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Analysis of Lipoprotein(a) Receptors: Roles in Catabolism and Pericellular Plasminogen Activation

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by

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I hereby declare that this dissertation incorporates material that is result of joint research, as follows:

This dissertation incorporates the outcome of joint research in collaboration with Dr. Santica Marcovina, Dr. Nabil Seidah, and Dr. Ross Milne under the supervision of Dr. Marlys Koschinsky. In all cases, key ideas, primary contributions, experimental design, execution, data analysis, and interpretation was performed by the author. Additionally, manuscripts were written initially by the author and revised and edited by Dr. Michael Boffa and Dr. Marlys Koschinsky for Chapters 2, 3, and 4 and revised and edited by Dr. Michael Boffa, Dr. Marlys Koschinsky, Dr. Santica Marcovina, and Dr. Nabil Seidah for Chapter 5.

- Collaboration with Dr. Santica Marcovina is covered in Chapters 2 and 5 of this dissertation; this contribution was through providing purified Lp(a) and anti-apo(a) antibody.
- Collaboration with Dr. Nabil Seidah is covered in Chapter 5 of this dissertation; this contribution was through providing PCSK9 and LDLR constructs.
- Collaboration with Dr. Ross Milne is covered in Chapter 5 of this dissertation; this contribution was through providing LDLR blocking antibody 5G2 and 7H2.

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Dissertation Chapter	Publication Title and Full Citation	Publication Status
Chapter 2	Romagnuolo R, Marcovina SM, Boffa MB, Koschinsky ML. Inhibition of plasminogen activation by apo(a): role of carboxyl-terminal lysines and identification of inhibitory domains in apo(a). J Lipid Res. 2014; 55(4):625-34.	Published
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Chapter 4	Romagnuolo R, Boffa MB, Koschinsky ML. Inhibition of pericellular plasminogen activation by apolipoprotein(a): Roles of urokinase plasminogen activator receptor and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$ in vascular cells.	In preparation
Chapter 5	Romagnuolo R, Scipione C, Boffa MB, Marcovina SM, Seidah NG, Koschinsky ML. Lipoprotein(a) catabolism is regulated by proprotein convertase subtilisin/kexin type 9 through the low density lipoprotein receptor. J Biol Chem. 2014; ID: JBC/2014/611988.	Under revision

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ABSTRACT

Elevated plasma concentrations of lipoprotein(a) (Lp(a)) have been identified as an independent, causal risk factor for coronary heart disease. Lp(a) resembles low-density lipoprotein (LDL), but is distinguished by the covalent addition of apolipoprotein(a) (apo(a)) to the apolipoproteinB-100 (apoB-100) moiety of LDL. Apo(a) shares homology with the zymogen plasminogen and Lp(a)/apo(a) can inhibit plasminogen activation to the serine protease plasmin on the fibrin surface. In addition, apo(a)/Lp(a) can inhibit fibrinolysis through attenuation of Glu^{1}-plasminogen to Lys^{78}-plasminogen conversion: a key positive feedback mechanism in accelerating plasmin formation. Cellular receptors for Lp(a)/apo(a) are suggested to contribute to the pathophysiological mechanisms of Lp(a) such as inhibition of pericellular plasminogen activation as well as Lp(a) clearance from plasma. The roles and identities of these receptors, however, remain elusive. The work in this dissertation evaluates the roles of receptors implicated in the ability of apo(a)/Lp(a) to inhibit pericellular plasminogen activation as well as in Lp(a) clearance by hepatocytes. Apo(a)/Lp(a) was found to potently inhibit pericellular plasminogen activation on vascular and blood cells through attenuating Glu^{1}- to Lys^{78}-plasminogen conversion. For both these effects, critical roles for the strong lysine binding site in kringle IV type 10 as well as the kringle V domain within apo(a) were identified; there was also no dependency on apo(a) isoform size. We found that the urokinase receptor and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$ all contribute to plasminogen activation and apo(a) mediated inhibition of plasminogen activation on the cell surfaces of vascular and blood cells, with only a minor role for receptors containing carboxyl-terminal lysines. *In vivo* evidence suggests a potential role for proprotein convertase subtilisin/kexin type 9 (PCSK9) in Lp(a) clearance through an unidentified receptor. Effects on the number of LDL-receptor (LDLR) molecules were found to underlie the ability of PCSK9 to modulate Lp(a) catabolism in hepatic cells and fibroblasts. This process was dependent on the apoB-100 component of Lp(a) and on clathrin-mediated endocytosis. Taken together, our data contribute importantly to our understanding of the mechanisms of Lp(a) pathogenicity with regard to inhibition of pericellular plasminogen activation and highlight a novel role for PCSK9 activity and the LDLR in modulating Lp(a) catabolism.

I dedicate this work to my family and friends for their encouragement and love

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Definition
apo(a)	apolipoprotein(a)
apoB-100	apolipoproteinB-100
apoE	apolipoprotein E
ApoER2	apolipoprotein E receptor 2
ARH	autosomal recessive hypercholesterolemia
ASO	antisense oligonucleotide
AVS	aortic valve stenosis
BHK	baby hamster kidney
BSA	bovine serum albumin
CAC	coronary artery calcification
CAD	coronary artery disease
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CHRD	cysteine-histidine-rich domain
CpB	carboxypeptidase B
EBM-2	endothelial basal medium-2
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGM-2	endothelial growth medium-2
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FBS	fetal bovine serum
FC	free cholesterol
FH	Familial hypercholesterolemia
GOF	gain-of-function

gp330	megalin/glycoprotein 330
GPI	glycosyl phosphatidylinositol
GWA	genome-wide linkage and association
HBS	HEPES-buffered saline
HDL	high-density lipoprotein
HEK293	human embryonic kidney
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HepG2	human hepatocellular carcinoma cell
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HUVEC	human umbilical vein endothelial cell
IL	interleukin
K_D	dissociation constant
KI	kringle I
KII	kringle II
KIII	kringle III
KIV	kringle IV
KV	kringle V
LBS	lysine-binding site
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LOF	loss-of-function
Lp(a)	lipoprotein(a)
LPDS	lipoprotein-depleted serum
LRP	low-density lipoprotein-related protein
mAb	monoclonal antibody
MAC-1	macrophage-1 antigen (integrin $\alpha_M\beta_2$)
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
MI	myocardial infarction
MMP	matrix metalloproteinases
mRNA	messenger RNA

NCS	newborn calf serum
NO	nitric oxide
P	protease
PAD	peripheral arterial disease
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCSK9	proprotein convertase subtilisin/kexin type 9
PEG	polyethylene glycol
Pg	plasminogen
PL	phospholipid
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsufonyl fluoride
r-apo(a)	recombinant apo(a)
RFU	relative fluorescent unit
sc-tPA	single-chain tissue-type plasminogen activator
sc-uPA	single-chain urokinase-type plasminogen activator
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sLBS	strong lysine-binding site
SmBM	smooth muscle basal medium
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SR-BI	scavenger receptor class B type I
TAFI	thrombin-activatable fibrinolysis inhibitor
tc-tPA	two-chained tissue-type plasminogen activator
tc-uPA	two-chained urokinase-type plasminogen activator
TFPI	tissue factor pathway inhibitor
TG	triglycerides
TGF-β	transforming growth factor-β

TGN	*trans* Golgi network
THP-1	human acute monocytic leukemia cells
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor
VTE	venous thromboembolism
wLBS	weak lysine-binding site
ε-ACA	ε-aminocaproic acid

CHAPTER 1

General Introduction

1.1 LIPOPROTEIN(a)

Lipoprotein(a) (Lp(a)) is an enigmatic lipoprotein that has been identified as a causal risk factor for coronary heart disease (CHD) [1,2]. Recent evidence suggests that Lp(a) is the strongest genetic risk factor for CHD [Reviewed in ref. 3]. Lp(a) is a complex lipoprotein that resembles low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoproteinB-100 (apoB-100); however, Lp(a) is a unique lipoprotein class that contains a highly glycosolayted hydrophilic component called apolipoprotein(a) (apo(a)) (Fig. 1.1) [4]. Glycosylation accounts for approximately 28% of apo(a) by mass, with both N- and O-linked glycans present on the molecule [5]. Apo(a) is predominantly synthesised in the liver [6], after which it becomes covalently attached to apoB-100 through a single disulfide bond to form an Lp(a) particle [7] (see Section 1.1.3).

Analysis of the sequence of apo(a) in 1987, following cloning of apo(a) from liver, revealed striking similarities to the fibrinolytic zymogen plasminogen [8]. Apo(a) consists of multiple copies of a plasminogen-like kringle IV (KIV) domain, followed by single copies of plasminogen kringle V (KV) and protease-like domains (Fig. 1.2) [8,9]. Unlike plasminogen, the protease domain of apo(a) is inactive [10]. The apo(a) KIV domain is further classified into 10 distinct subtypes (KIV_1 to KIV_{10}) based on amino acid sequence [8]. Isoform size heterogeneity is observed within the population based on differing numbers of identically repeated copies of the sequence encoding KIV_2 in *LPA*, the gene encoding apo(a) [11-13]. The number of KIV_2 domains can vary from 3 to greater than 30 identically repeated copies [13].

The similarities between apo(a) and plasminogen represent a potential link between the areas of atherosclerosis and thrombosis. Plasmin, the active form of the zymogen plasminogen, is a key enzyme involved in fibrinolysis. Therefore, the plasminogen-like apo(a) moiety of Lp(a) has been linked to thrombotic events. On the other hand, the atherogenic LDL-like component of Lp(a) can contribute to the development and progression of atherosclerosis. However, many physiological/ pathophysiological proposed functions of Lp(a) have also been shown to be unique to this class of lipoprotein independent of its similarities to the LDL- and plasminogen-like properties.

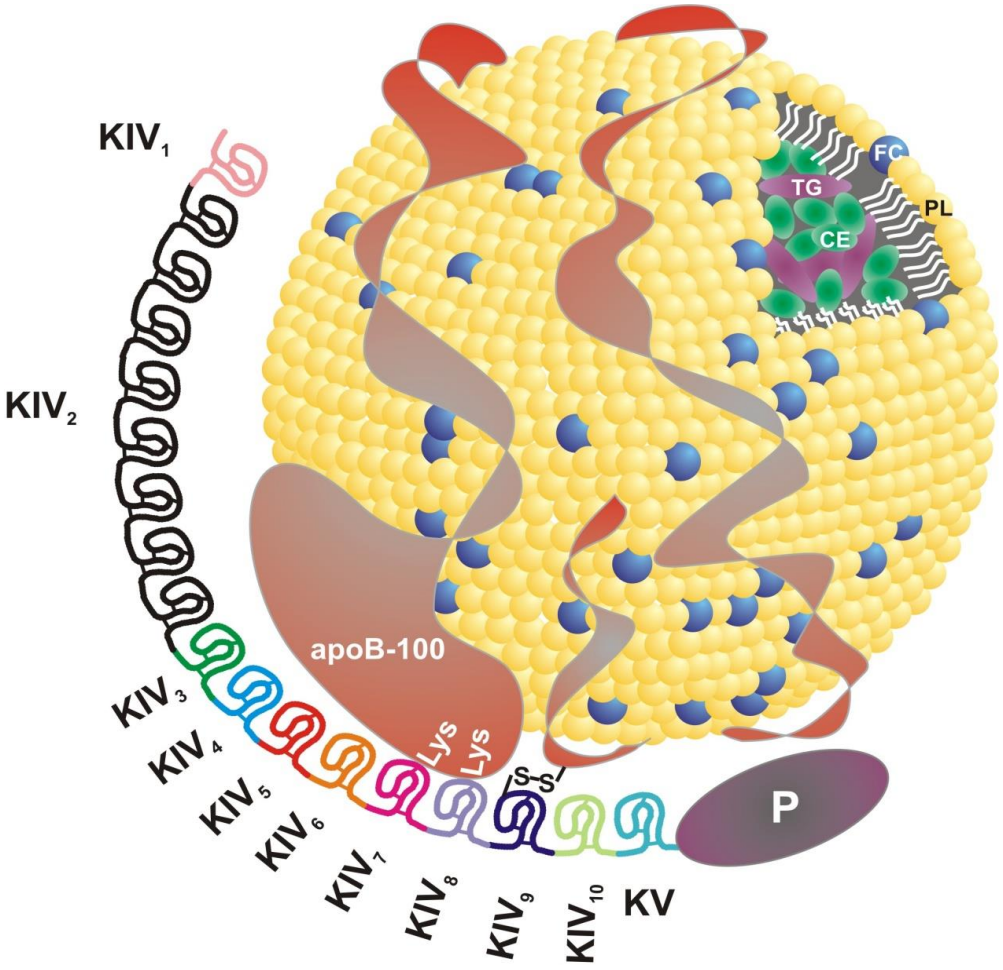

Figure 1.1: ***Representation of the structure of Lp(a).*** Lipoprotein(a) (Lp(a)) resembles low-density lipoprotein (LDL) in both lipid composition and presence of apolipoproteinB-100 (apoB-100), but is distinguished from LDL by the presence of apolipoprotein(a) (apo(a)). The LDL-like moiety is composed of a central core containing triglycerides (TG) and cholesteryl esters (CE), surrounded by an outer shell of phospholipids (PL) and free cholesterol (FC). Apo(a) consists of multiple copies of a plasminogen-like kringle IV (KIV) domain, followed by single copies of a plasminogen-like kringle V (KV) and protease (P) domains. KIV domains in apo(a) can be further classified into 10 distinct subtypes (KIV_1 to KIV_{10}) based on amino acid sequence with the number of identically repeated KIV_2 domains varying in the population from 3 to >30. Lysine binding sites (LBSs) in KIV_7 and KIV_8 play a key role in non-covalent association with two Lys residues in the amino-terminal region of apoB-100. This initial non-covalent interaction between apo(a) and apoB-100 allows for disulfide bond formation between a free cysteine in KIV_9 of apo(a) and apoB-100. Adapted from reference 4.

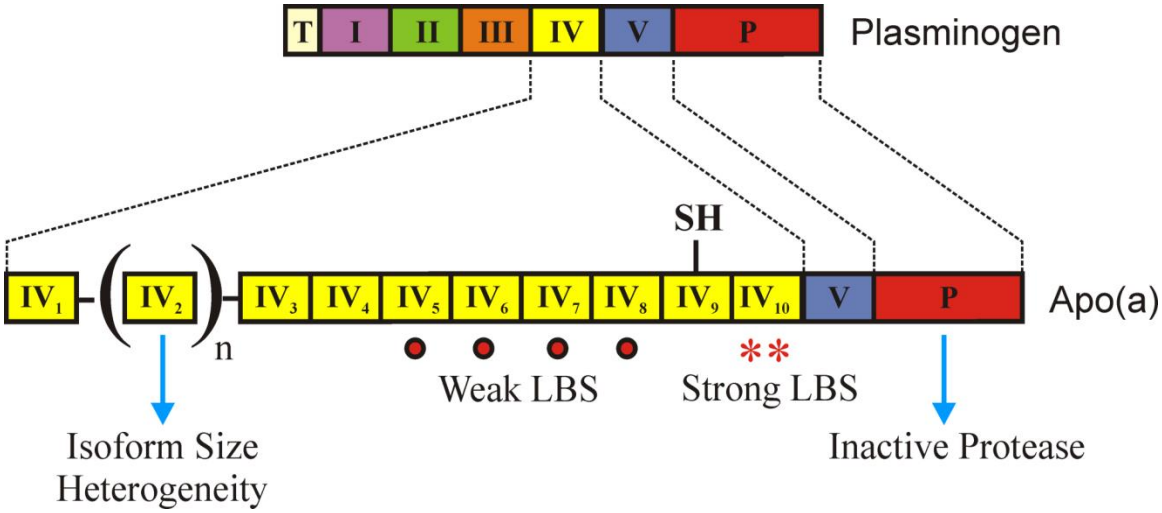

Figure 1.2: ***Structural similarities between plasminogen and apo(a).*** Plasminogen consists of an amino terminal tail sequence (T) followed by five kringle domains (KI-KV) and a serine protease domain (P). Apo(a) contains a number of repeated domains which are highly similar to that of plasminogen kringle IV (KIV), followed by single copies of a plasminogen kringle V (KV) and protease-like (P) domains. Kringle IV domains of apo(a) can be grouped into 10 distinct types on the basis of amino acid sequence with each present in a single copy except for KIV type 2 (KIV_2). The number of KIV_2 domains ranges from 3 to greater than 30 identically repeated copies which gives rise to the Lp(a) isoform size heterogeneity observed in the population. Unlike plasminogen, the protease domain of apo(a) is catalytically inactive. The weak lysine binding sites (LBSs) and strong LBS of apo(a) (indicated with circles and asterisks resepctively) are important for many physiological interactions between apo(a) and various substrates. The cysteine (SH) present in KIV_9 of apo(a) is required for disulfide bond formation to the C-terminal region of apoB-100. Adapted from reference 9.

Apo(a) consists of multiple kringle units that are tri-looped structures containing three invariant intramolecular disulfide bonds [9]. Kringle domains are generally found in many coagulation and fibrinolytic proteins and are recognized as binding motifs. Apo(a) KIV_{5-8} each contain "weak" lysine binding sites (LBS) whereas KIV_{10} contains a "strong" LBS based on its ability to bind to free lysine or lysine analogues [14]. Apo(a) also contains a weak LBS in the KV domain. The LBS acts as a pocket that allows for effective binding to lysine or lysine analogues. The pocket contains a cationic and anionic subsite at either end that acts by stabilizing the carboxylate and ammonium group of lysine respectively, separated by a hydrophobic trough [15]. The hydrophobic trough of the LBS pockets found in plasminogen and apo(a) are generally composed of Trp or Phe and act to stabilize the aliphatic backbone of lysine [15]. The LBS present in the KIV_{10} domain of apo(a) bears the greatest sequence similarity to that of plasminogen KIV [15]. The only exception is a conservative substitution of Arg^{35} in plasminogen KIV to Lys^{35} in apo(a) KIV_{10} [8]. Key amino acids in KIV_{10} that mediate binding to lysine include Arg^{35}, Asp^{54}, Phe^{62}, and Asp^{56} [15]. Key amino acid substitutions in each of the KIV_{5-8} domains result in a lower affinity of these kringles for lysine and its analogues compared to KIV_{10}. For example, KIV_7 of apo(a) contains a Tyr at amino acid position 62 compared to a Phe at the corresponding position in KIV_{10}. This results in a unique network of electrostatic interactions and hydrogen bonding in KIV_7 leading to reduced flexibility and adaptability in accommodating lysine and its analogues compared to KIV_{10} [16]. Plasminogen not only contains a strong LBS present in KIV but also in KI (two orders of magnitude greater than KIV for lysine affinity) and a weak LBS present in KV [15, 17]. The LBSs present in plasminogen KI and KIV are generally responsible for binding to fibrin [15].

Plasma Lp(a) levels vary widely within the population from nearly undetectable to greater than 100 mg/dL [18]. The majority of apo(a) is found associated with Lp(a) particles in the plasma with very little free apo(a) detected [19]. The greater than 1000-fold variation in plasma levels of Lp(a) observed in the population is predominantly determined (up to 90%) by the *LPA* gene [20]. The largest contributor (up to 60%) of this variation resides in the genetically determined isoform size heterogeneity of Lp(a) observed within the population [reviewed in ref. 3]. A general inverse relationship exists between Lp(a) plasma levels and apo(a) size [21, 22]. This phenomenon is due in part to less efficient secretion of larger apo(a) isoforms from hepatocytes [21]. This occurs as a result of the longer retention time for larger apo(a) species within the endoplasmic reticulum (ER) due to the additional time required to fold these species compared to smaller isoform sizes [23, 24]. The longer retention time results in increased quality control mediated degradation within the ER, leading to reduced secretion efficiency [23, 24].

Currently, there is no accepted pharmaceutical agent that can consistently or specifically lower elevated plasma Lp(a) levels. Of note, plasma Lp(a) levels are primarily dictated by the rate of production rather than catabolism. Currently, however, the route of catabolism of Lp(a) from the circulation is unclear. Understanding the catabolic fate of Lp(a) may prove vital in potentially lowering elevated plasma levels of the proatherogenic and antifibrinolytic/prothrombotic Lp(a) particle.

1.1.1 Lp(a) pathophysiology: potential mechanisms of action

The pathophysiological mechanism of how elevated Lp(a) levels contribute to atherothrombotic diseases remains largely speculative. Elevated levels of Lp(a), greater than 30-50 mg/dL, confer risk for a variety of atherosclerotic disorders including CHD [reviewed in ref. 2, 25], ischemic stroke [26-28], and peripheral vascular disease [29]. Lp(a) has been proposed to contribute to not only proatherogenic but also prothrombotic/antifibrinolytic processes (Fig. 1.3). Elevated levels of plasma Lp(a) result in accumulation of Lp(a) within the arterial wall at sites of atherosclerotic lesions [30]. Lp(a)/apo(a) can be retained within atherosclerotic lesions through interactions with components of the extracellular matrix (ECM) including fibronectin, fibrinogen, tetranectin, proteoglycans and DANCE [31, 32].

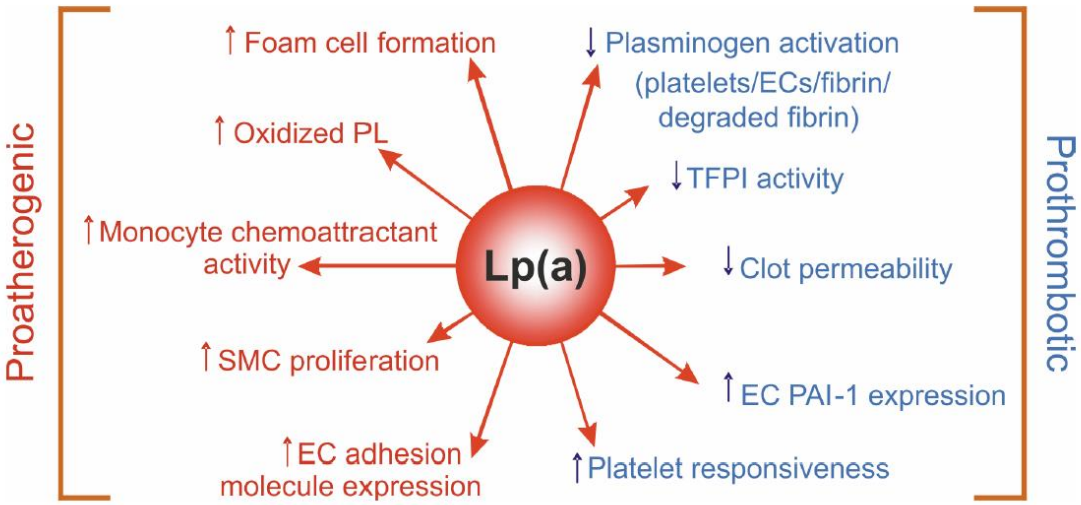

Figure 1.3: ***Proposed proatherogenic and prothrombotic mechanisms of Lp(a) action.*** Lp(a) has been identified as an independent risk factor for CHD as well as purely thrombotic events such as stroke and venous thromboembolism (VTE). Illustrated here are some processes that may be mediated by Lp(a) based on *in vitro* and *in vivo* studies. The mechanisms are categorized as either proatherogenic, illustrated in red on the left, or prothrombotic, illustrated in blue on the right. PL, phospholipids; SMC, smooth muscle cells; EC, endothelial cells; TFPI, tissue factor pathway inhibitor; PAI-1, plasminogen activator inhibitor-1. Adapted from reference 33.

1.1.1.1 Development of atherosclerosis

Atherosclerosis is a progressive, inflammatory disease resulting in the accumulation of lipids and fibrous elements in arteries [reviewed in ref. 34]. The progression of atherosclerotic lesions initially occurs through subendothelal lipoprotein retention. Accumulation of the retained lipoproteins is greater in individuals with elevated levels of circulating LDL and Lp(a). Lipoproteins in the subendothelium can interact with the ECM and are subsequently retained. The retained lipoproteins undergo modifications such as oxidization, aggregation, or lipolysis. The oxidized lipoproteins trigger a response from endothelial cells (ECs) resulting in the production of pro-inflammatory molecules that recruit monocytes and lymphocytes to the arterial wall. In this milieu, monocytes are subsequently differentiated into macrophages and can engorge oxidized lipoproteins leading to foam cell formation [34]. Over time, foam cell death occurs resulting in formation of a necrotic core, which is evident in late stages of atherosclerosis. Macrophages can also release various cytokines and growth factors which promote smooth muscle cell (SMC) migration and proliferation. The SMCs undergo phenotypic switching from a quiescent to a proliferative state leading to migration from the medial layer to the intima. In the intima, SMCs synthesize ECM proteins, resulting in the formation of a fibrous cap over the plaque [35]. The plaque may become vulnerable over time through events including a reduction in SMCs proliferation and production of ECM proteins, secretion of proteases from macrophages, and by the calcification of advanced lesions. The weakening of the fibrous cap predisposes the lesion to plaque rupture or erosion leading to acute thrombosis and vascular occlusion. This, in turn, causes a myocardial infarction, unstable angina, or stroke due to reduced

blood flow [34]. High-density lipoprotein (HDL) has been shown to be protective against atherosclerosis through removal of excess cholesterol from peripheral tissues or by protecting lipoproteins from oxidation [34].

1.1.1.2 Proposed proatherogenic/prothrombotic properties of Lp(a)

The accumulation of Lp(a) at the site of atherosclerotic lesions can have many consequences for the process of lesion development. Lp(a) can be retained within the subendothelium to a greater extent than LDL as the apo(a) component of Lp(a) can contribute to binding to arterial wall components as mentioned above. Lp(a) can be readily oxidized in atherosclerotic lesions and engulfed by macrophages to form foam cells [36]. The apo(a) component of Lp(a) contains unique functions that promote atherogenesis that are not related to plasminogen or LDL homology, such as the ability to bind to oxidized phospholipids.

Pro-inflammatory oxidized phospholipids have been shown to preferentially bind to Lp(a) compared to LDL [37]. It has been speculated that Lp(a) deposition at sites of lesions results in the targeting of pro-inflammatory oxidized phospholipid species to developing plaques. The site of oxidized phospholipid addition to apo(a) has been previously shown to occur in the KV domain potentially through covalent attachment to a lysine within this domain [38]. However, a later study determined that oxidized phospholipids are present upon removal of the KV domain of apo(a) [39]. Removal of the strong LBS in KIV_{10} of apo(a) abolishes the ability to bind to oxidized phospholipids [39]. The exact location and mechanism of addition for the oxidized phospholipid covalent attachment to apo(a) remains elusive. Interestingly, the KV domain of apo(a) can also stimulate macrophages to produce and secrete the pro-inflammatory cytokine

interleukin (IL)-8 [38]. The importance of oxidized phospholipids present on apo(a) in mediating this process is unknown. In addition, the oxidized phospholipid moiety on apo(a) can induce apoptosis in ER-stressed macrophages through a CD36-TLR2-dependent mechanism which may contribute to plaque necrosis [40]. Overall, these findings illustrate that oxidized phospholipids associated with Lp(a) may contribute to the process of atherosclerosis.

Lp(a) can also contribute to endothelial dysfunction through multiple mechanisms. Endothelial dysfunction, in which the normal endothelium exhibits increased permeability as well as pro-coagulative and pro-inflammatory properties, is implicated in the early stages of atherosclerosis [41]. It has been demonstrated that Lp(a) can contribute to endothelial dysfunction through its ability to elicit cytoskeletal rearrangement in human umbilical vein endothelial cells (HUVECs) [42]. The rearrangement of the cytoskeleton results in the loss of cell-cell contact and subsequent increase in EC permeability [43]. This process may, in turn, result in increased LDL and Lp(a) deposition in the arterial wall. Furthermore, apo(a) can elicit independent pro-inflammatory effects through stimulation of the nuclear translocation of β-catenin, resulting in increased prostaglandin synthesis, and stimulating the expression of pro-inflammatory mediators such as cell adhesion molecules [44, 45]. Lp(a) can also impair vasodilation through reducing nitric oxide (NO) availability by inhibiting eNOS expression [46].

Angiogenesis, which is the process of development of new blood vessels, is also a key contributor to the development of atherosclerosis. An important regulator of this process is the ability of various enzymes, such as matrix metalloproteinases (MMPs) and

plasmin, to degrade the ECM in order for remodeling to occur. The role of Lp(a) in angiogenesis remains controversial with a number of conflicting reports. For example, Lp(a)/apo(a) was able to stimulate EC growth and migration through decreasing the total and active transforming growth factor-β (TGF-β) levels secreted by these cells [47]. The ability of apo(a) to mediate this effect was shown to be dependent on the presence of the LBS in KIV_{10} of apo(a). In this study, the activation of the ECs was dependent on the integrin $\alpha_V\beta_3$ [46]. Conversely, Lp(a) can inhibit *in vitro* tube formation through inhibition of plasmin formation by apo(a) KV [48]. Further work is needed to explore the potential distinct mechanism of how Lp(a) elicits its affects on EC migration/proliferation and tube formation *in vivo*.

As noted above, SMC migration and proliferation is also a key step in the development of atherosclerosis. Lp(a) has been found to be colocalized with macrophages and SMCs in patients with coronary atheroma [49]. Human apo(a) transgenic rabbits were found to promote SMC proliferation and dedifferentiation [50]. This study determined that the accumulation of SMCs in advanced atherosclerotic lesions was often associated with Lp(a) deposition [50]. Further, it has been demonstrated that apo(a)/Lp(a) can stimulate SMC migration and proliferation through the apo(a) KIV_9 domain [51]. The KIV_9 domain inhibits the activation of TGF-β resulting in increased SMC migration and proliferation [51]. The ability of apo(a) to inhibit this process was dependent on the presence of plasminogen, suggesting that apo(a) may inhibit pericellular plasmin formation.

The ability of apo(a) to inhibit plasminogen activation is directly correlated with prothrombotic/antifibrinolytic properties of Lp(a). A number of studies have shown that

apo(a)/Lp(a) can inhibit fibrinolysis and contribute to thrombus formation [52-56]. Apo(a) shares a high degree of structural similarity to plasminogen and therefore it is possible that apo(a) may bind to plasminogen receptors and result in a decrease in plasmin formation. More detail on the mechanism of how apo(a)/Lp(a) inhibits fibrinolysis and potentially pericellular plasminogen activation can be found in Section 1.2.3. It has been shown that the apo(a) component of Lp(a) can interact with the plasminogen receptor $\alpha_M\beta_2$ resulting in the recruitment of inflammatory cells to atherosclerotic plaques [57]. Further work will be needed to identify the role of other cell surface plasminogen receptors in mediating apo(a)/Lp(a) pathogenicity.

Mechanistically, there is some evidence to suggest that small apo(a) (<22 KIV domains) isoform sizes may in fact confer increased CHD risk (reviewed in ref. 1). Small apo(a) species have been shown to be associated more readily with small, dense LDL particles which are more atherogenic than larger, less dense LDL species [58]. There is also an inverse relationship between apo(a) size and fibrin binding affinity [53]. The increased affinity of smaller apo(a) isoforms to bind to fibrin may result in an increased risk for thrombotic events. Furthermore, an inverse relationship exists between small apo(a) isoforms and oxidized phospholipid binding which may contribute to the atherogenicity of these species [59].

Taken together, there is the potential for Lp(a)/apo(a) to contribute to the development and progression of atherosclerosis through multiple mechanisms that affect vascular cell phenotype. Lp(a)/apo(a) may also be implicated in purely thrombotic processes as demonstrated by its ability to inhibit fibrinolysis.

1.1.2 Lp(a) pathophysiology: evidence from clinical studies

Retrospective case-control studies consistently have shown that elevated levels of plasma Lp(a) are a risk factor for CHD [60-63]. However, these studies were criticized since they cannot be interpreted to prove a causal role for Lp(a) in CHD, rather than simply a marker for the process. Stronger evidence in this regard has come from prospective studies [reviewed in ref. 1, 2]. A large meta-analysis encompassing 36 prospective studies and over 125,000 patients demonstrated that elevated plasma Lp(a) is an independent risk factor for CHD [60]. This study indicated that elevated Lp(a) concentrations are continuously associated with CHD risk and that risk is primarily associated with vascular outcomes [60]. This is in contrast to the previously held notion for the risk threshold of above 30 mg/dL for Lp(a) levels [3].

Genetic studies have shown that elevated plasma Lp(a) levels are a causal risk factor for CHD [62, 63]. For example, a Mendelian randomization study design has been utilized to definitively demonstrate causality [63]. A Mendelian randomization study is a method used to measure the random variation in genes from parents to offspring and to estimate a casual effect from observational data generated by random assignment [63]. The Mendelian randomization study by Kamstrup and coworkers [63] demonstrated that small *LPA* allele size is strongly associated with both elevated Lp(a) levels and increased risk for myocardial infarction (MI), thereby demonstrating that elevated Lp(a) levels are a causal risk factor for CHD.

Genome-wide linkage and association (GWA) studies further emphasized that the *LPA* gene is a major determinant for Lp(a) plasma levels [64, 65]. The association between gene variants and CHD in these GWA studies suggest that genetic variation in

LPA is the strongest predictor of CHD [66]. The major determinant of elevated Lp(a) levels is the number of KIV_2 repeats encoded by *LPA*, and single nucleotide polymorphisms (SNPs) present in *LPA*. Clarke and colleagues [62] identified two SNPs that are strongly associated with both an increased risk for CHD as well as Lp(a) levels [62]. The SNPs identified were rs3798220, corresponding to an Ile to Met substitution at position 4399 in the protease domain of apo(a), and rs10455872, which is intronic [62]. The I4399M mutation was found to be strongly associated with elevated oxidized phospholipids [67] and individuals with this mutation showed greater benefit from aspirin treatment for the prevention of CHD [68]. The relationship of the SNPs identified with an increase in Lp(a) concentration as well as risk for CHD also support a causal role for elevated Lp(a) levels and CHD risk.

It is clear that small Lp(a) isoform sizes (<22 KIV domains) confer risk for CHD. However, the relationship between apo(a) isoform size and risk for CHD independent of Lp(a) concentration remains controversial. Conflicting results have been reported on the risk associated with small *LPA* allele size independent of elevated plasma Lp(a) concentration. A meta-analysis of over 58,000 participants suggested that small isoforms of apo(a) (<22 KIV_2 repeats) are associated with an increased risk for vascular disease, although an independent role for isoform size could not be concluded [26]. In fact, the only clinical study that demonstrated an independent role for Lp(a) isoform size and risk was the Bruneck study [reviewed in ref. 1]. Conversely, the two recent genetic studies described above revealed that the risk association for CHD in these specific studies was abolished following adjustment of *LPA* allele size to plasma concentration [62, 63].

Taken together, it is still unclear if small *LPA* allele size confers risk for atherogenic or thrombogenic processes independent of plasma Lp(a) levels.

Interestingly, elevated Lp(a) levels have been shown to confer increased risk for thrombotic events such as venous thromboembolism (VTE) [69, 70] and ischemic stroke [26-28]. A more recent prospective population study revealed that Lp(a) was associated with coronary artery disease (CAD) but not with ischemic stroke [29]. However, an increased risk for reoccurrence of ischemic stroke was determined in patients with elevated Lp(a) levels [28]. Elevated levels of plasma Lp(a) (>30 mg/dL) have been shown to be a risk factor for VTE in children, with a small population size of 186 patients in this study [70]. Also, Lp(a) levels >300 mg/dL were found to be an independent risk factor for VTE in adults, again in a small study of 603 patients [69]. However, larger studies revealed that elevated plasma Lp(a) levels are not a risk factor for primarily thrombotic phenotypes including VTE [71-73]. Most recently, analysis of 21,483 women indicated no risk of VTE in patients with elevated plasma Lp(a) levels, except for those with Lp(a) concentrations in the top 1% [73].

The role of elevated plasma Lp(a) levels has also been directly correlated with coronary artery calcification (CAC), which is an atherosclerotic process, and aortic valve stenosis (AVS), which is not an atherosclerotic process. However, the role for elevated Lp(a) levels as a risk factor for CAC remains controversial, with evidence both for [74, 75] and against [76, 77] such a relationship. Three recent genetic studies revealed a clear causal association between elevated plasma Lp(a) levels and AVS [78-80]. Interestingly, a threefold increased risk of AVS was not only associated with small *LPA* allele size, but also with SNPs rs10455872 and rs3798220 [79]. The mechanism by which elevated

Lp(a) levels contribute to CAC and development of AVS is poorly understood and remains a subject for future investigation.

Overall, current evidence suggests that elevated Lp(a) levels are an independent causal risk factor for CHD, with a more disputed role in purely thrombotic processes *in vivo*. The exact pathophysiological contribution of Lp(a) to the development and progression of CHD remains unclear, with recent evidence also suggesting a role for elevated plasma Lp(a) levels in the development of CAC and AVS. The role of apo(a) isoform size as an independent contributor to risk for atherothrombotic disorders also remains to be conclusively determined.

1.1.3 Lp(a) assembly

It is generally accepted that Lp(a) assembly occurs via a two-step mechanism [81, 82]. First, an initial non-covalent interaction occurs between the weak LBS present in apo(a) and N-terminal lysine residues in apoB-100 [81, 82]. Using point mutations to systematically abolish the weak LBS in apo(a), it was identified that the weak LBS in KIV_7 and KIV_8 were essential in mediating the non-covalent step in Lp(a) assembly [81]. The non-covalent interaction is required to facilitate the disulfide linkage between the free cysteine in KIV_9 of apo(a) and apoB-100 through correct alignment of the two molecules [81].

These preponderance of evidence suggests that Lp(a) assembly occurs extracellularly [7, 83-84], rather than within the cell [85-88]. For example, a previous study indicated that extracellular Lp(a) formation occurred within the medium of hepatic cells (HepG2) and that apo(a) was not associated covalently with apoB-100 within the cell [7]. Furthermore, extracellular Lp(a) assembly was demonstrated in primary cultures

of baboon hepatocytes, perhaps on the cell surface, with no indication of intracellular Lp(a) formation [83]. The latter study also identified the importance of LBS in assembly as the process was inhibited by the addition of the lysine analogue ε-aminocaproic acid (ε-ACA) to the cells [83].

Spontaneous disulfide bond formation occurs extremely slowly *in vitro* between purified apo(a) and LDL despite the high affinity of the non-covalent interaction between apo(a) and apoB-100 [84]. However in the presence of a putative Lp(a) oxidase secreted by hepatic cells, the ability of apo(a) to form a disulfide bond with apoB-100 is greatly accelerated [84]. Interestingly, apo(a) adopts a closed conformation that becomes more open upon binding to lysine analogs which allows for more rapid disulfide bond formation between apo(a) and apoB-100 [84]. It is possible that non-covalent interaction between apo(a) and apoB-100 occurs within the cell followed by extracellular disulfide bond formation either on hepatic cell surface, in space of Disse, or within the plasma.

The prevailing view of extracellular assembly of Lp(a) particles is difficult to reconcile with Lp(a) kinetic data generated in humans using stable isotope labeling. An early kinetic study reported that production rates for apo(a) and apoB-100 in Lp(a) are identical, and that Lp(a) is formed from newly-synthesized apo(a) and apoB-100, rather than the association of newly-synthesized apo(a) with circulating LDL as suggested by the extracellular Lp(a) assembly model [85]. A recent kinetic study in human subjects reported that the production rate of apoB-100 associated with Lp(a) particles is very different from apoB-100 associated with LDL or very-low-density lipoprotein (VLDL), but similar to the production rate of apo(a) itself [86]. This has been taken as evidence for intracellular assembly of Lp(a), which has not been directly observed except for a study

using a very small and non-physiological isoform of apo(a) [87]. However, the kinetic studies cannot exclude the possibility that Lp(a) forms extracellularly from a special pool of newly-synthesized apoB-100 containing lipoprotein. It is also possible that a pool of apoB-100 containing lipoprotein assembles non-covalently with apo(a) intracellularly and that the covalent complex forms upon secretion. Further studies are required to re-visit the site of Lp(a) assembly in the context of *in vivo* human kinetic data.

1.1.4 Lp(a) catabolism

The mechanism of Lp(a) catabolism remains unclear despite many studies directed at studying how Lp(a) is cleared from plasma. Various reports have suggested that the liver plays a crucial role in catabolising Lp(a) with a more minor role for the kidney. A study by Cain [89] and coworkers determined that Lp(a) was found to be cleared predominantly by the liver (34.6%) with only a small fraction detected within the kidney (1.3%). These findings coincide with a previous report indicating that Lp(a) clearance in a rat model was 28.5% by the liver and 0.5% by the kidney indicating a clear role for hepatic rather than renal cells in the clearance of Lp(a) [90]. However, patients with renal disease have been shown to have elevated Lp(a) plasma levels [91, 92]. Frischmann and coworkers [93] used an *in vivo* turnover study in hemodialysis patients and determined that the increase in plasma Lp(a) levels observed is due to the rate of Lp(a) catabolism rather than production. Overall, these results indicate that the kidney plays an indirect role in Lp(a) clearance from plasma with potentially a more significant role in patients with kidney disease.

Of note, plasma Lp(a) levels have been shown to be dictated primarily by the rate of production rather than catabolism [94]. A study by Rader and coworkers determined that the catabolic rate of different isoforms of apo(a) were similar *in vivo*, whereas the production rate was significantly greater for the smaller isoform compared to the larger isoform of apo(a) [94]. Understanding how Lp(a) is cleared from the plasma presents a useful strategy to potentially lower elevated plasma Lp(a) levels.

1.1.4.1 Potential receptors for Lp(a) catabolism

A variety of receptors have been proposed to be involved in Lp(a) clearance likely through the apo(a) or the apoB-100 components of Lp(a) [89, 95-109]. Initially, clearance of Lp(a) was assumed to occur in a similar manner to that of LDL through the LDL-receptor (LDLR). This is because Lp(a) contains an LDL-like moiety and therefore it was assumed that Lp(a) can be catabolised through binding of the apoB-100 component of Lp(a) to the LDLR similar to what is observed with LDL. However, statin therapy, which increases the number of hepatic LDLRs, generally has little to no effect on the clearance of Lp(a), but has a significant effect on LDL removal from plasma [reviewed in ref. 9]. Therefore, presence of the apo(a) component in Lp(a) may interfere with the binding of apoB-100 to the LDLR. This hypothesis was validated using a mouse model in which excess apo(a) was able to compete for clearance of radiolabeled Lp(a); this was interpreted to suggest that Lp(a) clearance is predominantly driven by the apo(a) rather than the apoB-100 component of Lp(a) [89]. However, a study by Tam and coworkers [95] determined that apo(a) complexed with LDL can bind to HepG2 cells via high and low affinity receptors. The results demonstrated that the apo(a) component was responsible for binding to low affinity plasminogen receptors on these cells, whereas the

apoB-100 component of Lp(a) appeared to mediate binding to the higher affinity LDLR [95]. Further studies need to be conducted to determine the physiological route of Lp(a) catabolism in plasma.

In addition to the LDLR [95-103] and plasminogen receptors [53, 95], a number of other possible receptors have been identified which may play a role in Lp(a) catabolism. These include the low-density lipoprotein-related protein (LRP-1) [101], VLDL-receptor (VLDLR) [107], megalin/glycoprotein 330 (gp330) [108], and scavenger receptor class B type I (SR-BI) [109]. As noted above, the liver is the predominant organ which clears plasma levels of Lp(a) with only a minor role for the kidney [89, 90]. Only the LDLR, LRP-1, and SR-BI are prominently expressed in the liver which suggests a relatively minor role for the VLDLR and megalin/gp330 receptor in Lp(a) clearance *in vivo* [89, 109].

1.1.4.2 The role for the LDLR in Lp(a) catabolism

The main physiological role for the LDLR is to bind and internalize LDL from the circulating plasma into hepatic cells. The LDLR is traditionally divided into five domains; (1) the ligand-binding domain (composed of seven cysteine-rich domains, R1-R7); (2) two epidermal growth factor (EGF) subunits (EGF-A and EGF–B), a six-bladed β-propeller, and an EGF-C domain; (3) an O-linked sugar domain; (4) a transmembrane domain; (5) and a cytoplasmic tail (Fig 1.4) [110]. The ligand binding domain can bind to various lipoproteins through interactions with apoB-100 and apolipoprotein E (apoE) [111]. The apoB-100 component of LDL binds to the LDLR and the complex undergoes clathrin-mediated internalization [112]. In the acidic environment of sorting endosomes, the LDLR adopts a closed conformation which weakens the LDLR:LDL interaction

[112]. Subsequently, LDL is released from the LDLR and is transported to lysosomes for degradation, whereas the LDLR is recycled back to the cell membrane [112].

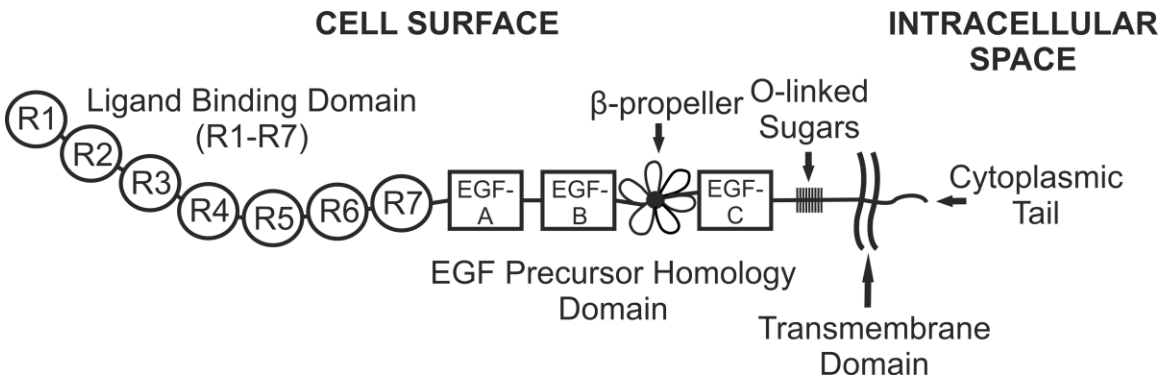

Figure 1.4: ***Representation of the domains of the LDLR*****.** The LDLR is divided into five domains; (1) the ligand-binding repeats (composed of seven cysteine-rich domains, R1-R7); (2) an EGF precursor homology domain (composed of an EGF-A and EGF–B, a six-bladed β-propeller, and an EGF-C domain); (3) an O-linked sugar domain; (4) a transmembrane domain; and (5) a cytoplasmic tail. The ligand binding domain can bind to apoB-100 and apoE. The EGF-A domain is required for binding to PCSK9. Image adapted from reference 110.

