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ORIGINAL ARTICLE

Experimental Animal Models Evaluating the Causal Role of Lipoprotein(a) in Atherosclerosis and Aortic Stenosis

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Abstract Lipoprotein(a) [Lp(a)], comprised of apolipoprotein(a) [apo(a)] and a low-density lipoprotein-like particle, is a genetically determined, causal risk factor for cardiovascular disease and calcific aortic valve stenosis. Lp(a) is the major plasma lipoprotein carrier of oxidized phospholipids, is pro-inflammatory, inhibits plasminogen activation, and promotes smooth muscle cell proliferation, as defined mostly through *in vitro* studies. Although Lp(a) is not expressed in commonly studied laboratory animals, mouse and rabbit models transgenic for Lp(a) and apo(a) have been developed to address their pathogenicity *in vivo*. These models have provided significant insights into the pathophysiology of Lp(a), particularly in understanding the mechanisms of Lp(a) in mediating atherosclerosis. Studies in Lp(a)-transgenic mouse models have demonstrated that apo(a) is retained in atheromas and suggest that it promotes fatty streak formation. Furthermore, rabbit models have shown that Lp(a) promotes atherosclerosis and vascular calcification. However, many of these models have limitations. Mouse models need to be transgenic for both apo(a) and human apolipoprotein B-100 since apo(a) does not covalently associated with mouse apoB to form Lp(a). In established mouse and rabbit models of atherosclerosis, Lp(a) levels are low, generally <20 mg/dL, which is considered to be within the normal range in humans. Furthermore, only one apo(a) isoform can be expressed in a given model whereas over 40 isoforms exist in humans. Mouse models should also ideally be studied in an LDL receptor negative background for atherosclerosis studies, as mice don't develop sufficiently elevated plasma cholesterol to study atherosclerosis in detail. With recent data that cardiovascular disease and calcific aortic valve stenosis is causally mediated by the *LPA* gene, development of optimized Lp(a)-transgenic animal models will provide an opportunity to further understand the mechanistic role of Lp(a) in atherosclerosis and aortic stenosis and provide a platform to test novel therapies for cardiovascular disease.

Keywords Lipoprotein(a) · apo(a) · Oxidized phospholipids · Atherosclerosis · Aortic stenosis · Animal models

Introduction

Lipoprotein(a) is an unique plasma particle composed of a low-density lipoprotein (LDL)-like particle where apolipoprotein B-100 is covalently attached to the hydrophilic, carbohydrate-rich apolipoprotein(a) [apo(a)]. Since its discovery in 1963 by Berg [1], its physiologic role remains unknown, although accumulated evidence suggests that Lp(a) is a genetically determined, independent, causal risk factor for atherosclerotic cardiovascular disease and aortic valve stenosis.

Encoded by the *LPA* gene on chromosome 6, apo(a) is a large, highly polymorphic, glycoprotein with over 40 different isoforms in human populations. The size of the apo(a) protein is inversely related to plasma Lp(a) levels, which can vary >1000 fold between individuals. More than 90 % of the inter-individual variation of Lp(a) levels is genetically determined [2]. A large proportion of this variation is determined by the length of the *LPA* gene, while other overlapping

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contributions include *LPA* single nucleotide polymorphisms (SNPs), primarily rs10455872 and rs3798220 in Caucasians [3–5] and rs9457951 in Blacks [6], ethnicity [7], and other unidentified elements.

Epidemiology of Lp(a) for Atherosclerotic Cardiovascular Disease and Calcific Aortic Stenosis

Lp(a) is a prevalent risk factor for cardiovascular disease, with 20 % of the population having Lp(a) >50 mg/dL. Such levels are consistently associated with approximately a two-fold increased risk of myocardial infarction (MI) [8, 9] and faster progression of aortic stenosis (AS) in prospectively followed cohorts [10]. Genome wide association studies established an association between *LPA* SNPs and coronary artery disease (CAD), MI [11–14], and calcific aortic valve disease [15] and coronary artery calcification in some but not all studies [16, 17]. Moreover, these findings have been corroborated by recent Mendelian randomization studies, demonstrating that *LPA* SNPs associated with elevated plasma Lp(a) levels also predict development of myocardial infarction [3, 18] and aortic valve stenosis [19, 20], supporting a role for Lp(a) as a genetically determined, independent, causal risk factor for these diseases.

Structure and Function of Lp(a)

LPA evolved through duplication of its neighboring *PLG* gene encoding plasminogen, is highly homologous to plasminogen and retains kringle IV (KIV), kringle V, and the protease-like domain (Fig. 1a) [21]. However, over the course of 40–80 million years since the first existence of *LPA* in primates [21, 22], KIV of plasminogen has been expanded into ten types (KIV_1-KIV_{10}) to form apo(a) with all kringles present in a single copy except for the variable number of identical tandem KIV_2 repeats between individuals, ranging from 3 to >40 copies [7]. Apo(a) isoform size as well as apo(a) heterogeneity is directly determined by the number KIV_2 repeats, as the remaining number of kringles are present equally on all isoforms. Separately, due to mutations, the apo(a) protease-like domain is catalytically inactive [23].

