High-level lipoprotein [a] expression in transgenic mice: evidence for oxidized phospholipids in lipoprotein [a] but not in low density lipoproteins

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Abstract Efforts to elucidate the role of lipoprotein [a] (Lp[a]) in atherogenesis have been hampered by the lack of an animal model with high plasma Lp[a] levels. We produced two lines of transgenic mice expressing apolipoprotein [a] (apo[a]) in the liver and crossed them with mice expressing human apolipoprotein B-100 (apoB-100), generating two lines of Lp[a] mice. One had Lp[a] levels of ~700 mg/dl, well above the 30 mg/dl threshold associated with increased risk of atherosclerosis in humans; the other had levels of ~35 mg/dl. Most of the LDL in mice with high-level apo[a] expression was covalently bound to apo[a], but most of the LDL in the low-expressing line was free. Using an enzyme-linked sandwich assay with monoclonal antibody EO6, we found high levels of oxidized phospholipids in Lp[a] from high-expressing mice but not in LDL from low-expressing mice or in LDL from human apoB-100 transgenic mice ($P < 0.00001$), even though all mice had similar plasma levels of human apoB-100. The increase in oxidized lipids specific to Lp[a] in high-level apo[a]-expressing mice suggests a mechanism by which increased circulating levels of Lp[a] could contribute to atherogenesis.—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, T. L. Innerarity, and R. E. Pitas. **High-level lipoprotein [a] expression in transgenic mice: evidence for oxidized phospholipids in lipoprotein [a] but not in low density lipoproteins. *J. Lipid Res.* 2005.** 46: **769–778.**

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Plasma levels of lipoprotein [a] (Lp[a]) greater than 30 mg/dl are associated with increased risk of myocardial infarction and stroke (1, 2). Lp[a] is composed of apolipoprotein [a] (apo[a]), a plasminogen-like glycoprotein linked to apolipoprotein B-100 (apoB-100) of LDL through a disulfide bond (3). Although LDL's role in atherosclerosis is well established (4–6), the mechanism by which Lp[a] contributes to atherogenesis has remained elusive.

Oxidation of LDL plays a key role in the initiation and progression of atherosclerosis (7–9). The presence of oxidized LDL (OxLDL) in the artery wall leads to the release of cytokines that attract monocytes to lesion-prone areas and to the upregulation of scavenger receptors on monocyte-derived macrophages. The uptake of oxidized lipoproteins by scavenger receptors leads to lipid accumulation and fatty streak formation. Immunohistochemical studies of human tissue with monoclonal antibody (MAb) EO6, which recognizes oxidized phospholipids and oxidized phospholipid-protein adducts (10), have identified OxLDL and oxidized lipids in atherosclerotic plaques (7).

The oxidation of Lp[a] has not been studied extensively, but oxidized Lp[a] is a ligand for scavenger receptors (8, 9) and might reasonably be expected to contribute to foam cell formation. In addition, in vitro oxidative

Abbreviations: apo[a], apolipoprotein [a]; apoB-100, apolipoprotein B-100; FPLC, fast-performance liquid chromatography; IDL, intermediate density lipoprotein; Lp[a], lipoprotein [a]; MAb, monoclonal antibody; OxLDL, oxidized low density lipoprotein; RLU, relative light units.

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modification increases the inhibitory effect of Lp[a] on plasminogen binding to cell surfaces, which could attenuate fibrinolytic activity by reducing plasminogen activation (11). Recently, Tsimikas et al. (12) showed that Lp[a] in human plasma contains oxidized phospholipid but free LDL does not. These results were confirmed and extended by Edelstein et al. (13), who demonstrated that some oxidized phospholipids in Lp[a] are also covalently bound to lysines in kringle V of apo[a]. Defining the physiological significance of these biochemical findings will be greatly facilitated by the availability of an animal model with high-level expression of Lp[a].

Studies of Lp[a] have been hampered by the lack of a suitable animal model. Although several models have been developed (4, 14, 15), all have significant limitations. Lawn et al. (14) identified small fatty streak lesions in the proximal ascending aorta of transgenic mice expressing low levels of apo[a]. However, the apo[a] was not covalently bound to mouse apoB-100, and the plasma did not contain bona fide Lp[a]. In other studies, transgenic mice coexpressing apo[a] and human apoB-100, which had Lp[a] levels of 25 mg/dl, had no more atherosclerosis than mice expressing human apoB-100 alone (4). However, the interpretation of that study was somewhat clouded by different genetic backgrounds and by the fact that the control mice had very high levels of LDL, which contributed to a high background level of atherosclerosis. More recently, Sun et al. (15) expressed human apo[a] in wild-type and Watanabe heritable hyperlipidemic rabbits. The Watanabe rabbits expressing Lp[a] had more atherosclerosis than their nontransgenic littermates. But the plasma level of Lp[a]-like particles was only 15 mg/dl, and most of the apo[a] in the plasma was not covalently linked to rabbit apoB-100.

Here, we describe the development of transgenic mice with high (~700 mg/dl) and low (~35 mg/dl) levels of Lp[a] expression. The Lp[a] in these mice contains high levels of oxidized phospholipid, whereas the LDL in these mice or in control mice expressing only apoB-100 does not. These Lp[a] mice provide a new and exciting model in which to study Lp[a] metabolism and to examine the mechanism by which Lp[a] and oxidized phospholipids contribute to the development of atherosclerosis.

