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Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a)[1]

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Abstract **Oxidized phospholipids (OxPLs) are present on apolipoprotein (a) [apo(a)] and lipoprotein (a) [Lp(a)] but the determinants influencing their binding are not known. The presence of OxPLs on apo(a)/Lp(a) was evaluated in plasma from healthy humans, apes, monkeys, apo(a)/Lp(a) transgenic mice, lysine binding site (LBS) mutant apo(a)/Lp(a) mice with $Asp^{55/57}$→$Ala^{55/57}$ substitution of kringle (K) IV_{10})], and a variety of recombinant apo(a) [r-apo(a)] constructs. Using antibody E06, which binds the phosphocholine (PC) headgroup of OxPLs, Western and ELISA formats revealed that OxPLs were only present in apo(a) with an intact KIV_{10} LBS. Lipid extracts of purified human Lp(a) contained both E06- and nonE06-detectable OxPLs by tandem liquid chromatography-mass spectrometry (LC-MS/MS). Trypsin digestion of 17K r-apo(a) showed PC-containing OxPLs covalently bound to apo(a) fragments by LC-MS/MS that could be saponified by ammonium hydroxide. Interestingly, PC-containing OxPLs were also present in 17K r-apo(a) with Asp^{57}→Ala^{57} substitution in KIV_{10} that lacked E06 immunoreactivity. In conclusion, E06- and nonE06-detectable OxPLs are present in the lipid phase of Lp(a) and covalently bound to apo(a). E06 immunoreactivity, reflecting pro-inflammatory OxPLs accessible to the immune system, is strongly influenced by KIV_{10} LBS and is unique to human apo(a), which may explain Lp(a)'s pro-atherogenic potential.**—Leibundgut, G., C. Scipione, H. Yin, M. Schneider, M. B. Boffa, S. Green, X. Yang, E. Dennis, J. L. Witztum, M. L. Koschinsky, and S. Tsimikas. **Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a).** ***J. Lipid Res.*** **2013.** 54: **2815–2830.**

Supplementary key words kringles • lipoproteins • plasminogen

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Lipoprotein (a) [Lp(a)] is composed of apolipoprotein (a) [apo(a)] covalently bound to apolipoprotein B-100 (apoB) via a single disulfide bond on kringle (K) IV type 9 (KIV_9) to a site near the low density lipoprotein (LDL) receptor binding site of apoB (1–3). The *LPA* gene encoding apo(a) is present on chromosome 6q26 and is highly homologous to the plasminogen (*PLG*) gene (**Fig. 1**). PLG is a zymogen that is activated to plasmin and contains five Ks and a protease domain. Apo(a) contains only KIV and KV and has an inactive protease domain due to a Ser^{561}-Ile^{562} substitution for Arg^{561}-Val^{562} that prevents PLG activators from converting PLG to plasmin (4). In addition, apo(a) contains 10 subtypes of KIV; the KIV_2 subtype is present in variable numbers of identically-repeated copies. Unlike the *PLG* gene that is present widely across species, the *LPA* gene appeared late during primate evolution and is present only in humans, nonhuman primates, and old world monkeys. An apo(a) variant exists in European hedgehogs, where it is present only as multiple copies of KIII and thus likely arose independently during evolution (5).

Recent studies demonstrate that genetically elevated Lp(a) levels independently predict cardiovascular disease (CVD) and peripheral arterial disease (6–8). Mendelian randomization studies have also provided strong supporting

Abbreviations: ALDO-PC, 1-palmitoyl-2-(9′-oxo-nonanoyl)-sn-glycero-3-phosphocholine; AzPC, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine; CAD, coronary artery disease; CVD, cardiovascular disease; DLPC, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine; K, kringle; LBS, lysine binding site; Lp(a), lipoprotein (a); LPA, gene encoding apo(a); OxPL, oxidized phospholipid; PC, phosphocholine; PEIPC, epoxy-isoprostane-phosphocholine; PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine; PIS, precursor ion scanning; PLG, plasminogen; r-apo(a), recombinant apo(a); POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; WT, wild-type.

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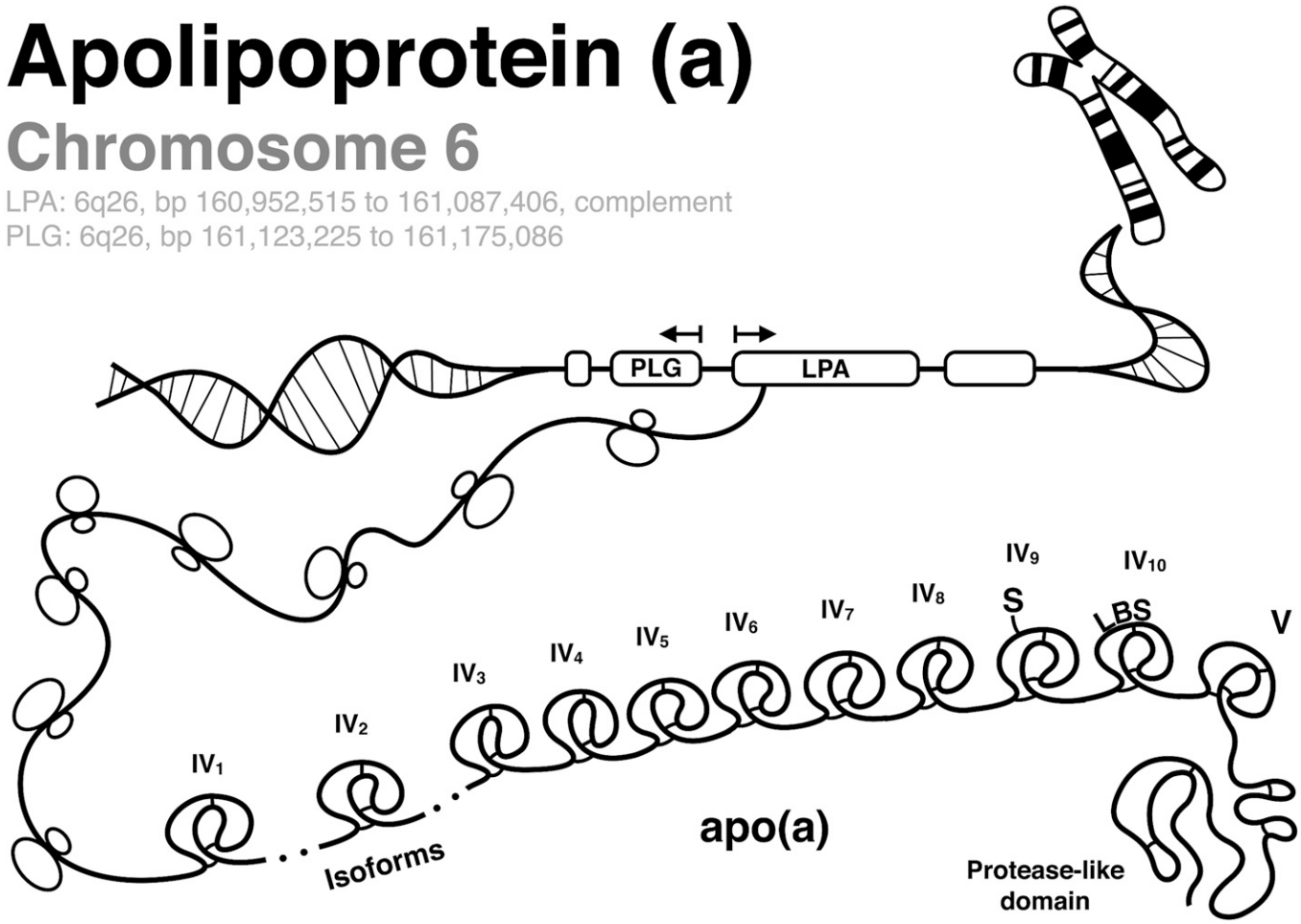

Fig. 1. Genetic architecture of PLG and apo(a). The illustration depicts chromosome 6q26 containing the genes for PLG and apo(a) (LPA), which is transcribed into apo(a) containing KIV_1, various repeats of KIV_2, KIV_3 to KIV_8, KIV_9 that contains an additional cysteine which covalently binds to apoB-100 of Lp(a) via a disulfide bond (S), KIV_{10} that contains the LBS, and the inactive protease domain.

evidence that Lp(a) is a genetic risk factor that may causally mediate CVD (9, 10). However, the underlying mechanisms by which Lp(a) mediates atherogenicity are not well understood (11).

We made the observation that Lp(a) is a preferential lipoprotein carrier of oxidized phospholipids (OxPLs) using a variety of experimental and clinical approaches (12–16). Furthermore, we developed an ELISA that quantitates phosphocholine (PC)-containing OxPLs on human apoB lipoproteins (OxPL/apoB), which primarily reflects the presence of OxPLs on the most atherogenic Lp(a) particles (17). OxPLs are highly prevalent in human vulnerable plaques (18) and in total chronic coronary occlusions (19). We have demonstrated that plasma OxPL/apoB levels identify angiographically-determined coronary artery disease (CAD) (14), predict the presence and progression of carotid and femoral atherosclerosis (20) and development of symptomatic peripheral arterial disease (21), and are elevated following acute coronary syndromes (12) and following percutaneous coronary intervention (22). Importantly, increased baseline levels of OxPL/apoB predict 15 year occurrence of new CVD events in previously healthy subjects independent of traditional risk factors and their Framingham risk score (6, 23), and allow reclassification of a significant number of subjects in intermediate Framingham risk category into higher or lower risk categories (24). Thus, OxPL/apoB appears to reflect the adverse consequences of highly atherogenic Lp(a) particles on CVD outcomes, but is also independently associated with CVD risk above and beyond Lp(a) levels in certain populations (6, 14). More recently, we also demonstrated that PLG of a variety of species contains covalently bound OxPLs (25). In contrast to the pro-atherogenic effects of OxPLs on Lp(a), OxPLs on PLG promote fibrinolysis, whereas the absence of OxPLs on PLG result in delayed fibrinolysis, which would be predicted to be atheroprotective (25).

In this study, we evaluate the potential determinants of OxPL binding on apo(a)/Lp(a) using several techniques, including isolated Lp(a) from humans, plasma from apes and monkeys, and recombinant apo(a) [r-apo(a)] constructs with a variety modifications encompassing apo(a) differences in various species. We also examine unique transgenic murine models expressing human Lp(a), including an apo(a) with mutations in a canonical lysine binding site (LBS) on KIV_{10}, which is based on the sequences derived from KIV of PLG (26).

METHODS

Composition of apo(a) of various species and of r-apo(a) constructs

Figure 2A displays the composition of apo(a) in various species used in this study. Compared with humans, all species except baboons and orangutans have differences in the seven amino acid LBS of KIV_{10}. The r-apo(a) constructs studied contain a variety of modifications of Ks and KIV_{10} LBS, including the Asp^{57}→Ala^{57} variant found in the chimpanzee and gorilla.

Figure 2B displays the r-apo(a) variants representing naturally-occurring isoforms as well as various deletion and point mutants. These r-apo(a) constructs, which were derived from the original published human cDNA of apo(a), were generated from the expression plasmid pRK5 ha17 and their construction, expression, and purification have been previously described (27–30). Additionally, site-directed mutagenesis of pRK5 ha17 was used to generate new variants in which Lys^{12} or Lys^{42} in KV (17KΔLys^{12}, 17KΔLys^{42}) or both (17KΔ$Lys^{12/42}$) were mutated to alanine with similar techniques as for the above r-apo(a) constructs. A construct comprised of the 8K-IV wild-type (WT) and 8K-IV lysine-binding defective mutant (LBS^-) r-apo(a), used to generate transgenic mice with a truncated apo(a) as previously described (31–33), was also studied and their construction is described below.