The role of the LDLR in Lp(a) clearance remains controversial with many studies indicating a role [95-103, 113] while other studies reporting no role for this receptor [89,104-106, 114]. Certainly, the relative ineffectiveness of statin therapy on Lp(a) levels supports a minimal role, if any, for the LDLR in mediating Lp(a) clearance. Statins inhibit the rate limiting step in cholesterol biosynthesis, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reducatase, which is highly regulated by the supply of cholesterol [115]. This effectively reduces the production of cholesterol within the cell and results in the upregulation of LDLR hepatic levels with a concomitant lowering of plasma LDL levels.

Several reports have shown a role for the LDLR in Lp(a) catabolism using both *in vitro* [95-98, 101] and *in vivo* methods [103]. Cell culture models using hepatic HepG2 cells or fibroblasts illustrate that the LDLR can bind to Lp(a) and mediate Lp(a) uptake [95, 103]. Lp(a) and LDL can also compete with each other for binding to fibroblasts [98, 103]. Familial hypercholesterolemia (FH) fibroblast cells with a defective LDLR were also shown to bind significantly less Lp(a) compared to wild-type fibroblasts [98, 102]. Another study using fibroblasts indicated, in a more indirect manner, that larger isoforms of Lp(a) bound to LRP-1 whereas smaller isoforms of Lp(a) bound to the LDLR in these cells [101]. These results may suggest that larger isoforms of apo(a) interfere to a greater extent with the ability of the apoB-100 component of Lp(a) to bind to the LDLR. The most significant finding illustrating the importance of the LDLR was conducted *in vivo* by Hofmann and coworkers using a mouse model [103]. In this study, overexpression of the LDLR in mice significantly increased the clearance of injected purified Lp(a) compared to wild-type mice [103].

In contrast, several studies have reported that there is no role for the LDLR in Lp(a) clearance [89, 104, 114]. For example, Lp(a) clearance was not significantly reduced in LDLR$^{-/-}$ mice compared to control mice whereas a significant decrease in LDL clearance was observed in the knockout mice [89]. In another study, radiolabeled Lp(a) was injected into FH homozygous human subjects with no signifcant difference in Lp(a) clearance in the FH patients compared to control subjects [104]. These *in vivo* models suggested that the apo(a) rather than the apoB-100 component of Lp(a) is responsible for its clearance from plasma in an LDLR-independent manner.

1.1.4.3 Familial hypercholesterolemia and Lp(a) levels

FH is an autosomal dominant disorder in which mutations in the LDLR cause elevated plasma LDL levels and premature CHD [116]. FH can also arise from mutations in the genes encoding the ligand apoB-100 or proprotein convertase subtilisin/kexin type 9 (PCSK9) [116, 117]. Mutations within apoB-100 may result in the inability of LDL to be cleared from the plasma whereas gain-of-function (GOF) mutations of PCSK9 result in an increased ability to target the LDLR for degradation (see Section 1.1.4.5.1). Autosomal recessive hypercholesterolemia (ARH) is phenotypically similar to FH but is generally less severe [118, 119]. ARH is caused by a mutation in the gene encoding the putative adaptor protein, ARH, which is important for recruiting the LDLR:LDL complex into clathrin-coated pits [118, 119].

There is contradictory evidence as for the role of the LDLR in Lp(a) catabolism from studies looking at endogenous levels of Lp(a) in FH subjects. Individuals that are heterozygous or homozygous for a mutation in the LDLR resulted in either an increase [99, 100, 113] or had no effect [105, 106] on Lp(a) plasma levels. However, the most

recent analysis of 1,960 patients with FH and 957 non-FH relatives revealed that Lp(a) plasma levels were significantly higher in FH patients compared to their unaffected relatives [99]. Overall, the aforementioned results underscore the contradictory evidence that exists for the role of the LDLR in Lp(a) catabolism.

1.1.4.4 Other potential Lp(a) catabolic receptors

LRP-1 [101] and SR-BI [109] are both expressed in the liver and have been shown to potentially regulate Lp(a) clearance. Previous studies found that Lp(a) can bind to LRP-1 [101, 108] but this was later disproven in subsequent studies [95, 114]. Recently, SR-BI was identified as a receptor for Lp(a) catabolism *in vivo* [109]. SR-BI was previously shown to bind to a variety of lipoproteins including HDL and LDL [120]. Yang and coworkers [109] determined that Lp(a) clearance was significantly greater in SR-BI transgenic mice and delayed in SR-BI knockout mice compared to control mice. However, 65% of the injected Lp(a) was cleared over 24 hours in the SR-BI knockout mice suggesting a role for other receptors in the clearance of Lp(a) [109].

VLDLR [107], megalin/gp330 receptor [101, 108], and the family of plasminogen receptors [53, 95] have all been shown to bind to Lp(a). However, VLDLR and megalin/gp330 are not expressed in the liver which is the predominant organ involved in plasma Lp(a) catabolism [89, 90]. As such, it is possible that the VLDLR and megalin/gp330 receptor may play a minor role in Lp(a) clearance in a non-hepatic setting, such as the kidney. Various studies have shown that apo(a) can compete for plasminogen for binding to cell surfaces [53, 95]. However, there is no evidence to suggest that plasminogen receptors play a role in Lp(a) uptake and degradation in hepatic cells.

Taken together, the mechanism of Lp(a) catabolism remains elusive. This may reflect the fact that the apo(a) gene is only found in humans and Old World monkeys. This complicates the interpretation of data generated for Lp(a) catabolism using traditional animal models [121]. Future experiments clearly need to focus on defining the mechanism for Lp(a) clearance from human plasma. This could pave the way for the development of therapeutic strategies that can be utilized to lower plasma Lp(a) levels.

1.1.4.5 Therapeutic strategies to lower plasma Lp(a) levels

There is currently no accepted pharmaceutical agent that can specifically reduce elevated levels of Lp(a). Many of the treatments that do exist lower both LDL and Lp(a) levels. It is important to identify a specific therapeutic agent that can lower elevated plasma Lp(a) at either the rate of production, assembly, or catabolism in order to address the benefits of Lp(a)-lowering on CHD risk in prospective studies.

Lp(a) levels are relatively resistant to traditional lipid-lowering strategies such as statin therapy, or diet and exercise [reviewed in ref. 9]. The most effective method of lowering Lp(a) is through lipid apheresis procedures [122]. Specific Lp(a)-apheresis for 18 months in 30 subjects with normal LDL levels and elevated Lp(a) levels resulted in a clear regression in CHD and a significant decrease of 73% in Lp(a) levels [123]. However, Lp(a)-apheresis is costly, invasive and not a practical long term therapy option. Several strategies have or are currently being explored to determine their effectiveness in lowering plasma Lp(a) levels; these include statins, niacin, cholesteryl ester transfer protein (CETP) inhibitors, antisense oligonucleotide (ASO) approaches, and the use of monoclonal antibodies blocking PCSK9.

Niacin treatment is perhaps the most popular choice for lowering Lp(a) levels. Currently, the European Atherosclerosis Society recommends niacin treatment for individuals with Lp(a) levels >50 mg/dL [3]. Niacin can lower Lp(a) levels by approximately 20-30% [124, 125] but is also associated with severe side effects including flushing and hepatotoxicity [126]. Two clinical trials have recently been terminated as niacin treatment did not reduce the overall risk of cardiovascular disease [125, 127]. Specifically, Lp(a) levels were reduced in both trials, but this decrease could not be correlated with improved outcomes.

CETP facilitates the transport of cholesteryl esters (CE) and triglycerides between lipoproteins and is important for lipoprotein metabolism in the circulation [128]. Inhibition of CETP function results in an increase in HDL-cholesterol and a decrease in LDL-cholesterol levels [129]. Recently, the CETP inhibitor, anacetrapib, has shown success in raising HDL levels as well as lowering plasma Lp(a) levels by approximately 40% [130]. The mechanism by which anacetrapib can lower Lp(a) is unknown but may potentially involve a reduction in the efficiency of Lp(a) assembly due to alterations in LDL lipid composition.

Promising results have also been found with the use of ASO therapy to lower Lp(a) levels [131, 132]. ASOs are short, single-stranded, synthetic oligonucleotides that are designed to specifically bind to and degrade a target messenger RNA (mRNA) [133]. Mipomersen, an ASO targeting apoB, resulted in the reduction in apoB synthesis and therefore the production of all apoB containing lipoproteins including VLDL, LDL, and Lp(a) [131]. Phase 2 clinical trials with mipomersen for 13 weeks revealed a significant reduction in Lp(a) levels [131]. Conversely, an ASO targeting the KIV_2 domain of apo(a)

resulted in a significant decrease in Lp(a) levels in transgenic mice expressing human apo(a)/apoB-100 [132]. The use of ASO targeting either apoB or apo(a) may prove to be an attractive method for lowering of Lp(a) levels.

1.1.4.5.1 PCSK9 Based Therapies

PCSK9 was first discovered in 2003 and is the ninth and most recently identified member of the proprotein convertase family. Since its discovery, it has been under intense investigation in both basic research and clinical studies as it can drastically affect LDL cholesterol levels [134]. PCSK9 is highly expressed in the liver and to a lesser extent in the intestine and kidney [135]. The 692 amino acid glycoprotein contains a pro-domain, followed by a catalytic domain, hinge region, and a C-terminal cysteine-histidine-rich domain (CHRD) (Fig. 1.5) [136, 137]. The CHRD can be further classified into three modules, M1-M3 [138]. Once the signal peptide is cleaved from PCSK9, the zymogen proPCSK9 remains in the ER until intramolecular cleavage occurs [139]. The proPCSK9 cleaves itself at Gln^{152}-Ser^{153} to generate the mature enzyme and prosegment [139, 140]. Interestingly, the prosegment remains non-covalently bound to the mature PCSK9 which renders it enzymatically inactive since the prosegment occupies the active site cleft [136]. The cleaved prosegment domain remains associated with PCSK9 and the complex is transported to the Golgi complex and subsequently secreted from the cell.

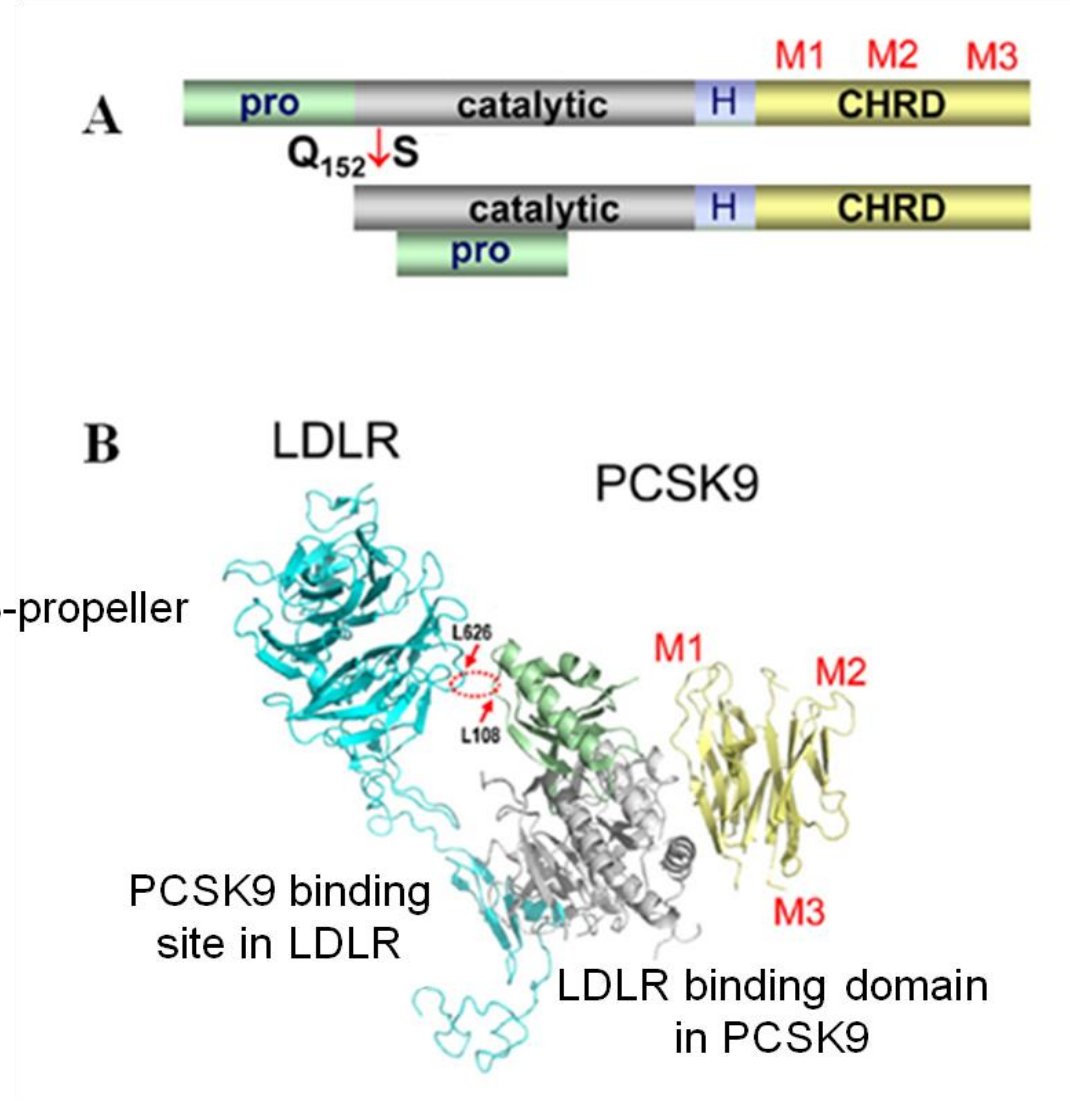

Figure 1.5: ***Representation of the domains of PCSK9 and its binding to the LDLR*****. A**, PCSK9 zymogen contains a prosegment (pro), catalytic domain, hinge region (H), and a C-terminal Cys-His-rich domain (CHRD). The zymogen is autocatalytically cleaved within the ER at $Q_{152}\downarrow S_{153}$. The cleaved prosegment (pro) remains attached to the mature PCSK9 which renders it catalytically inactive. **B**, Crystal structure of the ectodomain of the LDLR bound to PCSK9. The catalytic domain of PCSK9 (LDLR binding domain in PCSK9) interacts with the EGF-A domain of the LDLR (PCSK9 binding site in LDLR). A predicted weak hydrophobic interaction between Leu_{108} of the prosegment domain of PCSK9 and Leu_{626} of the β-propeller domain of the LDLR is also illustrated. The CHRD domain can be classified into three modules, M1-M3. Image modified from reference 137.

The main physiological target of PCSK9 in lipoprotein homeostasis is the LDLR. In addition to the LDLR, PCSK9 is also an endogenous regulator of other lipoprotein receptors including the VLDLR [141], LRP-1 [142], and apolipoprotein E receptor 2 (ApoER2) [141]. The catalytic domain of PCSK9 can bind to the EGF-A domain of the LDLR and target the complex for degradation (Fig. 1.5 and Fig. 1.6) [110, 143]. As mentioned above, when the LDLR binds to its ligand LDL, the complex is internalized through clathrin-coated vesicles, with the internalized LDL directed to lysosomes and the LDLR recycled back to the cell surface [144]. However, in the acidic environment of the endosome, the PCSK9:LDLR interaction becomes stronger and, through an as yet identified mechanism, the complex is targeted to lysosomes for degradation [145, 146]. The M2 module of PCSK9 has been shown to be required for PCSK9 to target the LDLR for degradation through the extracellular pathway [138]. Removal of the M2 module from PCSK9 still allows for extracellular binding to occur between PCSK9 and the LDLR, but the complex is unable to be targeted for lysosomal degradation. This suggests that PCSK9 interacts with a putative protein/receptor which allows for targeting of the complex for degradation. PCSK9 can also target the LDLR for degradation through an intracellular pathway without requiring secretion [147]. In this intracellular pathway, PCSK9 can interact with the LDLR in the *trans* Golgi network (TGN) and the complex is shuttled to lysosomes through clathrin-mediated endocytosis [148]. Interestingly, LDL acts as a regulator of PCSK9 activity by binding to the N-terminal region of PCSK9 [149]. PCSK9 cannot function in targeting the LDLR for degradation when LDL is bound to PCSK9. [149].

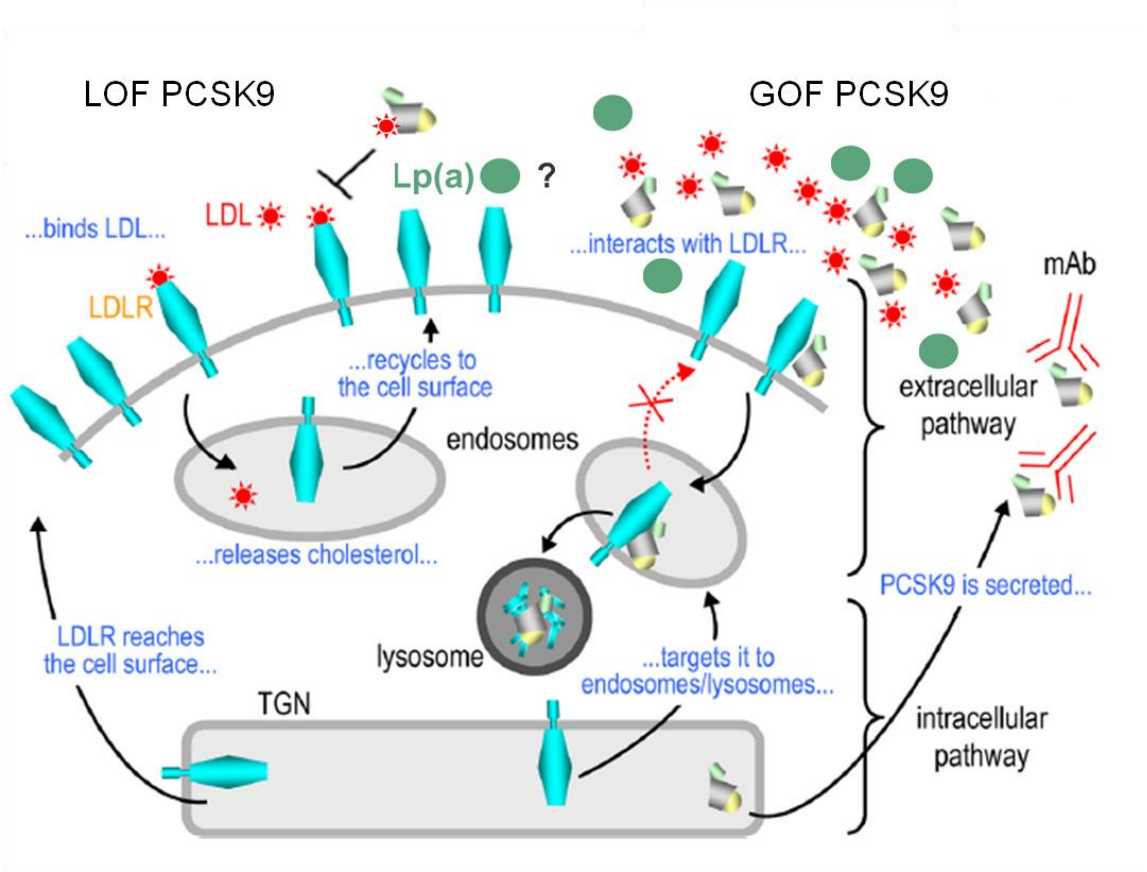

Figure 1.6: ***Representation of the LDLR degradation pathway mediated by PCSK9.*** At low concentrations of PCSK9, or with a LOF PCSK9 mutant, LDLR levels are high, which allows for LDL to bind to the LDLR and enter into clathrin-coated vesicles. The LDL is released from the LDLR in the low acidic environment of the endosomes and is targeted for degradation whereas the LDLR is recycled. Conversely, at high concentrations of PCSK9, or with a gain-of-function (GOF) PCSK9 mutant, PCSK9 binds to the LDLR and targets this complex for degradation in either an extracellular (cell surface) or intracellular pathway (*trans* Golgi network; TGN). This results in low levels of hepatic LDLR number and concomitant high plasma LDL levels. PCSK9 monoclonal antibodies (mAb) can bind to PCSK9 (extracellular pathway) and prevent its binding to the LDLR, thereby increasing hepatic LDLR number. Also, LDL can bind to PCSK9 and prevent PCSK9 from targeting the LDLR for degradation. The potential role of PCSK9 and the LDLR in Lp(a) clearance remains undetermined. Image modified from reference 137.

Natural GOF and loss of function (LOF) mutations of PCSK9 occur within the population that result in drastically elevated or lowered plasma LDL levels, respectively. The highly active PCSK9 GOF mutation D374Y results in a 10- to 25- fold increased binding affinity to the LDLR which leads to decreased hepatic LDLR levels and increased circulating LDL levels [136, 137]. Conversely, a patient heterozygous for the LOF Q152H mutation (corresponding to the site at which proPCSK9 is autocatalytically cleaved in the ER), exhibited up to 80% and 50% decreases in circulating PCSK9 and LDL levels respectively [150]. This is in keeping with reports that PCSK9-knockout mouse models exhibit an ~80% decrease in LDL levels [151]. On this basis, several pharmaceutical companies are developing strategies to decrease the ability of PCSK9 to bind to and target the LDLR for degradation.

Several preclinical and clinical trials are underway to test various approaches to inhibit PCSK9 [reviewed in ref. 137]. The most promising approach has been with the use of monoclonal antibodies against PCSK9 which block PCSK9 from binding to the LDLR [152-156]. Recent clinical trials with a PCSK9 monoclonal antibody resulted in decreases of approximately 30% and 70% for Lp(a) and LDL levels respectively [152-156]. The mechanism by which PCSK9 can regulate Lp(a) catabolism with respect to the involvement of the LDLR or other potential Lp(a) receptors that can be targeted by PCSK9 is unknown. The decrease of only ~30% for Lp(a) compared to the 70% decrease observed for LDL may suggest that Lp(a) may have more than one receptor responsible for its clearance, and that the LDLR is not the preferred route for catabolism of Lp(a).

Targeting PCSK9 may present a useful method to lower elevated plasma Lp(a) levels. However, the mechanism of how PCSK9 can effect Lp(a) catabolism is unknown.

Identifiying the receptor(s) responsible for the PCSK9-mediated ability to lower Lp(a) levels is essential in advancing the therapeutic options to lower Lp(a) catabolism.

1.2 PLASMINOGEN: STRUCTURE, FUNCTION, AND ACTIVATION

Plasminogen is the zymogen form of the serine protease plasmin and is predominantly synthesised and secreted from the liver [157]. Plasminogen is composed of a tail domain, followed by five kringle domains (KI-KV) and a serine protease domain (Fig. 1.2). The kringle domains are important for mediating the interaction of plasminogen with substrates such as fibrin, cell surfaces, and plasminogen activators. Generally, these binding interactions are dependent on the lysine binding properties of plasminogen [reviewed in ref. 157].

Native, circulating plasminogen is 791 amino acids with an N-terminal glutamic acid (Glu-plasminogen). The zymogen Glu-plasminogen can be activated to form Glu-plasmin through proteolytic cleavage by plasminogen activators at Arg^{561}-Val^{562} [47]. Following cleavage, the N-terminal heavy chain remains covalently linked to the carboxy-terminal light chain of plasminogen via two disulfide bonds. Cleavage of Arg^{561}-Val^{562} results in the catalytically active serine protease with the active site, His^{603}, Asp^{646}, Ser^{741}, in the light chain of plasmin [158] (Fig. 1.7). Plasmin plays a key role in many physiological processes including fibrinolysis, cell migration and proliferation, angiogenesis, inflammation, wound healing, and tumor cell invasion and metastasis [157-164].

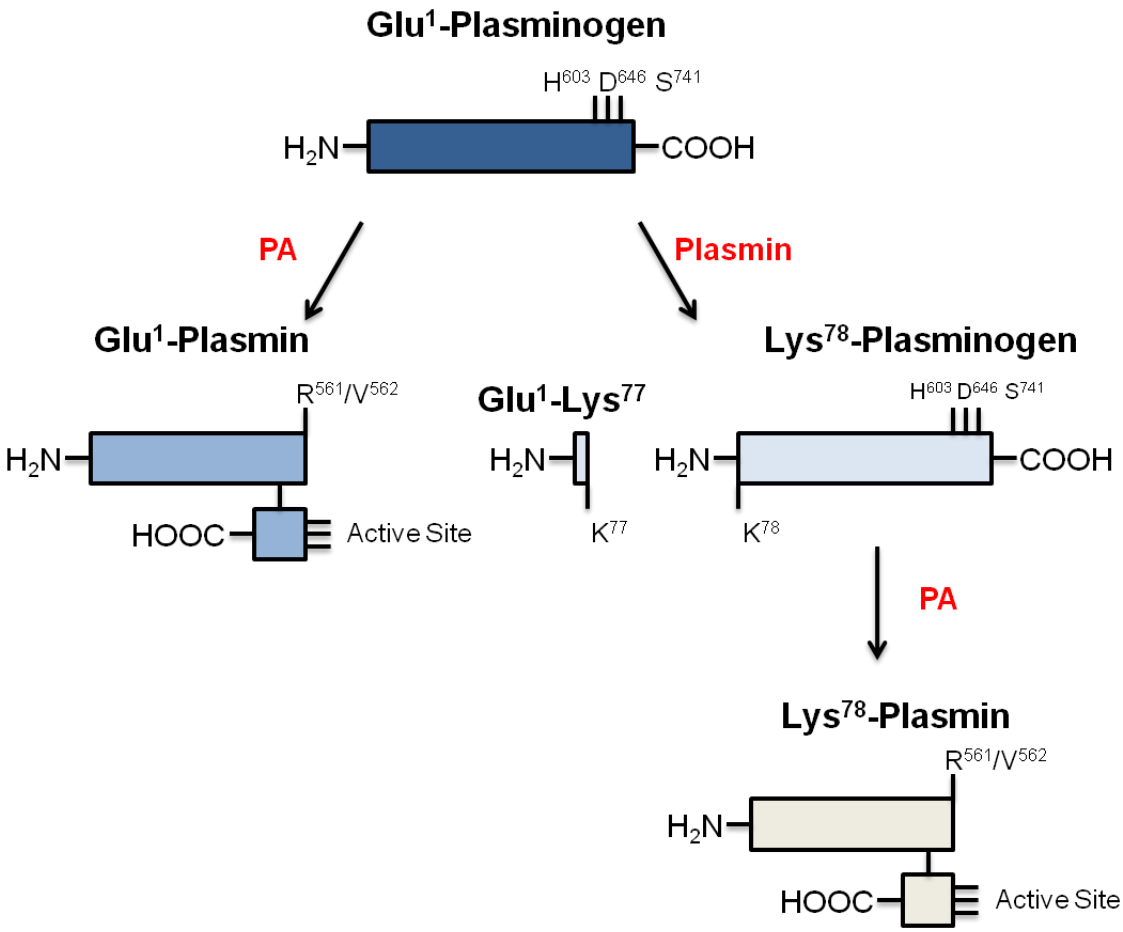

Figure 1.7: ***Schematic representation of plasminogen activation and Glu- to Lys-plasminogen conversion.*** Glu[1]-plasminogen is the native circulating form of plasminogen and is in a closed conformation. The closed conformation does not allow for rapid activation by plasminogen activators (PA) tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Once bound to fibrin or cell surfaces, Glu[1]-plasminogen adopts a more open, extended conformation which allows it to be more readily activated through cleavage at Arg[561]-Val[562] by PA to form Glu-plasmin. Plasmin can cleave Glu[1]-plasminogen at Lys[77]-Lys[78], releasing the N-terminal peptide fragment to form Lys[78]-plasminogen (contains an N-terminal Lys). Lys[78]-plasminogen is in a more extended, open conformation which allows for accelerated activation by PA to form Lys[78]-plasmin. Conversion of Glu[1]- to Lys[78]-plasminogen greatly accelerates the overall rate of plasminogen activation on the cell or fibrin surfaces or in solution. The active site (His[603], Asp[646], Ser[741]) of Glu- or Lys-plasmin is necessary for its catalytic activity.

The two main plasminogen activators are urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). tPA is predominantly expressed in ECs and SMCs [165] whereas uPA is predominantly expressed in the kidney, lung, and tumor cell lines and does not bind to fibrin [166, 167]. The role of tPA and uPA varies within the vasculature, and is primarily dictated by the cells that express these activators. As such, tPA plays a key role in fibrinolysis or EC/SMC migration. Conversely, uPA plays a minor role in fibrinolysis and a more primary role in ECM degradation in the process of tumor cell invasion and metastasis [46-53].

1.2.1 Positive feedback mechanisms in the process of plasminogen activation

The rate of plasminogen activation can be greatly accelerated through a variety of positive feedback mechanisms. The circulating form of plasminogen, Glu-plasminogen, adopts a closed, spiral conformation that limits its accessibility to be activated by plasminogen activators [168, 169]. In order for accelerated plasminogen activation to occur, Glu-plasminogen must bind to the cell or fibrin surfaces. Once bound to these cell surfaces, Glu-plasminogen adopts a more open conformation and is therefore more accessible to plasminogen activators [168, 169]. As a result, an initial, small amount of Glu-plasminogen can be activated to form Glu-plasmin. Glu-plasmin remains bound to the cell and fibrin surfaces in order to prevent rapid inhibition through its natural inhibitor α_2-antiplasmin [170]. The initial production of Glu-plasmin formed can then directly contribute to the positive feedback mechanism of plasminogen activation on these surfaces.

Plasmin can proteolytically cleave Glu-plasminogen at the carboxyl side of Lys^{62}, Arg^{68}, or Lys^{77} to form a product termed “Lys-plasminogen” [170-172]. The most

common site of cleavage by plasmin is at Lys^{77}-Lys^{78} resulting in an N-terminal Lys residue. Lys-plasminogen adopts a more extended/opened conformation that significantly lowers its half-life in plasma [173]. However, the benefit of converting Glu- to Lys-plasminogen is that the more open conformation allows for greater accessibility to plasminogen activators in order to accelerate the rate of plasmin formation [174, 175]. In solution, the rate of tPA-mediated plasminogen activation increases 11-fold for Lys-plasminogen compared to Glu-plasminogen, illustrating the importance of the open conformation in accelerating plasminogen activation [174-176].

Cell surface receptors (see Section 1.2.2) play a crucial role in mediating plasminogen activation and the positive feedback mechanisms assocaiated with plasmin formation. Binding of Glu-plasminogen to cell surface receptors allows for a more open conformation that promotes cleavage by plasmin to form Lys-plasminogen [177]. Indeed, it has been reported that conversion of Glu- to Lys-plasminogen is necessary for optimal stimulation of plasminogen activation on HUVECs and monocytoid cells [178, 179]. Gong and coworkers illustrated the importance of Glu- to Lys-plasminogen conversion on HUVECs [178]. In this study, a mutant form of plasminogen was used, [D646E] Glu-plasminogen, that does not contain plasmin activity but can still be cleaved by plasminogen activators [178]. This mutant was far less effective in the enhancement of plasminogen activation on HUVEC surfaces compared to wild-type plasminogen [178]. This underscores the importance of plasmin activity in cleaving Glu- to Lys-plasminogen for the enhancement of plasminogen activation.

Furthermore, the initial production of Glu-plasmin can further contribute to the positive feedback mechanism of plasminogen activation through cleavage of both tPA

and uPA. Plasmin cleaves single-chain tPA (sc-tPA) at Arg^{275}-Ile^{276} resulting in a two-chain form with slightly better activity than sc-tPA [180]. Conversely, uPA is secreted as single-chain uPA (sc-uPA) with minimal activity until it is cleaved by plasmin at Lys^{158}-Ile^{159} resulting in a two-chained form (tc-uPA) [181]. Converting sc-uPA to the tc-uPA form lowers the K_M for plasminogen from 20-50 μM to approximately 0.2 μM [182].

Overall, the positive feedback mechanism of plasminogen activation is crucial for the enhanced production of plasmin. If in fact apo(a)/Lp(a) can inhibit pericellular plasminogen activation it would be imperative to determine if it is through inhibition of the positive feedback mechanism involving Glu- to Lys-plasminogen conversion.

1.2.2 Plasminogen receptors

Plasminogen receptors play a pivotal role in localizing plasmin generation on the cell surfaces. These plasminogen binding proteins play an essential role in both physiological and pathological processes as discussed above (see Section 1.2). Plasminogen receptors are broadly distributed on many cell types including monocytes [183], macrophages [184], ECs [185], fibroblasts [186], platelets [187], and carcinoma cells [188]. Plasminogen binding occurs with low affinity to these cellular receptors (K_D ~ 1 μM) but with high capacity, ranging from ~10^4 sites on platelets [189] to >10^7 sites on ECs [190].

Many cell-surface plasminogen receptors contain a carboxyl-terminal lysine or an exposed lysine residue that can mediate their interaction with plasminogen through LBS within plasminogen kringles [reviewed in ref. 157]. The LBS present in KI, KIV, and KV have all been implicated in the ability of plasminogen to bind to fibrin [191], whereas interaction with ECs is primarily through the LBS present in KIV and KV of

plasminogen [192]. As such, plasminogen binding to cells can be blocked by the addition of the lysine analogue ε-ACA [189]. Furthermore, treatment of monocytoid cells with carboxypeptidase B (CpB), which removes carboxyl-terminal Arg or Lys residues from cell surfaces, resulted in a decrease in plasminogen binding and plasminogen activation by 60% and 95% respectively [193]. Plasminogen receptors are also important due to their ability to protect plasmin from its natural inhibitor α_2-antiplasmin. Plasmin generated in solution is rapidly inhibited by α_2-antiplasmin, whereas cell surface-bound plasmin is protected from its inhibitor.

Plasminogen receptors identified to date can be categorized into three subtypes: i) those that contain an exposed carboxyl-terminal lysine; ii) those that undergo post-translational cleavage to expose a carboxyl-terminal lysine; or iii) those that do not contain a carboxyl-terminal lysine. The majority of plasminogen receptors identified do contain a carboxyl-terminal lysine residue [157]. Some of the more well-known plasminogen receptors which contain a carboxyl-terminal lysine include enolase-1 (present on monocytoid cells), S100A10 (present on ECs), TATA-binding protein-interacting protein (present on monocytoid cells) and Plg-R_{KT} (present on catecholaminergic cells) [157, 194]. An example of a receptor of the second subtype is annexin II, which is present on ECs [157]. Annexin II undergoes limited proteolysis on the cell surface that results in the exposure of a carboxyl-terminal lysine [195]. The modified annexin II can bind to plasminogen but is unable to protect plasmin from α_2-antiplasmin inhibition [195]. However, binding of annexin II with S100A10 to form a heteroterameric complex result in an increased ability of this complex to enhance plasminogen activation as S100A10 contains a carboxyl-terminal lysine [157]. The

annexin II S100A10 heterotetramer can also protect both plasmin and tPA from their natural inhibitors α_2-antiplasmin and plasminogen activator inhibitor (PAI1) respectively [196]. Plasminogen receptors in the third subtype do not contain a carboxyl-terminal lysine. These include $\alpha_M\beta_2$ (present on phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils) and glycoprotein IIb/IIIa (present on platelets) [157].

Another important receptor for the production of plasmin is the glycosyl phosphatidylinositol (GPI)-anchored uPA receptor (uPAR). The primary ligand of uPAR is uPA, and this receptor plays a key regulatory role in mediating plasminogen activation and ECM degradation (Fig. 1.8) [197]. Binding of the zymogen form of uPA (sc-uPA) to uPAR promotes its cleavage by plasmin to form tc-uPA [198]. Conversion of sc-uPA to tc-uPA results in a more catalytically active enzyme that can accelerate plasminogen activation to a greater extent. Plasmin can also activate MMPs that can then degrade ECM components [199]. Activated tc-uPA can also cleave uPAR resulting in a soluble form of the receptor and a membrane-associated fragment [200]. The membrane-associated fragment cannot bind to uPA, thereby inactivating the function of uPAR in uPA-mediated plasminogen activation [200]. uPAR can also interact with the ECM protein vitronectin which is a ligand for integrins such as $\alpha_V\beta_3$ [201, 202]. uPAR does not contain a transmembrane domain and therefore interacts with integrins, such as $\alpha_V\beta_3$, to transmit intracellular signals (reviewed in ref. 203). Interestingly, apo(a)/Lp(a) has been shown to stimulate cell growth and migration in ECs through the $\alpha_V\beta_3$ integrin [46] (see Section 1.1.1.2).

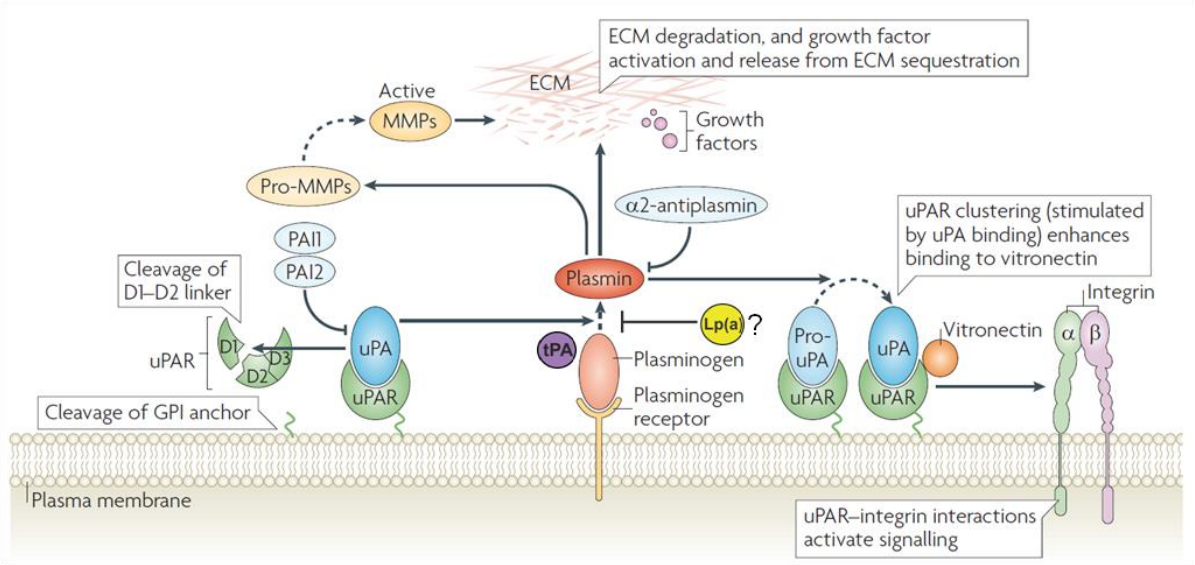

Figure 1.8: ***The role of the uPAR in the plasminogen activation system.*** The uPAR functions by binding to pro-uPA or active uPA to accelerate plasminogen activation. The inactive pro-uPA binds to uPAR followed by plasmin cleavage of pro-uPA to its active form. Active uPA then has increased catalytic activity to cleave the zymogen plasminogen to form plasmin. tPA can also cleave plasminogen to its active form plasmin. Binding of both uPA to uPAR as well as plasminogen to its cognate receptors greatly accelerates the rate of plasmin formation. The plasmin generated can then activate pro-matrix metalloproteinases (pro-MMPs) to their active form. Active MMPs and plasmin can degrade the ECM to promote cell proliferation and the release of growth factors sequestered from the ECM. uPA can also cleave uPAR at the D1-D2 domain and renders the membrane bound uPAR unable to bind to uPA thereby inactivating its function. uPAR can also interact with vitronectin, a component of the ECM, which can in turn interact with integrins. Binding of uPAR/uPA to integrins, through vitronectin, allows for intracellular signal transduction pathways to be activated. Plasmin can be inhibited by its natural inhibitor α_2-antiplasmin whereas plasminogen activator inhibitor (PAI1 or PAI2) can inactivate uPA. The role of Lp(a)/apo(a) in potentially inhibiting pericellular plasminogen activation is unknown. Image modified from reference 203.

1.2.3 The role of Lp(a)/apo(a) in plasminogen activation

The apo(a) component of Lp(a) has been previously shown to stimulate SMC migration and proliferation through inhibition of plasminogen activation [50, 51]; this, in turn, results in decreased activity of TGF-β [51]. This process was shown to be dependent on the ability of the KIV_9 domain of apo(a) to mediate inhibition of plasminogen activation [51]. Apo(a)/Lp(a) can also attenuate fibrinolysis through inhibition of tPA-mediated plasminogen activation [55]. In the latter study, critical roles for the strong LBS in KIV_{10} and the amino terminal of apo(a) were identified as important in apo(a)-mediated inhibition of plasminogen activation on fibrin [55]. Inhibition of plasminogen activation on the fibrin surface occurred because of reduced turnover number of the quaternary complex (cofactor, tPA, plasminogen, and apo(a)) compared to the ternary complex (cofactor, tPA, and plasminogen) [55]. Apo(a) has been shown to inhibit Glu- to Lys-plasminogen conversion, which contributes to the inhibition of fibrinolysis [56]. In the latter study, critical domains in KIV_{5-9} as well as KV were found to be required for maximal inhibition of the Glu- to Lys-plasminogen conversion [56]. As previously noted, conversion of Glu- to Lys-plasminogen is a critical positive feedback mechanism which greatly accelerates the production of plasmin on both fibrin and cell surfaces.

The role of apo(a)/Lp(a) in inhibition of pericellular plasminogen on vascular cell surfaces has not been fully explored. Lp(a) and apo(a) can compete with plasminogen for binding to EC and monocyte surfaces [54, 204]. Lp(a) was reported to inhibit the generation of plasmin on ECs, although the data were not shown and the role of apo(a) in this inhibition was not studied [54]. Further experimentation needs to be conducted to identify the direct role, if any, that apo(a)/Lp(a) has on the process of pericellular

plasminogen activation. The potential ability of Lp(a)/apo(a) to inhibit pericellular plasminogen activation may effect the dissolution of mural fibrin clots as well as cell migration and proliferation, angiogenesis, wound healing, and tumor cell invasion and metastasis.

1.3 RATIONALE AND OBJECTIVES

Elevated levels of Lp(a) are an independent risk factor for CHD. Determining the mechanism of how Lp(a) functions is imperative for developing strategies for the treatment of individuals who have elevated plasma levels. Identification of the receptor(s) responsible for the binding of Lp(a) to cells is key to understanding the route of Lp(a) catabolism, as well as the basis of its pathogenic mechanisms of action.

Many studies have shown that apo(a)/Lp(a) can interfere with the process of fibrinolysis in part through attenuation of the positive feedback mechanism of Glu-plasminogen to Lys-plasminogen conversion. However, the ability of apo(a)/Lp(a) to inhibit pericellular plasminogen activation and Glu- to Lys-plasminogen conversion on vascular cells is unclear at present.

Determination of the receptor(s) responsible for Lp(a) catabolism may provide new therapeutic strategies to lower plasma Lp(a) levels in high risk patients. Studies using anti-PCSK9 monoclonal antibody have shown promise for reducing plasma Lp(a) levels. However, the mechanism by which PCSK9 can regulate Lp(a) levels is unknown, which may limit the utility of this approach.

Our hypothesises is that apo(a)/Lp(a) can inhibit pericellular plasminogen activation through attenuation of Glu- to Lys-plasminogen conversion on vascular cells and that the ability of apo(a)/Lp(a) to inhibit this process will be dependent on the

competition of the apo(a) component of Lp(a) for binding to known plasminogen receptors. Moreover, we hypothesize that Lp(a) can be bound and internalized by the LDLR, and that this forms the basis by which inhibition of PCSK9 lowers plasma Lp(a) levels.

To address these hypotheses, the research objectives of this dissertation are:

1) To identify the role of apo(a)/Lp(a) in the inhibition of pericellular plasminogen activation on vascular cells;
2) To elucidate the mechanism by which apo(a)/Lp(a) inhibits pericellular plasminogen activation on vascular cells;
3) To identify candidate vascular receptor(s) that can bind to apo(a);
4) To identify the mechanism by which PCSK9 can lower Lp(a) levels in hepatic cells.

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CHAPTER 2

Inhibition of plasminogen activation by apolipoprotein(a): role of carboxyl-terminal lysines and identification of inhibitory domains in apolipoprotein(a)

2.1 SUMMARY

Apolipoprotein(a) [apo(a)], the distinguishing protein component of lipoprotein(a), exhibits sequence similarity to plasminogen and can inhibit binding of plasminogen to cell surfaces. Plasmin generated on the surface of vascular cells plays a role in cell migration and proliferation, two of the fibroproliferative inflammatory events that underlie atherosclerosis. The ability of apo(a) to inhibit pericellular plasminogen activation on vascular cells was therefore evaluated. Two isoforms of apo(a), 12K and 17K, were found to significantly decrease tPA-mediated plasminogen activation on HUVECs and THP-1 monocytes and macrophages. Lp(a) purified from human plasma decreased plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages. Removal of kringle V or the strong lysine binding site in kringle IV_{10} completely abolished the inhibitory effect of apo(a). Treatment with carboxypeptidase B (CpB) to assess the roles of carboxyl-terminal lysines in cellular receptors lead in most cases to decreases in plasminogen activation as well as plasminogen and apo(a) binding; inhibition of plasminogen activation by apo(a) was unaffected, however. Our findings directly demonstrate that apo(a) inhibits pericellular plasminogen activation in all three cell types, although binding of apo(a) to cell-surface receptors containing carboxyl-terminal lysines does not appear to play a major role in the inhibition mechanism.

2.2 BACKGROUND

Lipoprotein(a) [Lp(a)] was first discovered in 1963 by Kåre Berg [1] and has since been under intense investigation in both basic research and clinical studies [2]. Lp(a) has a complex structure consisting of an LDL moiety, which contains apoB-100, and the unique glycoprotein apolipoprotein(a) [apo(a)] [3]. The levels of Lp(a) vary over 1000 fold within the human population ranging from less than 1 to greater than 100 mg/dL [4]. Lp(a) possesses both proatherogenic and prothrombotic properties due to the LDL-like moiety and apo(a) component of the lipoprotein respectively [5, 6]. Elevated plasma concentrations of Lp(a) have been identified as an independent risk factor for cardiovascular diseases such as coronary heart disease, ischemic stroke, and peripheral arterial disease [7-11]. Lp(a) has also been associated with purely thrombotic events, such as venous thromboembolism [12]. The molecular cloning of a cDNA encoding apo(a) revealed that it contains remarkable homology to the fibrinolytic zymogen plasminogen [13].

