

**INTERACTIONS OF LIPOPROTEIN(A) WITH THE
PLASMINOGEN SYSTEM: MECHANISMS AND
PATHOPHYSIOLOGICAL CONSEQUENCES**

by

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Abstract

Elevated plasma concentrations of lipoprotein(a) (Lp(a)) are associated with increased risk of atherothrombotic disease. Lp(a) is a unique lipoprotein consisting of a low density lipoprotein-like moiety covalently linked to apolipoprotein(a) (apo(a)), a homologue of the fibrinolytic proenzyme plasminogen. Apo(a) is extremely heterogeneous in size with small isoforms being independently associated with increased cardiovascular risk.

Several *in vitro* and *in vivo* studies have shown that Lp(a)/apo(a) can inhibit tissue-type plasminogen activator (tPA)-mediated plasminogen activation on fibrin surfaces, although the mechanism of inhibition by apo(a) remains controversial. Essential to fibrin clot lysis are a number of plasmin-dependent positive feedback reactions that enhance the efficiency of plasminogen activation, including the plasmin-mediated conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen.

Additionally, abnormal fibrin clot structures have been associated with both an increased risk of cardiovascular disease and elevated Lp(a) levels. Similarly, oxidized phospholipids have been implicated in the development of cardiovascular disease, and are not only preferentially carried by Lp(a) in the plasma but have also been shown to covalently-modify both apo(a) and plasminogen.

In this thesis, we built upon the understanding of the role of apo(a) in plasminogen activation on the fibrin/degraded fibrin surface by determining that: (i) apo(a) inhibits plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion and identifying the critical domains in apo(a) responsible for this effect, (ii) apo(a) isoform size does not affect either the inhibition of tPA-mediated plasminogen activation or the inhibition of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion, (iii) apo(a) modifies fibrin clot structure to form more dense clots

with thinner fibers and reduced permeability, modifications that enhance the ability of apo(a) to inhibit tPA-mediated plasminogen activation and (iv) the phosphorus content of apo(a) affects its ability to inhibit tPA-mediated plasminogen activation and the phosphorus content of plasminogen affects its ability to be activated by tPA.

By understanding these individual reactions, each of which has the potential to affect the broader fibrin clot lysis process, we have expanded our understanding of the overall effect of Lp(a)/apo(a) in the inhibition of plasminogen activation on the fibrin/degraded fibrin surface and thus broadened our understanding of how Lp(a)/apo(a) may mediate the inhibition of thrombolysis *in vivo*.

Co-Authorship

The experiments presented herein were the work of the author under the guidance and direction of Dr. Marlys Koschinsky and Dr. Michael Boffa.

The recombinant apo(a) stable lines were provided by Dr. Marlys Koschinsky with additional isoform constructs generated by Susan Johnston as outlined in Chapter 2. The recombinant plasminogen stable line was provided by Dr. Michael Nesheim.

Confocal experiments were performed with the assistance of the Jalna Meens, Facility Technician at the Cancer Research Institute Imaging Facility at Queen's University. The scanning electron microscopy experiments were performed with the assistance of Sharon Lackey, Facility Technician at ESEM Facility at the GLIER Institute at the University of Windsor.

All manuscripts were written initially by the author and revised and edited by Dr. Marlys Koschinsky and Dr. Michael Boffa; Figures 1.3-1.5 were designed by and are used with the permission of Dr. Marlys Koschinsky and Dr. Michael Boffa.

The protocols employed for the quantification of the phosphorus content of the proteins, and for the enzymatic digestion and the subsequent purification of the enzymatically-digested plasminogen were modified for our purposes by Corrado Scipione.

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Table of Contents

Abstract	ii
Co-Authorship	iv
Acknowledgements.....	v
List of Figures	viii
List of Abbreviations	x
Chapter 1 <i>Introduction</i>	1
1.1 Lipoprotein(a)	1
1.2 Pathogenicity of Lp(a)	2
1.3 Lp(a) & Plasminogen Activation on Fibrin/Degraded Fibrin Surfaces	4
1.4 Plasmin-Mediated Positive Feedback Reactions in Plasminogen Activation.....	7
1.5 Fibrin Clot Structure	8
1.6 Lipoprotein(a) & Oxidation	9
1.7 Hypothesis	11
1.8 Objectives	11
1.9 Figures	12
Chapter 2 <i>Apolipoprotein(a) Inhibits the Conversion of Glu-Plasminogen to Lys-Plasminogen: A Novel Mechanism for Lipoprotein(a)-Mediated Inhibition of Plasminogen Activation</i>	17
2.1 Summary	17
2.2 Introduction.....	18
2.3 Materials & Methods	21
2.4 Results.....	24
2.5 Discussion.....	27
2.6 Figures	32
Chapter 3 <i>Apolipoprotein(a) Induces Prothrombotic Alterations to Fibrin Clot Structure that Exacerbates its Inhibition of tPA-Mediated Plasminogen Activation</i>	39
3.1 Summary	39
3.2 Introduction.....	40

3.3 Materials & Methods	42
3.4 Results.....	46
3.5 Discussion.....	51
3.6 Figures	55
Chapter 4 <i>Phosphatidylcholine-Adducts on Both Plasminogen and Apolipoprotein(a) Affect tPA-Mediated Plasminogen Activation</i>	67
4.1 Summary.....	67
4.2 Introduction.....	68
4.3 Materials & Methods	70
4.4 Results.....	74
4.5 Discussion.....	77
4.6 Figures	81
Chapter 5 <i>Discussion</i>	89
5.1 General Findings.....	89
5.1.1 CHAPTER 2: Glu ¹ -Plasminogen to Lys ⁷⁸ -Plasminogen Conversion	89
5.1.2 CHAPTER 3: Clot Structure.....	90
5.1.3 CHAPTER 4: Isoform Size & Oxidized Phosphatidylcholine-Adducts in tPA-Mediated Plasminogen Activation	92
5.2 Pathophysiological Implications.....	94
5.2.1 Glu ¹ -Plasminogen to Lys ⁷⁸ -Plasminogen Conversion	94
5.2.2 Fibrin Clot Structure	95
5.2.3 Oxidative Modification of Lp(a) & Apo(a)	96
5.3 Future Directions	98
References.....	101

List of Figures

Chapter 1 Introduction

Figure 1.1 <i>The Structure of Lipoprotein(a)</i>	12
Figure 1.2 <i>Structural homologies between apo(a) and plasminogen</i>	13
Figure 1.3 <i>Schematic diagram indicating structural features and functional domains of apo(a)</i>	14
Figure 1.4 <i>Potential pathogenic mechanisms of Lp(a)</i>	15
Figure 1.5 <i>Equilibrium template model for the inhibition of tPA-mediated plasminogen activation by apo(a)</i>	16

Chapter 2 Apolipoprotein(a) Inhibits the Conversion of Glu-Plasminogen to Lys-Plasminogen: A Novel Mechanism for Lipoprotein(a)-Mediated Inhibition of Plasminogen Activation

Figure 2.1 <i>Recombinant apo(a) variants utilized in study</i>	32
Figure 2.2 <i>Dose-dependent inhibition of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸- plasminogen conversion by apo(a)</i>	33
Figure 2.3 <i>Role of individual domains in apo(a) in inhibition of Glu¹-plasminogen to Lys⁷⁸- plasminogen conversion</i>	35
Figure 2.4 <i>The role of apo(a) isoform size in apo(a)-mediated inhibition of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion</i>	37

Chapter 3 Apolipoprotein(a) Induces Prothrombotic Alterations to Fibrin Clot Structure that Exacerbates its Inhibition of tPA-Mediated Plasminogen Activation

Figure 3.1 <i>The effect of increasing 17KIV r-apo(a) concentration on the permeability of lipoprotein-deficient plasma clots</i>	55
Figure 3.2 <i>Box plots of clot permeability parameters for clots derived from lipoprotein- deficient plasma, plasma-purified fibrinogen and commercial (Calbiochem®) fibrinogen in the presence and absence of 17KIV r-apo(a)</i>	56
Figure 3.3 <i>Apo(a) induces a change in fibrin clot ultrastructure</i>	59
Figure 3.4 <i>Quantification of the effect of 17KIV r- apo(a) on the ultrastructure of a fibrin clot</i>	60
Figure 3.5. <i>The effect of apo(a) on fibrin polymerization</i>	63

Figure 3.6. <i>The effect of apo(a) on plasmin-mediated clot lysis</i>	64
Figure 3.7. <i>The effect of decreasing thrombin concentration on the inhibition of tPA-mediated plasminogen activation by apo(a) on fibrin</i>	65
Supplementary Figure 3.1. <i>Clot generated using commercially-available (Calbiochem®) fibrinogen in the absence of apo(a)</i>	66

Chapter 4 *Phosphatidylcholine-Adducts on Both Plasminogen and Apolipoprotein(a) Affect tPA-Mediated Plasminogen Activation*

Figure 4.1. <i>tPA-mediated activation of fluorescently-labeled recombinant plasminogen on fibrin degradation products as a function of apo(a) isoform size</i>	81
Figure 4.2. <i>tPA-mediated activation of fluorescently labeled recombinant plasminogen on fibrin degradation products as a function of apo(a) isoform size subdivided by isoform preparation and plotted in association with protein phosphorus content</i>	82
Figure 4.3. <i>Immunoblot analysis of E06 reactivity and phosphorus quantification for apo(a) proteins</i>	83
Figure 4.4. <i>Average initial rate of tPA-mediated plasminogen activation on fibrin degradation products as a function of the moles of oxidized phosphotidylcholine per mole of r-apo(a) protein</i>	85
Figure 4.5. <i>Immunoblot analysis of E06 reactivity and phosphorus quantification for r-plasminogen proteins</i>	86
Figure 4.6. <i>Average initial rate of tPA-mediated plasminogen activation on fibrin degradation products as a function of the moles of oxidized phosphotidylcholine per mole of r-plasminogen protein</i>	88

List of Abbreviations

Apo(a)	Apolipoprotein(a)
ApoB-100	ApolipoproteinB-100
C-terminal	Carboxy-Terminal
cDNA	Complementary Deoxyribonucleic Acid
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
ER	Endoplasmic Reticulum
ESEM	Environmental Scanning Electron Microscope
FDPs	Fibrin Degradation Products
Glu ¹ -Pg	Glu ¹ -Plasminogen
HMW	High Molecular Weight
K1	Kringle 1 Domain of Plasminogen
K2	Kringle 2 Domain of Plasminogen
K3	Kringle 3 Domain of Plasminogen
K4	Kringle 4 Domain of Plasminogen
K5	Kringle 5 Domain of Plasminogen
KIV	Kringle 4-like Domain of Apolipoprotein(a)
KIV ₁₋₁₀	Kringle 4-like Domain of Apolipoprotein(a) Subtypes 1-10
KV	Kringle 5-like Domain of Apolipoprotein(a)
LBS	Lysine Binding Site
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LMW	Low Molecular Weight
Lp(a)	Lipoprotein(a)
Lys ⁷⁸ -Pg	Lys ⁷⁸ -Plasminogen
MI	Myocardial Infarction
N-terminal	Amino-Terminal
OxLp(a)	Oxidized Lp(a)
OxPL	Oxidized Phospholipids

OxPtdC	Oxidized Phosphatidylcholine
PAD	Peripheral Artery Disease
PAI-1	Plasminogen Activator Inhibitor-1
sc-tPA	Single-Chain Tissue-Type Plasminogen Activator
SEM	Scanning Electron Microscope
SNPs	Single Nucleotide Polymorphisms
TAFI	Thrombin-Activable Fibrinolysis Inhibitor
tPA	Tissue-Type Plasminogen Activator
SMC	Smooth Muscle Cells

Chapter 1

Introduction

1.1 Lipoprotein(a)

Lipoprotein(a) (Lp(a)) constitutes a distinct class of lipoprotein particles found in human plasma. It is very similar to low-density lipoprotein (LDL) in terms of lipid composition—both contain a cholesteryl ester and triglyceride core surrounded by a monolayer of phospholipid and unesterified cholesterol—and apolipoproteinB-100 (apoB-100) content. Lp(a) is distinguishable from LDL in that it contains an additional large glycoprotein, apolipoprotein(a) (apo(a)), that is covalently linked by a single disulfide bond to apoB-100 [1,2] (Figure 1.1). Analysis of the apo(a) cDNA from a human liver library in 1987 revealed that apo(a) is highly similar to the fibrinolytic proenzyme plasminogen [3].

Plasminogen is the single-chain zymogen of the serine protease plasmin. It is composed of 791 amino acids arranged in seven domains: an N-terminal pre-activation peptide followed by five tandem *kringle* domains (K1-5), and a catalytic domain including the serine protease catalytic triad of His⁶⁰³, Asp⁶⁴⁶, and Ser⁷⁴¹ [4,5]. The *kringle* domain, named based on its resemblance to the danish pastry, is an autonomous domain found in a number of proteins involved in blood coagulation and fibrinolysis, and is known to be important in mediating protein-protein interactions [6-8]. It is a structural motif characterized by a triple-loop structure of approximately 80 amino acids held together by three invariant disulphide bonds [7]. K1, K4, and K5 of plasminogen contain lysine-binding site (LBS) substructures [7-9]. The LBS is a cleft or pocket with a cationic and anionic subsite at either end separated by a hydrophobic region of highly conserved aromatic residues. The cationic and anionic subsites form ion pair interactions

with the carboxylate and ammonium groups, respectively, of ω -amino acids such as lysine [10]. The active site of plasminogen is distorted and therefore catalytically inactive [11]. Cleavage of the Arg⁵⁶¹-Val⁵⁶² peptide bond by plasminogen activators gives rise to the catalytically active serine protease, plasmin, in which the heavy chain (containing the kringle domains) is bound by two interchain disulphide bridges to a light chain (containing the catalytic domain) [6].

Apo(a) contains three of the structural domains present in plasminogen: single copies of a plasminogen-like protease domain and K5 domain (KV), as well as multiple copies of a plasminogen-like K4 domain (KIV) (Figure 1.2) [3]. The protease domain of apo(a) shares a high degree of sequence similarity (94%) with the protease domain of plasminogen, including the catalytic triad [3]. However, the protease domain of apo(a) is catalytically inactive. This is due to an Arg to Ser substitution in apo(a) at the activation cleavage site (Arg⁵⁶¹-Val⁵⁶²) that prevents its cleavage by plasminogen activators, as well as the presence of a nine-amino acid deletion, which alters the conformation of the active site [12,13]. Apo(a) contains 10 types of plasminogen-like K4 domains (KIV₁₋₁₀), which are distinguishable from each other by small variations in amino acid sequence, and exhibit up to 75% identity with K4 of plasminogen [3]. Each KIV subtype is present in a single copy except for KIV₂, which is present in 3 to >30 identically repeated copies attributable to allelic variations in the number of sequences encoding KIV in the apo(a) gene [3,14]. This forms the basis of the Lp(a) size heterogeneity evident in the human population, wherein at least 34 isoforms have been detected [15] (Figure 1.3).

1.2 Pathogenicity of Lp(a)

Numerous studies have identified high plasma Lp(a) concentrations (>25-30mg/dL) as a risk factor for a variety of atherosclerotic disorders, including peripheral vascular disease [16-20],

cerebrovascular disease [21-27], and coronary heart disease [24,28-32]. Additionally, a number of studies have also identified elevated Lp(a) plasma levels as a risk factor for purely thrombotic disorders (*i.e.*, not secondary to atherosclerosis). Specifically, clinical studies have identified Lp(a) as a risk factor for venous thromboembolism in adults [33-35] and children [36-38], and for spontaneous [39-41] and recurrent [39,42] ischemic stroke in children. As a result of the studies over the past few decades, Lp(a) is now conclusively considered a cardiovascular risk factor [43]. Although the mechanisms by which Lp(a) exerts its proatherogenic and prothrombotic effects have not been conclusively determined, evidence for multiple potential pathogenic mechanisms have been reported (Figure 1.4) [44].

An additional important mediator of Lp(a) pathogenicity may be apo(a) isoform size. As mentioned, apo(a) is highly polymorphic due to the genetically determined KIV₂ repeat number [3,14], resulting in apo(a) proteins of variable size (167 to >650 kDa) [45]. Plasma levels can vary by over 1000-fold in the human population, ranging from <0.1 to >100 mg/dL [46] between individuals with different isoforms of apo(a) [47]. In particular, apo(a) isoform size is inversely related to plasma levels such that smaller apo(a) isoforms are associated with higher plasma Lp(a) levels [47]. At the genetic level, the major determinant of the variation in Lp(a) plasma levels is attributed to the number of KIV repeats [48] in the *LPA* gene that encodes apo(a) [49], wherein an inverse relationship has been established. At the level of the protein, Lp(a) plasma levels are correlated with the rate of production rather than catabolism [50,51], and small apo(a) isoforms have been shown to have substantially higher production rates [52]. Also, White and colleagues [53] reported that large apo(a) isoforms had longer ER retention times than small apo(a) isoforms.

Apo(a) isoform size has also been implicated as a risk factor for cardiovascular disease independent of its association with plasma Lp(a) levels [54-57]. Specifically, low molecular

weight [LMW] apo(a) isoforms, defined as <22 KIV domains [58], have been found to be associated with an increased risk of cardiovascular disease [55,56,59,60]. This suggests a functional heterogeneity among the apo(a) isoforms, particularly between isoforms of high and low molecular weight. In support of this notion, Hervio and colleagues [61] reported that smaller isoforms bind fibrin with greater affinity than larger isoforms of apo(a). However, neither the mechanistic basis of this observation nor that of the increased associated risk with LMW apo(a) isoforms has been investigated.

1.3 Lp(a) & Plasminogen Activation on Fibrin/Degraded Fibrin Surfaces

Impaired fibrinolysis has been identified as a risk factor for the development of atherosclerosis [62]. It is postulated that impaired fibrinolysis plays a role in the pathogenesis of atherosclerosis by promoting a predisposition to fibrin deposits, and to atherothrombosis by contributing to the formation of an occlusive thrombus upon plaque rupture [63]. Lp(a)/apo(a) has been shown to inhibit fibrinolysis [64] and more specifically, tissue-type plasminogen activator (tPA)-mediated plasminogen activation *in vitro* [65-67] and thrombolysis *in vivo* [68,69].

Fibrinolysis, the process of fibrin solubilization, mediates the regulation of coagulation to prevent inappropriate or excessive clotting of the blood [70] and one of the ways in which apo(a) exerts its pathogenic effects is by interfering with the activation of plasminogen to plasmin, the enzyme responsible for fibrin dissolution. Tissue-type plasminogen activator (tPA) is mainly synthesized in the vascular endothelial cells and is secreted as a single-chain form into the circulating blood in response to various stimuli including thrombin, the terminal protease of the coagulation cascade [4,71]. The conversion of fibrinogen to fibrin is accompanied by conformational changes that result in the exposure of binding sites for tPA and plasminogen [72-

74]. Plasminogen and tPA then bind to fibrin, forming a ternary complex; efficient plasminogen activation requires the formation of this ternary complex [75,76]. The peptide bond between Arg⁵⁶¹-Val⁵⁶² is then cleaved by tPA, thereby activating plasminogen to the serine protease plasmin. Plasmin in turn catalyzes the digestion of the insoluble fibrin clot to soluble fibrin degradation products (FDPs) [77].

As mentioned, several *in vitro* and *in vivo* studies have shown that Lp(a)/ apo(a) can inhibit fibrinolysis [64,68,69]. However, the mechanism by which apo(a) inhibits plasminogen activation remains controversial. This is because both the classical competitive [65] and uncompetitive [66] mechanisms have been reported. More recently, our laboratory described an equilibrium template model to describe the inhibitory mechanism [67]. In this study by Hancock and colleagues, it was found that the inhibition of plasminogen activation by apo(a) could be adequately described by modifying the equilibrium template model of plasminogen activation previously described by Horrevoets and co-workers [76] to include the inhibitor, apo(a) (Figure 1.5). According to the plasminogen activation model, tPA and plasminogen can bind to fibrin in either order to form the binary complexes of fibrin-tPA or fibrin-plasminogen. The remaining third component of the plasminogen activation complex then binds, and through the interactions not only of fibrin-plasminogen and fibrin-tPA individually, but also interactions between tPA-plasminogen, a stable ternary complex of fibrin-tPA-plasminogen is formed. It is from this ternary complex that plasmin is generated efficiently. In this model, the catalytic efficiency of plasminogen activation is dependent upon the stability of the ternary complex as a whole rather than the affinity with which plasminogen and tPA bind to fibrin.

In our modified model (Figure 1.5), apo(a) binds to the fibrin surface. This interaction forms an apo(a)-bound fibrin surface to which tPA and plasminogen once again bind in either

order to form the complexes of apo(a)-fibrin-tPA or apo(a)-fibrin-plasminogen. The fourth component then binds to form a quaternary complex of apo(a)-fibrin-tPA-plasminogen. It is from this quaternary complex that plasmin is formed, albeit much less efficiently than from the corresponding ternary complex of fibrin-tPA-plasminogen. The inhibitory effect of apo(a) is accounted for by the fact that the quaternary complex is more stable, but exhibits a lower turnover number than the corresponding ternary complex. Importantly, in this model, the inhibitor apo(a) interacts with all three components of the plasminogen activation system simultaneously: fibrin (or FDPs), tPA and plasminogen [67].

An additional important finding reported by Hancock and colleagues [67] was the identification of structural domains in apo(a) that are critical to the ability of apo(a) to mediate the inhibition of plasminogen activation. Specifically, the KV, the strong LBS in KIV₁₀ as well as the amino-terminus of apo(a) were found to be required for maximum apo(a)-mediated inhibition of plasminogen activation.

The equilibrium template model of the inhibition of plasminogen activation by apo(a) introduces the idea of the quaternary complex of apo(a)-fibrin-tPA-plasminogen, in which apo(a) can interact with the three remaining components of the complex simultaneously. Notably, isoform size has been reported to affect one of the binary complexes; as mentioned, LMW Lp(a) isoforms have been reported to bind to fibrin more avidly than HMW species [12][78]. This introduces the possibility that LMW and HMW isoforms of apo(a) may have different effects with respect to plasminogen activation, however this has not been studied. These findings, together with the prospective and epidemiological studies that have correlated LMW isoforms of apo(a) with risk for cardiovascular disease [55,56,59,60,79], suggest that LMW apo(a) isoforms may interact with fibrin in the context of the quaternary complex differently than HMW isoforms

and that this could underpin part of the observation of increased risk associated with LMW apo(a) isoforms.

1.4 Plasmin-Mediated Positive Feedback Reactions in Plasminogen Activation

Plasminogen activation mediated by tPA involves a number of plasmin-dependent positive feedback reactions. The small amount of plasmin formed during the initial phase can modify the properties of single-chain tPA (sc-tPA), native Glu¹-plasminogen (Glu¹-Pg) and fibrin to make the activation process more efficient [76].

Plasmin cleaves the peptide bond between Arg²⁷⁵ and Ile²⁷⁶ of the sc-tPA, generating a two-chain form of the molecule, which is a slightly better activator of plasminogen in solution relative to sc-tPA [4,80].

The circulating form of plasminogen is the native Glu¹-Pg [9,76,81]. It exhibits a very tight, spiral structure that is resistant to activation because the activation cleavage site (Arg⁵⁶¹-Val⁵⁶²) is not easily accessible to tPA [76,81]. Plasmin hydrolyses the Lys⁷⁷-Lys⁷⁸ peptide bond of native Glu¹-Pg releasing the N-terminal activation peptide to generate Lys⁷⁸-plasminogen (Lys⁷⁸-Pg). Release of the N-terminal peptide disrupts intramolecular interactions resulting in a shift to an open extended conformation in which the activation peptide bond is readily accessible to tPA. Consequently, Lys⁷⁸-Pg is a better substrate for tPA, which accelerates its rate of activation [9,76,81].

Initial plasmin-mediated cleavage of fibrin produces a modified form referred to as 'plasmin-modified fibrin', which is characterized by newly exposed C-terminal lysine residues. Plasmin-modified fibrin is a better cofactor for tPA-mediated plasminogen activation because the

newly exposed C-terminal lysine residues can act as high affinity binding sites for plasminogen and tPA through interaction with their respective LBS-containing kringle domains [77,82].

These positive feedback reactions constitute important mechanisms that enhance the efficiency of plasminogen activation. As such, the ability of apo(a) to interfere with these reactions could constitute an additional and significant mechanism by which apo(a) could inhibit plasminogen activation. However, the effect of apo(a) on these reactions has not yet been investigated.

1.5 Fibrin Clot Structure

Fibrin is the main structural component of both the physiological fibrin clot and the pathological thrombus [83]. The conversion of fibrinogen to fibrin by thrombin results in the production of fibrin fibers that grow both longitudinally and laterally, and then branch and interconnect to form a three-dimensional network [83]. The process of fibrin polymerization is generally described in two-stages. First, thrombin-cleaved fibrin monomers assemble into half-staggered, double-stranded protofibrils; and second, protofibrils associate laterally to form thicker fibrin fibers [84].

A variety of factors have been shown to alter clot structure [85], which is significant because variations in clot structure have been correlated with both bleeding and thrombotic tendencies [86]. Epidemiological studies have also identified an association between altered clot structure and thromboembolic diseases, including coronary artery disease, ischemic stroke, peripheral artery disease and venous thromboembolism (reviewed in [85]).

Both the stability and the fibrinolytic potential of the fibrin clot can be regulated by its structure [87]. Fiber diameter and the size of the pores in the fiber network are the most

thoroughly explored parameters. Collet and colleagues [88] demonstrated that dense clots with reduced inter-fiber pores were formed by fibers with a thinner diameter and that these clot structures were resistant to fibrinolysis due to a decrease in the binding of tPA. However, they also observed that this was despite the fact that the individual thin fibrin fibers were dissolved at a faster rate than the thick fibers. The conclusion was therefore that the fibrin network architecture (dense vs. loose) was the primary determinant of the rate of fibrinolysis. It has since been postulated that the decreased permeability afforded by the smaller pore size results in attenuated fibrinolysis because of restricted access of the dissolved lytic enzymes to the fiber network [87]. Notably, the aforementioned epidemiological studies have most frequently associated dense clots with abnormally thin fibers, reduced permeability, and hypofibrinolysis with the incidence and/or risk of cardiovascular diseases.