MATERIALS AND METHODS

Apo[a] transgenic mice

Wild-type human apo[a] cDNA encoded kringles IV-1, IV-2, a fusion of IV-3 and IV-5, IV-6 to IV-10, V, and the protease domain as described (16). The vector, pRK5 ha8, was digested with *Xho*I, polished with *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and cut with *Eco*RI. The apo[a] fragment was inserted into a liver cDNA expression vector (17, 18) that had been digested with *Kpn*I and polished and cut as described above. *Eco*RI sites were introduced into 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 apo[a] cDNA. The 8.6 kb apo[a] expression vector (containing the *Apoe* promoter, *Apoe* intron 1, the apo[a] cDNA, and LE6) was excised from pLIVha8 with *Sac*II, purified, and microinjected into C57BL/6×SJL zygotes. Transgenic founders were identified by PCR of DNA isolated from tail biopsies.

Breeding of apo[a] and Lp[a] mice

Hemizygous mice expressing human apo[a] were crossed with hemizygous mice expressing human apoB-100 only (19, 20), owing to a mutation in codon 2,153 that prevented apoB-48 synthesis (19). 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).

Northern blot analysis

RNA was isolated from several tissues of an apo[a] transgenic mouse (from founder 23), and 10 μg was electrophoresed on a 1% agarose gel containing 18% formaldehyde. Liver mRNAs from the transgenic mouse (10.0, 7.5, 5.0, and 2.5 μg) and from a nontransgenic littermate (10 μg) were also examined. After transfer to a nylon membrane (Schleicher and Schuell, Keene, NH), the blot was hybridized to an apo[a] cDNA probe labeled with [^{32}P]dCTP in Quickhyb solution (Stratagene) at 65°C for 2 h. The blot was washed in 2× standard saline citrate (150 mmol/l NaCl, 15 mmol/l sodium citrate) and 0.1% SDS at 55°C for 30 min, in 0.1× standard saline citrate and 0.1% SDS at 60°C for 1 h, and exposed to X-ray film overnight. Signals were quantified with a phosphorimager and quantification software (Bio-Rad Quantity One, Philadelphia, PA).

Detection of apo[a], Lp[a], and oxidized phospholipid

Plasma concentrations of human apo[a] and Lp[a] in mouse plasma were estimated by SDS-PAGE and Western blotting with rabbit polyclonal antibodies against human apo[a] (Cortex, San Leandro, CA) and human apoB (HB2). Mouse plasma and control human plasma (apo[a] ~500 kDa) were subjected to SDS-PAGE with 5% gels under reducing and nonreducing conditions, transferred to nitrocellulose, and incubated first with the primary antibodies (1:5,000 for anti-human apo[a] and 1:15,000 for anti-human apoB) and then with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Little Chalfont, UK).

To detect oxidized phospholipids bound to the proteins, Western blotting was performed with the IgM MAb EO6 (13). Mouse plasma (10 μl) was incubated for 30 min with 100 μl of protein A-agarose (Boehringer, Mannheim, Germany) and centrifuged to remove mouse antibodies. The supernatant fraction of the plasma was then size-fractionated by SDS-PAGE. The nitrocellulose blots were blocked with Superblock (Pierce, Rockford, IL) at 22°C for 60 min followed by incubation first with EO6 (1:1,000) in 10% Superblock at 4°C for 18 h and then with horseradish peroxidase-conjugated anti-mouse IgG (Amersham). Signals were generated by incubating the membranes with chemiluminescent reagent (Amersham) and exposing them to X-ray film (Fuji, Tokyo, Japan).

Plasma apo[a] and Lp[a] concentrations were measured with a direct-binding, MAb-based ELISA (21). The capture antibody (a-6) is directed to an epitope in apo[a] kringle IV type 2, and the detection antibody (a-40) is directed to a unique epitope in kringle IV type 9. Because each apo[a] molecule contains only one kringle IV type 9, this assay is insensitive to apo[a] isoform size heterogeneity. This assay was used to quantitate plasma apo[a] levels (nanomoles per liter) in transgenic mice expressing human apo[a] or both human apo[a] and human apoB-100.

Formation of Lp[a] in vitro

Disulfide bond formation between apo[a] and apoB in vitro was assessed as described (22). Different dilutions of plasma from

human apo[a] transgenic mice (in 150 mmol/l NaCl) were mixed with 4 μl of plasma from human apoB-100 transgenic mice for 5 min at 37°C. Lp[a] formation was assessed by size-fractionating the mixtures by SDS-PAGE under nonreducing conditions and performing Western blot analysis with antibodies against human apo[a] and human apoB-100. Lp[a] was identified as an apoB-100- and apo[a]-containing band with a higher molecular mass than the apoB-100 in free LDL.

Lipid and lipoprotein determination

Blood was obtained from tail veins of 8–14 week old mice after a 4 h fast, and plasma was obtained by centrifugation at 16,000 *g* for 10 min at 4°C. Lipoprotein electrophoresis of plasma was performed on 1% agarose gels in 1.3% barbital buffer. The gels were dried and stained with Fat Red 7B (Helena Laboratories, Beaumont, TX) to identify lipoprotein bands. To determine the distribution of proteins and lipids within the plasma lipoproteins, 120 μl of mouse plasma was size-fractionated by fast-performance liquid chromatography (FPLC) on a Superose 6 column (Amersham) that had been equilibrated with PBS containing 1 mmol/l EDTA. The distribution of apo[a] and Lp[a] was determined by Western blot analysis; the distribution of cholesterol and triglycerides was determined with colorimetric assays (Roche, Mannheim, Germany).