Apo(a) contains repeated copies of a sequence similar to that of plasminogen kringle IV (KIV) followed by sequences similar to the kringle V (KV) and protease domain of plasminogen [13]. The kringle IV-like domain in apo(a) can be further classified into ten subtypes (KIV_1 to KIV_{10}), according to differences in amino acid sequence. The number of KIV_2 repeats determines the isoform size heterogeneity of apo(a) [14, 15] with the number of repeats ranging from 3 to >30 [16]. The protease domain in apo(a), unlike that of plasminogen, does not exhibit plasmin-like activity and is not proteolytically active [17]. Both apo(a) and plasminogen contain lysine binding sites (LBSs) which mediate their respective interactions with various cell surface

receptors or the fibrin surface. Apo(a) KIV types 5 to 8 each contain a weak LBS, with KIV types 7 and 8 forming a noncovalent association with apoB-100 [18-20]. This precedes covalent formation of the Lp(a) particle by facilitating disulfide bond formation between a free cysteine in KIV_9 of apo(a) and the C-terminus of apoB-100 [21, 22]. Apo(a) KIV_{10} contains a strong LBS that resembles the LBS present in plasminogen kringle 4 [23]. Apo(a) may therefore potentially interfere with plasminogen binding to or activation on vascular cells.

Plasminogen is the zymogen form of the serine protease plasmin. The active enzyme plays a key role in the processes of fibrinolysis, cell migration and proliferation, angiogenesis, and inflammation [24-29]. Plasminogen activation is mediated through proteolytic cleavage of Arg^{561}-Val^{562} of plasminogen by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). This process occurs more readily on the cell surface and following conversion of native Glu-plasminogen to Lys-plasminogen [30-32]. Apo(a) has been shown to inhibit plasminogen activation on the fibrin surface through KV and the strong LBS in KIV type 10 [33]. Moreover, apo(a) directly inhibits Glu- to Lys-plasminogen conversion on the fibrin surface with critical roles identified for kringle IV types 5-9 and KV [34]. An apo(a)-mediated decrease in plasmin generation, dependent on apo(a) KIV_9, has also been shown in the context of smooth muscle cells (SMCs) which results in the inhibition of activation of transforming growth factor β (TGF-β) [35]; this leads to the stimulation of SMC migration and proliferation [35-37].

The strong LBS present in plasminogen allows for its binding and subsequent activation to occur on various receptors containing carboxyl-terminal lysines including

annexin A2 S100A10 heterotetramer, enolase-1, and Plg-R_{KT} [38]. For example, treatment of monocytoid cells with carboxypeptidase B (CpB), which removes carboxyl terminal lysines, leads to a decrease of 64% for plasminogen binding and greater than 95% for plasminogen activation [39]. On catecholaminergic cells, binding of plasminogen decreased following CpB treatment by approximately 70% whereas a decrease of 90% was observed for plasminogen activation [40]. In another study. binding of plasminogen to human umbilical vein endothelial cells (HUVECs) was also inhibited by ε-aminocaproic acid (ε-ACA), a lysine analogue, by 70-80% [29].

CpB and active thrombin-activatable fibrinolysis inhibitor (TAFIa) have also been shown to reduce plasminogen activation by 80% on the annexin A2 S100A10 heterotetramer *in vitro* [41]. In a rat primary hepatocyte model, TAFI knockdown increased the amount of plasmin formed on the cells and promoted proliferation, suggesting the ability of TAFIa to decrease plasminogen binding to hepatocytes [42]. Previous studies have suggested that apo(a) may inhibit pericellular plasminogen activation based on the observation that apo(a) decreases plasminogen binding to endothelial cells and monocytes [43]. Moreover, Lp(a) was reported to inhibit the generation of plasmin activity on endothelial cell surface and not in the fluid phase [44]. The effect of apo(a) on plasminogen activation on vascular cells has not otherwise been explored. Most notably, the role of CpB with respect to the effect of apo(a) and plasminogen binding and activation has not been shown for relevant vascular and blood cells such as endothelial cells, monocytes and macrophages.

2.3 METHODS

2.3.1 Construction, expression, and purification of recombinant apo(a)

The construction and expression in human embryonic kidney (HEK293) cells of the r-apo(a) variants utilized in this study (KIV_{5-8}, 12K, 12ΔV, 17K, 17KΔLBS_{10}, and 17KΔV) has been previously described [17, 33, 34, 45-4]. Variants were purified from conditioned medium, harvested from stably-expressing cell lines, using lysine-Sepharose affinity chromatography as previously described [17, 33, 34, 45-48]. Protein concentrations were determined spectrophotometrically [17, 33, 34, 45-48] and assessed for purity by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

2.3.2 Isolation of Lp(a)

Blood was collected from one healthy volunteer homozygous for a 16K apo(a) isoform. Blood was collected into BD Vacutainer blood collection tubes containing sodium polyanethol sulfonate and acid citrate dextrose. Plasma was obtained through centrifugation of whole blood at 3,000 × g for 15 min at 4°C. Phenylmethylsufonyl fluoride (PMSF) was added to a final concentration of 0.1 mM and the plasma was rotated at 4°C for 15 min. The plasma was adjusted to a density of 1.02 g/mL with solid sodium bromide and centrifuged at 45,000 × g for 24 hrs at 6°C. The top fraction was removed and the infranatant was adjusted to a density of 1.21 g/mL with solid sodium bromide. The sample was centrifuged at 45, 000 g for 24 hrs at 6°C and the top layer was isolated and extensively dialyzed against 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.01% NaN_3, and 0.01% EDTA (Buffer A). The sample was then subjected to gel-

filtration chromatography over Sepharose CL-4B (Sigma-Aldrich) (2.5 cm × 80 cm column) using buffer A containing 0.1% (v/v) Tween 20 and 0.1 M proline. Fractions were collected and samples with an absorbance over 0.1 at 280 nm were subjected to western blot analysis using an anti-apo(a) antibody to determine Lp(a) containing fractions. Samples containing Lp(a) were pooled and diluted 3-fold with distilled water and loaded onto a DEAE-Sepharose Fast Flow (Pharmacia) ion exchange column (2.5 × 3 cm column). Lp(a) was eluted with an NaCl concentration gradient (50 to 300 mM NaCl in 20 mM Tris-HCl, pH 7.4). Lp(a) containing fractions, as determined by western blot analysis, were pooled and dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4, 150 mM NaCl). Concentration was determined by bicinchoninic acid assay (Pierce) using BSA as a standard. The integrity of the purified Lp(a) was assessed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining.

2.3.3 Cell culture

HUVECs were obtained from Clonetics and grown in EGM Complete Medium (Clonetics). All HUVEC experiments were conducted using cells at passage five. Human acute monocytic leukemia (THP-1) cells were grown in RPMI complete growth medium 1640 (GIBCO) adjusted to contain 4.5 g/L glucose (Sigma-Aldrich), 10 mM HEPES (Fisher Scientific), 1.0 mM sodium pyruvate (GIBCO), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) and supplemented with 10% FBS (GIBCO) and 1% antibiotic-antimycotic (GIBCO). Cells were maintained at a density of 0.3- 1 × 10^6 cells/ mL and subculturing was performed by centrifuging cells at 100 × g for 5 min and resuspending in the appropriate volume of fresh medium. PMA (Sigma-Aldrich) was added to THP-1

cells at a final concentration of 0.1 μM for 72 hours to differentiate them into macrophage-like cells.

2.3.4 Plasminogen activation assay

HUVECs were grown to confluency in a 96-well microtiter plate (Corning). THP-1 cells were seeded at a density of 150 000 – 200 000 cells per well and differentiated for 72 hours with 0.1 μM PMA. THP-1 monocytes were seeded at a density of 200 000 cells per well. Cells were washed three times with HBS containing 0.4% (w/v) BSA (HBS-BSA) prior to adding reaction mixture. Reaction mixtures contained: 60 nM plasma derived plasminogen (purified as per (49)), 15 nM tPA (Alteplase; Kingston General Hospital Pharmacy), H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (Bachem), and various concentrations of apo(a) (0, 100, 400, or 800 nM) in HBS-BSA. RPMI complete growth medium minus FBS and antibiotic and supplemented with 0.4% (w/v) BSA (RPMI-0.4%BSA) was used instead of HBS-BSA for THP-1 macrophages. Control experiments were conducted in the absence of tPA. Plasmin formation was monitored over 2 hours at 37°C at an excitation wavelength of 370 nm and an emission wavelength of 470 nm and emission cutoff filter of 455 nm using a plate reading fluorescence spectrometer (SpectraMax M5^e, Molecular Devices). The rate of plasminogen activation was taken as the initial slope of the plot of RFU against min^2 from 10 to 40 mins. Rates obtained for each individual experiment performed in triplicate were normalized to the rate of plasminogen activation in the absence of apo(a). Plasminogen activation in the absence of cells was performed with the same reaction mixture described above. Plasminogen activation with Lp(a) was conducted as above with minor changes; 25 nM

of plasma derived plasminogen, instead of 60 nM, was utilized with 75 nM of Lp(a) or apo(a).

2.3.5 Cell binding assay

Assays utilized purified r-apo(a) fluorescently labeled using Alexa Fluor 488 protein labeling kit (Invitrogen) or fluorescein isothiocyanate-labeled human plasminogen (Molecular Innovations). Briefly, cells were washed three times with HBS-BSA and fluorescently labeled plasminogen or r-apo(a), at a concentration of 400 nM, was incubated with cells for one hour at 37°C. Following incubation, cells were washed twice with HBS-BSA and the fluorescence was measured at an excitation wavelength of 494 nm and emission wavelength of 519 nm and cutoff of 515 nm using a plate reading fluorescence spectrometer. For THP-1 macrophages, the buffer used was RPMI-BSA instead of HBS-BSA. Experiments in which two to three acid wash steps (0.2 M acetic acid pH 2.5, 0.5 M NaCl) were included prior to measuring the fluorescence showed that at least 80% of the labeled proteins were surface-bound (as opposed to internalized; Suppl. Fig. 2.1). Binding of apo(a) and plasminogen to the cells appeared to be specific and reversible (Suppl. Fig. 2.2).

2.3.6 Carboxypeptidase treatment

CpB (Calbiochem) treatment of HUVECs and THP-1 monocytes was performed in HBS-BSA and for THP-1 macrophages in RPMI-BSA. Cells were washed three times and incubated with CpB for one hour at 37°C. Cells were then washed three times followed by assay of plasminogen activation or apo(a)/plasminogen binding as described above. The activity of CpB was measured using anisylazoformylarginine (Calbiochem).

2.3.7 Statistical methods

Comparisons between data sets were performed using one-way ANOVA with SPSS version 22 software. Statistical significance was presumed at $p<0.05$.

2.4 RESULTS

2.4.1 Apo(a) directly inhibits plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs

We examined two different isoforms of apo(a) (12K and 17K) and both were found to inhibit pericellular plasminogen activation in THP-1 monocytes (Fig. 2.1A, B). Both isoforms exhibit a similar concentration-dependent effect on inhibition. At 800 nM, 12K and 17K inhibited plasminogen activation by 57% and 46% respectively. At 800 nM, 12K was not significantly more effective at inhibiting activation compared to 17K. Removal of kringle V in both 12K and 17K not only abolished the inhibitory effect of apo(a) but, surprisingly, caused an increase in plasminogen activation (up to 3.6- and 2.4-fold, respectively; Fig. 2.1A, B). Similarly, inactivation of the strong LBS in apo(a) (17KΔLBS_{10} variant) led to a maximal 2.1 fold increase in plasminogen activation (Fig. 2.1A). The weak LBSs of apo(a), present in the KIV_{5-8} variant, do not exhibit a significant inhibitory effect at any of the tested concentrations (Fig. 2.1B). These results indicate that apo(a) can directly inhibit pericellular plasminogen activation in THP-1 monocytes and imply roles for kringle V and the strong LBS in KIV type 10 in mediating this effect.

Comparing plasminogen activation in the presence or absence of THP-1 monocytes revealed a 74% decrease in activation in the absence of cells, and this rate was not affected by 17K, 12K or KIV_{5-8} (Fig. 2.1A, B). However, 17KΔ$LBS_{10,}$ 17KΔV, and 12KΔV shows a significant increase in plasminogen activation with increasing concentration similar to that in the presence of cells, suggesting that the cell-independent

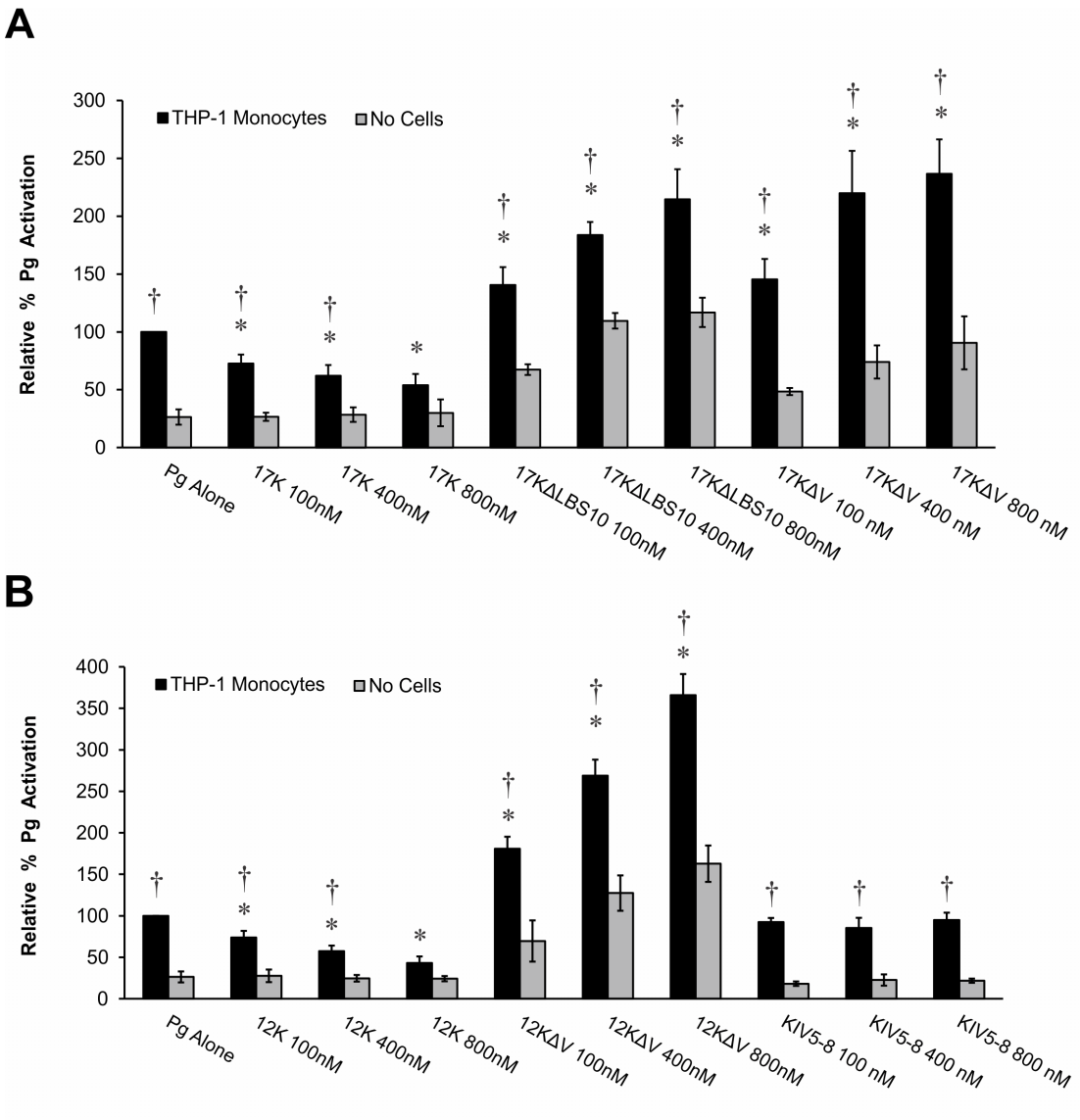
stimulation of plasminogen activation by these variants largely accounts for the increase in plasminogen activation occurring in the presence of cells.

Treatment of THP-1 cells for 72 hours with a phorbol ester results in the differentiation of these cells into macrophage-like cells [50]. Apo(a) inhibits plasminogen activation on THP-1 macrophages in a dose-dependent manner, with a maximal inhibition of 57% and 65% for 12K and 17K, respectively, observed at 800 nM (Fig. 2.1C, D). At 800 nM, 12K and 17K were not significantly different in its ability to inhibit activation. Both the strong LBS in KIV type 10 of 17K (17KΔLBS_{10}) (Fig. 2.1C) and the kringle V domain (12KΔV) (Fig. 2.1D) are required for the inhibitory effect of apo(a); as with the THP-1 monocytes, both of these variants resulted in increased, rather than decreased, plasminogen activation. For the 17KΔLBS_{10} and 12KΔV variant, this increase again can be ascribed to stimulation of cell-independent plasminogen activation (Fig. 2.1C, D). In the absence of THP-1 macrophages, the rate of plasminogen activation was significantly lower (71%) and was unaffected by the presence of 12K and 17K, as was the case for THP-1 monocytes.

Similar experiments were also performed with HUVECs, and with similar results. Apo(a) directly inhibits pericellular plasminogen activation on the surface of HUVECs. A maximal inhibitor effect of 48%, for 12K, and 58%, for 17K was observed at a concentration of 800 nM (Fig. 2.1E, F). At 800 nM, 12K was not significantly more effective at inhibiting activation compared to 17K. As with THP-1 monocytes and macrophages, loss of the strong LBS in KIV_{10} or the loss of KV resulted in a cell-independent increase in plasminogen activation (Fig. 2.1E, F).

Taken together, the effect of apo(a) on cell-dependent plasminogen activation is remarkably similar for all three cell types tested; moreover, the effect of loss or mutation of specific domains of apo(a) is consistent in all three cell types. These results indicate a potential common mechanism through which apo(a) inhibits pericellular plasminogen activation in each cell type.

To demonstrate that activation of plasminogen observed was dependent on the added tPA and plasminogen and not from the cells themselves or contamination of the r-apo(a), experiments were conducted in the absence of tPA or plasminogen. Omission of either of these components virtually eliminated detectable plasminogen activation in the presence of all three cell types, either in the presence or absence of various r-apo(a) variants (Fig. 2.2).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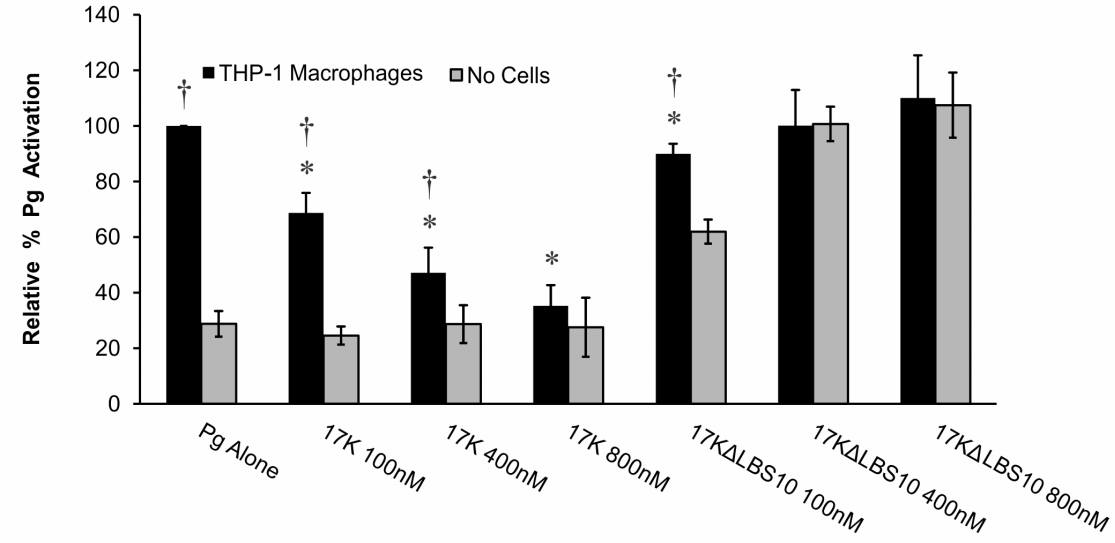
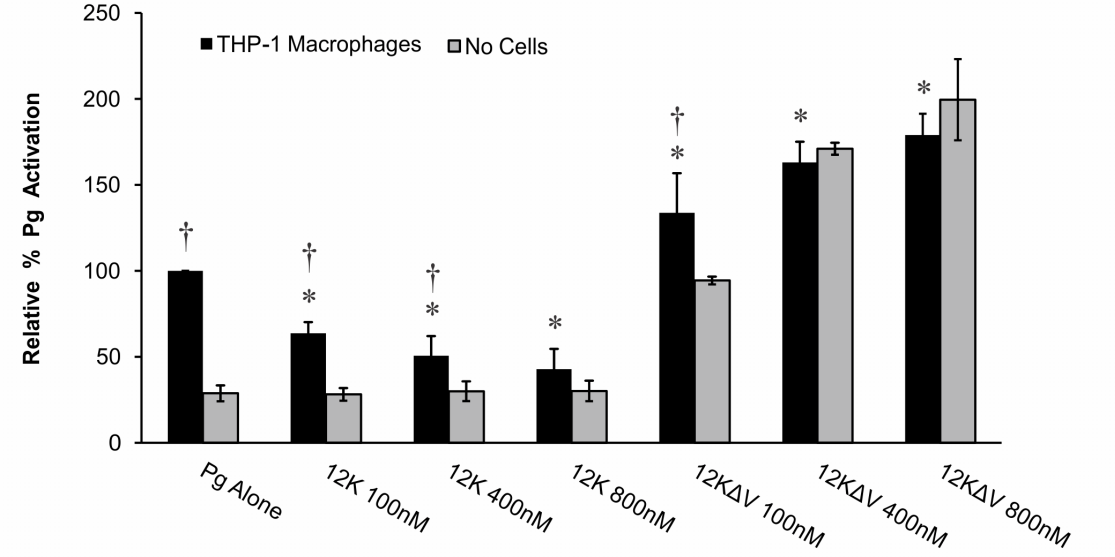
KIV5-8 800 nM

C


Relative % Pg Activation
140
120
100
80
60
40
20
0
THP-1 Macrophages
No Cells
Pg Alone
17K 100nM
17K 400nM
17K 800nM
17KΔLBS10 100nM
17KΔLBS10 400nM
17KΔLBS10 800nM

D


Relative % Pg Activation
250
200
150
100
50
0
THP-1 Macrophages
No Cells
Pg Alone
12K 100nM
12K 400nM
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12KΔV 800nM

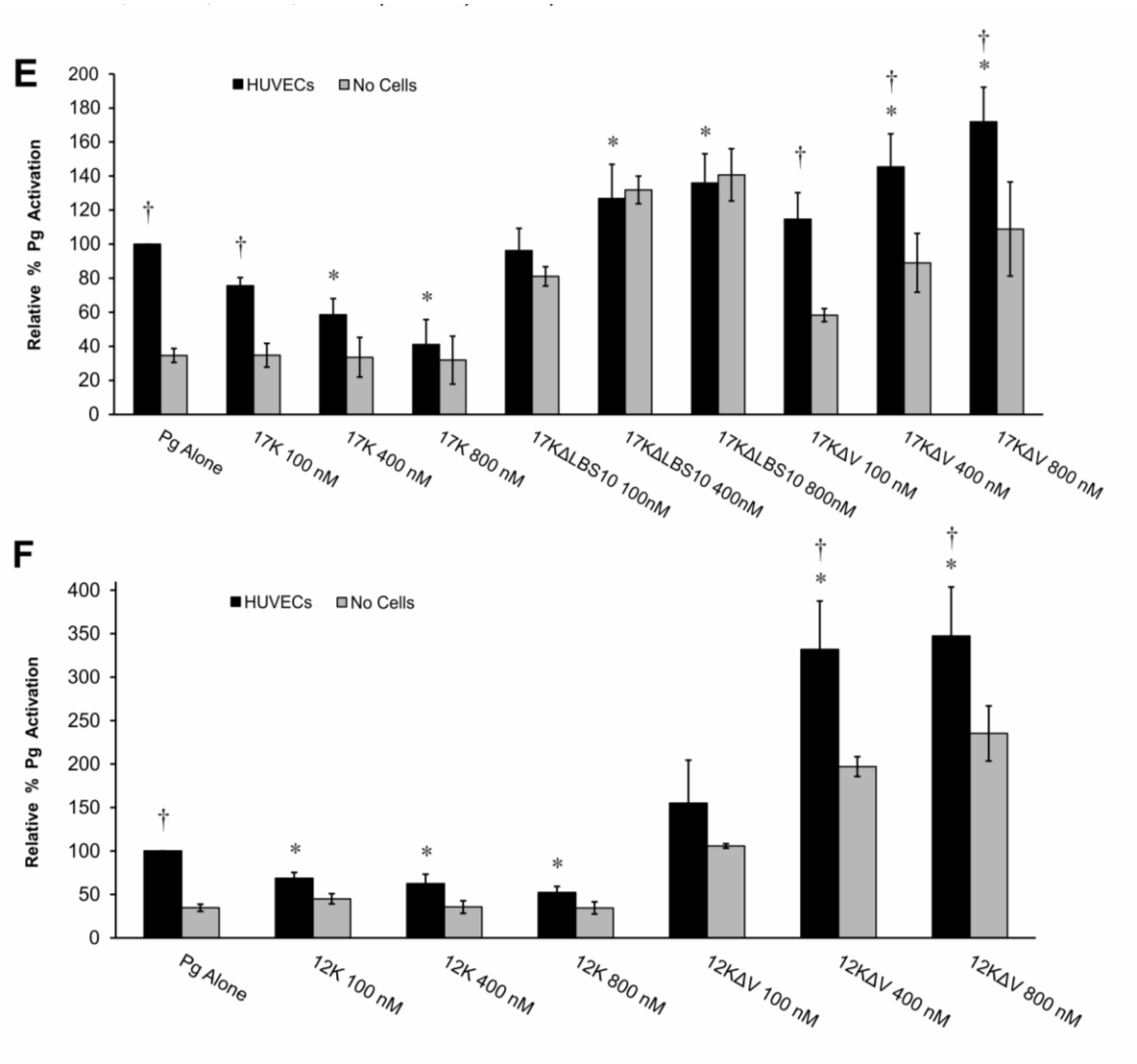


Figure 2.1: ***Effect of apo(a) on pericellular plasminogen activation***. THP-1 monocytes (**A, B**), THP-1 macrophages (**C, D**), or HUVECs (**E, F**) were incubated with various concentrations of r-apo(a) (from 100 nM to 800 nM) as well as plasma-derived plasminogen (60 nM), tPA (15nM), and H-D-Val-Leu-Lys-AMC; control reactions lacked apo(a) or cells. Plasmin generation was monitored over 2 hours at 37°C and the slope of RFU from 10-40 min vs min^2 was determined. The data represent the means ± standard deviations from 3 - 16 independent experiments performed in triplicate for cells and 3 - 6 independent experiments for no cells. Asterisks: $p<0.05$ versus absence of apo(a) on cells; daggers: $p<0.05$ versus absence of cells.

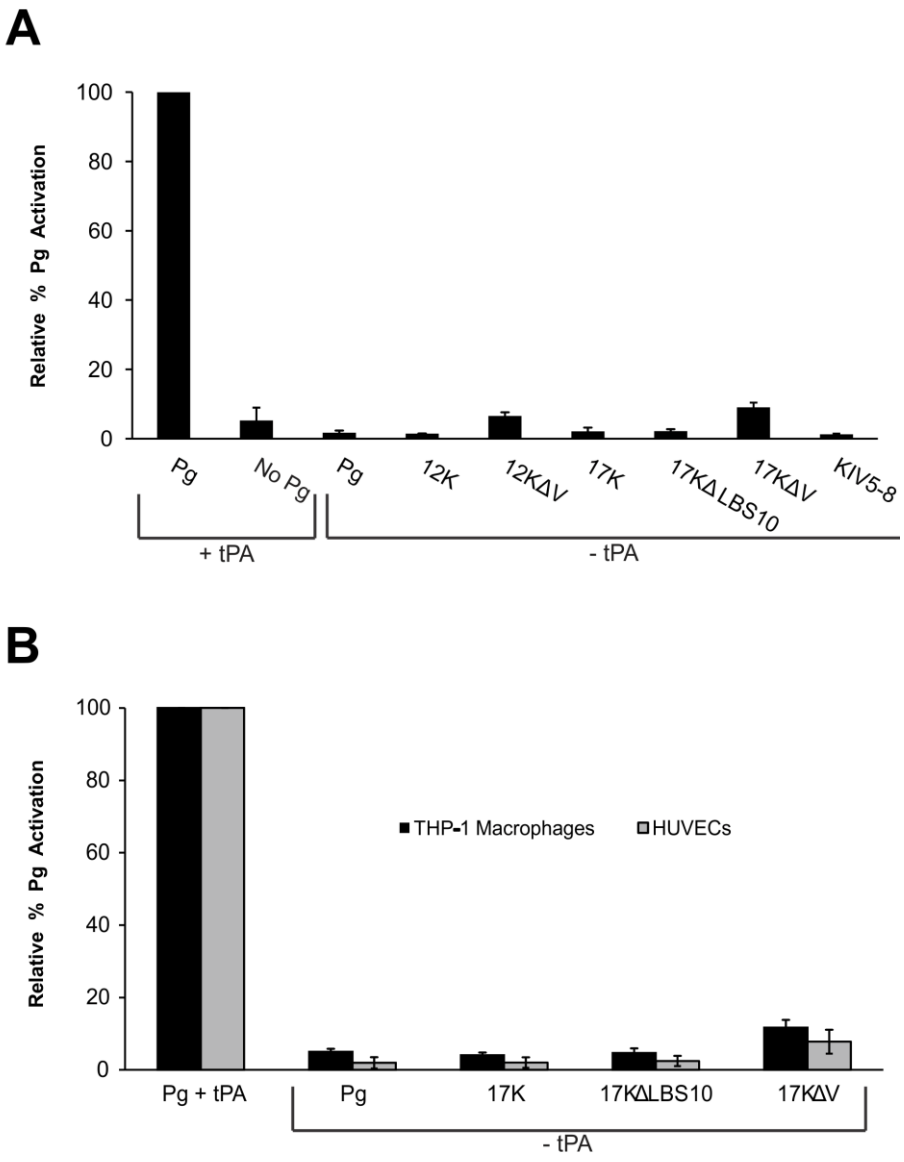


Figure 2.2: ***Plasminogen activation in the absence of tPA on THP-1 monocytes, THP-1 macrophages, and HUVECs***. Plasminogen activation experiments were conducted as described in the legend to Fig. 2.1, but in the absence of plasminogen and tPA **A**: Effect of plasminogen activation in the absence of plasminogen and tPA for plasminogen alone, 12K, 12KΔV, 17K, 17KΔLBS$_{10}$, 17KΔV, and KIV5-8 at 400 nM each on THP-1 monocytes. **B**: Effect of plasminogen activation in the absence of tPA for plasminogen alone, 17K, 17KΔLBS$_{10}$, and 17KΔV at 400 nM each on THP-1 macrophages and HUVECs. Data represent the means ± standard deviations from 3-7 independent experiments performed in triplicate. In all cases, the relative plasminogen activation was significantly different ($p<0.05$) from control (in the presence of plasminogen and tPA combined).

2.4.2 Lp(a) inhibits plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages

We examined the ability of Lp(a), purified from human plasma, to inhibit pericellular plasminogen activation. Experiments were conducted using 25 nM plasminogen and 75 nM of Lp(a) or apo(a). Lp(a) and 17K significantly inhibited plasminogen activation by 15% and 35% on THP-1 monocytes respectively (Fig. 2.3A). However, Lp(a) did not inhibit plasminogen activation on THP-1 macrophages whereas a decrease of 24% was observed with 17K (Fig. 2.3B). Conversely, both Lp(a) and 17K could inhibit plasminogen activation to the same extent, 30% and 28% respectively, on HUVECs (Fig 2.3C). As observed above, plasminogen activation was largely abolished in the absence of tPA or cells, and was not altered with the addition of Lp(a) or 17K (Fig. 2.3)

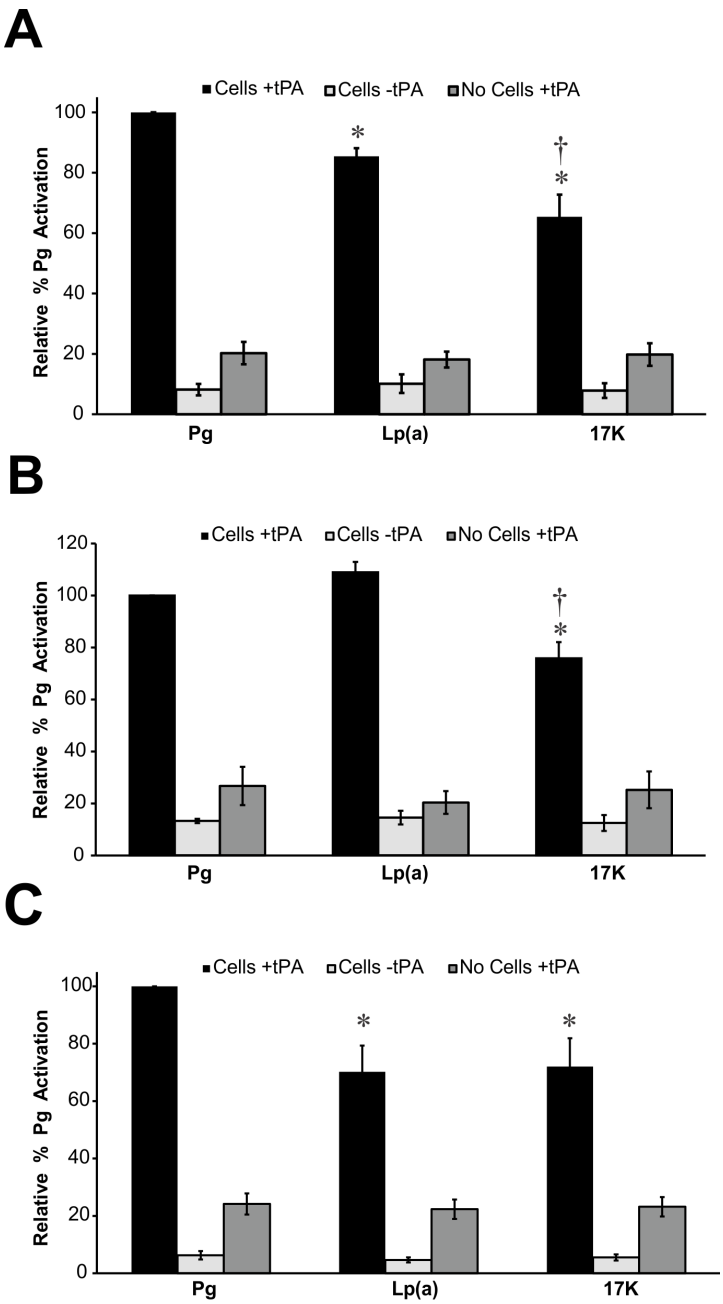


Figure 2.3: ***Effect of Lp(a) on plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs***. Plasminogen activation experiments were performed as described in the legend to Fig. 2.1 with slight modifications: 25 nM of plasminogen and 75 nM of Lp(a)/ apo(a) was utilized. **A**: Effect of Lp(a) on plasminogen activation in the presence or absence of THP-1 monocytes and tPA. **B**: Effect of Lp(a) on plasminogen activation in the presence or absence of THP-1 macrophages and tPA. **C**: Effect of Lp(a) on plasminogen activation in the presence or absence of HUVECs and tPA. The data represent the means ± standard deviations from 3 - 6 independent experiments performed in duplicate. Asterisks: $p<0.05$ versus plasminogen alone in the presence of cells and tPA; daggers: $p<0.05$ versus Lp(a) in the presence of cells and tPA.

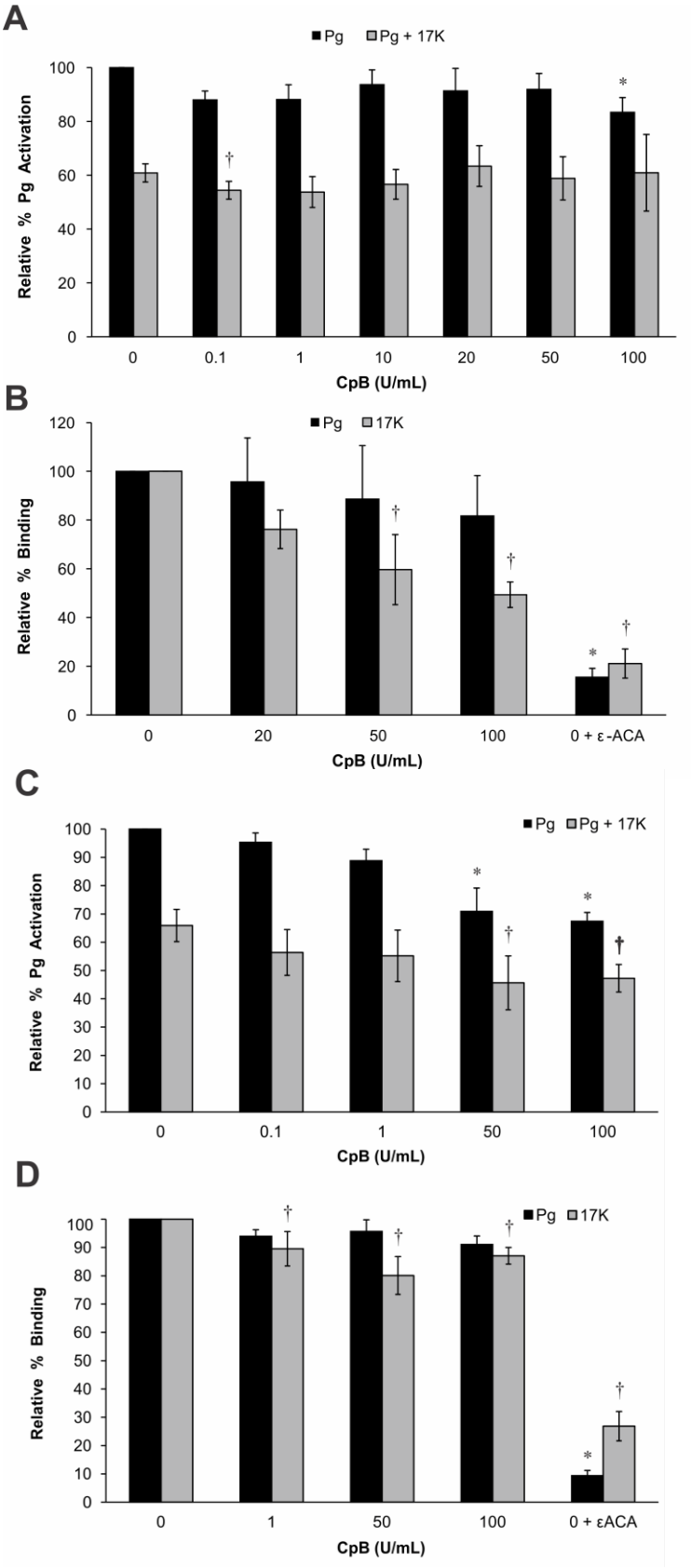
2.4.3 Role of carboxyl terminal lysines in cell-dependent plasminogen activation and its inhibition by apo(a)

It has been previously reported that CpB treatment of U937 monocytoid cells reduced plasminogen binding by 64% and cell-enhanced plasminogen activation by 95% [39]. These results have been interpreted to mean that plasminogen binding to these residues on cell-surface receptors is required for the acceleration of plasminogen activation by cells. Treatment of THP-1 monocytes with various concentrations of CpB ranging from 0.1 to 100 U/mL results in a significant decrease in plasminogen activation (by a modest 16%) at 100 U/ mL (Fig 2.4A, B). Addition of 17K, at 400 nM, at any concentration of CpB results in a decrease in plasminogen activation at all concentrations of CpB; CpB no longer has any significant effect at any concentration in the presence of 17K (Fig. 2.4A). Similar results were observed for THP-1 macrophages (Fig. 2.4C), except that CpB treatment had a greater effect of plasminogen activation, with significant decreases occurring at 50 and 100 U/mL both in the presence and absence of 17K (Fig. 2.4C). Receptors with carboxyl terminal lysines also influence plasminogen activation on HUVECs as a decrease of 24% is observed following treatment with 100 U/mL CpB; a decrease in plasminogen activation owing to CpB was also observed in the presence of 17K although statistical significance was not reached (Fig. 2.4E).

2.4.4 Role of carboxyl terminal lysines in plasminogen and apo(a) binding by cells

To attempt to rationalize the effect of CpB on the plasminogen activation on the surface of cells, and its inhibition by apo(a), we examined the ability of plasminogen and apo(a) to bind to THP-1 monocytes, macrophages, and HUVECs, after CpB treatment or in the presence of the lysine analogue ε-ACA. Plasminogen and apo(a) binding (using

400 nM) decreased by 18% and 51% respectively following CpB treatment (100 U/mL) on THP-1 monocytes (Fig. 2.4B). Binding of plasminogen and apo(a) decreased by 84% and 79% respectively with the addition of 200 mM ε-ACA (Fig. 2.4B). Similarly, in THP-1 macrophages, the addition of ε-ACA reduced plasminogen and apo(a) binding by 91% and 73% respectively (Fig. 2.4D). However, a more modest decrease in plasminogen and apo(a) is observed following CpB treatment at 100 U/mL (9% and 13% respectively) (Fig. 2.4D). Plasminogen binding to HUVECs also decreases following CpB (100 U/mL) treatment and the addition of ε-ACA by 29% and 75% respectively (Fig. 2.4F). Surprisingly, however, apo(a) binding actually increases by 25% and 3% following CpB (100 U/mL) treatment and the addition of ε-ACA respectively (Fig. 2.4F). These results indicate that apo(a) binding is partially mediated through receptors with carboxyl terminal lysines on THP-1 macrophages and monocytes but not on HUVECs. However, since CpB treatment had little or no effect on the ability of apo(a) to inhibit plasminogen activation, these lysine-dependent binding events play a minor role, if any, in inhibition of plasminogen activation by apo(a).


A
Pg
Pg + 17K
Relative % Pg Activation
100
80
60
40
20
0
0
0.1
1
10
20
50
100
CpB (U/mL)
B
Pg
17K
Relative % Binding
120
100
80
60
40
20
0
0
20
50
100
0 + ε-ACA
CpB (U/mL)
C
Pg
Pg + 17K
Relative % Pg Activation
100
90
80
70
60
50
40
30
20
10
0
0
0.1
1
50
100
CpB (U/mL)
D
Pg
17K
Relative % Binding
100
90
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CpB (U/mL)

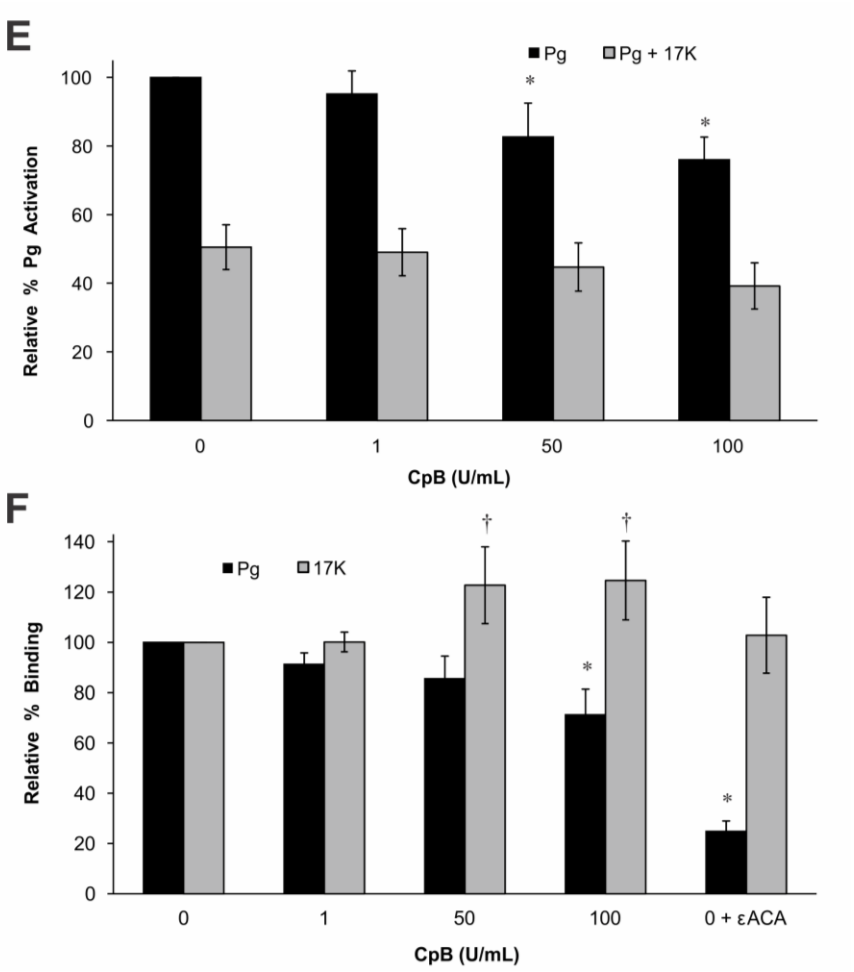

Figure 2.4: ***Effect of removal of carboxyl terminal lysines using CpB on plasminogen activation and plasminogen and apo(a) binding***. **A, C, E**: Effect of CpB treatment in plasminogen activation on THP-1 monocytes (**A**), THP-1 macrophages (**C**), or HUVECs (**E**) in the presence or absence of r-apo(a). Cells were washed three times followed by incubation with CpB, at various concentrations, for one hour at 37°C. Cells were then washed three times following which plasminogen activation was measured as described in the legend to Fig 2.1. Asterisks: $p<0.05$ versus absence of CpB for plasminogen activation in absence of apo(a); daggers: $p<0.05$ versus absence of CpB for plasminogen activation in presence of apo(a). **B, D, F**: Effect on plasminogen and apo(a) binding following CpB treatment of THP-1 monocytes (**B**), THP-1 macrophages (**D**), or HUVECs (**F**). Cells treated with CpB were washed three times and incubated with fluorescently labeled plasminogen or 17K at 400 nM, in the presence or absence of 200 mM ε-ACA for one hour at 37°C. Cells were washed two times and the amount of bound fluorescent proteins was measured using a plate-reading fluorimeter. The data represent the means ± standard deviations from 4 - 11 independent experiments performed in triplicate for plasminogen activation and 3-7 independent experiments for binding experiments. Asterisks: $p<0.05$ versus absence of CpB (or ε-ACA) for plasminogen; daggers: $p<0.05$ versus absence of CpB (or ε-ACA) for apo(a).

