Oxidative events cause degradation of apoB-100 but not of apo[a] and facilitate enzymatic cleavage of both proteins

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Abstract **Lipoprotein [a] (Lp[a]) contains equimolar amounts of apoB-100 and apolipoprotein [a] (apo[a]). Both proteins are amenable to degradation in vivo by mechanisms yet to be clearly defined. In this study, we examined the in vitro susceptibility of LDL and Lp[a], obtained from the same donor, to oxidation by either Cu^{2+} or the combined *Crotalus adamanteus* phospholipase A_2 and soybean lipoxygenase system, monitoring the course of the reaction by the generation of conjugated dienes and fatty acids. In some experiments, treatment with leukocyte elastase (LE) or matrix metalloproteinase 12 (MMP-12) was administered before and after the oxidative step. In the case of Lp[a] we found that with both oxidizing systems, conditions that caused the breakdown of apoB-100 did not degrade apo[a] although oxidation-mediated changes were detected in the latter by intrinsic tryptophan fluorescence spectroscopy. Similar results were obtained with a reassembled Lp[a] obtained by incubating free apo[a] with LDL. Both apo[a] and apoB-100 were cleaved by LE and MMP-12 but the enzymatic cleavage was more marked when the preoxidized proteins were used as a substrate. Taken together, our in vitro studies indicate that apo[a] but not apoB-100 resists oxidative fragmentation, whereas both proteins are cleaved by enzymes of the serine and metalloproteinase families. We speculate that the fragments of apo[a] observed in vivo may be preferentially generated by proteolytic rather than oxidative events, whereas apoB-100 can be degraded by both mechanisms.** —Edelstein, C., K. Nakajima, D. Pfaffinger, and A. M. Scanu. **Oxidative events cause degradation of apoB-100 but not of apo[a] and facilitate enzymatic cleavage of both proteins.** ***J. Lipid Res.*** **2001.** 42: **1664–1670.**

Supplementary key words apolipoprotein [a] • apolipoprotein [a] fragments • apolipoprotein B-100 • proteolysis • oxidation

Lipoprotein [a] (Lp[a]) is a particle in which apolipoprotein [a] (apo[a]), a specific multikringle structure, is linked by a single disulfide bridge to an apoB-100-containing lipoprotein, which in the plasma of normotriglyceridemic subjects is mainly represented by an LDL (1). Apo[a] can also occur in an unbound form, either intact (2) or fragmented (3–7). The mechanisms underlying the generation of these products are not understood, except that in the case of fragments, an enzymatic participation has become apparent on the basis of the results of in vitro and in vivo studies (3, 8, 9). Regarding the apoB-100 constituent of authentic LDL, its breakdown has been shown to occur in "maximally" oxidized particles (10) and also following the action of biologically occurring enzymes of the elastase and metalloproteinase families (3, 9). In the case of apoB-100 as a constituent of Lp[a], information about the mechanisms underlying its degradation as a function of oxidative versus proteolytic events is comparatively limited, and this also applies to apo[a]. At present it is unclear whether the LDL constituent of Lp[a] is susceptible to oxidative and proteolytic changes as is authentic LDL and whether apo[a], shown to be cleaved by proteolytic enzymes, can also be degraded by oxidative events. We reasoned that an understanding of these processes in vitro would provide a useful background for investigating the mechanism(s) of formation of Lp[a] fragments in vivo. To this end, in the current study we used in vitro systems to study the effect of oxidative and proteolytic conditions on purified preparations of human LDL, Lp[a], and free apo[a], all obtained from the same subject. We show here that oxidative events that in Lp[a] cause the breakdown of apoB-100 do not affect the cleavage of apo[a], whereas both proteins are readily cleaved by the action of proteolytic enzymes of the serine and metalloproteinase families.

MATERIALS AND METHODS

Materials

ε-Aminocaproic acid (EACA), (4-amidinophenyl)-methanesulfonyl fluoride (APMSF), human leukocyte elastase (LE) (EC

Abbreviations: apo[a], apolipoprotein [a]; APMSF, (4-amidinophenyl)-methanesulfonyl fluoride; DENP, diethyl *p*-nitrophenyl phosphate; EACA, ε-aminocaproic acid; LE, leukocyte elastase; Lp[a], lipoprotein [a]; MMP-12, matrix metalloproteinase 12; PAH-AH, platelet-activating factor hydrolase; PLA_2, phopholipase A_2; SLO, soybean lipoxygenase.