Density gradient ultracentrifugation

Discontinuous density gradient ultracentrifugation was performed as described (23). Briefly, a nonlinear salt gradient was constructed to maximize the separation of LDL, Lp[a], and HDL classes. VLDL remained at the top of the tube. The gradient consisted of 2 ml of 20.7 mol/l NaCl (d = 1.21 g/ml), 3 ml of 4 mol/l NaCl, and a mixture of 0.5 ml of mouse plasma and 0.5 ml of 150 mmol/l NaCl. The remainder of the tube was filled to a total of 13.2 ml with 670 mmol/l NaCl. The gradients were centrifuged in an SW41 swinging-bucket rotor (Beckman, Fullerton, CA) at 35,000 rpm for 64 h at 15°C. At the end of the run, the tube was pierced at the bottom and 20 fractions of 660 μl each were collected. The density of each fraction was calculated from the refractive index (Bausch and Lomb, Rochester, NY), and the distribution of Lp[a] was determined by Western blot analysis.

Analysis of oxidized phospholipid content

The oxidized phospholipid content of human or mouse apoB-100 lipoproteins was assessed with a "sandwich" chemiluminescence immunoassay (24, 25) in which MAb MB47 was used to capture human apoB-100 and biotin-labeled MAb EO6 was used to detect oxidized phospholipids on the captured particles. MB47 is specific for human apoB-100 and does not bind to mouse apoB-100 (26). EO6 binds to OxLDL and specifically to oxidized phospholipids containing phosphorylcholine (10, 27). MB47 (5 μg/ml) was added to 96-well microtiter plates (Microlite2; Dynex Technologies, Chantilly, VA) in TBS (50 nmol/l Tris-HCl, pH 7.4) containing 150 nmol/l NaCl, 0.27 mmol/l EDTA, and 0.02% NaN_3 and incubated overnight at 4°C. The plates were washed three times with TBS washing buffer containing aprotinin (0.001%) in an automated plate washer, and 1% BSA in PBS was added to all wells for 45 min at room temperature to block nonspecific binding sites. The plates were then washed, and 50 μl of the mouse plasma diluted 1:100 in PBS containing 1% BSA was added for 1 h at room temperature. In preliminary experiments, a 1:100 dilution of mouse plasma containing human apoB-100 did not saturate the bound MB47. To determine the relative amounts of human apoB-100 bound by MB47, biotin-labeled goat anti-human apoB-100 (Biodesign International, Kennebunk, ME) was added to each well for 1 h at room temperature. The plates were washed with TBS and incubated with alkaline phosphatase-labeled NeutrAvidin (Pierce; diluted 1:10,000) in TBS containing 1% BSA, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$ for 1 h at room temperature. The plates were then washed four times with washing buffer and incubated with 50% Lumi-Phos 530 in distilled water (25 μl/well) for 1.5 h at room temperature in the dark. The chemiluminescence was read on a MLX microtiter plate luminometer (Dynex Technologies). Data are expressed in relative light units (RLU) measured over 100 ms.

To detect endogenous mouse apoB-100, the same procedure was used except that a MAb (LF3; 5 μg/ml) specific for mouse apoB-100 (28) was used to capture mouse apoB-100 and biotin-labeled LF5 (28) (1 μg/ml) was used for detection. LF5 was biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce). LF3 and LF5 are specific for mouse apoB-100 and do not bind to human apoB-100.

Oxidized phospholipid epitopes present on human or mouse apoB-100

Phosphorylcholine-containing oxidized phospholipid epitopes on the captured human or mouse apoB-100 were detected with MAb EO6 (12). Biotinylated EO6 (conjugated with EZ-Link Biotin-LC-Hydrazide; Pierce) was added to microtiter wells containing captured human or mouse apoB-100 particles at 1.5 μl/well (human) or 50 μl/well (mouse) in TBS with 1% BSA. The amount of bound EO6 was then determined with the chemiluminescence technique described above and expressed in RLU. The relative number of EO6 epitopes (oxidized phospholipids) bound per apoB-100 particle was then determined by dividing the bound EO6 RLU by the apoB-100 RLU in parallel wells. All samples were measured in a single assay; the intra-assay coefficient of variation was 8–10%.

Determination of apo[a] on human or mouse apoB-100

Human apoB-100 was captured with MB47 and mouse apoB-100 was captured with LF3, as described above. LPA4, an apo[a]-specific MAb that does not cross-react with plasminogen, was biotinylated with EZ-Link Sulfo-NHS-Biotin and added to each well at a concentration of 0.5 μg/ml in TBS with 1% BSA and then detected with the chemiluminescence technique described above.

Statistical analysis

Statistical analysis was done by one-way ANOVA with MicroCal Origin version 6.1 (MicroCal Software, Northampton, MA).

RESULTS

Generation of apo[a] mice

Mice expressing human apo[a] were created with an apo[a] cDNA containing eight plasminogen kringle IV-like domains followed by a kringle V-like domain and the protease-like domain (16). This small apo[a] isoform is more likely to yield high levels of Lp[a] in the plasma (29, 30). The human cDNA was inserted into a liver expression vector containing 3.9 kb of the *Apoe* promoter and 0.77 kb of the *Apoe* hepatic control region (LE6) (**Fig. 1A**). To create transgenic mice, an 8.6 kb fragment containing the apo[a] cDNA was microinjected into fertilized mouse eggs. Genomic DNA of each human apo[a] founder mouse was identified by PCR (Fig. 1B).

