

# A novel function of lipoprotein [a] as a preferential carrier of oxidized phospholipids in human plasma

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**Abstract** Oxidized phospholipids (OxPLs) on apolipoprotein B-100 (apoB-100) particles are strongly associated with lipoprotein [a] (Lp[a]). In this study, we evaluated whether Lp[a] is preferentially the carrier of OxPL in human plasma. The content of OxPL on apoB-100 particles was measured with monoclonal antibody E06, which recognizes the phosphocholine (PC) headgroup of oxidized but not native phospholipids. To assess whether OxPLs were preferentially bound by Lp[a] as opposed to other lipoproteins, immunoprecipitation and ultracentrifugation experiments, *in vitro* transfer studies, and chemiluminescent ELISAs were performed. Immunoprecipitation of Lp[a] from human plasma with an apolipoprotein [a] (apo[a])-specific antibody demonstrated that more than 85% of E06 reactivity (i.e., OxPL) coimmunoprecipitated with Lp[a]. Ultracentrifugation experiments showed that nearly all OxPLs were found in fractions containing apo[a], as opposed to other apolipoproteins. *In vitro* transfer studies showed that oxidized LDL preferentially donates OxPLs to Lp[a], as opposed to LDL, in a time- and temperature-dependent manner, even in aqueous buffer. Approximately 50% of E06 immunoreactivity could be extracted from isolated Lp[a] following exposure of plasma to various lipid solvents. These data demonstrate that Lp[a] is the preferential carrier of PC-containing OxPL in human plasma. This unique property of Lp[a] suggests novel insights into its physiological function and mechanisms of atherogenicity.—Bergmark, C., A. Dewan, A. Orsoni, E. Merki, E. R. Miller, M.-J. Shin, C. J. Binder, S. Hörkkö, R. M. Krauss, M. J. Chapman, J. L. Witztum, and S. Tsimikas. A novel function of lipoprotein [a] as a preferential carrier of oxidized phospholipids in human plasma. *J. Lipid Res.* 2008. 49: 2230–2239.

**Supplementary key words** lipoproteins • atherosclerosis • lipids • cholesterol • oxidation

Lipoprotein [a] (Lp[a]) was discovered more than 40 years ago by Kare Berg (1) and consists of an LDL particle to which is attached the carbohydrate-rich apolipoprotein [a] (apo[a]). The apo[a] gene encodes a variable number of tri-loop structures called kringles (K) stabilized by three disulfide bonds. Apo[a] consists of KIV, KV, and an inactive protease-like domain. Cysteine 4057 of KIV-9 of apo[a] and cysteine 4326 of apolipoprotein B-100 (apoB-100) form a disulfide bond to create an Lp[a] particle (2–6). The clinical interest in Lp[a] emanates from its association with cardiovascular disease (CVD) when present in high plasma concentrations. A meta-analysis of prospective studies demonstrated that elevated levels of Lp[a] are an independent risk factor for CVD (7). The pro-atherogenic influence of Lp[a] seems to be particularly enhanced in subjects with elevated levels of LDL cholesterol (8, 9).

The gene for apo[a] appeared recently on the evolutionary scale and is present only in humans and nonhuman primates. An unrelated apo[a]-like gene consisting only of KIII repeats is also present in hedgehogs and is postulated to have evolved independently through divergent evolution (10). The physiological role of Lp[a] and the underlying mechanisms through which it contributes to CVD are unknown. One hypothesis suggests that Lp[a] promotes thrombosis by inhibiting thrombolysis. Apo[a] has

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Abbreviations: apo[a], apolipoprotein [a]; CAD, coronary artery disease; CVD, cardiovascular disease; Lp[a], lipoprotein [a]; MDA, malondialdehyde; OxLDL, oxidized LDL; OxPL, oxidized phospholipid; OxPL/apoB, oxidized phospholipid epitopes per apolipoprotein B-100; PC, phosphocholine; PCI, percutaneous coronary intervention; RLU, relative light unit.

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high homology to plasminogen, but lacks the active catalytic protease domain of plasmin. Thus, it has been postulated to competitively inhibit the conversion of plasminogen to plasmin, and in vitro, Lp[a] inhibits thrombolysis (11). However, there is little evidence to support such a role in vivo.

We have recently proposed the alternative hypothesis that a unique physiological role of Lp[a] may be to bind and transport proinflammatory oxidized phospholipids (OxPLs) (8, 12–19). Thus, when present at low plasma concentrations, Lp[a] would actually be anti-inflammatory via its ability to bind OxPLs. The corollary to this is that one potential physiological role of Lp[a] is to bind OxPLs and either directly prevent their proinflammatory properties, or perhaps even enhance their degradation. In that regard, it may have some potential benefit at low plasma levels. This is supported by recent observations from our group in the Bruneck study (19) and by two prior studies from Berg et al. (20, 21) showing a J-shaped relationship of Lp[a] to cardiovascular events, where patients with the lowest quartile of Lp[a] had a higher risk for cardiovascular events compared with subjects in the second quartile, but subjects in the third and fourth quartiles had much higher risk. Thus, when present at high plasma concentrations, Lp[a] would be more atherogenic than native LDL, because it binds with increased affinity to arterial intimal proteoglycans (22), resulting in an increased intimal concentration of LDL, with associated proinflammatory OxPL. There is also indirect evidence from Tsironis et al. (23) demonstrating reduced lipoprotein-associated phospholipase A2 activity on Lp[a], as opposed to on LDL from the same patients, suggesting that inability to degrade OxPL on Lp[a] may lead to coronary artery disease (CAD).