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3.4.21.37), soybean lipoxygenase (EC 1.13.11.12) type V (SLO), diethyl *p*-nitrophenyl phosphate (DENP), and PBS packets were from Sigma (St. Louis, MO). Phospholipase A_2 (PLA_2) (EC 3.1.1.4) isolated from *Crotalus adamanteus* venom was obtained from Worthington Biochemical (Freehold, NJ). Immobilon-P membranes were from Millipore (Bedford, MA) and an enhanced chemiluminescence kit (ECL Western blotting detection kit) was from Amersham (Arlington Heights, IL).

Antisera to purified preparations of apo[a], Lp[a], and LDL were raised in rabbits and affinity-purified antibodies to apo[a], Lp[a] (anti-Lp[a]), and LDL (anti-apoB) were prepared as previously described (9). Anti-Lp[a] was shown to be devoid of immunoreactivity to LDL and plasminogen; anti-apoB was unreactive to apo[a]. All other chemicals were of reagent grade.

Preparation of human Lp[a], apo[a], and LDL

Lp[a], having a single apo[a] isoform with 14 kringle IV-2 repeats, was isolated from the plasma of a normal donor by combining ultracentrifugation and lysine-Sepharose column chromatography as described previously (11). Apo[a] was separated from the purified Lp[a] under mild reductive conditions as previously described (8). Authentic LDL free of apo[a] was isolated at d 1.030–1.050 from the plasma by sequential flotation (12). The final products were homogeneous by 1% agarose and 4% polyacrylamide gel electrophoresis and shown not to be cross-contaminated by immunoblot analyses using polyclonal antisera against apoB-100 and apo[a].

Oxidation studies

Two conditions were used: *1*) enzymatic, making use of the combined action of *C. adamanteus* PLA_2 and SLO; and *2*) action of the transition metal Cu^{2+} in the form of $CuSO_4$. All studies were conducted in triplicate and normalized to the concentration of apoB.

PLA_2-SLO system. Aliquots of 0.5 mg/ml of either LDL or Lp[a] in terms of apoB protein were incubated with PLA_2 (mass ratio, 325:1, protein:enzyme) and SLO (mass ratio, 325:25, protein: enzyme) at 37°C in a 10 mM Tris buffer containing 1 mM $CaCl_2$, 150 mM NaCl, and 0.02% NaN_3, pH 7.4. In some experiments the reactions were conducted in the presence of either APMSF or DENP as inhibitor of the LDL-associated PLA_2 or platelet-activating factor hydrolase (PAH-AH). The reaction was stopped by the addition of 10 mM EDTA as a final concentration.

Cu^{2+} system. Both Lp[a] and LDL contained 0.1 apoB at mg/ml. The oxidation was conducted in PBS, pH 7.4, in the presence of 50 μM APMSF and aprotinin (2 μg/ml) at 37°C with 5, 10, 20, and 30 μM $CuSO_4$ as a function of time.

In both systems the progress of the reaction was monitored at timed intervals by the release of fatty acids, using a commercial kit (NEFA C; Wako, Richmond, VA). This kit utilizes a colorimetic method for the quantitation of nonesterified (or free) fatty acids. The method relies on the acylation of CoA by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase, permits the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)-aniline with 4-aminoantipyrene to form a purple-colored adduct that can be measured colorimetrically at 550 nm. Diene generation was measured by absorbance at 234 nm. Aliquots were taken for Western blot analyses in order to identify apo[a] and apoB.

Oxidation of reassembled Lp[a]

According to our previous studies (8), free apo[a] was added to LDL at a molar ratio of 50:1 (LDL to apo[a]) in either the presence or absence of 0.2 M EACA, an agent known to prevent the first step of Lp[a] assembly (13). The system was then subjected to the action of PLA_2-SLO or Cu^{2+} and the course of the reaction was monitored as described above. At each time point, immunoblot analyses were also conducted.

Isolation and properties of apo[a] from oxidized Lp[a]

For this purpose, we used an Lp[a] oxidized with 5 μM Cu^{2+} for 3 h after verifying that there was no apo[a] breakdown up to 9 h as assessed by 4% SDS-PAGE. Apo[a] was isolated from Lp[a] as described previously (8).

Direct interaction of free apo[a] with Cu^{2+}

Studies were conducted at 37°C as a function of Cu^{2+} concentration (0–30 μM) at an apo[a] concentration of 0.05 mg/ml in 10 mM phosphate buffer, pH 7.4.

Fluorescence spectroscopy

Relative fluorescence intensity was measured at 22°C in a Perkin-Elmer (Norwalk, CT)) LS50B spectrofluorimeter with an excitation at 290 nm (bandwidth of 5 nm for both excitation and emission slits) in 10 mM phosphate buffer, pH 7.4. For each sample, an average of 10 scans was analyzed.