Similarly, elevated Lp(a) levels have been associated with an altered clot structure characterized by reduced permeability and hypofibrinolysis [89]. However, a direct causal relationship between Lp(a)/apo(a) and altered clot structure has not been determined.

1.6 Lipoprotein(a) & Oxidation

The cellular and molecular mechanisms underlying the atherogenic action of Lp(a) has been attributable, in part, to the occurrence of an oxidative modification of both the lipid and protein (apo(a) and apoB-100) components of Lp(a) [90-93]. This is because the oxidative modification of Lp(a) causes diverse biochemical [91,94] and functional changes that promote atherogenesis [95]. The oxidative modification of Lp(a) has been reported to: (i) increase Lp(a)-mediated plasminogen activator inhibitor-1 (PAI-1) production in vascular endothelial cells [96], (ii) impair endothelium-dependent vasodilation [97], (iii) allow its uptake by macrophages

through scavenger receptors [98], (iv) stimulate vascular smooth muscle cell proliferation and (v) induce the adhesion of monocytic cells to endothelial cells [99].

Lp(a) is not normally found in the arterial intima, however it has been shown to be capable of entering into human atherosclerotic plaques [100] and has been identified in atherosclerotic lesions [94]. The mechanisms by which Lp(a) enters and leaves the arterial wall are reported to be similar to LDL [101,102], but Lp(a) is retained more avidly in the vasculature than is LDL [90] and therefore accumulates at sites of arterial injury to a greater extent than LDL. The prolonged residence time of Lp(a) in the subintima relative to LDL has been postulated to be due to its higher affinity for the extracellular matrix and for fibrin(ogen) [66,103,104]. Additionally, Lp(a) is associated with a lower amount of antioxidants relative to LDL [105], which together with its increased retention time in the subintima, promotes its oxidative modification.

Additionally, Lp(a) has been demonstrated to be a preferential carrier of oxidized phospholipids (OxPL) in human plasma [106-109]; of the OxPL associated with Lp(a), half is associated with the protein components, i.e. apo(a) and apoB-100. OxPLs can modify proteins through a process in which the phospholipid is oxidized to an aldehyde, which can then react with ϵ -amino groups of lysine residues to form Schiff base adducts [110]. It has been directly demonstrated that apo(a) is modified by OxPLs, in particular, by the OxPL oxidized phosphatidylcholine (OxPtdC) [111]. The functional consequences of this modification have not been investigated, although, as outlined, there are reasons to suspect that it may be functionally important (reviewed in [112,113]). More recently, plasminogen was also identified to contain OxPtdC-adducts [114], but as with apo(a), the functional consequences are not yet known.

1.7 Hypothesis

Epidemiological studies have indicated a role for Lp(a) in atherosclerosis and its thrombotic sequelae, as well as for purely thrombotic events (reviewed in [43,115-117]). Furthermore, Lp(a)/apo(a) has been shown to inhibit fibrinolysis [64] and tissue-type plasminogen activator (tPA)-mediated plasminogen activation *in vitro* [65-67], and thrombolysis *in vivo* [68,69]. It is our view that by gaining a thorough understanding of the mechanisms by which Lp(a)/apo(a) can inhibit plasminogen activation on the fibrin/degraded fibrin surface *in vitro* we can integrate these findings into a model that can bring us much closer to understanding Lp(a)/apo(a)-mediated inhibition of thrombolysis *in vivo* and the mechanisms underlying the epidemiological observations.

1.8 Objectives

In this thesis, we have sought to build upon the understanding of the role of apo(a) in plasminogen activation on the fibrin/degraded fibrin surface by: (i) investigating the affect of apo(a) on plasmin-mediated Glu¹-Pg to Lys⁷⁸-Pg conversion, (ii) investigating the effect of apo(a) isoform size in the context of tPA-mediated plasminogen activation and the plasmin-mediated conversion Glu¹-Pg to Lys⁷⁸-Pg, (iii) determining the effect of apo(a) on fibrin clot structure, and (iv) determining the effect of the OxPL-modification on both apo(a) and plasminogen in the context of tPA-mediated plasminogen activation. By understanding the role of apo(a) in each of these individual reactions, each of which has the potential to affect the broader fibrin clot lysis process, we can extend our knowledge of the overall effect of Lp(a)/apo(a) in fibrin clot lysis. This may bring the reality of an integrated model a little closer, thereby contributing to our understanding of the role of Lp(a)/apo(a) in thrombolysis *in vivo*.

1.9 Figures

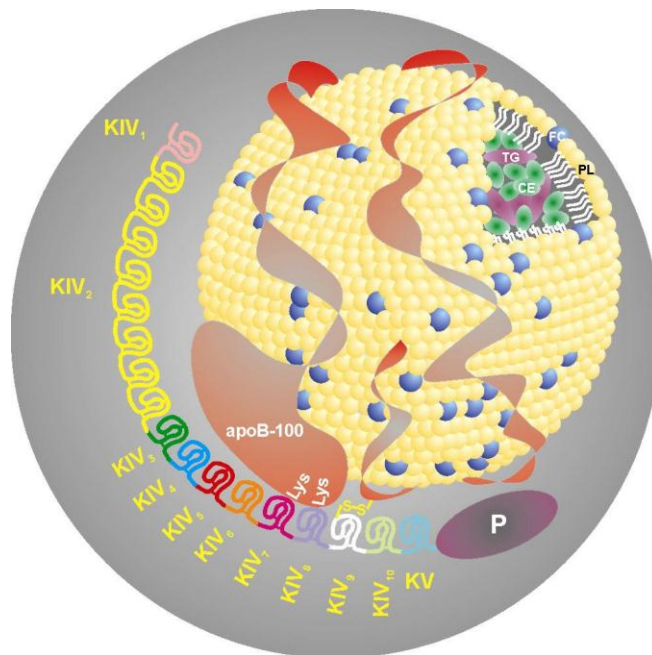


Figure 1.1 *The Structure of Lipoprotein(a)* [118]. Lp(a) resembles LDL with respect to lipid composition and the presence of apolipoprotein B-100. Lp(a) is distinguishable from LDL by the presence of an additional large glycoprotein, apolipoprotein(a) [apo(a)]. Apo(a) contains multiple copies of a domain closely resembling plasminogen kringle 4 that can be divided into 10 distinct subtypes on the basis of amino acid sequence (designated as KIV₁₋₁₀), as well as single copies of the plasminogen-like kringle 5 (KV) and protease-like (P) domains. Apo(a) is covalently linked to apoB-100 by a single disulphide bond. Also indicated is the lysine-dependent non-covalent interaction of lysine residues in apoB-100 with the KIV_{7,8} domains of apo(a) that is important for Lp(a) assembly.

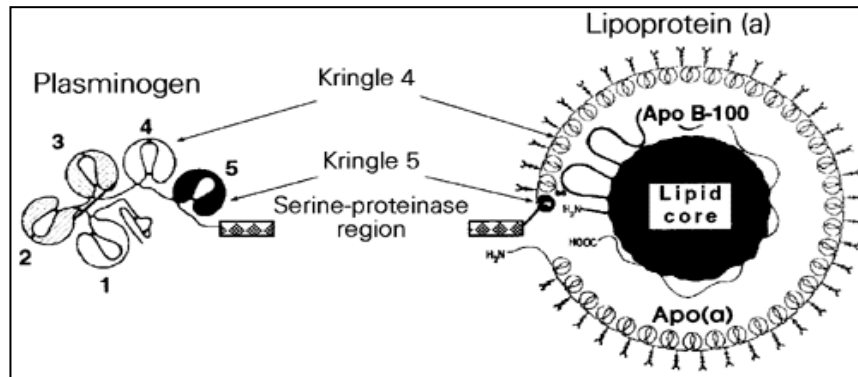


Figure 1.2 *Structural homologies between apo(a) and plasminogen* [119]. Apo(a) contains three of the structural domains present in plasminogen: single copies of a plasminogen-like serine protease domain and a K5-like domain, as well as multiple copies of a plasminogen-like K4 domain. Note that the protease domain in apo(a) is catalytically inactive.

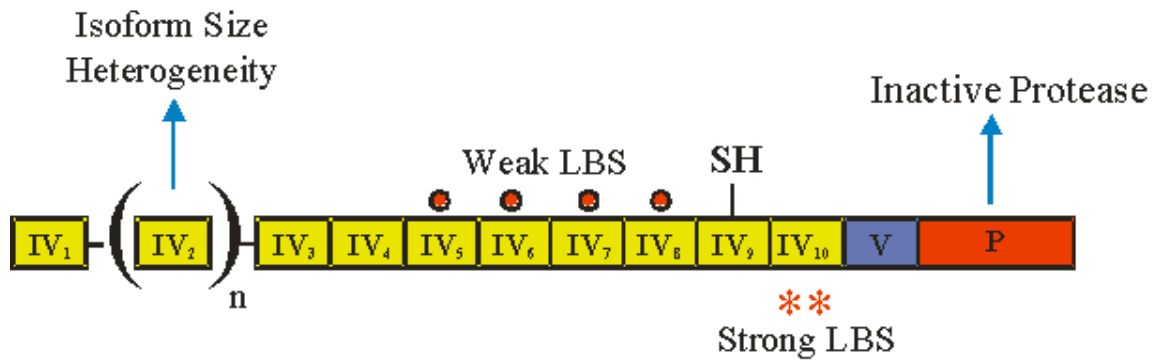


Figure 1.3 Schematic diagram indicating structural features and functional domains of apo (a). Apo(a) consists of 10 types of plasminogen-like kringle 4 domains [IV₁₋₁₀], as well as single copies of plasminogen-like kringle 5 [V] and protease-like [P] domains. Also shown in the diagram is the variability in the IV₂ repeat number that gives rise to apo(a) species of variable size, the location of the weak and strong lysine-binding sites [LBS] in each of IV₅₋₈ and IV₁₀ respectively, as well as the free cysteine [SH] by which apo(a) is covalently bonded to apolipoproteinB-100 in the lipoprotein(a) molecule.

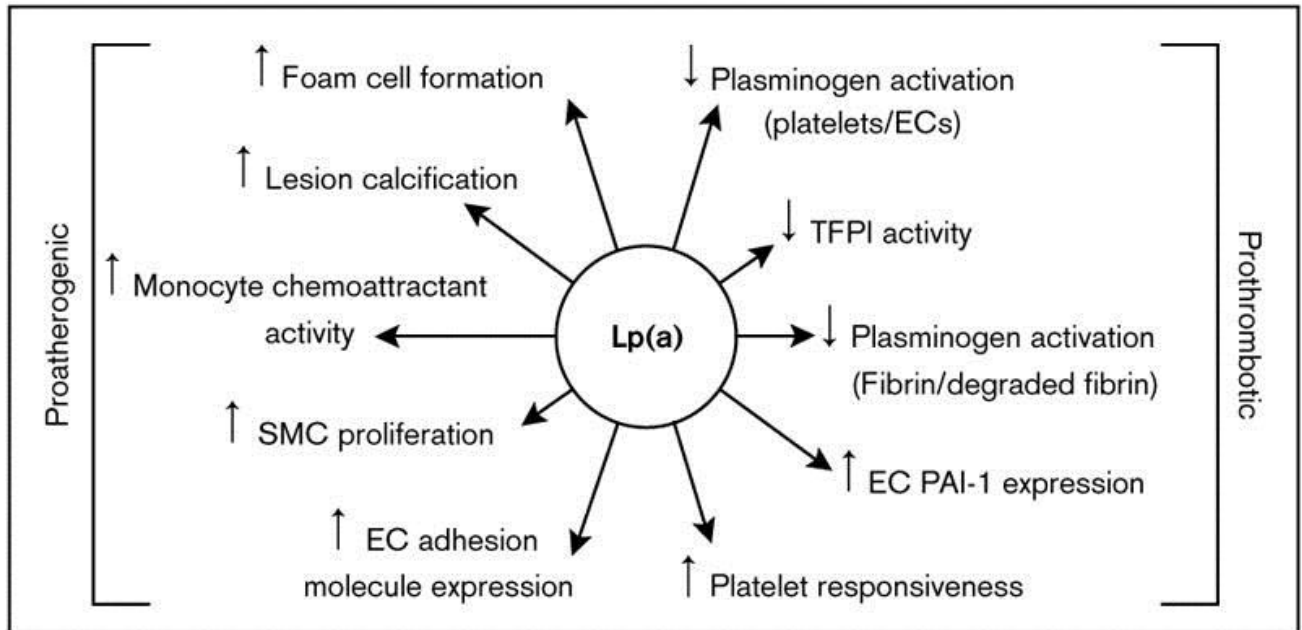


Figure 1.4 Potential pathogenic mechanisms of Lp(a). *In vitro* studies and animal models have demonstrated a number of potential mechanisms by which Lp(a) exerts its pathogenic effects. These mechanisms can be categorized as either proatherogenic (*left* side of figure) or prothrombotic (*right* side of figure) [44]. EC, endothelial cell; SMC, smooth muscle cell; PAI-1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor.

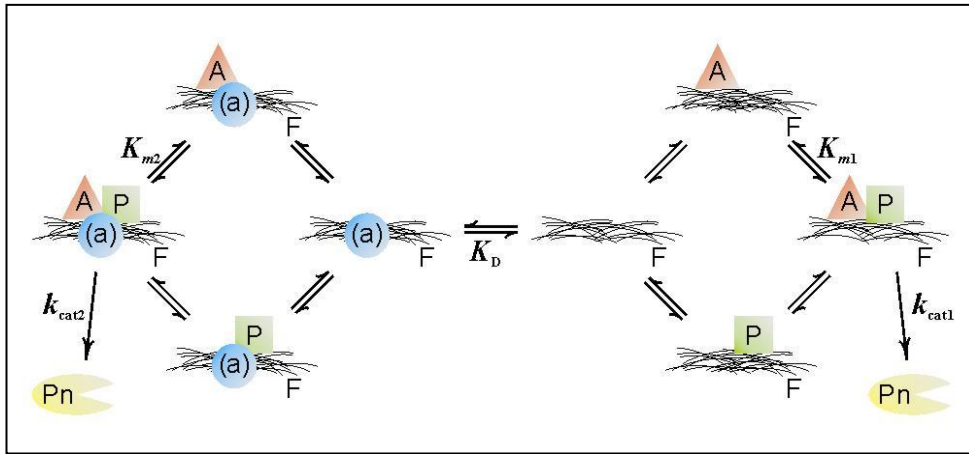


Figure 1.5 *Equilibrium template model for the inhibition of tPA-mediated plasminogen activation by apo(a).* According to the model, Lp(a)/apo(a) [(a)] interacts with fibrin [F], tPA [A], and plasminogen [P] to form a quaternary complex from which plasmin [Pn] is generated. Plasmin is formed less readily from the quaternary complex of (a)FAP than from the corresponding ternary complex of FAP because (a)FAP is more stable ($K_{m2} < K_{m1}$) but has a lower turnover number ($k_{cat2} < k_{cat1}$) than FAP [67].

Chapter 2

Apolipoprotein(a) Inhibits the Conversion of Glu¹-Plasminogen to Lys⁷⁸- Plasminogen: A Novel Mechanism for Lipoprotein(a)-Mediated Inhibition of Plasminogen Activation

2.1 Summary

Background. Elevated plasma concentrations of lipoprotein(a) (Lp(a)) are associated with an increased risk for thrombotic disorders. Lp(a) is a unique lipoprotein consisting of a low density lipoprotein-like moiety covalently linked to apolipoprotein(a) (apo(a)), a homologue of the fibrinolytic proenzyme plasminogen. Several *in vitro* and *in vivo* studies have shown that Lp(a)/apo(a) can inhibit tissue-type plasminogen activator-mediated plasminogen activation on fibrin surfaces, although the mechanism of inhibition by apo(a) remains controversial. Essential to fibrin clot lysis are a number of plasmin-dependent positive feedback reactions that enhance the efficiency of plasminogen activation, including the plasmin-mediated conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen.

Objective. Using acid-urea gel electrophoresis to resolve the two forms of radiolabeled plasminogen, we determined whether apo(a) is able to inhibit Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion.

Methods. The assays were performed in the absence or presence of different recombinant apo(a) species, including point mutants, deletion mutants, and variants that represent greater than 90% of the known apo(a) isoform sizes.

Results. Apo(a) substantially suppressed Glu¹-plasminogen conversion. Critical roles were identified for the kringle IV types 5-9 and kringle V; contributory roles for sequences within the amino-terminal half of the molecule were also observed. Additionally, with the exception of the smallest naturally-occurring isoform of apo(a), isoform size was found not to contribute to the inhibitory capacity of apo(a).

Conclusion. These findings underscore a novel contribution to the understanding of Lp(a)/apo(a)-mediated inhibition of plasminogen activation: the ability of the apo(a) component of Lp(a) to inhibit the key positive feedback step of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion.

Keywords: apolipoprotein(a), fibrinolysis, kringles, plasminogen, plasminogen activation

2.2 Introduction

Numerous studies have identified high plasma lipoprotein(a) (Lp(a)) concentrations (>25-30 mg/dL) as a risk factor for a variety of atherosclerotic and thrombotic disorders (reviewed in refs. [30,44,120]). Lp(a) constitutes a unique class of lipoprotein particles. It is very similar to low-density lipoprotein (LDL) in terms of lipid composition and the presence of apolipoproteinB-100 (apoB-100) but is distinguishable from LDL by the presence of an additional large glycoprotein, apolipoprotein(a) (apo(a)), which is covalently linked to apoB-100 via a single disulfide bond [1,2]. Cloning of human apo(a) revealed it to be highly similar to the fibrinolytic proenzyme plasminogen [3]. Apo(a) consists of multiple copies of a sequence resembling plasminogen kringle 4 (KIV) followed by single copies of domains homologous to the plasminogen kringle 5 (KV) and protease domains [3,121,122]; the protease-like domain in apo(a) is catalytically inactive [13]. The multiple KIV domains in apo(a) can be divided into 10

distinct subtypes (designated KIV₁₋₁₀), which are distinguishable from each other on the basis of amino acid sequence [3]. Each KIV subtype is present in a single copy except for KIV₂, which can be present in 3 to >40 identically repeated copies, attributable to allelic variations in the number of sequences encoding KIV in the apo(a) gene [15]. This forms the basis of the Lp(a) size heterogeneity evident in the human population, in which at least 34 isoforms have been detected [15].

While the presence of apo(a) clearly confers unique properties onto Lp(a), the mechanisms by which elevated concentrations of Lp(a) cause vascular disease have not been definitively identified. Lp(a), through its apo(a) moiety, is able to contribute to macrophage foam cell formation, endothelial dysfunction, inflammation, and smooth muscle cell migration and proliferation, all of which would exacerbate atherosclerosis (reviewed in [44,120]). Furthermore, owing to its similarity to plasminogen, Lp(a) has been hypothesized to possess an anti-fibrinolytic function. Indeed, several *in vitro* and *in vivo* studies have shown that Lp(a)/apo(a) can inhibit fibrin clot lysis [64,68,69], as well as tissue-type plasminogen activator (tPA)-mediated plasminogen activation on the surface of fibrin [65-67,123]. However, the mechanism by which apo(a) inhibits tPA-mediated plasminogen activation has been controversial, with both competitive [65] and uncompetitive [66,123] mechanisms invoked to describe the observed inhibition. More recently, we developed an equilibrium template model to describe the inhibitory mechanism [67]. In this model, apo(a) forms a quaternary complex with plasminogen, tPA, and fibrin that is catalytically inefficient in plasminogen activation, relative to the ternary complex formed in the absence of apo(a). Despite these advances, much more remains to be learned concerning the impact of Lp(a)/apo(a) in the fibrinolytic process.

The fibrinolytic cascade features a number of plasmin-dependent positive feedback reactions. The small amount of plasmin formed during the initial phase can modify the properties of single-chain tPA (sc-tPA), native Glu¹-plasminogen, and fibrin to make the activation process more efficient [75,82,124-126]. Of particular interest in the present study is the plasmin-mediated conversion of Glu¹-plasminogen, the circulating form of plasminogen, to Lys⁷⁸-plasminogen. Native Glu¹-plasminogen exhibits a very tight, spiral structure that binds poorly to fibrin [127,128]. In addition, because the activation cleavage site (Arg⁵⁶¹-Val⁵⁶²) is not readily accessible, Glu¹-plasminogen is a poor substrate for tPA [128]. Plasmin hydrolyses the Lys⁷⁷-Lys⁷⁸ peptide bond of native Glu¹-plasminogen releasing the N-terminal activation peptide, thereby generating Lys⁷⁸-plasminogen. The release of the amino-terminal peptide disrupts intramolecular interactions and results in a shift to an open extended conformation. The open conformation of Lys⁷⁸-plasminogen enables it to be a better substrate for tPA and to bind to fibrin with a higher affinity, which accelerates its rate of activation [75,126]. Therefore, the conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen is a key step in the fibrinolytic cascade. The impact of apo(a) on this step has not been ascertained.

In the present study, we describe the effect of apo(a) on the efficiency of the plasmin-mediated conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen, using acid-urea polyacrylamide gel electrophoresis to resolve the two forms of plasminogen [126]. We were able to demonstrate a substantial inhibitory effect of apo(a) on the plasmin-mediated conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen. This finding is significant since it represents a novel mechanism by which Lp(a)/apo(a) can inhibit plasminogen activation on fibrin surfaces.

2.3 Materials & Methods

Construction and Expression of Recombinant Apo(a) Variants—The topology of the recombinant apo(a) (r-apo(a)) variants utilized in this study are shown in Figure 2.1. The construction of plasmids in the pRK5 expression vector [129] that encode the following r-apo(a) variants have been previously described: 17K [130], 12K [130,131], 6K and KIV₈-P [132], KIV₁₀-P and 17K(D56A) [133], 17KΔV [67], KIV₁₋₄ [134], and (KIV₂)₅ [135]. Variants representing physiological apo(a) isoforms with different numbers of KIV₂, other than the 12K and 17K r-apo(a), were constructed as follows. A series of intermediate vectors were prepared consisting of pRK5 containing an *EcoRI-XmaI* fragment encompassing KIV₁, different numbers of copies of KIV₂, as well as KIV₃ and KIV₄. The intermediate vectors were made by digestion of r-apo(a) expression plasmids with *XmaI* followed by reclosure to remove all but the aforementioned sequences. The intermediate vectors were partially digested with *BamHI* to create a single insertion site within a KIV₂ sequence in the vector, into which was inserted concatamers of KIV₂ (obtained by partial digestion of a plasmid derived from the phage clone λa6 [3] with *BamHI*). The complete apo(a) coding sequences were then reconstructed by sequential insertion of an *XmaI/XbaI* fragment from a variant of pRK5ha17 [129] (lacking the *XmaI* site in the multiple cloning site) encompassing KIV₆ through the protease, and an *XmaI* fragment encompassing KIV₅. All expression plasmids were used to generate corresponding stably-expressing 293 (human embryonic kidney) cell lines as previously described [129].

Protein Purification—All r-apo(a) variants containing lysine-binding sites were purified from the conditioned medium (CM) of stably-expressing 293 cell lines by lysine-Sepharose affinity chromatography as described previously [67]. CM harvested from the cell line overexpressing the KIV₁₋₄ r-apo(a) variant (lacking lysine binding sites) was applied to a 5 mL ConcanavalinA-

Sepharose (Amersham Biosciences) column, pre-equilibrated in HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4 containing 150 mM NaCl). The column was first washed with HBS, then with HBS containing 0.5 M NaCl. Bound proteins were eluted by the sequential addition of HBS containing 0.5 M NaCl and either 0.05 M, 0.25 M, or 0.5 M *N*-acetyl-D-glucosamine (Sigma-Aldrich). Apo(a)-containing fractions, as determined by western blot analysis, were pooled, dialyzed extensively against 20 mM Tris-HCl, pH 8.0, and applied to a 5 mL Q-Sepharose (Amersham Biosciences) column. Bound proteins were eluted with a continuous salt gradient from 250 mM to 2 M NaCl (100 mL total gradient volume) and the protein-containing fractions were identified by SDS-PAGE, followed by silver staining. Apo(a) KIV₁₋₄ eluted between 350 and 600 mM NaCl. The appropriate fractions were pooled, dialyzed extensively against HBS, and concentrated using a 10 000 MWCO Amicon Ultra Centrifugal Filter Device (Millipore). Protein purity was assessed by SDS-PAGE followed by silver staining. The r-apo(a) variant (KIV₂)₅ (also lacking lysine binding sites) was purified from the conditioned medium of stably-expressing 293 cells by immunoaffinity chromatography as previously described [135] using an anti-apo(a) polyclonal antibody raised in rabbits [14].

A recombinant variant of plasminogen containing a serine to cysteine mutation at the active site was purified from the conditioned medium of stably-expressing baby hamster kidney (BHK-21) cells by affinity chromatography over lysine-Sepharose [136].

Fibrinogen was purified from units of citrated, fresh frozen human plasma, obtained from Kingston General Hospital (Kingston, Ontario), essentially as described previously [67].