Our hypothesis arose as a consequence of observations made when we initially developed a method to measure one class of OxPL in human plasma that was associated with apoB-100 (OxPL/apoB). This was accomplished using the murine monoclonal antibody E06, which is an IgM natural antibody that binds the phosphocholine (PC) headgroup of oxidized but not native phospholipids (24, 25). E06 recognizes PC on an equimolar basis when present as a PC salt, or as PC present in OxPL, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine, present as a lipid, or if covalently attached (via the sn2 side chain) to a variety of different peptides, irrespective of amino acid sequence (25). Using this assay, we have shown that plasma OxPL/apoB levels provide independent predictive value in quantitating the presence and extent of angiographically determined CAD (8), identifying the presence and progression of carotid and femoral atherosclerosis (18), and also predicting CVD events over a 10 year interval (19). The OxPL/apoB ratio was independent of all known risk factors, except for Lp[a], and, remarkably, in all clinical studies performed thus far, there was an unusually strong correlation of OxPL/apoB with Lp[a] ( $R = \sim 0.80\text{--}0.90$ ) (8, 12, 14–18). However, differences exist between Lp[a] mass and OxPL/apoB within different apo[a] isoform classes, with the strength of this correlation being weakest for the largest isoforms and strongest with the lowest number of kringle repeats ( $>29$  repeats,  $r = 0.66$ ; 23–

29 repeats,  $r = 0.88$ ; and  $<22$  repeats,  $r = 0.93$ ;  $P = 0.001$  each) (18). These clinical observations suggest that OxPL may be bound to Lp[a] and may thereby mediate a common biological influence on CVD. However, the physical association of OxPL with Lp[a] has not been studied in detail. Therefore, the purpose of this study is to better define the association of OxPL with Lp[a] in human plasma using a variety of immunoprecipitation and ultracentrifugation experiments, in vitro transfer studies, and chemiluminescent ELISAs.

## METHODS

### Study subjects

Three groups of subjects were studied in the various experiments (Table 1). In study 1, plasma was repeatedly obtained from 1 subject with elevated Lp[a] levels, but without CVD, for immunoprecipitation and ultracentrifugation experiments; in study 2, plasma was obtained from 14 subjects without CVD for detailed ultracentrifugation experiments; and in study 3, plasma was obtained from 12 subjects with familial hypercholesterolemia undergoing apheresis, 9 of whom had a history of CVD.

Each of these studies was approved by the respective institutional review boards of the University of California San Diego, the Dyslipidemia and Atherosclerosis Research INSERM Unit 551, and the Children's Hospital Oakland Research Institute, respectively, and all subjects gave written informed consent.

### Assessment of the presence of OxPLs on Lp[a] using immunoprecipitation

To assess whether OxPLs were physically associated with Lp[a] compared with other lipoproteins, plasma from the subject in study 1 with Lp[a] mass 112 mg/dl was diluted 1:50 in PBS in 1.5 ml conical, siliconized tubes in the presence of increasing amounts of LPA4 (molar ratio was based on molecular mass of LPA4 at 165 kDa and apo[a] at 500,000 kDa) to preferentially precipitate Lp[a]. LPA4 is a monoclonal IgG antibody binding the sequence TRNYCRNPDAEIRP on apo[a] and does not cross-react with plasminogen (15). After a 24 h incubation at 4°C, samples were centrifuged for 20 min at 10,000 g, and 50  $\mu$ l of the supernatant was added to microtiter wells coated with MB47, a murine monoclonal antibody that binds human apoB-100 (15). To measure the amounts of OxPL or apo[a] remaining in the superna-

TABLE 1. Baseline clinical characteristics of the three study groups

	Study 1	Study 2	Study 3
Age (years)	38	51 $\pm$ 10	44 $\pm$ 19
Male/female	1/0	11/4	6/6
Body mass index	25.1	26 $\pm$ 1.9	23 $\pm$ 4.5
Cardiovascular disease, yes/no	0/1	0/15	9/3
Lipid parameters (mg/dl)			
Total cholesterol	188	189 $\pm$ 33	225 $\pm$ 43
LDL-cholesterol	121	116 $\pm$ 32	106 $\pm$ 54
HDL-cholesterol	56	41 $\pm$ 9.3	38 $\pm$ 10
Triglycerides	53	164 $\pm$ 97	165 $\pm$ 33
Apolipoprotein A	125	N/D	119 $\pm$ 23
Apolipoprotein B	90	85 $\pm$ 12	125 $\pm$ 19
Lp[a] (median, 95% CI)	112	11.2 (7.6, 22.3)	49.5 (40.0–75.7)

Lp[a], lipoprotein [a]; N/D, not determined. Results are given as mean  $\pm$  SD.

tant, biotinylated E06 or LPA4 was then added to wells in parallel plates, and the amounts bound were determined using chemiluminescent techniques, as previously described (15). The data are expressed in relative light units (RLUs)/100 ms. To validate that equal numbers of apoB-100 particles were captured in each well, aliquots of the supernatants were added to MB47-coated wells in parallel control plates, and the amount of apoB-100 bound was measured using a biotinylated goat anti-human apoB-100 antibody (Bioscience International) as described above.

### Assessment of the presence of OxPL on Lp[a] using ultracentrifugally isolated fractions of plasma

Ultracentrifugation of plasma lipoprotein fractions was performed to assess which fractions contained E06 immunoreactivity. For this set of experiments, we used plasma from all three study groups using different isolation techniques: First, in study 1, lipoprotein fractions were separated into fractions with density <1.006, 1.006–1.020, 1.020–1.040, 1.040–1.060, 1.060–1.080, 1.080–1.110, and 1.110–1.210 g/ml and lipoprotein-deficient serum using sequential ultracentrifugation (26). This subject's lipid profile was: total cholesterol, 189 mg/dl; LDL-cholesterol (LDL-C), 121 mg/dl; HDL-C, 56 mg/dl; triglyceride, 53 mg/dl; and apoB-100, 90 mg/dl (Table 1). The densities of eluted fractions were verified using a DMA 45 Digital Density Meter (Anton Parr, Graz, Austria). Aliquots of equal amounts of protein (10 µg/ml) from each fraction were used to coat microtiter wells by overnight incubation at 4°C. The wells were then washed and the content of apoB-100, OxPL, and apo[a] determined with biotinylated goat anti-human apoB-100, E06, and LPA4, respectively, as described above.