Amino-terminal sequence analyses

The oxidized apo[a] samples were subjected to automated Edman degradation on an Applied Biosystems (Foster City, CA) 477A unit, using procedures recommended by the manufacturer. All sequencing procedures were carried out in the core laboratory of the Macromolecular and Structural Analysis Facility at the University of Kentucky (Lexington, KY).

Studies with proteolytic enzymes

These studies were designed to determine whether the apo[a], after oxidation of Lp[a], could be cleaved to the same extent as apo[a] from native Lp[a], by LE and matrix metalloproteinase 12 (MMP-12), enzymes that we showed previously to cleave this apolipoprotein whether free or bound to Lp[a] (3, 4, 9). In the current studies, we oxidized Lp[a] with 5 μM Cu^{2+} for 3 h at 37°C and terminated the reaction by the addition of 5 mM EDTA. The samples were subsequently dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, 0.02% NaN_3, pH 8.0, with 0.05% Brij 35 for MMP-12 or without this detergent for LE.

Limited proteolysis with LE. The enzyme (1 unit = 1 nmol of *p*-nitrophenol per s from *N*-1-BOC-L-Ala *p*-nitrophenol ester) was diluted 1,000-fold in 50 mM Tris-HCl, 100 mM NaCl, pH 8.0. One microliter of the diluted enzyme was incubated with 7.5 μg of Lp[a] or LDL protein or with 15 μg of apo[a] at 37°C for 30 min. The reaction was terminated by the addition of 5 mM APMSF for 20 min at 22°C.

Limited proteolysis with MMP-12. The active enzyme was incubated with human Lp[a]/apo[a] (final apo[a] concentration, 0.2 mg/ml) or LDL at a 25:1 weight ratio of protein to enzyme at 37°C as a function of time (0–7 h) in 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3; EDTA was added to a final concentration 50 mM to stop the reaction. Thereafter, SDS-PAGE was performed under reduced conditions.

Electrophoretic methods

SDS-PAGE (4% polyacrylamide) was performed on a Novex (San Diego, CA) system for 1.5 h at constant voltage (120 V) at 22°C as previously described (3). Immediately after electrophoresis, the gels were placed onto Immobilon-P sheets (Millipore) that had been previously wetted with a buffer containing

48 mM Tris, 39 mM glycine, pH 8.9. Blotting was performed on a horizontal semidry electroblot apparatus (Pharmacia-LKB, Piscataway, NJ) at 0.8–1 mA/cm^2 for 45 min at 22°C.

Immunoblotting

After electroblotting, the Immobilon-P sheets were blocked in PBS containing 5% nonfat dry powdered milk and 0.3% Tween 20 followed by incubation with anti-apo[a] or anti-apoB-100 antibody (3). Subsequently, the blots were developed with the ECL Western detection reagent (Amersham) according to the manufacturer instructions.

Lipoprotein and apolipoprotein analyses

Lp[a] and LDL protein were quantitated by a sandwich ELISA essentially as previously described (14) except that anti-Lp[a] IgG was used as the capture antibody and anti-apoB-100 IgG conjugated to alkaline phosphatase was used as the detection antibody. For the ELISA quantitation of apo[a], anti-apo[a] IgG conjugated to alkaline phosphatase was used as the detection antibody. Protein determinations were performed by the Bio-Rad (Hercules, CA) DC protein assay.

RESULTS

Studies of Lp[a]

Cu^{2+} system. PRODUCTION OF DIENES AND FFA FROM LP[A] AND LDL. On incubation with 5 μM Cu^{2+} there was a lag phase of about 120 min for Lp[a] and 110 min for LDL (**Fig. 1**). In addition, the maximum amount of dienes produced before the onset of decomposition was higher for LDL than Lp[a]. These values were 57.5 μM for LDL and 33.8 μM for Lp[a], assuming a molar absorbance of $\varepsilon_{234\ nm}$ for conjugated dienes of 29,500 L · mol^{-1} · cm^{-1}. On the other hand, there was no significant production of FFA in both lipoproteins. Essentially the same results were obtained when the reactions were carried out in the presence of APMSF or DENP, both inhibitors of PAH-AH, indicating that our preparations had no significant amount of active enzyme (see also below).

IMMUNOBLOT ANALYSES. On reduced 4% SDS-PAGE, during the 5-h incubation, there was no degradation of apo[a] (**Fig. 2**, left). On the other hand, under the same conditions, apoB-100 underwent degradation that increased as a function of time. The resistance of apo[a] to degradation was observed up to the 9-h point, the maximum time examined (data not shown).