Apo[a] mice

To determine the tissue-specific expression pattern of apo[a], Northern blot analysis was performed with RNA samples from transgenic mice. As expected, an apo[a]

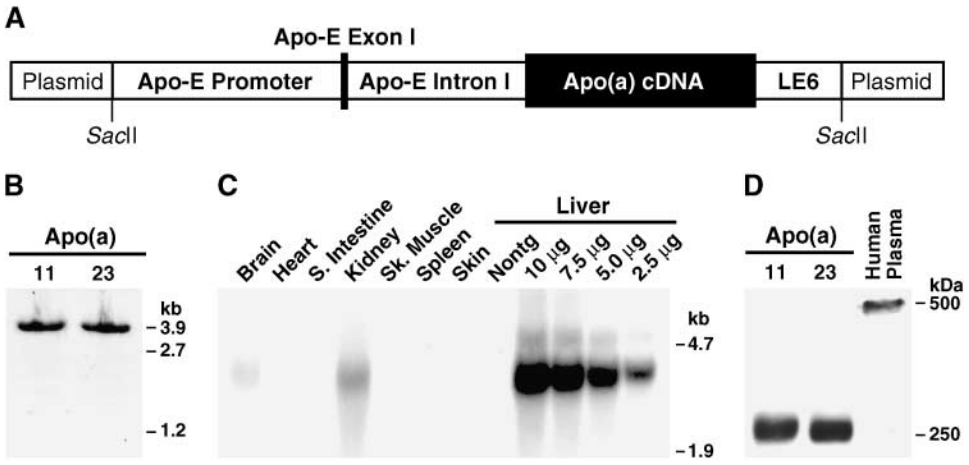

Fig. 1. Generation of transgenic mice expressing human apolipoprotein [a] (apo[a]). A: Construct for producing human apo[a] transgenic mice, in the 5′ to 3′ orientation. Expression of apo[a] was driven by a 3.0 kb *Apoe* promoter with a 0.9 kb *Apoe* intron; a 0.77 kb hepatic control region (LE6) was placed downstream of the apo[a] gene. An 8.6 kb *Sac*II fragment was microinjected into mouse embryos. B: Genomic DNA from apo[a] transgenic mice was amplified by PCR and analyzed by agarose gel electrophoresis. Apo[a]-expressing mice (11 and 23) were identified by a single DNA fragment of ~3.9 kb. C: Northern blot demonstrating the tissue specificity of apo[a] mRNA expression. Total RNA from various tissues of one transgenic offspring of founder 23 and total liver RNA of a nontransgenic (Nontg) littermate mouse were examined for the apo[a] transcript. Total RNA (10 μg) was used in all cases, except for the liver from the transgenic mouse, in which increasing amounts (2.5, 5.0, 7.5, and 10 μg) of mRNA was used. A ^{32}P-labeled human apo[a] cDNA was used as a probe. D: Relative levels of apo[a] in two lines of human apo[a] transgenic mice. Apo[a] in plasma (1 μl) from mice expressing apo[a] (11 and 23) was resolved by SDS-PAGE and detected by Western blotting. Apo[a] in plasma (1 μl) from a human subject expressing a high-molecular-mass form of apo[a] is shown as a positive control. Sk muscle, skeletal muscle.

transcript (~3.9 kb) was detected mainly in the liver (Fig. 1C). Low levels of expression were observed in the kidney and the brain. Plasma samples from selected lines of transgenic apo[a] mice were tested for protein expression by Western blots (Fig. 1D). The apo[a] in human plasma (control) was a high-molecular-mass isoform, whereas the mouse plasma contained a small apo[a] isoform (~250 kDa). The plasma cholesterol level was higher in apo[a] transgenic mice (112 ± 5 mg/dl; n = 7) than in their nontransgenic littermates (70 ± 4 mg/dl; n = 6, $P < 0.0001$). The triglyceride levels were not significantly different (71 ± 11 mg/dl versus 45 ± 6 mg/dl, respectively). The distribution of lipids within the lipoprotein fractions of chow-fed mice was assessed by FPLC. Most of the cholesterol was associated with HDL in both transgenic and nontransgenic mice (**Fig. 2A**), but the HDL cholesterol peak was higher in the transgenic mice. Triglycerides were found mainly in VLDL in nontransgenic mice and in VLDL and intermediate density lipoprotein (IDL)-sized particles in the apo[a] transgenic mice (Fig. 2B).

Formation of Lp[a] in vitro

To test the ability of apo[a] to bind covalently to human apoB-100 and form Lp[a], increasing amounts of plasma from an apo[a]-expressing mouse were incubated with plasma from a human apoB-100 transgenic mouse, separated by SDS-PAGE, and subjected to immunoblot analysis. In the presence of β-mercaptoethanol, increasing amounts of apo[a] were observed (**Fig. 3**, left). In the absence of a reducing agent, free apoB-100 was observed at 500 kDa and Lp[a] was evident as a higher-molecular-mass band (Fig. 3, right).

Generation of mice expressing Lp[a]

To generate mice expressing Lp[a] in vivo, we bred mice expressing high or low levels of apo[a] with transgenic mice that were hemizygous for human apoB-100 expression. Offspring that carried both transgenes expressed high or low levels of Lp[a]. The high- and low-expressing apo[a] transgenic mice (**Fig. 4A**) had similar amounts of human apoB-100 in their plasma (Fig. 4B). In the high expresser, most of the apoB-100 was covalently bound to apo[a] (Fig. 4C). In the low expresser, apo[a] was limiting and therefore only a small amount of Lp[a] was formed; most of the apoB-100 was free (Fig. 4C). The high-molecular-mass Lp[a] band contained apo[a], as judged by immunoblotting (not shown). To determine if the protein moiety of Lp[a] contains oxidized phospholipid, we examined Western blots of plasma for EO6 immunoreactivity (Fig. 4D). A high level of oxidized phospholipid was detected in the Lp[a] of the high-expresser mouse and only a trace in the low expresser (Fig. 4D). No oxidized phospholipid was associated with free apoB-100 in Lp[a] or nontransgenic mice.