Human subjects

All samples were acquired according to a study protocol approved by the University of California, San Diego Human Subjects Protection program and all study subjects gave their written informed consent before entering the study.

Source of animal plasma samples

Plasma samples from bonobos (*Pan paniscus*) (n = 4), chimpanzees (*Pan troglodytes*) (n = 7), gorillas (*Gorilla gorilla*) (n = 6), and baboons (*Papio species*) (n = 3) were provided by the San Diego Wild Animal Park, and plasmas from cynomolgus monkeys (*Macaca fascicularis*) (n = 4) were obtained as previously described (34). WT C57BL/6×SJL, $Apoe^{-/-}$, and $Ldlr^{-/-}$ mice were derived from existing mouse colonies. Apo(a) and Lp(a) transgenic mice with an apo(a) construct containing the apoE promoter and 8 KIV units (KIV_1, a single copy of KIV_2, a fusion of KIV_3 and KIV_5, and KIV_6 to KIV_{10}), KV, and the inactive protease-like domain on a C57BL/6×SJL background (n = 15) were previously described [termed 8K-IV apo(a)/Lp(a) mice] (33). These mice express both human apo(a) and human apoB-100 and have both elevated apo(a) and Lp(a) levels and increased levels of OxPLs on their apo(a)/Lp(a) particles (31–33). All animal studies were conducted using protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Generation of 8K-IV apo(a)/Lp(a) mice with defective KIV_{10} LBS

To generate 8K-IV apo(a)/Lp(a) mice with defective KIV_{10} LBS [8K-IV LBS^- apo(a)/Lp(a) mice], the Asp^{55} and Asp^{57} residues in the KIV_{10} LBS apo(a) cDNA construct were replaced by Ala^{55} and Ala^{57} residues, as previously described (35). The vector, pRK5 ha8Lysmuta, was digested with *Bgl*II, polished with *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and cut with *Eco*RI. The LBS mutant apo(a) fragment was inserted into a liver cDNA expression vector that had been digested with *Kpn*I and polished and cut as described above. *Eco*RI sites were introduced into the 5′ and 3′ ends of the *Apoe* hepatic control region (LE6) by PCR with primers 5′-CGGGAATTCTGCAGGCTCAGAG-3′ and 5′-GGGAATTCGAGCTCCGCGGCAGCCTGACCA-3′ (the 3′ primer contained a nested *Sac*II site). The LE6 was then ligated to the 3′ end of the LBS mutant apo(a) cDNA. The 8.6 kb LBS mutant apo(a) expression vector (containing the *Apoe* promoter, *Apoe* intron 1, the LBS mutant apo(a) cDNA, and LE6) was excised from pRK5 ha8Lysmuta with *Sac*II, purified, and microinjected into C57BL/6×SJL zygotes. The resultant mice were designated LBS^- apo(a) mice.

Breeding of 8K-IV WT and LBS^- r-apo(a)/Lp(a) mice

Hemizygous LBS^- apo(a) mice were crossed with hemizygous mice expressing human apoB-100 only, which were previously generated with an apoB mutation in codon 2,153 that prevented apoB-48 synthesis (36). All mice were on a C57/SJL background, weaned at 28 days of age, housed in a barrier facility with a 12 h light/12 h dark cycle, and fed a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO). Mice were genotyped by obtaining DNA from the tip of the tail and performing PCR using MyTaq HS Red Mix (BIOLINE, Inc., Taunton, MA). Forward and reverse primers for apo(a) were 5′-GACGGGAGACAGAGTGAAGC-3′ and 5′-TACCTAAACCACGCCAGGAC-3′, respectively, and for apoB 5′-GAAGAACTTCCGGAGAGTTGCAAT-3′ and 5′-CTCTTAGCCCCATTCAGCTCTGAC-3′, respectively. DNA samples were placed in a 1.5% agarose subgel containing ethidium bromide, run at 100 V for about 45 min, and visualized in a fluorescence reading chamber.

Expression of 8K-IV WT apo(a) and 8K-IV LBS^- r-apo(a) constructs in mammalian cells

Recombinant WT 8K-IV apo(a) was expressed in human embryonic 293 kidney cells (HEK293) and purified with a lysine Sepharose column as previously described (32). The original 8K construct in the pRK5 ha vector contained no selection gene. To introduce the 8K-IV LBS^- r-apo(a) cDNA into HEK293 cells, a cotransfection assay was performed using pcDNA3 containing a G418 Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) using a 3:1 ratio in order to ensure that the gene of interest was expressed. After several rounds of selection, individual colonies were selected and grown. Each colony was tested for expression by concentrating the media with 10,000 NMWL Amicon Ultra Centrifugal Filter Units from Millipore (Billerica, MA), purifying the 8K-IV LBS^- r-apo(a) by size exclusion chromatography (SW400 resin), and analyzing by both ELISA and Western blot. Nontransfected cells were also analyzed as a negative control.

Antibodies

Monoclonal antibodies MB47 specific for human apo B-100, LPA4 specific for apo(a), and E06 specific for the PC headgroup of OxPLs were previously described in detail (22).

Tandem LC-MS/MS analysis of OxPLs on r-apo(a) constructs

LC-MS/MS was utilized to assess the covalent binding of PC-containing OxPLs on the WT 17K r-apo(a) as well as the r-apo(a) 17KΔAsp^{57}, which has a substitution of Asp^{57} to Ala^{57} in the LBS of KIV_{10}. The 17K and 17KΔAsp^{57} r-apo(a) were thermally denatured, the disulfide bonds reduced by dithiothreitol (DTT), alkylated by iodoacetamide, and then digested with trypsin. In brief, to 100 μl of r-apo(a) (1 μg/μl in deionized water) was added 100 μl of ammonium bicarbonate buffer (50 mM pH 8) and 2 μl of 1 M DTT. The mixture was incubated at 50°C for 15 min. Twenty-five microliters of a saturated solution of iodoacetamide in DI water was added to the reaction mixture, which was kept in the dark at room temperature for 15 min. One half of the reaction mixture was then hydrolyzed in 30% NH_4OH for 14 h and the reaction mixture subjected to vacuum overnight to remove NH_4OH. The residue was reconstituted in 100 μl ammonium bicarbonate buffer and digested in trypsin with a molar ratio of 50:1 (r-apo(a):trypsin) overnight. The digested peptides were purified by solid phase

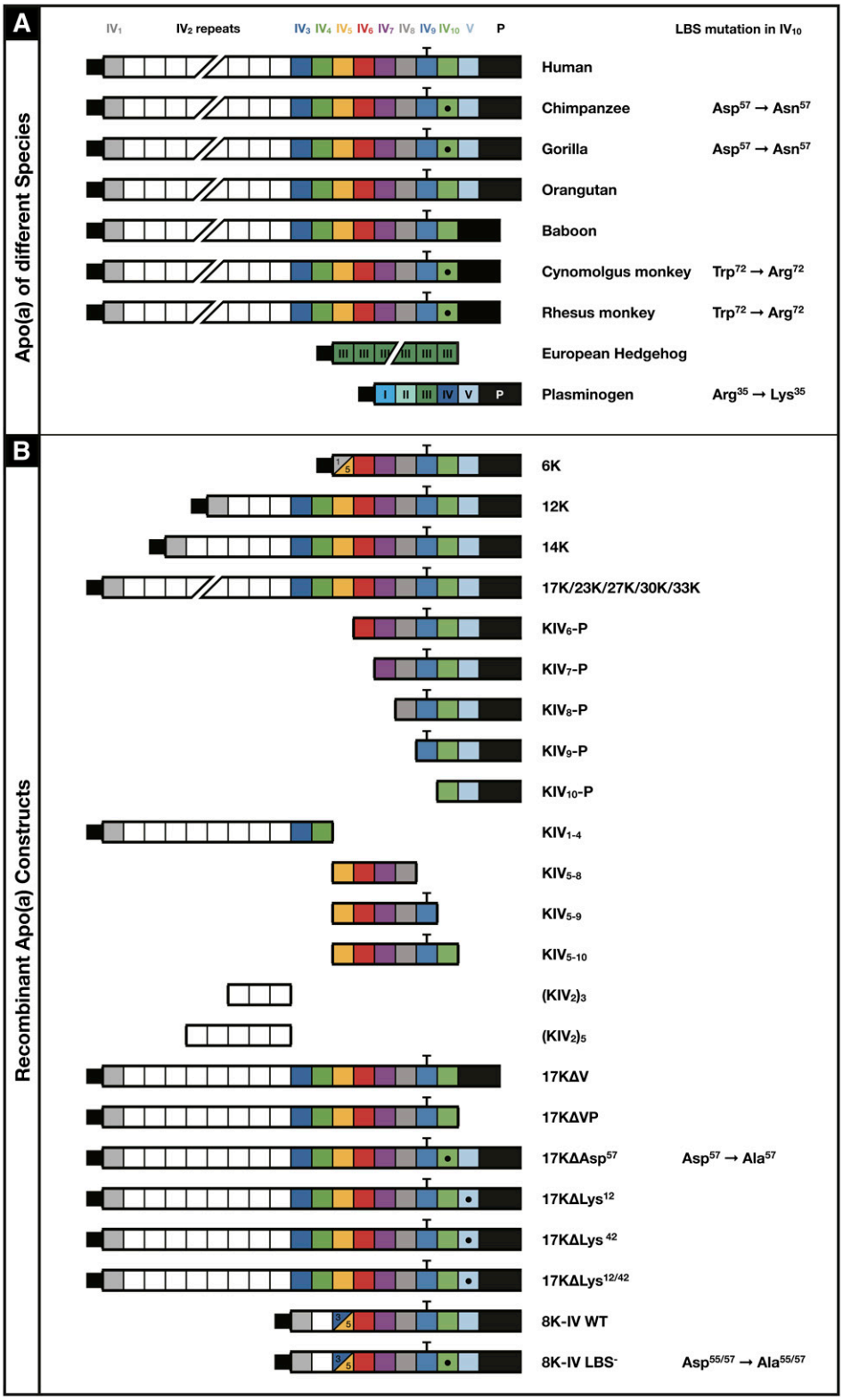

Fig. 2. Description of the species differences in apo(a) at the various apo(a) Ks (A) and the various r-apo(a) peptides (B) used in this study. The domain structure of the full-length r-apo(a) construct comprising 17 kringles (17K) is presented at the top, where squares resemble individual Ks, subscripts 1 to 10 denote the subtypes of KIV sequences, KV denotes the KV-like sequence and P is the inactive protease domain. The T-bar over KIV_9 indicates the location of the free-cysteine by which apo(a) is covalently bound to apoB-100 in Lp(a) via a disulfide bond. The dot within KIV_{10} indicates disruption of the LBS in this domain through mutagenesis and involved amino acids are indicated to the right. Variations include the 17K r-apo(a) without KV alone (17KΔV, baboon variant), also without the protease domain (17KΔVP), point mutations within the LBS ($17K\Delta Asp^{57}$, chimpanzee and gorilla variants), or deletions of key amino acids of KV ($17K\Delta Lys^{12}$,

extraction. A Waters SepPak Vac C18 1 cc cartridge was preconditioned with 1 ml of CH_3CN and 1 ml water; the digested peptides were loaded on the cartridge and washed with 1 ml of DI water. The peptides were eluted with 1 ml CH_3CN. The solvent was evaporated and the residue reconstituted in 100 μl of DI water.