EFFECT OF MMP-12 ON OXIDIZED LP[A]. In these experiments native Lp[a] treated with MMP-12 served as a control. In each experiment Lp[a] was incubated with the enzyme for up to 5 h. Oxidation by 5 μM Cu^{2+} promoted an increase in proteolysis by MMP-12 as shown by the progressive decrease in the intensity of the apo[a] (**Fig. 3**, upper middle panel) and the appearance of bands with a migration corresponding to F1 (kringles KIV 1–4) and F2 (kringles KIV 5–10, KV, and the protease region) as previously reported with this enzyme (4). In contrast, proteolysis of native Lp[a] progressed slower than that of the oxidized lipoprotein, reaching a plateau at 3 h. The action of MMP-12 was also more marked with oxidized than nonoxidized apoB-100 (Fig. 3, lower left and middle panels). Comparable results were obtained with elastase (data not shown).

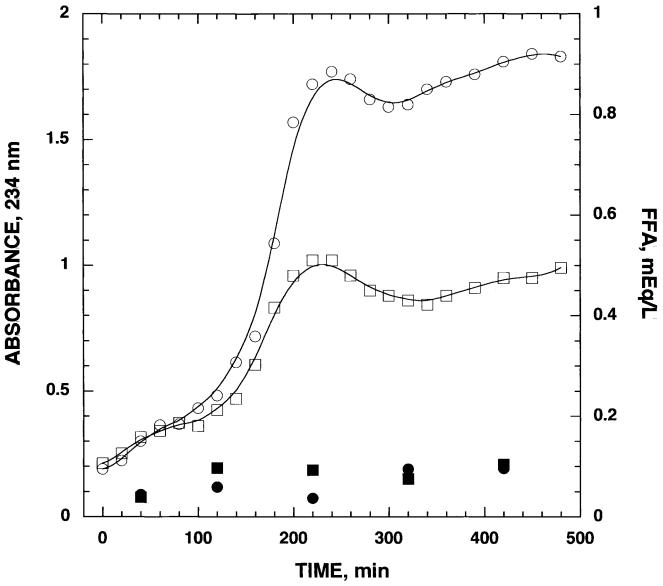

Fig. 1. Time course of the generation of dienes and FFA from LDL and Lp[a] after oxidation by $CuSO_4$. LDL and Lp[a] were incubated at 37°C with 5 μM $CuSO_4$ at an apoB-100 protein concentration of 0.1 mg/ml. At the indicated time points aliquots were taken for analyses and the reaction was stopped by adding 5 mM EDTA (final concentration). Dienes were measured by absorbance at 234 nm; FFA was measured by a commercial kit (see Materials and Methods). Open circles: dienes generated by LDL; open squares: dienes generated by Lp[a]; closed circles and squares: FFA from LDL and Lp[a], respectively.

STUDIES OF APO[A] ISOLATED FROM OXIDIZED LP[A]. Sequence analyses showed an N-terminal amino acid (glutamate) identical to that of the N terminus of native apo[a] and consistent with the absence of protein degradation as shown by the immunoblot data (Fig. 2). However, Cu^{2+} oxidation caused changes in tryptophan fluorescence spectra (**Fig. 4**). Compared with control apo[a] (solid line), a 20% decrease in fluorescence intensity was

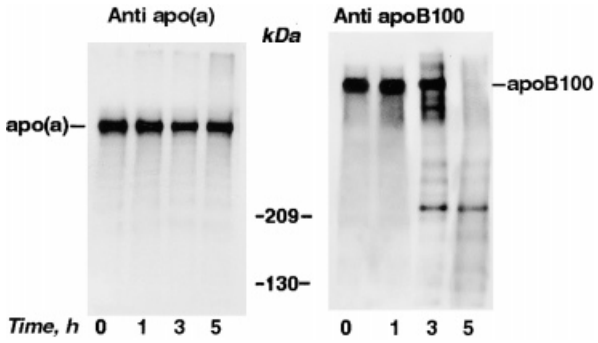

Fig. 2. Time course of the oxidation of Lp[a] by $CuSO_4$. Lp[a] was incubated with Cu^{2+} as described in the legend to Fig. 1 and, after stopping the reaction at each time point with EDTA, aliquots of the samples were prepared for gel analysis, reduced with 3% 2-mercaptoethanol, separated by 4% SDS-PAGE, and Western blotted. Rabbit anti-apo[a] (left) and rabbit anti-apoB-100 (right) were used to probe the blots.