Isotopic Labeling—Recombinant Glu¹-plasminogen was radiolabeled with Na¹²⁵I (Perkin Elmer) as described previously [126], with some modifications. Recombinant Glu¹-plasminogen (0.5 mg) in 250 μ L of 200 mM Tris-HCl pH 7.4 containing 100 mM NaCl was radiolabeled at 22°C using

iodo-beads (Pierce). Two IODO-BEADS were washed once with 1 mL of buffer and then suspended in 0.5 mL buffer and 1 mCi of Na¹²⁵I was added. After 5 minutes incubation, the solution containing the Na¹²⁵I was added to the solution of protein and the mixture was incubated at room temperature for 10 minutes. The reaction was quenched by the addition of sodium metabisulfite and the radiolabeled protein was separated from the free label using a 10 mL Econo-Pac 10 DG column (BioRad). Protein-containing fractions were pooled and the protein concentration was determined spectrophotometrically (corrected for Rayleigh scattering). The radiolabeled protein was stored at 4°C prior to use.

Conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen—Solutions containing CaCl₂, labeled and unlabeled ¹²⁵I-plasminogen, fibrinogen, and different concentrations of r-apo(a) were prepared in 20 mM Tris-HCl pH 7.4, 100 mM NaCl. These solutions were added to microtiter wells containing human plasmin and human thrombin (both from Hematologic Technologies Inc.) such that the final concentrations of each reactant were: 5 mM CaCl₂; 50 nM ¹²⁵I-plasminogen; 950 nM unlabeled plasminogen; 3 μM fibrinogen; 50 nM plasmin; and 30 nM thrombin. The reactions in individual wells were quenched at various time points with an equal volume of sample buffer containing 1.8 M acetic acid, 30% sucrose, 50 mM ε-aminocaproic acid (ε-ACA), 5 units/mL aprotinin, and basic fuchsin as an electrophoresis indicator. Aliquots of the samples (15 μL) were subjected to urea/acetic acid gel electrophoresis [126] on 10% polyacrylamide gels for 80 minutes at 120V. After electrophoresis, gels were fixed, dried, and exposed to a phosphorimager screen. After scanning, band intensities were determined using a Molecular Images FX phosphorimager running Quantity One Software (BioRad). Percent conversion values were calculated by dividing the intensity of the Lys⁷⁸-plasminogen band by the sum of the intensities of the Lys⁷⁸-plasminogen and Glu¹-plasminogen bands.

2.4 Results

We previously reported that apo(a) can inhibit lysis of fibrin clots containing Glu¹-plasminogen, but not clots in which Lys⁷⁸-plasminogen was substituted (i.e. when the plasmin-mediated positive feedback step of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion was bypassed) [64]. In subsequent studies using a fluorescently-labeled variant of recombinant Glu¹-plasminogen, we investigated the mechanism by which apo(a) inhibits tPA-mediated activation of Glu¹-plasminogen to Glu¹-plasmin in the presence of fibrin or high-molecular weight soluble fibrin degradation products, and devised an equilibrium template model to describe the inhibitory behaviour of apo(a) [67]. In this model system, however, no active plasmin is generated and so the plasmin-dependent positive feedback reactions do not occur.

Therefore, in order to address the key question of whether apo(a) inhibits the positive feedback step of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion, we directly examined the effect of apo(a) on the rate of this reaction in the context of a lysing fibrin clot. Fibrinogen was clotted in the presence of plasminogen (a proportion of which was radioiodinated) and plasmin, and in the presence or absence of a r-apo(a) variant containing 17 KIV domains (17K; representing a physiologically-relevant isoform of apo(a)). Based on the use of similar radioiodinated plasminogen preparations to track the fate of Glu¹-plasminogen during fibrinolysis [126], the labeled plasminogen likely represents the behaviour of native plasminogen. The clots were quenched with acetic acid at different times, and the extent of conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen was determined by acetic acid/urea gel electrophoresis. The data show a substantial, dose-dependent inhibition of conversion by apo(a) in the presence of fibrin (Figure 2.2A,B), with little or no inhibition at the lowest concentration of apo(a) tested (0.5 μ M) and no apparent saturation of the inhibitory effect of apo(a) at the highest concentration of

17K tested (3 μ M). Interestingly, for the 17K isoform, an apo(a) concentration of 0.5 μ M corresponds to an Lp(a) concentration of approximately 25 mg/dL, which is in the vicinity of the risk threshold for plasma Lp(a) concentrations that has been identified in epidemiological studies [30,44,120].

It was also found that apo(a) inhibits Glu¹-plasminogen conversion only in the presence of fibrin. As expected, the extent of conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen was very much higher in the presence of fibrin (Figure 2.2C) with or without the addition of 17K r-apo(a). This is because Glu¹-plasminogen is a better substrate for plasmin when it is bound to partially-degraded fibrin [124]. However, in the absence of fibrin, the presence of 17K increased the extent of conversion (Figure 2.2C), which may relate to the ability of Glu¹-plasminogen to bind to apo(a) in solution [133]. Thus, since inhibition of conversion is observed only in the presence of fibrin, it confirms that apo(a) inhibits Glu¹-plasminogen conversion in a fibrin-dependent manner.

In order to assess which domains in apo(a) mediate its ability to inhibit the conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen, we employed a battery of recombinant apo(a) encompassing deletions of one or more kringle domains, or containing point mutations in individual kringles (Figure 2.1). In order to assess if the domains involved in inhibition reside at the amino- or carboxyl-terminal end of the molecule, we compared the effects of the KIV₁₋₄ and 6K variants (Figure 2.3A). Interestingly, both of these species resulted in inhibition. Specifically, the 6K variant was found to be slightly more effective and KIV₁₋₄ slightly less effective than 17K (Figure 2.3A). To investigate the ability of the major repeat kringle (KIV₂) to mediate inhibition, we studied a variant ((KIV₂)₅) that contains five copies of this kringle exclusively (Figure 2.3A).

This variant only marginally inhibited conversion, except at early time points, and thus it appears that the KIV types 1, 3, and 4 also present in KIV₁₋₄ may be playing a more important role.

The carboxyl-terminal region of apo(a) contains several classes of lysine binding sites, including weak sites in KIV₅₋₈ and a stronger site in KIV₁₀ [44]. KIV₉ contains the free cysteine that mediates covalent coupling of apo(a) to apoB in the Lp(a) particle [1], as well as an amino acid binding site that can accommodate lysine or phenylalanine [137]. Notably, the KIV₈-P variant displays a partially reduced inhibitory activity, whereas the KIV₁₀-P completely lacks inhibitory activity (Figure 2.3B). Similar to the effect of KIV₈-P, a variant of 17K in which the strong lysine binding site in KIV₁₀ has been abolished by mutagenesis (17K Δ LBS₁₀) has only partially reduced inhibitory activity.

Our previous studies of plasminogen activation in the context of fibrin have shown a clear role for KV in inhibition of plasminogen activation by tPA in this milieu [67]. To investigate whether this motif might also influence inhibition of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion, we studied a variant in which KV was removed (17K Δ V)) (Figure 2.3C). The results show that removal of the entire KV motif virtually abolishes inhibitory activity.

Smaller apo(a) isoforms, which have been associated with an increased incidence of cardiovascular disease independent of plasma Lp(a) concentrations [44,120], bind more avidly to fibrin and thus have been suggested to inhibit fibrinolysis more effectively than larger apo(a) isoforms [138,139]. In order to assess if apo(a) isoform size also affects the conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen, we utilized a series of r-apo(a) species, differing only in the number of KIV₂ repeats, which encompass over 95% of the observed isoforms in the human population (ranging between 12 and 33 KIV repeats) (Figure 2.1). With the exception of the 12K

species, a very small isoform observed in only a very tiny proportion of the population (< 1%) [15], all of the different apo(a) isoforms inhibited conversion to a similar extent (Figure 2.4); the 12K species is a more potent inhibitor of conversion than the other isoforms (Figure 2.4).

2.5 Discussion

It is generally accepted that Lp(a)/apo(a) can inhibit tPA-mediated plasminogen activation although the mechanism remains controversial. The present study contributes to the understanding of this mechanism by demonstrating, for the first time, that apo(a) interferes with the important positive feedback loop of plasmin-mediated conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen. By interfering with the self-propagating phase of plasminogen activation that this feedback reaction affords, Lp(a)/apo(a) could effectively downregulate the activation process, resulting in a reduction in the amount of plasmin generated. The effect of apo(a) is substantial—more than a two-fold decrease in conversion at the highest apo(a) concentration tested—indicating that this inhibitory effect would have a marked effect on fibrinolysis.

In order to gain insights into the inhibition mechanism, therefore, we have investigated the domains in apo(a) that are required for this inhibitory effect. In our previous study focusing on tPA-mediated activation of Glu¹-plasminogen to Glu¹-plasmin [67], we investigated both the mechanism of apo(a)-mediated inhibition as well as the domains in apo(a) responsible for the inhibitory effect. The KIV₁₀ LBS, KV domain, and the amino terminus of apo(a) were identified as critical for inhibition of tPA-mediated plasminogen activation, as determined by domain disruption or removal. Specifically, the strong lysine-binding site in the KIV₁₀ domain and the amino-terminus (KIV₁₋₄) were determined to be necessary for maximal inhibition, whereas no inhibition was observed in the absence of KV, indicating an essential role for this domain. The

domain requirements for inhibition of Glu¹-plasminogen conversion observed in the present study show some similarities to the above trends, but also underscore some important differences. In both studies, the apo(a) KV plays an essential role, with removal of this domain essentially abolishing the inhibitory effect (Figure 2.3C) [67]. Further studies will be required to assess if the effect of KV on inhibition in the respective systems is dependent on the weak LBS present in this kringle.

An additional point of comparison is that while both studies found that removal of the lysine binding site in KIV₁₀ only partially abolished inhibitory activity, contrasting results were found with respect to the role of the amino-terminal domain of apo(a). The 6K variant, which lacks this region, was an effective inhibitor of Glu¹-plasminogen conversion (Figure 2.3A), however, was greatly compromised with respect to inhibition of Glu¹-plasminogen activation [67]. Although the ability of the amino-terminal domain on its own (i.e. the KIV₁₋₄ variant) was not tested with respect to its ability to inhibit Glu¹-plasminogen activation [67], we found in the current study that it did possess a significant ability to inhibit Glu¹-plasminogen conversion. It should be noted that the underlying mechanisms of Lp(a)/apo(a)-mediated inhibition of Glu¹-plasminogen activation and conversion are likely to be somewhat different, as different enzymes catalyze the reactions and target a different cleavage site on plasminogen.

In our previous study, we devised a model to explain the data acquired for the inhibition of tPA-mediated Glu¹-plasminogen activation by apo(a). According to the model, apo(a) forms a quaternary complex with fibrin, plasminogen, and tPA that turns over very inefficiently compared to the ternary catalytic complex lacking apo(a). Although we do not yet know the precise nature of the interactions of apo(a) with the other components of the quaternary complex, evidence exists for binary interactions between apo(a) and fibrin, plasminogen, and tPA [67].

In the current study, apo(a) interferes with the ability of plasmin to cleave fibrin-bound Glu¹-plasminogen. Based on the pattern of domains required for inhibition, the data can not be explained by simple bimolecular interactions between apo(a) and fibrin, or Glu¹-plasminogen, or plasmin. We do not believe that the observed inhibition can be accounted for by competition between apo(a) and plasminogen for plasminogen binding sites on fibrin, since some apo(a) species that contain intact fibrin-binding sites (i.e the KIV₁₀-P variant) and are able to bind plasminogen in solution [133] do not inhibit conversion (Figure 2.3B). Conversely, the KIV₁₋₄ variant, which binds neither plasminogen nor fibrin, was also capable of inhibiting Glu¹-plasminogen conversion. This inhibitory activity appears to be largely dependent on KIV₁, KIV₃, and KIV₄, as the concatamer of KIV₂ domains was a very weak inhibitor of conversion. Interestingly, this is the first report of a potential function for the apo(a) KIV₁, KIV₃, and/or KIV₄ domains. Furthermore, it is also unlikely that apo(a) is binding plasmin (which is predominantly in the Lys⁷⁸-plasmin form) as apo(a) binds only very weakly to Lys⁷⁸-plasminogen (Kd > 50 μM) [140]. We therefore propose that apo(a) binds to fibrin-bound plasminogen in such a way as to sterically prevent plasmin cleavage in a manner that differs from its solution-phase interaction with plasminogen, as the latter requires only the protease-like domain of apo(a) [133]. Also, based on the domains involved, the mechanism may involve similar interactions between apo(a)-fibrin or apo(a)-plasminogen as participate in the inhibition of tPA-mediated Glu¹-plasminogen activation by apo(a) [67], as a number of similar domain requirements are observed for both processes. Further studies will be required to definitively determine the nature of the binding interactions and to identify which domains in apo(a) contribute to binding versus steric hindrance of plasmin cleavage.

Although an inverse correlation has been well-established between apo(a) isoform size and plasma Lp(a) levels, a number of studies have shown that small apo(a) isoform size (defined as <22 KIV domains [54]) is associated with risk for coronary heart disease independent of plasma Lp(a) levels [54-56]. However, the molecular basis underlying these observations remains unclear. In the present study, all 7 isoforms ranging from 14 through 33 KIV domains were found to inhibit plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion to a similar extent (Figure 2.4), while the smallest physiologically-relevant isoform tested (12K) was superior to the others as an inhibitor. This suggests that isoform size does not materially influence the inhibition process, and hence that the increased risk associated with low molecular weight isoforms of apo(a) cannot be ascribed to the ability of apo(a) to inhibit Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion. Nonetheless, it remains possible that apo(a) isoform size does have an effect on the overall process of tPA-mediated plasminogen activation inhibition, as suggested by previous studies [138,141].

It has been found that the conversion of Glu¹-plasminogen to Lys⁷⁸-plasminogen is the mechanism by which pericellular plasminogen activation is enhanced [142]. Cells bind Glu¹-plasminogen through cell-surface receptors containing carboxyl-terminal lysines, and this binding presumably results in a conformational change in Glu¹-plasminogen that facilitates its cleavage by plasmin. The resultant Lys⁷⁸-plasminogen that is formed is a much better substrate for plasminogen activators than Glu¹-plasminogen in the solution phase [142], resulting in an enhanced rate of activation. This is in contrast with plasminogen activation on fibrin, where production of Lys⁷⁸-plasminogen favors formation of a ternary complex with the plasminogen activator and fibrin, thereby accelerating plasmin generation [126]. It will be interesting to assess if apo(a) also affects pericellular plasminogen activation through inhibition of plasmin-mediated

Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion, and to contrast the mechanism of the effect of apo(a) in these two contexts.

Several mechanisms accounting for the ability of apo(a) to inhibit fibrinolysis have now been proposed, including inhibition of plasminogen and tPA binding to fibrin [65,66,138] and inhibition of tPA-mediated Glu¹-plasminogen activation through binding to the ternary catalytic complex [67]. The current study is the first to determine that apo(a) can inhibit one of the key positive feedback reactions, that of Glu¹-plasminogen conversion to Lys⁷⁸-plasminogen by plasmin, which greatly influences plasminogen activation efficiency. Future studies will need to integrate these observations in the context of fibrinolysis and in animals models of thrombotic diseases; the knowledge of the domains in apo(a) involved in the respective mechanisms will greatly facilitate resolution of the outstanding mechanistic questions.

2.6 Figures

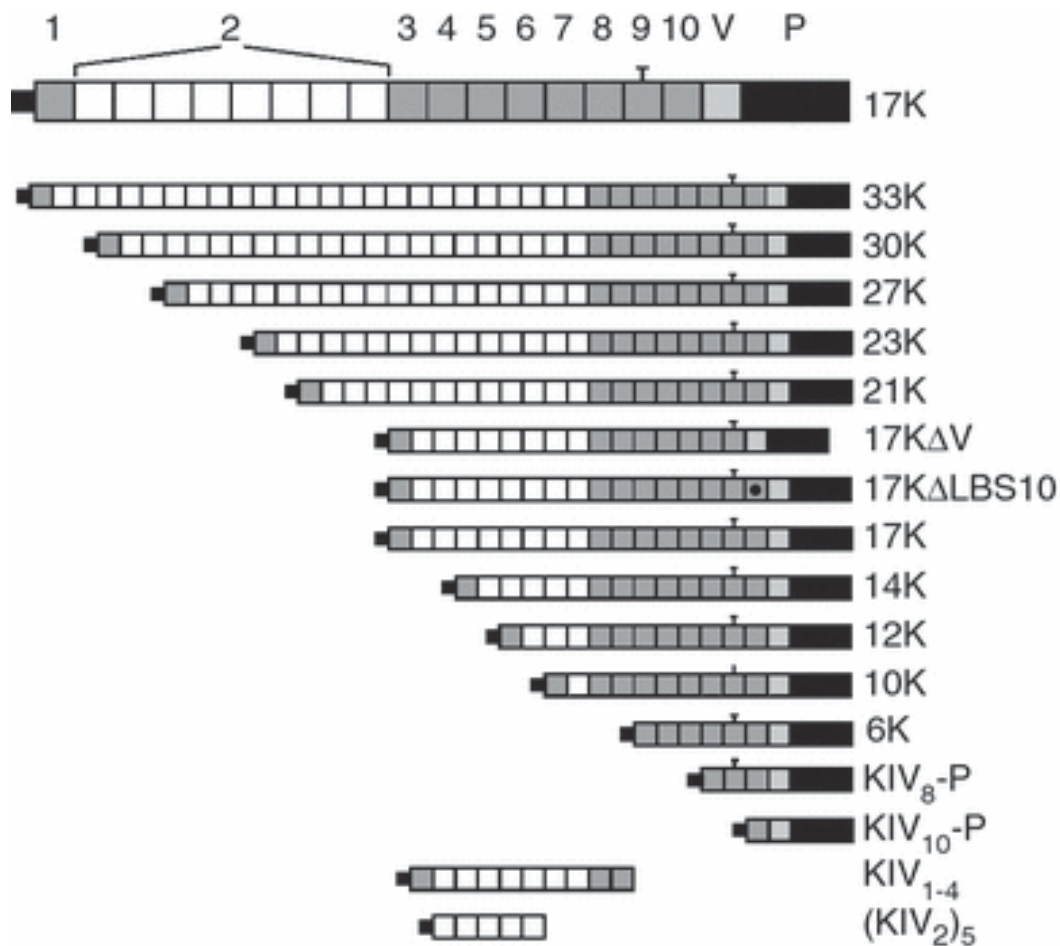


Figure 2.1 *Recombinant apo(a) variants utilized in study.* The domain structure of the full-length r-apo(a) constructs is presented at the top, where 1-10 denote the subtypes of the plasminogen-like kringle IV sequences, KV denotes the V-like sequences, and P is the protease domain. The bar over KIV₉ indicates the location of the free-cysteine by which apo(a) is covalently bound to apoB-100 in the Lp(a) molecule and the circle within a kringle indicates disruption of the lysine binding site in this domain through mutagenesis. The range of variants between 12K and 33K represent naturally-occurring isoforms of apo(a).

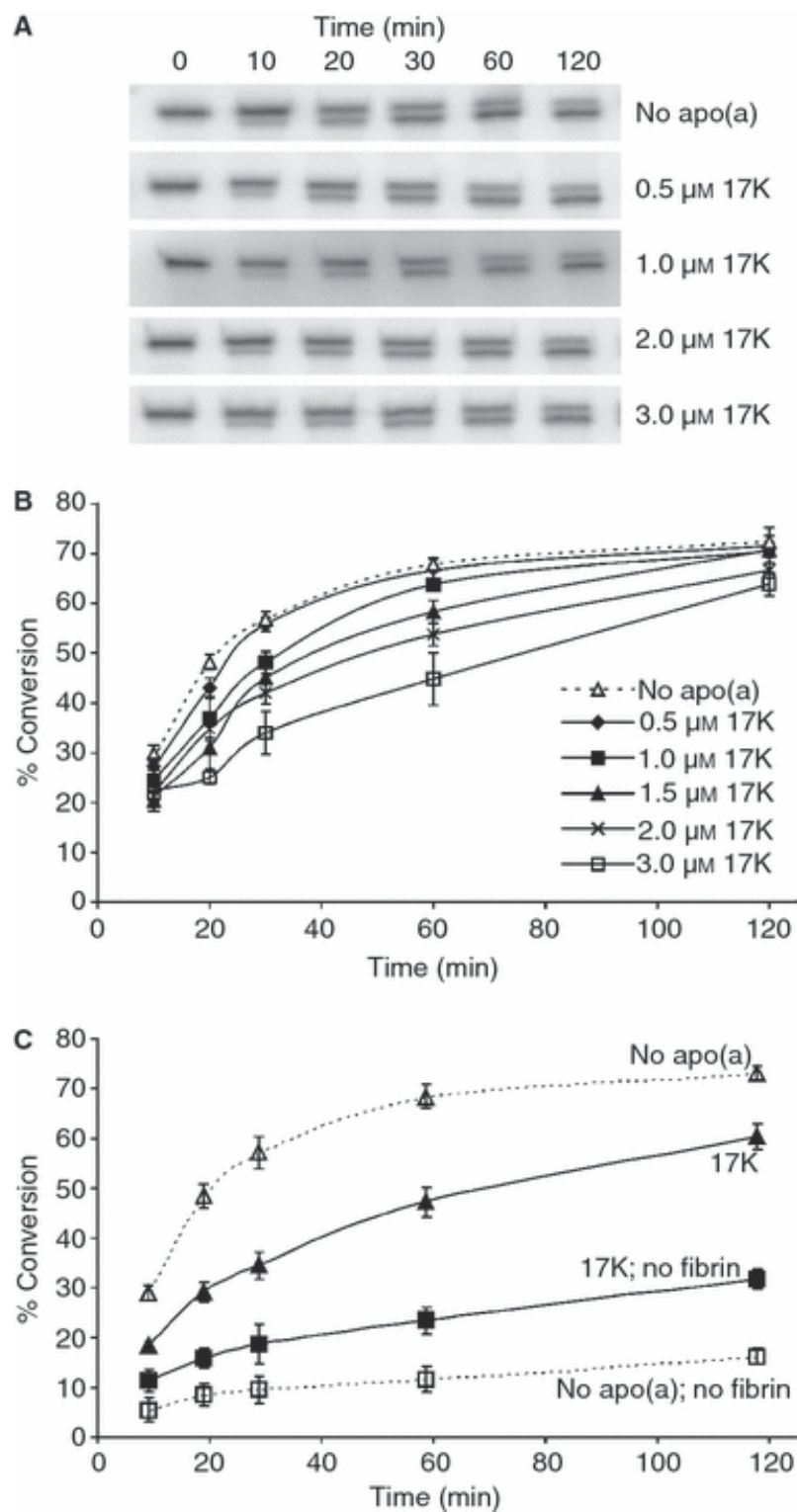


Figure 2.2 *Dose-dependent inhibition of plasmin-mediated Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion by apo(a).* *Panel A.* Representative autoradiograms illustrating the effect of apo(a) on Lys-plasminogen formation. Reactions contained 3 μ M fibrinogen (clotted by 30 nM thrombin in the presence of 5 mM CaCl₂), 1 μ M recombinant plasminogen (in which the active site serine has been replaced with a cysteine) of which 5% was labeled with ¹²⁵I, 50 nM plasmin, and different concentrations of 17K r-apo(a). Reactions were quenched at different times by the addition of acetic acid, and Glu¹- and Lys⁷⁸-plasminogen were resolved by acetic acid/urea gel electrophoresis, with the plasminogen bands visualized using a phosphor screen. Glu¹-plasminogen and Lys⁷⁸-plasminogen are the upper and lower bands, respectively, on the autoradiograms. *Panel B.* Following quantification of the intensity of the Glu¹- and Lys⁷⁸-plasminogen bands using a phosphorimager, the percent conversion was calculated by dividing the intensity of the Lys⁷⁸-plasminogen bands by the sum of the intensities of the Glu¹- and Lys⁷⁸-plasminogen bands. The graph shows these values plotted versus time for each of the indicated r-apo(a) concentrations. The data shown are the means of at least 4 independent experiments; the error bars are the standard errors of the mean. *Panel C.* Parallel conversion experiments were assembled in the presence or absence of fibrin (i.e. in the presence or absence of fibrinogen, thrombin, and CaCl₂). The data shown are the means of 4 to 5 independent experiments; the error bars are the standard errors of the mean.