LC-MS/MS analysis was carried out on a triple quadrupole instrument Thermal TSQ Vantage mass spectrometer coupled with a Waters NanoAcquity autosampler/UPLC system, as previously described (25, 37). The LC separation was achieved on a trapping column with 5 cm of 5 μm Jupiter C18 reverse phase material pressure-packed into a fused silica capillary (360 μm o.d. × 100 μm i.d.) and a resolving (analytical) column with 18 cm of 3 μm Jupiter C18 material pressure-packed into a capillary (360 μm o.d. × 100 μm i.d.) with a laser-pulled tip for nano-electrospray ionization. Full scan monitoring was carried out to scan a mass range of *m/z* 350 to 1,500. Precursor ion scanning (PIS) monitoring was carried out to identify a product ion of *m/z* 184, which is characteristic for the PC headgroup.

Identification of OxPLs in r-apo(a) constructs and plasma of humans and animals by chemiluminescent ELISA

The presence of apo(a), Lp(a), apoB, OxPL/apo(a), and OxPL/apoB in the various purified recombinant proteins and mouse, animal, and human plasma was performed by a variety of immunoassays, as previously described (17, 31, 32). Values are reported as relative light units (RLU) per 100 ms using chemiluminescent ELISA. Both direct plating of material and double antibody sandwich assays were utilized. Briefly, for direct plating assays, r-apo(a) constructs (5 μg/ml) or plasma (1:100 dilution) were plated on microtiter well plates overnight and apo(a) detected with antibody LPA4, OxPLs with antibody E06, and human apoB detected with antibody MB47, which does not cross-react with mouse apoB. For OxPL/apo(a) sandwich ELISA, LPA4 (5 μg/ml) as capture antibody was plated overnight and constructs (5 μg/ml) or plasma (1:100 to 1:400 dilution) added and OxPLs detected with biotinylated E06 (2 μg/ml). The human, ape, and monkey plasma samples were usually used at a 1:400 dilution, which was established as the concentration at which the relationship is linear. For OxPL/apoB sandwich ELISA, MB47 (5 μg/ml) as capture antibody was plated overnight and constructs (5 μg/ml) or plasma (1:100 dilution) added and OxPLs detected with biotinylated E06 (2 μg/ml). To determine Lp(a) levels in transgenic 8K-IV Lp(a) and 8K-IV LBS^- Lp(a) mice, MB47 was immobilized on microtiter well plates for capture of human apoB, plasma added (1:400), and Lp(a) detected with antibody LPA4. To determine apo(a) levels in 8K-IV WT and 8K-IV LBS^- r-apo(a) constructs, apo(a) was captured on microtiter well plates with LPA4 (5 μg/ml), constructs (5 μg/ml), and apo(a) detected with antibody LPA4 (the TRNYCRNPDAEIRP epitope for LPA4 is present on KIV-5, KIV-7, and KIV-8).

Identification of OxPLs in human Lp(a) by LC-MS/MS

Lp(a) from subjects with hypercholesterolemia was isolated using density gradient ultracentrifugation followed by size exclusion chromatography with a Sephacryl S-400 or lysine Sepharose chromatography (16). We extracted the OxPLs from intact purified Lp(a) with 2:1 chloroform:methanol and analyzed the extract for the presence of 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) (38–40), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), and epoxy-isoprostane-phosphocholine (PEIPC), which are highly bioactive OxPLs (41, 42). To identify specific species of OxPLs in the lipid phase of Lp(a) (i.e., extractable OxPLs), we employed reversed phase liquid chromatography directly coupled to a triple quadrupole MS (LC-MS/MS), as recently described (40, 43, 44). In brief, for PC, lyso-PC, and oxidized PC containing OxPLs (PC-OxPL) analysis, protonated adducts $(PC+H)^+$, $(lyso\text{-}PC+H)^+$, and $(PC\text{-}OxPL+H)^+$ are formed when operating the ion source in positive electrospray mode. The MS is operated in PIS mode using a collision energy setting of 35 V and a mass scan range of 500 to 900 atomic mass units. In a specified PIS mode, the MS detects all precursor ions within the mass scan range that produce the specified fragment. PC and lyso-PC species produce the PC headgroup with *m/z* = 184, regardless of their parent mass or moiety. Applied Biosystems MS software (Analyst 1.5.1) using the extracted ion current (XIC) option allows a search for specified precursor masses detected during the run.

Immunoblot analyses

Nonreducing SDS-PAGE of various apo(a) constructs, purified Lp(a), and PLG (all at 1 μg/ml) was carried out using precast gradient gels with 4–12% polyacrylamide concentrations and performed on a Xcell SureLock Mini-Cell system (Invitrogen) for 1.5 h in 1× MOPS SDS (NuPAGE MES SDS Running Buffer). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and Tween for 1 h. Incubation with biotinylated LPA4 and biotinylated EO6 was performed on separate membranes in 1% nonfat dry milk in Tris-buffered saline and Tween overnight at 4°C. Protein detection was performed with streptavidin conjugated to horseradish peroxidase (HRP) from R and D Systems (Minneapolis, MN). For visualization Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific Inc., Rockford, IL) was used as substrate and fluorescence detected at 428 nm wavelength on a BioSpectrumAC imaging system using VisionWorksLS imaging acquisition and analysis software from UVP (Upland, CA). In these immunoblots, OxPLs are denoted to reflect only E06-detectable OxPLs and not all OxPLs.

Statistics

For grouped numerical values, a mean value ± standard deviation (mean ± SD) was calculated. Analysis of quantitative parameters between groups was performed by two-tailed Student's unpaired *t*-test and within the same group by paired *t*-test or with repeated-measures one-way ANOVA. $P \leqslant 0.05$ was considered significant. Data presented in the text and tables are mean ± SD and in figures mean value ± standard error of the mean (mean ± SEM).

RESULTS

The LBS of KIV_{10} apo(a) consists of seven amino acids as follows by convention using the nomenclature of KV of PLG: Arg^{35}, Asp^{55}, Asp^{57}, Trp^{62}, Phe^{64}, Arg^{71}, and Trp^{72}. Because of the presence of several naming systems in the literature and differences in the number of amino acids in Ks between species, we provide a comparative summary of PLG KIV and KV, and apo(a) KIV_{10} of various species in **Fig. 3**.

17KΔLys^{42}, 17KΔ$Lys^{12/42}$), as well as fragments of the 17K construct including KIV_6-P, KIV_7-P, KIV_8-P, KIV_9-P, KIV_{10}-P, KIV_6-P, $KIV_{1–4}$, $KIV_{5–8}$, $KIV_{5–9}$, and $KIV_{5–10}$, and longer (23K, 27K, 30K, 33K) and shorter versions (6K, 12K, 14K) with variations of the KIV_2 repeats. 8K-IV indicates a short r-apo(a) version with a fusion of KIV_3 to KIV_5 that is identical to human apo(a) (WT) or has a disrupted LBS (LBS^-).

Amino acid number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Human Plasminogen Kringle V		M	F		N		K	G				K	R	A			V			T	P		Q	D		A	A	Q	E					S	I	F				T
Human Plasminogen Kringle IV	C	Y	H	G	D	G	Q	S	Y	R	G	T	S	S	T	T	T	T	G	K	K	C	Q	S	W	S	S	M	T	P	H	R	H	Q	K	-	T	P	E	N
Apolipoprotein (a) Kringle IV_{10} Human					N								F				V			R	T														R					
8K-IV WT mice					N								F				V			R	T														R					
8K-IV LBS^{-} mice					N								F				V			R	T														R					
Cynomolgus					N								F				V			R	T										Q		K	R					
Rhesus monkey					N								F				V			R	T										Q		K	R					
Baboon					N								F				V			R	T										Q		K	R					
Bonobo					N								F				V			R	T										Q			R					
Chimpanzee					N								F				V			R	T										Q			R					
Gorilla					N								F				V			R	T													R					
Orangutan					N								F				V			R	T			P							Q			R					

Amino acid number	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Human Plasminogen Kringle V	N		R				E	K								G		V		G				Y			N		R	K	L	Y	D			D	V	P	Q	
Human Plasminogen Kringle IV	Y	P	N	A	G	L	T	M	N	Y	C	R	N	P	D	A	D	K	G	-	P	W	C	F	T	T	D	P	S	V	R	W	E	Y	C	N	L	K	K	C
Apolipoprotein (a) Kringle IV_{10} Human				D														T								M				I							T	R	
8K-IV WT mice				D														T								M											T	R	
8K-IV LBS^{-} mice				D											A		A	T								M											T	R	
Cynomolgus	H			D														T								M					R						T	R	
Rhesus monkey	H			D														T								M					R						T	R	
Baboon	H			D														T								M											T	R	
Bonobo				D													N	T																			T	R	
Chimpanzee				D													N	T																			T	R	
Gorilla				D													N	T																			T	R	
Orangutan				D														T												I							T	Q	

Determination of OxPLs on Lp(a) and apoB from humans, apes, and monkeys using ELISA techniques

OxPL/apoB and OxPL/apo(a) were measured in five humans with widely different Lp(a) levels (data from >15,000 analyses of human plasma for OxPL/apoB and Lp(a) have been published previously (17), therefore we only show data from five representative humans for comparison), chimpanzees, bonobos, gorillas, baboons, and cynomolgus monkeys, and presented as mean levels and individual data points. **Figure 4A, C** demonstrates the levels of apoB and Lp(a) captured on the plates from each of the different samples. Figure 4B, D demonstrates the levels of OxPLs on apoB-100 particles and on apo(a) for each of these samples. It can be appreciated that OxPL/apoB and OxPL/apo(a) are mainly present in the humans with high Lp(a) levels, as previously well documented (17). However, all other species had essentially undetectable OxPL/apoB and OxPL/apo(a) levels, with values at or below the sensitivity of these assays (Fig. 4B, D). Importantly, E06 immunoreactivity was not present in chimpanzees and gorillas whose Lp(a) is known to contain KV.

Determination of OxPLs on 8K-IV WT apo(a) and 8K-IV LBS$^-$ apo(a) recombinant constructs

Immunoblot analysis of supernatants of HEK293 cells expressing 8K-IV WT r-apo(a) and 8K-IV LBS$^-$ r-apo(a) constructs demonstrates apo(a) immunoreactivity, detected by antibody LPA4, as expected (**Fig. 5A**). However, OxPLs detected by antibody EO6 is only present in the 8K-IV WT r-apo(a) but not in the LBS$^-$ r-apo(a) construct. There are no other E06 positive bands throughout the gel, suggesting that there are no other expressed proteins containing significant OxPLs. ELISA shows similar apo(a) levels for both variants, but OxPLs are only present in the WT 8K-IV r-apo(a) and not the LBS$^-$ 8K-IV r-apo(a) construct (Fig. 5B).

Determination of the presence of OxPLs on 8K-IV WT Lp(a) and 8K-IV LBS$^-$ Lp(a) transgenic mice

Circulating Lp(a) and apo(a) levels were not statistically different between 8K-IV WT Lp(a) and 8K-IV LBS$^-$ Lp(a) mice (**Fig. 6A, C**). However, OxPL/apoB (Fig. 6B, $P < 0.0001$) and OxPL/apo(a) (Fig. 6D, $P < 0.0001$) levels were significantly higher in the 8K-IV WT Lp(a) mice compared with the 8K-IV LBS$^-$ Lp(a) mice, where levels were essentially nondetectable.