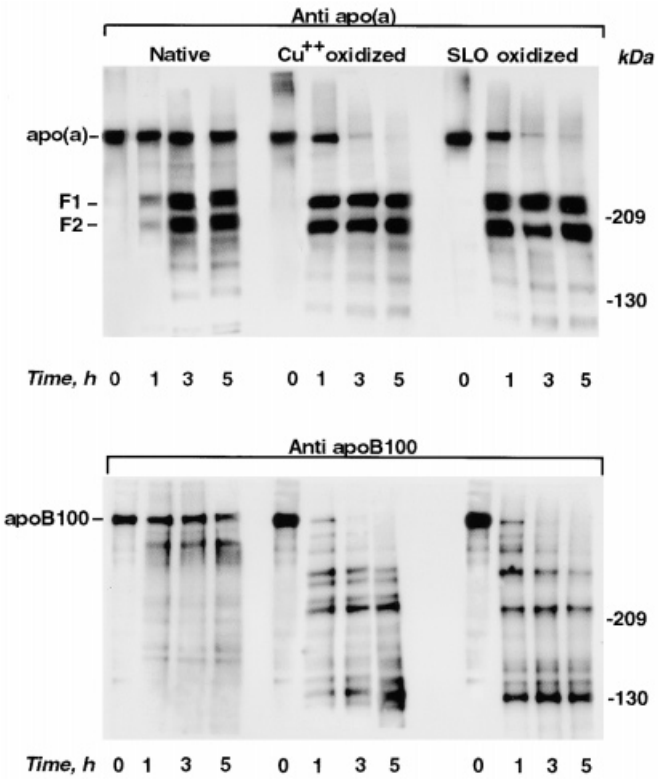

Fig. 3. Time course of proteolysis of Lp[a] by MMP-12 before and after oxidation. Lp[a] was oxidized by either Cu^{2+} or SLO and native unoxidized or oxidized Lp[a] was digested by incubation with MMP-12 for the indicated time periods at 37°C. Each reaction was terminated by the addition of EDTA (50 mM, final concentration), prepared for gel analysis under reduced conditions, separated by 4% SDS-PAGE, and Western blotted. Top: Rabbit anti-apo[a]-immunostained blots. Bottom: Rabbit anti-apoB-100-immunostained blots.

observed for oxidized apo[a] along with a small red shift in the tryptophan emission peak (from 343 to 345 nm). The results at 3 h were not significantly different from those examined at the 9-h time point.

SLO system. PRODUCTION OF DIENES AND FFA FROM LP[A] AND LDL. For both Lp[a] and LDL the generation of dienes paralleled that of FFA (**Fig. 5**). However, in each instance the generation of the two analytes was more marked in LDL than Lp[a]. The results were not affected by the presence of APMSF, an inhibitor of PAH-AH, indicating that the FFA were produced by the venom PLA_2.

IMMUNOBLOT ANALYSES. Within the 9-h incubation there was no breakdown of apo[a] (**Fig. 6**). However, at the 5- and 9-h points some apo[a] aggregates had formed as exhibited by the high molecular weight bands. On the other hand, at the same time points, apoB-100 exhibited a marked degradation. With this protein there was no evidence of aggregation.

EFFECT OF MMP-12 ON OXIDIZED LP[A]. Both apo[a] (Fig. 3, upper right panel) and apoB-100 (Fig. 3, lower right panel) were more readily hydrolyzed by MMP-12 compared with their native counterparts. There was a progressive decrease in undegraded apo[a] and the appearance

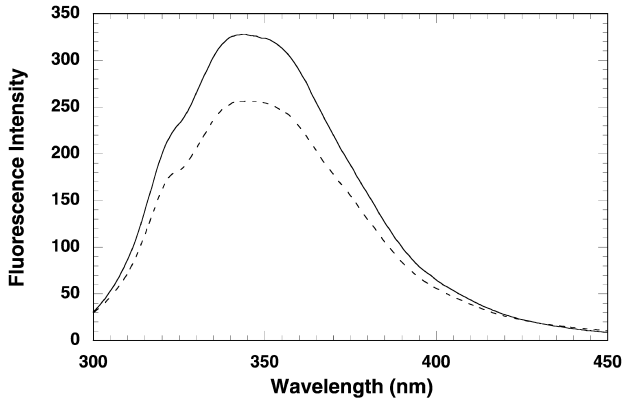

Fig. 4. Intrinsic tryptophan fluorescence emission spectra (uncorrected) of apo[a] isolated from native and oxidized Lp[a]. Lp[a] was oxidized with 5 μM Cu^{2+} as described in the legend to Fig. 1 for 3 h at 37°C. After the reaction was terminated with EDTA, apo[a] was isolated and purified from Lp[a]. Solid line, apo[a] isolated from unoxidized native Lp[a]; dashed line, apo[a] isolated from oxidized Lp[a].

of two major staining bands corresponding to the mobility of apo[a] fragments F1 and F2 along with bands of lower intensity and faster mobility.