The kringle domains on apo(a) confer the ability of Lp(a) to interact with proteins and lipids. For example, the proposed mechanism for Lp(a) assembly involves two steps, the first mediated by non-covalent interactions between lysine binding sites (LBS) in KIV_7-KIV_8 and Lys680 and Lys690 on apoB-100 [24]. Subsequently, a disulfide bond between Cys4057 in KIV_9 of apo(a) and Cys4326 of apoB-100 is formed [25]. Another important apo(a) domain is KIV_{10}. This domain, which shares 88 % amino acid sequence homology with plasminogen KIV (ligand binding domain) [26], has been implicated in lysine binding and competitive interaction between Lp(a) and plasminogen.

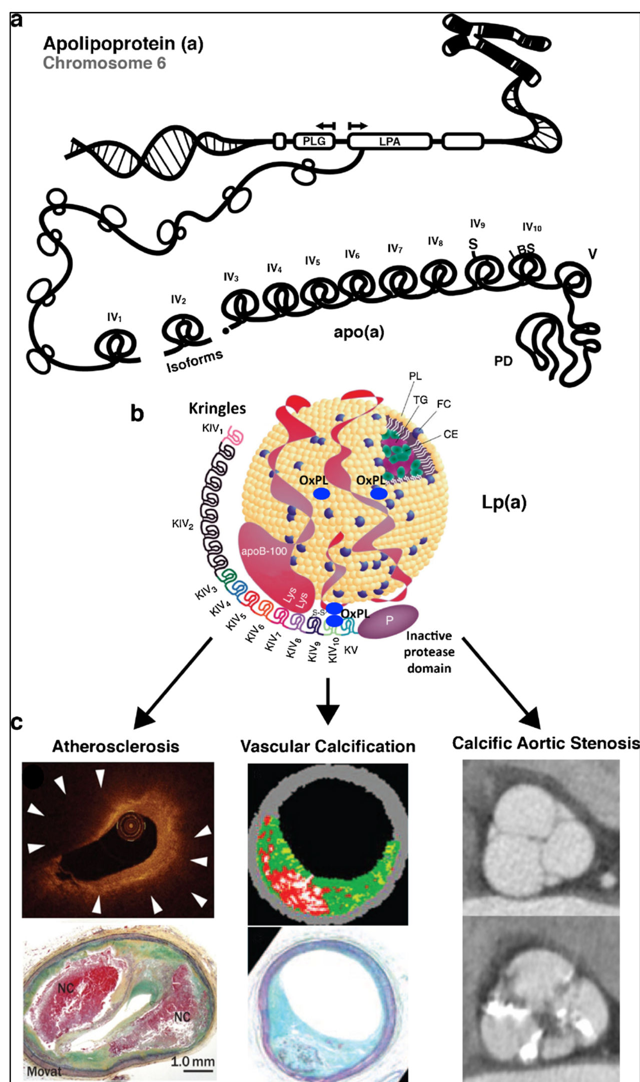
Fig. 1 Summary of pathological consequences of elevated Lp(a). **a** Genetic architecture of apo(a). Panel **a** The genes for plasminogen (*PLG*) and apo(a) (*LPA*) are depicted on chromosome 6. *LPA* is transcribed into apo(a) consisting of kringle domains KIV_1-KIV_{10}, KV, and a protease-like domain, with variable number of tandem KIV_2 repeats that determine apo(a) isoform size. A strong lysine binding site (LBS) is present in KIV10. Panel **b** Structure of Lp(a) and its content of oxidized phospholipids. Lp(a) consists of apo(a) covalently bound to apoB-100 through a disulfide bone in KIV_9. Oxidized phospholipids (OxPL) are present within the lipid phase as well as covalently bound to Lp(a). Panel **c** Cardiovascular diseases associated with elevated Lp(a) include atherosclerosis (*left panel*), vascular calcification (*middle panels*) and aortic stenosis (*right panels*). *Left panels* depict optical coherence tomography (OCT) imaging of a thin fibrous cap atheroma "vulnerable plaque" with large necrotic cores (NC) and hemorrhage. The corresponding histologic specimen is shown below the OCT image. The overlying fibrous cap (FC) is thin (70 μm) and heavily infiltrated by macrophages. The middle panels depict intravascular ultrasonographic (IVUS) images using "virtual histology", show fibrous (*dark green*) and fibrofatty (*light green*) necrotic core (*red*) and calcified (*white*) tissue. The corresponding histologic specimen is shown below the IVUS image. The *right panels* depict a non-calcified tricuspid aortic valve (top) and the presence of aortic valve calcifications using multislice computed tomography. Figures were reproduced with permission from **a** Leibundgut et al. [75], **b** Rao et al. [48], **c** *left panel*, Otsuka et al. [131], *middle panel*- Konig et al. [132], *right panel* Tops et al. [133]