For study 2, we recruited 14 healthy normolipidemic male volunteers who were not on hypolipidemic agents, and were without overt CVD. Three density fractions were isolated over the LDL density interval of 1.033–1.064 g/ml to more finely assess the presence of OxPL in this density range. Blood samples were collected after an overnight fast into tubes containing 1 mg/ml EDTA and 10 µM Trolox. Plasma was adjusted to a density of 1.030 g/ml with sodium chloride-D<sub>2</sub>O solution and centrifuged at 40,000 rpm for 18 h at 10°C in a Beckman 40.3 Ti rotor. The top 2.0 ml was decanted, and the supernatant fluid was adjusted to a density of 1.063 g/ml with sodium chloride-D<sub>2</sub>O solution. After centrifugation at 10°C for 18 h at 40,000 rpm in a Beckman 40.3 Ti rotor, the LDL was withdrawn in the top 1.0 ml and dialyzed in a NaBr solution of 1.04 g/ml overnight with two changes of dialyzing solution. The dialyzed LDL (2.0 ml) was layered above a NaBr solution of density 1.0540 g/ml (2.5 ml), and 2.5 ml of a NaBr solution of density 1.0275 g/ml was layered above the LDL. The tubes were then centrifuged to equilibrium at 40,000 rpm for 40 h in a Beckman SW 45 rotor at 18°C. A blank tube containing salt with D<sub>2</sub>O was included with each ultracentrifugation for densitometric analysis of subfractions. The contents of each tube were then withdrawn by pipetting, and the first 2.5 ml (density <1.033 g/ml) and bottom 0.5 ml (density >1.064 g/ml) were discarded. The three sequential fractions used for compositional analysis were of density 1.033–1.038 g/ml (1.0 ml), 1.038–1.049 g/ml (1.5 ml), and 1.049–1.064 g/ml (1.5 ml). ApoB-100 levels were then determined in these fractions with a commercial assay. To directly determine the content of OxPL and apo[a] on apoB-100 particles in each fraction, MB47 was plated and equal amounts of apoB-100 (10 µg/ml) from each subfraction were then added. The amount of OxPL and apo[a] on apoB-100 was determined with biotinylated E06 and LPA4, respectively, as described above. The amount of apoB-100 bound in each well was determined using biotinylated goat anti-human apoB-100.

As a third approach to assess the presence of OxPL over the entire lipoprotein density range, we recruited 12 patients with familial hypercholesterolemia, documented by both clinical and genetic testing, who were undergoing apheresis every 2–3 weeks. Density gradient ultracentrifugation was used to separate very narrow density bands of lipoproteins. These patients had a homogeneous lipoprotein profile except for differences in Lp[a] levels (Table 1). Plasma was fractionated on the basis of the hydrated density by the isopycnic ultracentrifugal density gradient procedure as described previously (27), with the modification that 2.5 ml (instead of 3 ml) of the 1.006 g/ml NaCl solution was added to the top of each gradient. Gradients were constructed in Ultraclear (Beckman) tubes of the Beckman SW41-Ti rotor (27). Gentamycin (final concentration 50 mg/ml) and an antioxidant, EDTA (final concentration 0.26 mM), were also added to each “plasma-KBr” sample. Ultracentrifugation was performed in a Beckman L-80 ultracentrifuge at 40,000 rpm for 44 h at 15°C, using max acceleration and deceleration modes. Twenty-four fractions of 0.4 ml were collected successively from the meniscus of each tube with a Gilson precision pipette; however, the first fraction ( $d < 1.015$  g/ml) was removed in the same volume with a narrow-bore Pasteur pipette in order to permit a more satisfactory separation of VLDL, which tended to adhere to the tube walls. Fifty µl aliquots from each fraction were used to coat microtiter wells by overnight incubation at 4°C. The wells were then washed and the content of apoB-100, OxPL, and apo[a] determined with biotinylated goat anti-human apoB-100, E06, and LPA4, respectively, as described above.

### Assessment of the net transfer of OxPL to Lp[a] in vitro

To determine the relative capacity of LDL versus Lp[a] to bind OxPL, we compared the net transfer of OxPL, as measured by E06, from oxidized LDL (OxLDL) to either LDL or Lp[a] in a time- and temperature-dependent manner. LDL (with no Lp[a]) and Lp[a] to be used as acceptors were obtained using a lysine-sepharose column, as previously described (13). Each of these acceptor lipoproteins was biotinylated by standard methods (EZ-Link Sulfo-NHS-Biotin; Pierce, Inc.). Donor lipoproteins consisted of LDL (3 µg/ml), or copper-oxidized LDL (Cu-OxLDL, 3 µg/ml), prepared as described (15, 26). Previous studies have shown that this level of oxidation results in approximately 75 mol of OxPL on Cu-OxLDL (28–30). Donors were incubated with acceptor biotinylated lipoproteins (b-LDL or b-Lp[a]) in PBS in siliconized tubes in the presence of EDTA and butylated hydroxytoluene to prevent oxidation in the incubation. In control experiments, we verified that under the conditions of the transfer assay, no oxidation of native LDL occurred. Following time- and temperature-controlled incubations, aliquots were transferred to microtiter wells coated with NeutrAvidin (5 µg/ml), which selectively bound the biotinylated acceptor lipoprotein. The aliquots were added in sufficient amounts to ensure that a saturating content of the acceptor lipoprotein was added to the NeutrAvidin-coated wells, and control experiments with biotinylated goat anti-human apoB-100 and apo[a] confirmed that equal amounts of the biotinylated acceptor lipoproteins were captured per well in each experiment. The content of OxPL on the bound acceptor lipoprotein was then determined by the binding of E06 as described above.

### Determination of whether malondialdehyde-lysine epitopes exist on Lp[a]

To determine whether the presence of OxPL on Lp[a] represented a preferential and specific pattern of binding versus generalized oxidation, we evaluated whether malondialdehyde (MDA) epitopes were present on Lp[a]. MDA is a common epi-



tope resulting from lipid peroxidation, and the presence of MDA epitopes on LDL and autoantibodies to MDA-LDL have been demonstrated in human plasma and in atherosclerotic lesions (15). Microtiter wells were coated with 10  $\mu\text{g}/\text{ml}$  LDL, MDA-LDL, Lp[a], and apo[a]. The presence of OxPL was measured with biotinylated E06, and MDA epitopes were measured with biotinylated E014, a murine monoclonal antibody that binds MDA-LDL but does not bind OxPL epitopes (24).

#### Determination of lipid-extractable OxPL epitopes on isolated Lp[a]

To determine whether the OxPLs on Lp[a] were susceptible to extraction by organic agents, we either directly plated ultracentrifugally isolated Lp[a] on microtiter wells or we captured Lp[a] into microtiter wells from plasma using antibody LPA4. We then determined the content of OxPL before and after extraction with various organic solvents.

In the first method, Lp[a] was used to coat microtiter wells by overnight incubation as described above. To each well was added either 100% isopropanol, ether, or methanol to extract the lipids. The plates were washed, and the procedure was repeated four times. Following extraction of the lipids, biotinylated E06 or biotinylated LPA4 was added to determine the content of OxPL and Lp[a] remaining on the plate. Similar experiments were performed on the same samples without lipid extraction. In the second method, antibody LPA4 (5  $\mu\text{g}/\text{ml}$ ) was plated to microtiter wells, and plasma (1:100 dilution) with known Lp[a] values was added to capture Lp[a] and the lipids extracted as above. Biotinylated E06 or biotinylated LPA4 was then added to assess the presence of OxPL and Lp[a]. The antibody LPA4 has more than

one binding site on apo[a]; thus, it may be used in a sandwich ELISA in this format.