2.5 DISCUSSION

It is generally accepted that apo(a) can inhibit pericellular plasminogen activation on vascular and blood cells, although the mechanism is not understood. As well, the domains in apo(a) which may potentially mediate this inhibitory effect and the relative role of apo(a) in various cell types is unknown. The present study contributes to the understanding of the role of apo(a) in inhibiting pericellular plasminogen activation on vascular and blood cells, specific domains in apo(a) which mediate this inhibitory effect, as well as the role of carboxyl terminal lysines in plasminogen activation and plasminogen and apo(a) binding.

In this study, we have provided direct evidence that (i) apo(a) inhibits pericellular plasminogen activation on HUVECs, THP-1 monocytes, and THP-1 macrophages; (ii) the kringle V domain and strong LBS in kringle IV type 10 play a crucial role in inhibiting activation whereas kringle 5-8 does not; (iii) Lp(a), at ~7 mg/ dL or 75 nM, can inhibit plasminogen activation on THP-1 monocytes and HUVECs but not on THP-1 macrophages (iv) plasminogen binding to the cells and cell-dependent plasminogen activation are dependent to a partial extent on receptors containing carboxyl-terminal lysines on all cell types; (v) apo(a) binding to THP-1 monocytes and macrophages is partially dependent of these receptors, but apo(a) binding to HUVECs is independent of these receptors and of lysine binding in general; and (vi) the binding by apo(a) to carboxyl-terminal lysine-containing sites does not appear to account for its ability to inhibit plasminogen activation.

A previous study has shown that Lp(a)/apo(a) can compete with plasminogen for binding on the endothelial cell surface and hence inhibit plasminogen activation [44].

Binding of plasminogen to the cell surface initiates pericellular plasminogen activation through interaction with its respective plasminogen activators: tPA and uPA. Both tPA and uPA have been shown to be expressed in a variety of cell types, including endothelial cells for tPA [51] and lung, kidney, and several tumor cell lines for uPA [52, 53]. It has been shown that tPA can directly bind to various cell types other than endothelial cells, such as platelets, monocytes, and monocytoid cells [30, 39, 54, 55]. Lp(a) was reported to inhibit plasminogen activation on the surface of resting platelets by inhibiting plasminogen and tPA binding [56]. Therefore, the effects of apo(a) and Lp(a) on tPA-mediated pericellular plasminogen activation on vascular cells were evaluated.

Plasminogen receptors are a heterogeneous and ubiquitously present group of cell surface proteins [38]. Plasminogen receptors have been shown to contain carboxyl-terminal lysine residues that bind plasminogen [38]. Removal of these carboxyl-terminal lysines destroys the ability of this subset of receptors to bind to plasminogen and support its activation [38]. However, not all plasminogen receptors contain a carboxyl-terminal lysine, with examples of this type being actin, amphoterin, $\alpha_V\beta_3$, $\alpha_M\beta_2$, and $\alpha_{IIb}\beta_3$ [57]. Binding of plasminogen to these receptors presumably converts plasminogen from its native closed conformation into a more open and highly activatable form [38]. The initial plasmin generated on the cell surface begins to catalyze the positive feedback mechanism of converting Glu- to Lys-plasminogen, the latter of which is itself more readily activatable [32]. Furthermore, the plasmin generated on the surface of the cells is protected from its natural inhibitor α_2-antiplasmin [31]. In the context of fibrin, carboxyl terminal lysines have been shown to play a crucial role in regulating plasminogen

activation [25, 58]. Hence, the role of carboxyl-terminal lysines on the cell surface was also of interest in this study.

Our studies, for the first time, have directly demonstrated that apo(a) is capable of inhibiting pericellular plasminogen activation. We showed this to be the case with two different recombinant apo(a) species, 12K and 17K, representing physiologically-relevant apo(a) isoforms. Interestingly, the 12K and 17K were similar in their ability to inhibit pericellular plasminogen activation. This suggests that the number of KIV type 2 repeats do not dictate the level of inhibition which is in agreement with the findings of Hancock and co-workers in the context of fibrin [33]. On the other hand, studies using a wider array of apo(a) sizes in the context of an intact Lp(a) particle have reported an inverse relationship between isoform size and the ability of Lp(a) to inhibit fibrinolysis and interfere with plasminogen binding to fibrin [59, 60], while larger Lp(a) isoforms were found to bind with less affinity to THP-1 monocytes [61]. Therefore, additional studies are required to assess the possibility that inhibition of pericellular plasminogen activation by apo(a)/Lp(a) is also isoform-size dependent.

The concentration of circulating plasminogen in plasma is 2.2 μM [62] whereas the concentration of Lp(a) varies from 1 to greater than 100 mg/dL, which is approximately equal to 0.01 to 1 μM [4]. The molar concentration of plasminogen circulating in the blood is generally greater than that of apo(a); however apo(a) has been shown to accumulate within atherosclerotic lesions and carotid plaques, likely due to its ability to interact with a wide variety of extracellular matrix elements [63-65]. Importantly, we showed that the ability of apo(a) and Lp(a) to inhibit pericellular plasminogen activation was strictly dependent on the presence of cells (Figs. 2.1, 2.3).

This likely reflects the ability of apo(a) to influence binding of plasminogen and/or tPA to the cells, although in our own binding studies we were not able to conclusively determine the extent to which apo(a) and plasminogen compete for binding to the cells (Suppl. Fig. 2.3).

Previous studies have identified specific domains of apo(a) that mediate its inhibitory effect on plasminogen activation as well as Glu-plasminogen to Lys-plasminogen conversion on the fibrin surface [33, 34]. Ablation of the strong LBS in kringle IV type 10 was shown to substantially reduce the inhibitory effect of apo(a) whereas removal of kringle V abolished the effect in plasminogen activation on fibrin [33]. Critical roles for kringle IV types 5-9 and kringle V were identified in inhibiting Glu- to Lys-plasminogen conversion on fibrin [34]. Interestingly, apo(a) from baboons and rhesus monkeys do not contain kringle V and are not prone to atherosclerosis [66, 67]. In the case of baboons, the absence of KV renders the Lp(a) particle incapable of binding to lysine despite the presence of an intact strong LBS in KIV_{10} [66]. On the other hand, in our previous studies of SMCs, we found that KIV_9, but not the strong LBS in KIV_{10}, mediated a decrease in TGF-β activation in a mechanism that was dependent on the presence of plasminogen (and thus, presumably, an effect on plasminogen activation) [35]. Therefore, there may be cell-type differences in the mechanisms through which apo(a)/Lp(a) interferes with plasminogen activation. This may in turn reflect different cohorts of plasminogen – or apo(a) – receptors on difference cell types.

We show here that the strong LBS in kringle IV type 10 as well as kringle V are essential for the inhibitory effects of apo(a) on pericellular plasminogen activation on THP-1 monocytes, THP-1 macrophages, and HUVECs. In fact, removal of theses

domains had the unexpected effect of enhancing plasminogen activation on the vascular cells tested. We suspect that these effects are related to the ability of apo(a) to bind to plasminogen in solution [46]. Indeed, from experiments conducted in the presence and absence of cells, it appears this enhancing effect of 17KΔLBS$_{10}$, 17KΔV and 12KΔV is unaffected by the presence or absence of cells. However, 12K and 17K r-apo(a) do not enhance plasminogen activation in the absence of cells. Accordingly, we must hypothesize that 17KΔLBS$_{10}$ and 12KΔV bind plasminogen in such a way that promotes its solution-phase activation, while intact 12K or 17K do not.

The ability of Lp(a) to inhibiting plasminogen activation was also evaluated (Fig. 2.3). Lp(a) was found to inhibit plasminogen activation, to the same extent as 17K, on HUVECs and to a much lesser extent on THP-1 monocytes. Plasminogen activation was not inhibited with the low concentration of Lp(a) utilized, ~7 mg/ dL, on THP-1 macrophages. We were restricted to a relatively low concentration of Lp(a) by the concentration of the preparation. The concentration utilized is less than the cardiovascular risk factor of Lp(a) levels >50 mg/ dL and could account for the lack of effect on THP-1 macrophages, possibly due to more rapid uptake of Lp(a) by these cells for example by the apo(a)/Lp(a) receptor identified by Keesler and coworkers [48].

The importance of carboxyl terminal lysine receptors was determined in vascular and blood cells. Overall, CpB treatment had modest but significant decreasing effects on plasminogen activation and on plasminogen and apo(a) binding (Fig. 2.4), with CpB treatment never decreasing binding or activation to below 50% of control. The exception to this was in HUVECs, where apo(a) bound in a manner completely independent of carboxyl-terminal lysines (Fig. 2.4F). Addition of the lysine analogue ε-ACA had a more

profound effect on plasminogen or apo(a) binding, which may suggest that some binding sites may have represented internal, as compared to carboxyl-terminal, lysine residues. Because of its profound effects on plasminogen conformation, we were unable to assess the effects of ε-ACA on plasminogen activation. Nonetheless, the available data suggest that the inhibition of plasminogen activation by apo(a) is not dependent on carboxyl-terminal lysines, since CpB treatment of the cells had only a small effect, if any, on the extent of the inhibition. If carboxyl-lysines played a role (such as mediating the binding of apo(a), plasminogen, or tPA required for the inhibition) inhibition by apo(a) would have been eliminated, when in fact it was only minimally impacted (Fig. 2.4A, C, E). Indeed, in HUVECs, where apo(a) is able to inhibit plasminogen activation over 50%, apo(a) binding is completely independent of carboxyl-terminal lysines (Fig. 2.4). It is reasonable to hypothesize, then, that if lysines do play a role, they would be internal lysines in cell-surface receptors.

Apo(a) has been shown in this current study to play a crucial role in regulating plasminogen activation on vascular cells attributable to kringle V and the strong LBS site in kringle IV type 10. What remains to be determined are the identities of the receptors that presumably mediate these effects. Moreover, it must be evaluated if the inhibition of plasminogen activation by apo(a) results from competition between these proteins for binding sites on cells and/or is a consequence of the ability of apo(a) to inhibit plasminogen activation via interference with the positive feedback mechanism of Glu- to Lys-plasminogen conversion.

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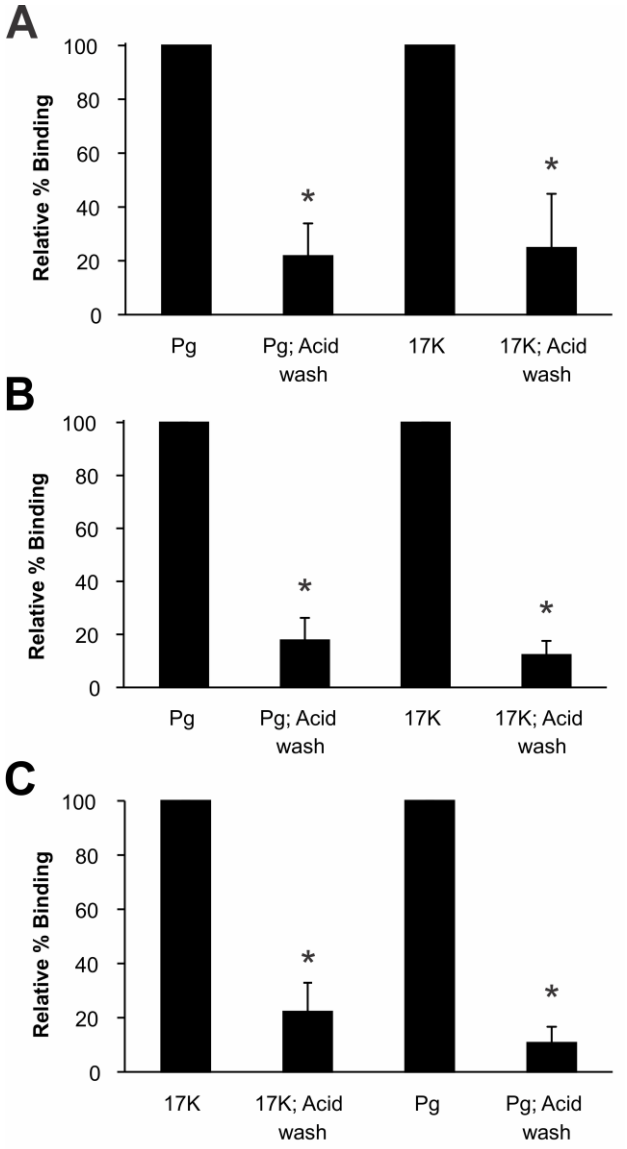
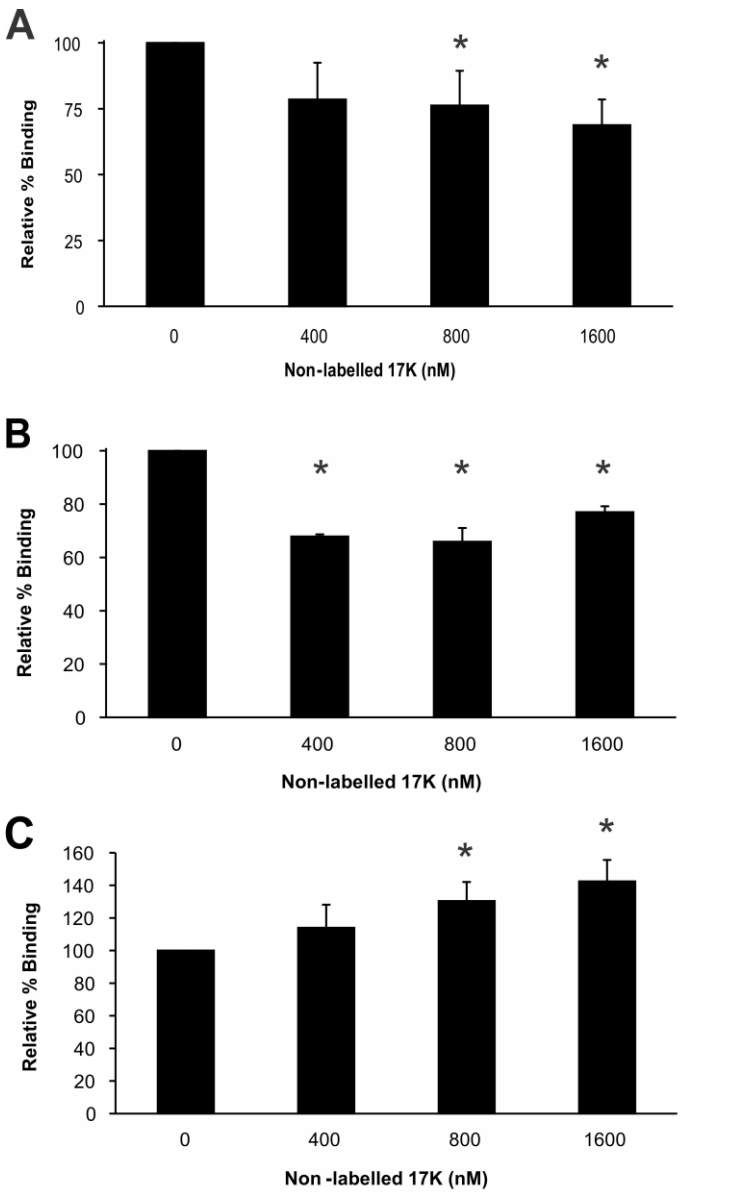
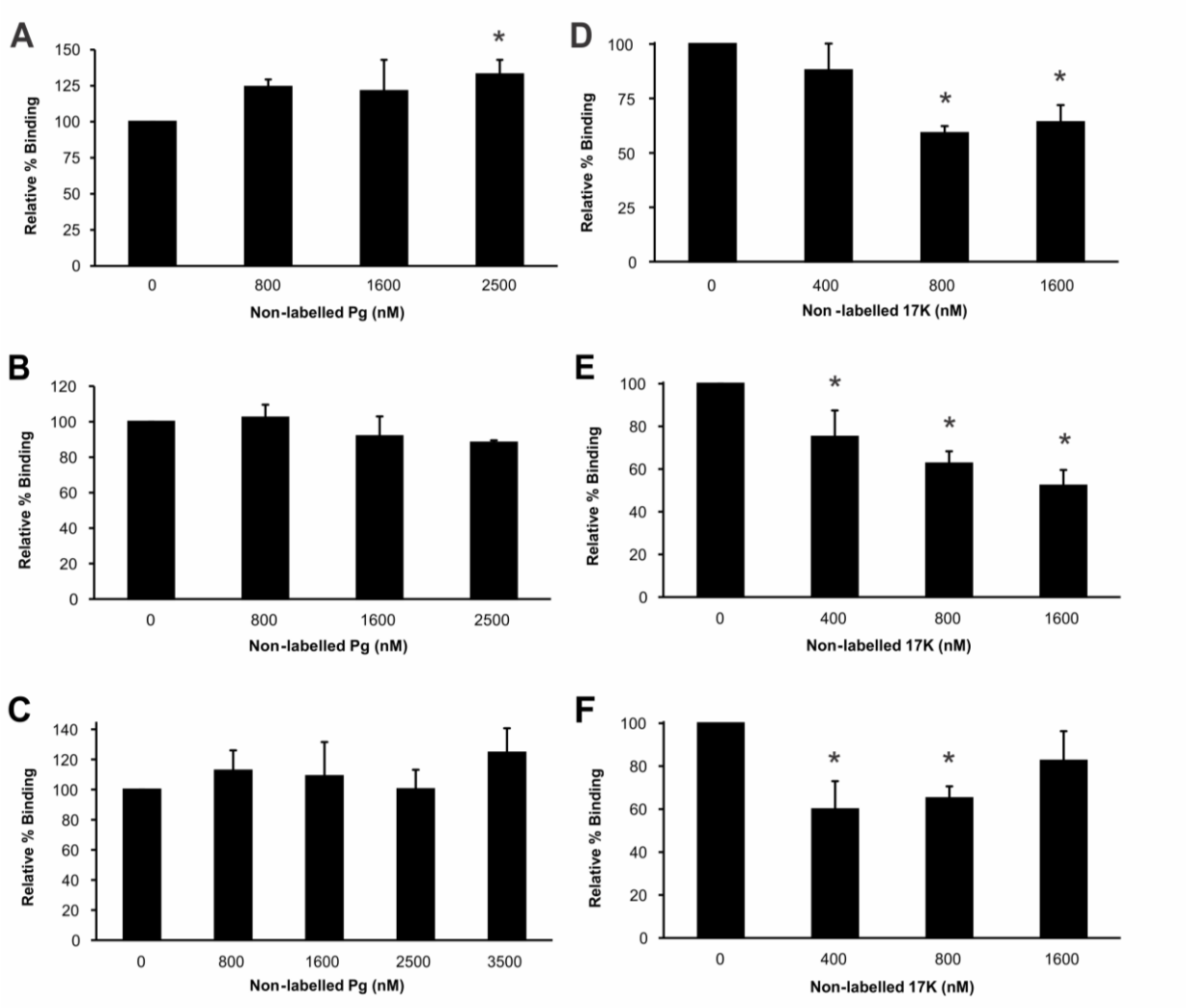
2.7 SUPPORTING INFORMATION



Supplementary Figure 2.1: ***Effect of acid washing on the binding of 17K apo(a) or plasminogen to THP-1 monocytes***. (**A**), THP-1 macrophages (**B**), or HUVECs (**C**). After incubating fluorescently-labeled apo(a) or plasminogen (400 nM) with the cells, we conducted a series of acid washes in order to assess whether we are detecting bound or internalized plasminogen or apo(a). The acid wash (two or three washes with 0.2 M acetic acid pH 2.5, 0.5 M NaCl) will remove any surface bound plasminogen or apo(a). Upon acid wash of the three cell types, a substantial decrease in the amount of bound plasminogen/apo(a) was detected indicating that the majority is surface bound and not internalized. The minimal amount detected following acid wash could either be residual bound plasminogen/ apo(a) (i.e. non-specific binding) or a small amount that was internalized. The results shown are the mean ± s.e.m. of 3 – 6 independent experiments. Asterisks: $p < 0.05$ versus absence of acid wash.



Supplementary Figure 2.2: Apo(a) *competition binding experiments.* Binding of Alexa-labeled 17K apo(a) (400 nM) to THP-1 monocytes (**A**), THP-1 macrophages (**B**), or HUVECs (**C**) was conducted in the absence or presence of unlabeled 17K apo(a) (400 to 1600 nM; i.e. up to a 4-fold molar excess). Based on our experiments with ε-ACA, we believe that at least 80% of the apo(a) binding is specific in THP-1 monocytes and macrophages (see Fig. 2.4B, D). As ε-ACA did not inhibit apo(a) binding to HUVECs (nor did CpB), we were not able to make the same estimation, although an acid-wash step similarly eliminated at least 80% of the binding (see above). We thus attempted to "self-compete" the binding to HUVECs of Alexa-labeled apo(a) with unlabeled apo(a). The results show a decrease in labeled apo(a) binding in THP-1 monocytes or macrophages, although the extent of the decrease was small at the relatively small molar excesses that we attempted. The results shown are the mean ± s.e.m. of 3 – 6 independent experiments. Asterisks: $p < 0.05$ versus absence of unlabeled apo(a).


A
Relative % Binding
150
125
100
75
50
25
0
0
800
1600
2500
Non-labelled Pg (nM)
*
B
Relative % Binding
120
100
80
60
40
20
0
0
800
1600
2500
Non-labelled Pg (nM)
C
Relative % Binding
140
120
100
80
60
40
20
0
0
800
1600
2500
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Non-labelled Pg (nM)
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F
Relative % Binding
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Supplementary Figure 2.3: ***Plasminogen competition binding experiments.*** Effect of unlabeled plasminogen on the binding of Alexa-labeled 17K apo(a) to THP-1 monocytes (**A**), THP-1 macrophages (**B**), or HUVECs (**C**). Effect of unlabeled 17K apo(a) on the binding of Alexa-labeled plasminogen to THP-1 monocytes (**D**), THP-1 macrophages (**E**), or HUVECs (**F**). Binding experiments were conducted with 400 nM Alexa-labeled 17K apo(a) or plasminogen in the absence or presence of unlabeled plasminogen or 17K apo(a). Assessment of the effect of plasminogen on apo(a) binding is perilous because of the ability of apo(a) and plasminogen to bind [Sangrar, W., B. R. Gabel, M. B. Boffa, J. B. Walker, M. A. Hancock, S. M. Marcovina, A. J. Horrevoets., M. E. Nesheim, and M. L. Koschinsky. 1997. The solution phase interaction between apolipoprotein(a) and plasminogen inhibits the binding of plasminogen to a plasmin-modified fibrinogen surface. Biochemistry. 36: 10353-10363] such that addition of an unlabeled excess of one may not decrease the binding of the other. In any event, whether the competitor genuinely competes for a binding site or merely prevents binding to the cell surface through a solution-phase interaction cannot be distinguished. Lack of competition may indicate binding to different binding sites. The data show that unlabeled plasminogen does not compete for binding of labeled apo(a), while unlabeled apo(a) does compete for binding of labeled plasminogen. It is possible that the putative apo(a)/plasminogen complex binds to apo(a) binding sites but not to plasminogen binding sites, or that apo(a) binds to sites that either do or do not binding to plasminogen. The results shown are the mean ± s.e.m. of 3 – 6 independent experiments. Asterisks: $p<0.05$ versus absence of unlabeled competitor.

CHAPTER 3

Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen on the surface of vascular endothelial and smooth muscle cells

3.1 SUMMARY

Lipoprotein(a) [Lp(a)] is an enigmatic lipoprotein which has been identified as an independent, causal risk factor for coronary heart disease. Lp(a) consists of a low-density lipoprotein (LDL) moiety covalently linked to the unique glycoprotein apolipoprotein(a) [apo(a)]. Apo(a) is homologous to the fibrinolytic zymogen plasminogen and thus may interfere with plasminogen activation. Conversion by native Glu-plasminogen by plasmin to the more readily activatable Lys-plasminogen greatly accelerates plasminogen activation and is necessary for optimal stimulation of plasminogen activation on endothelial cells. Lp(a)/apo(a) has been previously shown to inhibit pericellular plasminogen activation on vascular cells, but the mechanism underling these observations is unknown. We therefore explored whether apo(a) can inhibit pericellular Glu- to Lys-plasminogen conversion on cell surfaces. A physiologically relevant recombinant version of apo(a) (17K) significantly inhibits plasmin-mediated Glu- to Lys-plasminogen conversion on human umbilical vein endothelial cells (HUVECs) and smooth muscle cells (SMCs). All isoforms of apo(a) that were analyzed, ranging in size from 3 to 21 kringle IV type 2 repeats, were able to inhibit conversion to a similar extent. Removal of the kringle V and protease domain of apo(a) strongly reduces the ability of apo(a) to inhibit conversion on HUVECs and SMCs. Removing the strong lysine binding site in KIV_{10} of apo(a) abolishes its ability to inhibit conversion on HUVECs and, to a lesser extent, on SMCs. These results indicate a novel mechanism in which apo(a) inhibits the positive feedback mechanism that accelerates plasmin formation on vascular cells.

3.2 BACKGROUND

Plasma lipoprotein(a) [Lp(a)] levels vary dramatically within the human population, ranging between individuals from undetectable to greater than 100 mg/dL [1, 2]. Elevated levels of Lp(a) – greater than 30-50 mg/dL – have been established as a causal, independent risk factor for atherothrombotic disorders including coronary heart disease (CHD) [3-5]. Lp(a) is similar in lipid composition to low-density lipoprotein (LDL) and contains apolipoproteinB-100 (apoB-100) [6]. However, Lp(a) constitutes a distinct lipoprotein class in that it also contains the unique glycoprotein apolipoprotein(a) [apo(a)] [7]. Apo(a) is evolutionarily related to the fibrinolytic proenzyme plasminogen [8]: apo(a) consists of multiple copies of a plasminogen kringle IV (KIV)-like domain followed by a plasminogen kringle V (KV)-like domain and an inactive protease domain [9]. Unlike plasminogen, apo(a) consists of multiple copies of KIV that are classified as 10 distinct subtypes based on amino acid sequence (KIV_1 to KIV_{10}) [8]. Apo(a) isoform size is genetically determined based on variation in allele size of *LPA* (the gene encoding apo(a)) which results in varying numbers of identically repeating copies of KIV_2 [10-12]. The number of identically repeated copies of KIV_2 can vary from 3 to >40 which gives rise to the isoform size heterogeneity of Lp(a) observed in the human population [12].

Lp(a)/apo(a) has been previously reported to inhibit both *in vitro* and *in vivo* fibrin clot lysis [13-15] and plasminogen activation on fibrin or degraded fibrin [16]. This is partially attributable to the ability of apo(a) to inhibit the positive feedback mechanism of Glu-plasminogen to Lys-plasminogen conversion [17]. Recently, it has been shown that Lp(a)/apo(a) can inhibit pericellular plasminogen activation on human umbilical vein endothelial cells (HUVECs), human acute monocytic leukemia (THP-1) cells, and THP-1

cells differentiated into macrophages [18]. Plasmin generation is crucial for fibrinolysis, angiogenesis, cell migration and proliferation, wound healing, and tumor cell invasion and metastasis [19-24].

Binding of native Glu-plasminogen to fibrin or cell surfaces greatly accelerates plasminogen activation [25-26]. Glu-plasminogen exhibits a tight, spiral structure [27] and is thought to adopt a more open conformation that is more readily activated when bound to fibrin or cell surfaces [28]. Glu-plasminogen can also be converted by plasmin cleavage at Lys^{77} to form Lys-plasminogen which can be more readily activated by plasminogen activators as it is in a more open, extended conformation compared to the native circulating Glu-plasminogen [25, 27]. Lys-plasminogen can also bind with a higher affinity to substrates such as fibrin which further accelerates plasmin formation [29-31]. Conversion of Glu- to Lys-plasminogen is also necessary for optimal activation of plasminogen on HUVECs and monocytoid cells [26, 32]; stimulation of Glu- to Lys-plasminogen conversion is the main mechanism by which cell surfaces accelerate plasminogen activation. Once Lys-plasminogen is formed, it is more readily converted to plasmin in solution by tPA or uPA [26, 32, 33].

In the present study, we evaluated the mechanism by which apo(a) can inhibit pericellular plasminogen activation on HUVECs and smooth muscle cells (SMCs), focusing specifically on the effect of apo(a) on plasmin-mediated Glu- to Lys-plasminogen conversion on these cell surfaces.

3.3 METHODS

3.3.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and maintained in EBM-2 media supplemented with the EGM-2 bulletkit (Lonza). Smooth muscle cells (SMCs) were obtained from Clonetics and maintained in smooth muscle basal medium (SmBM) medium (Lonza) supplemented with the SmGM-2 bullet kit (Lonza). Human embryonic kidney cells (HEK293) cells were maintained in MEM (GIBCO) containing 5% fetal bovine serum (FBS; GIBCO) and 1% antibiotic-antimycotic (GIBCO). Baby hamster kidney (BHK-21) cells were maintained in DMEM (GIBCO) containing 5% newborn calf serum (NCS; Sigma) and 1% antibiotic-antimycotic (GIBCO). All cells were maintained in a humidified incubator at 37°C and 95% air/5% CO2 atmosphere. All experiments were conducted at passage five for both HUVECS and SMCs.

3.3.2 Construction, expression, and purification of recombinant apo(a)

The recombinant apo(a) (r-apo(a)) variants used in this study are shown schematically in Fig. 3.1. The construction and expression of the r-apo(a) variants have been previously described [9, 16, 34-36]. Briefly, the expression plasmids used to express the r-apo(a) variants were constructed in the pRK5 vector containing a cytomegalovirus promoter and SV40 transcription termination signal. Expression plasmids encoding the recombinant apo(a) (r-apo(a)) variants 12K, 12KΔV, 14K, 17K, 17KΔLBS$_{10}$, 17KΔP, 17KΔV, 23K, and 30K were transfected into HEK293 and stably-expressing cell lines derived. Recombinant proteins were purified from cell-conditioned medium using lysine-

Sepharose affinity chromatography. Protein concentrations were determined using absorbance measurements taken at 280 nm and extinction coefficients corresponding to each respective variant as previously described [9, 16, 34-36]. Purity of the proteins was confirmed by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

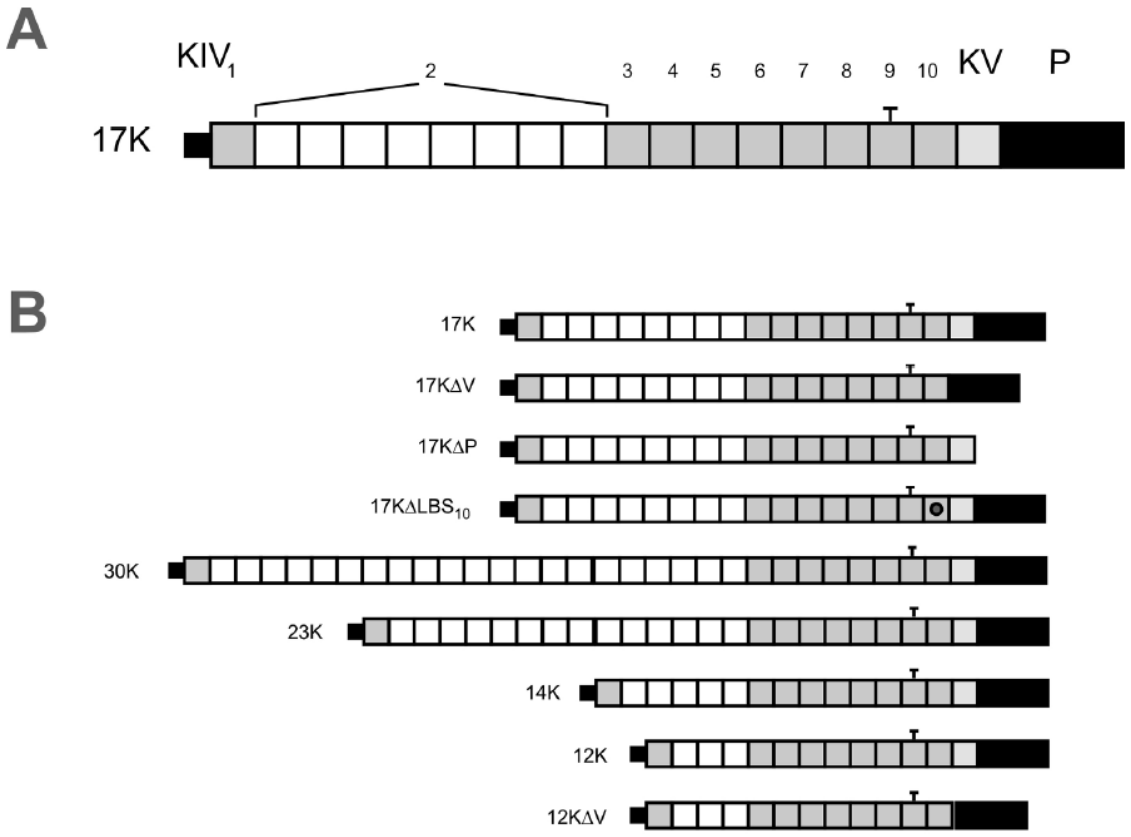


Figure 3.1: ***Recombinant-apo(a) constructs utilized in study***. The domain structure of the full-length r-apo(a) constructs is presented, where KIV and KV denote kringle homologous to plasminogen kringles 4 and 5, respectively, and P represents the plasminogen-like protease domain. The bar indicates the location of the free-cysteine by which apo(a) is covalently bound to apoB-100 in the Lp(a) particle. Mutations and deletions of specific domains are schematized, with the circle representing an Asp.→ Ala mutation at position 57 of KIV_{10} that abolishes the strong LBS in this kringle.

3.3.3 Construction, expression, purification, and labeling of recombinant plasminogen

The construction and expression of plasminogen has been previously described [16, 36]. Briefly, full length plasminogen cDNA was inserted into the multiple-cloning site of the pNUT expression vector, followed by site-directed mutagenesis to achieve a recombinant variant of plasminogen containing a serine to cysteine mutation (S741C) in the active site. The plasmid was then transfected into BHK-21 cells to produce a stably-expressing cell line. Recombinant plasminogen in cell-conditioned medium was purified using affinity chromatography over lysine-Sepharose. Purified plasminogen was fluorescently labeled using the cysteine-specific fluorescent probe, 5-iodoacetamidofluorescein (5'-IAF). Protein concentrations were determined using absorbance measurements taken at 280 nm (extinction coefficient of 1.6 M^{-1} $cm^{-}1$) and corrected for the contribution of fluorescein ($A_{280} = 0.19 \times A_{495}$). Purity of the protein was confirmed by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

3.3.4 Measurement of Plasmin-Mediated Conversion of Glu-Plasminogen to Lys-Plasminogen

Passage five HUVECs and SMCs were grown to confluence in a 24-well cell culture plate and washed two times with Hanks buffered salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 1 g/L glucose, pH 7.4) containing 0.1% (w/v) BSA (HBSS-BSA). The cells were treated with 200 μL of a solution containing 50 nM fluorescently labelled recombinant-Glu-plasminogen, 5 nM Lys-plasmin (Haematologic Technologies

Inc.), and various concentrations of r-apo(a) (ranging from 0 to 400 nM) in HBSS-BSA. Reactions were terminated by rapidly washing the cells once with HBSS-BSA then adding 100 μL of reducing sample buffer (31.2 mM Tris, pH 7.2, 2% SDS, 10% sucrose, 0.002% bromophenol blue, 15 mM dithiothreitol, 10mM EDTA, 10 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor (Sigma), 0.02% NaN_3, 5 units/ml Trasylol, 50 mM ε-aminocaproic acid (ε-ACA)). This technique has been previously shown to capture greater than 90% of the cell-associated plasminogen with minimal contamination by unbound plasminogen [26]. Samples were subjected to 7% SDS-PAGE under reducing conditions. The resulting gels were exposed under a cy2 lens using the FluoroChem Q system (AlphaInnotech) to visualize Glu- and Lys-plasminogen utilizing the fluorescent tag on plasminogen. Band intensities were determined using Alpha View software (AlphaInnotech). Percent conversion values were calculated by dividing the Lys-plasminogen band intensity with the sum of the Glu- and Lys-plasminogen band intensities. Experiments were performed in the absence of cells in a 1.5 mL amber tube (Eppendorf) at 37°C, with the same concentrations of plasminogen, plasmin, and r-apo(a) as above. At specific time points, a sample of the solution was removed followed by the addition of reducing sample buffer in a 1:1 ratio. The samples were then subjected to SDS-PAGE and imaging as above.

3.3.5 Statistical Methods

Comparisons between data sets were performed using two-tailed Student t-test assuming unequal variance. Statistical significance was presumed at $p<0.05$.

3.4 RESULTS

Previous work has shown that plasmin-mediated conversion of Glu- to Lys-plasminogen is necessary for optimal stimulation of plasminogen activation on HUVECs [26]. We have previously reported that apo(a) can inhibit plasminogen activation as well as the conversion of Glu- to Lys-plasminogen on the fibrin surface [16, 17]. We have also reported that apo(a) can inhibit pericellular plasminogen activation on the surface of vascular cells [18]. In this study, we determined the effect of apo(a) on pericellular Glu- to Lys-plasminogen conversion by plasmin. We utilized a recombinant variant of plasminogen that contains a Ser to Cys mutation in the active site such that no plasmin activity develops upon cleavage of the variant with plasminogen activators [36]. Therefore, no plasmin beyond that added to the reaction is formed which prevents positive feedback in the conversion reaction. Moreover, the mutation allows labeling of the recombinant plasminogen with a fluorophore containing a sulfhydryl-directed functional group, allowing for detection specifically of the Glu-plasminogen substrate and its Lys-plasminogen product following electrophoretic separation of the reactions. We used a washing technique that specifically captures the cell-associated plasminogen forms with minimal contamination from the reactants in the cell medium [26].

HUVECs and SMCs were treated with 50 nM labeled recombinant plasminogen, 5 nM plasmin, and various concentrations of r-apo(a) and at specific time points the reaction was terminated through the addition of reduced sample buffer directly to the cells. Following resolution of Glu- and Lys-plasminogen by SDS-PAGE, the extent of conversion was determined by dividing the Lys-plasminogen band intensity with the sum of the Glu- and Lys-plasminogen bands intensities. Shown in Fig. 3.2 are representative

gels showing the effect of apo(a) on Glu- to Lys-plasminogen conversion on the surface of HUVECs (Fig. 3.2A), SMCs (Fig. 3.2B), and in the absence of cells (Fig. 3.2C).

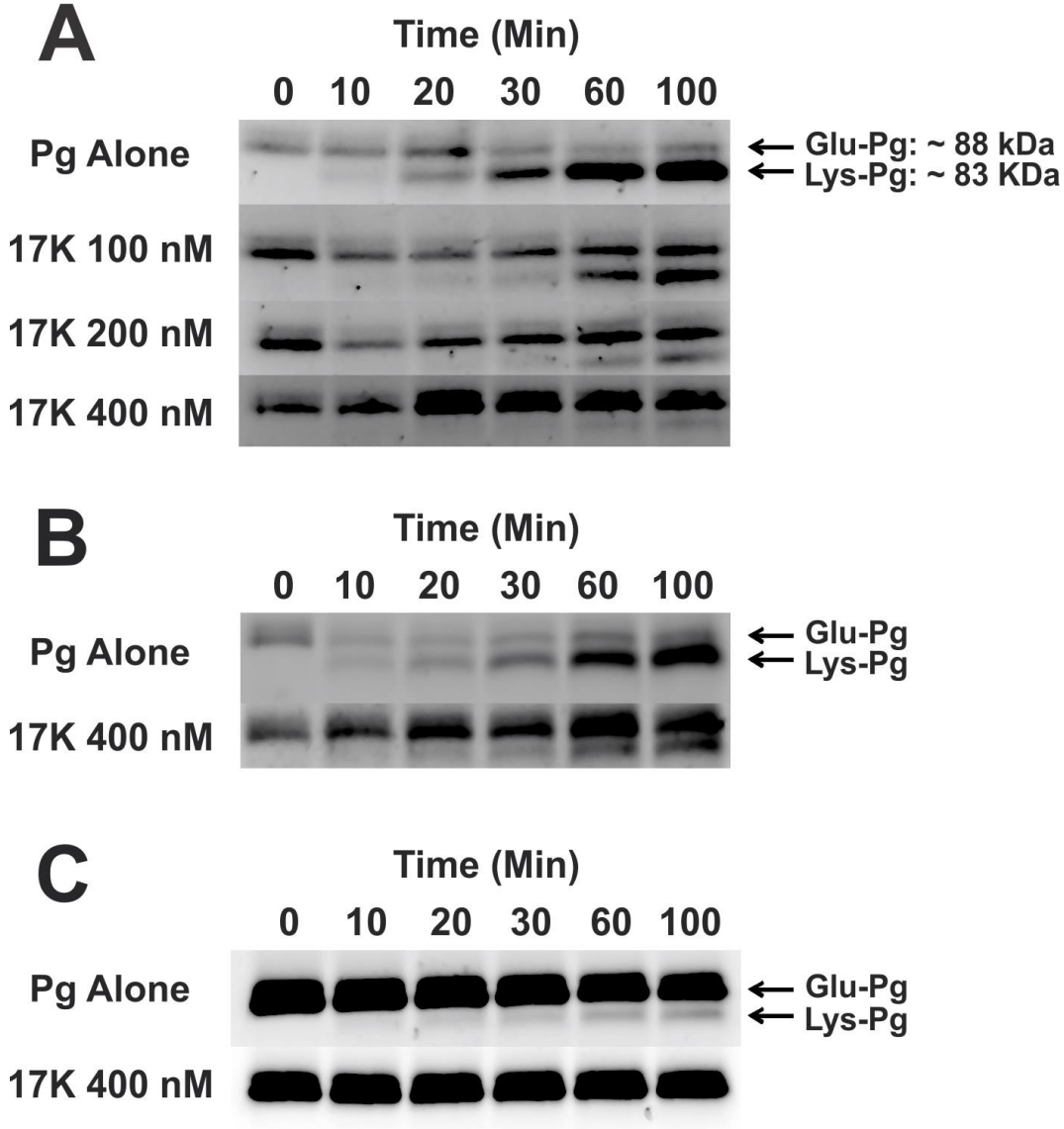
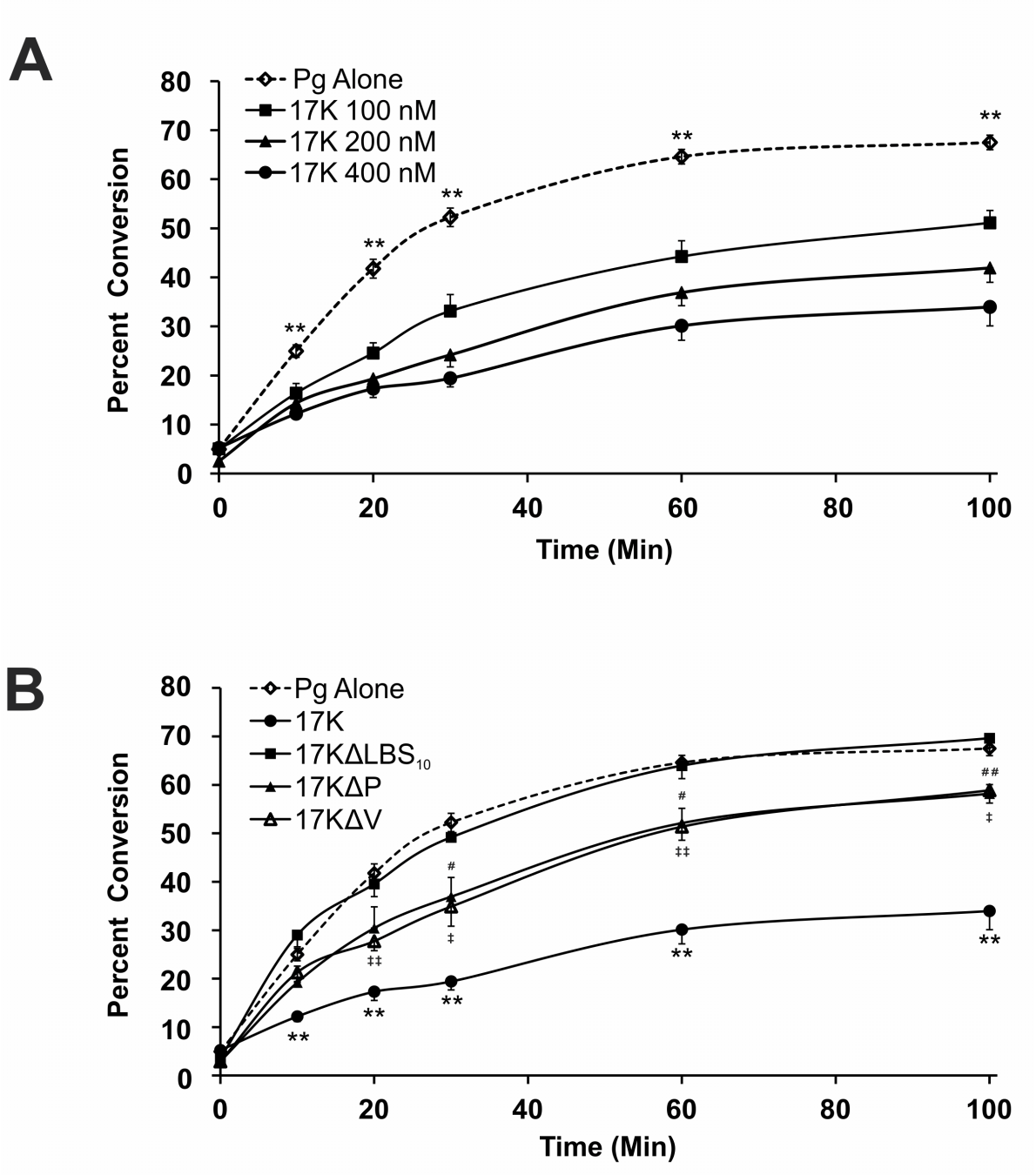


Figure 3.2: ***Inhibition of plasmin-mediated Glu- to Lys-plasminogen conversion by apo(a)***. Reactions contained 50 nM fluorescently labeled Glu-plasminogen (in which the active site serine is mutated to a cysteine and fluorescently labeled), 5 nM plasmin, and various concentrations of r-apo(a). At specific time points, the cells were washed and reactions were terminated by the addition of SDS-PAGE sample buffer. In the absence of cells, sample buffer was added in a 1:1 ratio to reaction mixture to terminate reactions at specific time points. Samples were subjected to SDS-PAGE and Glu- and Lys-plasminogen bands were observed and quantified by fluorescence image analysis Representative gels are shown for the effect of apo(a) on Glu- to Lys-plasminogen conversion on (**A**) HUVECs, (**B**) SMCs, and (**C**) in the absence of cells. Glu- and Lys-plasminogen (Pg) are the upper and lower bands, respectively, on the gels.