Lp(a) is an Inhibitor of Plasminogen Activation *In Vitro*

Lp(a) interferes with plasminogen binding to fibrin [27] and endothelial cells [28] and competitively inhibits tissue plasminogen activator (tPA) mediated activation of plasminogen *in vitro* [27, 29]. Three-dimensional molecular models of KIV_{10} and KV, based on X-ray crystallographic structures of plasminogen, predict that these domains will bind lysine [26], competing with plasminogen for an interaction of this amino acid residue on fibrin and endothelial cells. Using recombinant apo(a), Hancock et al. demonstrated that a single point mutation in the lysine binding site of KIV_{10} or total deletion of KV significantly diminishes the inhibition of plasminogen activation by tPA in the presence of fibrin [29].

Lp(a) is the Major Lipoprotein Carrier of Oxidized Phospholipids

Oxidized phospholipids (OxPL) play a central role in the development of atherosclerosis, particularly in pro-inflammatory pathways [30, 31]. Elevated levels of oxidized phospholipids on apolipoprotein B-100 (OxPL-apoB), detected using murine monoclonal antibody E06 that binds to the phosphocholine head group on oxidized but not native phospholipids, predict death, MI, and stroke in unselected populations followed prospectively [9, 32–35]. Furthermore, OxPL-apoB correlate with endothelial dysfunction and progression of coronary



calcification [36, 37], predict the progression of femoral/carotid disease [38], coronary artery disease (CAD) [39], and are elevated in patients with ACS [40] and following PCI [41]. Interestingly, the predictive value of OxPL-apoB and Lp(a) for CAD and CVD events is conditioned by the presence of a pro-inflammatory haplotype genetically defined at the IL-1 gene locus [34]. This finding confirms a strong link between lipoprotein oxidation and development of atherosclerotic lesions and adverse CVD events.

In humans, more than 85 % of plasma lipoprotein-associated OxPL are bound to Lp(a) while the remaining exists primarily on apoB containing lipoproteins, although a very small amount is present on HDL [42–44]. A second large and independent pool of OxPL present on other proteins is primarily present on plasminogen [45, 46]. Interestingly, when OxPL are present on plasminogen, they potentiate fibrinolysis *in vitro*, suggesting that the risk of OxPL on (lipo)proteins, is context dependent and that they are pro-atherogenic on Lp(a) but potentially anti-thrombotic on plasminogen [46, 47]. Consistent with the role of this apoB-100 containing lipoprotein as the preferential lipoprotein carrier of OxPL, OxPL-apoB levels correlate with Lp(a) levels in the population as a whole. However, this is dependent on genetics and underlying apo(a) isoform composition, irrespective of race. For example, the strongest association (r-values up to 0.85) is in patients with elevated Lp(a) levels and concomitantly small apo(a) isoforms while the weakest correlations (r-values as low as 0.13) are found in subjects with low Lp(a) levels along with large apo(a) isoforms [7, 35, 48, 49]. Further genetic evidence that Lp(a) levels determine plasma OxPL levels include the high genetic covariance of Lp(a) and OxPL-apoB in a study examining these parameters in monozygotic versus dizygotic twins [48], and that the *LPA* single nucleotide polymorphisms (SNPs) rs3798220 [49] and rs10455872 [48] are also associated with elevated levels. Lastly, in trials with pharmacologic [10, 43, 50–53] or dietary [37, 54, 55] interventions that raise Lp(a), OxPL-apoB is increased as well. Conversely, lipid apheresis [42], and antisense to apo(a) [56] and niacin (unpublished data), all lower OxPL-apoB.

Mechanisms of the Atherogenicity of Lp(a) and apo(a), Insights from Animal Models

The pathophysiologic basis of Lp(a) in atherosclerotic and aortic valve disease has been attributed to both its LDL moiety and apo(a) component. Both apoB-100 and apo(a) have been co-localized in human coronary and carotid atheromas [57], coronary bypass grafts [58, 59] as well as stenotic aortic valves [60], suggesting a local role of Lp(a) in the development and/or progression of these disease.

LDL [61] and apoB [62], the obligate apolipoprotein present on atherogenic non-HDL particles, are both well-established risk factors for cardiovascular disease. However, there are several atherogenic features of Lp(a) and apo(a), which include enhancing monocyte entry and retention in the vessel wall and macrophage foam cell formation [63–65], promoting apoptosis in endoplasmic reticulum-stress macrophages [66], promoting release of pro-inflammatory IL-8 [67], promoting smooth muscle cell proliferation [68], and antifibrinolytic effects [27, 28, 69] have been proposed (reviewed in [70, 71]). Moreover, a key component of the atherogenicity of Lp(a) may be due to its role as a carrier of OxPL (Fig. 1b).