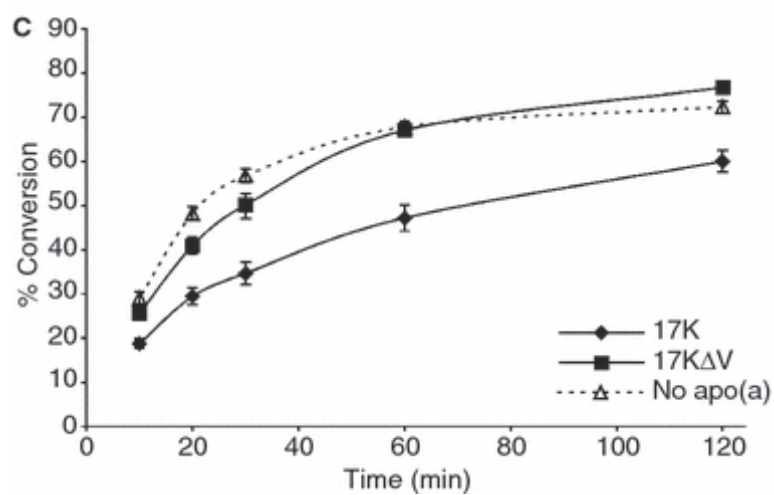
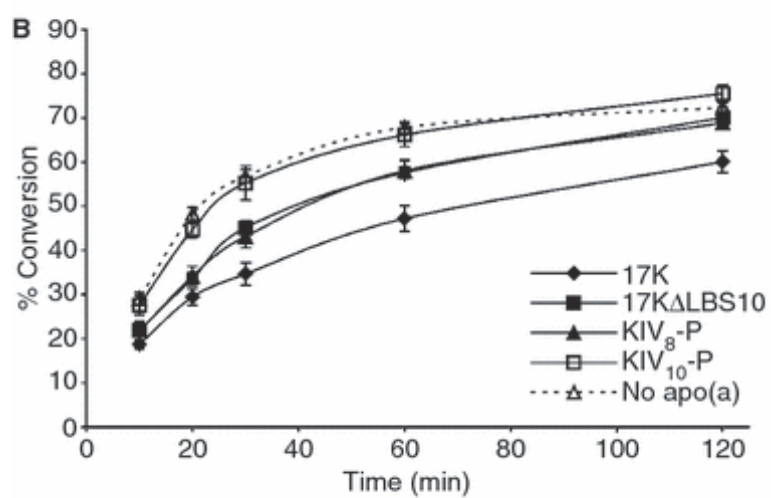
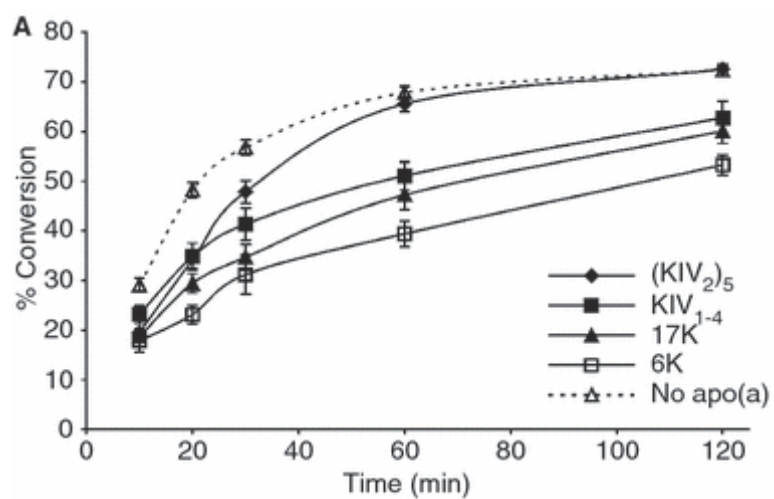


Figure 2.3 *Role of individual domains in apo(a) in inhibition of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion.* Conversion experiments were conducted as described in the legend to Figure 2.2, with 3 μ M of the respective r-apo(a) variants. The data shown are the means of at least 4 independent experiments; the error bars are the standard errors of the mean. *Panel A.* Comparison of the roles of the amino- and carboxyl-terminal domains of apo(a). *Panel B.* Roles of the weak (KIV₅₋₈) and strong (KIV₁₀) lysine binding sites in apo(a). *Panel C.* Role of KV.

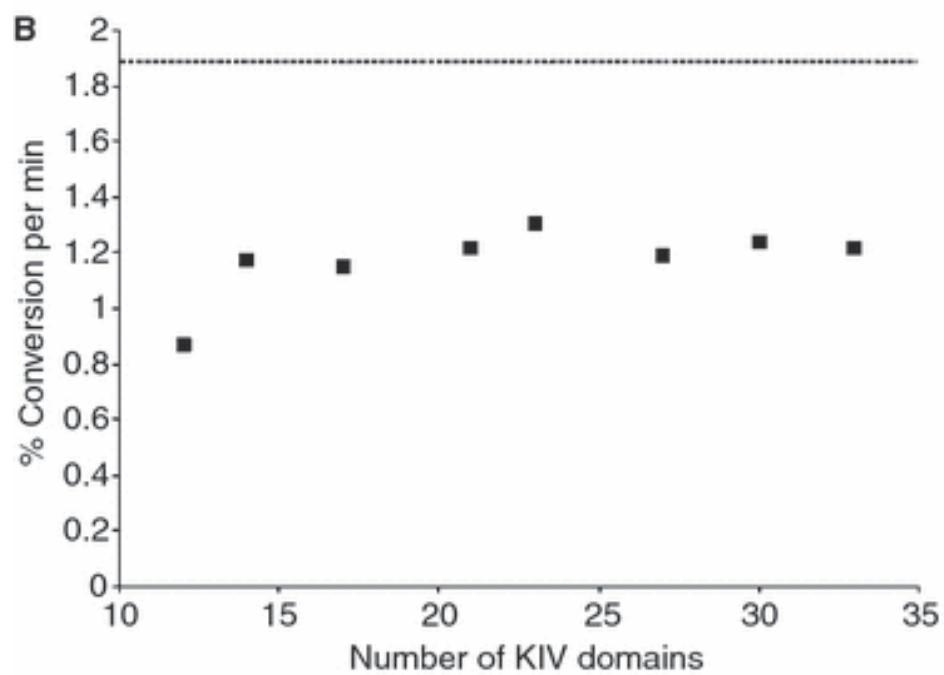
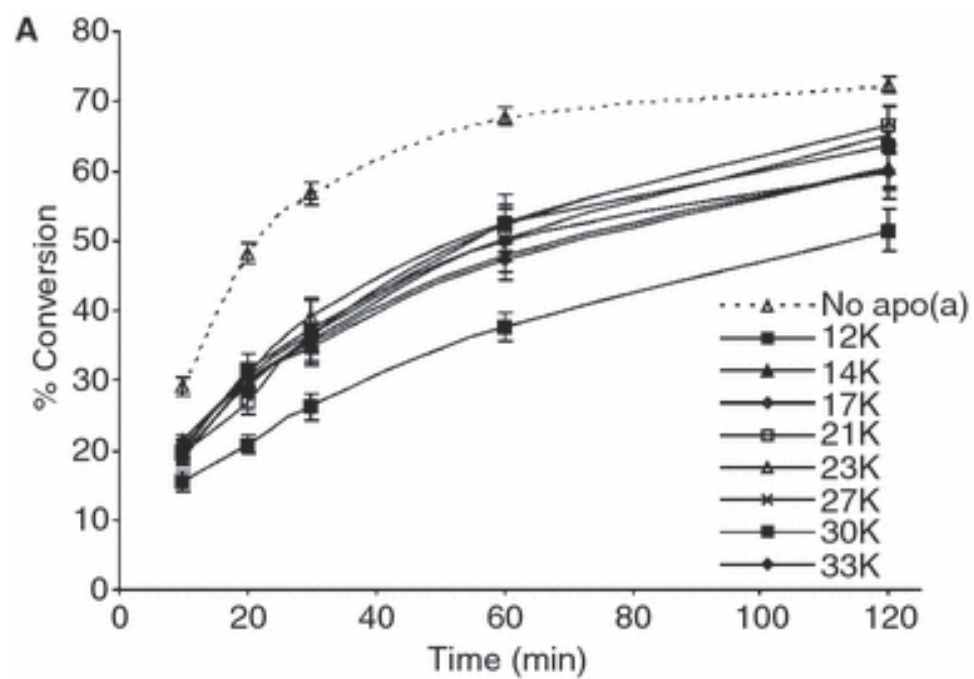


Figure 2.4 *The role of apo(a) isoform size in apo(a)-mediated inhibition of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion. Panel A* Conversion experiments were assembled as described in the legend to Figure 2.2, in the presence or absence of the indicated r-apo(a) variants (3 μM) which represent almost the entire size range of naturally-occurring apo(a) isoforms. The data shown are the means of at least 4 independent experiments; the error bars are the standard errors of the mean. *Panel B.* The rate of Glu¹-plasminogen conversion over the first 20 minutes was determined for each r-apo(a) isoform size from the data in *Panel A*, and plotted against the number of KIV repeats in the respective isoforms. The dashed line represents the rate of conversion in the absence of apo(a).

Chapter 3

Apolipoprotein(a) Induces Prothrombotic Alterations to Fibrin Clot Structure that Exacerbate its Inhibition of tPA-Mediated Plasminogen Activation

3.1 Summary

Background: Both elevated plasma lipoprotein(a) (Lp(a)) concentrations and abnormal fibrin clot structures have been associated with an increased risk of cardiovascular disease. Elevated Lp(a) levels have also been found to correlate with abnormal fibrin structures. Together these findings suggest that a possible mechanism of Lp(a) pathogenicity may be mediated through altering the fibrin clot structure.

Objective: The present study sought to determine whether the structure and/or function of the fibrin clot is affected by apolipoprotein(a) (apo(a)).

Methods: Plasma or purified fibrinogen clots were formed in the presence and absence of purified recombinant apo(a) (r-apo(a)) protein. Clot structures were assessed by permeation and turbidity assays, an assay for fluorescent-plasminogen activation, and confocal and scanning electron microscopy.

Results: Clots formed in the presence of apo(a) had significantly decreased permeability ($P = 0.002$), increased density (confocal: $P < 0.001$; SEM: $P = 0.03$) and decreased fiber diameter (confocal: $P < 0.001$; SEM $P = 0.003$). Maximum absorbance was unaffected by the presence of r-apo(a), however the initial rate of fibrin polymerization was increased. Additionally, the extent

of inhibition of tissue-type plasminogen activator (tPA)-mediated plasminogen activation by r-apo(a) was increased under conditions of altered clot structure.

Conclusions: Recombinant apo(a) directly influences fibrin architecture generating denser clots composed of thinner fibers with reduced permeability. The thinner fibrin fibers are likely the consequence of the observed increase in the rate of fibrin polymerization in the presence of apo(a). Importantly, alterations in the fibrin clot structure were observed to increase the extent to which apo(a) inhibits tPA-mediated plasminogen activation.

KEYWORDS: apolipoprotein(a); fibrin clot structure; fibrinolysis; plasminogen

3.2 Introduction

Cardiovascular diseases account for more deaths than any other major cause of death in the industrialized world [143]; and both lipoprotein(a) (Lp(a)) [43,115] and altered fibrin structure [85,89] have been repeatedly implicated as cardiovascular risk factors, particularly with respect to diseases that are associated with thromboembolic events. Moreover, it has been reported that elevated Lp(a) levels correlate with an abnormal fibrin structure that is resistant to fibrinolysis [89].

Lp(a) is a plasma lipoprotein consisting of a cholesterol-rich low density lipoprotein (LDL)-like lipid component that includes a molecule of apolipoproteinB-100 (apoB-100); and a unique high molecular weight glycoprotein, apolipoprotein(a) (apo(a)) that is covalently linked to the lipid element through a single disulphide-bond formed with apoB-100 protein [1,2,144]. Upon characterization of the cDNA sequence [3], it was determined that apo(a) is composed of domains homologous to the kringle 4, kringle 5 and protease domains of the fibrinolytic proenzyme plasminogen. In particular, apo(a) is composed of multiple copies of a plasminogen-like kringle 4

domain (KIV), which can be subdivided into 10 distinct subtypes (KIV₁₋₁₀) based on amino acid sequence, along with single copies of a plasminogen-like kringle 5 domain (KV) and an inactive plasminogen-like protease domain [3]. Each of the apo(a) KIV subtypes is present in a single copy with the exception of KIV₂, which can be present in 3 to >30 identically repeated copies [15], an attribute determined by the copy number of a 5.5 kb sequence—corresponding to one KIV₂ encoding sequence—in the *LPA* gene [122]; this gives rise to isoforms of Lp(a)/apo(a) ranging in apparent molecular mass from <400 kDa to >800 kDa [15].

The exact mechanisms by which Lp(a) contributes to cardiovascular risk remain unclear, however a variety of potential mechanisms have been investigated (reviewed in [145]). Numerous studies, both *in vivo* [68,69] and *in vitro* [65-67], have identified Lp(a)/apo(a) as an inhibitor of fibrinolysis, and there is a body of epidemiological, genetic and molecular evidence to support that impaired fibrinolysis contributes to the development and/or progression of atherosclerosis [63]—the underlying etiology of the thromboembolic events of myocardial infarction and stroke [146]—by contributing to the development and/or persistence of thrombi in the vasculature.

Fibrinolysis is the process in which the circulating proenzyme plasminogen is activated to the enzyme plasmin by tissue-type plasminogen activator (tPA) on the surface of fibrin. Plasmin then mediates the dissolution of the insoluble fibrin to soluble fibrin degradation products by cleaving Arg-X and Lys-X bonds in fibrin fibers. The most common mechanism by which Lp(a)/apo(a) has been reported to interfere with fibrinolysis is in the attenuation of tPA-mediated plasminogen activation [65-68], however a plasminogen-independent prothrombotic effect of apo(a) has also been observed [147]. Notably, tPA-mediated plasminogen activation is affected by fibrin structure in that thin fibers organized in a dense fibrin network have not only been shown to be resistant to fibrinolysis but also to have decreased affinity for both tPA and

plasminogen [88,148], prerequisites for efficient plasminogen activation on the fibrin surface [75].

Based on the observation that Lp(a) levels are correlated with abnormal fibrin structure and function, it had been surmised that Lp(a)/apo(a) would have an effect on clot structure; however, a direct effect has not been shown. The current study sought to determine the direct effect of a 17 KIV recombinant apo(a) on clot structure by comparing clots generated in a purified system in the presence and absence of r-apo(a) protein in terms of permeability, turbidity, lytic potential, tPA-mediated plasminogen activation and ultrastructure.

3.3 Materials & Methods

Purification of Recombinant Apo(a)—The recombinant apo(a) variant containing 17 kringle 4-like (KIV) domains was cloned and stably expressed in human embryonic kidney (HEK) 293 cells as previously described [129] and purified from the conditioned medium of stably-expressing 293 cell lines by lysine-Sepharose affinity chromatography as previously described [129]. Protein purity was assessed by SDS-PAGE followed by silver staining.

Purification of Recombinant Plasminogen—A recombinant variant of Glu¹-plasminogen containing a serine to cysteine mutation (S741C) at the active site was cloned and expressed in baby hamster kidney (BHK-21) cells as previously described [136]. It was purified from the conditioned medium of stably-expressing BHK-21 cells by affinity chromatography and labeled with 5'-iodoacetamidofluorescein at the mutated residue (5'IAF; Molecular Probes, Eugene, OR) as previously described [67].

Purification of Fibrinogen—Fibrinogen was purified from units of citrated, fresh frozen human plasma, obtained from Kingston General Hospital (Kingston, ON), as described previously [67].

Lipoprotein-Deficient Plasma—Human plasma was obtained from Kingston General Hospital (Kingston, ON). Lipoprotein-deficient plasma was prepared by adjusting the density of the plasma to 1.22 g/mL with sodium bromide and then centrifuging the plasma at 60 600 rpm in polycarbonate tubes in a Beckman Ti 70.1 rotor (Beckman Coulter, Mississauga, ON) for 16 hours at room temperature. The bottom layer was extracted and dialyzed 3-times for 1 hour at 4°C in 20 mM HEPES, 0.15 M NaCl, pH 7.4.

Clot Permeability Assay—Clot permeation was determined in a pressure-driven tube system as previously described, with some modification [149]. Lipoprotein-deficient plasma was diluted 1:1 with Tris-Buffered Saline (TBS; 0.05 M Tris-HCL, 0.1 M NaCl, pH 7.5) and clotted with 20 mM CaCl₂ and 2 U/ml human α -thrombin (Hematologic Technologies Inc., Essex Junction, VT, USA) in the presence or absence of recombinant apo(a) protein. After incubation in a moist chamber for 2 hours at room temperature, the tube containing the clot was connected to a reservoir containing TBS and the flow rate through the fibrin clot was measured, as previously described [149]. Plasma-purified fibrinogen was diluted to 3 mg/mL with TBS and clotted with 20 mM CaCl₂ and 1 U/mL thrombin, whereas plasminogen-depleted, plasma-purified fibrinogen (EMD-Calbiochem, Mississauga, ON, Canada) was diluted to 3 mg/mL with TBS and clotted with 20 mM CaCl₂ and 0.01 U/mL thrombin. The resulting K_s values in the presence or absence of r-apo(a) were compared using a one-tailed or two-tailed Mann-Whitney *U* test.

Coagulation (Clot Turbidity)—Fibrinogen was diluted to 3 mg/mL in TBS in the presence or absence of 3.0 μ M 17KIV r-apo(a) and was then added to 96-well polystyrene plates (Corning, Troy, MI) containing 1 U/mL thrombin (Haematologic Technologies Inc., Vermont, USA) and 20 mM CaCl₂. Absorbance was read at 340 nm every 30 seconds for 1 hour with a SpectraMax Plus

spectrophotometer (Molecular Devices, Downington, PA). The maximum absorbance values in the presence or absence of r-apo(a) were compared using a one-tailed homoscedastic t-test.

Fibrinolysis Assays—Fibrinolysis assays were conducted in the same way as the clot turbidity assays with the addition of 30 nM human plasmin (Haematologic Technologies Inc., Vermont, USA) to each well. The 50% lysis times in the presence or absence of r-apo(a) were compared using a one-tailed homoscedastic t-test.

Fluorescent Plasminogen Activation Assays—5'IAF-labeled r-plasminogen(S741C) was cleaved by tPA (Cathflo® Activase®, Kingston General Hospital, Kingston, ON) resulting in a decrease in fluorescent intensity that was monitored using a SpectraMax M5e fluorescent plate reader (Molecular Devices, Downington, PA, USA) in a 96-well solid black polystyrene microplate (Corning, Troy, MI), as previously described [67]. Briefly, 80 μ L volumes containing 5'IAF-labeled plasminogen (1.8 μ M final concentration) and FDPs (0.6 μ M final) in the presence or absence of r-apo(a) (3.0 μ M final) in HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4 containing 150 mM NaCl) containing 0.02% (v/v) Tween 80 were added to wells containing 20 μ L volumes of CaCl₂ (10 mM final) and tPA (50 nM final) in HBS with 0.02% Tween 80. The fluorescence was monitored for 1 hour at 30 second intervals. The resultant curves were corrected for the buffer blank and then corrected for the internal filter effects as described [67].

In order to correct for the internal filter effect, standard curves were first generated for every combination of 5'IAF r-plasminogen, using final concentrations ranging from 0-3 μ M and FDPs, using a single final concentration of 0.6 μ M. From the standard curves, the parameters for the fluorescence per mole of 5'IAF-labeled plasminogen (*i*) and the exponential coefficient (*a*) were determined by fitting the curves (in relative fluorescent units, RFU) by non-linear regression to the equation,

$$\text{RFU} = i \times [\text{Plasminogen}] \times \exp(-a \times \text{RFU}/i) \quad [67]$$

using the SOLVER tool from Microsoft Excel (Microsoft Corporation, Mississauga, ON) as described [150]. The a and i values were then used to correct for the internal filter effect according to the equation:

$$\text{RFU}_{\text{corrected}} = \text{RFU}_{\text{raw}} \times \exp(a/i \times \text{RFU}_{\text{raw}}) \quad [67]$$

In order to determine the initial rate of plasmin formation per mole of tPA, first the slope and intercept of the initial linear region of the corrected curves was calculated using the SLOPE and INTERCEPT functions from Microsoft Excel (Microsoft Corporation, Mississauga, ON). Then, these values were used to calculate the rate according to the equation:

$$\text{Rate} = (\text{slope}) (1/(0.5 \times \text{intercept})) ([\text{Plasminogen}]_{\text{initial}}/[\text{tPA}]_{\text{initial}}) \quad [67]$$

FITC-Labeling of Fibrinogen—Plasma-purified fibrinogen was labeled with a FITC fluorescent tag (Sigma, St. Louis, MO, USA) according to the manufacturer's instruction, with some modification. Briefly, 2 mg of fibrinogen was labeled using a 1 mg/mL stock solution FITC at a final concentration of 50 $\mu\text{g}/\text{mL}$. The reaction was incubated for 1 hour at room temperature.

Confocal Microscopy—FITC-labeled fibrinogen (3 mg/mL) in the presence of absence of 17 KIV r-apo(a) was incubated with 2 U/mL thrombin and 20 mM CaCl_2 on microscope slides. The clots were allowed to mature for 3 hours in a moist chamber at room temperature at which point coverslips were placed on top of the clots and secured. Samples were visualized using a Leica TCS SP-2 laser scanning confocal microscope (Leica Microsystems, Guelph, ON, Canada). Clots were observed and photographed digitally in at least 3 different areas per clot at 3-4 different magnifications. The diameter of fibrin fibers and the percent area was measured using ImageJ software (National Institutes of Health, Bethesda, MD). At least five fibers per clot region per clot was measured and averaged to determine the average fiber diameter for each clot. Percent area

was determined by adjusting the greyscale threshold to encapsulate only the superficial fibers in the image; the software was then used to calculate 'Area Fraction'. Fiber diameter and area fraction values in the presence and absence of r-apo(a) were compared using a heteroscedastic t-test.

Scanning Electron Microscopy—Fibrinogen in the presence or absence of 3 μ M 17 KIV r-apo(a) was incubated with 0.01 U/mL thrombin and 20 mM CaCl_2 on top of 22 mm coverslips (Fisher Scientific, Nepean, ON). After 3 hours at room temperature in a moist chamber, clots were fixed for 2 hours in 2.5% (vol/vol) glutaraldehyde diluted in phosphate buffered saline (PBS; 0.14 M NaCl, 3 mM KCL, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4). Clots were then washed 3 times with PBS for 30 minutes, followed by 3 times with ddH₂O for 30 minutes. Clots were left overnight in ddH₂O and only removed from the water when they were to be visualized. Samples were analyzed with the use of a field-emission environmental scanning electron microscope (ESEM, FEI QUANTA-200 FEG type, Holland FEI Company). Images were taken at a pressure of 70 kPa, operating at an accelerating voltage of 10 kV and a 10 mm working distance. Image analysis was conducted as with confocal microscopy experiments with the slight modification that fiber diameter was determined by measuring every discernable fiber at a magnification of 30,000 times. Fiber diameter and area fraction values in the presence and absence of r-apo(a) were compared using a heteroscedastic Student t-test.

3.4 Results

Plasma was clotted in the presence or absence of a recombinant apolipoprotein(a) (r-apo(a)) protein with 17 kringle 4-like (KIV) domains and the average clot pore size was measured rheologically by means of a permeability assay. In this assay, the flow rate through the individual

fibrin clots was measured and used to calculate the permeability constant (K_s) [151], an intrinsic property of the clot. Clots formed using lipoprotein-deficient plasma in the presence of r-apo(a) showed a significant 2-fold decrease in permeability relative to clots formed in the absence of r-apo(a) (Figure 3.1; 0 nM 17KIV r-apo(a): $7.39 \pm 1.35 \times 10^{-9} \text{ cm}^2$; 100 nM 17KIV r-apo(a): $3.51 \pm 0.77 \times 10^{-9} \text{ cm}^2$; $P = 0.002$). However, there was not an observed dose response with increasing concentrations of r-apo(a) (Figure 3.1; 100 nM 17KIV r-apo(a): $3.51 \pm 0.77 \times 10^{-9} \text{ cm}^2$; 200 nM: $3.29 \pm 1.08 \times 10^{-9} \text{ cm}^2$; 400 nM: $3.70 \pm 0.64 \times 10^{-9} \text{ cm}^2$).

We observed a wide range of K_s values for clots generated from different preparations of lipoprotein-deficient plasma in the absence of r-apo(a) (data not shown), which made it difficult to directly compare data sets. Attempts were then made to approximate similar K_s values in the absence of r-apo(a) by varying the thrombin (enzyme) concentration and/or by supplementing the plasma with a known amount of fibrinogen (substrate), as both thrombin and fibrinogen concentrations influence fibrin structure [151]. However, we found it impossible to establish a consistent baseline condition using the different lipoprotein-deficient plasma preparations. We therefore attempted to recapitulate the assay using fibrinogen purified from plasma. It was found that the plasma-purified fibrin clots formed in the presence of either 0.5 μM ($K_s = 3.47 \pm 1.08 \times 10^{-7} \text{ cm}^2$; $P = 0.05$) or 2.0 μM r-apo(a) ($K_s = 2.68 \pm 0.66 \times 10^{-7} \text{ cm}^2$; $P = 0.007$) were significantly less permeable than clots formed in the absence of r-apo(a) ($K_s = 3.61 \pm 0.84 \times 10^{-7} \text{ cm}^2$), however clots generated in the presence of 1.0 μM r-apo(a) were not significantly different ($K_s = 4.81 \pm 1.36 \times 10^{-7} \text{ cm}^2$; $P = 0.3$). The discrepancy in these results is likely the consequence of the extremely large range over which this data falls and the highly skewed nature of the data (Figure 3.2B), features that are exaggerated when compared with K_s values acquired using the lipoprotein-deficient plasma (Figure 3.2A). In a final attempt to observe meaningful and

consistent permeability data and to address the possibility that the skewness of the data was a consequence of our purification protocol, a commercially-available fibrinogen product was used to generate clots in the presence or absence of 17KIV r-apo(a). No significant difference was observed between the means in the presence or absence of r-apo(a), however once again the data were highly skewed and had a large range (Figure 3.2C; 0 μM r-apo(a): $K_s = 1.86 \pm 0.25 \times 10^{-8} \text{ cm}^2$; 3 μM r-apo(a): $K_s = 2.61 \pm 0.36 \times 10^{-8} \text{ cm}^2$; $P = 0.18$, two-tailed). Interestingly, when visualized using scanning electron microscopy, clots generated using the plasma-purified fibrinogen had different morphological appearances compared to clots formed using commercial fibrinogen. The plasma-purified clots had long straight fibers in an ordered arrangement (Figure 3.3C-D), whereas the commercial fibrinogen produced clots with very fine fibers that were bent, disorganized and formed nodular structures (Supplementary Figure 3.1).

Confocal microscopy (low resolution) and scanning electron microscopy (high resolution) were used to study the ultrastructure of clots formed in the presence or absence of r-apo(a). In both sets of microscopy experiments, fibrin clots prepared in the presence of r-apo(a) had thinner fibers and a denser fibrin network (Figure 3.3B,D) than clots prepared in the absence of r-apo(a) (Figure 3.3A,C). The images were quantified with respect to average fiber diameter and percent area, a measure of clot density. From the confocal images it was determined that the average percent area increased significantly in the presence of even 0.5 μM r-apo(a) (Figure 3.4A; 0 μM r-apo(a): $25.2 \pm 3.6\%$; 0.5 μM r-apo(a): $41.5 \pm 7.3\%$ cm^2 ; $P < 0.001$). Although there was not a significant increase in the average percent area of clots formed in presence of 1.0 μM r-apo(a) relative to 0.5 μM r-apo(a) (Figure 3.4A; 1.0 μM r-apo(a): $45.7 \pm 4.6\%$; $P = 0.1$), there was a significant increase in the average percent area of clots formed in the presence of 2.0 μM r-apo(a) relative to 1.0 μM r-apo(a) (Figure 3.4A; 2.0 μM r-apo(a): $56.0 \pm 5.6\%$; $P = 0.002$).