STUDIES OF APO[A] ISOLATED FROM OXIDIZED LP[A]. As with the copper-oxidized product, PLA_2/SLO-oxidized apo[a] showed a single N-terminal amino acid compatible with the retention of its full length. Moreover, compared with control apo[a], there was a 35% decrease in tryp-

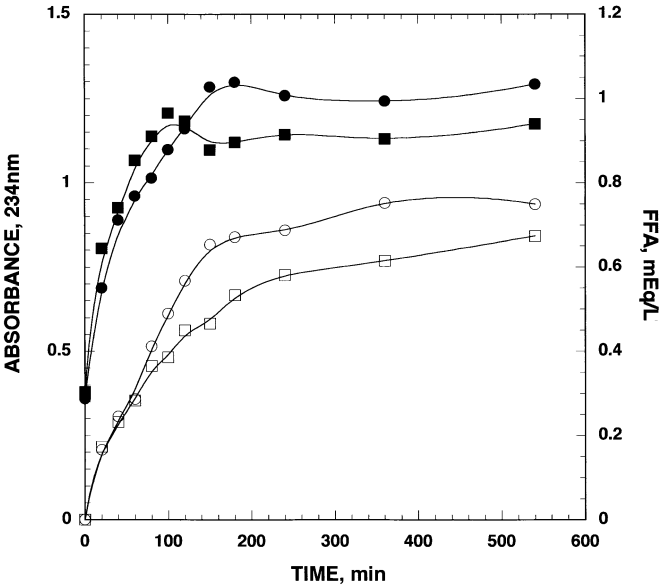

Fig. 5. Time course of the production of dienes and FFA from LDL and Lp[a] on oxidation by PLA_2/SLO. LDL and Lp[a] were incubated with PLA_2/SLO at 37°C . At the indicated time points aliquots were taken for analyses and the reaction was stopped by adding 10 mM EDTA (final concentration). Dienes were measured by absorbance at 234 nm; FFA by a commercial kit. Open circles, dienes generated by LDL; open squares, dienes generated by Lp[a]; closed circles and squares, FFA from LDL and Lp[a], respectively.

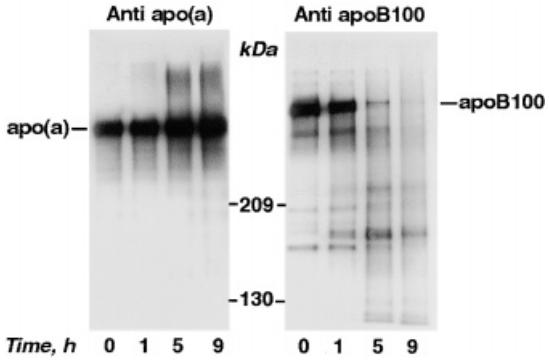

Fig. 6. Time course of the oxidation of Lp[a] by PLA_2/SLO. Lp[a] was incubated with PLA_2/SLO and after stopping the reaction at each time point with EDTA, aliquots of the samples were prepared for gel analysis, reduced with 3% 2-mercaptoethanol, separated by 4% SDS-PAGE, and Western blotted. Rabbit anti-apo[a] (left) and rabbit anti-apoB-100 (right) were used to probe the blots.

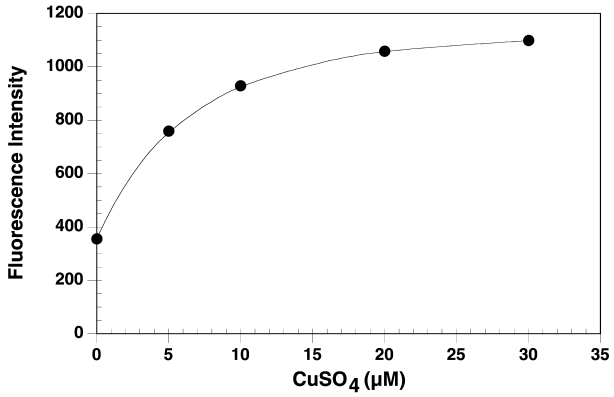

Fig. 8. Change in intrinsic fluorescence on addition of Cu^{2+} to apo[a]. Apo[a] (50 μg/ml) was incubated in buffer (10 mM phosphate, pH 7.4) containing 0, 5, 10, 20, and 30 μM $CuSO_4$ at 37°C. After 3 h, the reactions were terminated by the addition of EDTA (5 mM, final concentration). After dialysis against 10 mM phosphate buffer, pH 7.4, tryptophan fluorescence intensity was measured at 343 nm with excitation at 290 nm.

tophan fluorescence intensity indicating oxidation of tryptophan residues (**Fig. 7**). This change was accompanied by a small blue shift in wavelength of the tryptophan emission peak (from 343 to 340 nm), apparently resulting from a subtle conformational change of the oxidized apo[a].