Determination of the presence of OxPLs on r-apo(a) constructs

ELISA of various r-apo(a) constructs was performed to detect the presence of OxPLs using E06. The r-apo(a) constructs were initially captured on microtiter well plates with LPA4 and the amount of apo(a) captured then detected by biotinylated LPA4. For all constructs shown, the levels of r-apo(a) captured in the wells were fairly similar (**Fig. 7A**). All constructs have evidence of OxPL immunoreactivity, except the 17KΔAsp^{57} and the $(KIV_2)_5$ constructs (Fig. 7B). OxPLs were clearly present in apo(a) captured from the 17K and 17KΔVP constructs, which are missing KV and KV and the protease domain respectively, the I4399M r-apo(a) construct, which represents the LPA single nucleotide polymorphism (SNP) rs3798220 with an isoleucine to methionine substitution associated with CAD (45, 46) and high OxPL/apoB levels (47), and human plasma. No OxPLs were detectable on 17KΔAsp^{57} r-apo(a) (chimpanzee and gorilla variant) that contains KV but has a substitution of alanine to aspartate 57 on KIV_{10}.

Of note, Lys^{12} and Lys^{42} of KV had previously been postulated to bind OxPLs in both native apo(a) and r-apo(a) (13) based on computer modeling. However, constructs containing either mutation of Lys^{12} (17KΔLys^{12}) or Lys^{42} (17KΔLys^{42}) or both (17KΔLys$^{12/42}$) on KV contained measurable levels of OxPLs, though the amounts bound were lower than other constructs. Indeed, constructs lacking KV altogether (17KΔV and 17KΔVP) had similar E06 reactivity as 17K.

Because the amounts of apo(a) captured on the plates were not identical for all constructs, we also determined the OxPL/apo(a) ratio (Fig. 7C) by dividing the OxPL content on the plate by apo(a) content on separate plates (i.e., data in Fig. 6B divided by data in Fig. 6A), which shows similar results to the raw data.

Determination of the presence of OxPLs on r-apo(a) constructs using immunoblotting

Immunoblotting of various r-apo(a) constructs on SDS-PAGE identified the key determinant of OxPL binding to be KIV_{10}. OxPLs were present on Lp(a) and PLG, as expected, and on the r-apo(a) constructs 6K, 12K, 14K, 17K, 23K, 27K, 30K and 33K, 17KΔV, 17KΔVP, 17KΔLys$^{12/42}$ as well as truncated variants from KIV_6-P (KIV_6 through protease domain) to KIV_{10}-P. E06 immunoreactivity was not present on r-apo(a) consisting only of $KIV_{1–4}$, $(KIV_2)_5$, or 17KΔAsp^{57}, or on r-apo(a) constructs lacking KIV_{10} (**Fig. 8**). The presence of apo(a) in these constructs was confirmed in parallel gels.

Determination of OxPLs on 17K WT and 17KΔAsp^{57} r-apo(a) constructs using LC-MS/MS

We next utilized LC-MS/MS to identify PC containing OxPLs on r-apo(a) constructs 17K and 17KΔAsp^{57}. The presence of PC on OxPLs of trypsin digests of r-apo(a)s was assessed by PIS for $m/z = 184$, which is the signature of PC. A number of prominent peptide peaks containing PC were present in the LC-chromatogram in the 17K construct (**Fig. 9A**), indicating variously sized PC-modified peptides. Interestingly, the 17KΔAsp^{57} r-apo(a), despite having no E06 immunoreactivity, had a similar but not

Fig. 3. Amino acid composition of human PLG KIV and KV_{10} and human, ape, and monkey apo(a) KIV_{10}. One-letter symbols indicate amino acid changes and empty fields identical amino acids compared with KIV of human PLG. Blue: cysteine forming the disulfide bonds in the K structure. Green: amino acids of the LBS. Numbering according to KV of PLG. Note: KIV of PLG and IV_{10} subtypes of apo(a) are missing two amino acids compared with KV (indicated by a minus sign).

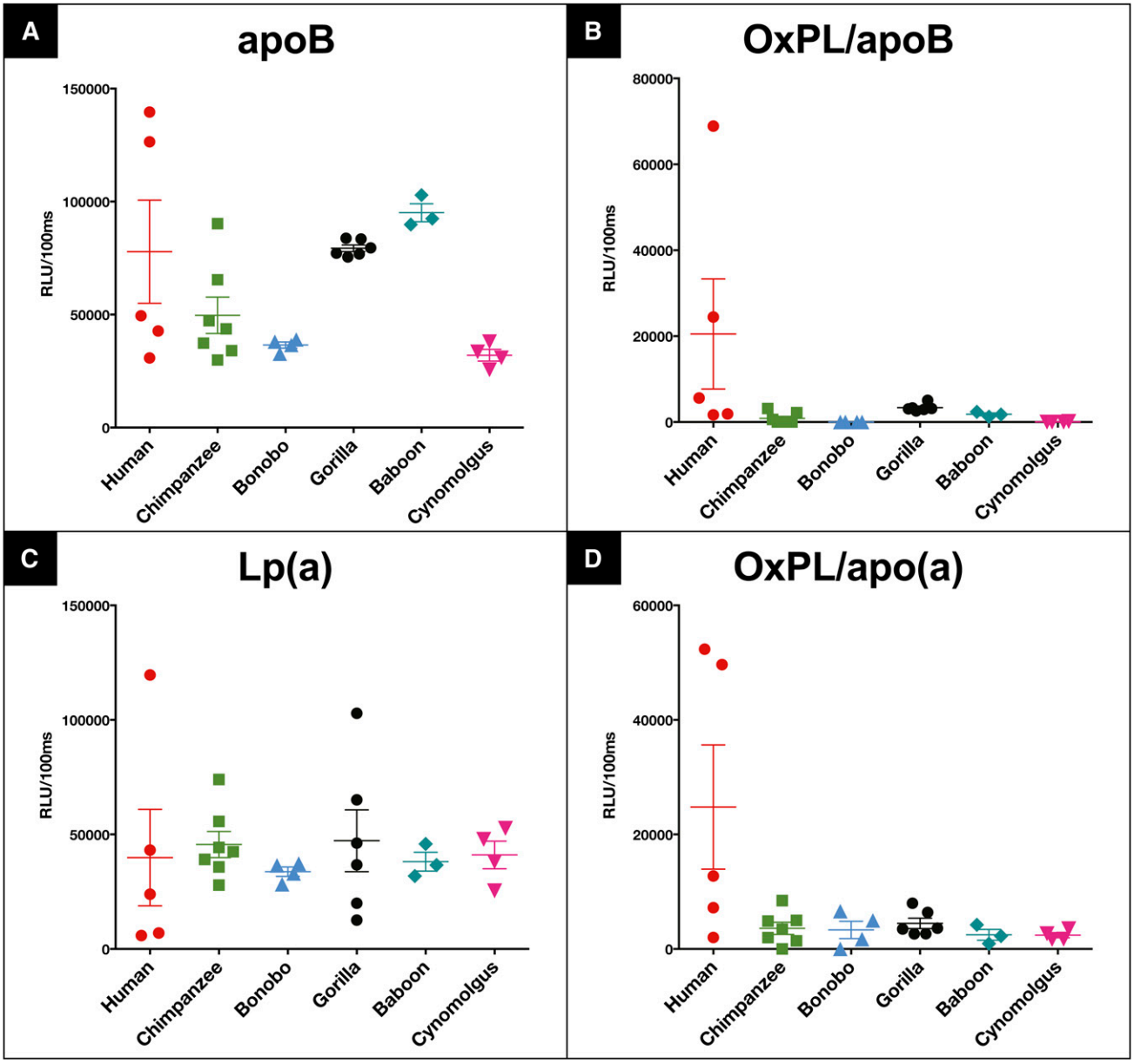

Fig. 4. ApoB-100 (A), OxPL/apoB (B), Lp(a) (C), and OxPL/apo(a) (D) of human (n = 5), chimpanzee (n = 7), bonobo (n = 4), gorilla (n = 6), baboon (n = 3), and cynomolgus monkey (n = 4) plasma. Bars indicate mean value and standard error of the mean. RLU, relative light units.

identical chromatogram (Fig. 9B). To rule out the possibility that the hydrolysis had destroyed the peptides themselves, we did full scan experiments on samples with and without NH_4OH treatment and similar patterns of peptides were observed (data not shown). These PC adducts can be hydrolyzed under basic conditions as shown in Fig. 9C, D. These data strongly suggest that the peaks shown in Fig. 9A, B were indeed peptides with covalently modified PC species, not peptides that happen to have fragments with *m/z* 184 and thus misidentified, otherwise, they would have shown up in Fig. 9C, D.

Determination of OxPLs in lipid phase of Lp(a) using LC-MS/MS following lipid extraction

We had previously demonstrated the ability to separate oxidized and nonoxidized species of varying polarities employing reverse phase LC-MS/MS, using a mixture of PC standards (Avanti Polar Lipids) such as POVPC, PGPC, PAzPC (1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine), ALDO-PC (1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine), and DLPC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine) (41, 43, 44). We have applied this methodology to identify noncovalently bound OxPLs on Lp(a) derived from three normal donors with Lp(a) levels ~90 mg/dl.

Lp(a) was isolated by density gradient ultracentrifugation followed by NaCl/ε-aminocaproic acid elution on lysine Sepharose. The purified Lp(a) was then extracted with 2:1 chloroform:methanol and the organic phase run on LC-MS/MS. Prominent peaks in the Lp(a) organic phase extract, but not in the aqueous phase, were positively identified as POVPC and PGPC using appropriate

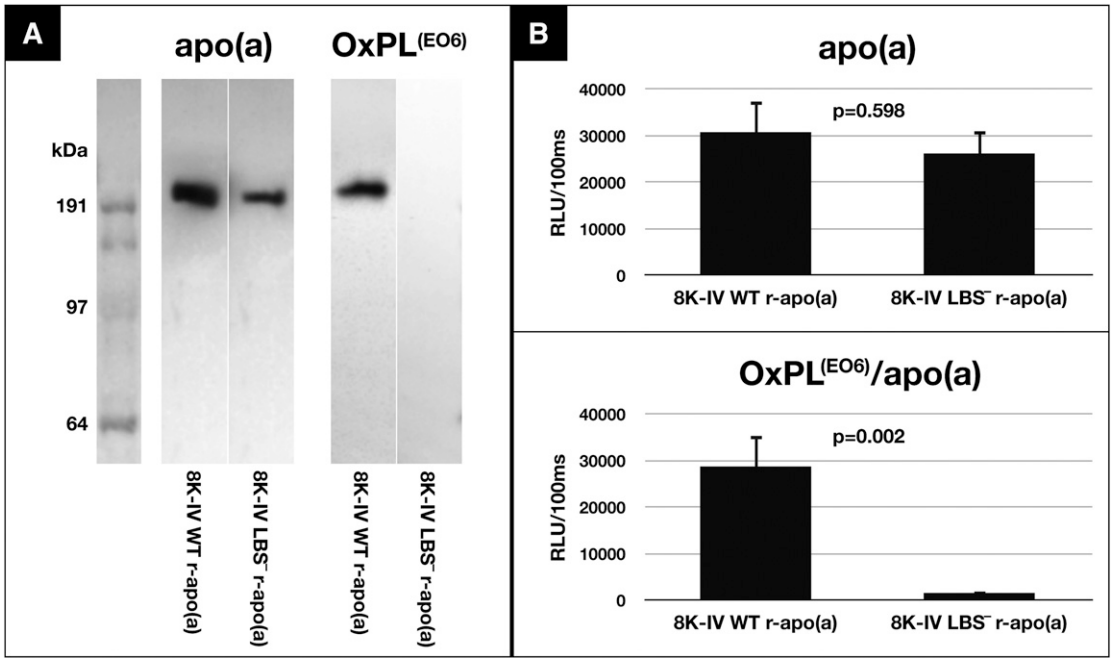

Fig. 5. WT versus LBS-deficient 8K-IV r-apo(a) from HEK293 cell line. A: Western blot: LPA4 detects both the WT and mutant r-apo(a), which were loaded after purification by lysine-Sepharose binding and by size exclusion chromatography. Only the WT r-apo(a) has EO6 reactivity, unlike the LBS-deficient r-apo(a) construct. B: ELISA: both versions of the r-apo(a) are detectable with LPA4, however only the WT version shows EO6 reactivity. RLU, relative light units.

standards (**Fig. 10**). POVPC is known to be detected by E06, but PGPC is not (38), confirming that Lp(a) contains lipid phase E06-detectable and E06-nondetectable OxPLs.