High-expressing Lp[a] mice

Lp[a] high-expresser mice that were hemizygous for both apo[a] and apoB-100 had plasma Lp[a] mass levels of 701 ± 59 mg/dl (1,928 ± 162 nmol/l). Total plasma cholesterol levels were higher in the Lp[a] mice than in

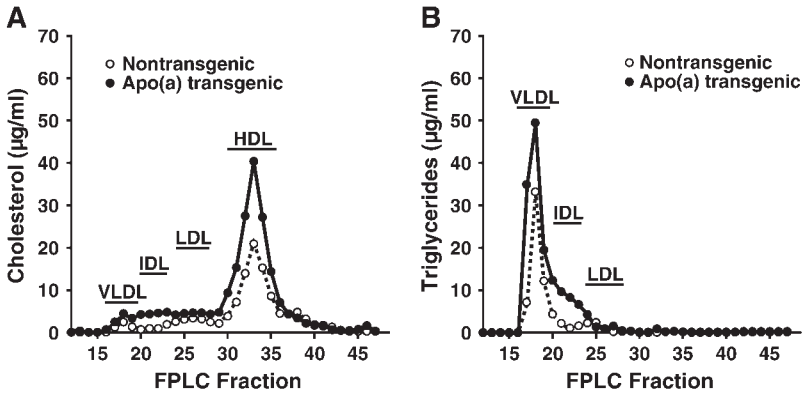

Fig. 2. Distribution of lipids within the plasma lipoproteins of nontransgenic and apo[a] transgenic mice. Pooled plasma from nontransgenic (n = 6) or apo[a] transgenic mice (n = 7) was size-fractionated by fast-performance liquid chromatography (FPLC), and the cholesterol (A) and triglyceride (B) contents of each fraction were measured. IDL, intermediate density lipoprotein.

the apoB-100-expressing mice [246 ± 22 mg/dl (n = 3) versus 169 ± 6 mg/dl (n = 8); $P < 0.001$], as were the plasma triglycerides (281 ± 66 versus 174 ± 11 mg/dl; $P < 0.05$). As shown by density gradient ultracentrifugation, the mean density of Lp[a] was 1.075 g/ml. As judged by FPLC fractionation, cholesterol was associated mainly with HDL and LDL in the human apoB-100 transgenic mice and with the Lp[a] fractions in the Lp[a] mice (**Fig. 5A**). HDL levels in the Lp[a] mice were similar to those in the human apoB-100 transgenic mice. The triglycerides in Lp[a] mice were in somewhat larger particles than in the human apoB-100 transgenic mice (Fig. 5B). The overall higher plasma lipid levels of the Lp[a] mice were also apparent by agarose gel electrophoresis (Fig. 5B, inset).

To confirm that the larger lipoproteins in the plasma of Lp[a]-expressing mice contained Lp[a], FPLC fractions were examined by immunoblot analysis. In the apo[a]-only mice, most of the apo[a] was located in LDL-sized particles (**Fig. 6**, upper panel), almost certainly because of a noncovalent association with murine LDL. Human apoB-100 in the human apoB-100 transgenic mice was also found predominantly in LDL fractions (Fig. 6, middle panel). In the Lp[a] mice, both apo[a] and human apoB-100 were shifted to somewhat larger lipoproteins (Fig. 6, bottom panel), consistent with the shift in cholesterol and triglyceride distribution to larger particles. The small amount of free human apoB-100 was also shifted in the same manner, suggesting that the small portion of the free apoB-100 might be noncovalently associated with apo[a] (Fig. 6, bottom panel).

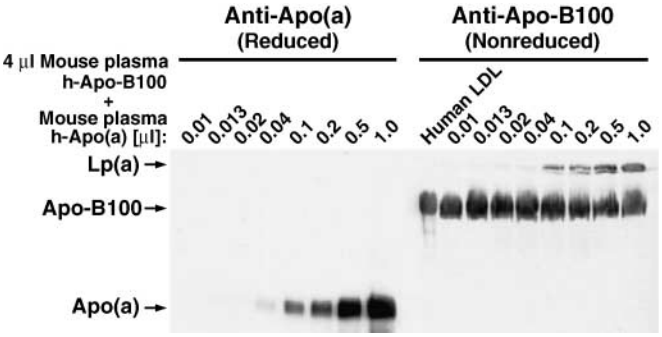

Fig. 3. Apo[a]-dependent formation of lipoprotein [a] (Lp[a]) in vitro. Increasing amounts of apo[a] transgenic mouse plasma were mixed with human apolipoprotein B-100 (apoB-100) transgenic mouse plasma (4 μl). After a 5 min incubation at 37°C, apo[a] and Lp[a] in the mixtures was assessed by immunoblot analysis (using anti-apo[a] or anti-apoB-100, as indicated) after SDS-PAGE, both with reducing (left) and nonreducing (right) conditions. Formation of the disulfide linkage between apo[a] and apoB-100 was evident from the larger Lp[a] complex (detected with anti-apoB-100) that migrated more slowly than LDL.

Oxidized phospholipid content of apoB-100-containing lipoproteins

To more fully characterize the oxidized phospholipids in the lipoproteins from our transgenic mice, we examined plasma of mice expressing human apo[a], human apoB-100, and low (36 ± 12 mg/dl or 98 ± 32 nmol/l) or high levels of Lp[a] with double-antibody sandwich immunoassays. First, human apoB-100-containing lipoproteins were captured with antibody MB47 and then detected with a polyclonal apoB-specific antibody. The amount of human apoB in high-expressing Lp[a] mice was similar to that of mice expressing low levels of Lp[a] or apoB-100 alone (**Fig. 7A**). As expected, no human apoB-100 was detected in plasma from mice expressing apo[a] alone. The captured human apoB-containing lipoproteins were then assessed for their content of oxidized phospholipid with antibody EO6 (12). A high level of EO6 binding to the apoB-100-containing lipoproteins was noted in mice with high levels of Lp[a] expression but not in mice with human apoB-100 alone (Fig. 7B). There was ~17.4 times more oxidized phospholipid in Lp[a] of mice expressing high levels of Lp[a] than in mice expressing low levels of Lp[a]. However, it is interesting that there was ~19.5 times more Lp[a] in mice expressing high levels than in mice expressing low levels of Lp[a]. Therefore, the ratio of oxidized phospholipid to Lp[a] was similar in mice with high or low Lp[a] expression.