Studies of free apo[a]

Effect of Cu^{2+}. Free apo[a] exposed to different copper concentrations from 5 to 30 μM exhibited no breakdown as shown by immunoblot analyses (data not shown). Evidence of the occurrence of chemical changes was provided by the fluorescence spectra exhibiting a progressive increase in tryptophan fluorescence intensity at the maximum emission wavelength of 343 nm as a function of Cu^{2+} concentration (**Fig. 8**). As compared with the native apo[a], the modified protein was also more susceptible to the action of LE (not shown) and MMP-12 (**Fig. 9**) as indicated by the relative amount of undigested apo[a] and the increase in the intensity of bands with a faster mobility. Although both the native and oxidized apo[a] appeared to reach a plateau after 1 h of incubation with the enzyme, the extent of degradation for oxidized apo[a] was greater than for the native protein.

Effect of oxidation on apo[a] after reincorporation into an Lp[a] particle. In these studies, apo[a] was added to LDL (1:50 molar ratio, apo[a]:apoB) in either the presence or absence of 0.2 M EACA. The mixture was then subjected to either Cu^{2+} or PLA_2-SLO oxidation under conditions described in Materials and Methods. In the absence of EACA, the course of diene and FFA generation was similar to that observed with Lp[a] subjected to the same oxidation conditions. Moreover, by immunoblot analyses, the apo[a] was intact. In the presence of EACA, the pattern of diene and FFA generation resembled that of LDL (data not shown).

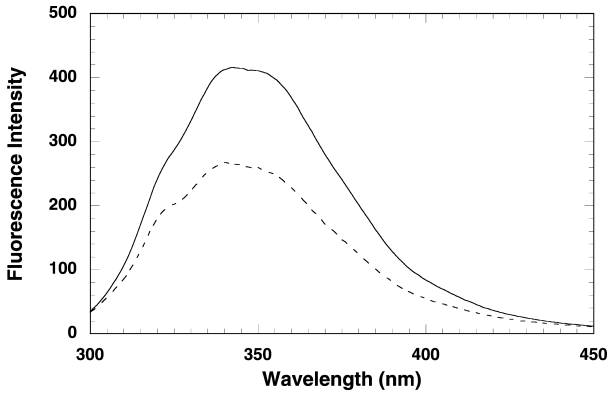

Fig. 7. Intrinsic tryptophan fluorescence emission spectra (uncorrected) of apo[a] isolated from native and oxidized Lp[a]. Lp[a] was oxidized with PLA_2/SLO and the reaction was terminated with EDTA, and apo[a] was isolated and purified from Lp[a]. Solid line, apo[a] isolated from unoxidized native Lp[a]; dashed line, apo[a] isolated from oxidized Lp[a].

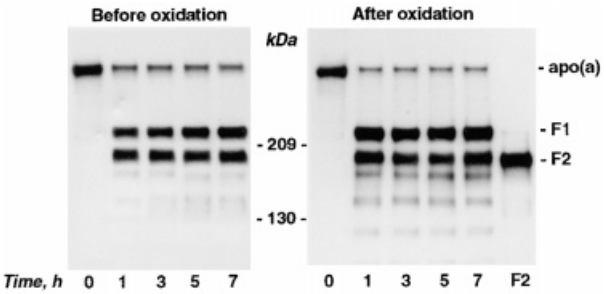

Fig. 9. Time course of proteolysis of apo[a] by MMP-12 before and after oxidation. Apo[a] was oxidized by 5 μM $CuSO_4$ for 3 h and dialyzed. Both the native and oxidized protein were incubated with MMP-12 for the indicated time periods at 37°C. The digestions were terminated by the addition of 50 mM EDTA (final), and then prepared for gel analysis under reduced conditions and separated on 4% SDS-PAGE. Immunoblotting was performed with rabbit anti-apo[a].

DISCUSSION

In the current study we have shown that Lp[a], under the pro-oxidant conditions created by either Cu^{2+} or PLA_2-SLO, undergoes cleavage of apoB-100 but not apo[a]. This difference in susceptibility to degradation may relate to the fact that apo[a], being nonlipophilic and projecting away from the lipoprotein surface (2), was inaccessible to the free radicals generated by lipid peroxidation. Against this view are the spectroscopic data indicating that the free apolipoprotein isolated from the oxidizing systems had undergone chemical modification as assessed by tryptophan fluorescence analyses. The more likely possibility is that, under our experimental conditions, the resistance to degradation by apo[a] was due to its intrinsic structural properties in either the kringle domains, nonkringle domains, or both. Of interest in this regard was our observation (C. Edelstein, K. Nakajima, D. Pfaffinger, and A. M. Scanu, unpublished observations) that plasminogen, a kringle-containing protein structurally similar to apo[a], retains its full length when incubated with an LDL undergoing oxidation by either Cu^{2+} or PLA_2-SLO. Whether resistance to oxidative degradation also applies to other kringle-containing proteins remains to be established.