DISCUSSION

In this study we demonstrate several novel observations in the relationship between OxPLs and Lp(a): *1*) Among all the species tested that have circulating Lp(a), only human Lp(a) contains OxPLs detectable by monoclonal antibody E06; *2*) The PC-OxPLs in human Lp(a) are both covalently bound to apo(a) and also extractable from the lipid phase; *3*) Lp(a) transgenic mice expressing a modified human Lp(a) with an intact KIV_{10} LBS and the presence of KV contained E06-detectable OxPLs, whereas Lp(a) transgenic mice expressing a very similar apo(a) construct, but in which two critical amino acids of the LBS of apo(a) were modified, did not contain E06-detectable OxPLs. These data, along with similar results in selected r-apo(a) constructs, support the hypothesis that the KIV_{10} LBS, along with its well-known property of mediating fibrin binding (35, 48), influences covalent binding of OxPLs to apo(a); *4*) Lp(a) contains both E06-detectable (POVPC) and nonEO6-detectable (PGPC) OxPLs, consistent with its ability to bind a variety of OxPLs; and *5*) PC-containing OxPLs were shown to be present by LC-MS/MS on the natural human 17K r-apo(a), consistent with the ability of E06 to bind to this construct. The observation that saponification removed the PC signal from the peptides suggests that this PC was indeed part of a covalently linked OxPL. Surprisingly, however, we also found peptides containing PC in the $17K\Delta Asp^{57}$ construct, which represents a mutation present in the chimpanzee and gorilla, and which were not detected by E06 binding, suggesting either the presence of nonE06-detectable OxPLs or a steric hindrance mediated by the change in the LBS preventing E06 from detecting such OxPLs. Overall, these observations have implications for understanding the role of OxPLs in binding to Lp(a) and mediating human CVD.

Prior studies in transgenic mice have shown that modification of the LBS in KIV_{10} results in reduced focal accumulation of apo(a) in mouse aorta following high cholesterol diet (35, 48). Modifications of this binding pocket are present in all apes and monkeys studied, except the baboon and orangutan, and the apo(a)/Lp(a) from these species does not have EO6 immunoreactivity on ELISA. In most baboons, an intact LBS on apo(a) is functionally present that is similar to the human apo(a) that binds lysine. However, baboon Lp(a), as opposed to apo(a), does not bind to lysine or to plasmin-modified fibrinogen (49). Interestingly, KV is absent in baboon apo(a) and thus, in baboon Lp(a) the absence of KV appears to prevent access to lysine resides in the KIV_{10} LBS due to altered apo(a) confirmation causing steric hindrance (49). Even in humans, significant lysine binding heterogeneity of apo(a) exists among different subjects, and part of this heterogeneity may be mediated by noncovalent associations of Lp(a) with other circulating proteins that may mask the KIV_{10} LBS (50, 51).

The role of KIV_{10} LBS in mediating binding of OxPLs was further documented by lack of E06 immunoreactivity in r-apo(a) constructs that were designed with various changes in the LBS and other K sites, as well by lack of E06

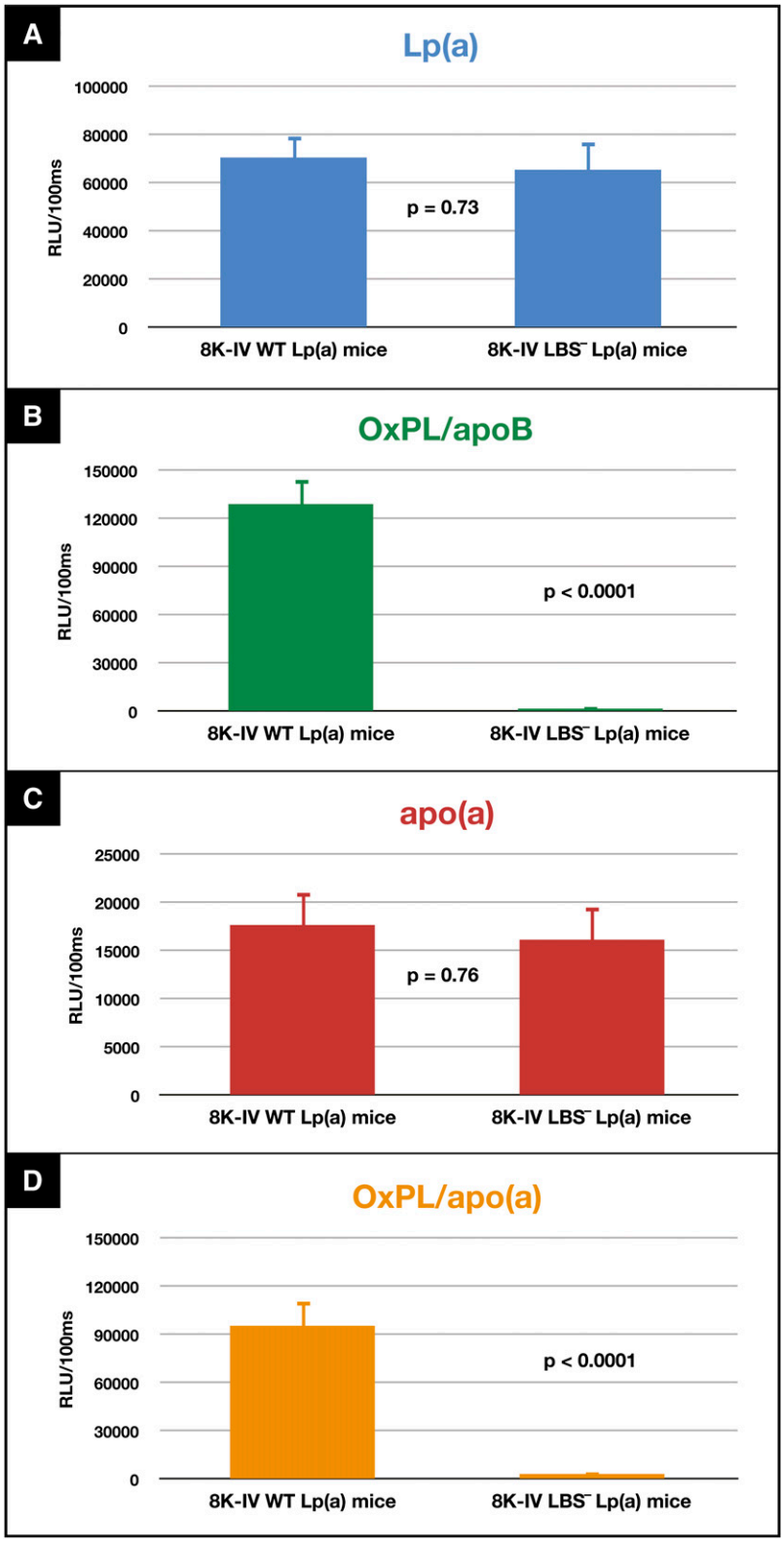

Fig. 6. Lp(a) (A), OxPL/apoB (B), apo(a) (C), and OxPL/apo(a) (D) measurements of plasma from WT Lp(a) transgenic mice and LBS-deficient Lp(a) transgenic mice. Plasma from each of these mice was subjected to the various assays described in Methods, and the results presented as relative light units (RLU)/100 ms. Plasma from 15 WT and 15 LBS-deficient mice were studied.

immunoreactivity in Lp(a) of mice expressing an LBS$^-$ apo(a) transgene. In previous studies (13), it was suggested by enzymatic digestion of human apo(a) that KV was the site containing contained OxPLs and that rhesus monkey apo(a), which does not contain KV, had no OxPLs, as demonstrated by lack of E06 immunoreactivity. Computer modeling further suggested that Lys^{12} and Lys^{42} of KV covalently bound OxPLs, but no experimental confirmation of the data were presented and nonhuman primate apo(a) containing KV was not evaluated. In contrast, our current results show that the presence of KV is not necessary for OxPL binding, as r-apo(a) constructs lacking KV had strong E06 immunoreactivity. Furthermore, chimpanzees (and bonobos) that have KV did not show E06

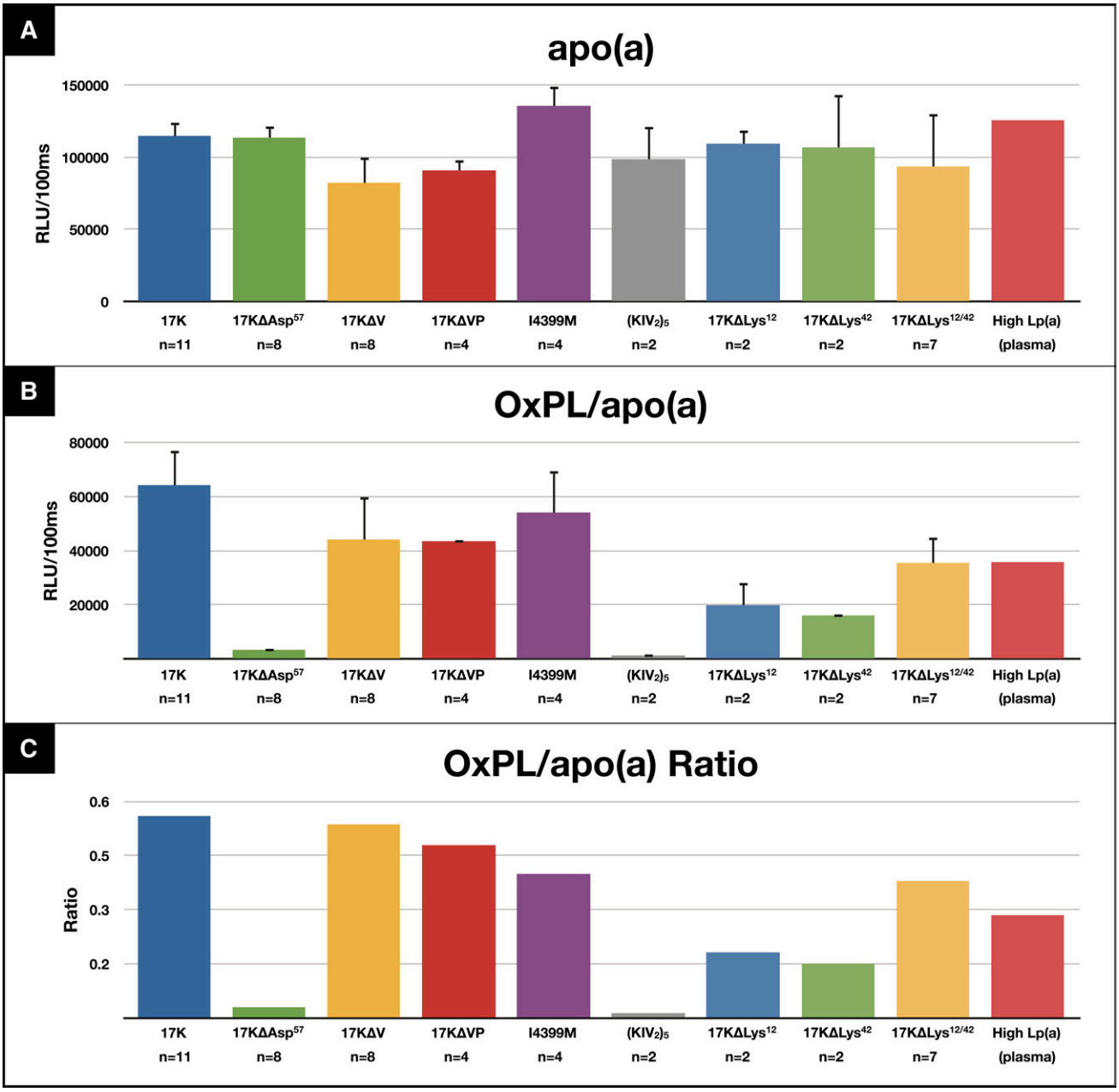

Fig. 7. Assessment of the presence of OxPL on various r-apo(a) constructs via ELISA as shown in Fig. 2. I4399M = LPA SNP rs3798220 (single nucleotide polymorphism of the LPA gene that is associated with increased Lp(a) levels and an increased risk for CAD). High Lp(a) (plasma) = plasma from a healthy subject with high Lp(a) plasma levels (70 mg/dl).

immunoreactivity, a finding that seems to be explained by their KIV_{10} Asp^{57}→Ala^{57} substitution compared with human apo(a). This observation does not appear to be due to lack of circulating OxPLs, as cholesterol- and fat-fed cynomolgus monkeys with total plasma cholesterol ∼700 mg/dl and abundant E06-detectable OxPLs in atherosclerotic lesions also do not have E06-detectable OxPLs on Lp(a), as shown previously (34), but they do on PLG which has an intact KIV_{10} LBS compared with Lp(a) (25). In addition, cynomolgus monkeys do carry OxPLs on apoB particles, as previously shown (34).