The ratio of OxPL/Lp[a] was then determined by dividing the E06 RLU with the LPA4 RLU. The difference in OxPL/Lp[a] before and after lipid extraction represents the amount of extractable OxPL from either plated or captured Lp[a].

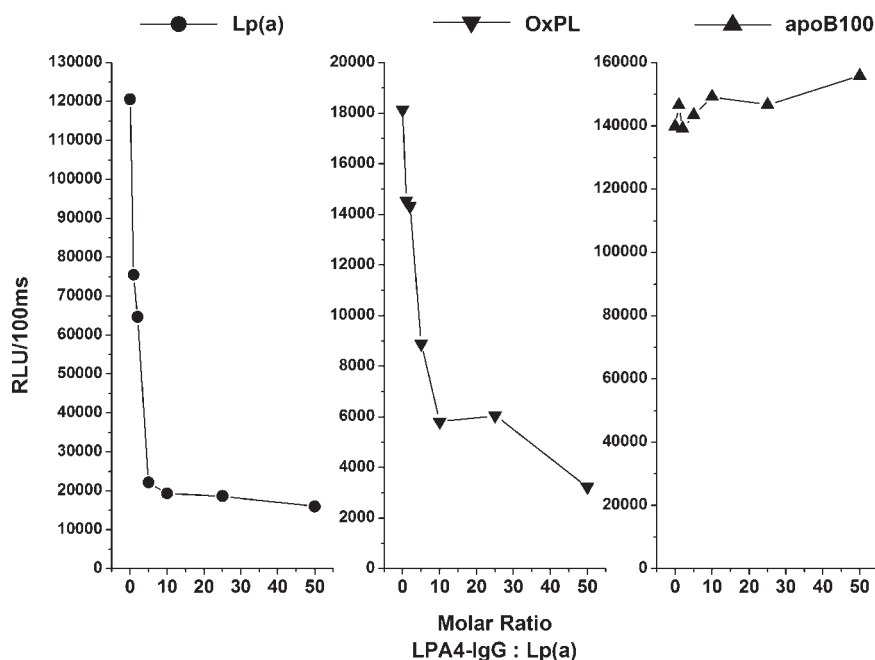
#### Statistics

Differences between the groups were assessed by paired Student's *t*-test. Analysis of quantitative parameters between the different ultracentrifugation fractions was performed with repeated-measures one-way ANOVA with the post hoc Bonferroni multiple comparison test as appropriate.  $P < 0.05$  was considered significant.

## RESULTS

#### Characteristics of study subjects

Table 1 demonstrates the baseline demographics and lipoprotein levels of the three study groups. The subject in study 1 had a very high Lp[a] level (112 mg/dl), the subjects in study 2 had essentially normal lipid profiles including Lp[a] levels, and the subjects in study 3 had familial hypercholesterolemia and were undergoing regular apheresis. This latter group had homogeneous lipid profiles, but they were recruited for this study based on different Lp[a] levels, which were equally distributed into four groups



**Fig. 1.** This figure displays a representative example of the immunoprecipitation of lipoprotein [a] (Lp[a]) from plasma in an individual with Lp[a] 112 mg/dl. Increasing amounts of the monoclonal IgG antibody LPA4 were incubated with plasma overnight to immunoprecipitate Lp[a]. MB47 was then used to capture apolipoprotein B-100 (apoB-100) from the supernatant, which was assessed for the presence of apo[a] with antibody LPA4 (left panel) and oxidized phospholipid (OxPL) with antibody E06 (middle panel). In parallel wells, we demonstrated that the amount of apoB-100 captured in each well was equal (right panel). To ensure that LPA4 had not immunoprecipitated all the apoB-100 from plasma, we assessed whether a saturating amount of apoB-100 remained in the supernatant of each sample. As shown in the right panel, a saturating amount of apoB-100 remained in the supernatants after the LPA4 immunoprecipitation. RLU, relative light unit.

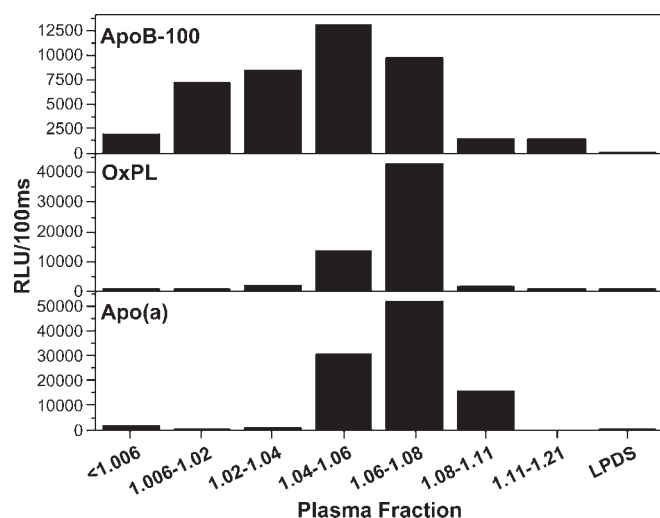
according to Lp[a] levels (mean  $\pm$  SD, mg/dl): normal ( $10 \pm 0.0$  mg/dl), mildly elevated ( $48 \pm 5$  mg/dl), modestly elevated ( $88 \pm 11$  mg/dl), and high ( $122 \pm 12$  mg/dl). Of these patients, 9 out of 12 also had clinical evidence of CAD (angina, myocardial infarction, or coronary revascularization).

#### Immunoprecipitation experiments demonstrate that OxPLs in human plasma are physically associated with Lp[a]

Monoclonal antibody LPA4 immunoprecipitated Lp[a] from plasma in a dose-dependent fashion. More than 85% of Lp[a] was precipitated at a molar ratio of LPA4/Lp[a]  $>5$  (Fig. 1, left panel). In general, there was a concomitant immunoprecipitation of E06 reactivity, albeit at slightly higher molar ratios (Fig. 1, middle panel). In this assay, there was no change in the amount of apoB-100 captured on the microtiter well plates (Fig. 1, right panel), indicating that the amount of apoB-100 remaining in the supernatant always saturated the MB47 capture antibody on the bottom of the microtiter plate.