Animal studies have added insight into the role of Lp(a), and specifically apo(a), in binding OxPL. In transgenic mice expressing human apoB-100, E06 immunoreactivity was detected on human apoB-100 containing lipoproteins only if apo(a) was concurrently expressed [72]. Furthermore, transgenic mice expressing apo(a) had OxPL associated with murine apoB-100 [72], which can non-covalently bind to apo(a)

[72, 73]. When apo(a) levels are lowered with antisense targeted against the *LPA* gene, OxPL on human and murine apoB-100 are diminished as well [74]. OxPL is thought to bind to apo(a) both covalently and non-covalently as 30–70 % of E06 immunoreactivity in Lp(a) from various human subjects was lipid soluble [44]. Comparisons between species with expression natural variants of Lp(a) have provided further clues on how apo(a) binds OxPL. Except for humans, primates naturally expressing Lp(a) lack E06 immunoreactivity and coincidentally contain loss of function mutations in the apo(a) KIV_{10} lysine binding site or lack KV, suggesting these domains are essential for binding to OxPL. Indeed, mice expressing a recombinant apo(a) with 2 amino acid substitutions ($Asp^{55/57} \rightarrow Asn^{55/57}$) in KIV_{10} abolished both lysine binding and OxPL binding as detected by E06 [75]. Recombinant human apo(a) containing $KIV_{8–10,}$ but not KV is unable to bind OxPL [76], demonstrating that the presence of both KIV_{10} LBS and KV are required for this function. Incidentally, this may explain why OxPL is not detected on baboon apo(a) which contains all amino acid residues in KIV_{10} predicted to be involved with lysine binding but not KV.

Transgenic mice expressing apo(a) have also provided insight into the role of this protein on atherosclerosis (summarized in Table 1). Unlike humans, where plasma apo(a) is almost entirely covalently bound to LDL, apo(a) circulates as non-lipoprotein associated, or "free" apo(a) in mice [77], as well as non-covalently bound to murine apolipoprotein B-100 [72–75, 78]. Therefore, apo(a) transgenic mice provide a platform to study the role of apo(a), independent of human LDL, on atherosclerosis. Cholesterol and cholate-fed transgenic mice expressing apo(a) developed more aortic fatty streaks compared to non-transgenic controls [77, 79, 80]. Similar findings were reported in study of old (66 weeks old at time of analysis) mice expressing apo(a), which were more likely to develop atheromas and larger lesions than control mice [81]. Interestingly, transgenic expression of human apolipoprotein AI in apo(a) mice prevents the development of fatty streaks in apo(a)-transgenic cholesterol-fed animals [79]. However, it should be noted that Mancini et al. [82] did not observe increased aortic atherosclerosis in cholesterol and cholate-fed mice expressing apo(a) compared to controls.

Moreover, transgenic mice which express human apo(a) with 17 total KIV repeats (17 K) and containing point mutations in two key anionic residues in KIV_{10} lysine binding site ($Asp^{55/57} \rightarrow Asn^{55/57}$) [LBS mut apo(a)], develop significantly less aortic fatty streaks compared to mice which express WT apo(a) [83]. This mutation in apo(a) renders the protein incapable of binding to OxPL as detected by E06 [75], lysine and fibrin [83]. In a diet induced mouse model of atherosclerosis, both fibrin and apo(a) co-localize with fatty streak lesions [84]. However, in fibrinogen (and therefore fibrin) deficient animals, apo(a) was not detected in the aorta [84]. Similarly, there was significantly less apo(a) detected immunologically in lesions from LBS mutant apo(a) mice compared to WT apo(a) expressing mice [83]. This observation was recapitulated in a separate mouse model, where human apo(a) with 8 total KIV repeats (8 K) and containing a separate KIV_{10} lysine binding site point mutation ($Tyr^{72} \rightarrow Arg^{72}$) expressed by adenovirus in animals transgenic for human apoB (h-apoB) also demonstrated less vessel wall accumulation compared to control mice expressing WT apo(a) [85]. Taken together, these experiments show that apo(a) can be targeted to the vessel wall by its KIV_{10} lysine binding site, which facilitates fibrin(ogen) binding. Once resident in the artery wall, the relative contributions of fibrin and OxPL, bound to apo(a), towards the development and progression of atherosclerosis remains to be determined.