Next, mouse apoB-100-containing plasma lipoproteins were captured with MAb LF3 and tested for EO6 epitopes

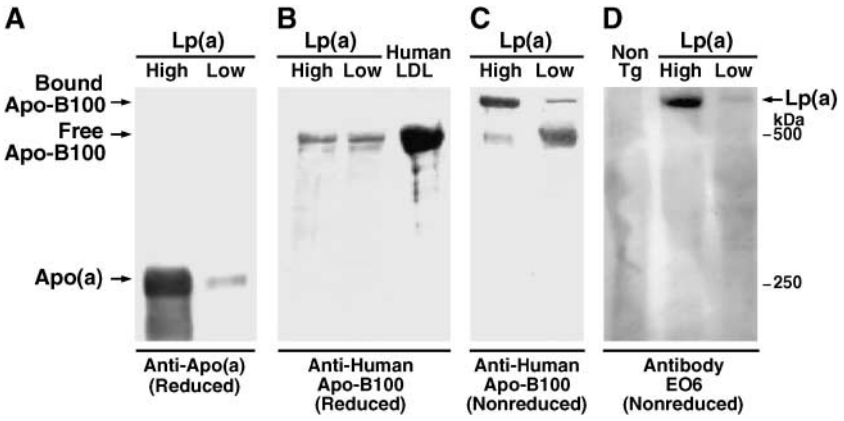

Fig. 4. Formation of Lp[a] and oxidized phospholipid-protein adducts in vivo in mice expressing high or low levels of apo[a]. Mice expressing high or low levels of apo[a] were crossed with transgenic mice expressing human LDL. Plasma proteins were subjected to SDS-PAGE with 5% gels under reducing and nonreducing conditions, transferred to nitrocellulose, and immunoblotted with antibodies against human apo[a], human apoB, or oxidized phospholipid. High-level expression of apo[a] (A) resulted in high-levels of Lp[a], with most of the apoB-100 bound to apo[a] (C). The Lp[a] contained high levels of oxidized phospholipid (D). Low-level expression of apo[a] (A) resulted in low levels of Lp[a], as indicated by the amount of free and bound apoB-100 in both mice (C); these mice had low levels of oxidized phospholipid-protein adducts (D). High- and low-expressing Lp[a] mice had similar amounts of apoB-100 (B). Oxidized phospholipid was not detected in the apoB-100 (D). Non Tg, nontransgenic.

(**Fig. 8**). Oxidized phospholipids were detected in the captured particles from mice that expressed apo[a] alone (Fig. 8B), suggesting that a covalent association between apo[a] and apoB is not required for oxidized phospholipids to accumulate. There was significant association of apo[a] with the captured mouse apoB-100 in the apo[a] mice (Fig. 8D). In addition, human apoB-100 was also identified on the mouse apoB-100 along with apo[a], demonstrating that intact Lp[a] associates with the mouse apoB-100 (data not shown). A similar association has been shown between Lp[a] and human LDL (31). The Lp[a] mice had a relatively lower level of EO6 epitopes on the mouse apoB-containing lipoproteins (Fig. 8B), probably because most of the apo[a] was covalently associated with human apoB-100 (Fig. 8C) and therefore less available for noncovalent association with mouse apoB-100. No oxidized phospholipid was found in the mouse apoB-containing lipoproteins in mice expressing human apoB-100 alone (Fig. 8B). These findings indicate that almost all oxidized phospholipids detected with EO6 are on apoB-100 particles to which apo[a] is covalently or noncovalently bound. The oxidized phospholipid content was significantly higher on Lp[a] particles than on LDL particles (Fig. 8A).

DISCUSSION

We generated mice expressing ~700 mg/dl of human Lp[a] in their plasma by crossing high-expressing apo[a] transgenic mice with mice expressing human apoB-100.

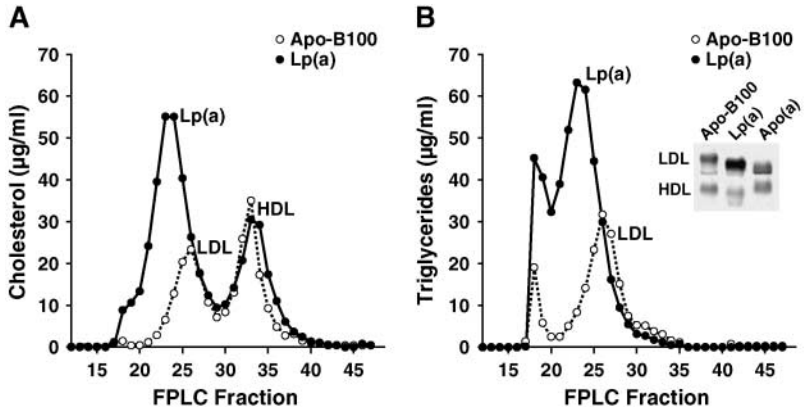

Fig. 5. Lipoprotein profiles of mice expressing human apoB-100 or high levels of Lp[a]. Pooled plasma from human apoB-100 transgenic mice (n = 8) or Lp[a] high-expressing mice (n = 3) was size-fractionated by FPLC, and the cholesterol (A) and triglyceride (B) contents of each fraction were determined. The inset shows agarose gels of apo[a], human apoB-100, and Lp[a] mouse plasma demonstrating the different lipid contents, as detected by staining with Fat Red 7B.