#### Ultracentrifugation experiments demonstrate that OxPLs in human plasma are present almost exclusively in fractions containing Lp[a]

As an additional approach to define the apoB-100 lipoproteins containing OxPL recognized by E06, we separated lipoproteins by ultracentrifugation, plated each fraction in microtiter wells either directly or on MB47 coated plates, and then directly assessed the presence of apo[a] and E06 immunoreactivity in each fraction immunochemically. E06 immunoreactivity was found predominantly in fractions containing apo[a] (Fig. 2). Lighter apoB-100 fractions not containing apo[a], e.g., density 1.006–1.040 g/ml, and the lipoprotein-deficient serum had minimal E06 immunoreactivity.



**Fig. 2.** From the same patient as in Fig. 1, plasma was sequentially ultracentrifuged into eight density fractions, and equal amounts of protein were directly plated into microtiter well plates. The presence of apoB-100, OxPL, and apo[a] in the various density fractions and lipid-deficient serum (LPDS) was assessed directly with antibodies MB47, E06, and LPA4, respectively, as described in the Methods section.

To specifically determine whether OxPLs were associated with non-Lp[a] apoB-100 lipoproteins, we performed density gradient ultracentrifugation of plasma from 14 subjects and collected multiple fractions in the classical LDL density range, focusing on the density gradients 1.033–1.038, 1.038–1.049, and 1.049–1.064 g/ml. We measured the content of OxPL in each fraction normalized to the amount of apoB-100 present. **Figure 3A** demonstrates that OxPL/apoB was present in all three fractions, although to a much greater extent in the 1.049–1.064 g/ml fraction. Apo[a] was found primarily in the densest fraction (Fig. 3B). Interestingly, there was no correlation between OxPL/apoB and apo[a] in the density fractions 1.033–1.038 g/ml (Fig. 3C) and 1.038–1.048 g/ml (Fig. 3D), but a very strong correlation in the 1.048–1.064 g/ml fraction ( $R = 0.80$ ;  $P = 0.0006$ ) (Fig. 3E), reflecting the predominance of Lp[a] in the densest fraction.

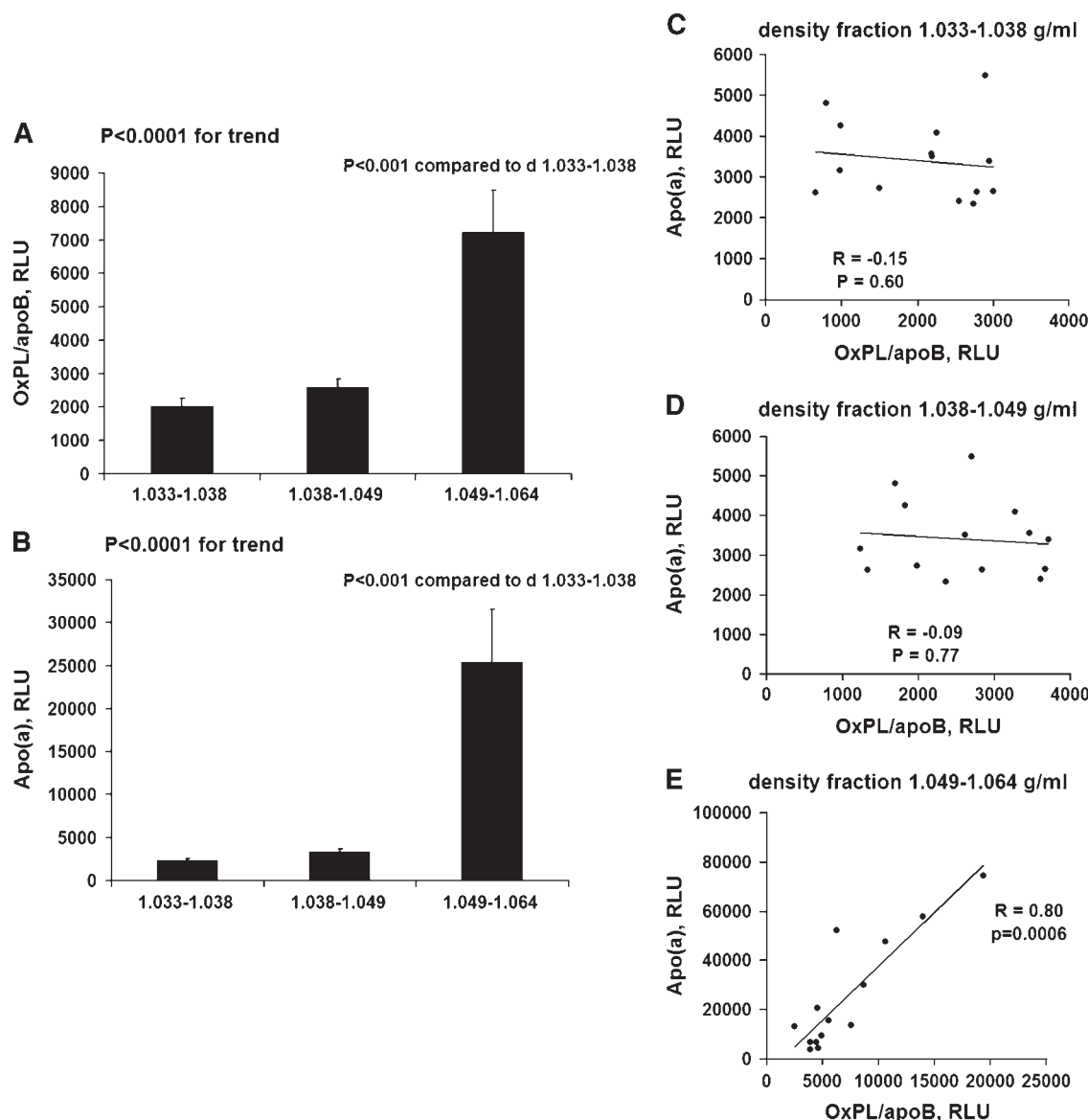
To obtain a more complete assessment of the presence of OxPL in the entire range of lipoproteins, a detailed ultracentrifugation experiment was performed. **Figure 4** shows the distribution of OxPL, apo[a], and apoB-100 in directly plated aliquots of the 24 fractions isolated from each subject. Note that OxPL is present exclusively in fractions containing apo[a], peaking at  $d \sim 1.075$ , whereas apoB-100 is present throughout the expected range. There is also a small amount of apo[a] in the VLDL range, which has been documented in other studies (31), that does not appear to have much associated OxPL.