Studies utilizing animal models have also attempted to assess the contribution of Lp(a) to the development of atherosclerosis relative to that of apoB-100 alone. In murine models expressing relatively low levels of Lp(a) levels, the addition of an *LPA* transgene did not appear to increase atherosclerotic burden in animals transgenic for h-apoB. Mancini et al. examined atherosclerosis in transgenic mice expressing cDNA encoding for 17 K apo(a), and double transgenic mice which also express high levels of h-apoB (Lp(a) mice) on a high cholesterol diet [82]. Lp(a) levels were 35–54 mg/dl in double transgenic mice and these animals had a two-fold higher lesion area in the proximal aorta although no difference in aortic lipid staining overall compared to single transgenic h-apoB mice [82]. Callow et al. [80] also compared proximal aorta lesion size between cholesterol-fed 17 K apo(a) mice, h-apoB mice and Lp(a) mice. Although Lp(a) levels were not reported in this study, Lp(a) mice had apo(a) levels of 9–14 mg/dl, determined relative to human Lp(a) standards on ELISA. Mice with high levels of h-apoB (>100 mg/dl) developed the largest lesions, and in this setting, the addition of the apo(a) was insignificant towards atherogenesis. However, mice which expressed apo(a) and low levels of h-apoB (14–17 mg/dl) developed 2.5 fold larger lesions than those which only expressed apo(a) [80], suggesting that Lp(a) promotes lesion development beyond the effect of apo(a). Sanan et al. developed transgenic mice expressing apo(a) and h-apoB (Lp(a) mice) or only h-apoB on an LDL-receptor deficient background [86]. In this model, both strains of mice had similar plasma h-apoB-100 levels while mice which also expressed apo(a) had average Lp(a) levels of 25 mg/dl. On a chow diet, both strains developed complex atherosclerotic lesions, although the aortic lesion burden was not different between the two groups [86]. Finally, Teivainen et al. [87] demonstrated that mice expressing h-apoB and a yeast artificial chromosome (YAC) containing *LPA* did not develop more atherosclerosis than those expressing *LPA* only.

Another mouse model examined the role of Lp(a) in chronic kidney disease, which is associated with accelerated atherosclerosis, increased cardiovascular death, and elevated Lp(a)

Table 1 Effect of Apo(a) and Lp(a) expression in experimental animal models of atherosclerosis

Study	Year	Model	Diet	apoB genotype	Apo(a) genotype	apo(a)/Lp(a) levels	Control animal	Atherosclerosis
Lawn et al. [77]	1992	Mouse	HC/HF	mouse	17 K	12 mg/dl	C57B6/SJL	↑
Callow et al. [80]	1995	Mouse	HC/HF	mouse	17 K	5 mg/dl	C57B6/SJL/FVB	↑
			HC/HF	mouse/human	17 K	9 mg/dl	apo(a) Tg	↑
			HC/HF	mouse/human	17 K	9 mg/dl	h-apoB Tg	No Δ
Mancini et al. [82]	1995	Mouse	HC/HF	mouse	17 K	11–15 mg/dl	C57B6/SJL	No Δ
			HC/HF	mouse/human	17 K	12–15 mg/dl	h-apoB Tg	No Δ
Boonmark et al. [83]	1997	Mouse	HC/HF	mouse	17 K	15 nM	FVB	↑
			HC/HF	mouse	17 K	35 nM	LBS mut 17 K	↑
Sanan et al. [86]	1998	Mouse	Chow	mouse	17 K	27 mg/dl	LDLR$^{-/-}$/C57B6/129Sv/Ev	No Δ
			Chow	mouse/human	17 K	25 mg/dl	h-apoB Tg/ LDLR$^{-/-}$	No Δ
Fan et al. [101]	2001	Rabbit	HC/HF	rabbit	17 K	27–29 nM	non-transgenic rabbit	↑
Berg et al. [81]	2002	Mouse	Chow	mouse	17 K	73 U/l	C57B6/SJL	↑
Sun et al. [97]	2005	Rabbit	Chow	rabbit	17 K	15 mg/dl	WHHL rabbit	↑ + vascular calcification
Teivainen et al. [87]	2004	Mouse	Chow	mouse	*LPA*-YAC, 12 K	7–59 mg/dl	FVB	↑ + vascular calcification
			HC/HF	mouse	*LPA*-YAC, 12 K	0.6–76 mg/dl	FVB	↑ + vascular calcification
			HC/HF	mouse/human	*LPA*-YAC, 12 K	NA	apo(a) Tg	No Δ
Schneider et al. [72]	2005	Mouse	Chow	mouse/human	8 K	700 mg/dl	NA	NA
Kitajima et al. [103]	2007	Rabbit	Chow	rabbit	17 K	3 mg/dl	WHHL rabbit	↑
Pedersen et al. [89]	2010	Mouse	Chow	mouse	12 K	45 nM	C57B6/SJL	↑
		Mouse	Chow	mouse/human	12 K	50 mg/dl	h-apoB Tg	↑

HC high cholesterol, *HF* high fat, *17 K* 17 kringle, *YAC* yeast artificial chromosome, *Tg* transgenic, *WHHL* Watanabe heritable hyperlipidemic; *NA* not available

levels in uremic patients (reviewed in [88]). In a nephrectomy model of uremia, mice transgenic for a 12 K cDNA construct encoding apo(a) or double transgenic for Lp(a) developed larger aortic root lesions compared to WT and h-apoB controls, respectively [89]. This model suggests that Lp(a) may be a therapeutic target in uremic or end-stage renal disease patients, a population which does not benefit from statin therapy [90, 91] has been difficult to manage for primary and secondary prevention of cardiovascular disease.