Fiber diameter was found to be significantly decreased in clots formed in the presence of r-apo(a) (Figure 3.4B; 0 μ M r-apo(a): $0.30 \pm 0.02\mu\text{m}$; 0.5 μ M r-apo(a): $0.23 \pm 0.01\mu\text{m}$; $P < 0.001$) but increasing concentrations of r-apo(a) did not produce significantly different fiber diameters (Figure 3.4B).

Scanning electron microscopy (SEM) experiments were also conducted in order to obtain high resolution images using a single concentration of 3.0 μ M 17KIV r-apo(a); this concentration was previously used in experiments investigating the effect of apo(a) on fibrinolysis [67,152]. Typically, there is a need for SEM samples to be dehydrated and fixed, which introduces the concern that the sample preparation may alter the network morphology [87]. Specifically, dehydration has been shown to shrink the native fibrin such that fibrin fiber diameter becomes artificially underestimated and fiber density overestimated [153]. However, in the present investigation, clots were visualized using an environmental SEM at low vacuum, which did not necessitate critical drying of the clots prior to visualization. In fact, the clots remained hydrated until introduced into the microscope and after visualization were observed to retain a degree of elasticity and moisture. It is therefore reasonable to assume that under these conditions, the clots were visualized in a more physiologically relevant state than those of traditional high vacuum SEM experiments, wherein samples are critical point dried and possibly also coated. From the SEM images it was calculated that the average percent area increased significantly in the presence of 17KIV r-apo(a) (Figure 3.4C; 0 μ M r-apo(a): $38.7 \pm 1.6\%$; 3 μ M r-apo(a): $46.5 \pm 3.4\%$; $P = 0.03$), whereas the fiber diameter significantly decreased in the presence of r-apo(a) (Figure 3.4D; 0 μ M r-apo(a): $0.136 \pm 0.008\mu\text{m}$; 3 μ M r-apo(a): $0.111 \pm 0.004\mu\text{m}$; $P = 0.003$).

We investigated whether the observed apo(a)-induced modifications in fibrin clot structure had functional consequences by measuring both clot formation and clot lysis in the

presence or absence of apo(a) using turbidity. The presence of r-apo(a) did not significantly affect the maximum absorbance measured at 1h (Figure 3.5; 0 μ M r-apo(a): $A_{340} = 0.534 \pm 0.07$, 3 μ M r-apo(a): 0.576 ± 0.06 , $P = 0.3$) but the absorbance profiles do have distinct appearances in that the clots formed in the presence of r-apo(a) have profiles characterized by a steeper initial slope than clots formed in the absence of r-apo(a) (Figure 3.5); this is interpreted as an increased rate of fibrin polymerization in the presence of r-apo(a) [154]. The presence of r-apo(a) was also found to significantly decrease the time required to reduce the absorbance by 50% by exogenously-added plasmin (Figure 3.6; 0 μ M r-apo(a): 590 ± 35 seconds, 3 μ M r-apo(a): 1035 ± 100 seconds; $P = 0.007$), which is a measure of clot lysis time.

As we have shown, apo(a) decreases fibrin fiber thickness and increases fiber density, which are parameters that have been shown to decrease the rate of fibrinolysis [88]. However we did not find evidence of impaired plasmin activity. Lp(a)/apo(a) is well known to inhibit tPA-mediated plasminogen activation on fibrin surfaces [65-67] and therefore simply measuring the rate of fibrinolysis through the addition of tPA and plasminogen to the clots would be uninterpretable. We attempted to work around this limitation by observing the effect of increasing thrombin concentration, itself a modifier of fibrin structure [88], on the activation of plasminogen by tPA in the presence and absence of r-apo(a). We used very low concentrations of thrombin to induce slow fibrin clotting in order to form clots with thicker fibers and a looser configuration [88], confirmed by turbidity measurements (data not shown), to overcompensate for the effects of r-apo(a) on fibrin structure. Using thrombin concentrations of 0.000067 U/mL to 0.0004 U/mL, the extent of apo(a)-mediated plasminogen activation inhibition did not change (at 0.000067 U/mL thrombin: 29.9% inhibition; 0.00013 U/mL thrombin: 33.5%; 0.0004 U/mL thrombin: 28.7%). However, at a thrombin concentration of 0.01 U/mL, there was an observed decrease in

plasminogen activation both in the presence and absence of r-apo(a) (Figure 3.7) and under these conditions, the extent of tPA-mediated plasminogen activation was increased (0.01 U/mL thrombin: 55.3% inhibition).

3.5 Discussion

There is increasing evidence that the structure of fibrin can regulate fibrinolysis and that clot structure has a role in determining the predisposition to atherothrombotic disease [85]. Lipoprotein(a) (Lp(a)), a cardiovascular risk factor, was found to be associated with abnormal fibrin structures in a comparison of healthy men to patients who had survived a myocardial infarction. It was observed that elevated Lp(a) levels correlated with abnormal fibrin structures and that these abnormal structures were more prevalent in patients than in healthy controls [89]. As a result, it was postulated that Lp(a) may cause the alterations observed in the fibrin network and in this way contribute to the pathogenesis of myocardial infarction. Herein we provide the first direct evidence that apo(a) affects fibrin clot structure to produce clots that are abnormally dense, 2-fold less permeable and composed of abnormally thin fibers. Additionally, we showed that these alterations in clot structure can contribute to reduced tPA-mediated plasminogen activation.

Permeability is a physiologically relevant estimate of the accessibility of dissolved agents, including lytic enzymes, to the fibrin fiber network [87]. We found that apo(a) significantly decreases the permeability constant of fibrin clots (Figure 3.1), which would suggest that apo(a) also decreases the fibrinolytic potential of the clot by decreasing the accessibility of lytic enzymes. However, we did not find any evidence that apo(a) down-regulates the ability of fibrin clots to be degraded by exogenously-added plasmin (Figure 3.6) but rather found the

process to be significantly up-regulated. There is an important caveat to this observation, which is that given the design of the experiment, wherein fibrinogen in the presence or absence of r-apo(a) was added to wells containing plasmin, it is possible that plasmin was distributed throughout the solution in advance of clotting such that permeability did not restrict the access of plasmin to the fibers as it may have had, had the enzyme had been added to the surface of the clot. Moreover, it has been shown that plasmin degrades thin fibrin fibers at a faster rate than thicker fibers [88]. Thus, if plasmin were to be distributed throughout the solution prior to the gel point, thereby eliminating the regulatory effect of the fibrin network architecture [88], it is possible that plasmin would degrade the r-apo(a)-modified clot structure at an increased rate because of its significantly thinner fibrin fibers.

Alternatively, it is possible that the apo(a)-modified fibrin structure could in fact enhance plasmin activity and yet attenuate fibrinolysis overall, as it is a multi-step process [75]. We therefore investigated the effect of r-apo(a)-modified fibrin structure on the activation of plasminogen to plasmin by tissue-type plasminogen activator (tPA). Lp(a)/apo(a) is known to inhibit the ability of tPA to activate plasminogen to plasmin [65-67], whereas plasmin activity is unaffected [64]. We determined that the extent to which apo(a) inhibited plasminogen activation was unaffected until, at the highest concentration of thrombin used, there was a notable change in the efficacy of fibrin as a cofactor (Figure 3.7); this is evident as a decrease in plasminogen activation both in the presence and absence of r-apo(a). Under these conditions, the extent of the inhibition of plasminogen activation by apo(a) increased ~15% (Figure 3.7). The impairment of the cofactor ability of fibrin can be interpreted as a change in fiber structure because it has been shown that decreased fibrin fiber diameter—a consequence of increased thrombin concentration—is associated with decreased tPA [88] and plasminogen binding [155].

Importantly, according to the most recent model of the inhibition of tPA-mediated plasminogen activation by apo(a), the interaction between apo(a) and fibrin is defined as a critical component of the inhibitory mechanism [67]. It is consistent with this model that by changing the interaction of apo(a) with fibrin and also the interactions of plasminogen and tPA with fibrin, the extent of inhibition could be modified.

Alternatively, it has been reported that apo(a) transgenic mice in a plasminogen-deficient background (apo(a):Plg^{-/-}) had a higher incidence of thrombotic events, characterized by thrombi with increased platelet and fibrin(ogen) content [147]. The absence of plasminogen from the mice implies a plasminogen-independent prothrombotic mechanism for apo(a). It is possible that the effect of apo(a) on fibrin clot structure is not solely dependent on the fibrinolytic system for its effects. It has been reported that clots formed with thin fibrin fibers and reduced porosity are also characterized by increased rigidity and an association with thrombotic and/or embolic events [156,157]. In these mice, therefore, the viscoelastic properties of abnormal clot structures induced by apo(a) may have contributed to the increased incidence of thrombosis.

The structure of fibrin clots has been shown to be affected by a variety of factors, including negatively charged substances [148,158]. Apo(a) has a high sialic acid content, which confers a net negative charge to the molecule [129]. Moreover, the identified high-affinity binding site for apo(a) in the fibrin(ogen) α C region [159] is both a positively-charged region and is involved in the lateral association stage of fibrin polymerization. The α C domain forms intermolecular associations with the α C domains of other fibrin monomers and in this way acts as an intermolecular bridge between fibrin protofibrils to form thick fibers [29]. The interaction between α C domains is believed to be dependent on electrostatic interactions between charge clusters in the C-terminal region [160] and it has been shown that efficient lateral association

does not occur if the charged cluster in the α C-domain is mutated or absent [161]. It is therefore possible that by interacting with this domain, apo(a) would affect the lateral association process, resulting in the formation of abnormally thin fibers. This is in fact consistent with the result we have reported in this study (Figure 3.4B,D).

In conclusion, our data indicate that apo(a) produces significant alterations in fibrin structure characterized by thin fibers, a dense structure and decreased permeability, properties typically referred to as prothrombotic. Furthermore, the alterations to the fibrin clot structure increase the inhibitory effect of apo(a) on tPA-mediated plasminogen activation. We have therefore identified an entirely novel mechanism by which apo(a) can down-regulate fibrinolysis through altering clot structure to one that restricts lytic enzyme access to fibrin fibers, as well as through a mechanism by which apo(a) can enhance its effect on tPA-mediated plasminogen activation.

3.6 Figures

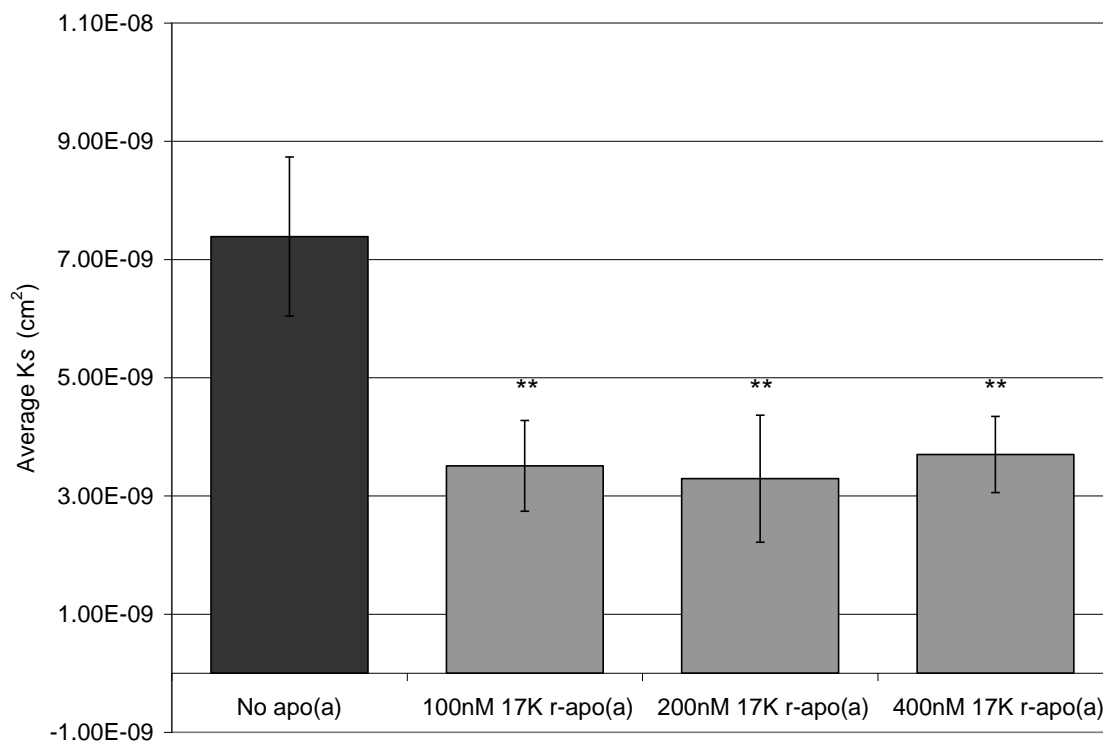
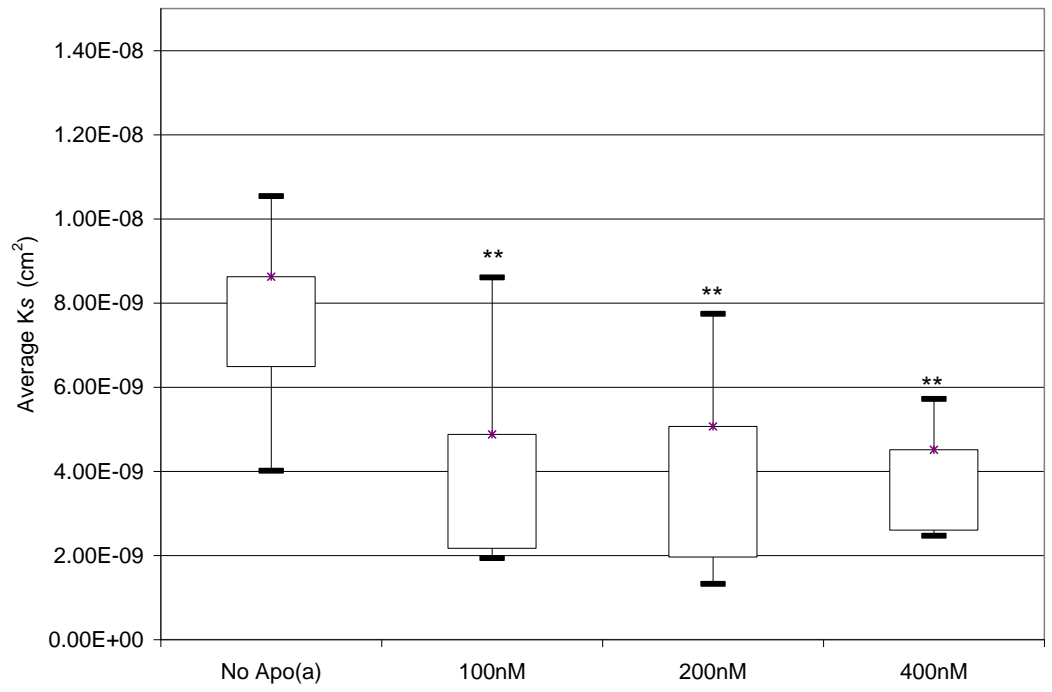
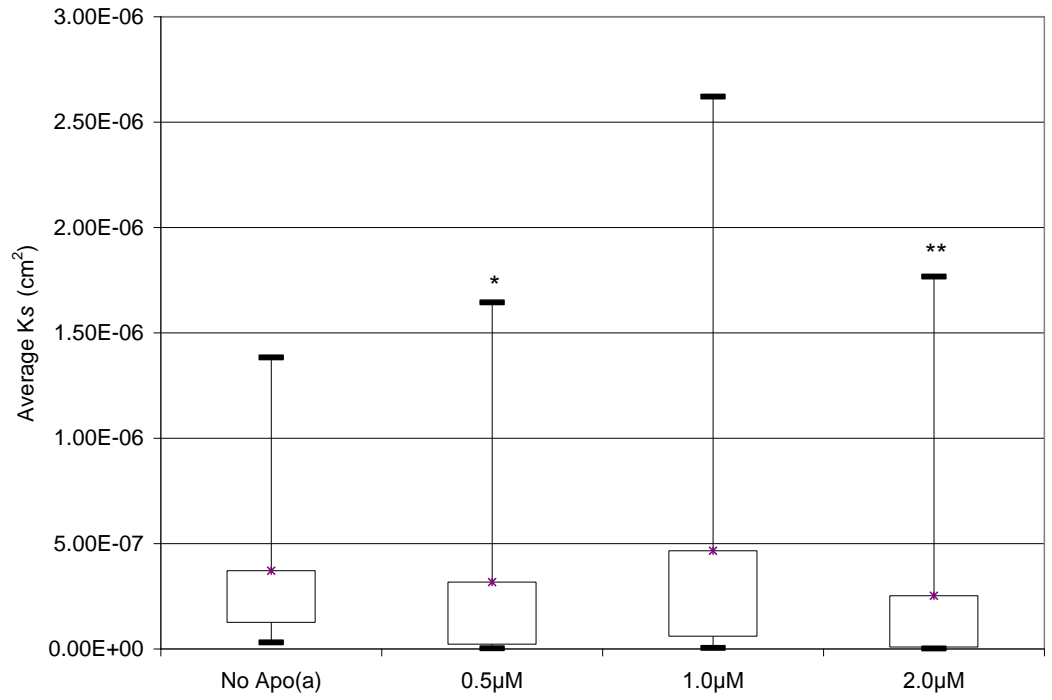


Figure 3.1 The effect of increasing 17KIV r-apo(a) concentration on the permeability of lipoprotein-deficient plasma clots. Lipoprotein-deficient plasma was clotted in the presence (dark grey) or absence (light grey) of r-apo(a) (100-400 nM) in the presence of CaCl₂ (20 mM) and thrombin (2 U/mL). The permeability constant (K_s) was measured and the results are expressed as mean ± standard error of the mean (n ≥ 6). The distributions of the clots formed in the absence and presence of r-apo(a) differed significantly (0 nM r-apo(a): 7.39 ± 1.35 × 10⁻⁹ cm²; 100 nM r-apo(a): 3.51 ± 0.77 × 10⁻⁹ cm², P = 0.002).

A



B



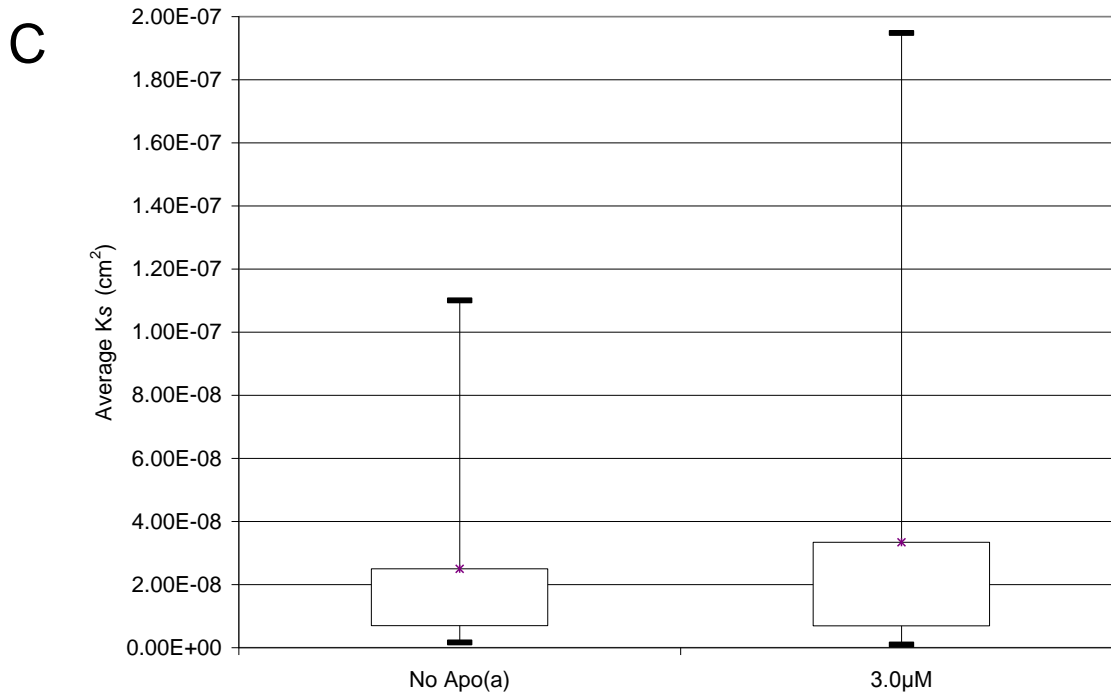


Figure 3.2 Box plots of clot permeability parameters for clots derived from lipoprotein-deficient plasma, plasma-purified fibrinogen and commercial (Calbiochem®) fibrinogen in the presence and absence of 17KIV r-apo(a).

Panel A. Clots were generated from lipoprotein-deficient plasma clotted with CaCl_2 (20 mM) and thrombin (2 U/ml) in the presence or absence of 100, 200 or 400 nM 17KIV r-apo(a). The distributions of the clots formed in the absence and presence of r-apo(a) differed significantly (0 nM r-apo(a): $7.39 \pm 0.135 \times 10^{-9} \text{ cm}^2$; 100 nM r-apo(a): $3.51 \pm 0.77 \times 10^{-9} \text{ cm}^2$ (Mann-Whitney $U = 11$, $n_1 = 8$, $n_2 = 13$, $P = 0.002$ one tailed); 200 nM r-apo(a): $3.29 \pm 1.08 \times 10^{-9} \text{ cm}^2$, ($U = 5$, $n_1 = 8$, $n_2 = 6$, $P = 0.008$ one tailed); 400 nM r-apo(a): $3.70 \pm 0.64 \times 10^{-9} \text{ cm}^2$ ($U = 3$, $n_1 = 8$, $n_2 = 6$, $P = 0.004$ one tailed)).

Panel B. Clots were generated from fibrinogen (3 mg/mL) purified from pooled fresh-frozen plasma clotted with CaCl_2 (20 mM) and thrombin (1 U/ml) in the presence or absence of 0.5, 1.0 or 2.0 μM 17KIV r-apo(a). The distributions of the clots formed in the absence or presence of either 0.5 μM or 2.0 μM r-apo(a) differed significantly, whereas there was no significant difference between the distribution of clots produced with 0 μM vs. 1.0 μM r-apo(a) (0 μM r-apo(a): $K_s = 3.61 \pm 0.84 \times 10^{-7} \text{ cm}^2$; 0.5 μM r-apo(a): $K_s = 3.47 \pm 1.08 \times 10^{-7} \text{ cm}^2$ (Mann-Whitney $U = 179$, $n_1 = 21$, $n_2 = 24$, $P = 0.05$ one-tailed); 1.0 μM : $K_s = 4.81 \pm 1.36 \times 10^{-7} \text{ cm}^2$ ($U = 278$, $n_1 = 21$, $n_2 = 278$, $P = 0.3$ one-tailed); 2.0 μM : $K_s = 2.68 \pm 0.66 \times 10^{-7} \text{ cm}^2$ ($U = 401$, $n_1 = 21$, $n_2 = 60$, $P = 0.007$ one-tailed)).

Panel C. Clots were generated from commercially-available (Calbiochem®) plasminogen-depleted fibrinogen from human plasma (3 mg/mL) clotted with CaCl_2 (20 mM) and thrombin (0.01 U/ml) in the presence or absence of 3.0 μM 17KIV r-apo(a). There was no observed significant difference between the distribution of clots produced in the presence or absence of r-

apo(a) (0 μ M: $1.86 \pm 0.25 \times 10^{-8}$ cm²; 3.0 μ M: $2.62 \pm 0.36 \times 10^{-8}$ cm² (Mann-Whitney $U = 2030$, $n_1 = 52$, $n_2 = 67$, $P = 0.18$ two-tailed)).