In general, the most likely amino acids binding OxPLs are cysteines, lysines, and histidines. The seven amino acids that define the KIV_{10} LBS do not have terminal amines or cysteines and are unlikely themselves to bind OxPLs. There are no lysines in KIV_{10} in human apo(a) (see Fig. 2) and all six cysteines are occupied in disulfide bonds. Therefore, it appears that histidines, of which there are three in KIV_{10}, are the likely sites that bind OxPLs in KIV_{10} in human apo(a). In particular, His^{31} and His^{33}, which flank Arg^{32}, are strong candidates for binding OxPLs. Compared with humans and gorillas, all other species with apo(a) have an Arg to Gln substitution at amino acid position 32 in KIV_{10}. Previous modeling studies (49) suggested that Arg^{32}, which is substituted in all other Ks in human apo(a) (4), quite markedly alters the conformation of this K that may allow the histidines to bind OxPLs. Further work removing the entire KIV_{10} and/or utilizing site-specific mutagenesis of likely

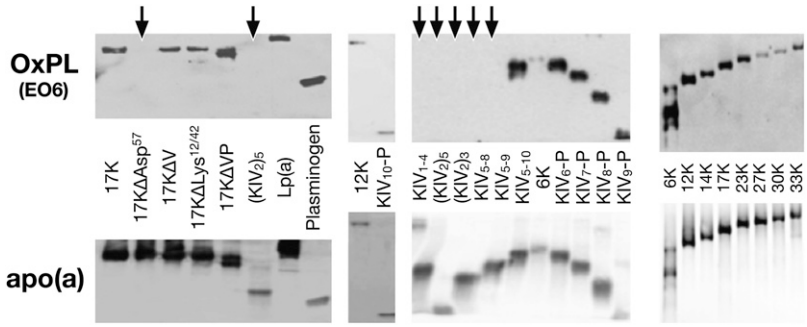

Fig. 8. Immunoblotting of various r-apo(a) constructs and human PLG with E06 for PC-OxPL and an anti-apo(a) antibody. Individual constructs are explained in detail in Fig. 2. Arrows indicate where E06 activity is not present. In these immunoblots OxPLs are denoted to reflect only E06-detectable OxPLs and not all OxPLs. RLU, relative light units.

sites will be needed to identify the exact amino acids involved in the covalent binding of OxPLs.

An important finding in this study was that OxPLs are not only present covalently on apo(a) but are also present in the lipid phase of Lp(a). We previously showed that E06 immunoreactivity was present in organic extracts of human Lp(a) (16). Now utilizing LC-MS/MS analyses of organic extracts of human Lp(a), we confirm that specific

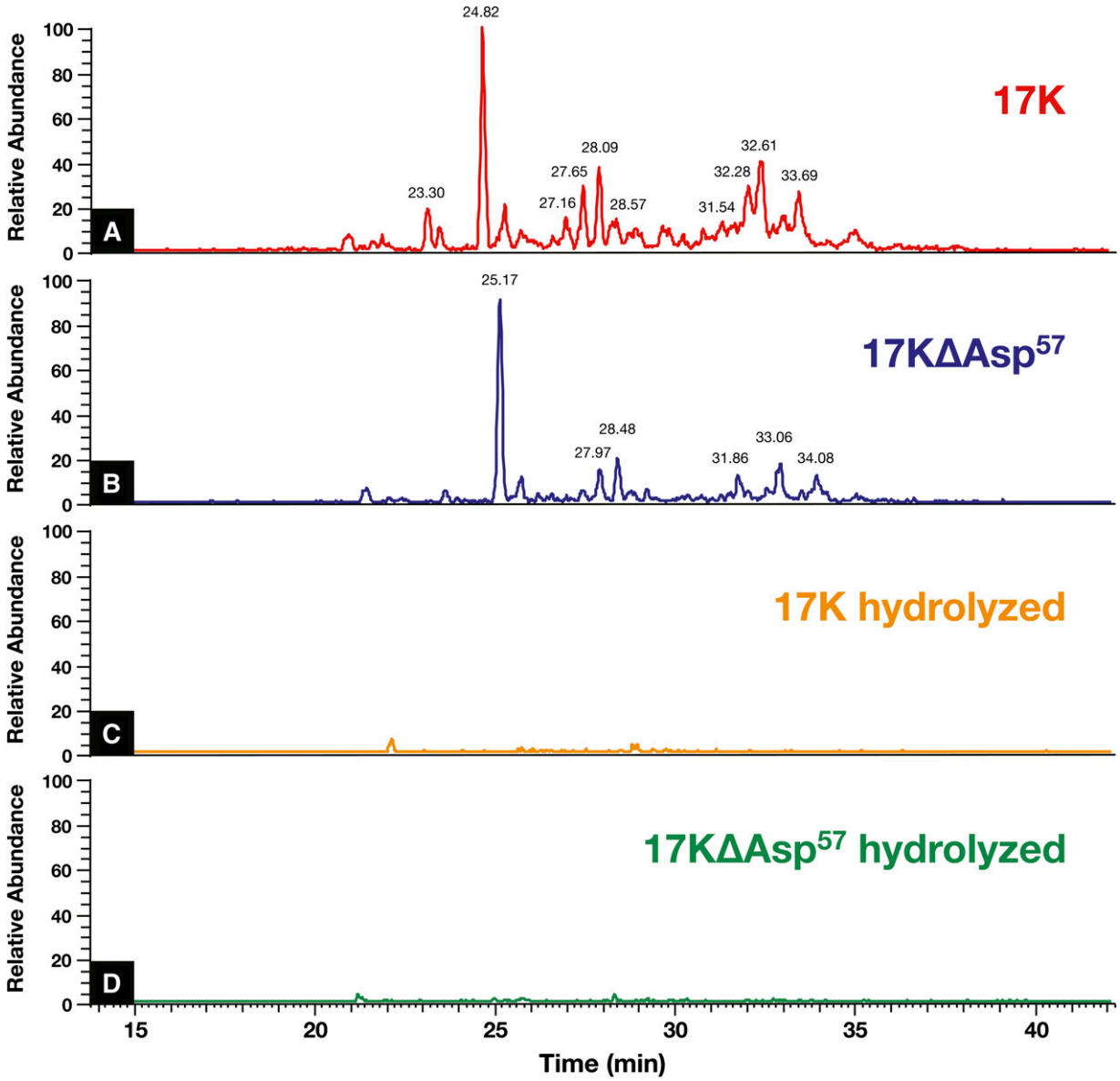

Fig. 9. Identification of OxPLs in r-apo(a) constructs. LC-MS/MS experiments on 17K (A), 17KΔAsp^{57} (B), and their hydrolyzed versions (C, D).

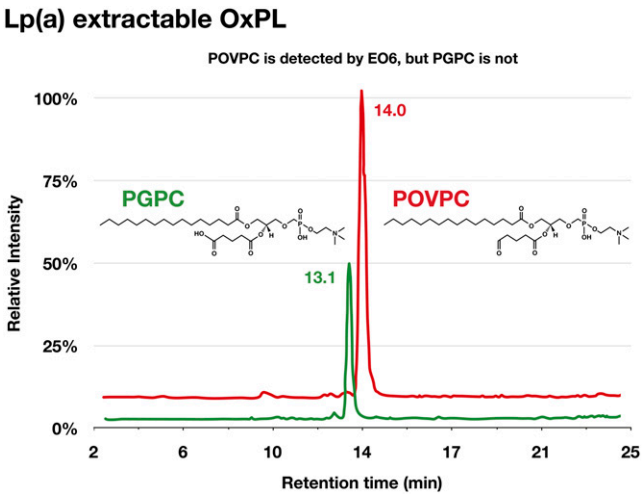

Fig. 10. Determination of PC containing OxPLs in organic phase of isolated of Lp(a) following lipid extraction using LC-MS/MS. Data represent findings from three normal donors with Lp(a) levels ~90 mg/dl.

OxPLs are present, such as POVPC, which is recognized by EO6, as well as PGPC, which is not recognized by E06 as previously documented (38). This demonstrates that there are OxPLs present in Lp(a) that are not recognized by E06 and suggests that the ability of Lp(a) to bind various OxPLs is a generalized property. The relative amounts of OxPLs on apo(a) versus Lp(a) have not been fully established and may vary according to underlying disease states. Indeed, we found previously that the amount of E06-detectable OxPLs in organic extracts of human Lp(a) varied considerably, with ~30–70% of E06 immunoreactivity in Lp(a) from various subjects being lipid soluble (16).

The r-apo(a) constructs contained covalently bound OxPLs. We speculate that the OxPLs on the r-apo(a) constructs are likely derived from OxPLs generated from apoptotic cells in cell culture (52). In vivo, OxPLs on Lp(a) may be derived from hepatocytes during Lp(a) assembly, particularly if there is enhanced hepatocyte oxidative stress, such as in nonalcoholic steatohepatitis (53), or transferred to Lp(a) in plasma from atherosclerotic plaques, as suggested by prior human studies in patients undergoing percutaneous coronary intervention (22, 54). Importantly, however, our studies of OxPLs on apo(a), expressed both in cell culture and found in mouse plasma, show a similar dependence on an intact LBS in KIV_{10}, suggesting that the OxPLs acquired by apo(a) in vitro are a relevant model for those acquired by the apo(a) component of Lp(a) in vivo.

A summary of the differences in Ks, attachment to LDL, protease activity, fibrin binding, and OxPL binding in PLG and apo(a) is given in **Fig. 11**. The evolution of Lp(a) in apes and monkeys from PLG has occurred concomitantly with losing the lysine binding properties of the KIV_{10} LBS. This has occurred through a variety of mechanisms, including loss of KV and/or modifications of KIV_{10} LBS (49, 55, 56). Along with loss of lysine binding, these species also do not have E06 immunoreactivity on Lp(a). Interestingly, we recently showed that humans, apes, monkeys, and lower species such as mice do have OxPLs on circulating PLG, which is not known to have similar LBS substitutions as apo(a), and that the presence of OxPLs on PLG actually mediates more optimal fibrinolysis (25). Thus, the presence of OxPLs on PLG would be expected to be protective against atherothrombosis, as opposed to OxPLs on Lp(a) being pro-atherogenic. This suggests that during evolution, human Lp(a), as opposed to Lp(a) on other species, has further evolved from ape and monkey Lp(a) by regaining the ability to both bind lysine through the KV_{10} LBS and also to overtly capture E06-detectable OxPLs.