One major limitation of the above mouse models, is that they express Lp(a) at levels short of the what is generally accepted as an "elevated" Lp(a) level in humans, which is >50 mg/dl [92]. Lp(a) levels less than this do not appear to confer as high increased risk of CVD after adjustment for traditional risk factors [32, 93]. To address this issue, Schneider et al. [72] generated transgenic mice with plasma Lp(a) levels of up to ~700 mg/dl based on the principle in humans that smaller apo(a) isoforms lead to higher Lp(a) levels due to faster synthesis of apo(a) [94]. This model expresses cDNA encoding an 8 K version of apo(a) [in contrast to previous animal models which expressed 17 K apo(a)], consisting of KIV_1, KIV_2, a fusion of $KVI_{3–5}$ and KIV_6–KIV_{10}, KV, and the protease domain, with an *Apoe* promoter regulating transcription of this transgene. These apo(a) mice were bred into those transgenic for a bacterial artificial chromosome containing a human apoB-100-only gene, which contains a mutation in codon 2,153 preventing mRNA editing and subsequent production of apoB-48. Two lines of double transgenic mice, expressing Lp(a) were described, one with lower levels of Lp(a) [~35 mg/dl] and another with very high levels [~700 mg/dl]. The high Lp(a) expressers have substantially higher plasma levels of OxPL present on human apoB-100 and mouse apoB-100 compared to low expressers and single transgenic human apoB-100-only mice. These findings likely reflect the role of apo(a) as a recruiter of OxPL to apoB containing particles associated with it. Although atherosclerosis studies have yet to be performed using this model, it provides the unique opportunity to examine the contributions of high Lp(a) levels and that of OxPL on Lp(a) towards this disease in mice.

It is also worth noting limitations beyond that of Lp(a) levels in mouse models. Despite the ability of human LDL to rapidly and readily covalently associate with apo(a) in mice, as demonstrated by infusion of human LDL in apo(a) transgenic mice [95], the majority of apo(a) can remain unbound to h-apoB-100 in double transgenic mice [78]. In this setting, it

will be difficult to exclude the possibility that "free" apo(a) acted competitively against Lp(a) for its role in atherosclerosis. To add another layer of complexity, an additional pool of apo(a), which is non-covalently associated with murine apoB-100 [72, 73] exists in Lp(a) mice. The overall atherosclerosis phenotype is most the net effect of contributions from each of these unique particles (Fig. 2) in Lp(a) mice.

Another animal model traditionally utilized to study atherosclerosis has been the rabbit model. This model has several advantages compared to the murine model. Human apo(a) is capable of forming a covalent bond with rabbit LDL [96] (Fig. 2) and various studies have reported that 20–80 % of apo(a) is linked to rabbit apoB-100 [96–99]. In addition, rabbits are more human-like with regards to lipoprotein metabolism in that they express cholesterol ester transfer protein (CETP) and apoB mRNA is not edited in the rabbit liver. However, rabbits have very low plasma levels of hepatic lipase and lack apoA-II [100]. Lastly, Watanabe heritable hyperlipidemic (WHHL) rabbits, which have defect LDL receptor function due to a spontaneous loss of function mutation, develop marked hypercholesterolemia, xanthomas, and atherosclerosis, resembling the phenotype of human familial hypercholesterolemia.

Cholesterol-fed transgenic (non-WHHL) rabbits expressing cDNA encoding for 17 K apo(a), with Lp(a) levels of 27–29 nmol/L (~11 mg/dL), developed more extensive atherosclerotic lesions in the aorta, carotid, iliac, and coronary arteries, compared to non-transgenic rabbits [101]. Interestingly, lesional apo(a) co-localized with immature smooth muscle-like cells, which were identified by positive vimentin and absent smooth muscle α-actin staining [101]. Prior work has implicated apo(a) in smooth muscle cell proliferation *in-vitro* via TGF-β activation [68] and shown that there is increased activated TGF-β, detected immunologically in aortas of mice expressing apo(a) [102]. WHHL rabbits expressing apo(a) with Lp(a) levels of 8–10 nmol/L (equivalent to ~3 mg/dl) developed more coronary atherosclerosis compared to controls [103]. A separate study examining transgenic WHHL rabbits with Lp(a) levels of 15 mg/dl, demonstrated that apo(a) transgenic animals develop much more complex atherosclerotic lesions compared to controls, despite having similar sudanophilic lesion burden [97]. Lesions in transgenic WHHL rabbits consisted of lipid core, fibrous cap, and striking vascular calcifications (rare in non-transgenic controls) which are intimately co-localized with Lp(a). Furthermore, the addition of Lp(a) to cultured vascular smooth muscle cells promoted