#### Demonstration of preferential transfer of OxPL to Lp[a] in vitro

The data thus far suggest that in plasma, the OxPL detected by E06 is predominantly bound to Lp[a], with only small amounts found on other apoB-100 particles. To test the possibility that Lp[a] preferentially binds such OxPLs, compared with LDL, we compared the net transfer of OxPL in OxLDL to Lp[a] and to LDL. Biotinylated LDL (as OxPL acceptor), free of any Lp[a], was incubated with OxPL donors OxLDL and native LDL and BSA for varying times and temperatures, and an assessment was made of the net accumulation of OxPL on the biotinylated LDL. There was essentially no net transfer of OxPL to LDL at 4°C from any donor lipoprotein, whereas there was apparent saturable transfer of a limited amount of OxPL to LDL from OxLDL at 37°C (Fig. 5, top row). In contrast, when biotinylated Lp[a] was the acceptor (Fig. 5, bottom row), there was apparent saturable transfer from OxLDL, even at 4°C. At 37°C, there was a rapid saturation and a  $>3$ -fold higher level of accumulation of E06 epitopes on the Lp[a], compared with LDL as the acceptor. In similar experiments, we also utilized Lp[a] as a donor and were unable to demonstrate a net transfer of OxPL to biotinylated LDL or Cu-OxLDL (data not shown).

#### Lp[a] does not contain malondialdehyde-lysine epitopes

To determine whether Lp[a] contains oxidation-specific epitopes in addition to OxPL, we directly plated various antigens on microtiter well plates and examined the binding of E06 compared with E014, a murine monoclonal



**Fig. 3.** Lipoproteins in the LDL density range 1.033–1.064 g/ml (three sequential fractions: 1.033–1.038, 1.038–1.049, and 1.049–1.064) from 12 subjects were isolated using density gradient ultracentrifugation. Microtiter well plates were coated with antibody MB47 to bind apoB-100, and an aliquot of each fraction was then added to assess the presence of OxPL (A) and Lp[a] (B). Panels C–E represent the correlation between OxPL/apoB and Lp[a] (apo[a]) in each density fraction, respectively. Error bars = mean SEM.

antibody binding only to MDA-lysine epitopes. In contrast to E06, which recognized Lp[a] and apo[a], E014 bound very well to MDA-LDL as expected, but not to any other antigens (Fig. 6).

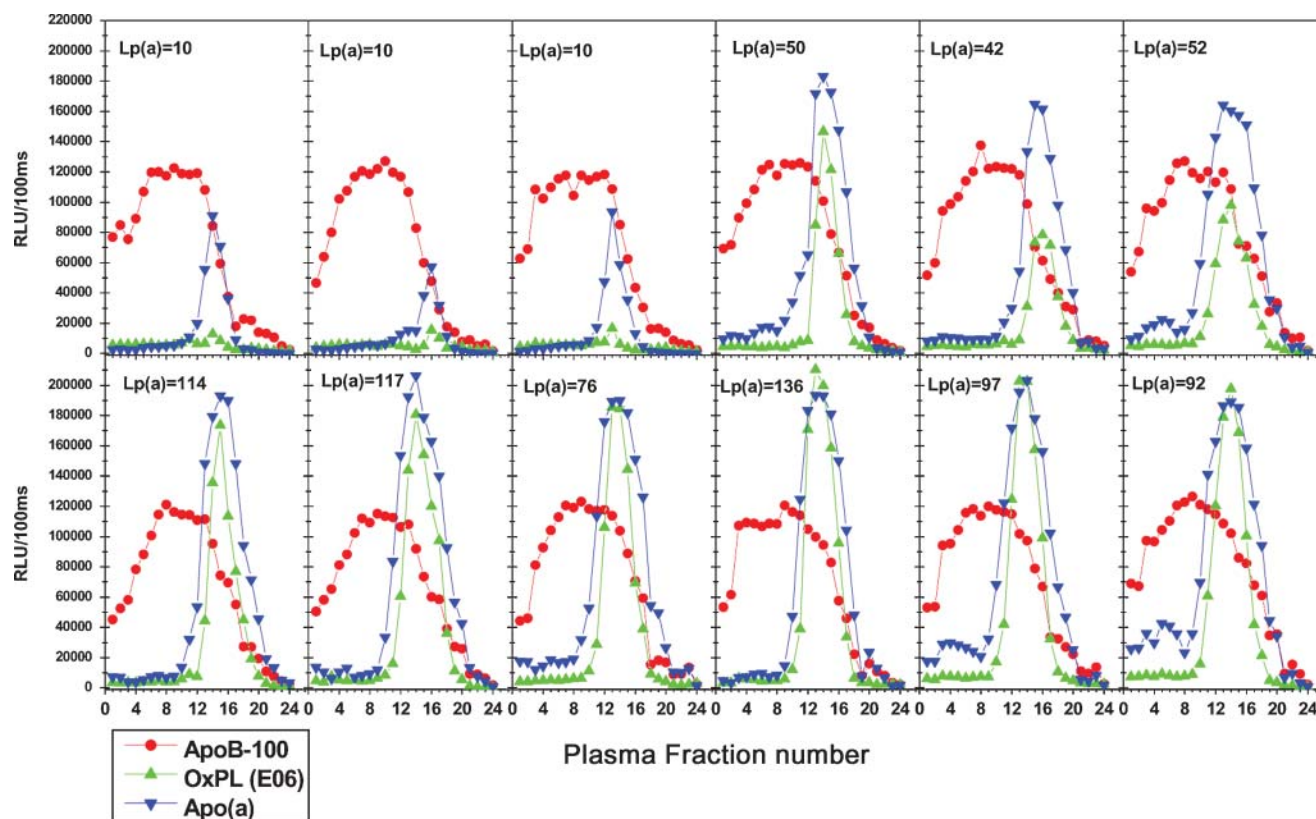
#### OxPLs are present in both the lipid and protein moieties of Lp[a]

Using different preparations of pure Lp[a] isolated from human plasma without concomitant contamination with LDL, we found that 30–70% of E06 immunoreactivity was lipid soluble, i.e., was removed from the bound Lp[a] following delipidation with different organic solvents such as ether, isopropanol, or methanol. There appeared to be variation between experiments, varying with the different Lp[a] preparations and with the solvents.

