 h also failed to result in specific formation of apoAI\textsubscript{16} and apoAI\textsubscript{32}, as indicated by the unaltered ratio of apoAI\textsubscript{32} to total apoAI (Fig. 7). Under these conditions oxidation of apoAI did not occur, the small amounts (typically < 5%) of apoAI\textsubscript{32} (eluting at 14.1 min in A) are present in the starting material, indicating that some apolipoprotein oxidation occurs during the isolation procedure.

**Role of α-Tocopherol in HDL Apolipoprotein Oxidation**—As oxidation of apoAI and apoAII was observed even when nearly normal levels of α-TOC were present (Figs. 1 and 3), the vitamin appeared not to protect HDL apolipoproteins from oxidative damage. Alternatively, the observed oxidation of apoAI and apoAII could have reflected events occurring in a subpopulation of HDL devoid of α-TOC since, on average, only one in two
HDL particles contained α-TOH. To distinguish between these two possibilities, HDL was enriched with α-TOH prior to oxidation. Such enrichment resulted in HDL which, on average, contained >1 molecule of α-TOH per particle (Table II). In α-TOH-enriched HDL exposed to AAPH, there was a striking increase in the extent of both loss of apoAI and apoAII and formation of oxidized apolipoproteins (Table II). This pro-oxidant effect of α-TOH was observed with in vitro and in vitro enriched HDL (Table II) and correlated directly with the extent of formation of CE-OH and CE-OH also increased with increasing α-TOH enrichment (r = 0.96 and 0.92 for CE-OH and CE-OH, respectively), consistent with HDL lipid peroxidation proceeding via TMP. The loss of Trp fluorescence also increased in α-TOH-enriched HDL although this effect was less pronounced than that observed for the formation of apoAI<sub>16</sub> and apoAI<sub>32</sub> (Table II).

To rule out the possibility that the pro-oxidant effect of α-TOH was a peculiarity associated with AAPH-induced oxidation, we also oxidized HDL with Cu<sup>2+</sup> and SLO. Similar to the situation with ROO·, supplementation of HDL with α-TOH increased the extent of CE-O(O)H formation regardless of the oxidant employed (Fig. 8). It has been shown previously that under the conditions employed, Cu<sup>2+</sup> and SLO oxidize lipoprotein lipids via TMP (17, 33). In all cases, increased lipid peroxidation was paralleled closely by increased levels of the Met(O)-containing forms of apoAI and AII in the α-TOH-supplemented HDL (Fig. 8).

**DISCUSSION**

Previous studies have shown that lipids in HDL can become oxidized before those in LDL (7) and that oxidation of HDL by Cu<sup>2+</sup> or lipid oxidation products derived from LDL can affect HDL functions related to reverse cholesterol transport (13). Mechanistic studies on the oxidation of HDL lipids and its relationship to apolipoprotein oxidation are therefore of potential physiological significance. The present study demonstrates that specific oxidation of HDL's apoAI and apoAII accompanies lipid peroxidation, occurs during the early α-TOH-containing stages of oxidation, is independent of direct reaction with the oxidants added, and can be promoted by α-TOH. The results demonstrate, for the first time, that in the absence of antioxidants, α-TOH can exert a pro-oxidant effect on proteins and that apolipoprotein oxidation represents an early event, even when mild oxidizing conditions are employed.

The HPLC method used for the measurements of apoAI and apoAII oxidation is based on a report by von Eckardstein et al. (24). These authors suggested that two (Met<sub>112</sub> and Met<sub>148</sub>) of the three Met residues in isolated apoAI are susceptible to oxidation and that neither or both of these two Met residues are oxidized (24). However, in the present study, using intact HDL rather than isolated apoAI, we detected a distinct modified form of apoAI with a molecular mass consistent with the addition of one oxygen atom to the native protein, i.e. one Met(O) as evidenced by amino acid analysis. Therefore, the previous result (24) that neither or both of the oxidation-susceptible Met residues in isolated apoAI become oxidized does not appear to hold when apoAI is oxidized in intact HDL. Future studies may reveal which of the Met residues is initially oxidized in apoAI or may confirm that both residues are equally susceptible to oxidation. It may be that oxidation of one of the Met residues in apoAI renders the second residue more susceptible to oxidation. Of possible significance, amino acid substitutions in apoAI peptides are known to affect Met oxidizability (24).