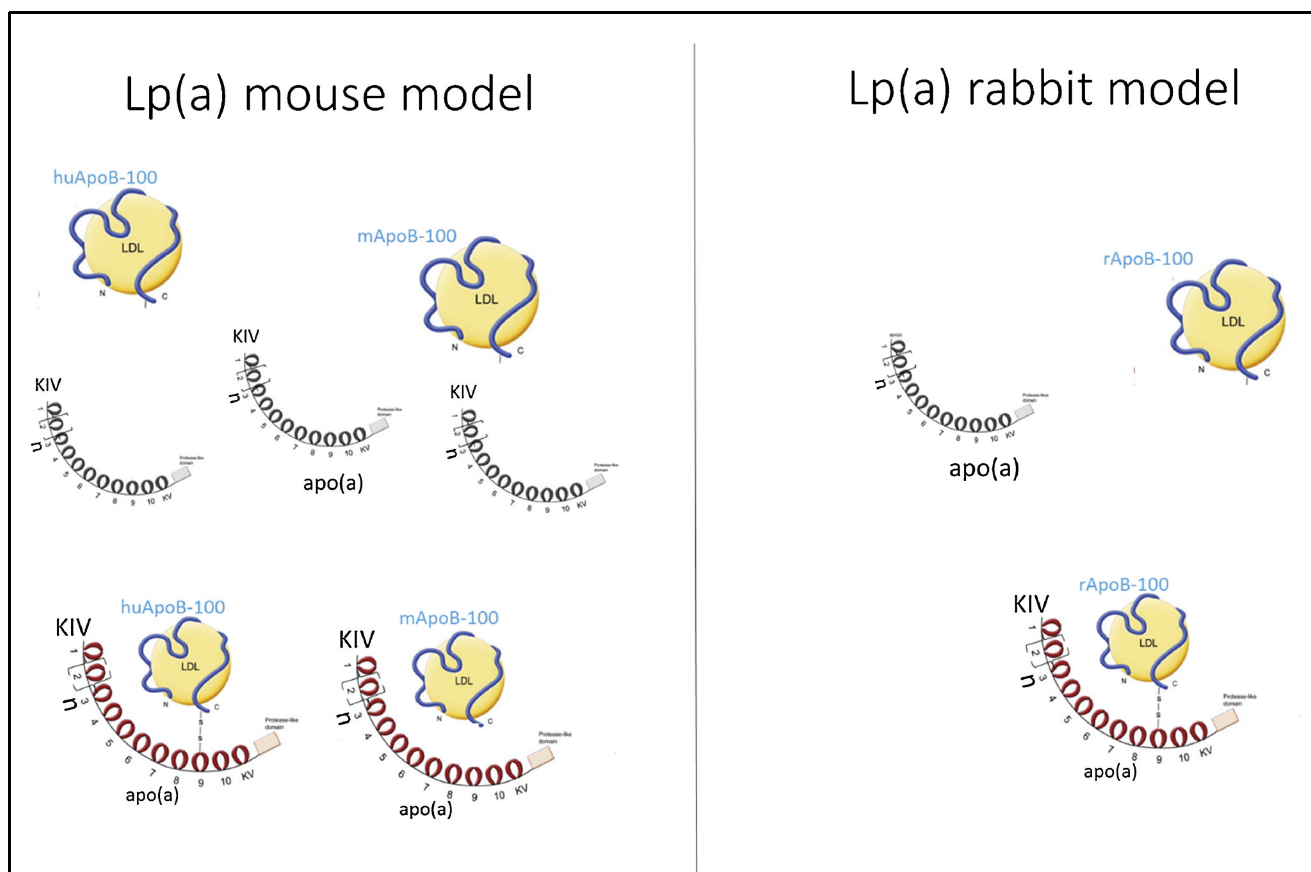


Fig. 2 Schematic depicting selected plasma apo/lipoproteins in the Lp(a) mouse (*left*) and rabbit (*right*) animal models. apo(a) = apolipoprotein(a); huApoB-100 = human apoB-100; mApoB-100 = murine apoB-100; rApoB-100 = rabbit apoB-100

calcium deposition in a dose dependent manner and was associated with dysregulation of calcium homeostasis with increased alkaline phosphatase activity and decreased osteopontin expression [97].

In summary, the overall work from animal models has complemented findings from large genetic studies in humans demonstrating that Lp(a) and apo(a) are risk factors for atherosclerotic cardiovascular disease. However, it is interesting to note transgenic mice with Lp(a) levels of 9–25 mg/dl did not have increased atherosclerosis compared to mice only transgenic for h-apoB, but in contrary, transgenic rabbits with Lp(a) levels from 3 to 11 mg/dL developed more severe and complex atherosclerosis compared to controls. These findings suggest that differences in species specific thresholds to the adverse effects of plasma Lp(a), and/or differences in the amount of "free" apo(a) relative to Lp(a) between these species may be involved in atherogenesis. As Lp(a) is not naturally expressed in mice or rabbits, these animals may not have evolved to develop the same downstream signaling targets that potentially exists in humans, and therefore Lp(a) transgenic animals may expectedly have phenotypes which do not perfectly align with that in humans. Also, with the exception of the model developed by Teivainen et al. [87] all transgenic animals express apo(a) cDNA driven by an exogenous promoter (Table 1). Though these model may be sufficient to address the question of whether apo(a) and Lp(a) contributes to cardiovascular disease, they do not reflect the complexity of *LPA* transcriptional regulation in humans. Finally, all mouse and rabbit models to date have expressed only one apo(a) isoform either 17 K which is present in humans or 8 K, which is not present naturally. With over 40 apo(a) isoforms present in humans, it has not been possible to fully represent the range of human Lp(a) variability.