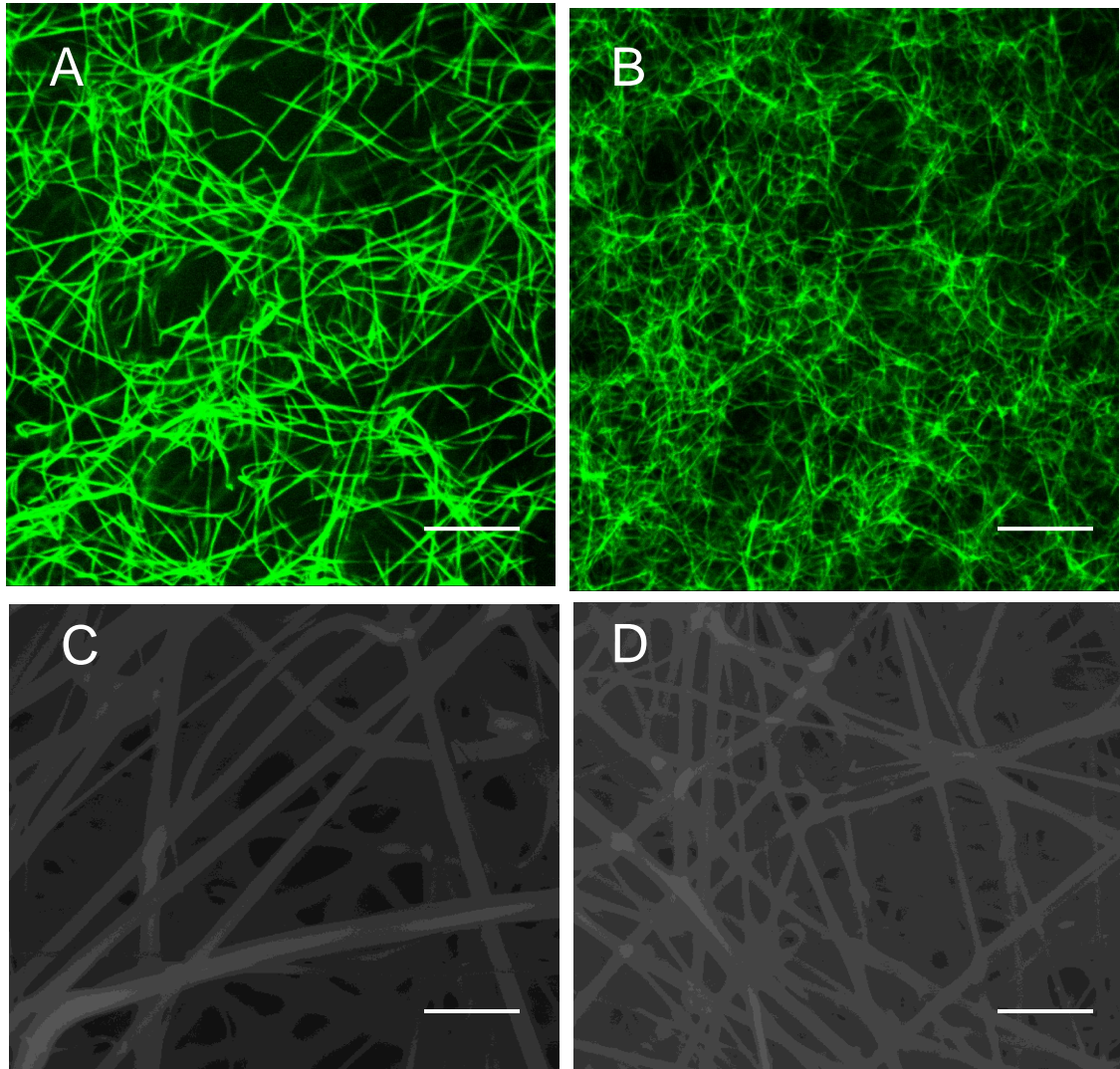
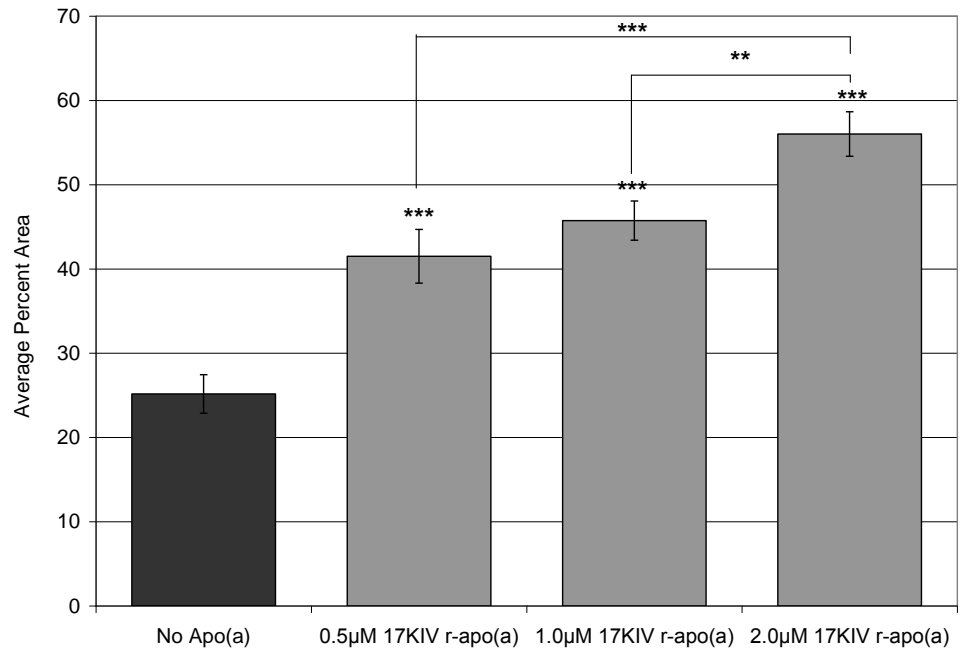
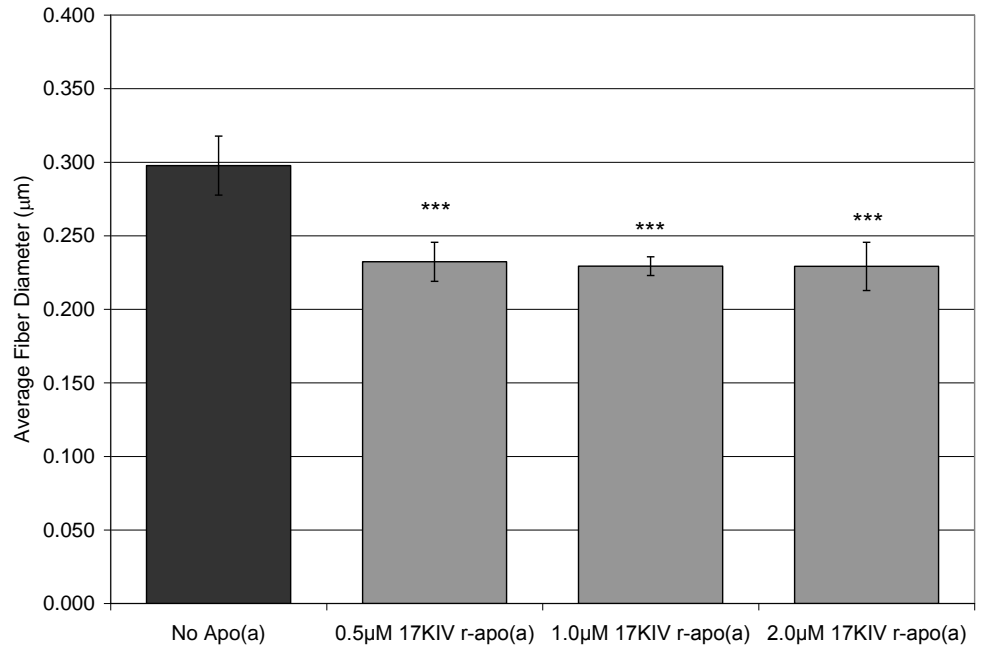


Figure 3.3 *Apo(a)* induces a change in fibrin clot ultrastructure. Fibrin clots were prepared by incubating plasma-purified fibrinogen (3 mg/mL) with thrombin (0.01 U/mL) and CaCl_2 (20 mM) in the absence (Panels A,C) and presence (B,D) of 17K r-apo(a) (B: 2 μM 17K r-apo(a) and D: 3 μM 17K r-apo(a)). Fibrin clots were analyzed by laser scanning confocal microscopy (A-B) and by scanning electron microscopy (C,D). A total of 3 different areas were visualized in 3 replicate clots, and representative images are shown. The scale bar in A and B = 15 μm . The scale bar in C and D = 2 μm

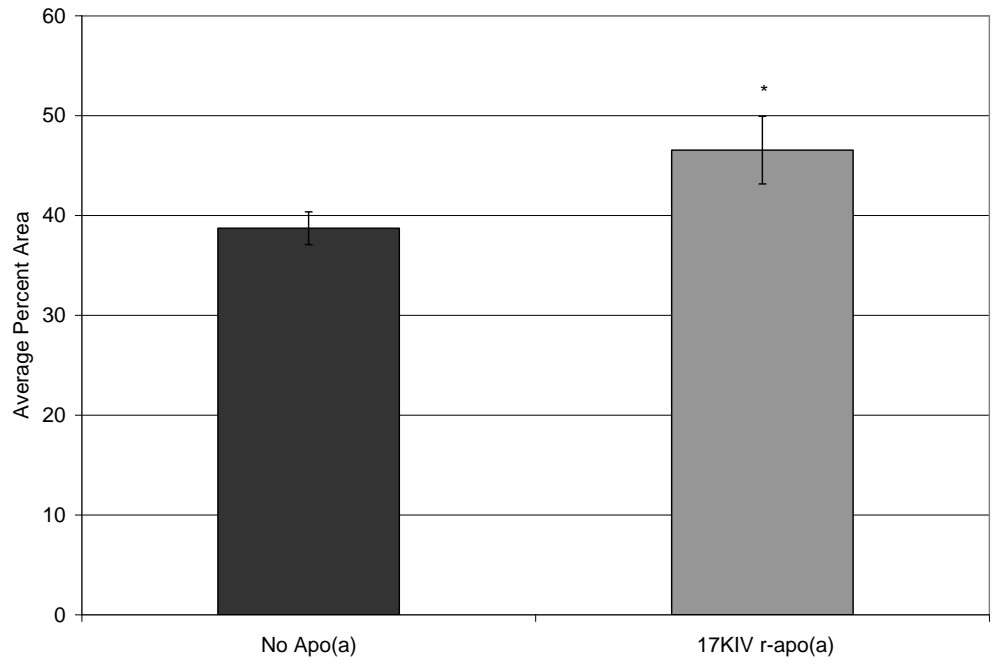
A



B



C



D

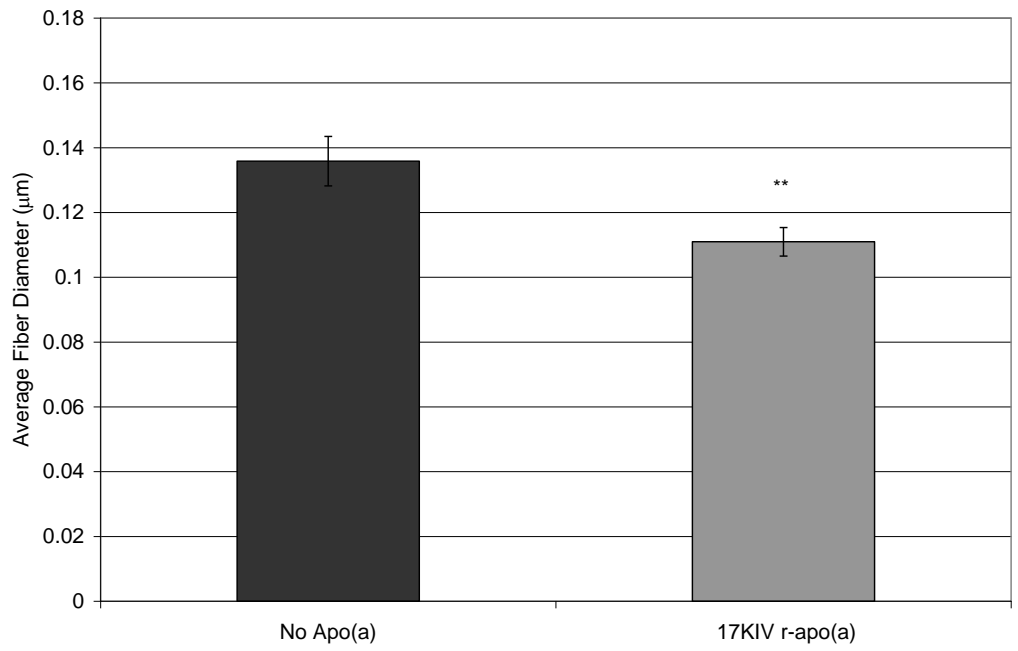


Figure 3.4 *Quantification of the effect of 17KIV r-apo(a) on the ultrastructure of a fibrin clot.* *Panel A.* The average percent area in the presence or absence of apo(a) as determined from confocal microscopy images. A one-tailed heteroscedastic Student t-test demonstrated a significant difference between the average fiber diameter of clots formed in the presence or absence of r-apo(a) ($P = 0.003$). *Panel B.* Average fiber length in the presence and absence of apo(a) determined by confocal microscopy. *Panel C.* The average percent area \pm standard error of the mean in the presence or absence of apo(a) by scanning electron microscopy of a representative clot. *Panel D.* Average fiber diameter \pm standard error of the mean in the presence and absence of apo(a) as determined by scanning electron microscopy of a representative clot.

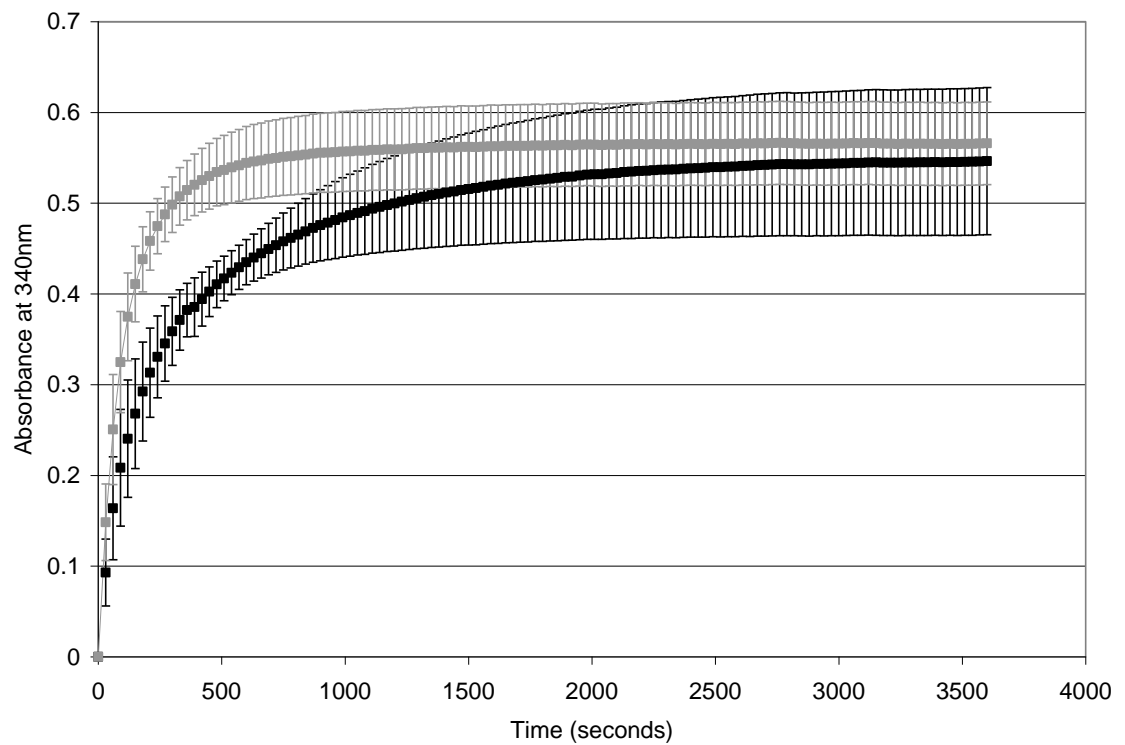


Figure 3.5 *The effect of apo(a) on fibrin polymerization.* Fibrinogen (3mg/mL) was clotted in the absence (black line) or presence (grey line) of recombinant apolipoprotein(a) (3.0 μ M) by an activation mix of thrombin (1 U/mL) and CaCl₂ (20 mM). The turbidity was monitored at 340 nm every 30 seconds. The results are presented as the mean \pm standard error of the mean (n = 5).

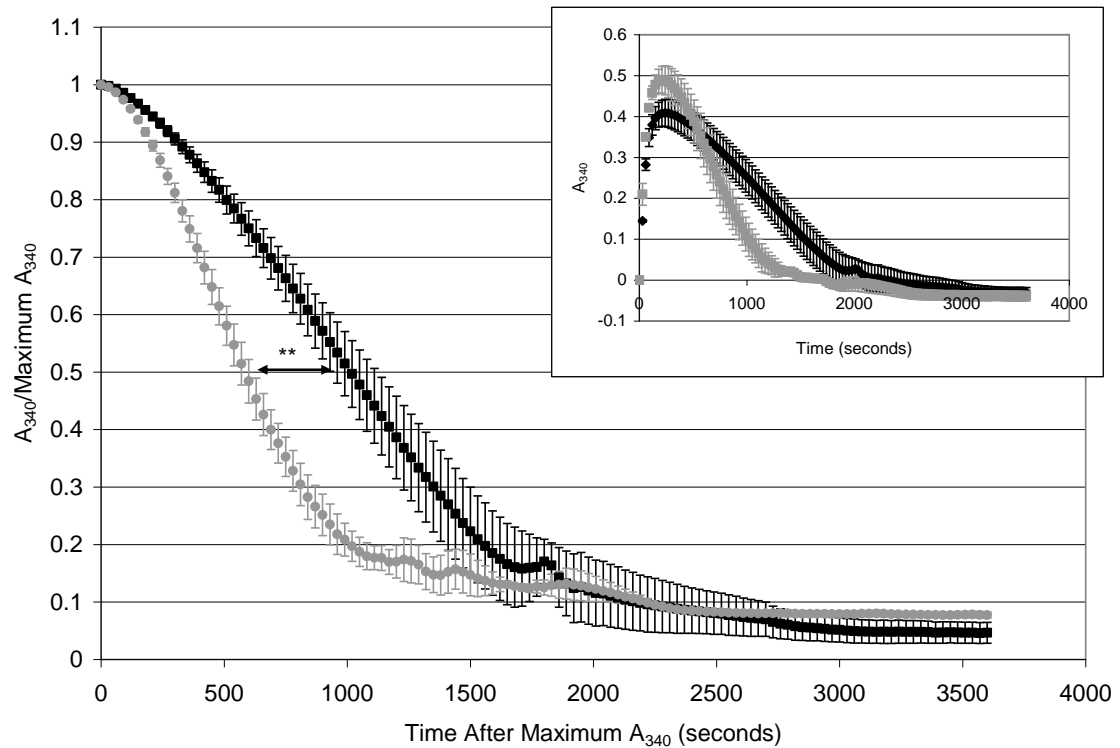


Figure 3.6 *The effect of apo(a) on plasmin-mediated clot lysis.* Plasma-purified fibrinogen (3 mg/mL) in the presence (grey) or absence (black) of r-apo(a) (3 μ M) was added to wells containing thrombin (1 U/mL), CaCl₂ (20 mM) and plasmin (30 nM). The A₃₄₀ values were normalized to the maximum A₃₄₀ and are presented as the mean \pm standard error of the mean. A one-tailed homoscedastic t-test identified a significant decrease in the time required to reduce the absorbance by 50% in the presence (590 \pm 35 seconds) or absence (1035 \pm 100 seconds) of r-apo(a) ($P = 0.007$, $n = 3$). *Inset.* The entire turbidity profile is presented as the mean \pm standard error of the mean.

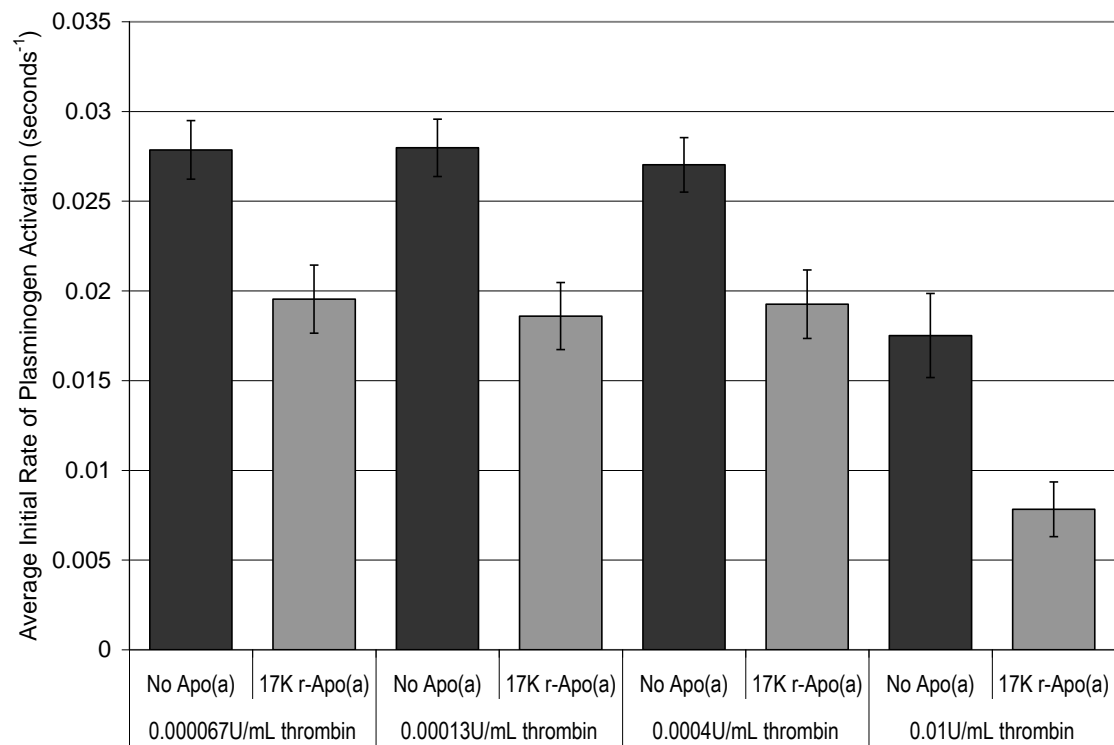
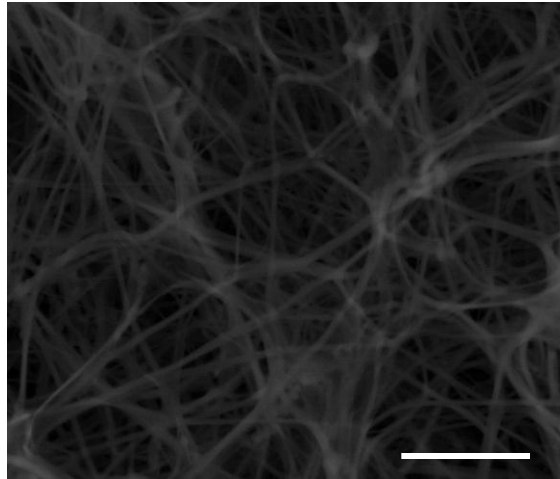


Figure 3.7 The effect of decreasing thrombin concentration on the inhibition of tPA-mediated plasminogen activation by apo(a) on fibrin. Fibrinogen (3.0 μM) and fluorescently-labeled recombinant plasminogen(S741C) (1.8 μM) in the presence or absence of 17KIV r-apo(a) (3.0 μM) was added to wells containing tPA (50 nM), CaCl_2 (10 mM) and an increasing amount of thrombin (0.000067-0.01 U/mL). The results are expressed as the mean \pm standard error of the mean.



Supplementary Figure 3.1 *Clot generated using commercially-available (Calbiochem®) fibrinogen in the absence of apo(a).* Representative images of fibrin clots prepared by incubating fibrinogen (3 mg/mL) with thrombin (0.01 U/mL) and CaCl₂ (20 mM) in the absence of recombinant apolipoprotein(a). Bar = 2 μm

Chapter 4

Phosphatidylcholine-Adducts on Both Plasminogen and Apolipoprotein(a) Affect tPA-Mediated Plasminogen Activation

4.1 Summary

Background: Elevated lipoprotein(a) (Lp(a)) levels have been identified as a risk factor for cardiovascular disease. Lp(a) is a distinct class of lipoprotein particles composed of a low density lipoprotein-like moiety covalently-linked to a large glycoprotein apolipoprotein(a) (apo(a)), a homolog of the fibrinolytic proenzyme plasminogen. Apo(a) is extremely heterogeneous in size and small isoforms are not only associated with elevated plasma levels of Lp(a) but also independently associated with increased cardiovascular risk. Several *in vitro* and *in vivo* studies have shown that Lp(a)/apo(a) can inhibit tissue-type plasminogen activator-mediated plasminogen activation on fibrin surfaces. Additionally, oxidized phospholipids (OxPL) have also been implicated in the development of cardiovascular disease. This is notable both because Lp(a) is a preferential carrier of OxPLs in the plasma and because it has been reported that not only apo(a) but also plasminogen is covalently modified by OxPLs.

Objective: We determined the effect of apo(a) isoform size on the ability of apo(a) to inhibit tPA-mediated plasminogen activation and investigated the effect of the reported oxidized phosphatidylcholine-protein modification of both plasminogen and apo(a) on this process.

Methods: A fluorescent recombinant plasminogen-based system was used to investigate the effect of apo(a) isoform size on the tPA-mediated activation of Glu¹-plasminogen to Glu¹-plasmin in the presence of degraded fibrin cofactors and in the absence of positive feedback reactions catalyzed by plasmin. Protein-phosphorus content and reactivity to the monoclonal antibody E06, which

recognizes oxidized phosphatidylcholine were determined for individual preparations of both recombinant apo(a) (r-apo(a)) and recombinant plasminogen (r-plasminogen) and correlated with initial rates of plasminogen activation.

Results: The phosphorus-content and E06 reactivity of individual preparation of r-apo(a) were found to correlate ($r = -0.66$) with the ability of apo(a) to inhibit tPA-mediated plasminogen activation; however, apo(a) isoform size did not. Similarly, the phosphorus content and E06 reactivity of individual plasminogen preparations were found to correlate with its initial rate of activation by tPA ($r = 0.95$) but did not correlate with the ability of apo(a) to inhibit its activation.

Conclusions: The ability of apo(a) to inhibit plasminogen activation is correlated with its phosphorus-content and E06 reactivity, and is a more important determinant of inhibitory capacity than apo(a) isoform size. Also, the phosphorus content and E06 reactivity of plasminogen does not affect the ability of apo(a) to inhibit its activation, however does correlate with the extent to which tPA can mediate its activation.