We performed both a titration of the physiologically relevant 17K r-apo(a) variant as well as a time course of the conversion reaction on HUVECs. Conversion in the absence of apo(a) is initially rapid, with the rate slowing considerably after 30 minutes (Fig. 3.3A). Addition of 17K r-apo(a) results in a dose-dependent decrease in the extent of conversion at all time points, with the maximal effect of 17K r-apo(a) being an almost 60% decrease in conversion at 400 nM 17K at 20 minutes.

We next utilized a series of r-apo(a) containing deletions of either specific domains or point mutations (Fig. 3.1) in order to assess which sequences of apo(a) mediate the ability of 17K to inhibit Glu- to Lys-plasminogen conversion on HUVECs. Removal of the protease domain and kringle V domain of 17K resulted in at least a 50% decrease in the ability to inhibit conversion on HUVECs (Fig. 3.3B). Removal of the strong lysine binding site (LBS) in kringle IV type 10 ($17K\Delta LBS_{10}$) abolishes the ability of 17K to inhibit Glu- to Lys-plasminogen conversion on HUVECs (Fig. 3.3B).

In order to determine if the effect of deleting certain domains is isoform-dependent, we analyzed the inhibitory effect of 12KΔV in comparison to 12K. As with 17K and 17KΔV, kringle V in 12K is required for maximal inhibition on HUVECs (Fig. 3.3C), with the loss of this kringle resulting in at least a 50% decrease in the extent of inhibition.


A
Pg Alone
17K 100 nM
17K 200 nM
17K 400 nM
Percent Conversion
Time (Min)
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100
B
Pg Alone
17K
17KΔLBS10
17KΔP
17KΔV
Percent Conversion
Time (Min)

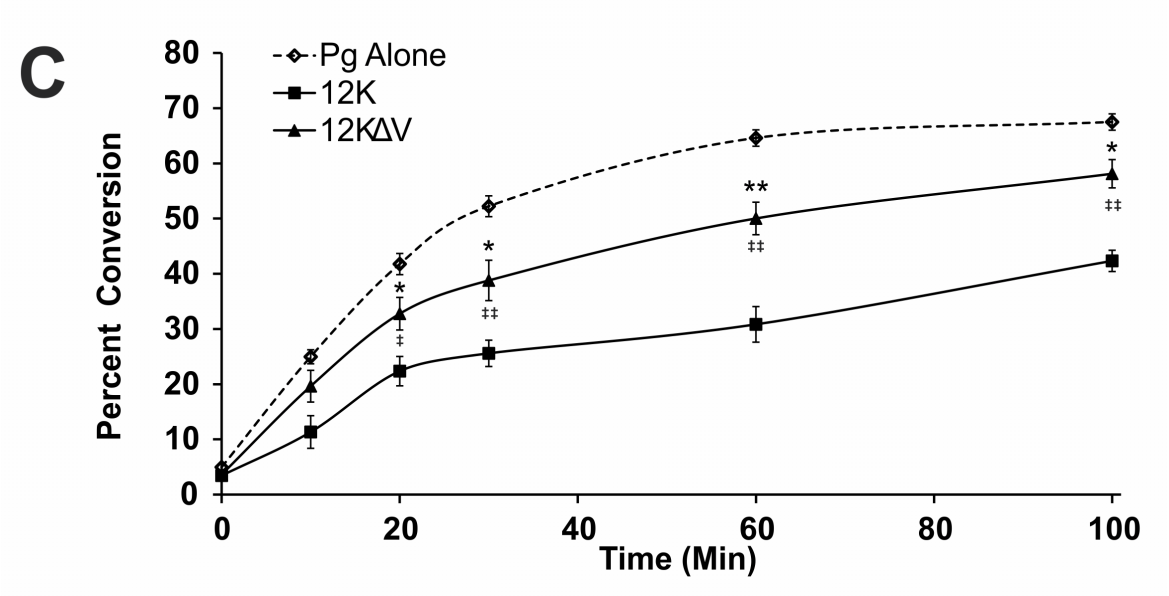


Figure 3.3: ***Inhibition by apo(a) of Glu- to Lys-plasminogen conversion on HUVECs***. HUVECs were grown to confluence in a 24 well plate and conversion experiments were conducted as described in the legend to Fig. 3.2. Percent conversion values were calculated by dividing the Lys-plasminogen band intensity with the sum of the Glu- and Lys-plasminogen band intensities. (**A**) Titration of 17K r-apo(a) on HUVECs. Data shown are the means ± standard error of the mean (SEM) from at least seven independent experiments. **: $p<0.01$ for each concentration of 17K (100, 200, and 400 nM) in comparison to plasminogen alone. (**B**) Examination of a series of domain deletions/mutations (see Fig. 3.1), each at 400 nM. Data shown are the means ± SEM from at least three independent experiments. **: $p<0.01$ for 17K in comparison to plasminogen alone. #:$p<0.05$ and ##: $p<0.01$ for 17KΔP in comparison to plasminogen alone. ǂ: $p<0.05$ and ǂ ǂ:$p<0.01$ for 17KΔV in comparison to plasminogen alone. (**C**) Comparison of the effects of 12K and 12KΔV. Data show are the means ± SEM from at least three independent experiments. *:$p<0.05$ and **: $p<0.01$ for 12KΔV in comparison to plasminogen alone. ǂ: $p<0.05$ and ǂ ǂ:$p<0.01$ for 12KΔV in comparison to 12K.

A series of r-apo(a) variants containing different numbers of KIV_2 repeats (Fig. 3.1) was utilized in order to assess whether apo(a) isoform size contributes to the inhibitory effect of Glu- to Lys-plasminogen conversion on HUVECs. All isoforms tested were able to inhibit Glu- to Lys-plasminogen conversion to a similar extent (Fig. 3.4), with no trend with respect to size apparent.

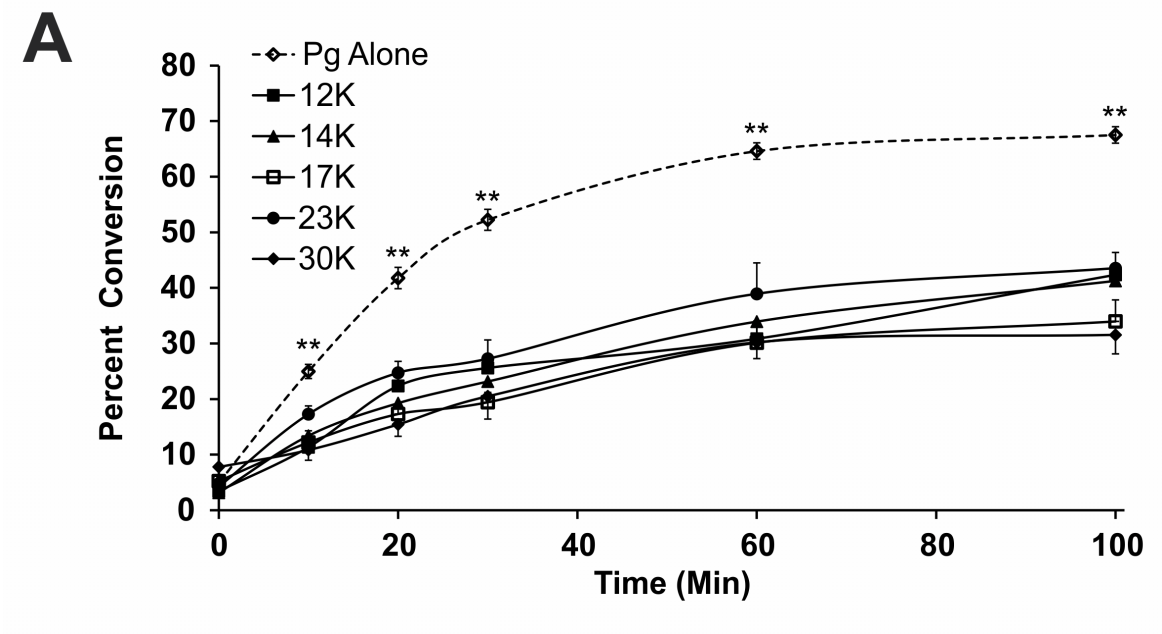


Figure 3.4: ***Effect of r-apo(a) isoform size on Glu- to Lys-plasminogen conversion on HUVECs***. Conversion experiments were conducted as described in the legend to Fig. 3.2 in the presence of 400 nM r-apo(a) variants differing in the number of KIV_2 repeats (see Fig. 3.1). Data shown are the means ± SEM from at least three independent experiments. **: $p<0.01$ for all isoforms tested (12K, 14K, 17K, 23K, and 30K) at each time points 10, 20, 30, 60 and 100 nM in comparison to plasminogen alone.

The ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on SMCs was also evaluated. The same dose-dependent decrease in Glu- to Lys-plasminogen conversion is observed on SMCs as was seen on HUVECs following the addition of 17K (Fig. 3.5A). Compared to on HUVECs, there is a more rapid initial increase in the rate of conversion in the absence of apo(a), and the maximal inhibitory effect of 17K occurs sooner and is greater in magnitude. Removal of the strong LBS significantly abrogates the inhibitory effect of 17K (Fig. 3.5B), but unlike in HUVECs this variant retains some inhibitory effect in SMCs, at least at early time points.

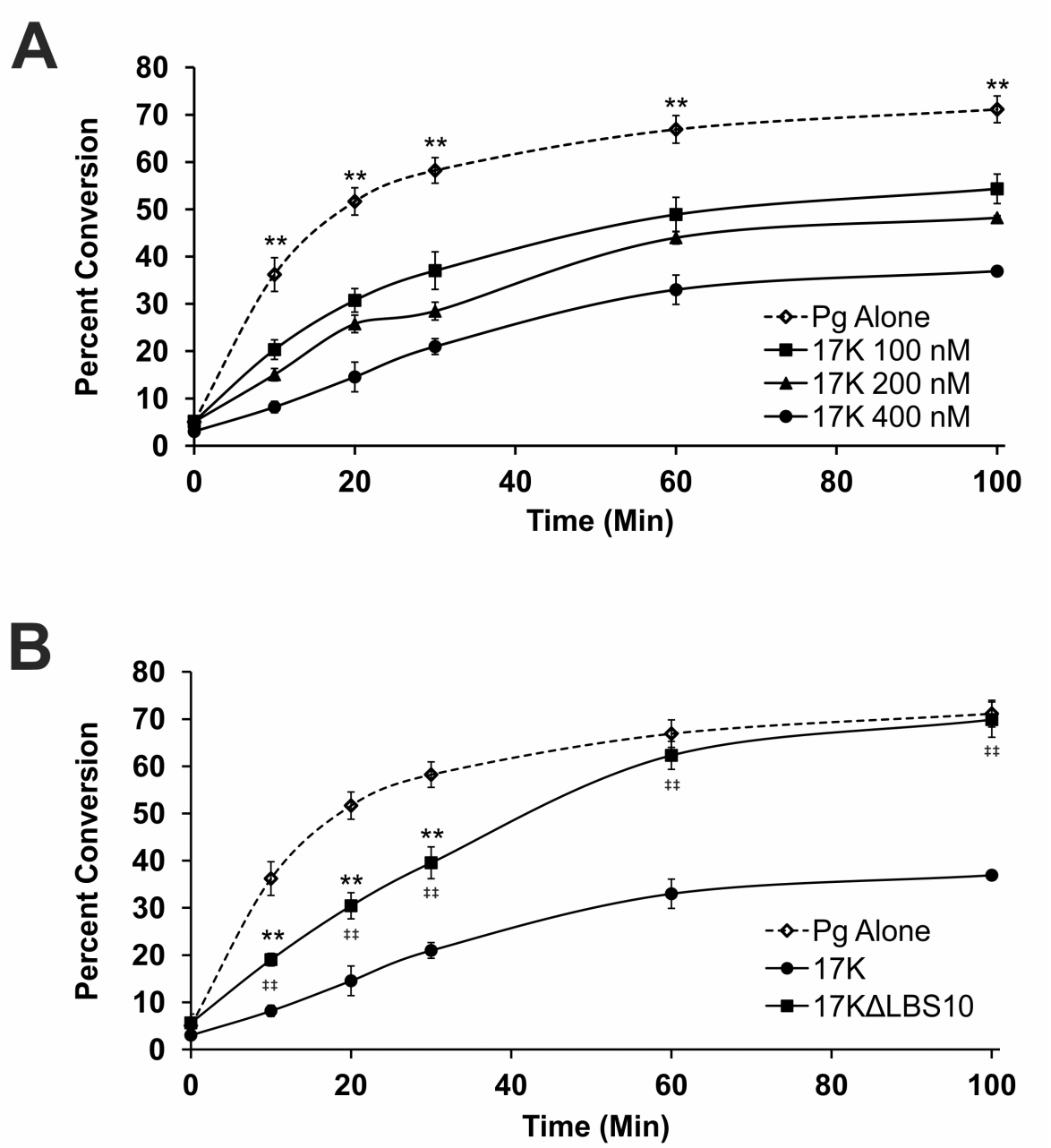


Figure 3.5: ***Inhibition by apo(a) of Glu- to Lys-plasminogen conversion on SMCs***. Conversion experiments were conducted as described in the legend to Fig. 3.2. (**A**) Titration of 17K r-apo(a) on SMCs Data shown are the means ± SEM from at least four independent experiments. **: $p<0.01$ for each concentration of 17K (100, 200, and 400 nM) in comparison to plasminogen alone. (**B**) Comparison of 17K and $KIV\Delta LBS_{10}$ (each at 400 nM). Data shown are the means ± SEM from at least four independent experiments. **: $p<0.01$ for $17K\Delta LBS_{10}$ in comparison to plasminogen alone. ǂ:$p<0.01$ for $17K\Delta LBS_{10}$ in comparison to 17K.

Previous work has also shown that plasminogen activation and Glu- to Lys-plasminogen conversion is greatly accelerated on fibrin or cell surfaces compared to in solution [17, 18]. In our assays, the percent Glu- to Lys-plasminogen conversion for HUVECs or SMCs compared to the reaction in solution was reduced by 73% and 82% respectively at the 10 min time point (Fig. 3.6). Interestingly, 17K retains the ability to inhibit Glu- to Lys-plasminogen conversion in the absence of cells, with the percentage decrease being of a similar magnitude to that observed on cells (Fig. 3.6).

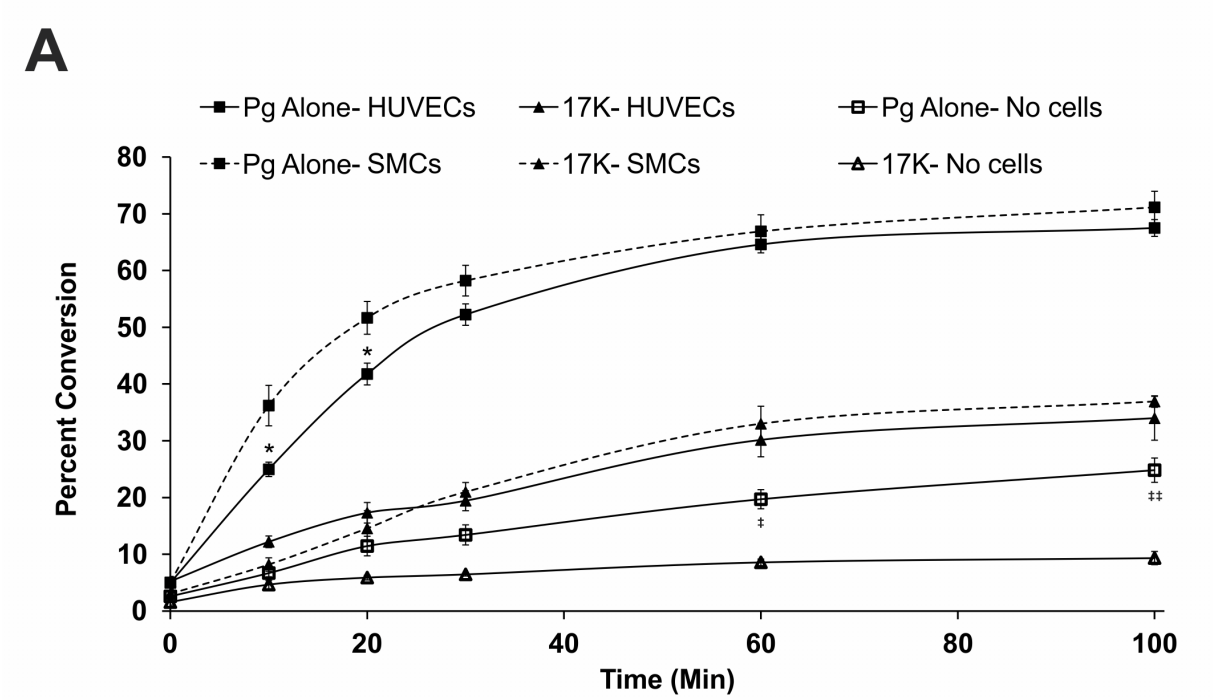


Figure 3.6: ***Comparison of Glu- to Lys-plasminogen conversion in the presence or absence of cells***. Conversion experiments were conducted as described in the legend to Fig. 3.2. Data shown are the means ± SEM from at least three independent experiments. *:p<0.05 for comparison of plasminogen alone between HUVECs and SMCs. ǂ: p<0.05 and ǂǂ:p<0.01 for comparison of plasminogen alone and 17K in the absence of cells.

3.5 DISCUSSION

It has been previously shown that Lp(a)/apo(a) can inhibit pericellular plasminogen activation on HUVECs, THP-1 monocytes, and THP-1 cells differentiated into macrophages [18]. However, the mechanism by which Lp(a)/apo(a) can inhibit this process is unknown. Lp(a)/apo(a) can inhibit plasminogen activation on the fibrin surface, in part, through inhibiting plasmin-mediated Glu- to Lys-plasminogen conversion [17]. This mechanism has also been shown to be crucial for the ability of cell surfaces to accelerate plasminogen activation [26, 32]. In this scenario, binding of Glu-plasminogen (closed conformation) to cells increases the rate at which plasmin cleaves the protein to form Lys-plasminogen (open conformation), which is then activated at a much higher rate by plasminogen activators in solution. Glu-plasminogen is in a closed, spiral conformation that limits accessibility of the plasmin cleavage site at Lys^{77} as well as the tPA/uPA cleavage site at Arg^{562}; conversion to Lys-plasminogen results in an open, extended conformation that relieves the latter conformational barrier. Presumably, binding of Glu-plasminogen to lysine-containing cell surface receptors similarly results in a more open conformation that promotes cleavage by plasmin to form Lys-plasminogen. Note that this model contrasts with earlier models that assumed that cell surfaces constitute a template to which Glu-plasminogen and plasminogen activators bind to form a ternary complex that accelerates plasminogen activation, in a manner analogous to the role of fibrin surfaces [26, 32].

Therefore, the current study set out to determine if apo(a) inhibits pericellular plasminogen activation specifically through inhibition of plasmin-mediated Glu- to Lys-plasminogen conversion. We found that, indeed, apo(a) can act as a potent inhibitor of

Glu- to Lys-plasminogen conversion on two vascular cell types: HUVECs and SMCs. The magnitude of the reduction (over 2-fold) is in keeping with our recent report of the effect of apo(a) on plasminogen activation on vascular and blood cells [18]. Together these findings suggest that inhibition of Glu- to Lys-plasminogen conversion is crucial to the overall mechanism of plasminogen activation inhibition by apo(a).

We found that Glu- to Lys-plasminogen conversion is significantly accelerated in the presence of cells compared to that observed in solution. Moreover, in the current study, apo(a) was able to inhibit conversion in the absence of cells. These results contradict previous findings which demonstrated that apo(a) accelerates Glu- to Lys-plasminogen conversion in the absence of fibrin [17]. The differences observed could be due to the contrast in the ratio of plasminogen:plasmin:apo(a) in the two systems. Nonetheless, apo(a) was not able to accelerate pericellular plasminogen activation in the absence of cells as was observed in the current study [18].

In order to gain mechanistic insights, we investigated the importance of specific domains of apo(a) which mediate its inhibitory capabilities in Glu- to Lys-plasminogen conversion. In the current study, we identified a crucial role for the strong LBS present in KIV type 10 in the inhibition of Glu- to Lys-plasminogen conversion. A mutant form of apo(a) lacking this LBS was completely unable to inhibit conversion on HUVECs and was considerably reduced in its ability to inhibit conversion on SMCs. As well, key roles were identified for the protease and KV domains. Removal of these domains resulted in a reduced ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on both cell types. The strong LBS and KV domain have been previously shown to be crucial for inhibiting plasminogen activation on HUVECs and THP-1 monocytes and macrophages,

with removal of either of these elements rendering apo(a) completely incapable of inhibition [18]. In addition, we found that apo(a) (at a concentration of 100 nM) inhibits plasminogen activation on SMCs and that removal of the KIV_{10} strong LBS abolished this effect (R.R. and M.L.K., unpublished data). It is notable, however, that removal of the KIV_{10} LBS and KV does not always abolish the ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion (Figs. 3.3, 3.5) suggesting that other mechanisms may also contribute to inhibition of pericellular plasminogen activation. We previously found that apo(a) can bind to plasminogen in solution [34, 37] which may inhibit the ability of tPA to cleave plasminogen in solution.

Previous results have shown that the protease domain of apo(a) is crucial for its ability to bind to plasminogen in solution, whereas the strong LBS in KIV_{10} had no effect on this interaction [34]. The role of KV in this solution phase interaction is not known. In the present study, removal of the strong LBS abolishes the inhibition of Glu- to Lys-plasminogen conversion on HUVECs and to a less extent on SMCs whereas removal of the protease domain only partially decreases inhibition of conversion on HUVECs. These results suggest that the predominant mechanism of the ability of apo(a) to inhibit conversion is not due to the interaction of apo(a) and plasminogen in solution. Furthermore, previous studies have shown that apo(a) can bind to Glu-plasminogen with high affinity (K_D ~0.3 μM) but to Lys-plasminogen with much lower affinity (K_D >50 μM) [37]. It is likely that apo(a) is inhibiting conversion either (i) through binding to Glu-plasminogen on the cell surface and inhibiting its ability to be cleaved by plasmin; or (ii) through preventing Glu-plasminogen from binding to the cell surface. Lp(a) has been previously shown to compete with plasminogen for binding to various cells [38]. Our

own work showed that 17K apo(a) could decrease binding of plasminogen to HUVECs and THP-1 cells, although plasminogen could not decrease 17K binding [18]. The role of the solution phase interaction between apo(a) and plasminogen on these effects is unclear. In light of the limited effect of removal of the protease domain in the current work, it is clear that there are complexities in the binding of apo(a) and plasminogen – both to each other and to cells – that will require a more detailed analysis with our r-apo(a) variants.

Various apo(a) isoform sizes were also analyzed in order to determine the importance of the number of KIV_2 domains in inhibiting conversion. Smaller isoforms of apo(a) – <22 KIV domains – have been identified as a risk factor for CHD [39]. While one study found that smaller isoforms are a risk factor for early atherosclerosis irrespective of Lp(a) concentration [40], the extent to which small apo(a) isoforms are a truly independent risk factor remains unclear. Lp(a) species containing smaller apo(a) isoforms bind more avidly to fibrin [41] and appear to cause hypofibrinolysis [42]. It has also been shown that smaller isoforms of Lp(a) bind with higher affinity to the surface of THP-1 monocytes [43]. However, the five isoforms analyzed in this study, ranging in size from 12 to 30 KIV domains, were able to inhibit conversion to similar extents on HUVECs. These results corroborate previous findings that showed no effect of isoform size on the ability of apo(a) to inhibit plasminogen activation on vascular cells [18] and Glu- to Lys-plasminogen conversion on fibrin surface [17].

We have described here a novel mechanism underlying the ability of apo(a) to inhibit pericellular plasminogen activation: attenuation of plasmin-mediated Glu- to Lys-plasminogen conversion on the vascular cell surface. The effect of apo(a) on conversion

appears to account for most, if not all, of the inhibitory effect of apo(a) on plasminogen activation. Therefore, our data generally support a model where the major effect of cell surfaces on plasminogen activation is to promote Glu- to Lys-plasminogen conversion [26, 32]. In the context of vascular cells, our findings would reflect the ability of apo(a) to influence dissolution of mural fibrin clots as well as cell migration and proliferation, angiogenesis, wound healing, and tumor cell invasion and metastasis.

3.6 REFERENCES

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CHAPTER 4

Inhibition of pericellular plasminogen activation by apolipoprotein(a): roles of urokinase plasminogen activator receptor and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$

4.1 SUMMARY

Lipoprotein(a) (Lp(a)) is an independent risk factor for numerous cardiovascular disorders including coronary heart disease. Apolipoprotein(a) (apo(a)), the unique glycoprotein component of Lp(a), contains sequences homologous to plasminogen. Plasminogen activation is markedly accelerated in the presence of cell surface receptors and can be inhibited in this context by apo(a). We evaluated here the role of potential receptors in regulating plasminogen activation and the ability of apo(a) to mediate inhibition of plasminogen activation on vascular cells as well as plasminogen and apo(a) binding to cells. The urokinase-type plasminogen activator receptor (uPAR) modulates plasminogen activation as well as plasminogen and apo(a) binding on human umbilical vein endothelial cells (HUVECs), human acute monocytic leukemia (THP-1) cells, and THP-1 macrophages as determined through uPAR knockdown and overexpression. Apo(a) variants lacking either the kringle V or the strong lysine binding site in kringle IV type 10 are not able to bind to uPAR to the same extent as wild-type apo(a). Plasminogen activation is also modulated, albeit to a lower extent, through the MAC-1 ($\alpha_M\beta_2$) integrin on HUVECs and THP-1 monocytes. Integrin $\alpha_V\beta_3$ can regulate plasminogen activation on THP-1 monocytes and to a lesser extent on HUVECs. The ability of apo(a) to inhibit plasminogen activation is abolished after blocking either the α_M, β_2, or $\alpha_V\beta_3$ integrins on HUVECs and THP-1 monocytes. These results indicate cell type-specific roles for uPAR, $\alpha_M\beta_2$, and $\alpha_V\beta_3$ in promoting plasminogen activation and in mediating the inhibitory effects of apo(a) on this process.

4.2 BACKGROUND

Elevated levels of plasma lipoprotein(a) (Lp(a)) have been identified as an independent risk factor for cardiovascular disorders such as coronary heart disease (CHD), ischemic stroke, and peripheral arterial disease (PAD) [1-5]. The structure of Lp(a) is similar to that of LDL, in that it contains apolipoprotein B-100 (apoB-100) as well as an identical lipid content [6], but differs from LDL by virtue of the unique glycoprotein apolipoprotein(a). Cloning of apo(a) revealed a high degree of sequence similarity to that of the plasma zymogen plasminogen [7]. Apo(a) contains repeated copies of a sequence similar to that of plasminogen kringle IV (KIV) followed by sequences similar to the kringle V (KV) and protease domain of plasminogen. Unlike that of plasminogen, the protease domain in apo(a) is catalytically inactive [8]. Apo(a) KIV can also be further classified into 10 subtypes (KIV_1 to KIV_{10}) based on amino acid sequence. Each KIV subtype is present in single copy number except for KIV_2 which is present in identical repeated copies from 3 to >40 giving rise to the isoform size heterogeneity present in apo(a) [9, 10]. Apo(a) is covalently linked to apoB-100 via a single disulfide bond whose formation is preceded by interactions between apoB-100 and the weak lysine binding sites (LBSs) of apo(a) kringle IV types 6-8 [11, 12].

The homology between plasminogen and apo(a) has been suggested to underlie the pathogenic effects of Lp(a) whereby the apo(a) component interferes with the plasminogen activation system [13]. Plasmin, generated through plasminogen activation, is a key component not only of fibrinolysis but also of cell migration and proliferation, angiogenesis, inflammation, wound healing, and tumor cell invasion and metastasis [14-20]. Apo(a) and plasminogen both contain LBSs which mediate their respective

interactions with fibrin and various cell surface receptors [21]. Plasminogen activation is inherently inefficient, and biologically meaningful rates of plasmin formation require a cofactor in the form of fibrin or a cellular surface [22, 23]. Apo(a) is able to inhibit plasminogen activation within the fibrin clot, leading to impaired fibrinolysis [24]. Moreover, Lp(a) and apo(a) have been recently shown to be capable of inhibiting pericellular plasminogen activation on monocytes, macrophages, and vascular endothelial cells (ECs) [25]. To date, however, the identity of lysine-containing receptors that bind apo(a) and mediate its inhibition of plasminogen activation remain obscure. Apo(a) has been shown to stimulate cell growth and migration in ECs through integrin $\alpha_V\beta_3$ and Lp(a) can recruit inflammatory cells through MAC-1 ($\alpha_M\beta_2$) integrin; direct binding of apo(a) to the latter integrin has been demonstrated [26, 27].

The uPA receptor (uPAR) has been shown to be a key regulator in mediating plasminogen activation and extracellular matrix (ECM) degradation [28]. The glycosyl phosphatidylinositol (GPI)-anchored uPAR can bind to uPA with high affinity (1 nM) as well as the ECM protein vitronectin, a ligand of $\alpha_V\beta_3$ [29, 30]. Activation of pro-uPA to its active form is mediated through the binding of pro-uPA to uPAR [28, 31].

A variety of receptors have been identified on different cell types which can mediate both plasminogen activation as well as plasmin binding [14, 32]. Most plasminogen receptors are synthesized with a carboxyl terminal lysine or expose a lysine on the cell surface which can mimic a carboxyl-terminal lysine. The receptors that bind plasminogen are redundant and broadly distributed on various cells with binding sites ranging from 10^5 to 10^7 per cell [33]. Known receptors for plasminogen on vascular cells include annexin A2/p11, actin, $\alpha_V\beta_3$ integrin, histone H2B, $\alpha_M\beta_2$ integrin, Plg-R_{KT}, α-

enolase, $\alpha IIb\beta_3$, and the TAT-binding protein-interacting protein [14, 32-40]. Collectively, these receptors serve to accelerate plasminogen activation as well as to protect plasmin from its natural physiological inhibitor, α_2-antiplasmin.

In the present study, we describe the role of various specific receptors – namely uPAR, $\alpha_M\beta_2$, and $\alpha_V\beta_3$ – in regulating plasminogen activation as well as plasminogen and apo(a) binding.

4.3 METHODS

4.3.1 Construction, expression, and purification of recombinant apo(a)

The construction and expression of the various r-apo(a) variants have been previously described. The pRK5 expression vectors encoding 10K, 12K, 12ΔV, 17K, 17KΔLBS$_{10}$ and 17KΔV were stably transfected into human embryonic kidney (HEK) 293 cells as previously described [8, 41-46]. All r-apo(a) variants were purified from conditioned media by lysine-Sepharose affinity chromatography as previously described [8, 41-46]. All r-apo(a) protein concentrations were determined spectrophotometrically and assessed for purity by SDS-PAGE under non-reduced and reduced conditions followed by silver staining.

4.3.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) and human acute monocytic leukemia (THP-1) cells were cultured, and the latter cell line differentiated into macrophage-like cells, as described previously [25].

4.3.3 Plasmid Mutagenesis

A plasmid containing the cDNA encoding carboxyl-terminally GFP tagged human uPAR, in the pCMV-AC-GFP vector, was purchased from Origene (catalog number RG201222). The plasmid was mutated to introduce a stop codon immediately upstream of the GFP tag via site directed mutagenesis, thereby restoring the natural carboxyl-termine Thr reside in uPAR. Site directed mutagenesis of the plasmid was conducted using the QuikChange II-E kit (Stratagene) as per the manufacturer's recommendations with the following primers: forward 5'-TCT CCT CTG GAC CTA

GCG TAC GCG GCC G-3' and reverse 5'-CGG CCG CGT ACG CTA GGT CCA GAG GAG A-3'. Base pairs AC were mutated to TA as indicated to introduce the stop codon. The resultant plasmid was used in overexpression experiments.

4.3.4 Transient transfection

HUVECs, THP-1 monocytes, and THP-1 macrophages were transfected with uPAR siRNA or control siRNA (Santa Cruz Biotechnology) at a concentration of 40-80 nM with transfection reagent and medium from Santa Cruz Biotechnology following the manufacturer's protocols. Transfection mixture was removed following 5-6 hour incubation and replaced with complete medium. Cells were assayed 72 hours following transfection. Percent knockdown was determined using qRT-PCR (see below). HUVECs, THP-1 monocytes, and THP-1 macrophages were transiently transfected with uPAR plasmid using Lipofectamine LTX and Plus Reagent (Invitrogen) as per the manufacturer's protocol. HUVECs were transfected with 0.3 μg/mL of DNA and incubated with transfection mixture for 4 hours, after which the cells were incubated in fresh medium for 48 hours prior to assay. THP-1 monocytes and macrophages were transfected with 0.75 μg/mL of DNA and incubated with transfection mixture for 24 hours, after which the cells were incubated in fresh medium for 48 hours prior to assay. Overexpression of uPAR was verified with western blot analysis.

4.3.5 Plasminogen activation and cell binding assays

Plasminogen activation experiments were performed as previously described [25], and utilized the fluorogenic plasmin substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (Bachem). The rate of plasminogen activation was taken between 10 and

40 min from the slope of relative fluorescent units (RFU) against min^2. Rates obtained for each individual experiment performed in triplicate were normalized to the rate of plasminogen activation in the absence of apo(a). For experiments using blocking monoclonal antibodies, prior to assay of plasminogen activation, cells were washed three times with blocking solution (10 mM Tris-HCl, pH 7.4,, 0.14 mM NaCl, 0.1% (w/v) BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) followed by incubation with mouse anti-human integrin $\alpha_V\beta_3$ (Millipore), mouse-anti human CD18 clone IB4 (Labs Inc Biotechnology), and/or mouse-anti human CD11b clone 44aacb (Imgenex) for one hour at 37°C. Cells were then washed three times with blocking solution followed by plasminogen activation assay as described above.

Cell binding assays used purified apo(a), fluorescently labeled using Alexa Fluor 488 protein labeling kit (Invitrogen), or fluorescein isothiocyanate-labeled human plasminogen (purchased from Molecular Innovations) as previously described [25].

4.3.6 qRT-PCR

Determination of uPAR knockdown efficiency was determined through iScript one-step RT-PCR kit with SYBR® green (Biorad). Total RNA was isolated from the transfected cells using RNeasy Plus Mini kit (QIAGEN). RT-PCR was executed using the following conditions: reverse transcription at 50°C for 30 min followed by heat inactivation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing/extension at 59°C for 1 min. The data were analyzed using Bio-Rad CFX Manager software version 3.1. The following primers were used: uPAR forward: 5'-GCC CAA TCC TGG AGC TTG A-3'; uPAR reverse: 5'-TCC CCT TGC AGC TGT AAC

ACT-3’; GAPDH forward: 5’-CTG CTC CTC CTG TTC GAC AGT-3’; GAPDH reverse: 5’-CCG TTG ACT CCG ACC TTC AC-3’.

4.3.7 Western Blotting

Determination of uPAR overexpression was determined through western blot analysis. Cells were lysed (50 mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) and samples (50 μg total protein per lane) were subjected to SDS-PAGE on 12% polyacrylamide gels under non-reducing conditions, after which the resolved proteins were transferred to an Immobilon-P transfer membrane (Millipore). Blocking of the membrane and incubation with primary and secondary antibodies was performed in 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween (TBST; 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and washing was performed with TBST. The primary antibody was polyclonal rabbit anti-human uPA Receptor (American Diagnostica) and secondary antibody was goat-anti rabbit IgG (Santa Cruz Biotechnology). SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biochemical) was used for the detection of bands, which were visualized with a FluorChem Q system (Alpha Innotech).

4.3.8 Statistical analysis

Comparisons were performed using the two-tailed Student's t-test assuming unequal variances.

4.4 RESULTS

4.4.1 Effect of uPAR knockdown on plasminogen activation on HUVECs, its inhibition by apo(a), and plasminogen and apo(a) binding

Analysis of uPAR mRNA expression indicates that uPAR knockdown was approximately 80% effective in HUVECs (Fig. 4.1A). Plasminogen activation decreases approximately 20-30% with the addition of 12K r-apo(a) (Fig. 4.1B). Removal of the strong LBS in KIV_{10} ($17K\Delta LBS_{10}$) or the kringle V domain ($17K\Delta V$) paradoxically increases the rate of plasminogen activation. As we have previously described [25], this effect is attributable to increased solution-phase (i.e. cell-independent) plasminogen activation promoted by these variants that does not occur with wild-type apo(a). The KV domain and strong LBS in KIV_{10} have also been previously shown to be required for optimal ability for apo(a) to inhibit plasminogen activation on the fibrin surface [24].

Knockdown of uPAR expression using siRNA results in a 22% decrease in plasminogen activation on HUVECs (Fig. 4.1B). However, no further significant decrease in activation is observed in the presence of apo(a) in the setting of uPAR knockdown. By contrast, uPAR knockdown had no effect on the increase in cell-independent plasminogen activation elicited by $17K\Delta LBS_{10}$ or $17K\Delta V$ (Fig. 4.1B).

Binding of plasminogen and apo(a) also decreased on HUVECs following uPAR knockdown (Fig. 4.1C-E). On the other hand, uPAR knockdown had no effect on $17K\Delta LBS_{10}$ or $17K\Delta V$ binding. Note that comparable binding was observed for wild-type apo(a) and the variants $17K\Delta LBS_{10}$ and $17K\Delta V$ in the absence of uPAR knockdown (data not shown), a result in keeping with previous findings which indicated that 17K and $17K\Delta LBS_{10}$ can bind equally well to HUVECs [47]. Interestingly, the lysine analogue ε-

ACA only decreased binding of plasminogen by about 50% (Fig. 4.1D) but appeared to increase 17K binding (Fig. 4.1E). Both apo(a) and plasminogen binding became insensitive to ε-ACA in the context of uPAR knockdown (Fig. 4.1D, E).

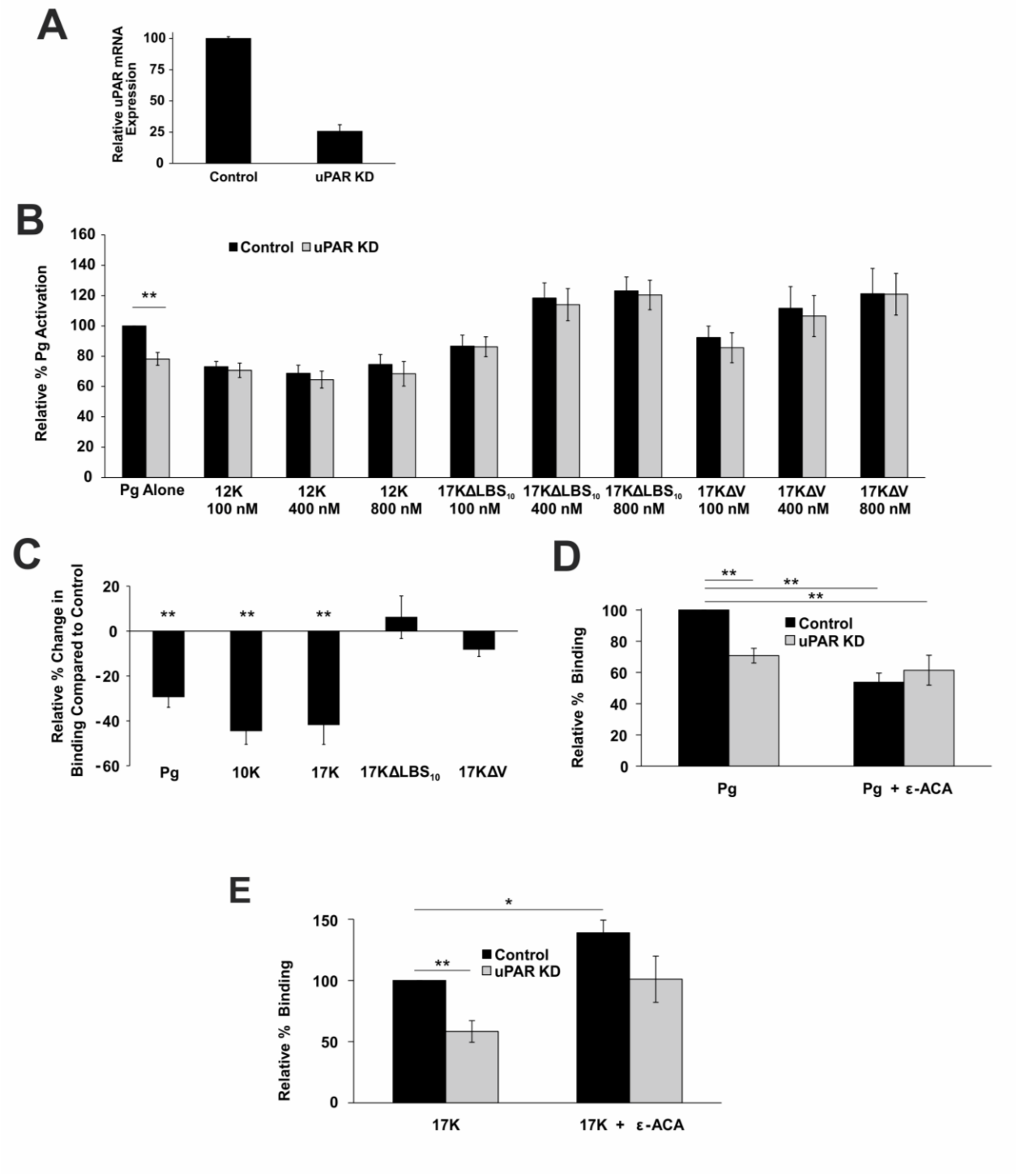

A
Relative uPAR mRNA Expression
100
75
50
25
0
Control
uPAR KD
B
Relative % Pg Activation
Control
uPAR KD
**
160
140
120
100
80
60
40
20
0
Pg Alone
12K 100 nM
12K 400 nM
12K 800 nM
17KΔLBS10 100 nM
17KΔLBS10 400 nM
17KΔLBS10 800 nM
17KΔV 100 nM
17KΔV 400 nM
17KΔV 800 nM
C
Relative % Change in Binding Compared to Control
20
0
-20
-40
-60
**
**
**
Pg
10K
17K
17KΔLBS10
17KΔV
D
Relative % Binding
**
**
**
100
80
60
40
20
0
Control
uPAR KD
Pg
Pg + ε-ACA
E
Relative % Binding
*
**
150
100
50
0
Control
uPAR KD
17K
17K + ε-ACA

Figure 4.1: ***Effect of uPAR knockdown in HUVECs on plasminogen activation and r-apo(a) and plasminogen binding***. Passage 5 HUVECs were transfected with uPAR siRNA for 72 hours followed by plasminogen activation and cell binding analysis. (**A**) Quantitative RT-PCR data showing a decrease in uPAR gene expression following uPAR knockdown. (**B**) The effect of uPAR knockdown on plasminogen activation on HUVECs was assessed in the absence of apo(a) or in the presence of the indicated quantities of 12K r-apo(a) or the mutants variants 17KΔLBS$_{10}$ or 17KΔV. (**C**) Effect of uPAR knockdown on the binding of plasminogen and r-apo(a) to HUVECs, expressed as the percent change in binding as a result of uPAR knockdown. (**D** and **E**) Binding data for plasminogen and 17K r-apo(a), respectively along with the effects of ε-ACA on the binding with uPAR knockdown. Error bars indicate standard error of the mean (SEM) with n≥3 independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$.

4.4.2 Effect of overexpression of uPAR in HUVECs on plasminogen activation, its inhibition by apo(a), and plasminogen and apo(a) binding

uPAR was successfully overexpressed in HUVECs as can be observed from the western blot against uPAR presented in Fig. 4.2A. Overexpression of uPAR results in a 31% increase in plasminogen activation (Fig. 4.2B) in the absence of apo(a). As was observed in Fig. 4.1B, apo(a) inhibited plasminogen activation. However, in the presence of apo(a) there was no increase in plasminogen activation in the context of overexpression of uPAR (Fig. 4.2B). These results corroborate our findings with uPAR knockdown: in both situations it is apparent that apo(a) regulates plasminogen activation via uPAR. Removal of the strong LBS in apo(a) abolishes the inhibitory effect on plasminogen activation, and overexpression of uPAR results in no significant change in plasminogen activation in the presence of this mutant (Fig. 4.2B). Overexpression of uPAR leads to increases in the binding of plasminogen, 12K, 17K and both mutants of 17K (Fig. 4.2C). Interestingly, the effect of uPAR overexpression on plasminogen binding to HUVECs is markedly reduced by the addition of ε-ACA (Fig. 4.2D) but not the effect of uPAR overexpression on 17K binding (Fig. 4.2E).