Future Directions

A consistent observation in atheromas from humans and apo(a) transgenic animals is that apo(a) is found in plaque. In mice, mutations in the apo(a) KIV_{10} LBS, a domain implicated in fibrin binding, plasminogen activation and OxPL binding, diminishes the effect of this protein on arterial retention and foam cell formation. Future *in vivo* studies, carefully delineating the role and relative contributions of each of these aspects of apo(a) function in the development of cardiovascular disease, are needed.

To further elucidate the relative contributions of OxPL on Lp(a) to atherogenesis, one could determine if an antagonist of the inflammatory properties of OxPL, such as E06 [104], would attenuate atherosclerosis in animals expressing Lp(a). The ideal model for such a study would be a rabbit model, however, transgenic mice expressing high levels (>50 mg/dl) of Lp(a) [h-apoB-100 and apo(a)], such as the one developed by Schneider et al. [72], may be appropriate as well.

An interesting phenomenon is that mice [87] and WHHL rabbits [97] expressing Lp(a) develop ectopic, vascular calcification. Elevated Lp(a) levels in humans is associated with coronary artery calcium [14, 105] in some studies but not all [16, 17] as well as uniformly in calcific aortic stenosis to date [10, 15, 19, 20]. In humans, calcified atheromas and aortic valves are associated with more advanced disease, which reinforces the pathogenic role of Lp(a).

Proposed Animal Models to Elucidate the Mechanisms of Lp(a) in the Development and Progression of Atherosclerosis and Aortic Stenosis

AS is a progressive disease affecting approximately 12 % of the population over the age of 75, for which there is currently no effective medical therapy [106]. Affected valves are characterized by progressive fibrosis, thickening and most importantly, calcification [107, 108]. As mentioned above, *LPA* SNPs associated with elevated plasma Lp(a) levels predict development of aortic valve calcification and stenosis [19, 20], and plasma Lp(a) levels of >50 mg/dl predicted progression of AS [10].

Early lesions on human stenotic aortic valve leaflets contain oxidized lipids, apoB and apo(a) [60, 109–111]. Similar to atherosclerosis, advanced aortic valve stenosis is characterized by ectopic calcification within this normally compliant tissue. Oxidized LDL (OxLDL) are highly enriched in OxPL [112–114] and have been implicated in promoting valvular ectopic calcification and bone formation, features that are pronounced in severe AS. Bone formation within the diseased AV is driven by the differentiation of vascular cells into osteoblasts [109], via bone morphogenic protein (BMP) signaling and upregulation of osteoblastic transcription factors including *RUNX2* and *MSX2*. BMP2 [114–117] as well as *RUNX2* [118] and *MSX2* [119] expression in vascular cells are upregulated following exposure to OxLDL. OxLDL exposure also suppresses osteoprotegerin [120], an inhibitor of vascular calcification via the RANK-L pathway [121, 122]. Finally, exposure to OxLDL *in vitro* stimulates extracellular matrix calcium deposition by vascular cells [118, 119, 123, 124] as well as upregulation of alkaline phosphatase [112, 119, 123, 125, 126], reminiscent of the observations with Lp(a) by Sun et al. [97] *in vitro* and in transgenic WHHL rabbits.

To better understand the role of OxPL on Lp(a) in atherosclerosis and AS and its therapeutic implications, an animal model will be necessary. Although WHHL transgenic rabbits expressing Lp(a) develop vascular calcification, WHHL rabbits do not develop hemodynamically significant AS [127]. To date, the only animal model that develops clinically-significant AS is the mouse apoB-100only/LDLR-deficient mouse fed a high cholesterol diet for 1 year [127, 128].

Therefore, LDLR-deficient mice which express high levels (>50 mg/dl) of Lp(a) would be the most clinically relevant. Moreover, one model will not be adequate to study the effect of apo(a) isoform sizes. Development of a number of animal models that express different sizes of apo(a) isoforms, from very small, such as 3 KIV_2 repeats which is the smallest isoform described in humans, to very large with >30 KIV_2 repeats will be highly informative. As these apo(a) constructs already exist [29, 75, 129, 130], the generation of transgenic mouse models can be accelerated. Within these models, the development and progression of aortic stenosis in Lp(a)/LDLR-deficient mice compared to Lp(a)-LBS-mut/LDLR-deficient mice or Lp(a)/LDLR-deficient mice which express high levels of E06 will provide further insight into the contribution of this pro-inflammatory and pro-calcific lipid towards Lp(a) mediated atherosclerosis and AS.

Compliance with Ethical Standards

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