KEYWORDS: apolipoprotein(a); fibrinolysis; oxidized phospholipids; plasminogen activation

4.2 Introduction

An elevated plasma Lp(a) concentration is considered as a cardiovascular risk factor [24,31,79]. Lp(a) is a plasma lipoprotein comprised of two components (i) a cholesterol-rich low density lipoprotein (LDL)-like particle that is covalently bound via apolipoproteinB-100 (apoB-100) to (ii) a large glycoprotein, apolipoprotein(a) (apo(a)) [1,2]. In 1987, apo(a) was identified as structurally similar to the fibrinolytic proenzyme plasminogen [3]. Specifically, apo(a) is composed of multiple repeats of a sequence resembling the plasminogen kringle 4 domain (apo(a) KIV), followed by single copies of a plasminogen-like kringle 5 (apo(a) KV) and protease-like

domain. The kringle 4-like domains of apo(a) can be divided into 10 subtypes based on amino acid substitutions (KIV₁₋₁₀) [3] and each subtype is present in a single copy with the exception of the KIV₂ subtype, which can be present in 3 to >30 identically-repeated copies [15]; this gives rise to a series of apo(a) isoforms of variable size. The protease domain of apo(a) shares 94% homology with the corresponding domain of plasminogen, however it is catalytically inactive [13].

Shortly after its characterization as a plasminogen-like molecule, apo(a) was found to impair fibrinolysis [162]. The central step in fibrinolysis is the breakdown of insoluble fibrin by the active enzyme plasmin. The generation of active plasmin involves the cleavage of the Arg⁵⁶¹-Val⁵⁶² bond in plasminogen by tissue-type plasminogen activator (tPA), which is a reaction that occurs most efficiently on the surface of fibrin [75,163]. Plasmin then cleaves the insoluble fibrin surface on which it was formed to soluble fibrin degradation products. It is now well established that Lp(a)/apo(a) attenuates tPA-mediated plasminogen activation on the fibrin surface [65-67], however there is not a consensus as to the mechanism. The most recent model suggests that apo(a) interacts with the fibrin (cofactor), plasminogen (substrate) and tPA (enzyme) to form a quaternary complex that is less catalytically efficient as an activation complex in the presence of apo(a) than in its absence [67]. In this way, the amount of the active plasmin enzyme that is generated in a given time is reduced, as is the subsequent digestion of fibrin to fibrin degradation products by plasmin.

Small Lp(a)/apo(a) isoforms have been identified to be an independent risk factor for cardiovascular disease [56,57]. Nonetheless, the effect of the length polymorphism of apo(a) has been the subject of only limited research to date. It has however been reported that low molecular weight isoforms of Lp(a) bind to fibrin with greater affinity than high molecular weight isoforms

[12,78]. This suggests a possible role for isoform size in the inhibition of tPA-mediated activation by apo(a) because the interaction of apo(a) with fibrin is reported in the aforementioned model of apo(a)-mediated inhibition to be an important component of the formation of the quaternary complex. We therefore sought to determine the effect of apo(a) isoform size on tPA-mediated plasminogen activation, using a series of recombinant apo(a) size isoforms in a fluorescence-based plasminogen activation assay.

There is increasing evidence that points to oxidized phospholipids (OxPLs) as both biomarkers and pathogenic factors in atherosclerosis [113]. Lp(a) has been identified as a major carrier of OxPLs in the circulation and of the OxPL associated with Lp(a), half are associated with its protein components, i.e. apo(a) or apoB-100 [112]. Apo(a) has been reported to be covalently modified by the specific OxPL, oxidized phosphatidylcholine (OxPtdC) by means of a Schiff base covalent linkage to specific lysine residues [164]. Interestingly, plasminogen has also been reported to have similar OxPtdC-modifications [111]. The formation of covalent OxPL-protein adducts has been reported for a variety of different proteins, wherein it has been shown to be functionally significant [165]; however, the OxPL-modifications of neither apo(a) nor plasminogen have been functionally characterized. We therefore investigated the effect of the OxPtdC-modification on both apo(a) and plasminogen in the context of the inhibition of tPA-mediated plasminogen activation.

4.3 Materials & Methods

Construction, Expression, and Purification of Recombinant Apo(a) Variants—The construction and expression of the various r-apo(a) size isoforms utilized in this study have been described previously [129,152]. Purification of all the r-apo(a) derivatives from the conditioned medium of

stably-expressing human embryonic kidney (HEK 293) cells by affinity chromatography has also been previously described [129].

Purification of Fluorescently-Labeled Recombinant Plasminogen—A variant of Glu¹-plasminogen, containing an active site serine to cysteine mutation (S741C) was cloned and expressed in baby hamster kidney (BHK-21) cells as previously described [136]. The recombinant plasminogen(S741C) protein (r-plasminogen) was purified from the conditioned medium of stably-expressing BHK-21 cells and labeled with 5'-iodoacetamidofluorescein at the mutated residue (5'IAF; Molecular Probes, Eugene, OR) as previously described [67].

Purification of Human Fibrinogen and Non-cross-linked FDPs—Fibrinogen was purified from citrated, fresh frozen, human plasma, obtained from Kingston General Hospital (Kingston, ON) as previously described [67]. Non-cross-linked FDPs were prepared by limited plasmin-mediated lysis of fibrin clots, as previously described [67].

Fluorescent Plasminogen Activation Assays—5'IAF-labeled r-plasminogen(S741C) was cleaved by tPA (Cathflo® Activase®, Kingston General Hospital, Kingston, ON) resulting in a decrease in fluorescent intensity that was monitored using a SpectraMax M5e fluorescent plate reader (Molecular Devices, Downington, PA, USA) in a 96-well solid black polystyrene microplate (Corning, Troy, MI), as previously described [67]. Briefly, 80 μ L volumes containing 5'IAF-labeled plasminogen (1.8 μ M final concentration) and FDPs (0.6 μ M final) in the presence or absence of r-apo(a) (3.0 μ M final) in HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4 containing 150 mM NaCl) containing 0.02% (v/v) Tween 80 were added to wells containing 20 μ L volumes of CaCl₂ (10 mM final) and tPA (50 nM final) in HBS with 0.02% Tween 80. The fluorescence was monitored for 1 hour at 30 second intervals. The resultant curves were corrected for the buffer blank and then corrected for the internal filter effects as described [67].

In order to correct for the internal filter effect, standard curves were first generated for every combination of 5'IAF r-plasminogen, using final concentrations ranging from 0-3 μM and FDPs, using a single final concentration of 0.6 μM . From the standard curves, the parameters for the fluorescence per mole of 5'IAF-labeled plasminogen (i) and the exponential coefficient (a) were determined by fitting the curves (in relative fluorescent units, RFU) by non-linear regression to the equation,

$$\text{RFU} = i \times [\text{Plasminogen}] \times \exp(-a \times \text{RFU}/i) \quad [67]$$

using the SOLVER tool from Microsoft Excel (Microsoft Corporation, Mississauga, ON) as described [150]. The a and i values were then used to correct for the internal filter effect according the equation:

$$\text{RFU}_{\text{corrected}} = \text{RFU}_{\text{raw}} \times \exp(a/i \times \text{RFU}_{\text{raw}}) \quad [67]$$

In order to determine the initial rate of plasmin formation per mole of tPA, first the slope and intercept of the initial linear region of the corrected curves was calculated using the SLOPE and INTERCEPT functions from Microsoft Excel (Microsoft Corporation, Mississauga, ON). Then, these values were used to calculate the rate according to the equation:

$$\text{Rate} = (\text{slope}) (1/(0.5 \times \text{intercept})) ([\text{Plasminogen}]_{\text{initial}}/[\text{tPA}]_{\text{initial}}) \quad [67]$$

Inorganic Phosphate Quantification (Phosphorus Assay)—Inorganic phosphate was quantified using a colorimetric method for the determination of phosphorus, as previously described [166]. Briefly, 50 μg of protein to be analyzed was added to a 12 mL borosilicate glass tubes (Fisher Scientific, Nepean, ON) and then 0.5 mL of 10 N H_2SO_4 was added to each tube. Marbles were placed atop the open end of the tubes and the tubes were placed into a 160°C pre-heated oven for 3 hours. After 3 hours, the tubes were removed and 3 drops of 30% H_2O_2 was added to each tube using a transfer pipette. The tubes with the marbles replaced were put back into the oven at 160°C

for 1.5 hours. After 1.5 hours, the tubes were removed from the oven and 0.2 mL of 5% ammonium molybdate (Sigma, St. Louis, MO) solution was added to each tube, followed by the addition of 4.4 mL ddH₂O to each tube. The solution was mixed by vortexing and then 0.2 mL of a freshly-made 1.6% solution of Fiske-SubbaRow reagent (Sigma, St. Louis, MO) was added and the solution was re-mixed by vortexing. The tubes were heated for 7 minutes in a 100°C water bath and the absorbance of the solutions was read at 830 nm. The sample absorbance values were compared with a phosphate standard curve generated for each experiment using known amounts of a phosphate standard solution.

Phospholipase A₂ Digestion of r-Plasminogen—Recombinant plasminogen was incubated with phospholipase A₂ (PLA₂ from *Naja mossambica mossambica*, Sigma, St. Louis, MO) to remove covalently-associated oxidized phospholipids. Briefly, 0.5 mg of r-plasminogen protein in 0.02 M HEPES, 0.15 M NaCl, pH 7.4 was supplemented with CaCl₂ to a final concentration of 5 mM CaCl₂. PLA₂ (17.5 units) was added to the pre-warmed protein solution and incubated at 37°C for 5 hours, with periodic mixing. Immediately following the digestion, the protein solution was loaded onto a pre-equilibrated 1 mL lysine-Sepharose column as described previously [129].

Dephosphorylation of r-Plasminogen—Phosphate groups were removed from r-plasminogen by incubation with shrimp alkaline phosphatase (SAP; Promega, Madison, WI). A 1 mL volume of 0.5 mg of recombinant plasminogen protein was washed 3-times with reaction buffer (50 mM Tris-HCl, pH 9.0, 10 mM MgCl₂) in an Amicon® centrifugal filter unit (Millipore, Billerica, MA). SAP (10 units) was added to the pre-warmed protein solution and incubated at 37°C for 30 minutes. Immediately following the digestion, the protein solution was loaded onto a pre-equilibrated 1 mL lysine-Sepharose column as described previously [129].

Western Blot Analysis—Recombinant proteins (apo(a) and plasminogen) were immunoblotted using the monoclonal antibody E06 (Avanti Polar Lipids Inc., Alabaster, AL) as previously described [114] but with some modification. SDS-PAGE gels (7%) were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) and blocked with 0.2 M Tris-HCl, 1.5 M NaCl, 0.02% sodium azide, 1% bovine serum albumin, pH 7.4 for 1 hour at 23°C prior to 18 hour incubation at 4°C with E06 diluted in the same Tris-based buffer. All washing and the dilution of the secondary antibody were done using the same Tris-based buffer. Following imaging, membranes were stripped with 0.2 M glycine, 0.1% SDS, 2% (vol/vol) Tween-20, pH 2.2 for 10 minutes, washed with phosphate buffered saline (PBS; 0.14 M NaCl, 3 mM KCL, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) for 10 minutes, with 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.0005% Triton X-100, pH 7.4 for 10 minutes, reactivated with methanol and blocked with 6% skim milk powder prior to re-probing the membranes using an anti-apo(a) monoclonal antibody raised in mice [167] or an anti-plasminogen antibody (goat-anti-human plasminogen, Affinity Biologicals Inc, Ancaster, ON).

4.4 Results

Quantitative assays of tissue-type plasminogen activator (tPA)-mediated Glu¹-plasminogen activation were performed using a catalytically inactive, fluorescently labeled form of recombinant human plasminogen (r-plasminogen) in the presence or absence of various recombinant apolipoprotein(a) (r-apo(a)) size isoforms, on the surface of soluble fibrin degradation products (FDPs). Soluble FDPs were used in lieu of fibrin, as they were observed to be a more consistent cofactor and because it had been shown that fibrin and FDPs serve as equivalent cofactors in this context [67]. Also, apo(a) affects the structure of the fibrin clot

(Chapter 3), which would add an additional variable to the assay; whereas the FDPs were polymerized and solubilized in the absence of apo(a). A series of r-apo(a) isoform proteins ranging from 12 to 30 KIV domains but differing only in the number of KIV₂ domains, were used to determine the effect of apo(a) isoform size on the initial rate of tPA-mediated plasminogen activation. All isoforms of r-apo(a) were found to inhibit tPA-mediated plasminogen activation (Figure 4.1). There also appears to be a bimodal trend wherein both the highest and the lowest molecular weight isoforms of r-apo(a) are more effective inhibitors of tPA-mediated plasminogen activation than the mid-sized r-apo(a) isoforms (Figure 4.1). However, when the data are subdivided into individual preparations of r-apo(a), within every isoform category there is a range of inhibitory effects, such that an overriding trend becomes less apparent (Figure 4.2). As a point of note, no effort was made on the part of the investigators to generate variable preparations of r-apo(a). Rather this variability was derived within the conditions of seemingly consistent culturing and purification.

In order to account for the variability between r-apo(a) preparations, the previously established oxidized phosphatidylcholine (OxPtdC) modification of r-apo(a) [114] was investigated because oxidized phospholipid (OxPL)-protein modifications can induce functional changes [165]. A selection of r-apo(a) isoform pairings that showed the most extreme values of average initial rates of tPA-mediated plasminogen activation (Figure 4.2) were subjected to immunoblotting with E06, a natural monoclonal auto-antibody shown to specifically recognize the phosphorylcholine head group of OxPtdC [114]. As a loading control, the blots were stripped and re-probed with an anti-apo(a) antibody. The E06 reactivity of individual r-apo(a) preparations was found to be both isoform-dependent with low molecular weight apo(a) isoforms reacting more readily with the E06 monoclonal antibody than high molecular weight apo(a) isoforms

(Figure 4.3A). With respect to the r-apo(a) preparations of the same isoform size, there was a reasonable association between increased E06 reactivity (Figure 4.3A) and a decreased average initial rate of plasminogen activation (Figure 4.2).

Recombinant apo(a) preparations were then quantified in terms of their phosphorus content. We found that the different preparations of the same isoform of apo(a) had different phosphorus content (Figure 4.3C). Moreover, the preparations with higher phosphorus content (Figure 4.3C) were associated with lower initial rates of plasminogen activation (Figure 4.2). This investigation was then expanded and every r-apo(a) preparation for which there was sufficient protein was quantified with respect to their phosphorus content. These values were plotted against the average initial plasminogen activation rates in the presence of r-apo(a) (Figure 4.2 & 4.4) in order to discern a relationship. The correlation coefficient was calculated to be -0.66 describing the association between decreased initial plasminogen activation rates and increased phosphorus-content of r-apo(a) (Figure 4.4).

It was observed additionally that there existed variability among the r-plasminogen preparations in terms of both their initial rate of activation by tPA and the extent to which apo(a) was able to inhibit their activation (data not shown). A similar investigation was therefore undertaken because, as mentioned, plasminogen has also been shown to be modified by OxPtdC-adducts [111]. In Figure 4.5A, it was observed that the plasminogen preparations have different reactivities to the E06 antibody. These plasminogen preparations were then quantified with respect to their phosphorus content, which similarly showed different phosphorus content for different plasminogen preparations (Figure 4.5C). Importantly, plasminogen has been reported to be phosphorylated [168,169] and therefore the phosphorylation of plasminogen could confound the interpretation of the phosphorus quantification results with respect to OxPtdC. As such, a r-

plasminogen preparation (#11) was subjected to both phospholipase A₂ (PLA₂) digestion to remove any associated OxPL from the protein, and to phosphatase digestion (shrimp alkaline phosphatase, SAP) to remove any phosphorylation modifications and then re-assessed as to their E06 reactivity and phosphorus content. Both enzyme treated plasminogen preparations showed reduced E06 reactivity (Figure 4.5A) but virtually no phosphorus content was detected from either treated protein (Figure 4.5C).

The ability of apo(a) to inhibit the tPA-mediated activation of a given preparation of r-plasminogen was found to be unrelated to both the E06 reactivity and to the phosphorus content of r-plasminogen (data not shown). However, in plotting the phosphorus content of the r-plasminogen preparations against the average initial rates of plasminogen activation in the absence of r-apo(a) protein, a direct relationship was found such that increasing phosphorus content correlated with increased tPA-mediated activation (Figure 4.6; $r = 0.95$).

4.5 Discussion

The apolipoprotein(a) (apo(a)) size polymorphism has been identified to be an independent risk factor for cardiovascular disease [54-56]. The investigation of the role of apo(a) isoform size is frequently complicated by the inverse relationship between isoform size and plasma lipoprotein(a) (Lp(a)) concentrations [47]. In the current study, the confounding factor of levels was circumvented by the use of a series of purified recombinant apo(a) (r-apo(a)) isoform proteins that were used at the same concentration irrespective of isoform size, and thereby provided direct evidence of the effect of apo(a) isoform size, independent of levels. Despite the difficulties, a limited number of studies have been conducted to investigate the effect of isoform size in the context of the anti-fibrinolytic effects of Lp(a)/apo(a). It was initially determined that

the ability of Lp(a)/apo(a) to inhibit plasminogen activation hinged on its ability to bind fibrin, which in turn was dependent on the kringle 4-like (KIV) repeats of apo(a) [170]. Shortly thereafter, it was shown that smaller Lp(a) isoforms bind to fibrin more avidly than larger isoforms [12,78]. These results suggested that given the dependence of the inhibition of tPA-mediated plasminogen activation by Lp(a)/apo(a) on the ability of apo(a) to bind fibrin, low molecular weight isoforms would be expected to be more potent inhibitors of plasminogen activation relative to their high molecular weight counterparts. However, more recently, using an array of r-apo(a) proteins in parallel with Lp(a) particles isolated from plasma, it was found that although native Lp(a) particles displayed fibrin affinities that were inversely related to apo(a) KIV number, all r-apo(a) preparations displayed similar affinities for fibrin [139]. Similarly, in the current study using r-apo(a) proteins, isoform size did not materially affect the ability of apo(a) to inhibit plasminogen activation (Figure 4.1 & 4.2). It is therefore possible that size-dependent effects are a property of the Lp(a) particle rather than of apo(a).

The products that are formed from the oxidation of phospholipids (PL) have become increasingly recognized to be of physiological and pathophysiological importance, and have specifically been implicated in the pathogenesis of atherosclerosis and plaque instability [171]. One of biological effects induced by OxPLs is the modification of proteins, wherein a polyunsaturated fatty acid can be oxidized to an aldehyde and can then form a Schiff base with an ϵ -amino group of a lysine residue [110] to generate a covalent OxPL-protein adduct. Such is the case with both apo(a) and plasminogen wherein it was demonstrated that OxPtdC can form a Schiff base with 1-2 lysine residues in the kringle 5-like (KV) domain of apo(a) [114], and with 1 lysine residue in each of the kringle 1-4 and kringle 5-protease segments of plasminogen [111]. However, little is known as to the functional consequences of these modifications. In one study,

comparing apo(a) fragments containing the proposed OxPtdC-modification site in apo(a) to fragments without, it was demonstrated that OxPtdC-modified-apo(a) could stimulate interleukin-8 secretion in cultured human macrophages [114]; and in a second study it was found that Lp(a) and apo(a) could induce apoptosis in ER-stressed macrophages through a mechanism that was dependent on the OxPL associated with apo(a) [172]. Herein, we provide evidence that the OxPtdC-modification of r-apo(a) is associated with the ability of apo(a) to inhibit plasminogen activation and that the OxPtdC-modification of plasminogen is associated with its ability to be activated by tPA.

Oxidized Lp(a) (OxLp(a)) has been reported to have additional specific biological properties compared with native Lp(a) [96-99]. Of particular interest, it has been postulated that OxLp(a) might attenuate the fibrinolytic activity to a greater extent than native Lp(a) [173] based on the finding that OxLp(a) inhibits plasminogen binding to U937 monocyte/macrophage cell surfaces [174]—an important component of cell-surface plasminogen activation [175]—to a greater extent than native Lp(a). Since OxPtdC has been reported to be equally distributed among the lipid and protein fractions of OxLp(a) [106], it is possible that at least part of the observed effects of OxLp(a) can be attributable to OxPtdC-modifications on apo(a).

A number of the effects attributable to Lp(a)/apo(a) have also been attributed to OxPL. Specifically, it has been reported that OxPLs stimulate both the differentiation and proliferation of smooth muscle cells [176,177]. Lp(a)/apo(a) has also been reported to enhance proliferation of human vascular smooth muscle cells by inhibiting the generation of the endogenous inhibitor of smooth muscle cell migration, transforming growth factor- β [178-180]. The inhibition of transforming growth factor- β in turn has been attributed to the ability of apo(a) to inhibit plasmin

generation [181]. It is therefore possible that these two sets of seemingly separate mechanisms are linked to OxPtdC- modified apo(a), and possibly even to OxPtdC-modified plasminogen.

Finally, in attempting to address the question of the source of the OxPtdC that modifies apo(a) and plasminogen and the possible location of this modification, it is of note that both recombinant cell lines used in the current study were stably transfected into kidney cells: human embryonic (293) in the case of apo(a) and baby hamster (BHK-21) in the case of plasminogen. The OxPtdC-modification of apo(a) or plasminogen is therefore not specific to the liver as was previously postulated [111]. Although, it was reported that the OxPtdC located on apo(a) did not derive from the circulating lipoproteins [164], there are also reports that OxPLs penetrate into the cell and covalently modify intracellular proteins [182]. The question therefore remains as to whether apo(a) and/or plasminogen associate with OxPtdC intracellularly or extracellularly and from where the OxPLs derive. However our findings do suggest that the mechanism does not appear to be liver specific, as previously postulated [111].

In conclusion, we report that the extent of the inhibition of tPA-mediated plasminogen activation on the surface of fibrin degradation products by r-apo(a) is primarily determined by the phosphorus content of the protein and not dependent upon r-apo(a) isoform size. Additionally, the phosphorus content of r-plasminogen directly correlates with its ability to be activated by tPA but does not affect its ability to be inhibited by r-apo(a). These results suggest that protein modification of both plasminogen and apo(a) are functionally significant regulatory factors in the tPA-mediated plasminogen activation system.

4.6 Figures

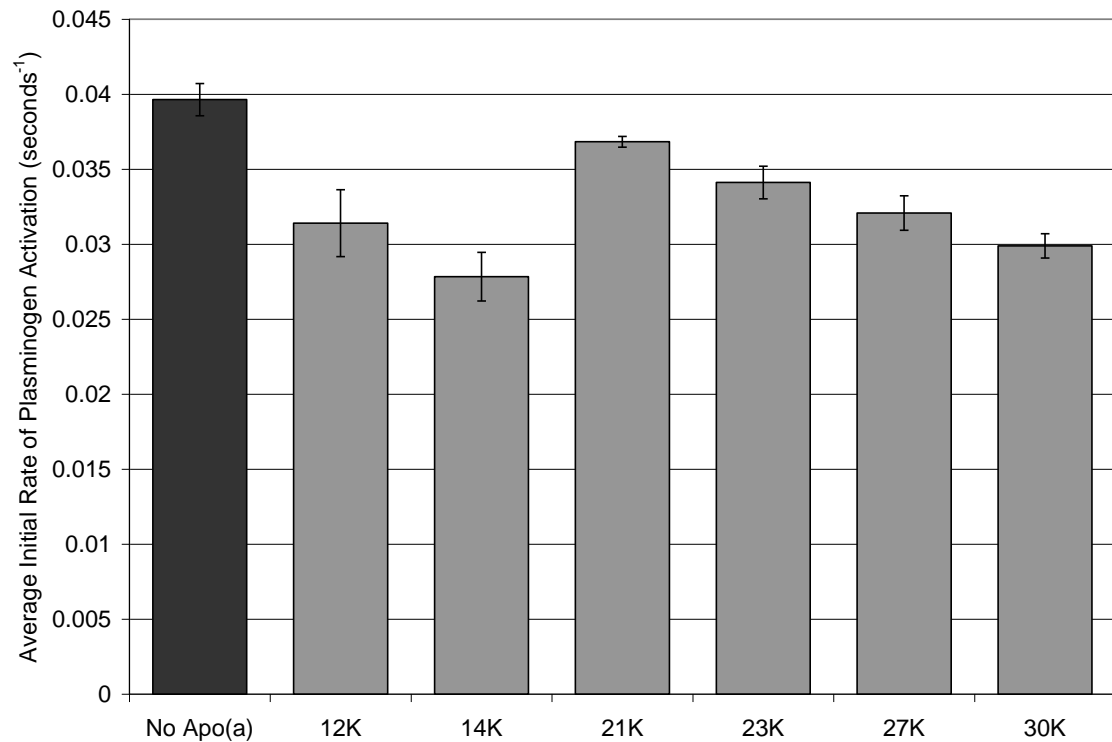


Figure 4.1 *tPA-mediated activation of fluorescently-labeled recombinant plasminogen on fibrin degradation products as a function of apo(a) isoform size.* The dark grey bar represents the mean \pm standard error of the mean (SEM) for the control condition (0 μ M r-apo(a)). The light grey bars represent the mean \pm SEM for plasminogen activation assays in the presence of 3 μ M r-apo(a) containing different numbers of KIV (K) domains.