We have previously shown that the PC of OxPLs present on OxPLs of OxLDL, or apoptotic cells or apoptotic blebs and debris represents a "danger associated molecular pattern" (57), which is recognized by a variety of innate "pattern recognition receptors" such as natural antibodies (58), scavenger receptors, e.g., CD36 (39) and C-reactive protein (59). Each of these pattern recognition receptors can bind and/or scavenge PC containing OxPLs, which are pro-inflammatory. In support of this, Lp(a) and its associated OxPLs were recently shown to trigger apoptosis in endoplasmic reticulum-stressed macrophages through a mechanism requiring both CD36 and Toll-like receptor 2 (40). In a similar manner, malondialdehyde epitopes, which represent another well-described oxidation-specific epitope resulting from lipid peroxidation, are bound by complement factor H, which protects against the oxidative stress that may mediate age-related macular degeneration (60). In future studies, demonstration of human "E06"-like antibodies may provide additional insights into the role of OxPLs in humans as danger-associated molecular patterns and the response of the adaptive and innate immune systems in removing them to protect the host (57, 61, 62).

It is now well-established that high levels of Lp(a) are a major risk factor for CAD (11). Lp(a) has an increased affinity for the extracellular matrix of the intima compared with LDL, and one can envision that the enhanced content of OxPLs would make Lp(a) even more pro-inflammatory. It was previously shown that the LBS of human Lp(a) is a key component in mediating the accumulation of Lp(a) in the intima as well, and in mediating enhanced fatty streak formation (35, 48). As shown in this study, such LBS intact Lp(a) as found in humans would also be enriched in OxPLs. This would lead to the hypothesis that an Lp(a) with loss of the LBS would also be associated with in an inability to bind OxPLs and this would be less atherogenic. Studies are underway in the LBS^{-} apo(a)/Lp(a) to assess whether they have less atherogenesis than WT apo(a)/Lp(a) mice with similar Lp(a) levels. Future studies will also need to dissociate LBS features of apo(a) along with the lack of E06 immunoreactivity to assess whether the reduced atherogenesis is due to the defective LBS or the failure to contain OxPLs.

Limitations of this study include the lack of adequate nonhuman primate plasma to perform comprehensive studies such as isolation of Lp(a), immunoblotting, or LC-MS/MS of lipid extracts to directly assess for the presence of OxPLs. Whether these species carry OxPLs on other proteins cannot be determined at present. However, the r-apo(a) constructs used covered the key differences in apo(a) present in apes

A

Species	Kringles III	Kringles IV	Kringles V	Attachment to LDL	Protease Domain	Protease Activity	Fibrin binding	OxPL binding (EO6)
Human	–	✓	✓	✓	✓	–	✓	✓
Bonobo	–	✓°	✓	✓	✓	–	–	–
Chimpanzee	–	✓°	✓	✓	✓	–	–	–
Gorilla	–	✓°	✓	✓	✓	–	–	–
Orangutan	–	✓	✓	✓	✓	–	?	?
Baboon	–	✓*	–	✓	✓	–	–	–
Cynomolgus	–	✓°	–	✓	✓	–	–	–
Rhesus monkey	–	✓°	–	✓	✓	–	–	–
Hedgehog	✓	–	–	✓	–	–	✓	–
Plasminogen	✓	✓	✓	–	✓	✓	✓	✓

? = data not available in the literature
* = contains all 7 amino acids in LBS but does not bind lysine-Sepharose
° = mutation in 1 amino acid of LBS and does not bind lysine

B

Lysine Binding Site	Key Amino Acid Differences
Plasminogen KIV	Trp^{62} I Phe^{64} I Trp^{72} Asp^{55} I Asp^{57} Lys^{35} I Arg^{71}
Human apo(a) KIV_{10}	Lys^{35} ➡ Arg^{35}
Cynomolgus & Rhesus monkey apo(a) KIV_{10} Rare human mutations	Trp^{72} ➡ Arg^{72}
Chimpanzee & Gorilla apo(a) KIV_{10}	Asp^{57} ➡ Asn^{57}
	Aromatic / Anionic / Cationic

Fig. 11. Summary of differences in E06 immunoreactivity of various species. A: Depicts the presence of KIII, KIV variants, KV, and the protease domain of apo(a) from various species and human PLG. B: Depicts the key amino acids of the functionally intact LBS of KIV of PLG, the conservative substitution of KIV_{10} of human apo(a), and two individual amino acid substitutions in monkeys, apes, and rare human mutations that render the LBS defective.

and monkeys compared with humans and may potentially be considered appropriate apo(a) surrogates.

In summary, E06- and nonE06-detectable PC containing OxPLs are present in the lipid phase of Lp(a) and covalently bound to apo(a). E06 immunoreactivity is strongly influenced by the KIV_{10} LBS and is unique to human Lp(a), which may explain its pro-atherogenic potential. Human clinical trials with potent Lp(a) lowering agents, that also may reduce their OxPL content (31, 32), will provide the ultimate evidence of the atherogenicity of Lp(a) (63).

REFERENCES

1. Dubé, J. B., M. B. Boffa, R. A. Hegele, and M. L. Koschinsky. 2012. Lipoprotein(a): more interesting than ever after 50 years. *Curr. Opin. Lipidol.* **23:** 133–140.
2. Hoover-Plow, J., and M. Huang. 2013. Lipoprotein(a) metabolism: potential sites for therapeutic targets. *Metabolism.* **62:** 479–491.
3. Kronenberg, F., and G. Utermann. 2013. Lipoprotein(a): resurrected by genetics. *J. Intern. Med.* **273:** 6–30.
4. McLean, J. W., J. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature.* **330:** 132–137.
5. Lawn, R. M., N. W. Boonmark, K. Schwartz, G. E. Lindahl, D. P. Wade, C. D. Byrne, K. J. Fong, K. Meer, and L. Patthy. 1995. The recurring evolution of lipoprotein(a). Insights from cloning of hedgehog apolipoprotein(a). *J. Biol. Chem.* **270:** 24004–24009.
6. Tsimikas, S., Z. Mallat, P. J. Talmud, J. J. Kastelein, N. J. Wareham, M. S. Sandhu, E. R. Miller, J. Benessiano, A. Tedgui, J. L. Witztum, et al. 2010. Oxidation-specific biomarkers, lipoprotein(a), and risk of fatal and nonfatal coronary events. *J. Am. Coll. Cardiol.* **56:** 946–955.
7. Erqou, S., A. Thompson, E. Di Angelantonio, D. Saleheen, S. Kaptoge, S. Marcovina, and J. Danesh. 2010. Apolipoprotein(a) isoforms and the risk of vascular disease: systematic review of 40 studies involving 58,000 participants. *J. Am. Coll. Cardiol.* **55:** 2160–2167.
8. Emerging Risk Factors Collaboration, S. Ergou, S. Kaptoge, P. L. Perry, E. Di Angelantonio, A. Thompson, I. R. White, S. M. Marcovina, R. Collins, S. G. Thompson, and J. Danesh. 2009. Lipoprotein(a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA.* **302:** 412–423.