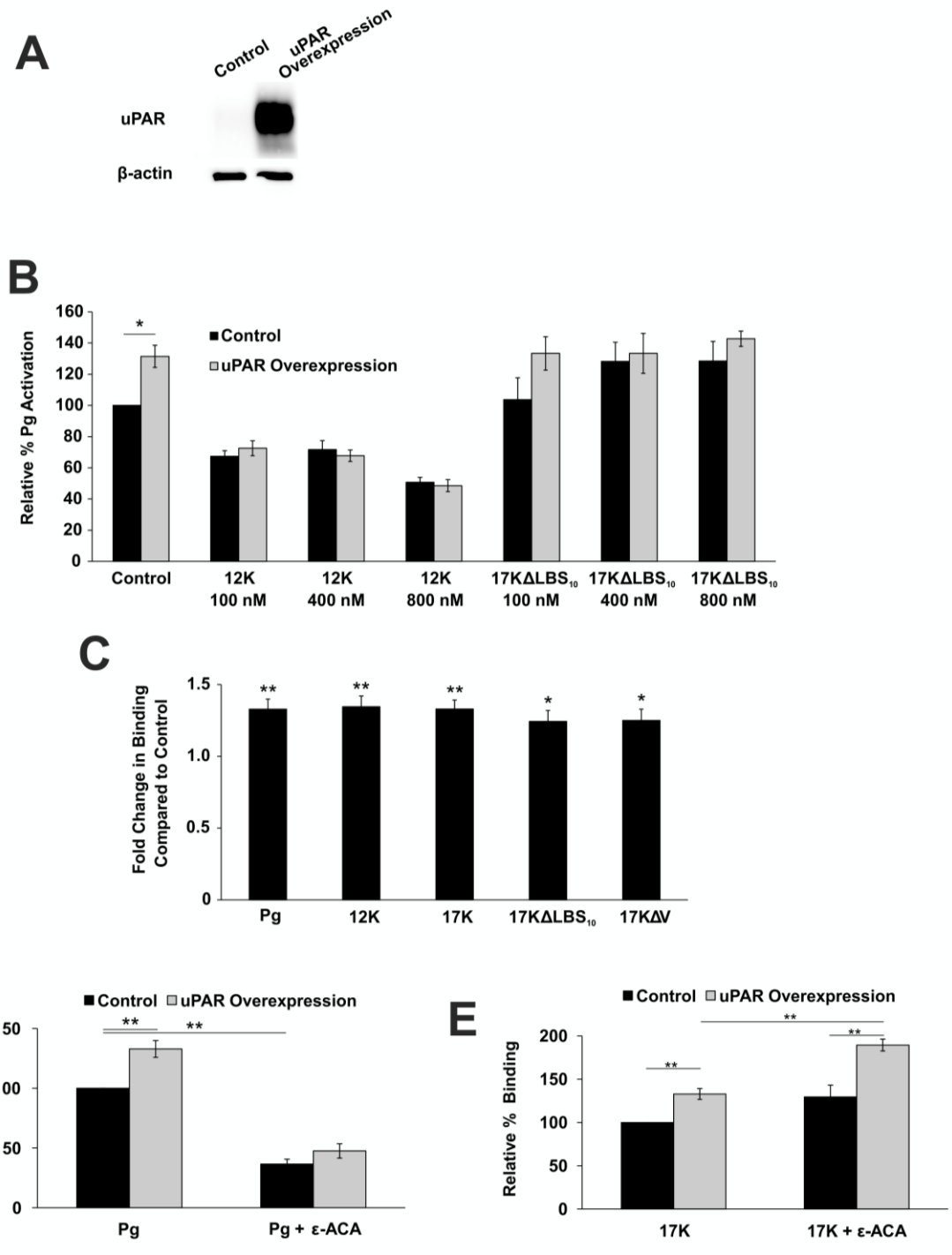

A
Control
uPAR Overexpression
uPAR
β-actin
B
Relative % Pg Activation
Control
uPAR Overexpression
Control
12K 100 nM
12K 400 nM
12K 800 nM
17KΔLBS10 100 nM
17KΔLBS10 400 nM
17KΔLBS10 800 nM
C
Fold Change in Binding Compared to Control
Pg
12K
17K
17KΔLBS10
17KΔV
D
Control
uPAR Overexpression
Relative % Binding
Pg
Pg + ε-ACA
E
Control
uPAR Overexpression
Relative % Binding
17K
17K + ε-ACA

Figure 4.2: ***Effect of uPAR overexpression in plasminogen activation and r-apo(a) and plasminogen binding on HUVECs***. Passage 5 HUVECs were transfected with uPAR for 48 hours followed by plasminogen activation and cell binding analysis. (**A**) Western blot showing uPAR overexpression in HUVECs with β-actin as loading control. (**B**) Plasminogen activation was measured in the presence or absence of the indicated concentrations of 12K r-apo(a) or the 17KΔLBS$_{10}$ variant. (**C**) Effect of uPAR overexpression on the binding of plasminogen and r-apo(a) variants to HUVECs, expressed as a fold change in binding as a result of uPAR overexpression compared to control. (**D** and **E**) Binding data for plasminogen and 17K r-apo(a), respectively, along with the effects of ε-ACA on the binding with uPAR overexpression. Error bars indicate SEM with n≥3 independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$.

4.4.3 Effect of overexpression of uPAR in THP-1 monocytes on plasminogen activation, its inhibition by apo(a), and plasminogen and apo(a) binding

uPAR was successfully overexpressed in THP-1 monocytes as can be observed from the western blot against uPAR shown in Fig. 4.3A. Overexpression of uPAR in these cells leads to a more than two-fold increase in plasminogen activation (Fig. 4.3B). Plasminogen activation is reduced with the addition of increasing concentrations of 12K or 17K whereas the inhibitory effect is abolished with the removal of KV or the strong LBS (Fig. 4.3B, C). In contrast to the results in HUVECs, an increase in plasminogen activation is observed following uPAR overexpression in the presence of apo(a) versus control. However, the extent of the increase declines at higher concentrations of 12K, but still remains significantly higher with 800 nM 12K. Similarly, increasing concentrations of 17K did not blunt the increase in activation observed upon uPAR overexpression (compare Figs. 4.3B and 4.3C). Addition of increasing concentrations of 12KΔV or $17K\Delta LBS_{10}$ attenuated the extent to which uPAR overexpression increased plasminogen activation compared to wild-type 12K or 17K (Fig. 4.3B, C).

Overexpression of uPAR substantially increases plasminogen, 12K and 17K binding in THP-1 monocytes (Fig. 4.3D), whereas only a non-significant increase in binding of $17K\Delta LBS_{10}$ and 17KΔV was observed. As with HUVECs, the mutants can bind to THP-1 monocytes in a manner comparable to wild-type apo(a) (data not shown). Addition of ε-ACA dramatically decreased binding of both plasminogen and 17K to THP-1 monocytes (Fig. 4.3E, F), in contrast to what we observed on HUVECs (Fig. 4.2D, E).

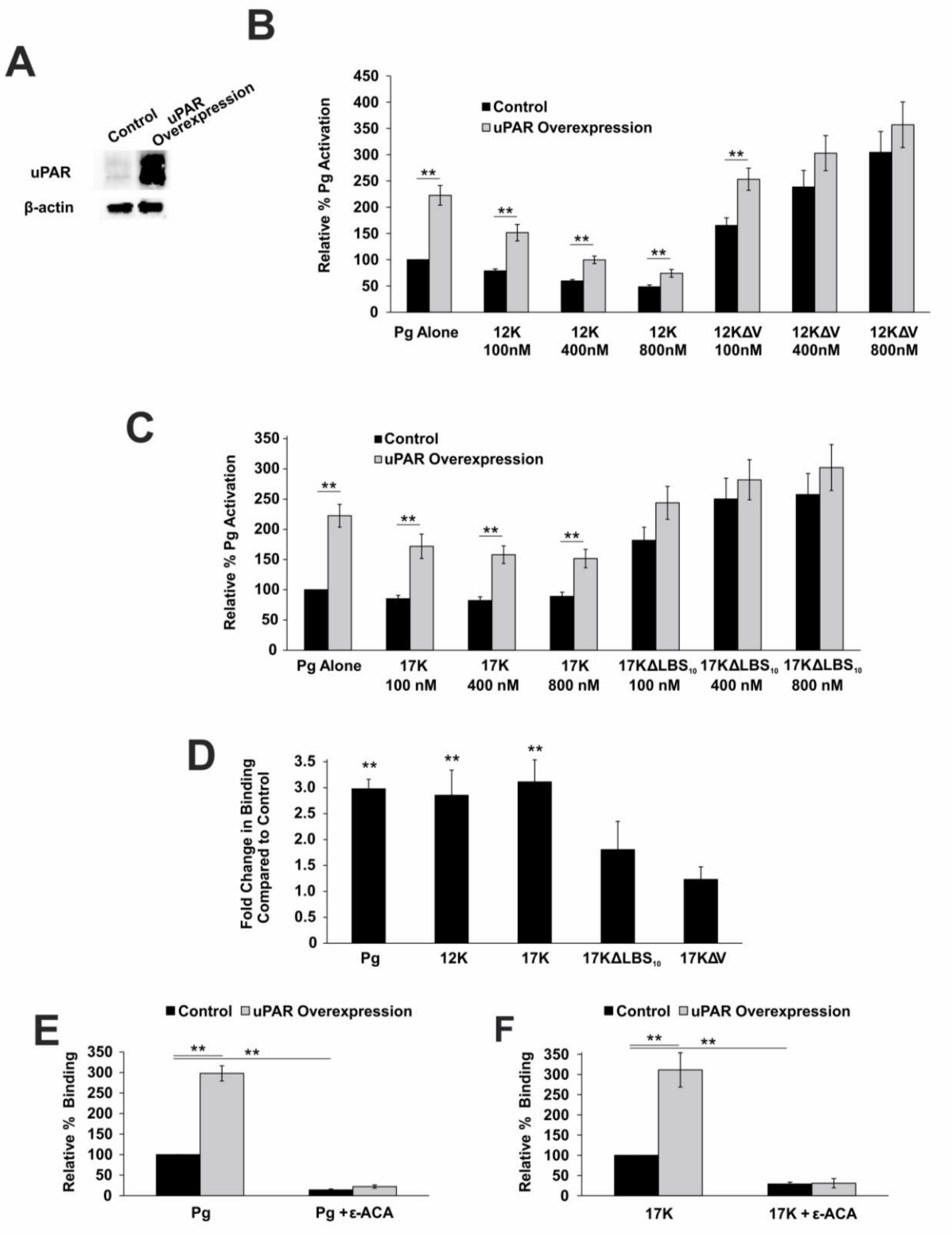
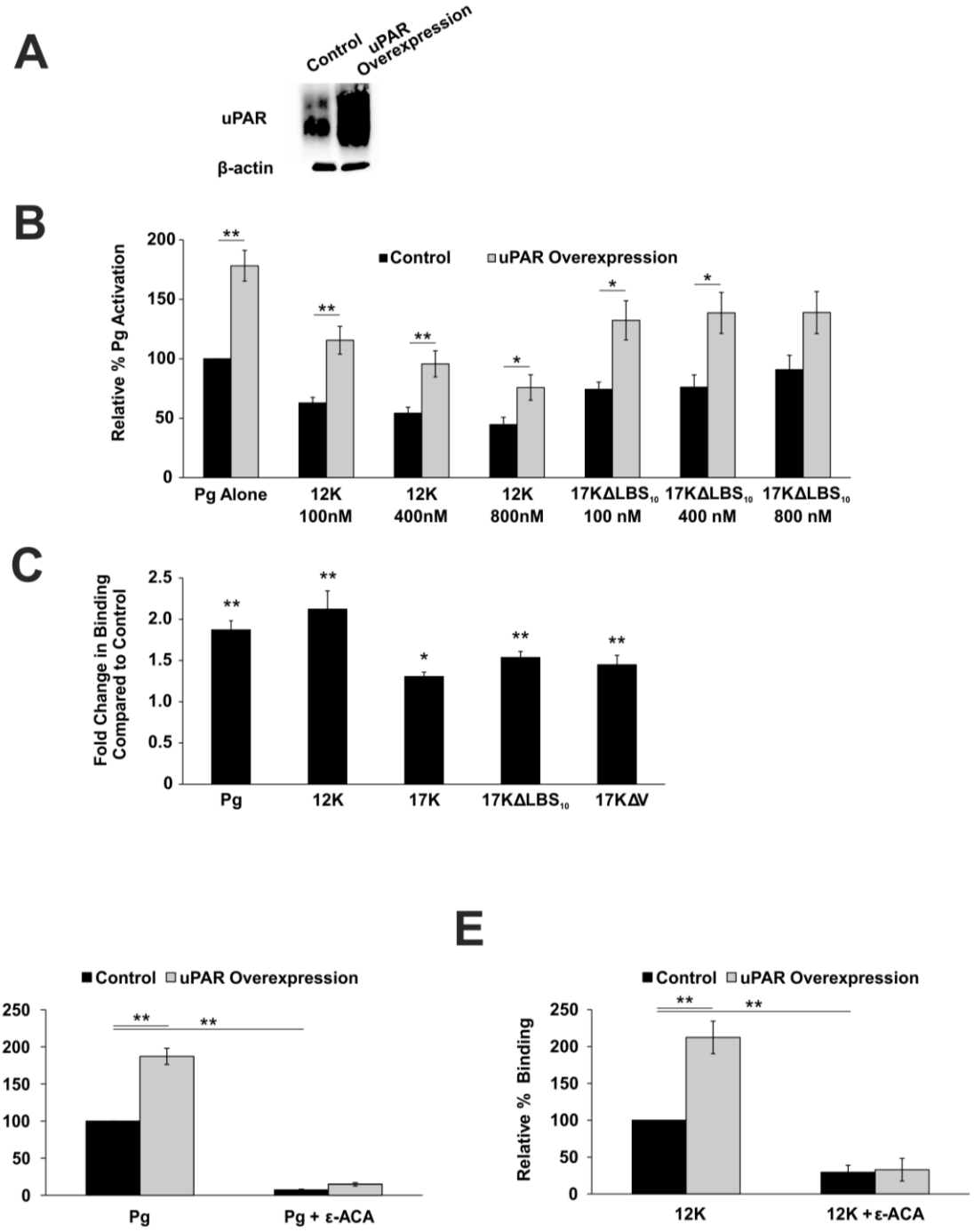

A
Control
uPAR Overexpression
uPAR
β-actin
B
Relative % Pg Activation
Control
uPAR Overexpression
Pg Alone
12K 100nM
12K 400nM
12K 800nM
12KΔV 100nM
12KΔV 400nM
12KΔV 800nM
C
Relative % Pg Activation
Control
uPAR Overexpression
Pg Alone
17K 100 nM
17K 400 nM
17K 800 nM
17KΔLBS10 100 nM
17KΔLBS10 400 nM
17KΔLBS10 800 nM
D
Fold Change in Binding Compared to Control
Pg
12K
17K
17KΔLBS10
17KΔV
E
Control
uPAR Overexpression
Relative % Binding
Pg
Pg +ε-ACA
F
Control
uPAR Overexpression
Relative % Binding
17K
17K + ε-ACA

Figure 4.3: ***Effect of uPAR overexpression in plasminogen activation and r-apo(a) and plasminogen binding on THP-1 monocytes***. THP-1 monocytes were transfected with uPAR for 48 hours followed by plasminogen activation and cell binding assay. (**A**) Western blot showing uPAR overexpression in THP-1 monocytes with β-actin as loading control. The data are shown for the effect of uPAR overexpression on plasminogen activation on THP-1 monocytes in the presence of various concentrations of 12K and 12KΔV (**B**) and 17K and 17KΔLBS$_{10}$ (**C**). (**D**) Effect of uPAR overexpression on the binding of plasminogen and r-apo(a) variants to THP-1 monocytes, expressed as the fold change in binding as a result of uPAR overexpression. (**E** and **F**) Binding data for plasminogen and 17K r-apo(a), respectively, along with the effects of ε-ACA on the binding with uPAR overexpression. Error bars indicate SEM with n≥3 independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$.

4.4.4 Effect of overexpression of uPAR in THP-1 macrophages on plasminogen activation, its inhibition by apo(a), and plasminogen and apo(a) binding

An increase in the expression of uPAR is known to occur following differentiation of THP-1 monocytes into macrophages [48]. Western blot analysis of lysates from control THP-1 macrophages (Fig. 4.4A) indicates a more prominent band than was observed in control HUVECs or THP-1 monocytes (Figs. 4.2A and 4.3A). Accordingly, plasminogen activation increased by a lesser extent following uPAR overexpression (Fig. 4.4B) (1.6-fold versus a 2-fold increase in THP-1 monocytes). As was observed in THP-1 monocytes, increasing concentrations of 12K decreased plasminogen activation but in THP-1 macrophages had a reduced effect on the extent to which plasminogen activation was enhanced by uPAR overexpression (Fig. 4.4B). Interestingly, addition of increasing concentrations of $17K\Delta LBS_{10}$ did not increase plasminogen activation as was observed in the other cell types (Fig. 4.4B) and higher concentrations of $17K\Delta LBS_{10}$ seemed to blunt the effect of uPAR overexpression on plasminogen activation. Also in contrast to THP-1 monocytes, binding of $17K\Delta LBS_{10}$ and 17KΔV was impacted by uPAR overexpression to a similar extent as 17K (Fig. 4.4C). The effect of ε-ACA on plasminogen and apo(a) binding was similar to that seen in THP-1 monocytes (Fig. 4.4D, E).


A
Control
uPAR Overexpression
uPAR
β-actin
B
Relative % Pg Activation
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17KΔLBS10 100 nM
17KΔLBS10 400 nM
17KΔLBS10 800 nM
C
Fold Change in Binding Compared to Control
Pg
12K
17K
17KΔLBS10
17KΔV
D
Control
uPAR Overexpression
Relative % Binding
Pg
Pg + ε-ACA
E
Control
uPAR Overexpression
Relative % Binding
12K
12K + ε-ACA

Figure 4.4: ***Role of uPAR in plasminogen activation and r-apo(a) and plasminogen binding on THP-1 macrophages as determined through uPAR overexpression***. THP-1 monocytes were differentiated into macrophage like cells with PMA for 72 hours followed by transfection of uPAR for 48 hours. (**A**) Western blot showing the successful overexpression of uPAR in THP-1 macrophages with β-actin as loading control. (**B**) The data are shown for the effect of uPAR overexpression on plasminogen activation on THP-1 macrophages in the presence of various concentrations of 12K r-apo(a) or the variants $17K\Delta LBS_{10}$. (**C**) Effect of uPAR overexpression on the binding of plasminogen and r-apo(a) variants to THP-1 macrophages, expressed as the fold change in binding as a result of uPAR overexpression. (**D** and **E**) Binding data for plasminogen and 12K r-apo(a), respectively, along with the effects of ε-ACA on the binding with uPAR overexpression. Error bars indicate SEM with $n \geq 3$ independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$.

4.4.5 Role of MAC-1 in regulating plasminogen activation on HUVECs and THP-1 monocytes

MAC-1 consists of the integrin subunits α_M and β_2. Both α_M and β_2 on HUVECs were blocked individually with monoclonal antibodies; this resulted in a reduction in plasminogen activation by a small but significant extent (Fig. 4.5A). Addition of 12K r-apo(a) resulted in a decrease in plasminogen activation compared to control, but addition of either monoclonal antibody resulted in no further significant decrease in plasminogen activation. These data suggest that the effect of 12K is mediated in part through MAC-1. Essentially identical results were obtained when the experiments were conducted using THP-1 monocytes (Fig. 4.5B).

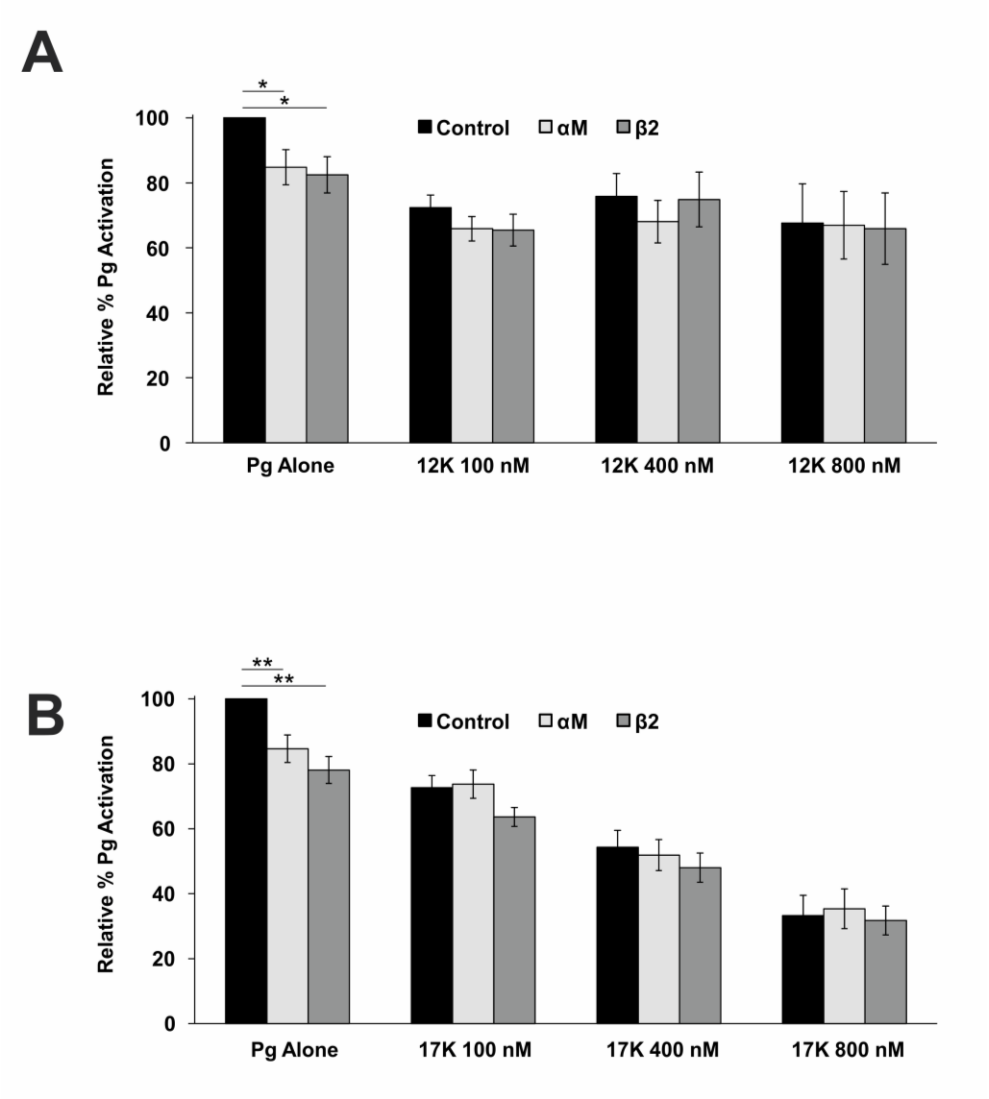

Figure 4.5: ***Role of α_M and β_2 in plasminogen activation on HUVECs and THP-1 monocytes***. The α_M and β_2 receptors were blocked with mouse-anti human CD18 and mouse-anti human CD11b, respectively, for 1 hour at 37°C. Plasminogen activation data for HUVECs (**A**) and THP-1 monocytes (**B**) are shown in the presence or absence of different concentrations of r-apo(a). Error bars indicate SEM with n≥3 independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$

4.4.6 Role of integrin $\alpha_V\beta_3$ in regulating plasminogen activation on HUVECs and THP-1 monocytes

Blocking $\alpha_V\beta_3$ with a monoclonal antibody reduced plasminogen activation by a very small but significant extent (10%) in HUVECs (Fig. 4.6A). Addition of the antibody in the presence of 17K resulted in no further effect. Using THP-1 monocytes, blocking of $\alpha_V\beta_3$ had a much greater effect on plasminogen activation (Fig. 46B). Apo(a) reduced plasminogen activation in a dose-dependent manner on these cells; the inhibitory effect of $\alpha_V\beta_3$ blockade disappeared at the higher concentrations of apo(a). These findings suggest a key role for this integrin in stimulating plasminogen activation on THP-1 monocytes and in mediating the inhibitory effects of apo(a) on pericellular plasminogen activation on these cells.

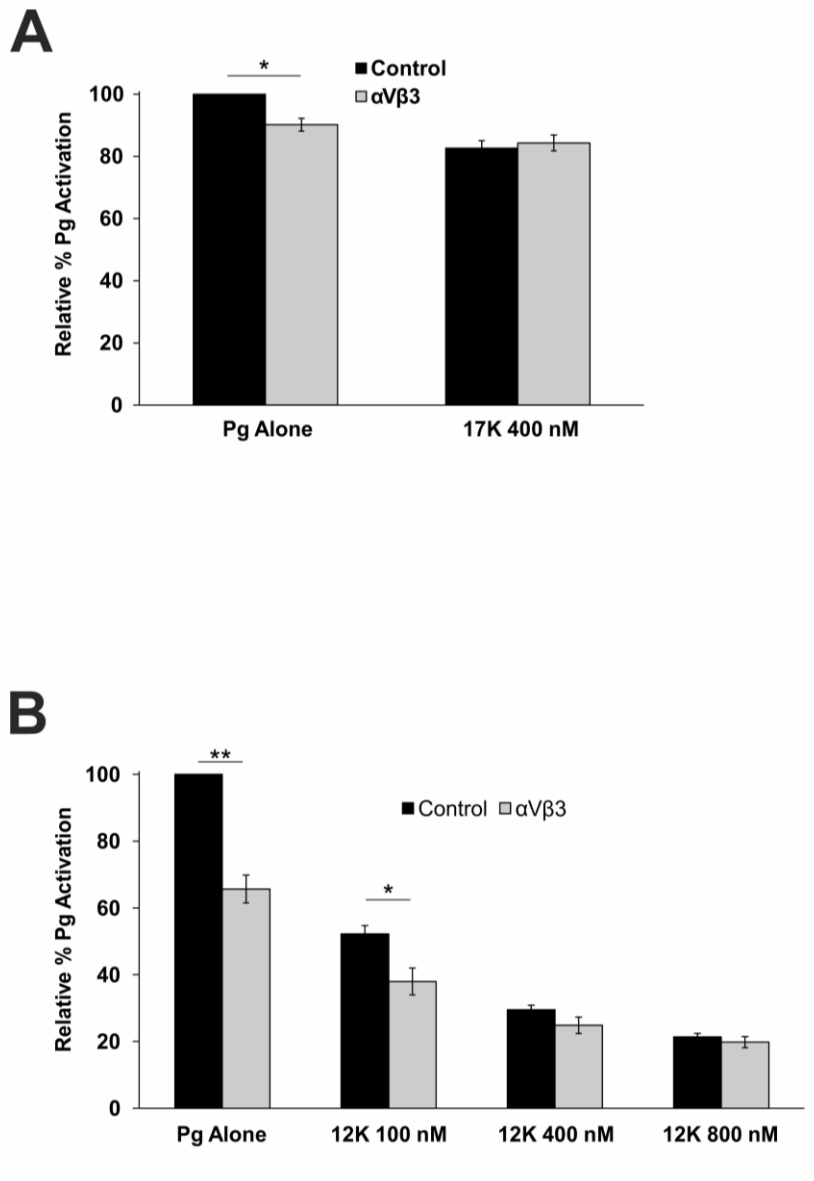

Figure 4.6: Role of $\alpha_V\beta_3$ in regulating pericellular plasminogen activation in THP-1 monocytes and HUVECs. Cells were treated with mouse anti-human integrin $\alpha_V\beta_3$ monoclonal antibody for one hour at 37°C to block this receptor. Plasminogen activation was then measured in HUVECs (**A**) or THP-1 monocytes (**B**). Error bars indicate SEM with n≥3 independent experiments performed in triplicates. *: $p<0.05$ **: $p<0.01$

4.5 DISCUSSION

Plasminogen receptors play a key role in regulating plasminogen activation leading to cell migration and proliferation, angiogenesis, inflammation, wound healing, and tumor cell invasion and metastasis [14-20]. To date, various plasminogen receptors have been identified on different vascular and blood cells and have been shown to be heterogeneous in nature. The roles of these receptors in apo(a)-mediated inhibition of plasminogen activation is less well-understood. The $\alpha_M\beta_2$ and $\alpha_V\beta_3$ integrins have been shown to mediate the effects of Lp(a) and apo(a), respectively, on recruitment of inflammatory cells and stimulation of cell growth and migration in ECs [26, 27]. Lp(a)/apo(a) has been previously shown to reduce plasminogen binding to monocytes and ECs [21, 49-51]. Moreover, binding of apo(a) to unidentified plasminogen receptors on hepatoma cells, fibroblasts, and platelets has been documented [52, 53]. It has been recently shown that Lp(a)/apo(a) can affect plasminogen activation on vascular cells [25]. However, the roles of specific plasminogen receptors in this process have yet to be explored. We have shown here that uPAR and the $\alpha_M\beta_2$ and $\alpha_V\beta_3$ integrins regulate plasminogen activation, and can mediate the ability of apo(a) to inhibit pericellular plasminogen activation.

We demonstrate that the GPI-anchored uPAR accelerates tPA-mediated plasminogen activation on the cell surface of HUVECs (Figs. 4.1 and 4.2), THP-1 monocytes (Fig. 4.3), and THP-1 macrophages (Fig. 4.4) as determined through either uPAR overexpression or knockdown. uPAR has been previously shown to bind to uPA, vitronectin, and β_3 integrins [54]. However, plasminogen, apo(a), and tPA have not previously been shown to bind to uPAR. Studies have indicated that apo(a) can bind to

the ECM protein fibronectin but not to vitronectin [55]. uPAR is involved in the remodeling of the ECM through regulation of plasminogen activation. This occurs through uPA binding to uPAR in close proximity to the cell surface where plasminogen binds to its respective receptors, thereby accelerating plasmin formation [54]. Our current results indicate that uPAR can directly regulate tPA-mediated plasminogen activation on vascular cells since knockdown or overexpression of uPAR altered the rate of plasminogen activation (Figs. 4.1-4.4). Furthermore, binding of both plasminogen and apo(a) increased following uPAR overexpression in HUVECs, THP-1 monocytes, and THP-1 macrophages. The mechanism underlying each of these observations remains to be fully elucidated: it is not clear if tPA, plasminogen or apo(a) directly bind uPAR or another protein (such as an integrin) to which uPAR may be bound.

We have recently directly demonstrated that apo(a) can inhibit plasminogen activation on the surface of ECs, monocytes and macrophages [25]. The mechanism by which apo(a) may inhibit plasminogen activation on the cell surface has not been fully explored to date. Previous studies indicated that apo(a) could decrease the binding of plasminogen to the cell surface by competition for binding sites [21, 49-51]. A key role for the strong LBS in KIV_{10} in binding to U937 monocytoid cells has been described [56]. Our previous study found only modest, cell-type-specific effects of carboxypeptidase B treatment on apo(a) and plasminogen binding as well as on plasminogen activation and its inhibition by apo(a) [25]. The current study integrates these findings by demonstrating roles in plasminogen activation and its inhibition by apo(a) of three receptors that lack carboxyl-terminal lysine residues: uPAR, MAC-1 and

$\alpha_V\beta_3$. It is possible, however, that these receptors contain internal lysines that modulate plasminogen activation and plasminogen and apo(a) binding.

In our previous findings we also identified key roles for apo(a) KV and the strong LBS in KIV_{10}, in that mutations in these sequences abolished the inhibitory effect of apo(a) and cell-surface plasminogen activation [25]. In HUVECs, uPAR knockdown or overexpression results in a decrease or increase, respectively, in plasminogen binding and activation as well as apo(a) binding (Figs. 4.1 and 4.2). Inhibition of plasminogen activation by apo(a) is dependent on kringle V and the strong LBS in KIV_{10} (Figs. 4.1 and 4.2). Previous studies have underscored the importance of both kringle V and the KIV_{10} strong LBS in inhibiting fibrinolysis [24]. While overexpression of uPAR increases plasminogen activation on HUVECs, this increase is abolished in the presence of apo(a) (Fig. 4.2). These results indicate that apo(a) can inhibit plasminogen activation via uPAR on HUVECs with critical roles for kringle V and the strong LBS in KIV_{10} in mediating this effect. It appears that the LBSs are not crucial for apo(a) to bind to uPAR on HUVECs since addition of the lysine analogue ε-ACA does not influence the ability of uPAR overexpression to increase apo(a) binding (Fig. 4.2). The role of uPAR seems to be cell-specific, since the effects of overexpression of uPAR in either THP-1 macrophages or monocytes are distinct from those documented in HUVECs. This suggests that uPAR is potentially interacting with another cell-specific receptor which varies the ability of apo(a) to both bind to and to inhibit plasminogen activation via uPAR. Both plasminogen and apo(a) binding increase following uPAR overexpression in all cell types studied (Figs.. 4.2C, 4.3D, 4.4C). However, the kringle V domain and the

strong LBS in KIV_{10} of apo(a) contribute to uPAR binding in HUVECs and THP-1 monocytes but not in THP-1 macrophages.

Interestingly, however, apo(a) does not inhibit plasminogen activation via uPAR on either THP-1 monocytes or macrophages (Fig. 4.3B, 4.3C, and 4.4B) as was shown using HUVECs. This is demonstrated by the percentage difference in plasminogen activation upon uPAR expression being unaffected by the addition of increasing concentrations of apo(a). The findings are surprising given the fact that apo(a) binding increased with uPAR overexpression in THP-1 monocytes and macrophages. These results suggest that the ability of apo(a) to inhibit plasminogen activation, through the uPAR, in THP-1 monocytes and macrophages is dependent on uPAR interacting with another cell-specific protein (such as an integrin).

Analysis of the role of the MAC-1 receptor on apo(a)-mediated plasminogen activation on HUVECs and THP-1 cells was also performed. The $\alpha_M\beta_2$ integrin is a prominent plasminogen receptor in neutrophils and has been reported to bind to apo(a) in monocytes through the β_2 integrin component [27]. Based on this report, plasminogen activation was analyzed in THP-1 monocytes and HUVECs in order to elucidate the contribution of this receptor to plasminogen activation on these cells. Results obtained using a monoclonal antibody targeting either the α_M or β_2 subunits indicate a small but significant role for the $\alpha_M\beta_2$ integrin in regulating plasminogen activation in the two cell types studied. However, blocking either α_M or β_2 did not further decrease plasminogen activation in the presence of increasing concentrations of apo(a), indicating that the effect of apo(a) on plasminogen activation may, in part, be mediated by the $\alpha_M\beta_2$ integrin.

Another candidate receptor, $\alpha_V\beta_3$ integrin, has been previously shown to elicit apo(a)/Lp(a) induced focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) activation in HUVECs [26]. In this study, apo(a) was shown to signal through $\alpha_V\beta_3$ resulting in downstream FAK and MAPK activation, stimulating EC migration and proliferation. We found that blocking the $\alpha_V\beta_3$ receptor with a monoclonal antibody in either THP-1 monocytes or HUVECs leads to a decrease in plasminogen activation with no difference in activation following the addition of apo(a). These results indicate that the $\alpha_V\beta_3$ integrin also plays a significant role in regulating plasminogen activation as well as enhancing the ability of apo(a) to inhibit plasminogen activation.

In conclusion, we have shown for the first time, the role of specific receptors in binding of apo(a) to vascular cell surfaces and in mediating the inhibitory effects of apo(a) on pericellular plasminogen activation. Our results suggest against an exclusive role for a particular receptor in mediating these effects, and instead are consistent with a model in which there are several contributing receptors in regulating plasminogen activation on various cell types. Moreover, there appear to be cell-type-specific differences in the roles of particular receptors. Recent, intriguing evidence suggest that Lp(a) levels <80 mg/dL is a significant risk factor for different forms of cancer including hepatocellular carcinoma, pancreatic cancer, and leukemia with no association of Lp(a) levels as a risk factor for lung cancer [57]. It is possible that apo(a) may inhibit plasminogen activation on cancerous cells via uPAR and reduce metastasis through attenuating cancer cell migration. It is tempting to speculate that Lp(a)/apo(a) may potentially play a role in inhibiting cancer progression. Our findings contribute significantly to unravelling the molecular basis of the physiological and

pathophysiological role of Lp(a)/apo(a) in the vasculature. Future work should explore the potential ability of Lp(a)/apo(a) to inhibit cancer metastasis and invasion through uPAR and the plasminogen activation system.

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CHAPTER 5

Lipoprotein(a) Catabolism is Regulated by Proprotein Convertase Subtilisin/Kexin Type 9 Through the Low Density Lipoprotein Receptor

5.1 SUMMARY

Elevated levels of Lipoprotein(a) (Lp(a)) have been identified as an independent risk factor for coronary heart disease. Plasma Lp(a) levels are reduced by monoclonal antibodies targeting proprotein convertase subtilisin/kexin type 9 (PCSK9). However, the mechanism of Lp(a) catabolism *in vivo* and the role of PCSK9 in this process are unknown. We report that Lp(a) internalization by hepatic HepG2 cells and primary human fibroblasts was effectively reduced by PCSK9. Overexpression of the low-density lipoprotein (LDL) receptor (LDLR) in HepG2 cells dramatically increased the internalization of Lp(a). Internalization of Lp(a) was markedly reduced following treatment of HepG2 cells with a function-blocking monoclonal antibody against the LDLR or the use of primary human fibroblasts from an individual with familial hypercholesterolemia; in both cases, Lp(a) internalization was not affected by PCSK9. Optimal Lp(a) internalization in both hepatic and primary human fibroblasts was dependent on the LDL rather than the apolipoprotein(a) component of Lp(a). Lp(a) internalization was also dependent on clathrin-coated pits and Lp(a) was targeted for lysosomal and not proteasomal degradation. Our data provide strong evidence that the LDLR plays a role in Lp(a) catabolism and that this process can be modulated by PCSK9. These results provide a direct mechanism underlying the therapeutic potential of PCSK9 in effectively lowering Lp(a) levels.

5.2 BACKGROUND

Lipoprotein(a) [Lp(a)] has been identified as an independent, causal risk factor for cardiovascular disease including coronary heart disease (CHD) [1, 2]. Lp(a) is similar to low-density lipoprotein (LDL) in lipid core composition and the presence of apolipoprotein B-100 (apoB), but also contains a unique glycoprotein apolipoprotein(a) (apo(a)) that has strong structural homology with the fibrinolytic zymogen plasminogen [3]. Apo(a) contains multiple copies of plasminogen-like kringle IV (KIV) sequences, followed by sequences closely resembling plasminogen kringle V (KV) and an inactive protease domain [3, 4]. The KIV domain can be further sub-divided into ten types (KIV_{1-10}) differing from each other in amino acid sequence. In Lp(a) particles, apo(a) is disulfide linked to the apoB component of the LDL-like moiety through a free cysteine residue in KIV_9 [5]; formation of Lp(a) requires initial non-covalent interactions between lysine residues in apoB with weak lysine binding sites present in apo(a) KIV_7 and KIV_8 [6]. Additionally, apo(a) contains a strong lysine binding site present in KIV_{10} which is important for its ability to interact with substrates such as fibrin [7].

Apo(a) can contain from 3 to >40 identically repeated KIV_2 domains which gives rise to the isoform size heterogeneity reported in the population [8]. A general inverse relationship between the size of apo(a) and Lp(a) plasma concentration has been well-established, with Lp(a) levels varying widely in the population [8]. It has been reported that the variation in concentration of Lp(a) is primarily controlled by the level of synthesis rather than catabolism [9, 10] with up to 90% of the variation being genetically determined based on variation in *LPA*, the gene encoding apo(a), including its size heterogeneity [11].

Many of the details of Lp(a) catabolism remain obscure. Various receptors have been suggested to mediate Lp(a) catabolism by the liver which include the LDL receptor (LDLR) [12-15], very low-density lipoprotein receptor (VLDLR) [16], low density lipoprotein receptor-related protein 1 (LRP-1) [17], megalin/gp330 [18], scavenger receptor class B type 1 (SR-B1) [19] and plasminogen receptors [12]. The role of the LDLR remains highly controversial, however. Hofmann and coworkers reported that Lp(a) clearance was significantly increased in mice overexpressing LDLR [20]. Additionally, several other studies both *in vitro* and *in vivo* have shown that the LDLR is capable of mediating Lp(a) binding and uptake [13-15]. A recent cross-sectional analysis of 1,960 patients with familial hypercholesterolemia (FH) revealed that Lp(a) levels were significantly higher in patients with a null LDLR allele compared to control subjects [21], a finding that is in agreement with an earlier report on this topic [22]. Conversely, Cain and colleagues reported that while plasma clearance of Lp(a) in mice occurs primarily through the liver and is mediated by apo(a), the catabolism of Lp(a) in $Ldlr^{-/-}$ mice was similar to that in wild-type mice [23]. Similar results were observed in metabolism studies of Lp(a) in human subjects with FH [24].

Recent studies have shown that Lp(a) levels in plasma can be reduced up to 30% using a proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitory monoclonal antibody [25-29]. In patients treated with a PCSK9 monoclonal antibody, the extent of Lp(a) lowering correlated with the extent of LDL lowering, although a more robust effect was observed for LDL levels which decreased up to ~70% [27, 28].

PCSK9 is an important regulator of hepatic LDLR number, and consists of a pro-domain, followed by a catalytic domain, a hinge region, and a carboxyl-terminal

cysteine/histidine-rich domain [30-32]. PCSK9 is synthesized as an inactive proenzyme that undergoes intramolecular autocatalytic cleavage in the endoplasmic reticulum (ER) [31, 32]. The cleaved prosegment remains associated with PCSK9, maintaining PCSK9 in a catalytically inactive form, and the complex is transported to the Golgi apparatus and subsequently secreted. PCSK9 acts as an endogenous regulator of LDLR levels and has been implicated in some cases of FH due to the dominant gain-of-function (GOF) mutations identified in the population [33]. GOF mutations lead to increased affinity of PCSK9 for the LDLR which results in a more rapid degradation of the LDLR and thus higher plasma LDL [34]. Conversely, loss-of-function mutations in PCSK9 result in dramatically lowered plasma LDL [33]. It is not yet known if PCSK9 mutation influence Lp(a) concentrations. PCSK9 can target the LDLR for degradation as well as the VLDLR, LRP-1, and apolipoprotein E receptor 2 (apoER2; LRP8) [35, 36]. However, plasma LDL is predominately cleared through the LDLR [37, 38].

In the current study, using a human hepatocellular carcinoma model system, we sought to understand the mechanistic basis of the ability of PCSK9 inhibitory antibodies to lower plasma Lp(a) concentrations, in the context of the ongoing controversy about the role of the LDLR in Lp(a) catabolism.

5.3 METHODS

5.3.1 Cell Culture

Human embryonic kidney (HEK293) cells were maintained in MEM (GIBCO) containing 5% fetal bovine serum (FBS; GIBCO) and 1% antibiotic-antimycotic (GIBCO). Human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC) and maintained in MEM supplemented with 10% FBS (ATCC) and 1% antibiotic-antimycotic (GIBCO). Primary familial hypercholesterolemic (FH) fibroblasts were obtained from Coriell Institute (catalogue numbers GM01386, GM01355, and GM00701) and maintained in MEM containing 10% FBS (ATCC). Experiments with FH fibroblasts were performed between passages 5 and 20.

5.3.2 Construction, Expression, and Purification of Recombinant Apo(a)

The construction of expression plasmids encoding the various recombinant apo(a) (r-apo(a)) variants utilized in this study (17K, 17KΔLBS$_{10}$, and 17KΔLBS$_{7,8}$), their transfection HEK293 cells and the purification of r-apo(a) from conditioned medium were previously described [6]. Briefly, the conditioned medium was subjected to lysine-Sepharose affinity chromatography and r-apo(a) was eluted using the lysine analogue ε-aminocaproic acid (ε-ACA). Following concentration and buffer exchange, protein concentrations were determined spectrophotometrically. The purity of r-apo(a) was assessed using SDS-PAGE followed by silver staining.

5.3.3 Construction, Expression, and Purification of Recombinant PCSK9

PCSK9 and PCSK9 D374Y expression plasmids in pIRES2-EGFP (Clontech) were previously-described [31, 32]. The PCSK9 cDNAs were excised from pIRES2-EGFP using AfeI and AgeI restriction endonucleases and ligated into pcDNA4C (Invitrogen), previously digested with EcoRV and AgeI, such that the expressed protein would contain a carboxyl-terminal 6× His tag. HEK293 cells at 1.8 $x10^6$ cells/well of a 6-well plate were seeded and transfected 24 hrs later with 2 µg of expression plasmid using MegaTran 1.0 (Origene) with a 3:1 ratio of reagent to DNA as per the manufacturer's instructions. Stable cells were selected with zeocin (150 µg/mL) 48 hrs post transfection. Stable cells were seeded into triple flasks with OPTI-MEM (Invitrogen) and conditioned medium was collected every 3 days with the addition of phenylmethylsulfonyl fluoride at a final concentration of 1 mM to the harvest. The harvested medium was adjusted to 50 mM phosphate buffer pH 8.0, 0.5 M NaCl, 1 mM β-mercaptoethanol, 5 mM imidazole, and 10% glycerol, applied to a Ni Sepharose excel (GE Healthcare) column, washed and eluted with 15 mM and 400 mM imidazole respectively. The eluted pool (4 column volumes) was extensively dialyzed against PCSK9 storage buffer (25 mM HEPES, pH 7.9, 150 mM NaCl, 0.1 mM $CaCl_2$, and 10% glycerol). The dialyzed samples were then concentrated with PEG-20,000 (Sigma) and dialyzed against storage buffer. Concentrations were determined through bicinchoninic acid assay (BCA assay; Pierce) using BSA as a standard. Purity of PCSK9 was assessed through SDS-PAGE followed by silver staining and stored in aliquots at -70°C until use.

5.3.4 Labeling of PCSK9

Purified PCSK9 was dialyzed against 0.1 M Na_2CO_3 pH 8.6, 0.2 M NaCl. PCSK9 was then incubated with a 5-fold molar excess of Alexa Fluor 488 carboxylic acid, succinimidyl ester mixed isomers dissolved in dimethyl sulfoxide at 10 mg/mL (Invitrogen). The reaction mixture was rocked for 4 hrs at 4°C to ensure complete labeling. The reaction was quenched with the addition of 0.01 volumes of 1 M Tris, pH 8.0 followed by incubation for 30 min at 4°C. Free dye was removed through extensive dialysis against 25 mM HEPES, pH 7.5, 300 mM NaCl, 50 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 10% glycerol. PCSK9 was concentrated using an Amicon Ultra-4 centrifugal filter with a 10 kDa membrane cutoff (Millipore). Concentration was determined spectrophotometrically with a moles dye per mole protein ratio of 2.8.

5.3.5 Transient Transfection

HepG2 cells were transfected with clathrin heavy chain siRNA or scrambled control siRNA (Santa Cruz Biotechnology) at a concentration of 80 nM as per the manufacturer's protocol. The transfection mixture was incubated on cells for 8 hrs followed by the addition of complete medium. Cells were assayed 48-72 hours post transfection. Percent knockdown was determined using qRT-PCR (see below). HepG2 cells were transiently transfected with v5 (empty vector), LDLR, or LDLRΔCT [36] using MegaTran 1.0 (Origene) as per the manufacturer's protocol. Briefly, HepG2 cells were seeded at a density of 2 $x10^5$ cells/6-well in antibiotic free media and transfected 24 hrs later with 1.3 µg of cDNA with a 3:1 ratio of reagent to DNA. Cells were assayed 72 hours post transfection.