A previous study suggested that oxidized forms of apoAI are present in isolated human HDL (8); however, we have found no evidence for this in HDL isolated rapidly (20) from non-fasted normolipidemic volunteers (n = 9) (see e.g. Fig. 2A). The differences between these studies could be due to the isolation procedures employed; in vitro storage of HDL is known to produce modified apolipoproteins (34). The data in Table I suggest that ~10% of the Met in native apoAI may already be present as Met(O). However, the method used for Met determination relies on the conversion of Met to homoserine and its lactone by CNBr, so that upon hydrolysis of the sample under reducing conditions, Met(O) is detected as Met. The reaction of Met with CNBr is known to be less efficient when Met residues are located adjacent to Ser, due to an N- to O-acyl shift (35). Since Met<sup>16</sup> is adjacent to Ser<sup>27</sup> in apoAI, the conversion of Met<sup>16</sup> to homoserine may not be complete and the remaining

**Table II**

HDL enriched with α-tocopherol exhibits increased sensitivity of cholesteryl esters and Met residues of apoAI and apoAII to AAPH-induced oxidation

| Exp. | α-TOH<sub>n</sub> | CE-OH | CE-OH | apoAI | apoAI<sub>16</sub> | apoAI<sub>32</sub> | apoAII | apoAII<sub>a</sub> | Tryp |
|------|-----------------|-------|-------|-------|-----------------|-----------------|-------|-----------------|------|
| 1    | 2.2             | 1.68  | 1.39  | 0.54  | 3.09            | 1.95            | 0.59  | 1.22            | 0.85 |
| 2    | 3.0             | 2.87  | 2.39  | 0.74  | 2.97            | 2.15            | 0.64  | 1.50            | 0.98 |
| 3    | 5.4             | 3.11  | 2.23  | 0.72  | 2.50            | 3.31            | 0.27  | 1.56            | 0.95 |
| 4    | 17.6            | 8.54  | 3.90  | 0.45  | 3.27            | 4.73            | 0.14  | 0.83            | 0.85 |

<sup>a</sup> α-TOH enrichment, expressed as the ratio of α-TOH content after to before enrichment, as measured before oxidation (t = 0 h).
Met erroneously assigned as Met(O), thereby overestimating the content of the latter in native apoAI. In support of this, it is difficult to create CNBr peptides of canine apoAI, where Met is the sole Met residue of the protein flanked by Ser87 (36). Thus the amount of Met(O)-containing apoAI present in circulating HDL has yet to be defined unequivocally.

An important finding of the present work is that apoAI and apoAII oxidation proceeds while HDL’s content of α-TOK remains largely intact. It was not possible to assess whether protein oxidation occurred in the presence of CoQ10H2 as the detection of protein oxidation by UV absorbance is much less sensitive than the electrochemical detection of CoQ10H2 (mean ± S.E.), respectively, before oxidation. CE-O(O)H (A and B) refers to CE-OH plus CE-OH. Apolipoprotein oxidation (C and D) is expressed as the ratio of the peak area of the oxidized apolipoproteins (i.e., apoAI16, apoAI132, apoAIIa) divided by the total peak area of oxidized and native apolipoproteins present in each sample. Data shown are means ± S.E. of three separate experiments.

**Fig. 8. Oxidation of HDL by Cu2⁺ or SLO.** HDL was oxidized at 37 °C by SLO (A and C, 4 × 10⁵ units/ml) or Cu2⁺ (B and D, at 1.51 molar ratio with HDL) and lipids and apolipoproteins analyzed as described in the legends to Figs. 1 and 2. Analyses were performed on in vitro α-TOK-enriched (C) and non-enriched HDL (○), which contained 52 ± 5 and 5 ± 1 μM α-TOK (mean ± S.E.), respectively, before oxidation. CE-O(O)H (A and B) refers to CE-OOH plus CE-OH. Apolipoprotein oxidation (C and D) is expressed as the ratio of the peak area of the oxidized apolipoproteins (i.e., apoAI16, apoAI132, and apoAIIa) divided by the total peak area of oxidized and native apolipoproteins present in each sample. Data shown are means ± S.E. of three separate experiments.
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