9. Clarke, R., J. F. Peden, J. C. Hopewell, T. Kyriakou, A. Goel, S. C. Heath, S. Parish, S. Barlera, M. G. Franzosi, S. Rust, et al.; PROCARDIS Consortium. 2009. Genetic variants associated with Lp(a) lipoprotein level and coronary disease. *N. Engl. J. Med.* **361:** 2518–2528.
10. Kamstrup, P. R., A. Tybjaerg-Hansen, R. Steffensen, and B. G. Nordestgaard. 2009. Genetically elevated lipoprotein(a) and increased risk of myocardial infarction. *JAMA.* **301:** 2331–2339.
11. Tsimikas, S., and J. H. Hall. 2012. Lipoprotein(a) as a potential causal genetic risk factor of cardiovascular disease: a rationale for increased efforts to understand its pathophysiology and develop targeted therapies. *J. Am. Coll. Cardiol.* **60:** 716–721.
12. Tsimikas, S., C. Bergmark, R. W. Beyer, R. Patel, J. Pattison, E. Miller, J. Juliano, and J. L. Witztum. 2003. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J. Am. Coll. Cardiol.* **41:** 360–370.
13. Edelstein, C., D. Pfaffinger, J. Hinman, E. Miller, G. Lipkind, S. Tsimikas, C. Bergmark, G. S. Getz, J. L. Witztum, and A. M. Scanu. 2003. Lysine-phosphatidylcholine adducts in kringle V impart unique immunological and potential pro-inflammatory properties to human apolipoprotein(a). *J. Biol. Chem.* **278:** 52841–52847.
14. Tsimikas, S., E. S. Brilakis, E. R. Miller, J. P. McConnell, R. J. Lennon, K. S. Kornman, J. L. Witztum, and P. B. Berger. 2005. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. *N. Engl. J. Med.* **353:** 46–57.
15. Tsimikas, S., and J. L. Witztum. 2008. The role of oxidized phospholipids in mediating lipoprotein(a) atherogenicity. *Curr. Opin. Lipidol.* **19:** 369–377.
16. Bergmark, C., A. Dewan, A. Orsoni, E. Merki, E. R. Miller, M. J. Shin, C. J. Binder, S. Horkko, R. M. Krauss, M. J. Chapman, et al. 2008. A novel function of lipoprotein [a] as a preferential carrier of oxidized phospholipids in human plasma. *J. Lipid Res.* **49:** 2230–2239.
17. Taleb, A., J. L. Witztum, and S. Tsimikas. 2011. Oxidized phospholipids on apolipoprotein B-100 (OxPL/apoB) containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark. Med.* **5:** 673–694.
18. van Dijk, R. A., F. Kolodgie, A. Ravandi, G. Leibundgut, P. P. Hu, A. Prasad, E. Mahmud, E. Dennis, L. K. Curtiss, J. L. Witztum, et al. 2012. Differential expression of oxidation-specific epitopes and apolipoprotein(a) in progressing and ruptured human coronary and carotid atherosclerotic lesions. *J. Lipid Res.* **53:** 2773–2790.
19. Fefer, P., S. Tsimikas, A. Segev, J. Sparkes, F. Otsuma, F. Kolodgie, R. Virmani, J. Juliano, T. Charron, and B. H. Strauss. 2012. The role of oxidized phospholipids, lipoprotein (a) and biomarkers of oxidized lipoproteins in chronically occluded coronary arteries in sudden cardiac death and following successful percutaneous revascularization. *Cardiovasc. Revasc. Med.* **13:** 11–19.
20. Tsimikas, S., S. Kiechl, J. Willeit, M. Mayr, E. R. Miller, F. Kronenberg, Q. Xu, C. Bergmark, S. Weger, F. Oberhollenzer, et al. 2006. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. *J. Am. Coll. Cardiol.* **47:** 2219–2228.
21. Gong, Y., and J. Hoover-Plow. 2012. The plasminogen system in regulating stem cell mobilization. *J. Biomed. Biotechnol.* **2012:** 437920.
22. Tsimikas, S., H. K. Lau, K. R. Han, B. Shortal, E. R. Miller, A. Segev, L. K. Curtiss, J. L. Witztum, and B. H. Strauss. 2004. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* **109:** 3164–3170.
23. Kiechl, S., J. Willeit, M. Mayr, B. Viehweider, M. Oberhollenzer, F. Kronenberg, C. J. Wiedermann, S. Oberthaler, Q. Xu, J. L. Witztum, et al. 2007. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1788–1795.
24. Tsimikas, S., P. Willeit, J. Willeit, P. Santer, M. Mayr, Q. Xu, A. Mayr, J. L. Witztum, and S. Kiechl. 2012. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J. Am. Coll. Cardiol.* **60:** 2218–2229.
25. Leibundgut, G., K. Arai, A. Orsoni, H. Yin, C. Scipione, E. R. Miller, M. L. Koschinsky, M. J. Chapman, J. L. Witztum, and S. Tsimikas. 2012. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. *J. Am. Coll. Cardiol.* **59:** 1426–1437.
26. Mulichak, A. M., A. Tulinsky, and K. G. Ravichandran. 1991. Crystal and molecular structure of human plasminogen kringle 4 refined at 1.9-A resolution. *Biochemistry.* **30:** 10576–10588.
27. Koschinsky, M. L., J. E. Tomlinson, T. F. Zioncheck, K. Schwartz, D. L. Eaton, and R. M. Lawn. 1991. Apolipoprotein(a): expression and characterization of a recombinant form of the protein in mammalian cells. *Biochemistry.* **30:** 5044–5051.
28. Sangrar, W., and M. L. Koschinsky. 2000. Characterization of the interaction of recombinant apolipoprotein(a) with modified fibrinogen surfaces and fibrin clots. *Biochem. Cell Biol.* **78:** 519–525.
29. Hancock, M. A., M. B. Boffa, S. M. Marcovina, M. E. Nesheim, and M. L. Koschinsky. 2003. Inhibition of plasminogen activation by lipoprotein(a): critical domains in apolipoprotein(a) and mechanism of inhibition on fibrin and degraded fibrin surfaces. *J. Biol. Chem.* **278:** 23260–23269.
30. Feric, N. T., M. B. Boffa, S. M. Johnston, and M. L. Koschinsky. 2008. Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen: a novel mechanism for lipoprotein(a)-mediated inhibition of plasminogen activation. *J. Thromb. Haemost.* **6:** 2113–2120.
31. Merki, E., M. J. Graham, A. E. Mullick, E. R. Miller, R. M. Crooke, R. E. Pitas, J. L. Witztum, and S. Tsimikas. 2008. Antisense oligonucleotide directed to human apolipoprotein B-100 reduces lipoprotein(a) levels and oxidized phospholipids on human apolipoprotein B-100 particles in lipoprotein(a) transgenic mice. *Circulation.* **118:** 743–753.
32. Merki, E., M. Graham, A. Taleb, G. Leibundgut, X. Yang, E. R. Miller, W. Fu, A. E. Mullick, R. Lee, P. Willeit, et al. 2011. Antisense oligonucleotide lowers plasma levels of apolipoprotein (a) and lipoprotein (a) in transgenic mice. *J. Am. Coll. Cardiol.* **57:** 1611–1621.
33. Schneider, M., J. L. Witztum, S. G. Young, E. H. Ludwig, E. R. Miller, S. Tsimikas, L. K. Curtiss, S. M. Marcovina, J. M. Taylor, R. M. Lawn, et al. 2005. High-level lipoprotein [a] expression in transgenic mice: evidence for oxidized phospholipids in lipoprotein [a] but not in low density lipoproteins. *J. Lipid Res.* **46:** 769–778.
34. Tsimikas, S., M. Aikawa, F. J. Miller, Jr., E. R. Miller, M. Torzewski, S. R. Lentz, C. Bergmark, D. D. Heistad, P. Libby, and J. L. Witztum. 2007. Increased plasma oxidized phospholipid:apolipoprotein B-100 ratio with concomitant depletion of oxidized phospholipids from atherosclerotic lesions after dietary lipid-lowering: a potential biomarker of early atherosclerosis regression. *Arterioscler. Thromb. Vasc. Biol.* **27:** 175–181.
35. Boonmark, N. W., X. J. Lou, Z. J. Yang, K. Schwartz, J. L. Zhang, E. M. Rubin, and R. M. Lawn. 1997. Modification of apolipoprotein(a) lysine binding site reduces atherosclerosis in transgenic mice. *J. Clin. Invest.* **100:** 558–564.
36. Skålén, K., M. Gustafsson, E. K. Rydberg, L. M. Hultén, O. Wiklund, T. L. Innerarity, and J. Borén. 2002. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature.* **417:** 750–754.
37. Szapacs, M. E., H. Y. Kim, N. A. Porter, and D. C. Liebler. 2008. Identification of proteins adducted by lipid peroxidation products in plasma and modifications of apolipoprotein A1 with a novel biotinylated phospholipid probe. *J. Proteome Res.* **7:** 4237–4246.
38. Friedman, P., S. Horkko, D. Steinberg, J. L. Witztum, and E. A. Dennis. 2002. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. Importance of Schiff base formation and aldol condensation. *J. Biol. Chem.* **277:** 7010–7020.
39. Boullier, A., P. Friedman, R. Harkewicz, K. Hartvigsen, S. R. Green, F. Almazan, E. A. Dennis, D. Steinberg, J. L. Witztum, and O. Quehenberger. 2005. Phosphocholine as a pattern recognition ligand for CD36. *J. Lipid Res.* **46:** 969–976.
40. Seimon, T. A., M. J. Nadolski, X. Liao, J. Magallon, M. Nguyen, N. T. Feric, M. L. Koschinsky, R. Harkewicz, J. L. Witztum, S. Tsimikas, et al. 2010. Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. *Cell Metab.* **12:** 467–482.
41. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, et al. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
42. Watson, A. D. 2006. Systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. *J. Lipid Res.* **47:** 2101–2111.
43. Fang, L., R. Harkewicz, K. Hartvigsen, P. Wiesner, S. H. Choi, F. Almazan, J. Pattison, E. Deer, T. Sayaphupha, E. A. Dennis, et al. 2010. Oxidized cholesteryl esters and phospholipids in zebrafish

larvae fed a high cholesterol diet: macrophage binding and activation. *J. Biol. Chem.* **285:** 32343–32351.
44. Harkewicz, R., K. Hartvigsen, F. Almazan, E. A. Dennis, J. L. Witztum, and Y. I. Miller. 2008. Cholesteryl ester hydroperoxides are biologically active components of minimally oxidized low density lipoprotein. *J. Biol. Chem.* **283:** 10241–10251.
45. Luke, M. M., J. P. Kane, D. M. Liu, C. M. Rowland, D. Shiffman, J. Cassano, J. J. Catanese, C. R. Pullinger, D. U. Leong, A. R. Arellano, et al. 2007. A polymorphism in the protease-like domain of apolipoprotein(a) is associated with severe coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **27:** 2030–2036.
46. Li, Y., M. M. Luke, D. Shiffman, and J. J. Devlin. 2011. Genetic variants in the apolipoprotein(a) gene and coronary heart disease. *Circ. Cardiovasc. Genet.* **4:** 565–573.
47. Arai, K., M. M. Luke, M. L. Koschinsky, E. R. Miller, C. R. Pullinger, J. L. Witztum, J. P. Kane, and S. Tsimikas. 2010. The I4399M variant of apolipoprotein(a) is associated with increased oxidized phospholipids on apolipoprotein B-100 particles. *Atherosclerosis.* **209:** 498–503.
48. Hughes, S. D., X. J. Lou, S. Ighani, J. Verstuyft, D. J. Grainger, R. M. Lawn, and E. M. Rubin. 1997. Lipoprotein(a) vascular accumulation in mice. In vivo analysis of the role of lysine binding sites using recombinant adenovirus. *J. Clin. Invest.* **100:** 1493–1500.
49. Belczewski, A. R., J. Ho, F. B. Taylor, Jr., M. B. Boffa, Z. Jia, and M. L. Koschinsky. 2005. Baboon lipoprotein(a) binds very weakly to lysine-agarose and fibrin despite the presence of a strong lysine-binding site in apolipoprotein(a) kringle IV type 10. *Biochemistry.* **44:** 555–564.
50. Xia, J., L. F. May, and M. L. Koschinsky. 2000. Characterization of the basis of lipoprotein [a] lysine-binding heterogeneity. *J. Lipid Res.* **41:** 1578–1584.
51. Scanu, A. M., D. Pfaffinger, J. C. Lee, and J. Hinman. 1994. A single point mutation (Trp72→Arg) in human apo(a) kringle 4-37 associated with a lysine binding defect in Lp(a). *Biochim. Biophys. Acta.* **1227:** 41–45.
52. Chang, M. K., C. J. Binder, Y. I. Miller, G. Subbanagounder, G. J. Silverman, J. A. Berliner, and J. L. Witztum. 2004. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *J. Exp. Med.* **200:** 1359–1370.
53. Bieghs, V., S. M. Walenbergh, T. Hendrikx, P. J. van Gorp, F. Verheyen, S. W. Olde Damink, A. A. Masclee, G. H. Koek, M. H. Hofker, C. J. Binder, et al. 2013. Trapping of oxidized LDL in lysosomes of Kupffer cells is a trigger for hepatic inflammation. *Liver Int.* **33:** 1056–1061.
54. Ravandi, A., R. Harkewicz, G. Leibundgut, Y. I. Miller, E. A. Dennis, J. L. Witztum, and S. Tsimikas. 2011. Identification of oxidized phospholipids and cholesteryl esters in embolic protection devices post percutaneous coronary, carotid and peripheral interventions in humans. (Poster P385 at ATVB 2011 Scientific Sessions. April 28–30, 2011; Chicago, IL).
55. Chenivesse, X., T. Huby, J. Wickins, J. Chapman, and J. Thillet. 1998. Molecular cloning of the cDNA encoding the carboxy-terminal domain of chimpanzee apolipoprotein(a): an Asp57→Asn mutation in kringle IV-10 is associated with poor fibrin binding. *Biochemistry.* **37:** 7213–7223.
56. Scanu, A. M., L. A. Miles, G. M. Fless, D. Pfaffinger, J. Eisenbart, E. Jackson, J. L. Hoover-Plow, T. Brunck, and E. F. Plow. 1993. Rhesus monkey lipoprotein(a) binds to lysine Sepharose and U937 monocytoid cells less efficiently than human lipoprotein(a). Evidence for the dominant role of kringle 4(37). *J. Clin. Invest.* **91:** 283–291.
57. Miller, Y. I., S. H. Choi, P. Wiesner, L. Fang, R. Harkewicz, K. Hartvigsen, A. Boullier, A. Gonen, C. J. Diehl, X. Que, et al. 2011. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ. Res.* **108:** 235–248.
58. Hartvigsen, K., M. Y. Chou, L. F. Hansen, P. X. Shaw, S. Tsimikas, C. J. Binder, and J. L. Witztum. 2009. The role of innate immunity in atherogenesis. *J. Lipid Res.* **50(Suppl):** S388–S393.
59. Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. USA.* **99:** 13043–13048.
60. Weismann, D., K. Hartvigsen, N. Lauer, K. L. Bennett, H. P. Scholl, P. Charbel Issa, M. Cano, H. Brandstatter, S. Tsimikas, C. Skerka, et al. 2011. Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. *Nature.* **478:** 76–81.
61. Lichtman, A. H., C. J. Binder, S. Tsimikas, and J. L. Witztum. 2013. Adaptive immunity in atherogenesis: new insights and therapeutic approaches. *J. Clin. Invest.* **123:** 27–36.
62. Leibundgut, G., J. L. Witztum, and S. Tsimikas. 2013. Oxidation-specific epitopes and immunological responses translational biotheranostic implications for atherosclerosis. *Curr. Opin. Pharmacol.* In press.
63. Kolski, B., and S. Tsimikas. 2012. Emerging therapeutic agents to lower lipoprotein (a) levels. *Curr. Opin. Lipidol.* **23:** 560–568.