5.3.6 qRT-PCR

Determination of clathrin heavy chain knockdown efficiency was determined through iTaq one-step RT-PCR kit with SYBR® green (Bio-Rad). The following primers were used: clathrin heavy chain forward: 5'-GGC CCA GAT TCT GCC AAT TCG TTT-3'; clathrin heavy chain reverse: 5'-TGA TGG CGC TGT CTG CTG AAA TTG-3'; GAPDH forward: 5'-GGA GCC AAA AGG GTC ATC ATC-3'; GAPDH reverse: 5'-GTT CAC ACC CAT GAC GAA CAT G-3'.

5.3.7 Internalization Assays

HepG2 cells (in some cases stably transfected with an expression vector for PCSK9) were seeded at 2 x 10^5 cells/well in a 24 well plate (precoated with 1 mg/mL gelatin), in media containing 10% LPDS for 16 hours. Cells were washed twice with OptiMEM (GIBCO) and treated with Lp(a) purified from human plasma (5-10 µg/mL) or r-apo(a) variants (100-200 nM) in the presence of 0, 1, 10, or 20 µg/mL purified recombinant PCSK9 in Opti-MEM for 4 hours at 37°C. For experiments using LDLR-blocking monoclonal antibodies, cells were pre-treated for 30 min with 50 µg/mL of 5G2 or 7H2 followed by incubation with Lp(a) or apo(a) in the continued presence of 50 µg/mL antibody for 2 hrs at 37°C. In some experiments, cells were co-treated with 10 µg/mL lactacystin (Cayman) or 150 µg/mL E-64d (Cayman) or vehicle (dimethyl sulfoxide) along with Lp(a) or apo(a) for 4 hrs at 37°C. For all internalization experiments, HepG2 cells were extensively washed: 3× with PBS-0.8% BSA, 2× with PBS-BSA-0.2M ε-ACA for 5 min each, 2× with 0.2 M acetic acid pH 2.5 containing 0.5 M NaCl for 10 min each, 2× with PBS. The cells were then lysed with lysis buffer (50

mM Tris pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1 mM PMSF, and 150 µg/mL benzamidine).

For experiments with fibroblasts, cells were seeded in a 24 well plate at 1.4 x 10^5 cells/well in medium containing 10% LPDS for 16 hrs. Cells were washed twice with OptiMEM and treated with Lp(a) (5 µg/mL) or apo(a) (100 nM) in the presence or absence of 20 µg/mL PCSK9 in OptiMEM for 4 hours at 37°C. Cells were extensively washed (3× with PBS-0.8% BSA, 2× with PBS containing 10 µg/mL heparin for 10 min, 1× PBS-BSA-0.2M ε-ACA for 5 min, 2× with 0.2 M acetic acid pH 2.5 containing 0.5 M NaCl for 10 min, 1× with 0.5 M HEPES pH 7.5, 100 mM NaCl for 10 min, and 1× with PBS) then lysed with lysis buffer. Concentrations of lysate samples were determined by BCA assay with BSA as a standard and analyzed by Western blotting.

5.3.8 Western Blotting

Cell lysates were subjected to SDS-PAGE on 5-15% (Lp(a)-treated cells) or 7-15% (apo(a)-treated cells) polyacrylamide gradient gels respectively. The gels were transferred onto PVDF membranes (Millipore) and immunoblotted with either mouse-anti human apo(a) a5 antibody [39], mouse-anti human β-actin (Sigma), or rabbit-anti human LDLR (GeneTex). Bands were visualized with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) and quantified using Alpha View software (Alpha Innotech).

5.3.9 LDLR Degradation Assay

HepG2 cells were seeded at 2 x 10^5 cells/well in a 24 well plate in medium containing 10% LPDS for 16 hours. PCSK9 (20 µg/mL) with 0, 100, 250 µg/mL of

plasma-purified Lp(a) or LDL or 0, 100, 250 nM apo(a) was added in OptiMEM and the cells incubated for 4 hours. Cells were washed three times with PBS and lysed. Concentrations of samples were determined by BCA assay and LDLR levels were analyzed by Western blotting.

5.3.10 Binding Study

Saturation binding curves were generated by incubating LDL or Lp(a), at 0.5 mg/mL, with increasing amounts of PCSK9-Alexa 488 (25-1200 nM) in binding buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 1% BSA) for one hour at 37°C. Glycerol was added to the samples to a final concentration of 10% and the samples were subjected to electrophoresis on 0.7% agarose gels (UltraPure Agarose, Invitrogen) for 2 hours at 40 V in 90 mM Tris pH 8.0, 80 mM borate, 2 mM calcium lactate. In-gel scanning and quantification of the anount of labeled PCSK9 free and bound to Lp(a) or LDL was achieved with a FluorChem Q imager (Alpha Innotech). Dissociation constants (K_D) were determined by fitting the data to an equation describing a rectangular hyperbola by nonlinear regression using Graph Pad Prism 6.

5.3.11 Purification of LDL and Lp(a) and Preparation of Lipoprotein-Deficient Serum (LPDS)

Blood was collected from a healthy human volunteer (with written informed consent) with no detectable Lp(a) into BD Vacutainers containing sodium polyanethol sulfonate and acid citrate dextrose. The blood was centrifuged at 2,000 × g for 15 min at 4°C and LDL was isolated from plasma through sequential ultracentrifugation (1.02 g/mL < d < 1.063 g/mL); the centrifugation steps were at 45,000 × g for 18 hrs at 4°C.

The isolated LDL was extensively dialyzed against 150 mM NaCl, 5.6 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 0.01% EDTA (pH 7.4). Lipoprotein-depleted serum (LPDS) was prepared through addition of NaBr to FBS (ATCC) to a final density of 1.21 g/mL followed by ultracentrifugation as described above. The top fraction was removed and the infranatant fraction containing LPDS was extensively dialyzed against HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4, 150 mM NaCl). Lp(a) was prepared from a single donor with high Lp(a) and a single 16-kringle apo(a) isoform as previously described [40]. Concentrations of LDL and Lp(a) were determined by BCA assay using BSA as a standard.

5.3.12 Purification of LDLR-Blocking Monoclonal Antibodies

Anti-human LDLR monoclonal antibodies 5G2 and 7H2 (a gift from Dr. Ross Milne, University of Ottawa Heart Institute) were purified from ascites fluid using Protein G Sepharose 4 Fast Flow affinity chromatography according to manufacturer's recommendations (GE Healthcare). Concentrations of antibodies were determined using BCA assay with BSA as a standard.

5.3.13 Statistical Methods

Comparisons between data sets were performed using two-tail Student's t-test assuming unequal variances.

5.4 RESULTS

5.4.1 PCSK9 inhibits Lp(a) and apo(a) internalization

PCSK9 can target the LDLR for degradation in an intracellular pathway by targeting the LDLR from the *trans* Golgi network directly to lysosomes [41]. Conversely, extracellular PCSK9 targets the LDLR for degradation through binding of PCSK9 to the EGF-A domain of the LDLR and subsequently targeting the complex to lysosomes for degradation [42, 43]. Herein, we evaluated the role of both the intra- and extracellular PCSK9-mediated degradation of LDLR in Lp(a)/apo(a) internalization by HepG2 cells. Overexpression of PCSK9, which would stimulate both the intracellular and extracellular pathway of targeting the LDLR for degradation, resulted in a significant decrease in the amount of Lp(a) internalized by HepG2 cells (Fig. 5.1A). Similar results were obtained for the internalization of a physiologically relevant recombinant apo(a) (r-apo(a)) species (17K) that contains 8 identically repeated KIV_2 domains (Fig. 5.1B). Addition of a lysine analog, ε-ACA, markedly inhibits the uptake of both Lp(a) and r-apo(a) (Fig. 5.1A, B) although PCSK9 still significantly reduced uptake of Lp(a). Likewise, mutating the strong lysine binding site present in KIV_{10} of 17K (17KΔLBS_{10} variant) results in a significant decrease in its ability to be internalized (Fig. 5.1B). Interestingly, however, PCSK9 is able to significantly decrease internalization of this variant but not internalization of either 17K or 17KΔLBS_{10} in the presence of ε-ACA (Fig. 5.1B).

To specifically determine the role of the extracellular PCSK9 degradation pathway, HepG2 cells were exposed to exogenous, purified PCSK9 or a gain-of-function (GOF) mutant of PCSK9 (D374Y) in the presence of Lp(a) or apo(a). Treatment of HepG2 cells with various concentrations of wild type (WT) PCSK9 resulted in a

significant decrease in Lp(a) and 17K internalization (Fig. 5.1C, D). The GOF mutant was found to have a more robust effect on Lp(a) (at 1 μg/mL) and 17K (at 1 and 10 μg/mL) internalization compared to WT PCSK9.

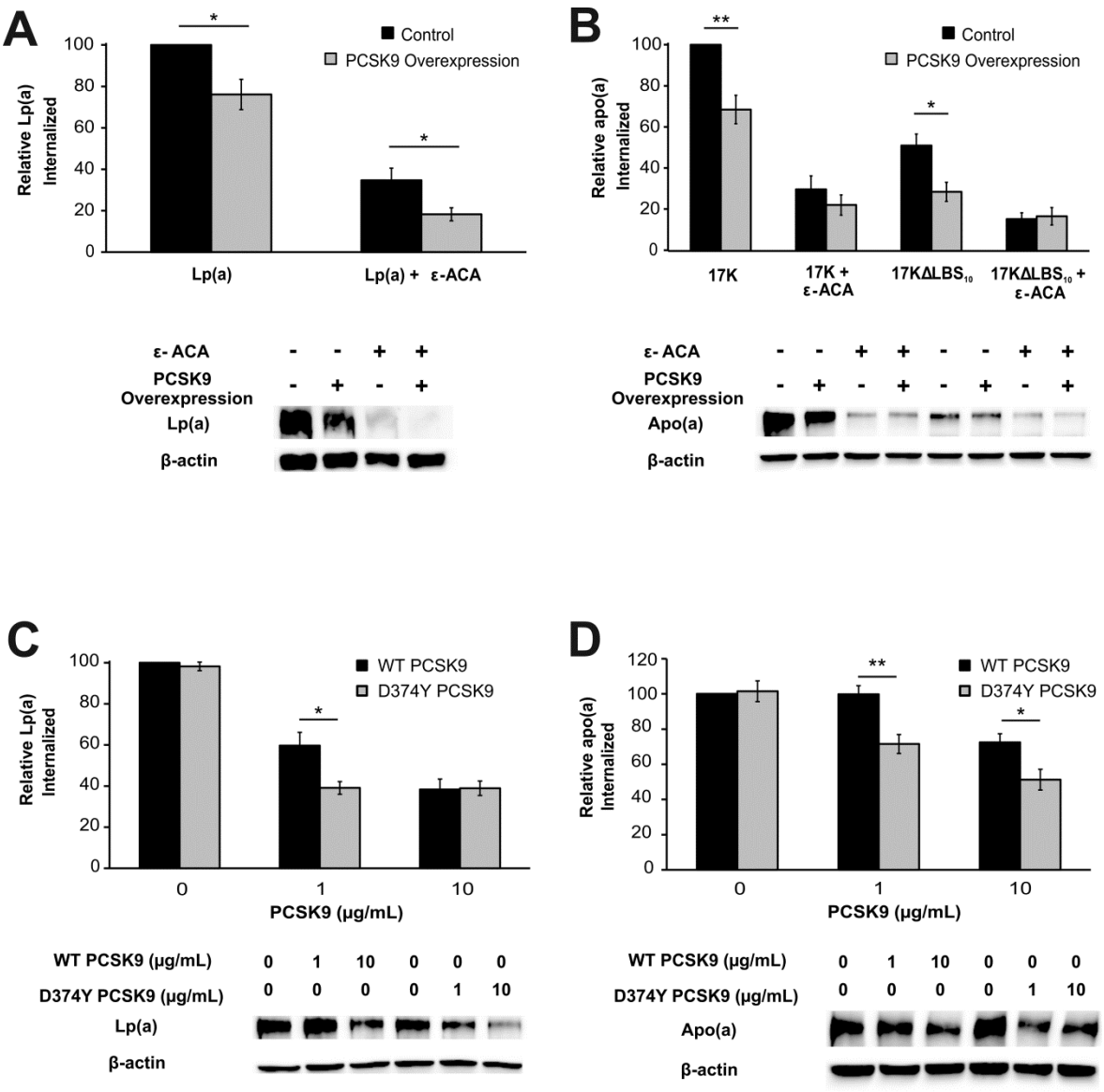

Figure 5.1: ***PCSK9 reduces the internalization of both Lp(a) and apo(a) in HepG2 cells***. **A** and **B**, HepG2 cells stably transfected with empty vector or PCSK9-v5 were grown for 16 hrs in LPDS media followed by treatment with either 10 μg/mL Lp(a) (**A**) or 200 nM apo(a) (**B**) in the presence or absence of 200 mM ε-ACA for 4 hours. The cells were extensively washed to remove any bound Lp(a)/apo(a) and lysed to determine the relative amount that was internalized, using β-actin as an internal control. Error bars indicate standard error of the mean (SEM) with n≥3 independent experiments. *: $p<0.05$ **: $p<0.01$. **C** and **D**, HepG2 cells were grown in LPDS media for 16 hrs followed by treatment with various concentrations of PCSK9 or PCSK9 D374Y in the presence of either 10 μg/mL Lp(a) (**C**) or 200 nM 17K (**D**). The cells were extensively washed to remove any bound Lp(a)/apo(a) and lysed to determine the relative amount that was internalized, using β-actin as an internal control. Error bars indicate SEM with n≥3 independent experiments. *: $p<0.05$; **: $p<0.01$.

5.4.2 PCSK9 does not bind to Lp(a)

It has been previously reported that PCSK9 can bind to LDL *in vitro* consistent with a one-site binding model with a K_D ~ 325 nM [44]. Furthermore, the binding of PCSK9 to LDL inhibits its ability to target the LDLR for degradation in HuH7 human hepatoma cells [44]. Hence, we determined whether Lp(a) can bind to PCSK9 *in vitro* and if Lp(a)/apo(a) can inhibit the ability of exogenous PCSK9 to target the LDLR for degradation. We found that LDL can bind to PCSK9 *in vitro* with a K_D of ~ 130 nM, a value close to that previously reported [44] (Fig. 5.2A, B). On the other hand, little or no binding of Lp(a) to PCSK9 was detected (Fig. 5.2A, C). Treatment of HepG2 cells with exogenous PCSK9 results in a substantial decrease in LDLR levels, while co-treatment of PCSK9 with LDL results in recovery of LDLR levels (Fig. 5.2D). These findings are also in agreement with previously reported data [42]. However, co-treatment of Lp(a) or 17K with PCSK9 results in no significant recovery in LDLR levels (Fig. 5.2E, F). Together, these results suggest that Lp(a) does not bind to PCSK9 and therefore cannot block the ability of PCSK9 to target the LDLR for degradation.

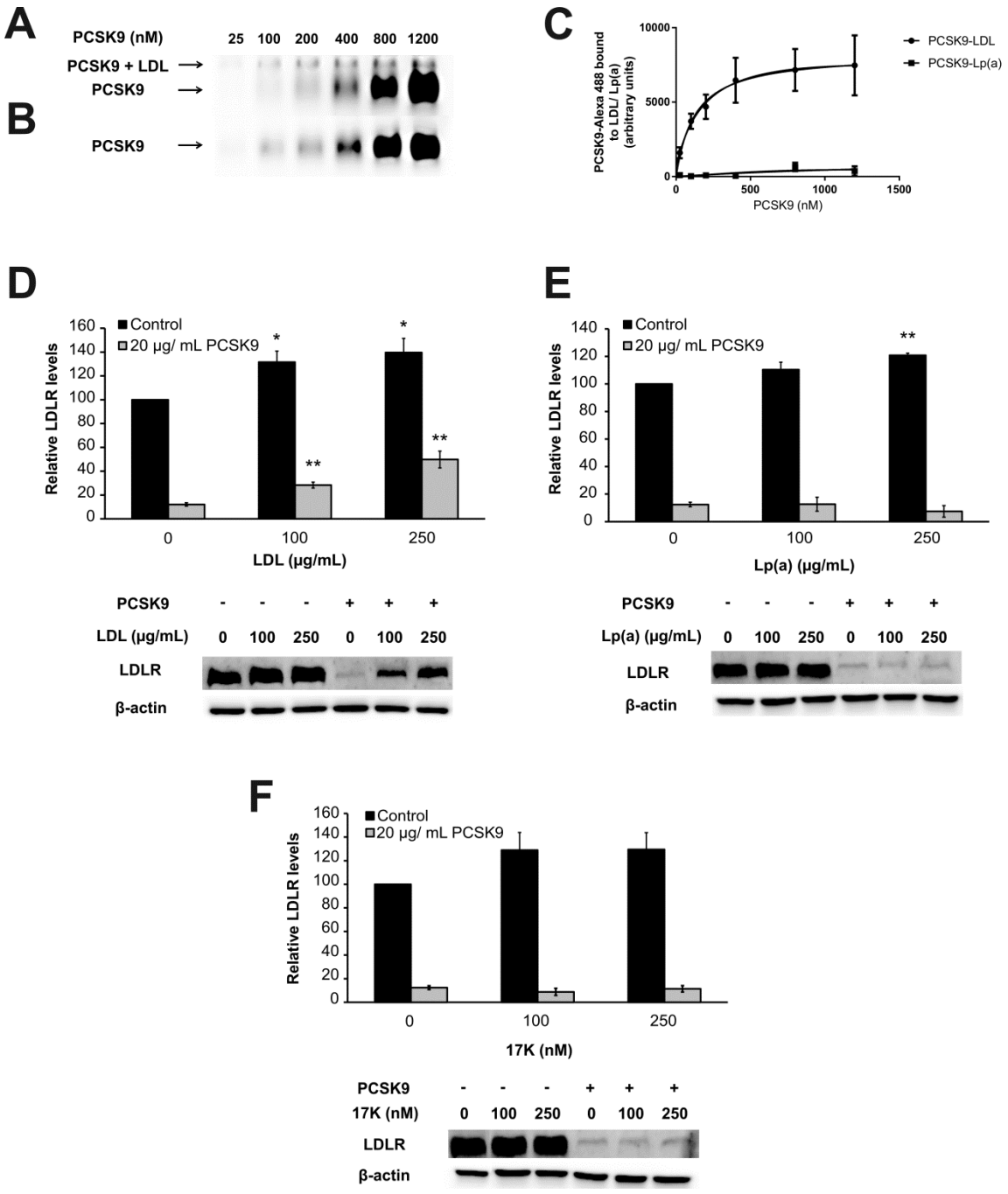
A
PCSK9 (nM)
25 100 200 400 800 1200
PCSK9 + LDL
PCSK9
B
PCSK9
C
PCSK9-Alexa 488 bound to LDL/ Lp(a) (arbitrary units)
PCSK9 (nM)
PCSK9-LDL
PCSK9-Lp(a)
D
Control
20 µg/ mL PCSK9
Relative LDLR levels
LDL (µg/mL)
PCSK9
LDL (µg/mL)
LDLR
β-actin
E
Control
20 µg/ mL PCSK9
Relative LDLR levels
Lp(a) (µg/mL)
PCSK9
Lp(a) (µg/mL)
LDLR
β-actin
F
Control
20 µg/ mL PCSK9
Relative LDLR levels
17K (nM)
PCSK9
17K (nM)
LDLR
β-actin

Figure 5.2: ***PCSK9 binds to LDL in vitro but not to Lp(a)***. LDLR levels are recovered from PCSK9 degradation by treatment with LDL but not Lp(a) or apo(a). Various concentrations of PCSK9-Alexa 488 were incubated with 0.5 mg/mL of purified LDL or Lp(a) for one hour at 37°C. **A** and **B**, Samples containing PCSK9 and either LDL (**A**) or Lp(a) (**B**) were resolved on 0.7% agarose gels. **C**, Bound and free levels of PCSK9 were quantified and fit to a saturation curve by nonlinear regression using Graph Pad Prism 6 to give a mean K_D for LDL of 127.6 ± 39.8 nM (n= 3). **D-F**, HepG2 cells were grown for 16 hrs in LPDS media followed by treatment with various concentrations of LDL (**D**), Lp(a) (**E**), or 17K r-apo(a) (**F**) in the presence or absence of 20 µg/mL PCSK9 for 4 hours. The relative LDLR levels were determined and normalized to β-actin. Error bars indicate SEM with n≥3 independent experiments. *: $p<0.05$; **: $p<0.01$ compared to control in the absence or presence of PCSK9.

5.4.3 Lp(a)/apo(a) internalization involves clathrin-mediated endocytosis and internalized Lp(a)/apo(a) is targeted to lysosomes

PCSK9 has been previously shown to target the LDLR for degradation via clathrin heavy chain-mediated endocytosis and subsequent targeting to lysosomes [45, 46]. We therefore determined whether Lp(a) and apo(a) undergo the same degradation pathway. Knockdown of clathrin heavy chain in HepG2 cells results in a significant decrease in Lp(a) and apo(a) internalization (Fig. 5.3). In both cases, while PCSK9 treatment results in a dose-dependent decrease in internalization in the absence of clathrin heavy chain knockdwon, no further decrease resulting from PCSK9 is observed in the presence of clathrin heavy chain knockdown (Fig. 5.3). These results indicate that the PCSK9-regulated internalization of Lp(a)/apo(a) is dependent on clathrin coated pits.

The degradation pathway that Lp(a)/apo(a) undergoes was further evaluated through inhibitors of both the lysosomal and proteosomal pathway. Treatment of HepG2 cells with a lysosomal inhibitor, E-64d, resulted in the intracellular accumulation of Lp(a) and apo(a) (Fig. 5.4). However, treatment with a proteosomal inhibitor, lactacystin, results in no accumulation of Lp(a) or apo(a). These results indicate that Lp(a)/apo(a) is internalized through clathrin-mediated endocytosis and is subsequently targeted for lysosomal degradation.

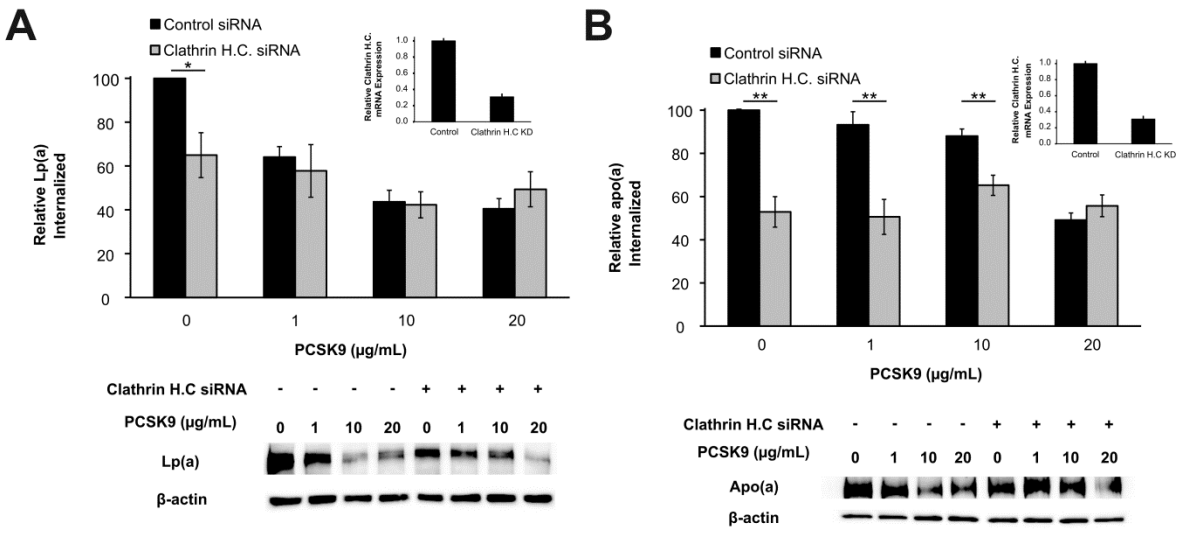

Figure 5.3: ***Lp(a) and apo(a) internalization is dependent on clathrin coated pits in HepG2 cells***. **A** and **B**, HepG2 cells were transfected with control or clathrin heavy chain siRNA for 48 hours followed by incubation of cells in LPDS media for 16 hrs. HepG2 cells were then treated with 5 μg/mL Lp(a) (**A**) or 200 nM 17K (**B**) in the presence or absence of PCSK9 for 4 hours. Internalization of Lp(a)/apo(a) was measured as in the Legend to Fig. 5.1. Error bars indicate SEM with n≥3 independent experiments. *: $p<0.05$; **: $p<0.01$. Insets: clathrin heavy chain mRNA was quantified using qRT-PCR following siRNA treatment to determine knockdown efficiency.

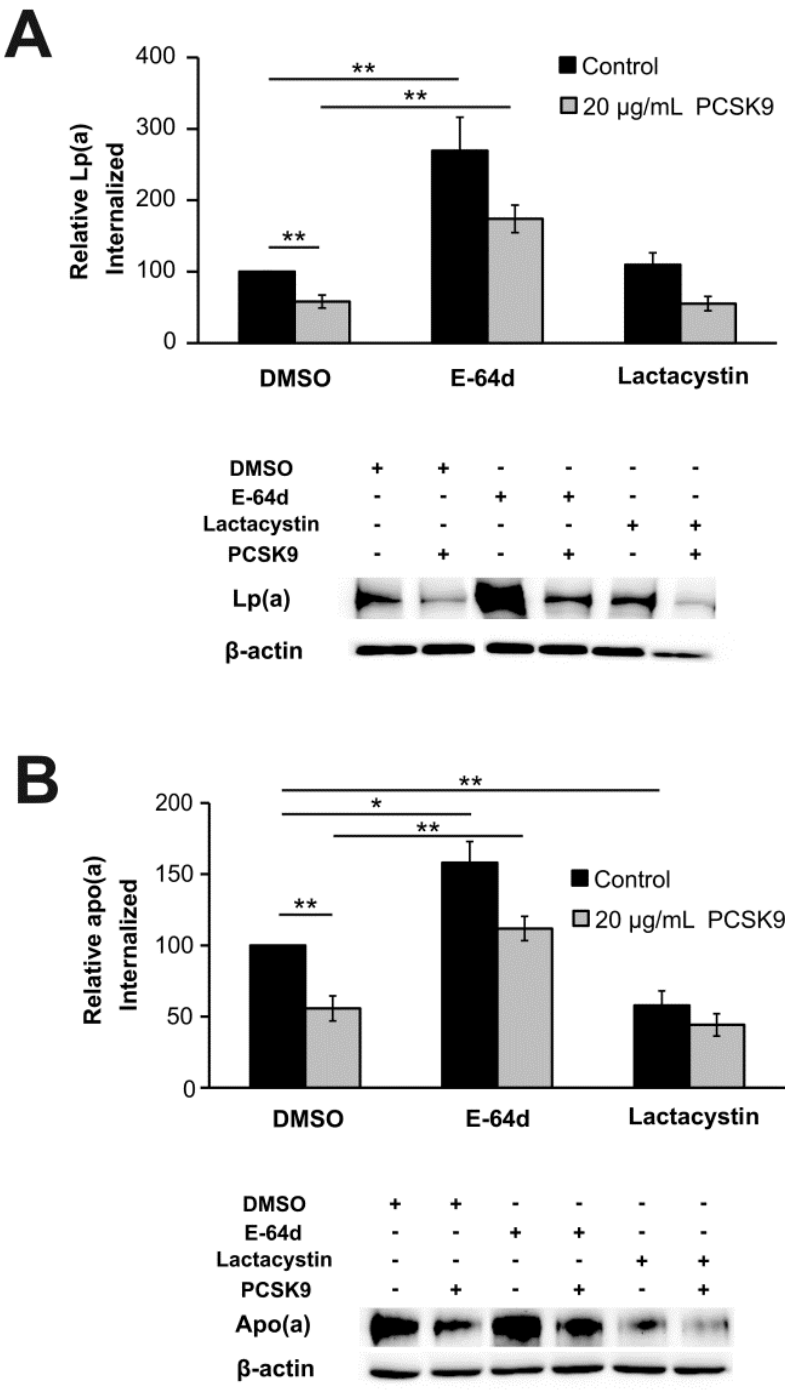

Figure 5.4: ***Lp(a) and apo(a) degradation in HepG2 cells occurs in lysosomes and not proteosomes***. **A** and **B**, HepG2 cells were grown for 16 hrs in LPDS media followed by treatment with either DMSO, E-64d (150 µg/mL), or lactacystin (10 µM) in the presence or absence of 20 µg/mL PCSK9 as well as Lp(a) (**A**) or 17K (**B**) for 4 hours. Internalization of Lp(a)/apo(a) was measured as in the Legend to Fig. 5.1. Error bars indicate SEM with n≥3 independent experiments. *: $p<0.05$; **: $p<0.01$.

5.4.4 PCSK9 regulates Lp(a) internalization through the LDLR

Previous studies have shown that apo(a) can be internalized into HepG2 cells through the LDLR or through lysine-dependent interactions with plasminogen receptors [12]. We therefore wanted to examine which of these routes might be sensitive to PCSK9. Apo(a) is not itself a ligand for LDLR, but r-apo(a) added to HepG2 cell medium binds non-covalently to apoB-containing lipoproteins secreted by the HepG2 cells which allows it to be internalized by the LDLR in a "piggybacking" manner [12]. The weak LBS in KIV type 7 and 8 mediate these non-covalent interactions and therefore, for internalization studies, we utilized a r-apo(a) variant in which both these LBS were mutated (17KΔLBS$_{7,8}$) [6]. We found that 17KΔLBS$_{7,8}$ was poorly internalized in HepG2 cells (Fig. 5.5A); although its internalization did not appear to be affected by PCSK9, this conclusion has to be tempered by the fact that the internalization of this species is at our limit of detection.

To determine more directly if the LDLR plays a role in Lp(a)/apo(a) internalization, the LDLR or the LDLR lacking its carboxyl tail (LDLRΔCT) were overexpressed in HepG2 cells. The ΔCT deletion occurs where the autosomal recessive hypercholesterolemia (ARH) adaptor protein binds and is important for recruiting the complex into clathrin-coated pits [47, 48]. Overexpression of LDLR in HepG2 cells results in a dramatic increase in Lp(a) internalization (Fig. 5.5B) and only a modest and not statistically significant increase in apo(a) internalization (Fig. 5.5C). Treatment of the cells overexpressing LDLR or LDLRΔCT with PCSK9 leads to a significant decrease in Lp(a) internalization (Fig. 5.5B).

Treatment of HepG2 cells with a blocking monoclonal LDLR antibody was also utilized to confirm that the LDLR is involved in Lp(a) catabolism and its regulation by PCSK9. Two LDLR-blocking monoclonal antibodies, 5G2 and 7H2, were used which were previously shown to specifically block the binding of LDL to the LDLR in cultured human fibroblasts [49]. Lp(a) internalization was markedly decreased by the addition of either antibody and PCSK9 had no effect on Lp(a) internalization in the presence of the antibodies (Fig. 5.5D). Furthermore, we found that 7H2 likewise markedly decreased 17K internalization (Fig. 5.5E). On the other hand, PCSK9 did not decrease 17K internalization in the presence of the antibody, and internalization of 17KΔLBS$_{7,8}$ appeared to be insensitive to both PCSK9 and the antibody (Fig. 5.5E). These results indicate that the LDLR mediates internalization of Lp(a) through the LDL component and in a manner that is regulated by PCSK9.

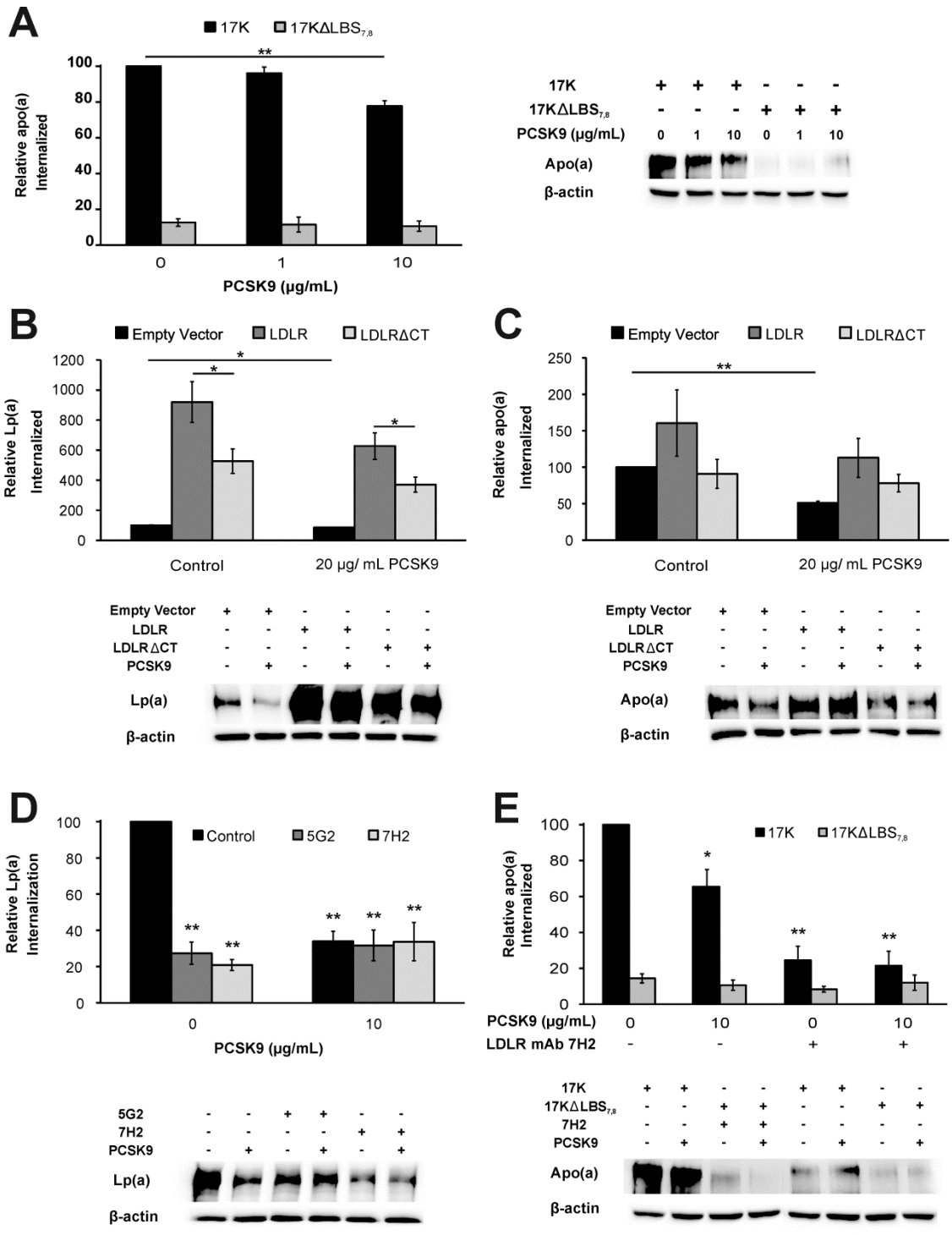
A
17K
17KΔLBS7,8
Relative apo(a) Internalized
PCSK9 (μg/mL)
17K
17KΔLBS7,8
PCSK9 (μg/mL)
Apo(a)
β-actin
B
Empty Vector
LDLR
LDLRΔCT
Relative Lp(a) Internalized
Control
20 μg/ mL PCSK9
Empty Vector
LDLR
LDLRΔCT
PCSK9
Lp(a)
β-actin
C
Empty Vector
LDLR
LDLRΔCT
Relative apo(a) Internalized
Control
20 μg/ mL PCSK9
Empty Vector
LDLR
LDLRΔCT
PCSK9
Apo(a)
β-actin
D
Control
5G2
7H2
Relative Lp(a) Internalization
PCSK9 (μg/mL)
5G2
7H2
PCSK9
Lp(a)
β-actin
E
17K
17KΔLBS7,8
Relative apo(a) Internalized
PCSK9 (μg/mL)
LDLR mAb 7H2
17K
17KΔLBS7,8
7H2
PCSK9
Apo(a)
β-actin

Figure 5.5: ***The LDLR contributes to Lp(a) and apo(a) internalization in HepG2 cells*****.** **A**, HepG2 cells were grown in LPDS media for 16 hrs followed by treatment with various concentrations of PCSK9 in the presence of 17K or 17KΔLBS$_{7,8}$ (200 nM). Internalization of apo(a) was measured as in the Legend to Fig. 5.1. Error bars indicate SEM with n≥3 independent experiments. **: p<0.01. **B** and **C**, HepG2 cells were transfected with expression plasmids encoding either LDLR or LDLRΔCT for 48 hours and were then grown for 16 hrs in LPDS media. The cells were incubated with 10 μg/mL Lp(a) (**B**) or 200 nM apo(a) (**C**) in the presence or absence of 20 μg/mL PCSK9 for 4 hours after which internalization of Lp(a)/apo(a) was measured. Error bars indicate SEM with n≥3 independent experiments. *: p<0.05; **: p<0.01. **D** and **E**, HepG2 cells were grown in LPDS media for 16 hrs followed by pretreatment with monoclonal LDLR blocking antibodies (mAb's 5G2 or 7H2; 50 μg/mL) for 30 min. Cells were then incubated with 5 μg/mL Lp(a) (**D**) or 100 nM apo(a) (**E**) in the presence or absence of 10 μg/mL PCSK9 and in continuing presence of mAb's for 2 hours, after which internalization of Lp(a)/apo(a) was measured. Error bars indicate SEM with n≥4 independent experiments. *: p<0.05 and **: p<0.01 for comparison of absence of antibody and PCSK9.

The role for the LDLR was also explored using primary fibroblasts isolated from individuals with or without FH. The three cell lines studied were GM01386 (fully functional LDLR), GM01355 (clinically affected with severe hypercholesterolemia with LDLR activity found to be partially negative), and GM00701 (LDLR activity <1% compared to normal cells). Lp(a) internalization is substantially decreased in cells deficient in LDLR, and the internalization was unaffected by PCSK9 in these cells (Fig. 5.6A). Conversely, no significant difference in 17K internalization is observed between LDLR-deficient and normal fibroblasts and there is no effect in any cell line of PCSK9 treatment (Fig. 5.6B). PCSK9 was able to dramatically decrease LDLR content of those fibroblasts that contained immunoreactive receptor (Fig. 5.6C). These findings underscore the requirement for apo(a) to couple to apoB-containing lipoproteins in order to internalize through the LDLR in a PCSK9-regulable manner as these cells do not express apoB-containing lipoproteins.

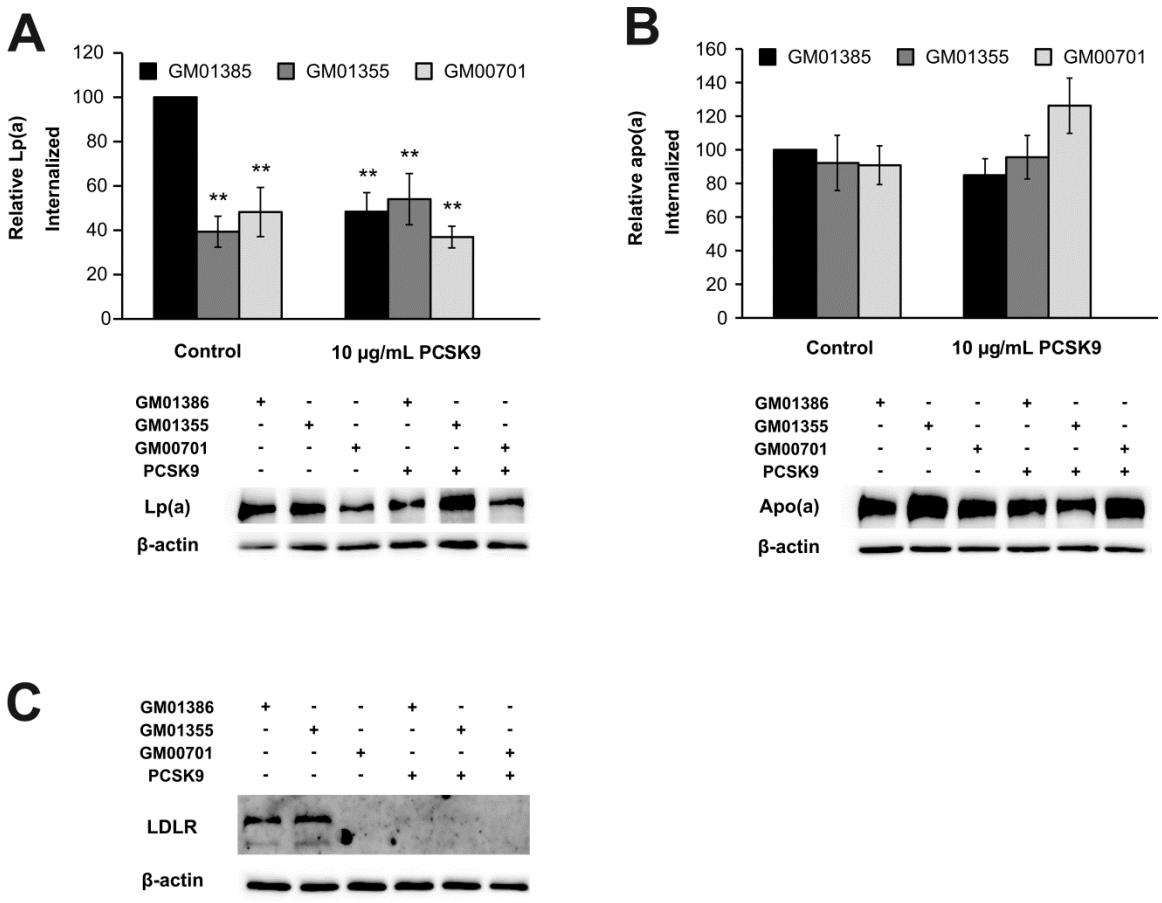

Figure 5.6: ***Lp(a), but not apo(a), internalization decreases in FH fibroblasts lacking LDLR***. **A** and **B**, FH Fibroblasts used were GM01386 (fully functional LDLR), GM01355 (clinically affected with severe hypercholesterolemia with LDLR activity found to be partially negative), and GM00701 (LDLR activity <1% compared to normal cells). The FH fibroblasts were grown in LPDS media for 16 hrs followed by incubation with 5 µg/mL Lp(a) (**A**) or 100 nM apo(a) (**B**) in the presence or absence of 10 µg/mL PCSK9 for 4 hours. Internalization of Lp(a)/apo(a) was measured as in the Legend to Fig. 5.1. Error bars indicate SEM with n≥5 independent experiments. *: $p<0.05$; **: $p<0.01$ for comparison of control in the absence of PCSK9 and ε-ACA. **C**, The LDLR content of the respective fibroblast cell lines in the presence or absence of PCSK9 treatment was assessed by Western blot analysis using a monoclonal antibody against LDLR.

5.5 DISCUSSION

Elevated plasma Lp(a) levels have been recently shown to be effectively reduced with the use of two different monoclonal antibodies against PCSK9 [25-29]. This therapy was conceived to lower LDL levels as inhibition of PCSK9 leads to upregulation of the LDLR. The ability of the therapy to lower plasma Lp(a) challenges the existing dogma that the LDLR does not play a major, if any, role in Lp(a) catabolism. Therefore, our aim was to directly assess the ability of PCSK9 to influence Lp(a) uptake in a cellular model and to explore the role of LDLR in this process.

We found that PCSK9 was indeed able to inhibit Lp(a) internalization in HepG2 cells (Fig. 5.1). This effect was observed whether PCSK9 was ectopically overexpressed (and hence active both intracellularly and extracellularly) or added as a purified protein to the culture medium along with Lp(a) (hence acting exclusively extracellularly). Notably, we also found that PCSK9 can stimulate internalization of apo(a) itself (Fig. 5.1). However, we conclude that the effect of PCSK9 on apo(a) internalization is dependent on the ability of free apo(a) to associate with apoB-containing lipoprotein particles in the culture medium, with internalization of the resultant complex being sensitive to PCSK9. This conclusion is based on the fact that internalization of apo(a) by fibroblasts, which do not express apoB, is insensitive to PCSK9 (Fig. 5.6). Moreover, internalization of the $17K\Delta LBS_{7,8}$ variant, which cannot associate non-covalently with apoB-containing lipoproteins [6], appears to be insensitive to the effects of PCSK9 (Fig. 5.5). Given these findings, and a previous report that demonstrated that apoB-100, not apo(a), is the ligand in Lp(a) for LDLR [12], we suspected that the LDLR, the major target of PCSK9, is mediating the PCSK9-sensitive component of Lp(a) internalization.

Importantly, apo(a), due to its structural similarities to plasminogen, may also potentially bind to and be internalized by plasminogen receptors, which contain carboxyl-terminal lysine residues [12]. Previous results have shown that removal of the strong lysine binding site in r-apo(a) (the 17KΔLBS$_{10}$ variant) results in an inability to effectively bind to fibrin surfaces [7]. In the current study, 17KΔLBS$_{10}$ internalization is significantly reduced, but not abolished, compared to wild-type 17K in HepG2 cells (Fig. 5.1). Treatment of HepG2 cells with the lysine analogue ε-ACA resulted in a significant decrease in both Lp(a) and apo(a) internalization, and PCSK9 was still able to inhibit Lp(a) (but not apo(a)) internalization in the presence of ε-ACA. Thus, removal of the strong LBS in apo(a) affects its ability to be internalized through lysine-dependent plasminogen receptors but not through non-covalent interactions with apoB and subsequent binding to LDLR. However, treatment with ε-ACA abolishes both the ability of apo(a) or Lp(a) to bind to lysine-dependent plasminogen receptors as well as the ability of apo(a) to couple to the apoB component of LDL [50]; the latter effect accounts for the inability of PCSK9 to inhibit apo(a) uptake in the presence of ε-ACA (Fig. 5.1B).

Recently, it has been reported that LDL can bind to PCSK9 and inhibit its ability to target the LDLR for degradation [44]. We therefore analyzed if Lp(a) can bind to PCSK9 in order to determine if (i) less Lp(a) is being internalized due to its ability to bind to PCSK9 and thus prevent its internalization; or (ii) Lp(a) binding to PCSK9 leads to a reduced ability for PCSK9 to target the LDLR for degradation, or other receptors, limiting the ability of Lp(a) to be internalized through those receptors. Through *in vitro* binding experiments, we have shown that PCSK9 cannot bind to Lp(a) (Fig. 5.2). As well, both Lp(a) and apo(a) do not inhibit the ability of PCSK9 to target the LDLR for

degradation in HepG2 cells. The exact site at which the apoB component of LDL binds to PCSK9 is currently unknown. Therefore, it is possible that the apo(a) component of Lp(a) is interfering with the interaction of PCSK9 and apoB.