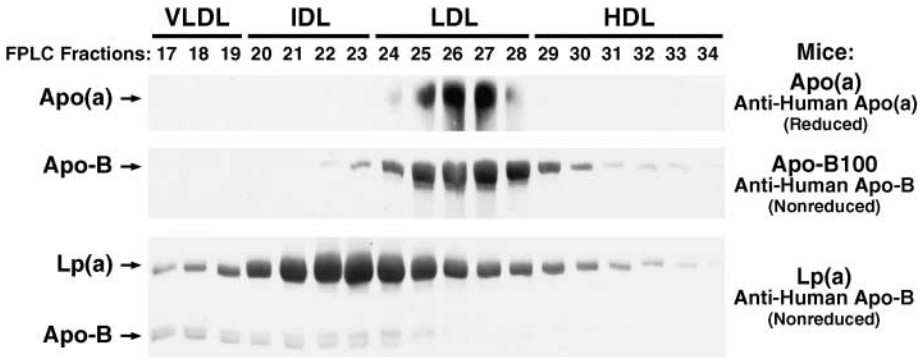

Fig. 6. Distribution of apo[a] and human apoB-100 in the plasma of transgenic mice expressing one or both proteins. Plasma from mice expressing apo[a], human apoB-100, or Lp[a] was size-fractionated by FPLC, and the fractions were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis. FPLC fractions of mouse plasma from the apo[a]-expressing mice were examined under reducing conditions with anti-human apo[a] (upper panel). Fractions of transgenic mouse plasma from mice expressing human apoB-100 (middle panel) or human Lp[a] (lower panel) were examined under nonreducing conditions with anti-human apoB-100. Disulfide bond formation in Lp[a]-expressing mice is indicated by the upper band containing human apoB-100 bound to apo[a]. Lp[a] is found in particles larger than apo[a] in apo[a] mice and in particles larger than human apoB-100 in apoB-100 mice.

The mice were produced with a construct encoding a relatively low-molecular-mass form of human apo[a] (~250 kDa), which is generally associated with high levels of Lp[a] in humans. This is the first animal model in which the Lp[a] levels greatly exceed 30 mg/dl, a level that is necessary to increase the risk of atherosclerosis in humans (32). Interestingly, apoB-100-containing lipoproteins from mice expressing high levels of Lp[a] contained significantly more oxidized phospholipids than those in mice expressing only human apoB-100.

The apo[a] transgenic mice were produced with a construct containing the apo[a] cDNA flanked by the *Apoe* promoter and the *Apoe* hepatic control region. The apo[a] in the plasma of those mice eluted from FPLC columns with an LDL-like size, consistent with an earlier report suggesting that apo[a] associates noncovalently with mouse apoB-100 (33). Interestingly, the apo[a]-expressing mice had higher plasma cholesterol levels than their nontransgenic littermates, even in the absence of the human apoB-100 transgene. Most of the increase in lipid was in the HDL fraction; however, lipoprotein levels were also increased in the IDL and LDL. It seems quite possible that the noncovalent association of apo[a] with mouse apoB-100 might lead to retarded clearance of mouse LDL, resulting in higher plasma lipid levels.

Breeding mice with high- or low-level apo[a] expression with mice expressing human apoB-100 resulted in offspring with ~700 or ~35 mg/dl of human Lp[a], respectively. In mice with a high level of Lp[a], virtually all of the LDL containing human apoB-100 was covalently bound to apo[a]. In mice with low levels of Lp[a], most apo[a] was covalently bound to human LDL, but LDL devoid of apo[a] was also present. Mice with high-level Lp[a] expression had increased lipid levels and altered lipoprotein profiles, as assessed by FPLC. Despite a peak density of 1.075 g/ml, Lp[a] was also present in larger, more buoyant lipoproteins, accounting for the increased plasma lipid levels.

The reason for the presence of larger and more buoy-

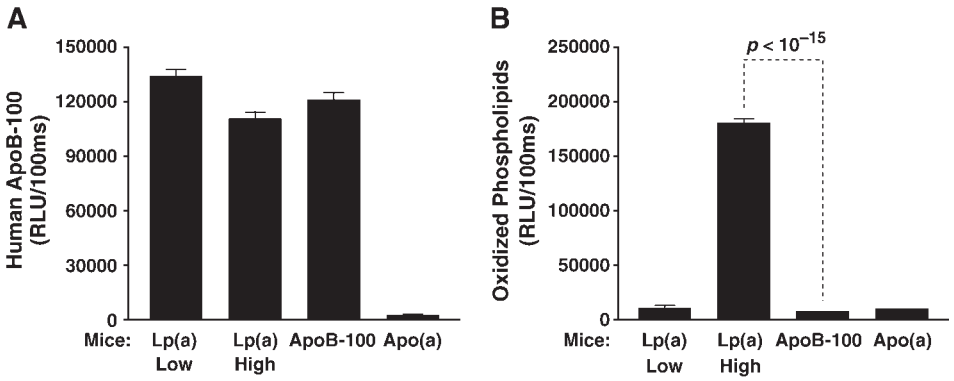

Fig. 7. Oxidized phospholipids on human apoB-100-containing lipoproteins. Human apoB-100-containing lipoproteins were captured from the plasma of mice expressing low (n = 4) or high (n = 5) levels of Lp[a], human apoB-100 (n = 9), or human apo[a] (n = 8) and then examined with chemiluminescence immunoassay for antibody recognition of human apoB-100 (A) and oxidized phospholipids with antibody EO6 (B). RLU, relative light units. Error bars represent mean ± SEM.