Similarly, when Lp[a] was captured on microtiter well plates with LPA4 and the lipids extracted with isopropanol, a significant reduction (50–70%) could be shown in the OxPL/apo[a] ratio. For example, in a subject with Lp[a] 115 mg/dl, the OxPL/apo[a] ratio decreased from  $0.93 \pm 27$  to  $0.29 \pm 0.01$  ( $P = 0.05$ ), and in a subject with Lp[a] 85 mg/dl, the OxPL/apo[a] ratio decreased from  $0.76 \pm 11$  to  $0.36 \pm 0.09$  ( $P = 0.007$ ).

#### DISCUSSION

This study documents that PC-containing OxPLs recognized by E06 are nearly exclusively bound to Lp[a], as opposed to other lipoproteins, in human plasma. Minor amounts of E06 immunoreactivity were bound to non-Lp[a]



**Fig. 4.** Using density gradient ultracentrifugation, lipoproteins in a very narrow density range were isolated throughout the entire range of lipoproteins from 12 subjects to generate 24 plasma aliquots in each subject. A fraction of each aliquot was plated directly onto microtiter well plates, and assessed for the presence of apoB-100 (red), OxPL (green), and apo[a] (purple). Data from individual patients are presented to emphasize the differences in subjects with differing plasma Lp[a] levels.

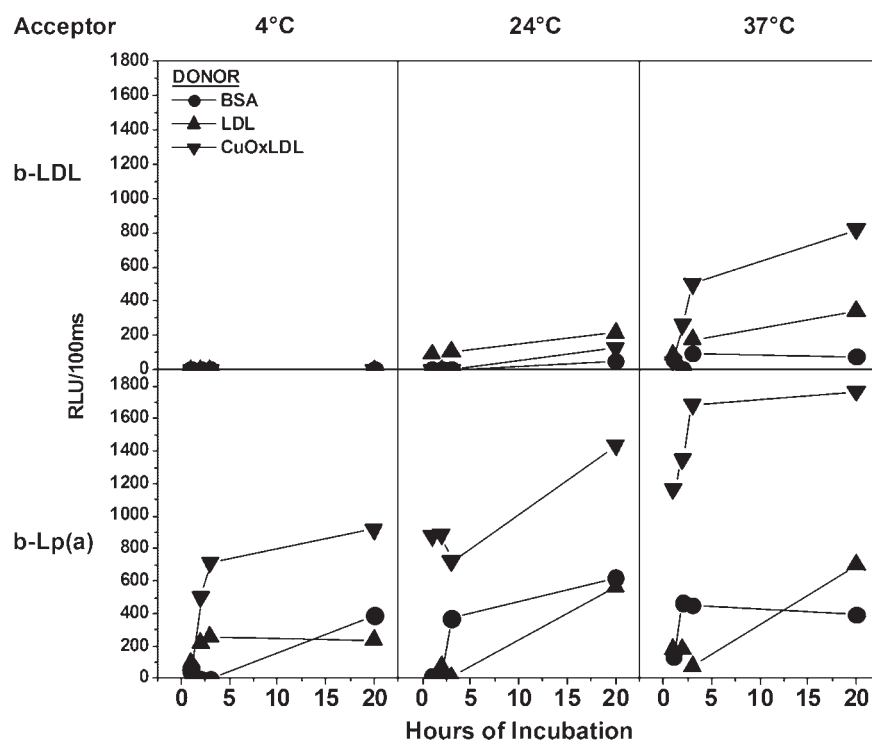
apoB-100 lipoproteins and to HDL, but with the techniques used, we could not detect OxPL in the lipoprotein-deficient compartment of plasma. In vitro transfer studies conducted in sample PBS buffer, in which OxLDL was a donor lipoprotein, demonstrated a time- and temperature-dependent accumulation of such OxPLs on Lp[a] and, to a much more limited extent, on LDL. We were unable to demonstrate transfer of such OxPLs from Lp[a] to LDL or biotinylated Lp[a]. This implies that these polar OxPLs were transferred from OxLDL to Lp[a] through the aqueous phase and were preferentially bound by Lp[a]. In addition, we were unable to show the presence on Lp[a] of another oxidation-specific epitope, MDA, using sensitive immunochemical techniques, which suggests that the Lp[a] is a specific carrier of OxPL and not simply oxidized in general. These data support the hypothesis that in vivo, OxPLs are transferred to plasma from other sources, such as oxidized lipoproteins, apoptotic cells, atherosclerotic lesions, or inflammatory tissues, where they are preferentially bound by Lp[a]. This study provides a mechanistic framework for understanding the strong association between OxPL/apoB and Lp[a] in human studies (8, 12–19).

Our epidemiological studies have shown a remarkable correlation of plasma levels of OxPL/apoB and Lp[a]. However, differences exist between Lp[a] mass and OxPL/apoB within different apo[a] isoform classes, with the strength of this correlation being weakest for the large-

est isoforms and strongest with the lowest number of kringle KIV repeats ( $>29$  repeats,  $r = 0.66$ ;  $23\text{--}29$  repeats,  $r = 0.88$ ; and  $<22$  repeats,  $r = 0.93$ ;  $P = 0.001$  each; data derived from the Bruneck study) (18). In individuals with low Lp[a] levels, there is a corresponding low level of OxPL/apoB, suggesting that in the absence of Lp[a], these OxPLs do not accumulate on plasma apoB-containing lipoproteins other than to a minor degree. A similar situation exists with most animals that we have studied (32, 33). For example, in mice with marked hypercholesterolemia, a situation in which OxPLs recognized by E06 are abundant in the arterial tissues (and probably elsewhere as well), the levels of OxPL/apoB in plasma are very low, and often just at the level of detection of our assay (33). In contrast, Lp[a]-transgenic mice have very high OxPL/apoB levels, even in a C57BL/6 background without obvious atherosclerosis (34). Presumably, this reflects the generation of such OxPLs as a component of normal physiological processes. Lp[a]-transgenic mice express both human apoB-100 and apo[a] and thus can form a true covalent Lp[a] similar to that found in humans (34). Mice expressing high levels of human apoB-100 alone, or apo[a] alone, nevertheless have very low levels of OxPL/apoB (34), suggesting the need for an intact Lp[a] to enable preferential binding of OxPL.