We chose the copper oxidation system on two accounts: *1*) as a model it can be compared with the extensive literature on copper-mediated oxidation of LDL; and *2*) copper is one of the components of the atherosclerotic plaque (15) and has also been implicated in the atherosclerotic process in animal models (16).

In the SLO system, the oxidized FFA were released by the action of a venom PLA_2, which appeared to be mainly responsible for the observed modifications of apoB-100 and apo[a] because inhibitors of PAH-AH had no effect on the course of the reaction. There was no significant production of FFA during Cu^{2+} oxidation, suggesting that the changes in apoB-100 and apo[a] were to a large extent related to the action of oxidized polyunsaturated fatty acid esterified to the phospholipid and cholesterol of the LDL constituent of Lp[a].

We consistently observed that the lipid-mediated oxidative events were significantly less marked in Lp[a] than in LDL. One plausible explanation for this finding is that apo[a] exhibited a partial protective effect by diverting from LDL a portion of the free radicals generated by fatty acid oxidation. This scavenging action would be consistent with the chemical changes detected by fluorescence analyses in the apo[a] isolated from both oxidized Lp[a] and oxidized reconstituted Lp[a]. This view is also consistent with the finding that apo[a] in Lp[a] modified by the exposure to oxidized fatty acid, was cleaved by either LE or MMP-12 more readily than untreated apo[a]. A partial "protective" action of apo[a] on LDL oxidation was also previously noted by Sattler et al. (17) using radiolabeled Lp[a]. Those authors attributed the effect to the sialic content of apo[a].

In the absence of lipid-mediated events, copper modified but did not degrade free apo[a]. Although our observations were centered around changes in tryptophan fluorescence, other modifications might have occurred, possibly involving the unpaired cysteine present in apo[a], as well as lysine and tyrosine residues, modifications that have been noted on oxidation of apo[a] by hypochlorite (18) and 2,2′-azobis-(amidinopropane)-HCl (19). It should also be noted that there was an important divergence between the fluorescence spectrum of free apo[a] in Fig. 6 and that of apo[a] in Lp[a] (Fig. 4), indicating that the type of structural modification induced in this protein by lipid peroxidation was different from that caused by the direct response of apo[a] to Cu^{2+}.

Our current results also confirm our previous observations that both LE (3) and MMP-12 (4) cleave apoB-100, which is also susceptible to degradation by other proteolytic enzymes: chymase (20), tryptase (20), plasmin (21), kallikrein (22), and thrombin (23). The novel information originating from our current work is that preoxidation renders apoB-100 as well as apo[a] more susceptible to enzymatic degradation, a sequence of events that has been previously observed in other systems (24, 25) and might be highly relevant to the atherosclerotic process.

Our current findings also indicate that there is a need to define the conditions of preparation and properties of oxidized Lp[a] before it can be meaningfully used in biological reactions. This particularly applies to conditions of "maximal" oxidation, in which both lipid-mediated and nonlipid mediated events are operative and lead to particle destabilization and aggregation (26).

In early atherogenesis, LDL particles entering the arterial intima undergo a set of modifications caused by the combined action of lipases, proteases, and oxidative agents. These events transform LDL into aggregated and fused particles as well as vesicular structures that have been suggested to have a high affinity for the extracellular matrix (26). Fragments of apoB-100 are also present and, as predicted from the in vitro studies, generated by both oxidative and proteolytic mechanisms. Of note, information about the potential cardiovascular pathogenicity of these fragments is not available. In the case of Lp[a], modifications of the type reported for LDL are likely to occur although, as suggested by our current results, apo[a] may partially interfere with some of the induced modifications. As previously shown by Hoff et al. (27), apo[a] fragments accumulate in the inflammatory milieu underlying atheromatous lesions; however, not in normal vessels. We have also reported that in surgical segments of vulnerable human carotid plaques, MMP-2 and -9 colocalize with the C-terminal fragment of apo[a] (fragment F2) (28), a domain that we have shown to have a high affinity for the extracellular matrix (29). Besides MMP-12, utilized in the current work, MMP-2, MMP-3, MMP-7, and MMP-9 also cleave apo[a] in vitro (C. Edelstein K. Nakajima, D. Pfaffinger, and A. M. Scanu, unpublished observations). The resistance to oxidation-dependent degradation of apo[a] shown in the current studies supports the conclusion that the fragments reported in body fluids (3–7) and in atheromatous lesions (28) are of a predominant proteolytic derivation and point at a close relationship between inflammation and structural stability of apo[a] in these lesions.

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