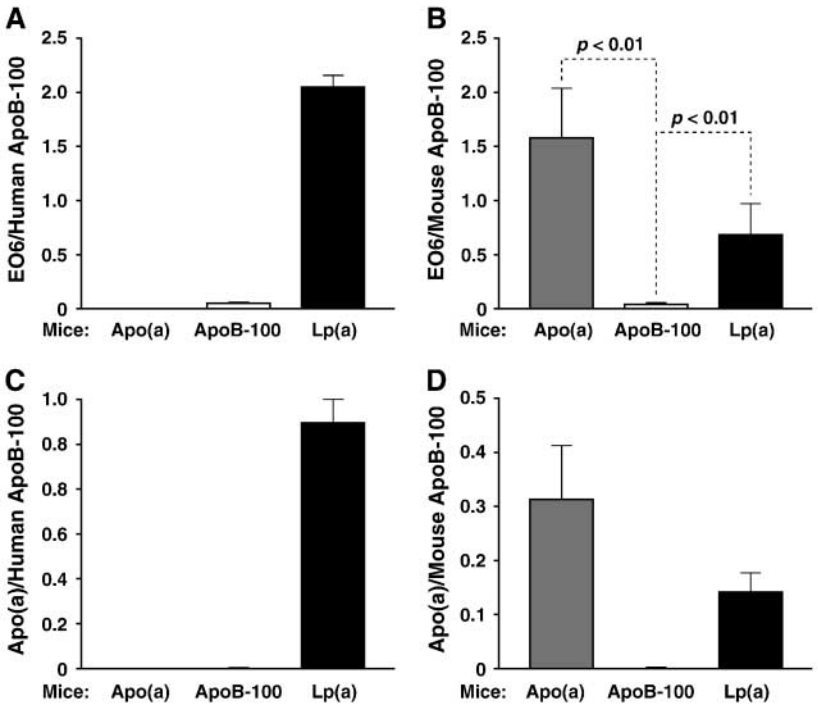

Fig. 8. Oxidized phospholipids and apo[a] in lipoproteins that contain human or mouse apoB-100. The oxidized phospholipid (A) and apo[a] (C) levels in captured human apoB-100-containing lipoproteins are compared with the oxidized phospholipid (B) and apo[a] (D) levels of captured mouse apoB-100-containing lipoproteins from plasma of mice expressing human apo[a] (n = 8), human apoB-100 (n = 10), or high levels of Lp[a] (n = 5). Error bars represent mean ± SEM.

ant Lp[a] particles is not entirely clear. Normally, apoB-100-containing VLDLs are hydrolyzed by lipases, with a portion being removed from the plasma by hepatic receptors and a portion being further metabolized to LDL. We suspect that the apo[a] binds preferentially to apoB-100 on LDL, but apo[a] can clearly bind to larger lipoproteins, including VLDL and IDL (34, 35). When the covalent apo[a]-apoB-100 interaction happens to occur on VLDL or IDL particles, the lipolytic processing of those particles may be retarded, leading to the accumulation of larger particles and increased plasma lipid levels in the Lp[a] mice.

Immunoblot analysis of plasma from a high-expresser Lp[a] mouse showed oxidized phospholipid in the Lp[a]. Interestingly, only mice expressing high levels of Lp[a] had high levels of oxidized phospholipid. No EO6 immunoreactivity was detected in apoB-100 from LDL in low-expressing Lp[a] mice or in the human apoB transgenic mice. To investigate this issue further, we examined the mice for the presence of oxidized phospholipids on the apoB-100-containing lipoproteins, LDL, and Lp[a] with an enzyme-linked sandwich assay. Oxidized phospholipid levels on human apoB-100-containing particles were significantly higher in mice expressing high levels of Lp[a] than in those expressing only human apoB-100. These high levels of oxidized phospholipids were also strictly associated with high levels of plasma Lp[a]. In addition, oxidized phospholipids were evident on mouse apoB-100-containing lipoproteins in the apo[a]-expressing mice. Therefore, covalent association of apo[a] and mouse apoB-100 was not required for EO6 epitopes to accumulate in the mouse apoB-100/apo[a] particles. These results are in agreement with studies in humans that showed the presence of oxidized phospholipid in Lp[a] (12, 13). The oxidized phospholipid is found both in the lipid phase (our unpublished observations) and covalently bound to apo[a], in particular to lysines of kringle V (13). What affects the level of oxidized phospholipid in Lp[a] is not clear; it is conceivable that the size of the apo[a] expressed might affect the accessibility of oxidized phospholipid to the Lp[a] and thus have an impact on the amount of oxidized phospholipid in the particle.

Based upon in vitro transfer studies, we believe that Lp[a] preferentially binds phosphorylcholine-containing oxidized phospholipids that are transferred from various tissues or lipoproteins. We have determined from in vitro transfer studies that oxidized phospholipids are preferentially transferred from OxLDL to Lp[a], compared with LDL, even in the absence of any lipid transfer proteins (our unpublished observations). As recently shown by Navab et al. (36), oxidized phospholipids can efflux from cells to a pre-β-HDL fraction. We speculate that the oxidized phospholipids might then be preferentially transferred to the Lp[a] particle. In support of this hypothesis is the recent observation that under ordinary circumstances in human plasma more than 95% of the oxidized phospholipids recognized by EO6 are found on Lp[a] (37). On the other hand, Lp[a] has been reported to bind better than

native LDL to the extracellular matrix of the arterial intima (38). If plasma Lp[a] levels were increased, for example for genetic reasons, an increased amount of Lp[a] would presumably accumulate in the arterial intimal matrix, where the enhanced content of oxidized phospholipids would be expected to be both proinflammatory and proatherogenic. Thus, the bound oxidized phospholipids might explain, in part, the increased atherogenicity of Lp[a]. Our mice with high levels of Lp[a], which have a greatly increased content of oxidized phospholipids, should be valuable for investigating the atherogenic potential of Lp[a] and the role of oxidized phospholipids in atherosclerosis.

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