The immunoprecipitation and ultracentrifugation studies demonstrated that Lp[a] is the main carrier of OxPLs in plasma, at least those detected by antibody E06. These data



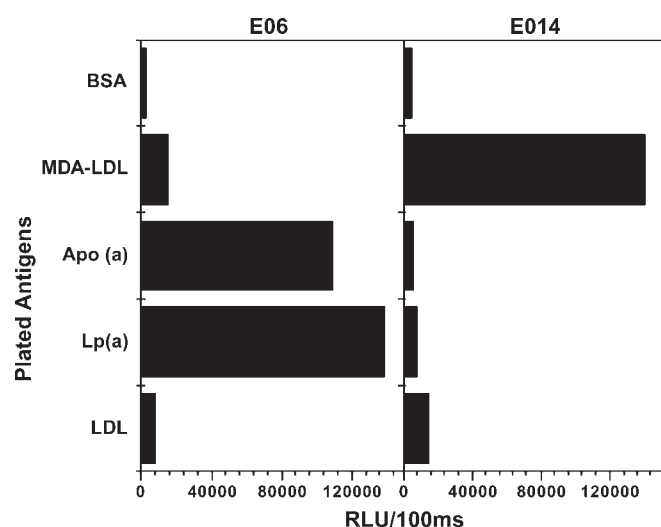


**Fig. 5.** This figure displays the relative capacity of LDL versus Lp[a] to bind OxPL. Biotinylated acceptor lipoproteins LDL or Lp[a] (b-LDL or b-Lp[a]) were incubated with donor lipoproteins LDL and copper-oxidized LDL (CuOxLDL). BSA was used as a control donor. Following time- and temperature-controlled incubations, aliquots were transferred to microtiter wells coated with NeutrAvidin (5  $\mu$ g/ml), which selectively bound the biotinylated acceptor lipoproteins b-LDL and b-Lp[a]. The content of OxPL on the bound acceptor lipoproteins was then determined by the binding of E06 as described above.

suggest that OxPL and Lp[a] are physically associated and that Lp[a] has a strong affinity for OxPL. In previous experiments, we showed that apo[a] covalently binds up to 2 mol of OxPL through Schiff-base formation (13). To

further determine which component of Lp[a] was responsible for this binding, we isolated Lp[a] and delipidated it with various solvents. These data are consistent with the fact that the lipid phase of Lp[a] contains at least as much OxPL as isolated apo[a] and most likely represents that fraction recently transferred to Lp[a]. We have also previously shown that in heavily oxidized LDL, generated by copper-induced oxidation, up to 75 mol of OxPL recognized by E06 can be covalently bound to apoB-100, and that this immunoreactivity is removed by saponification (25). However, we do not believe that the Lp[a] in plasma has been “oxidized” per se. This is supported by the observation that E014, which recognizes MDA-lysine epitopes, does not show immunoreactivity to Lp[a].

The *in vitro* transfer studies lend further support that Lp[a] is the preferential acceptor of OxPL, compared with LDL. This occurred in an *in vitro* system without any lipid transfer proteins present, suggesting that the more polar OxPL can exchange from the OxLDL donor to the Lp[a] particle, to which it preferentially binds. Interestingly, we previously reported a similar observation in patients undergoing percutaneous coronary intervention (PCI) (15). Immediately post-PCI, plasma levels of Lp[a] and OxPL/apoB increased by 65% and 35%, respectively. Immunoprecipitation experiments in selected patient samples at serial timepoints before and after PCI showed that immediately post procedure ( $\sim$ 1 h), only 50% of OxPLs detected in plasma on apoB were associated with Lp[a],



**Fig. 6.** Assessment of the presence of OxPL versus malondialdehyde (MDA) epitopes on Lp[a], detected by antibodies E06 and E014, respectively. Antigens consisting of BSA, MDA-LDL, apo[a], Lp[a], and LDL were directly plated at similar protein concentrations (10  $\mu$ g/ml) onto microtiter well plates, and the presence of OxPL and MDA epitopes was assessed with E06 and E014, respectively.



whereas the other 50% were associated with other apoB lipoproteins. However, by 6 h, nearly all of the OxPLs were again associated with Lp[a], suggesting either transfer of OxPL to Lp[a] or more-rapid clearance of non-Lp[a]-associated OxPL. These data are further supported by our in vivo observations made in Lp[a]-transgenic mice, where we demonstrated that Lp[a] in these mice contained high levels of OxPL, even in the absence of hypercholesterolemia or the presence of atherosclerosis (34). In future studies, we will use a combination of mass spectroscopy,  $^{13}\text{C}$ - and  $^1\text{H}$ -NMR and other techniques to positively identify some of the OxPLs found in ultracentrifugally isolated Lp[a] and those covalently adducted to apo[a]. These techniques should enable us to confirm the physical presence of PC-containing OxPL known to react with E06 in the Lp[a] and that bound to apo[a].

OxPLs are important contributors to early events in atherogenesis, inasmuch as they are present even in minimally oxidized forms of LDL and may activate proinflammatory genes in arterial wall cells, leading to inflammatory cascades in the vessel wall (35, 36). OxPLs are also present in advanced atherosclerotic lesions and apoptotic cells of animals and humans (37, 38). Natural defense immune mechanisms have evolved to bind and presumably detoxify OxPL, such as scavenger receptors (e.g., CD36), C-reactive protein, IgM autoantibodies, and perhaps Lp[a] (32, 39, 40). In fact, the IgM antibody E06 has the ability to inhibit OxLDL uptake by macrophages, preventing recognition by scavenger receptors (28, 41), and can inhibit a number of the proinflammatory properties of OxPL (35, 42). In a similar manner, we hypothesize that under certain settings, Lp[a] acts as an acute-phase reactant and its levels are increased in plasma within the context of the genetically determined set point. In turn, we postulate that the physiological role of Lp[a] may be to preferentially bind and transport OxPLs that are derived from apoptosis and cell death, as occurs during inflammation and oxidative stress, or when OxPLs are mobilized from tissues during iatrogenic plaque rupture during PCI or during lesion regression in response to therapeutic interventions. Lp[a] may possibly even detoxify OxPL. The latter possibility has relevance in light of the fact that Lp[a] is enriched in platelet-activating factor hydrolase (PAF-AH) (43, 44), an enzyme that not only inactivates PAF, but is known to hydrolyze *sn*2 short-chain fatty acids of phospholipids.

In conclusion, our studies demonstrate that Lp[a] is the main carrier of PC-containing OxPL in human lipoproteins, and studies are under way to determine the physiological and pathological consequences. It is important to emphasize that these data apply only to those OxPLs that are recognized by E06. Future studies will need to determine the exact OxPL actually present, the nature of the binding sites on Lp[a], and conceivably, whether other oxidized phospholipids are also bound by Lp[a]. ■

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