We also explored the degradation pathway of Lp(a)/apo(a) through PCSK9. Previous work has shown that PCSK9 can target the LDLR for degradation through clathrin-mediated endocytosis and is targeted for lysosomal degradation [43, 44]. We show here, through knockdown of clathrin heavy chain, that Lp(a) and apo(a) are also internalized through clathrin-mediated endocytosis. Treatment with PCSK9 results in no further decrease in Lp(a)/apo(a) internalization following clathrin heavy chain knockdown. This indicates that the receptors that internalize Lp(a)/apo(a) which can be regulated by PCSK9 are dependent on clathrin-mediated endocytosis. Furthermore, treatment with a lysosomal inhibitor, E-64d, but not a proteosomal inhibitor, results in a significant accumulation of intracellular Lp(a) and apo(a) with or without PCSK9 treatment. Both of these inhibitors are highly selective, potent, and irreversible inhibitors of their respective target proteases [51, 52]. E-64d inhibits calpain and the cysteine proteases cathepsins F, K, B, H, and L and acts by forming a thioester bond with the thiol of the active site cysteine without affecting cysteine residues in other enzymes. Lactacystin covalently modifies the amino-terminal threonine of specific catalytic subunits of the proteasome. Taken together, these results indicate that Lp(a)/apo(a) internalization alone or through PCSK9 occurs, in part, through clathrin-mediated endocytosis and Lp(a)/apo(a) is subsequently targeted for lysosomal degradation. We next determined which receptor is involved in Lp(a) catabolism which can be regulated by PCSK9.

The role for the LDLR in Lp(a) catabolism has been controversial. Compared to LDL, plasma Lp(a) concentrations are much less responsive to conventional lipid-lowering therapies including statins. Indeed, some studies have shown increased in plasma Lp(a) with statins, others no effect, and others decreases [53]. More recent, larger studies have found that statins modestly but significantly reduce Lp(a) in subjects with dyslipidemia or heterozygous FH [54, 55]. Moreover, in some studies of FH kindreds with a null LDLR, elevated plasma Lp(a) levels are observed in affected individuals [21, 22, 56], although this result has not been unanimously observed [22, 57, 58]. Although overexpression of LDLR in mice significantly increased Lp(a) clearance [20], plasma clearance studies in $Ldlr^{-/-}$ mice and human FH patients reported no significant difference in Lp(a) clearance compared to the presence of normal LDLR, although a non-significant decrease in fractional catabolic rate in the absence of LDLR of about 10% was reported in both studies [23, 24]. Plausible evidence therefore exists to indicate that the LDLR does participate in Lp(a) catabolism, which may account for the ability of PCSK9 inhibitors to lower plasma Lp(a). Accordingly, we directly examined the role of the LDLR in regulation of Lp(a) catabolism by PCSK9.

The following lines of evidence from our study very strongly support the concept of the LDLR being a PCSK9-regulable clearance receptor for Lp(a). (i) The GOF PCSK9 mutant D374Y, which can target the LDLR for degradation more rapidly, was more effective than WT PCSK9 in inhibiting both Lp(a) and apo(a) internalization in HepG2 cells (Fig. 5.1C, D). (ii) Overexpression of LDLR (and LDLRΔCT) dramatically increase Lp(a) clearance (Fig. 5.5B). Addition of PCSK9 in the context of LDLR overexpression decreased internalization, but the difference did not reach statistical significance. It is

possible that the dose of PCSK9 added was not sufficient to influence the very high concentrations of ectopically-expressed LDLR. (iii) Addition of blocking monoclonal antibodies against LDLR decreased Lp(a) internalization and PCSK9 had no effect in the setting of LDLR blockade with the antibodies (Fig. 5.5D). (iv) Human fibroblasts lacking LDLR showed decreased internalization of Lp(a) that was unaffected by the addition of PCSK9 (Fig. 5.6A).

It is notable that the LDLR lacking the cytoplasmic tail, which interacts with the ARH adaptor protein to promote endocytosis, retains a considerable fraction of the wild-type receptor's ability to internalize Lp(a) (Fig. 5.5B). It has been previously shown that PCSK9 cannot target the LDLR for degradation in primary hepatocytes isolated from $Arh^{-/-}$ mice [59]. However, PCSK9 can target the LDLR for degradation upon removal of the cytoplasmic tail in CHO cells [60], and the ARH adaptor protein is not necessary in PCSK9 mediated LDLR degradation in fibroblasts [46]. These results suggest a potential PCSK9-interacting partner in mediating endocytosis of the LDLR-PCSK9 in HepG2 cells.

Less of an increase is observed with apo(a) internalization following LDLR overexpression (Fig. 5.5C) indicating the requirement for apo(a) coupling to apoB for recognition by this receptor. Although HepG2 cells were deprived of LDL by growth in LPDS, these cells do express apoB and secrete apoB-containing lipoprotein particles in the LDL density range [12]. Formation of non-covalent complexes between these particles and apo(a) could be rate-limiting and therefore may account for why less of an increase in internalization is observed for apo(a) compared to Lp(a) with LDLR overexpression. Our results also show that Lp(a) internalization is significantly reduced

in human FH fibroblasts with a defective LDLR compared to fibroblasts with WT LDLR function (Fig. 5.6A). Fibroblasts do not express apoB and, not surprisingly therefore, the internalization of apo(a) is not affected by PCSK9 in either the control or LDLR defective fibroblast cells (Fig. 5.6B). Collectively, these results definitively indicate a role for the LDLR in internalization of Lp(a) through the apoB component rather than apo(a).

PCSK9 has been reported to downregulate other members of the LDLR, specifically the VLDLR and the apoE2 receptor [35]. It is not known if these are ligands for Lp(a) *in vivo*, but it does not appear that they are playing a role in Lp(a) internalization in our experiments, at least with respect to the PCSK9-dependent component. This conclusion stems from our observations that Lp(a) internalization is insensitive to PCSK9 in the presence of antibodies that block the LDLR (Fig. 5.5D) or in fibroblasts lacking functional LDLR (Fig. 5.6A).

It is notable that Lp(a) lowering by PCSK9 inhibitory antibodies is of a lower magnitude (~30%) compared to LDL (~70%) [25-29]. Because LDL concentrations are higher than Lp(a) on a particle number basis, LDL can compete with Lp(a) for binding to the LDLR. Considering the concentration of plasma LDL is generally far greater than that of Lp(a), it increases its likelihood of clearance through the LDLR compared to Lp(a). It is also notable that all study subjects receiving PCSK9 inhibitory antibodies were also receiving an optimal dose of statin [27], and an Lp(a) lowering effect was still observed. In this setting, by increasing hepatic LDLR to supraphysiological levels, there is a profound lowering of LDL levels which may in turn allow for the clearance of Lp(a) through the LDLR. This was also observed *in vivo* in which Lp(a) catabolism

significantly increased in mice overexpressing LDLR [20]. Our results clearly suggest that the effects of PCSK9 inhibitory antibodies on Lp(a) levels *in vivo* are the consequence of greater LDLR-mediated catabolism of Lp(a). Therefore, although the LDLR may not be a major route of clearance of Lp(a) under most circumstances, its importance increases in the setting of therapy to increase LDLR numbers, particularly by the use of inhibitory antibodies against PCSK9.

5.6 REFERENCES

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CHAPTER 6

General Discussion

Elevated levels of Lp(a) have been identified as an independent risk factor for CHD [1, 2]. However, the pathophysiological mechanisms by which Lp(a) mediates its harmful effects are not entirely understood. The aim of this work was to identify the mechanism of how Lp(a) can effect pericellular plasminogen activation, and to extend our understanding of Lp(a) catabolism. The role of Lp(a)/apo(a) in pericellular plasminogen activation was evaluated in vascular and blood cells. In addition, the role of novel apo(a) receptors in the context of plasminogen activation was assessed. Furthermore, the mechanism by which Lp(a)/apo(a) is catabolised and how this may potentially be regulated by PCSK9 was investigated. We found that Lp(a)/apo(a) can inhibit pericellular plasminogen activation through attenuating Glu- to Lys-plasminogen conversion. As well, a variety of receptors- uPAR, $\alpha_M\beta_2$, and $\alpha_V\beta_3$- were implicated in regulating plasminogen activation and mediating the ability of apo(a) to inhibit plasminogen activation on vascular and blood cells. Finally, we determined that Lp(a) catabolism can be regulated by PCSK9 through LDLR-dependent uptake of Lp(a).

6.1 LP(a)/APO(a) INHIBITS PERICELLULAR PLASMINOGEN ACTIVATION

Previous reports have established that apo(a) inhibits tPA-mediated fibrin clot lysis [3] and tPA-mediated plasminogen activation on the fibrin surface [4-7]. Apo(a) was found to inhibit fibrin clot lysis with tPA-mediated activation of Glu-plasminogen and to a less extent with either uPA as a plasminogen activator or with Lys-plasminogen [3]. These findings illustrate that the key inhibitory ability of apo(a) is to attenuate the conversion of Glu- to Lys-plasminogen on fibrin clots. Apo(a) was reported by our group to inhibit plasminogen activation through what was described as an equilibrium template

model with tPA, plasminogen, apo(a), and fibrin interacting in a quaternary complex [4]. Additionally, apo(a) was shown to attenuate the conversion of Glu-plasminogen to Lys-plasminogen on the fibrin surface [8]. Apo(a)-mediated inhibition of fibrinolysis, as well as Glu- to Lys-plasminogen conversion, was not dependent on apo(a) isoform size [4, 8]. Critical roles for the KV domain and the strong LBS in KIV_{10} were shown for both optimal inhibitory effects of apo(a) on fibrin mediated plasminogen activation as well as Glu- to Lys-plasminogen conversion [4, 8]. A N-terminally truncated version of apo(a) (missing KIV types 1-4) was unable to inhibit tPA-mediated Glu-plasminogen activation on the fibrin surface to the same extent as the full length version indicating that KIV types 1-4 of apo(a) are required for maximal inhibition of plasminogen activation [4]. However, the N-terminal domain of apo(a) was not required for apo(a) mediated inhibition of Glu- to Lys-plasminogen conversion on the fibrin surface [8]. Removal of the protease domain of apo(a) did not affect its ability to inhibit tPA-mediated Glu-plasminogen activation on the fibrin surface indicating that the interaction between apo(a) and plasminogen in solution is not required for this process [4]. Together these findings illustrate that there are common domains necessary for the ability of apo(a) to inhibit both plasminogen activation and Glu- to Lys-plasminogen conversion on the fibrin surface, although there are contrasting roles for some apo(a) domains in these two processes. This may reflect the fact that different enzymes are required in catalyzing the reactions for plasminogen activation (tPA) and for Glu- to Lys-plasminogen conversion (plasmin). A corresponding level of understanding of the ability of apo(a) to inhibit plasminogen activation and Glu- to Lys-plasminogen conversion on cell surfaces is critical as this is one of the potential pathophysiological roles for Lp(a). Plasmin

generated via cell surface receptors contributes not only to fibrinolysis and thrombolysis [9-11], but also to events involved in atherogenesis, cell migration and proliferation, angiogenesis, inflammation, wound healing, and cancer [12-20].

As a result, the role of apo(a) in potentially inhibiting pericellular plasminogen activation was explored in Chapter 2. As we report for the first time, the addition of apo(a) resulted in a significant dose-dependent decrease in plasminogen activation. A greater than 50% decrease in plasminogen activation was observed in the presence of the maximum concentration of apo(a) utilized on HUVECs, THP-1 monocytes, and THP-1 macrophages; the extent of the ability of apo(a) to inhibit plasminogen activation was similar in the three cell lines utilized. Critical roles for the KV domain and the strong LBS in KIV_{10} were required for optimal inhibitory ability of apo(a). These findings are in agreement with previous studies conducted on the fibrin surface with regard to the role of apo(a) isoform size and critical domains necessary for optimal inhibition of plasminogen activation and Glu- to Lys-plasminogen conversion on fibrin surface [4, 8].

We have shown that low concentrations of Lp(a) are effective at reducing plasminogen activation on HUVECs and THP-1 monocytes but not on THP-1 macrophages. Apo(a) was found to be more potent than Lp(a) for inhibition of plasminogen activation on THP-1 macrophages and HUVECs suggesting that the apo(a) component of Lp(a) is responsible for the inhibitory ability. These results are expected as apo(a) shares homology with plasminogen [21] and thus may compete with plasminogen for binding sites on cellular receptors. Similar to THP-1 monocytes, Lp(a) was able to inhibit plasminogen activation to the same extent as apo(a) on the fibrin surface [4]. This indicates that the differences in the receptors present on distinct cell types directly play a

role in the potency of Lp(a) for inhibition of plasminogen activation. For example, Lp(a) may potentially bind to non-plasminogen receptors through its LDL component to different extents on various cell types thereby diluting the effect of the apo(a) component of Lp(a) to inhibit plasminogen activation. In addition, different cell surfaces may bind and internalize Lp(a) to greater extents. For example, rapid uptake of Lp(a) has previously been shown in THP-1 macrophages [22]. This may explain the variation in the ability of Lp(a) to inhibit plasminogen activation on different cell surfaces. Although, higher concentrations of Lp(a) may have the ability to decrease plasminogen activation on THP-1 macrophages but were restricted to the relatively low concentration of the preparation.

The inhibitory ability of plasminogen activation by apo(a) in the current study was examined using the plasminogen activator tPA and not uPA. tPA was used as it is expressed in ECs and has been previously shown to bind to various cell types including monocytes, platelets, and monocytoid cells [23-26]. It would be interesting to determine if apo(a) is able to inhibit pericellular plasminogen activation using uPA as an activator. The uPAR plays a key role in uPA-mediated plasminogen activation [27] and it is possible that apo(a) not only interferes with plasminogen/uPA binding to cell surface receptors (such as uPAR) but also in the positive feedback mechanism of converting sc-uPA to its active tc-uPA form. Previous findings illustrated that apo(a), plasminogen, tPA, and fibrin interact as a quaternary complex which can attenuate the rate of plasminogen activation [4]. However, the current study did not quantitatively evaluate if the same mechanism occurs on the cell surface. Therefore, future experiments are needed to determine if apo(a) inhibits pericellular plasminogen activation through either: (i)

binding as a quaternary complex on the cell surface, (ii) simply competing with plasminogen for cell-surface binding sites, (iii) binding of apo(a) to plasminogen in solution and prevention of its subsequent activation, or (iv) a combination of the aforementioned events. If in fact inhibition of plasminogen activation is occurring through binding as a quaternary complex, than differences may be observed based on the cell-type (dependency of certain receptors such as uPAR) and the enzyme catalysing the reaction (tPA versus uPA). If apo(a) and plasminogen are exclusively competing for cell surface receptors, than the ability of apo(a) to inhibit plasminogen activation will be dictated by the receptors present on different cell types and not necessarily the type of plasminogen activator. If inhibition is occurring through simply binding of plasminogen and apo(a) in solution than no difference in the enzyme catalyzing the reaction or cell-type should dictate the ability of apo(a) to inhibit plasminogen activation.

The mechanism of how apo(a) can inhibit pericellular plasminogen activation was explored in Chapter 3. The native circulating form of plasminogen, Glu-plasminogen, is in a closed conformation that does not allow for rapid activation to plasmin by plasminogen activators [28, 29]. However, binding of Glu-plasminogen to cell surface receptors results in a more open conformation and accessibility to plasminogen activators or plasmin [30]. Cleavage of Lys^{77}-Lys^{78} by plasmin results in the removal of the N-terminal tail domain and formation of Lys-plasminogen [31]. Unlike Glu-plasminogen, Lys-plasminogen adopts a more extended, open conformation [28, 29]. This open form is a much better substrate for plasminogen activators. Previous results have identified that Glu- to Lys-plasminogen conversion is the primary driver of plasminogen activation on

ECs [32]. As well, our group has shown that apo(a) can inhibit Glu- to Lys-plasminogen conversion on the fibrin surface [8].

The inhibitory effect of apo(a) on pericellular plasminogen activation may potentially occur through attenuating Glu- to Lys-plasminogen conversion. In the current study, we have shown that apo(a) is a potent inhibitor of Glu- to Lys-plasminogen conversion on both HUVECs and SMCs. Isoform size of apo(a) did not affect its ability to inhibit Glu- to Lys-plasminogen conversion. The domains of apo(a) that are required to inhibit Glu- to Lys-plasminogen conversion are similar to that observed in Chapter 2 with respect to the ability of Lp(a)/apo(a) to inhibit pericellular plasminogen activation as well as to previous findings of Lp(a)/apo(a)-mediated inhibition of plasminogen activation and Glu- to Lys-plasminogen conversion on the fibrin surface [4, 8]. We have shown that removal of the strong LBS in KIV_{10} abolished the ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on vascular cells. The protease domain as well as the KV domain were also identified as necessary for the maximal ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on vascular cells. The importance of these domains was also illustrated with respect to their requirement for apo(a)-mediated inhibition of pericellular plasminogen activation on fibrin [4], as well as vascular and blood cells (Chapter 2). The KV domain was also required for the ability of apo(a) to inhibit Glu- to Lys-plasminogen conversion on the fibrin surface [8]. However, it is unknown if the LBS present in KV is required for the ability of apo(a) to inhibit this process. Apo(a) partially retained the ability to inhibit Glu- to Lys-plasminogen conversion in the absence of the protease domain on vascular cells. Previous results have shown that apo(a) can bind to plasminogen through the protease domain of apo(a) in solution [33]. As a result, the

predominant mechanism for which apo(a) inhibits Glu- to Lys-plasminogen conversion on vascular cells may not be through the binding of apo(a) to plasminogen in solution.

Binding of Lp(a)/apo(a) to cell surface receptors is assumed to occur predominantly through LBSs in apo(a) KIV types 5-8, KIV type 10, or KV in a similalr manner as plasminogen. Plasminogen has previously been shown to bind to cell surface receptors containing a carboxyl-terminal lysine residue [12]. Carboxyl-terminal lysines present on cell-surface receptors are responsible to a greater extent for plasminogen activation than for plasminogen binding. A previous study that removed carboxyl-terminal lysine residues on monocytoid cells resulted in a ~60% decrease in plasminogen binding and >95% decrease in plasminogen activation [26]. However, a previous report illustrated that apo(a) can effectively bind to ECs even with removal of the strong LBS present in KIV_{10} [34]. It has generally been assumed that Lp(a)/apo(a) can potentially disrupt the physiological functions of plasminogen by virtue of the high level of homology between the two, including the lysine-binding function. Nevertheless, apo(a) may bind to different cell surface receptors than plasminogen, or to receptors proximal to plasminogen receptors that result in steric hindrance of the ability of plasminogen to bind to cell surface receptors. The role of carboxyl-terminal lysine-containing receptors on the ability of apo(a) and plasminogen to compete for binding sites on vascular and blood cells was therefore explored.

As described in Chapter 2, removal of carboxyl-terminal lysines, through treatment with CpB, resulted in a small decrease in plasminogen activation on HUVECs, THP-1 monocytes, and THP-1 macrophages. A concomitant decrease in plasminogen binding was observed using these three cell lines. The addition of the lysine analogue ε-

ACA resulted in almost complete abolishment of the ability of plasminogen to bind to the cell surfaces. Conversely, apo(a) binding slightly decreased following CpB treatment in THP-1 monocytes and THP-1 macrophages, but not on the surface of HUVECs. Apo(a) binding was abolished by the addition of ε-ACA to THP-1 monocytes and THP-1 macrophages; this was not observed using HUVECs. These results suggest that apo(a) binding to ECs is most likely not dependent on receptors containing a carboxyl-terminal lysine residue, but that internal lysines in receptors may play a role.

Competition binding experiments were also conducted in order to determine if apo(a) or plasminogen can compete with one another for binding sites on vascular and blood cells. It is important to note that apo(a) and plasminogen can bind to each other in solution and may result in a "piggy-back" effect whereby apo(a) can still bind to cell or fibrin surfaces when plasminogen is bound to it, or *vice versa* [33]. The potential confounding effects of piggy-backing complicates the interpretation of competition experiments. We found that unlabelled plasminogen was unable to compete for binding of fluorescently labelled apo(a) to vascular and blood cells. However, unlabelled apo(a) effectively competed with fluorescently labelled plasminogen for binding to the cell surface. These results suggest that apo(a) can bind to plasminogen in solution, and that the complex retains the ability to bind to the cell surface through apo(a), but not through plasminogen. This may be a potential mechanism by which apo(a) interferes with both plasminogen activation and Glu- to Lys-plasminogen conversion. The lack of competition for one another may also indicate that apo(a) and plasminogen bind to different receptors. A previous study has shown that Lp(a) can compete with plasminogen for binding sites on monocytes and endothelial cells [35]. Plasminogen and

Lp(a) have similar affinities for plasminogen receptors (K_D~1-3 μM), but plasminogen can bind to approximately 10 times more binding sites than Lp(a) [35]. These results indicate that Lp(a) has the potential to compete with plasminogen for binding to cellular receptors, but Lp(a)/apo(a) can only bind to a subset of plasminogen receptors. Further experiments are required in order to convincingly determine if apo(a) and plasminogen are competing for the same cellular receptor(s) or if they are predominantly binding to independent receptor(s). *In vitro* experiments can be conducted to determine if apo(a) can bind to known plasminogen receptors such as Plg-R_{KT} and/or annexin II S100A10 [22, 36].

In conclusion, Lp(a)/apo(a) can inhibit pericellular plasminogen activation on vascular and blood cells through attenuating Glu- to Lys-plasminogen conversion. Apo(a) can potently inhibit tPA-mediated conversion of plasminogen to plasmin as well as plasmin mediated Glu- to Lys-plasminogen conversion, a key mechanism in accelerating the rate of plasminogen activation. The KV domain and the strong LBS in KIV_{10} were necessary for optimal inhibitory effect of apo(a) on plasminogen activation and Glu- to Lys-plasminogen conversion. The protease domain of apo(a) was required for maximal inhibition of Glu- to Lys-plasminogen conversion on vascular cells.

These findings extend our knowledge on the effect of Lp(a)/apo(a) on antifibrinolytic/prothrombotic and atherosclerotic processes. The ability of Lp(a)/apo(a) to inhibit pericellular plasminogen activation can ultimately lead to a prothrombotic state through inhibition of fibrinolysis *in vivo*. Lp(a)/apo(a) mediated inhibition of pericellular plasminogen activation can have additional implications within the vasculature. For example, plasmin generated on the surface of vascular and blood cells play a key role in

the dissolution of mural thrombi [10]. Mural thrombi are formed following plaque disruption and subsequent exposure of thrombogenic components within the plaque architecture to that of the blood within the lumen. The thrombus formed can then remain attached to the damaged endothelium and contribute to the development of atherosclerosis [10]. Ideally, the mural thrombus forms a plug at the site of the weakened fibrous cap and damaged endothelium and is subsequently dissolved by plasmin. However, the mural thrombus can also be incorporated into the atherosclerotic plaque through reorganization of the vascular cells if the thrombus is not dissolved [10]. This would ultimately result in plaque growth and further progression of atherosclerosis. We have shown here that Lp(a)/apo(a) can significantly reduce the formation of plasmin on vascular and blood cells which potentially has significant implications in promoting the stability of a mural thrombus and ultimately leading to the progression of atherosclerosis.

6.2 RECEPTORS INVOLVED IN APO(a) MEDIATED INHIBITION OF PLASMINOGEN ACTIVATION

Plasminogen receptors are expressed on many cell types with no single receptor responsible for binding of plasminogen on the cell surface [12]. Plasminogen binding occurs with low affinity to these cellular receptors (K_d ~ 1 μM) but with high capacity, ranging from ~10^4 sites on platelets to >10^7 sites on ECs [12]. Various plasminogen receptors have been identified which accelerate the rate of plasminogen activation [12]. The uPAR assists in uPA-mediated plasminogen activation by clustering uPA on the cell surface in close proximity to plasminogen receptors [37]. Binding of sc-uPA to uPAR allows for cleavage of sc-uPA by plasmin to its active two-chained form (tc-uPA) [38, 39]. As well, uPAR can also bind to vitronectin and β_3 integrins [40, 41]. uPAR does not

contain a transmembrane domain and therefore transmits intracellular signals through interacting with various integrins, such as $\alpha_V\beta_3$ [41]. The role of uPAR in the context of tPA mediated plasminogen activation and the ability of Lp(a)/apo(a) to inhibit this process was explored in Chapter 4. Knockdown of uPAR in HUVECs resulted in a significant decrease in plasminogen activation as well as plasminogen and apo(a) binding. The ability of apo(a) to inhibit plasminogen activation was partially attributable to the interaction with uPAR. Importantly, the KV domain and the strong LBS in KIV_{10} were required for apo(a) to bind to uPAR and to inhibit plasminogen activation. Similar to the results in Chapter 2, ε-ACA did not decrease binding of apo(a) on the cell surface of HUVECs, but did so on THP-1 monocytes and THP-1 macrophages.

Overexpression of uPAR was also conducted in HUVECs, THP-1 monocytes, and THP-1 macrophages to validate the role of uPAR in tPA-mediated plasminogen activation. Overexpression of uPAR in these cells resulted in an increase in plasminogen activation. Interestingly, apo(a) was able to inhibit uPAR-mediated plasminogen activation on HUVECs, but not on THP-1 monocytes or THP-1 macrophages. These results underscore the variation in the identity and role of plasminogen/apo(a) receptors on various cell types. Apo(a) and plasminogen binding increased with uPAR overexpression in HUVECs, THP-1 monocytes, and THP-1 macrophages. The KV domain and strong LBS in KIV_{10} of apo(a) were required for maximal binding of apo(a) to uPAR on HUVECs and THP-1 monocytes, but not on THP-1 macrophages. Interestingly, apo(a) did not inhibit plasminogen activation via uPAR on THP-1 monocytes or THP-1 macrophages even though an increase in apo(a) binding was observed following uPAR overexpression. These findings suggest that the ability of

apo(a) to inhibit plasminogen activation through uPAR is dependent on uPAR interacting with another cell-type specific protein such as an integrin. However, these findings do not directly address whether uPAR can in fact bind directly to tPA, plasminogen, or apo(a). Future experiments are needed to identify whether plasminogen or apo(a) can bind directly to uPAR or through interaction with another protein to which uPAR is bound. A more direct approach, using *in vitro* binding experiments, should be conducted with purified uPAR and apo(a) or plasminogen to determine if in fact they can bind with each other.

As noted above, uPAR can also interact with different integrins. Previous studies have shown that apo(a) can stimulate EC growth and migration through integrin $\alpha_V\beta_3$ [42], and that Lp(a) can bind to $\alpha_M\beta_2$ integrin and recruit inflammatory cells [43]. Based on these observations, the role of integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$ was explored in the ability of apo(a) to modulate the plasminogen activation system (Chapter 4). Plasminogen activation was reduced in the presence of a monoclonal antibody targeting either integrin α_M or integrin β_2 on THP-1 monocytes and HUVECs. A monoclonal antibody targeting $\alpha_V\beta_3$ also resulted in a minor decrease in plasminogen activation on HUVECs with a more substantial decrease observed using THP-1 monocytes. Addition of the highest concentration of apo(a) with THP-1 monocytes or HUVECs resulted in no difference in plasminogen activation in the absence or presence of monoclonal antibodies targeting α_M, β_2, or $\alpha_V\beta_3$ integrins. The collective data indicate that apo(a) may inhibit plasminogen activation to an extent through these receptors. In general, these findings confirm previous reports that indicate that a large subset of receptors contributes to plasminogen activation on various cell types. The receptors tested in the present study contributed to

plasminogen activation and the ability of apo(a) to inhibit this process; however, there is no single plasminogen receptor responsible for plasminogen activation or the ability of apo(a) to inhibit this process.

As mentioned above, the ability of apo(a) to inhibit pericellular plasminogen activation not only contributes to inhibition of fibrinolysis and thrombolysis [9-11], but also to events involved in atherogenesis, cell migration and proliferation, angiogenesis, inflammation, wound healing, and cancer [12-20]. This provides a basis for the suggested link between Lp(a) and atherosclerotic and thrombotic processes as well as nonvascular diseases such as cancer. The direct role of Lp(a)/apo(a) in these cellular processes, with respect to plasminogen activation, has yet to be fully elucidated, either in pathophysiological contexts (at high Lp(a) concentrations) or as a protective agent in normal physiology (at low Lp(a) concentrations). It would be interesting to determine if Lp(a)/apo(a) can directly inhibit plasminogen activation leading to attenuation of cancer metastasis and invasion. Recent intriguing evidence suggest that Lp(a) levels <80 mg/dL are a significant risk factor for different forms of cancer including hepatocellular carcinoma, pancreatic cancer, and leukemia with no association of Lp(a) levels as a risk factor for lung cancer [44]. It is possible that apo(a) can inhibit plasminogen activation on cancer cells. This would lead to less plasmin formation and subsequently delay degradation of the ECM, thereby preventing uncontrolled tissue remodelling. Considering that the uPAR plays a critical role in uPA-mediated plasminogen activation in the context of cell remodelling [41] and that apo(a) may regulate plasminogen activation via uPAR (Chapter 4), it is tempting to speculate that Lp(a)/apo(a) may play a role in impeding cancer progression. This may present a novel mechanism and potential

therapeutic option to delay cancer metastasis and invasion by the ability of Lp(a)/apo(a) to inhibit plasmin formation. This may present a protective physiological function of Lp(a). Future work should explore the potential ability of Lp(a)/apo(a) to inhibit cancer metastasis and invasion.

6.3 PCSK9 MEDIATED LP(a) CATABOLISM AND THE ROLE FOR THE LDLR

The most commonly used therapeutic option for treatment of individuals with elevated plasma Lp(a) has been through niacin therapy [45]. However, treating patients with niacin has been associated with often intolerable side effects [46]. Additionally, recent evidence suggests that niacin therapy may not further decrease cardiovascular risk, including that associated with elevated Lp(a) levels, compared to administration of statin therapy alone [47, 48]. Nevertheless, niacin can lower Lp(a) levels by 20-30% and is still recommended for administration to high-risk patients with elevated Lp(a) levels over 50 mg/dL [45].

A recent therapeutic approach to lowering Lp(a) levels is through blocking the function of PCSK9 [reviewed in ref. 49], although the mechanism by which PCSK9 may regulate Lp(a) levels is unknown. In Chapter 5, we present strong evidence that PCSK9 can decrease Lp(a) internalization in HepG2 hepatic cells and human primary fibroblasts through the LDLR. In this study, we showed that exogenous treatment of HepG2 cells with PCSK9 effectively and potently reduced the extent of Lp(a) and apo(a) internalization. These results are intriguing in that they corroborate *in vivo* findings demonstrating a role for PCSK9 in lowering Lp(a) levels [49]. Blocking PCSK9 with a monoclonal antibody results in ~30% and ~70% decrease in Lp(a) and LDL levels in humans respectively [50-52]. Clearly, LDL is a better ligand for the LDLR compared to

Lp(a). As a result, the decrease in Lp(a) levels observed through PCSK9 inhibition may be due in part to a drastic decrease in LDL levels and subsequently less competition for Lp(a) clearance via the LDLR. It is important to note that niacin treatment and blocking PCSK9 function is not specific in lowering Lp(a) as a significant decrease in LDL levels are also observed [46, 49].

The decrease observed for Lp(a) and apo(a) internalization in HepG2 cells following PCSK9 treatment may be the result of binding of Lp(a) to PCSK9 in solution, thereby preventing catabolism through the LDLR. This follows from a recent report that the apoB-100 component of LDL can bind to PCSK9 in solution, preventing the complex from catabolism through the LDLR [53]. As such, we conducted *in vitro* experiments to determine if PCSK9 can bind to Lp(a) and prevent the ability of Lp(a) to be catabolised through the LDLR similar to that observed for LDL. We found that Lp(a) does not bind to PCSK9 *in vitro*, nor does it protect the LDLR from degradation unlike what was observed for LDL suggesting that the apo(a) component of Lp(a) may interfere with the ability of PCSK9 to interact with apoB-100. It would be interesting to determine if the inability of Lp(a) to bind to PCSK9 is isoform size-dependent. Lp(a) has been previously shown to display differences in affinity to mononuclear cells with larger isoforms binding to the cell surface with lower affinity than small isoforms suggesting that smaller isoforms may potentially bind to other ligands such as PCSK9 [54]. Future work should be invested into determining if smaller Lp(a) isoforms can bind to PCSK9 *in vitro*. If binding of smaller isoforms of Lp(a) to PCSK9 does occur than it may prevent the function of PCSK9 to target the LDLR for degradation, which would lead to increased levels of LDL and potentially Lp(a).

The next step was to determine which receptor is responsible for the ability of PCSK9 to regulate Lp(a) catabolism. The GOF D374Y PCSK9 mutant is more active than wild-type PCSK9 at targeting the LDLR complex for degradation [55]. Interestingly, treatment with the GOF PCSK9 mutant D374Y resulted in a more robust effect in reducing Lp(a) and apo(a) internalization in HepG2 cells suggesting a role for the LDLR. Three other independent experiments were conducted in order to determine if the LDLR does in fact play a role in the effect of PCSK9 in Lp(a)/apo(a) catabolism. We found that overexpression of the LDLR in HepG2 cells resulted in a significant increase in Lp(a) internalization with a non-significant increase observed using apo(a). PCSK9 did not significantly reduce Lp(a) or apo(a) internalization with LDLR overexpression, possibly attributed to the significant enhancement in LDLR protein levels without increasing the concentration of PCSK9. These results suggest that the apoB-100 component of Lp(a) is responsible for the ability of Lp(a) to be catabolised by the LDLR. In another experiment, monoclonal antibodies targeting the LDLR were used to block the receptor to determine if an effect on Lp(a) catabolism is observed. A significant decrease in both Lp(a) and apo(a) internalization in HepG2 cells was observed using two different LDLR monoclonal antibodies, again suggesting a role for the LDLR. As a third line of evidence, human fibroblasts with either defective or no LDLR activity were utilized to determine the contribution of the LDLR to Lp(a) catabolism. We showed that Lp(a) internalization was significantly reduced in human primary fibroblasts with a defective or null LDLR compared to fibroblasts with normal LDLR activity. Crucially, apo(a) internalization was not affected by PCSK9 using either fibroblasts with normal LDLR or defective LDLR

activity. These results demonstrate conclusively that the effects of PCSK9 on Lp(a) catabolism are specifically attributable to the effect of PCSK9 on the LDLR.

The inability of apo(a) internalization to be modulated by PCSK9 in fibroblasts may indicate the importance for the non-covalent attachment of apo(a) to LDL. Unlike HepG2 cells, fibroblasts do not endogenously express apoB-100 and apo(a) is therefore unable to "piggy-back" on LDL in fibroblasts. The results indicate the importance for apo(a) to associate with apoB-100 in order for internalization through the LDLR to occur. Interaction between apo(a) and apoB-100 secreted by HepG2 cells is required to modulate apo(a) internalization by either PCSK9 treatment or the addition of monoclonal LDLR blocking antibodies. The importance of the apo(a) interaction with apoB-100 for LDLR-dependent uptake was further illustrated utilizing the 17KΔLBS$_{7,8}$ variant which has been previously shown to be unable to associate with apoB-100 [56]. Removal of the weak LBS in each of the KIV type 7 and 8 domains resulted in a significant decrease in the ability of this variant to be internalized compared to wild-type apo(a). Together, these results suggest that the non-covalent interaction between apo(a) and apoB-100 is necessary for internalization to occur through the LDLR and hence to be modulated by PCSK9.

The mechanism by which Lp(a)/apo(a) degradation occurs through PCSK9 was also explored in Chapter 5. The results indicate that Lp(a)/apo(a) internalization occurs through clathrin-mediated endocytosis in hepatic cells. Treatment of HepG2 cells with a lysosomal inhibitor, but not a proteasomal inhibitor, resulted in intracellular accumulation of Lp(a)/apo(a) consistent with a lysosomal degradation pathway. Collectively, these results suggest that Lp(a)/apo(a) internalization occurs through clathrin-coated pits and is

subsequently targeted for lysosomal degradation consistent with the LDLR internalization pathway. Future work should be conducted in order to determine if a co-receptor or protein is required for binding to the Lp(a):LDLR complex in order to target Lp(a) into clathrin coated pits for subsequent lysosomal degradation. This is suggested based on a report that a PCSK9 mutant lacking the M2 domain is unable to target the PCSK9:LDLR complex for degradation, but can still effectively bind to the LDLR [57]. It would also be interesting to determine if the LDLR is recycled following internalization with Lp(a) similar to the LDL:LDLR complex, or if the Lp(a):LDLR complex is targeted for degradation as has been described for the PCSK9:LDLR complex.

There still remain many questions with regard to how Lp(a) is catabolised in humans. The results in Chapter 5 indicate that PCSK9 can mediate Lp(a) catabolism through the LDLR, at least in a cell culture model. The LDLR may not necessarily represent the main Lp(a) receptor responsible for its clearance *in vivo*. Traditional statin therapy, which results in an increase in hepatic LDLR number, generally has minimal or no effect on Lp(a) levels. However, the LDLR may play a role in Lp(a) clearance in situations in which LDLR levels are furthere elevated, such as with the use of PCSK9 inhibitors with statins [49]. The role of the LDLR and PCSK9 must be explored in Lp(a) catabolism utilizing *in vivo* models. Currently, the role for the LDLR remains controversial with many studies conducted indicating a role for this receptor on Lp(a) catabolism [58-67], while others showing no role for this receptor [68-72]. A potential reason for the contradictory findings is that commonly used animal models, such as mice, do not contain the *LPA* gene, and may not be equipped with the optimal tools for Lp(a)

catabolism [73]. Therefore, caution must be used when studying Lp(a) catabolism using *in vivo* models other than in humans and Old World monkeys, which have the *LPA* gene [73]. Future experiments are required to determine if mouse models are appropriate for examining the role of the LDLR in Lp(a) clearance. For example, the role of the LDLR in binding and degradation of Lp(a) can be determined through *ex vivo* experiments using wild-type and $LDLR^{-/-}$ mice in the presence or absence of PCSK9. The findings can then be compared to those in Chapter 5 to determine if mice are an appropriate model for evaluating Lp(a) catabolism.

It would be interesting to study Lp(a) clearance in mice overexpressing human apo(a) and apoB-100 with or without the addition of either purified PCSK9 or a monoclonal antibody targeting the endogenous PCSK9. Additionally, determination of the role of the LDLR, or other receptors that can be targeted by PCSK9 in Lp(a) catabolism using knockout mouse models of these receptors. In this regard, PCSK9 has been shown to target not only the LDLR but also VLDLR, ApoER2, and LRP-1 for degradation [74, 75]. Since the liver has been shown to be the primary route for Lp(a) clearance [68, 76], study of the role of ApoER2 and LRP-1 should be prioritized since these receptors are expressed in the liver, unlike VLDLR.

Additional novel receptor(s) that are not regulated by PCSK9 may also be responsible for Lp(a) clearance. A more substantial decrease in LDL compared to Lp(a) levels is observed following PCSK9 inhibition which suggests that other receptors, in addition to the LDLR, can contribute to the clearance of Lp(a); these receptors would not be regulated by PCSK9. If in fact the LDLR, or other receptors targeted by PCSK9, are

the sole receptor(s) responsible for Lp(a) clearance, than similar lowering results should be observed to that of LDL for PCSK9 based therapies, which is not the case.

Owing to the high degree of homology between apo(a) and plasminogen [21], it is therefore possible that Lp(a) can be cleared from the circulation by plasminogen receptors via the apo(a) component of Lp(a). Previous studies have shown that Lp(a)/apo(a) can compete for binding to plasminogen receptors [77, 78]. Additionally, apo(a) has been shown to be taken up by FH fibroblasts through plasminogen receptors [58]. There may potentially be a role for the receptors identified in Chapter 4- uPAR and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$- in Lp(a) clearance. However, the role for this class of plasminogen receptors in Lp(a) binding and potentially its clearance from plasma *in vivo* is unknown. Previous work has shown that uPAR can interact with LRP and undergo clathrin-mediated endocytosis [79]. It may therefore be possible that Lp(a) interacts with uPAR and the Lp(a):uPAR:LRP complex is subsequently catabolised. Future work is needed to explore the possibility that Lp(a) is cleared through the apo(a) component by plasminogen receptors or other yet to be identified receptors. These findings will enable the generation of more specific therapeutic targets for lowering elevated Lp(a) plasma levels through modulation of Lp(a) catabolism.

6.4 CONCLUDING REMARKS

Lp(a) has been identified as an independent, causal risk factor for CHD, although the pathophysiological and physiological roles of Lp(a) remain elusive [1, 2]. The roles of Lp(a) isoform size, independent of Lp(a) concentration, and the impact on CHD still remain controversial. Lp(a) contains multifunctional properties attributable to both the

LDL and apo(a) components that result in a multitude of different mechanisms dependent on the vascular setting. For example, we have shown in the current study that elevated levels of Lp(a)/apo(a) can inhibit plasmin formation on vascular cells, which may promote atherosclerosis in the context of a mural thrombi. Conversely, a decrease in plasmin formation on cancer cells may be beneficial as it can potentially play a role in inhibiting cancer metastasis and invasion [44]. Lp(a) therefore possesses the ability to promote atherosclerosis and thrombosis as well as nonvascular processes, such as cancer, depending on the concentration and vascular setting.

Lp(a) is a challenging lipoprotein to study as it is only present in humans and Old World monkeys, which questions the validity of common *in vivo* models used to study Lp(a) [73]. The physiological role of Lp(a) is difficult to determine as knockout models are not feasible. The harmful effects of Lp(a) may be significantly reduced with the proper management for individuals with elevated plasma levels. Blocking the function of PCSK9 may present an effective method for reducing elevated levels of Lp(a) through increasing Lp(a) catabolism. Future work at reducing the rate of apo(a) synthesis, through ASO therapy, may offer a strategy to specifically lower Lp(a) levels at the rate of production and may provide an attractive therapeutic option. Many questions remain in determining the mechanisms of action and deciphering the harmful and potential protective roles of Lp(a). Further elucidation of the multiple roles of Lp(a) will allow for the evaluation the effects of targeting Lp(a) levels on cardiovascular and nonvascular outcomes.

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4. Seol HJ, Chang JH, Yamamoto J, **Romagnuolo R**, Suh Y, Weeks A, Agnihotri S, Smith CA, Rutka JT. Overexpression of CD99 increases the migration and invasiveness of human malignant glioma Cells. *Genes & Cancer*. 2012;3(9-10):535-49.
5. Diaz RJ, Guduk M, **Romagnuolo R**, Smith CA, Northcott P, Shih D, Berisha F, Flanagan A, Munoz DG, Cusimano MD, Pamir MN, Rutka JT. High-resolution whole-genome analysis of skull base chordomas implicates FHIT loss in chordoma pathogenesis. *Neoplasia*. 2012;14(9):788-98.
6. **Romagnuolo R**, Boffa MB, Koschinsky ML. Apolipoprotein(a)-dependent inhibition of pericellular plasminogen activation is mediated by specific cellular receptors. Blood (ASH Annual Meeting Abstracts). 2011;118:2236.

7. Nagai S, Moreno O, Smith CA, Ivanchuk S, **Romagnuolo R**, Golbourn B, Weeks A, Seol HJ, Rutka JT. Role of the cofilin activity cycle in astrocytoma migration and invasion. *Genes & Cancer*. 2011;2(9):859-69.

Manuscripts accepted/submitted

1. **Romagnuolo R**, Scipione C, Boffa MB, Marcovina SM, Seidah NG, Koschinsky ML. Lipoprotein(a) Catabolism is Regulated by Proprotein Convertase Subtilisin/Kexin Type 9 Through the Low Density Lipoprotein Receptor. *J Biol Chem*. 2014; (manuscript under revision; ID: JBC/2014/611988).

Manuscripts in preparation

1. **Romagnuolo R**, Boffa MB, Koschinsky ML. Inhibition of pericellular plasminogen activation by apolipoprotein(a): roles of urokinase plasminogen activator receptor and integrins αMβ2 and αVβ3 in vascular cells.
2. **Romagnuolo R**, DeMarco K, Boffa MB, Koschinsky ML. Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen on the surface of vascular endothelial and smooth muscle cells.

INVITED PRESENTATIONS

1. **Romagnuolo R**, Seidah NG, Koschinsky ML. (2014). Lipoprotein(a) internalization in HepG2 cells is regulated by proprotein convertase subtilisin kexin type 9 through the low-density lipoprotein receptor. American Heart Association (AHA) Scientific Sessions 2014. Chicago, IL, USA. November 15-19. Poster Presentation.

CONFERENCES

1. **Romagnuolo R**, Seidah NG, Koschinsky ML. (2014). Lipoprotein(a) internalization in HepG2 cells is regulated by proprotein convertase subtilisin kexin type 9 through the low-density lipoprotein receptor. 15th annual Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB) Conference. Toronto, Canada. May 1-3. Poster Presentation. Rated in the top 10% of accepted abstracts and invited to present in the Best of AHA Specialty Conference.
2. **Romagnuolo R**, Seidah NG, Koschinsky ML. (2013). Lipoprotein(a)/ apolipoprotein(a) internalization in HepG2 cells is regulated by PCSK9 through clathrin-mediated endocytosis. 38th Annual Canadian Lipoprotein Conference (CLC). Mont Tremblant, Canada. Sept 26-29. Oral presentation.
3. **Romagnuolo R**, DeMarco K, Boffa MB, Koschinsky ML. (2013) Apolipoprotein(a) inhibits the conversion of Glu-plasminogen to Lys-plasminogen on the surface of vascular endothelial and smooth muscle cells. XXIVth Congress of the International Society on Thrombosis and Haemostasis (ISTH). Amsterdam, Netherlands. June 29-July 4. E-poster oral presentation.
4. **Romagnuolo R**, Boffa MB, Koschinsky ML. (2012) Role of apolipoprotein(a) in angiogenesis and cell migration through plasminogen activation. First annual Windsor cancer research group (WCRG) conference. Windsor, ON, Canada. Oral presentation.
5. **Romagnuolo R**, Boffa MB, Koschinsky ML. (2011) Apolipoprotein(a)-dependent inhibition of pericellular plasminogen activation is mediated by specific cellular

receptors. 53rd annual American Society of Hematology (ASH) Annual Meeting. San Diego, CA, USA. Poster presentation.

6. **Romagnuolo R**, Boffa MB, Koschinsky ML. (2011) Inhibition of plasminogen activation through apolipoprotein(a) and determination of its candidate receptors. Graduate Student Conference: Bringing Together Communities. Windsor, ON, Canada. Poster presentation.