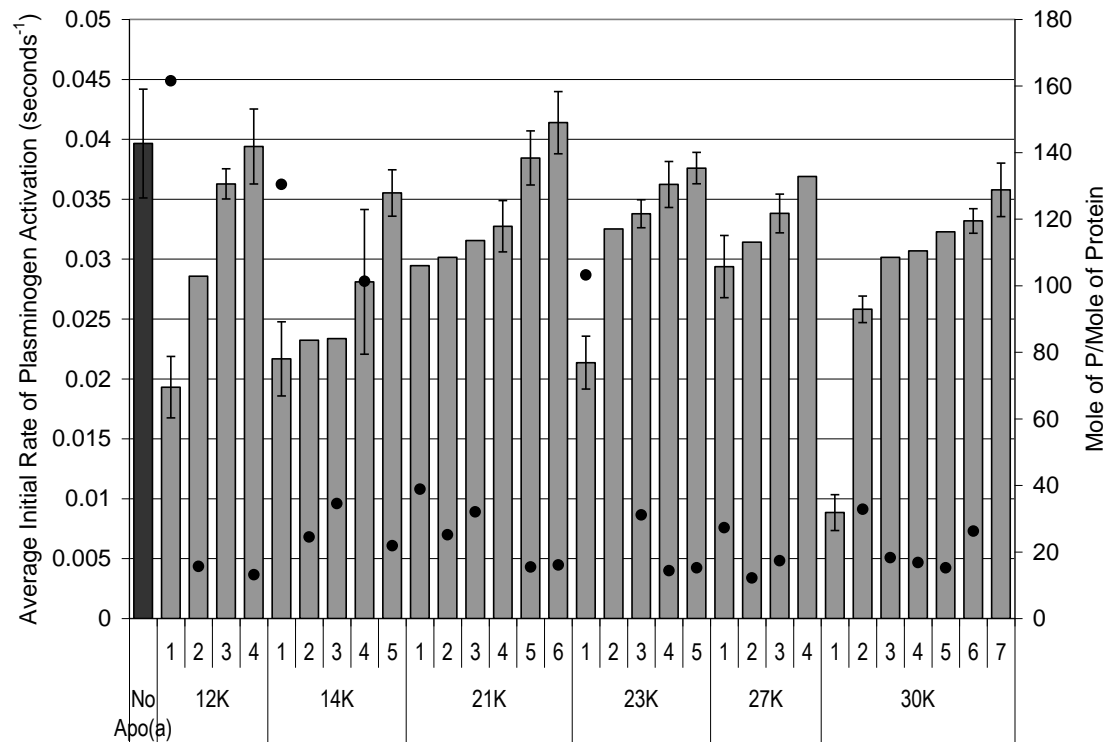


Figure 4.2 *tPA*-mediated activation of fluorescently labeled recombinant plasminogen on fibrin degradation products as a function of apo(a) isoform size subdivided by isoform preparation and plotted in association with protein phosphorus content. The dark grey bar represents the mean \pm standard error of the mean (SEM) for the control condition ($0\mu\text{M}$ r-apo(a)). The light grey bars represent the mean \pm SEM for plasminogen activation assays in the presence of $3\mu\text{M}$ r-apo(a) with differing numbers of KIV (K) domains. For each r-apo(a) isoform, individual preparations are labeled numerically and sorted, from lowest to highest, according to the average initial rate of plasminogen activation. The black dots represent the phosphorus content of individual preparations of r-apo(a) reported as moles of phosphorus per mole of r-apo(a) protein.

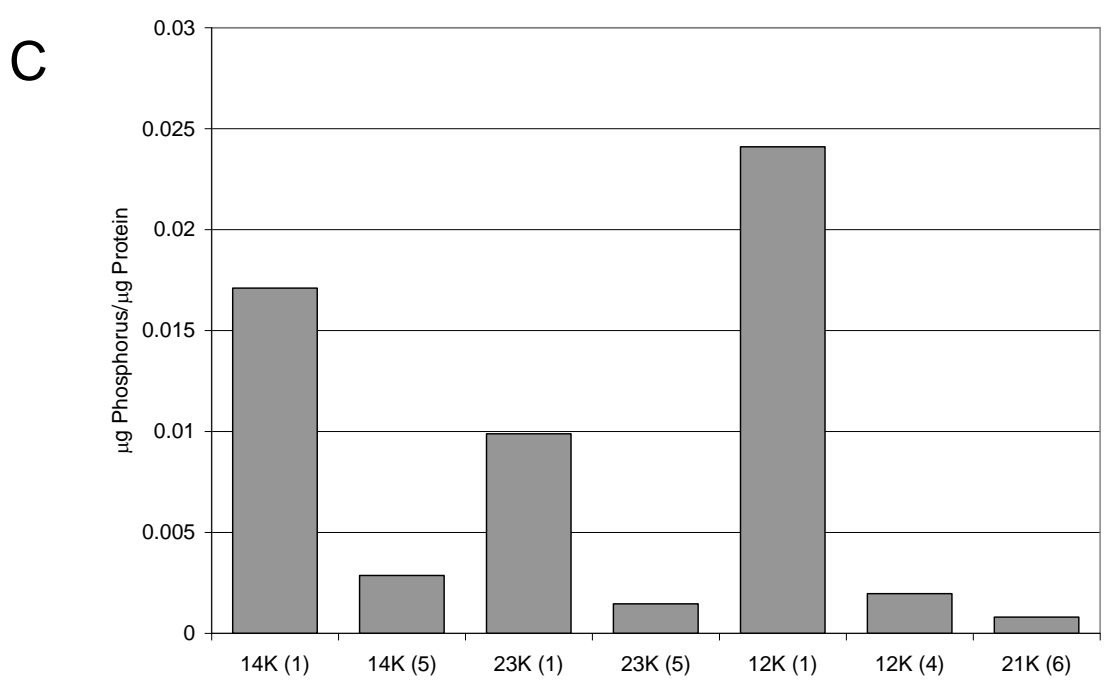
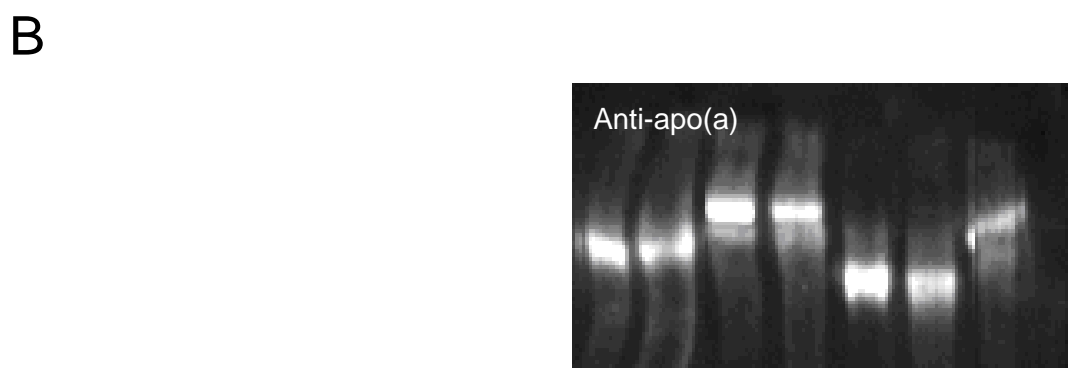
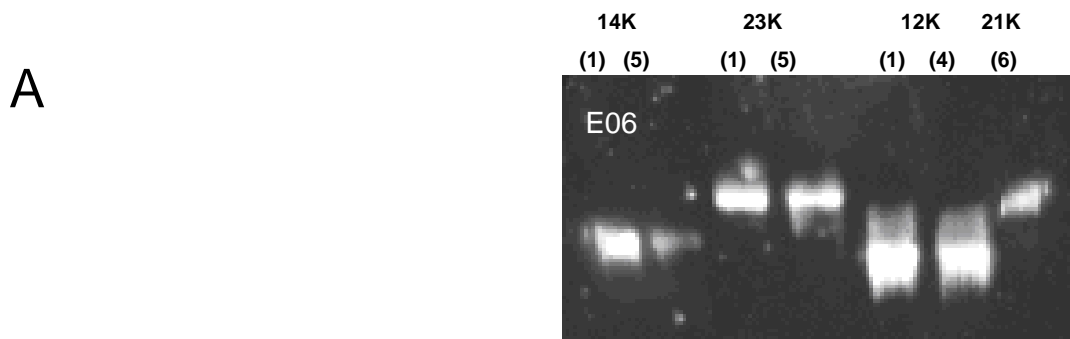


Figure 4.3 *Immunoblot analysis of E06 reactivity and phosphorus quantification for r-apo(a) proteins.* Recombinant apo(a) preparations for individual apo(a) isoforms displaying the most extreme effects on tissue-type plasminogen activation (Figure 4.2) were subjected to immunoblot analysis and phosphorus quantification. Labels coincide with those of Figure 4.2. Non-reduced samples (2 μ g of protein) were run on 7% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes incubated with the E06 monoclonal antibody to detect oxidized phosphatidylcholine products (*Panel A*). Following imaging, PVDF membranes were stripped re-probed with an anti-apo(a) antibody as a loading control (*Panel B*). The same r-apo(a) preparations were subjected to phosphorus quantification, which is reported as micrograms of phosphorus per microgram of r-apo(a) protein, reflecting the microgram-based loading of the immunoblot gel (*Panel C*). The data presented are representative of independent assays (n = 1-5).

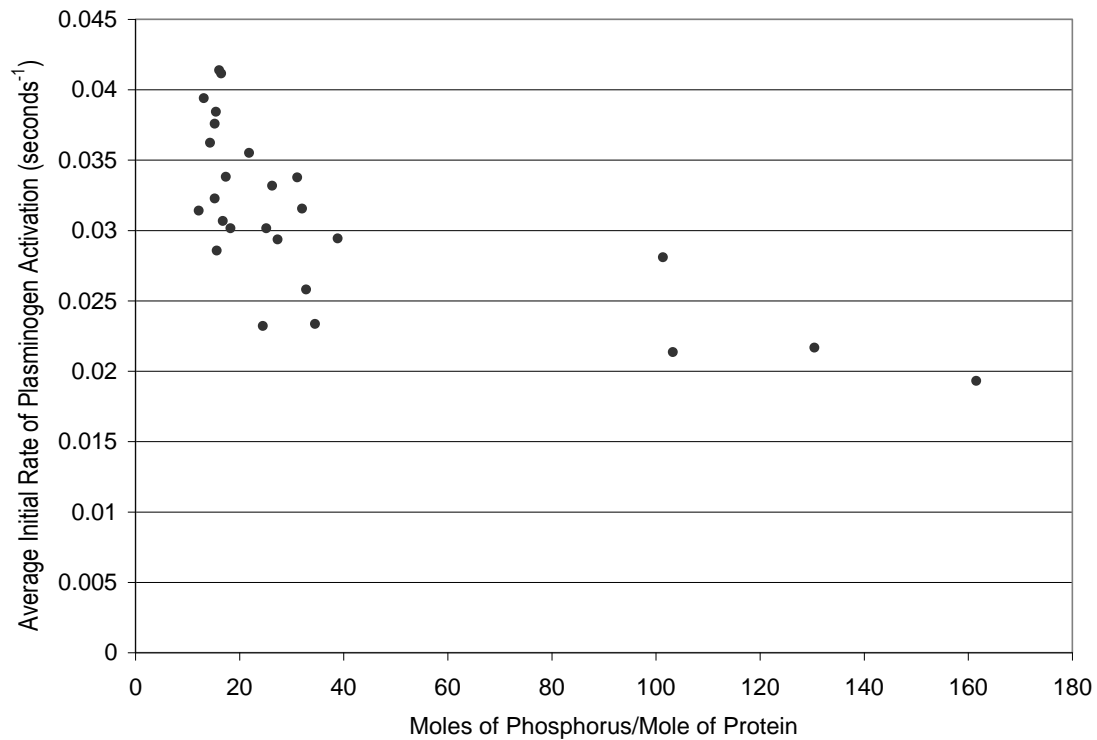


Figure 4.4 Average initial rate of tPA-mediated plasminogen activation on fibrin degradation products as a function of the moles of phosphate per mole of r-*apo(a)* protein. Each point represents the mean initial rate of plasminogen activation for individual preparations of r-*apo(a)* irrespective of isoform size.

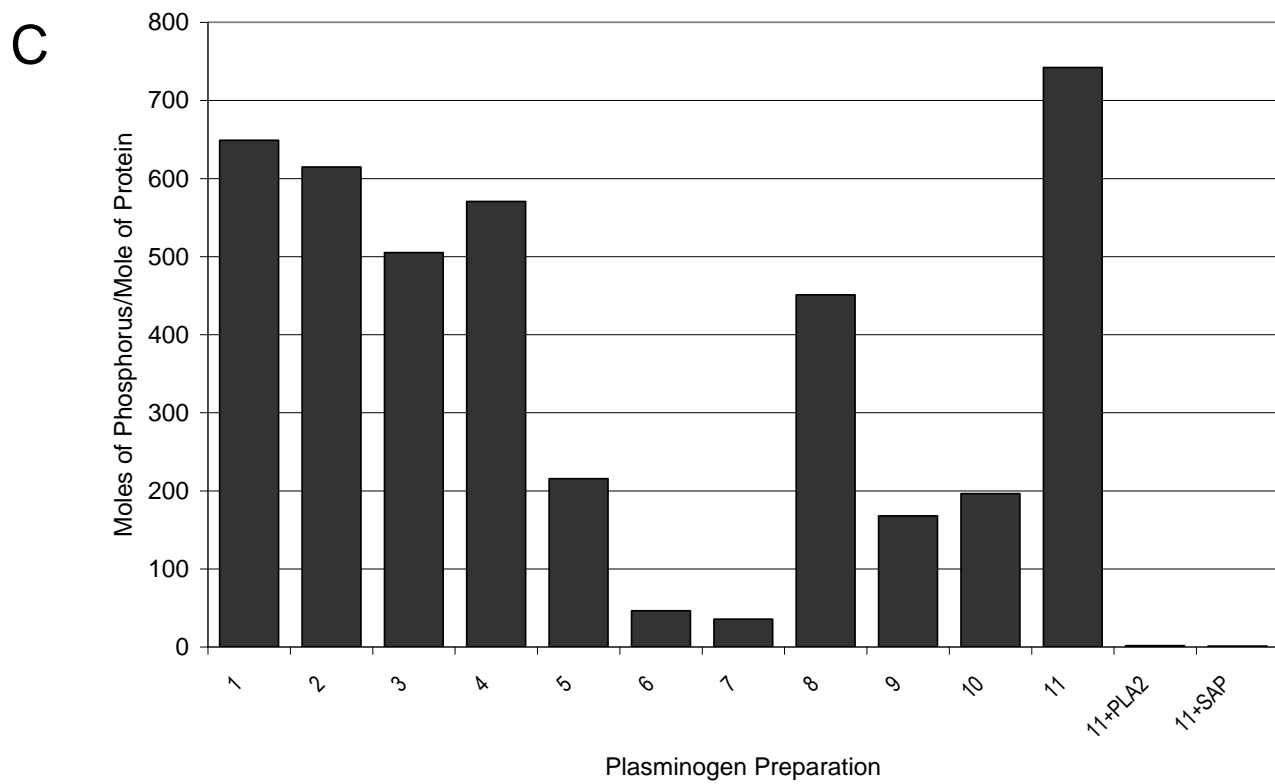
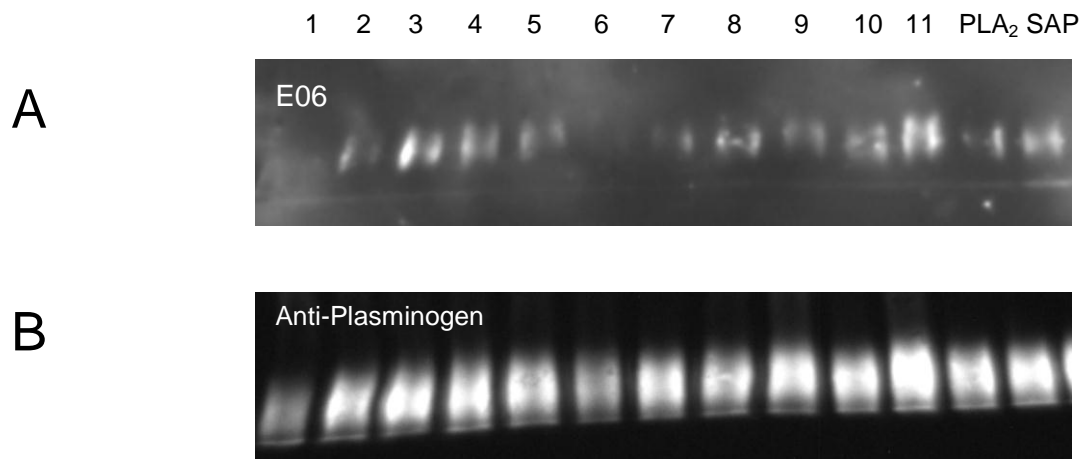


Figure 4.5 *Immunoblot analysis of E06 reactivity and phosphorus quantification for recombinant plasminogen (r-plasminogen) proteins.* An assortment of r-plasminogen preparations were subjected to immunoblot analysis and phosphorus quantification. Recombinant plasminogen preparation #11 was additionally digested with either phospholipase A₂ (PLA₂) or shrimp alkaline phosphatase (SAP) prior to being subjected to immunoblot and phosphorus analysis. Non-reduced samples were run on 7% SDS-PAGE gels with 0.2µg of protein and transferred onto polyvinylidene fluoride (PVDF) membranes incubated with the E06 monoclonal antibody to detect oxidized phosphatidylcholine products (*Panel A*). Following imaging, PVDF membranes were stripped re-probed with an anti-plasminogen antibody as a loading control (*Panel B*). The same r-plasminogen preparations were subjected to phosphorus quantification, which is reported as moles of phosphorus per mole of r-apo(a) protein (*Panel C*). The data presented are representative of ≥ 3 independent assays.

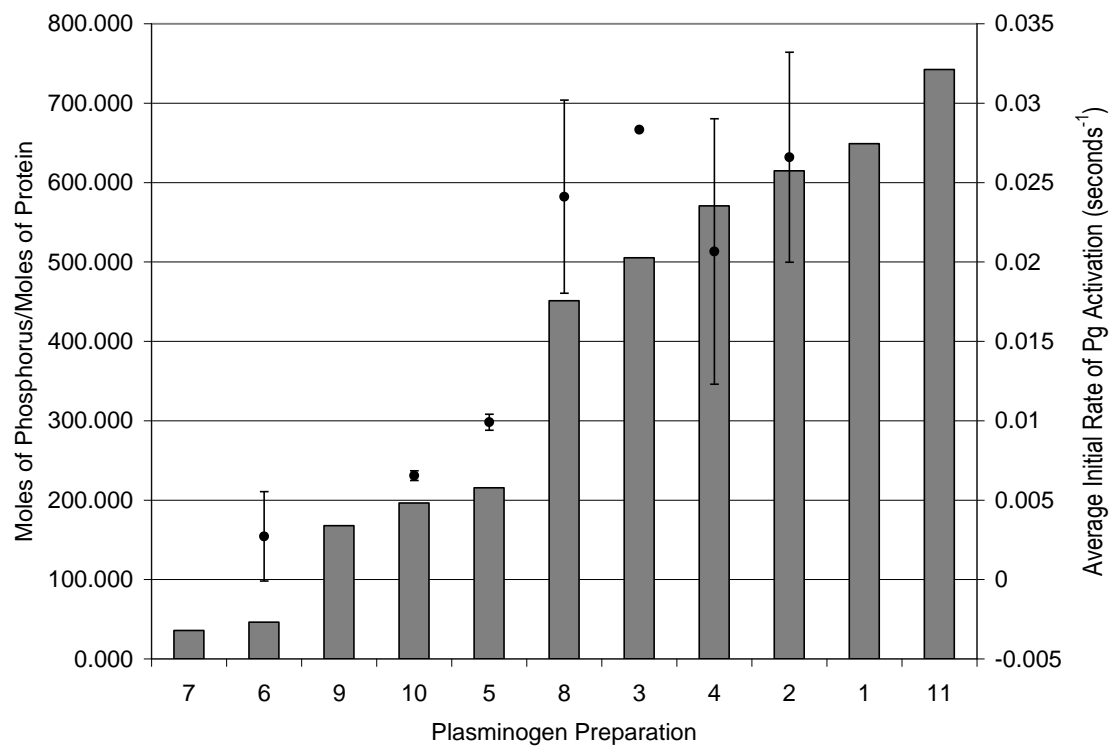


Figure 4.6 Average initial rate of tissue-type plasminogen activator-mediated plasminogen activation on fibrin degradation products as a function of the moles of oxidized phosphatidylcholine per mole of r-plasminogen protein. The dots represent the mean initial rate of plasminogen activation for individual preparations of r-plasminogen in the absence of apo(a) on fibrin degraded surfaces. The bars represent the moles of phosphorus per mole of protein. The data presented are representative of ≥ 3 independent assays.

Chapter 5

Discussion

5.1 General Findings

This thesis sought to build upon the understanding of the role of apolipoprotein(a) (apo(a)) in plasminogen activation on the fibrin/degraded fibrin surface with the objective of understanding the role of apo(a) in each of a number of individual reactions that affect the fibrinolytic process.

5.1.1 CHAPTER 2: Glu¹-Plasminogen to Lys⁷⁸-Plasminogen Conversion

It had been established that apo(a) inhibits the tissue-type plasminogen activator (tPA)-mediated activation Glu¹-plasminogen to Glu¹-plasmin by means of an equilibrium template model in which apo(a) interacts with each of tPA, plasminogen and fibrin to form a quaternary complex that is less effective at activating plasminogen [67]. It was also identified that the KV, the strong LBS in KIV₁₀ as well as the amino-terminus of apo(a) were required for maximum apo(a)-mediated inhibition of plasminogen activation [67].

In Chapter 2, we expanded upon this model by investigating whether apo(a) had an effect on the important plasmin-mediated positive feedback reaction of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion. Native Glu¹-plasminogen circulates in a closed conformation. Its amino-terminal tail domain is cleaved by plasmin and released, generating Lys⁷⁸-plasminogen, which has an open conformation and an increased associated rate of activation [76,81]. Apo(a) was found to inhibit this reaction 2-fold at a concentration of 3μM recombinant apo(a) (r-apo(a)). This is a novel finding in terms of an understanding of the mechanisms by which apo(a) can inhibit plasminogen activation, and how given the degree of the observed inhibition, apo(a) could

significantly contribute to the overall inhibition of fibrinolysis. Additionally, critical roles were identified for the KIV₈, KIV₉ and KV domains with contributory roles for KIV₁₀ and KIV_{5,7}, with no role for apo(a) isoform size. The apo(a) domains identified in this study relative to the domains identified in the Glu¹-plasminogen to Glu¹-plasmin tPA-mediated activation study by Hancock and colleagues [67] emphasizes that these two reaction schemes do not involve exactly the same protein-protein interactions. This is logical given that plasminogen conversion is mediated by the enzyme plasmin, whereas activation is mediated by tPA. Importantly, in both cases the KV domain was identified as critical for apo(a)-mediated inhibition and the KIV₁₀ domain was identified as important for maximal inhibition. These findings introduce the possibility that these domains could very well be critical to the ability of apo(a) to interact with plasminogen and/or fibrin, and therefore critical to the ability of apo(a) to inhibit plasminogen activation on the fibrin/degraded fibrin surface. This is because the apo(a)-plasminogen and apo(a)-fibrin interactions are common to both reaction schemes. Additional experiments would need to be conducted before such a conclusion could be drawn, however it may be the first step towards the identification of potential therapeutic targets for individuals at high risk of cardiovascular disease as a result of elevated Lp(a) levels.

5.1.2 CHAPTER 3: Clot Structure

Clot structure is recognized as a regulator of fibrinolysis [87] and Lp(a) is reported to be associated with abnormal clot structure [89]. These facts suggested the possibility that apo(a) could inhibit fibrinolysis by means of modifying fibrin clot structure. In Chapter 3, we present the first direct evidence that apo(a) does modify the structure of the fibrin clot in such a way as to produce a prothrombotic fibrin structure phenotype, characterized by dense clots with reduced permeability and thinner fibrin fibers. The observation of a 2-fold reduction in permeability in

and of itself suggests that apo(a) can regulate fibrinolysis by means of clot structure. This is because permeability determines the accessibility of plasmin to the fibrin fibers and therefore can determine the rate of clot dissolution [87,88]. Furthermore, we found that in the presence of r-apo(a), the rate of fibrin polymerization was increased, which has been shown to result in thinner fibers [88], also consistent with our results.

When we assessed the rate of fibrinolysis, initiated by exogenously added plasmin, we observed that it occurred at a faster rate in the presence of r-apo(a) than in its absence. This unexpected and seemingly contradictory observation may actually support our conclusion that apo(a) induces the formation of thin fibrin fibers. This is because as a consequence of the experimental design, wherein a larger volume of fibrinogen in the presence or absence of apo(a) was added to wells containing a smaller volume of plasmin and thrombin, the plasmin could have been distributed throughout the clot prior to the gelation point. As such, the effects of reduced permeability would have been irrelevant and plasmin could then cleave the thinner fibrin fibers formed in the presence of apo(a) at the accelerated rate that has been attributed to thin fiber dissolution [88]. This scenario would then appear as though lysis time were decreased by the presence of apo(a), which are the results we presented herein. Notably, this result is also consistent with previous reports that apo(a) does not affect the proteolytic activity of plasmin [64], and ultimately can provide an additional line of evidence that apo(a) induces the formation of thinner fibrin fibers.

Finally and most significantly, we observed that the extent of inhibition of tPA-mediated plasminogen activation by apo(a) is exacerbated by the apo(a)-induced modifications to the clot structure. Specifically, inhibition of plasminogen activation by apo(a) was increased by ~15% when the fibrin clot structure was modified by thrombin to a form in which it was a less efficient

cofactor for plasminogen activation. The effects of apo(a) on plasminogen activation and modified clot structure therefore appear to be additive.

Thus, in Chapter 3, we identified two additional mechanisms by which apo(a) can affect fibrin clot dissolution through modifying the fibrin clot structure: (i) by decreasing clot permeability thereby conferring a resistance to fibrinolysis and (ii) by enhancing the inhibition of tPA-mediated plasminogen activation on the fibrin surface.

5.1.3 CHAPTER 4: Isoform Size & Oxidized Phosphatidylcholine-Adducts in tPA-Mediated Plasminogen Activation

In Chapter 4, we returned to the study of the inhibition of tPA-mediated Glu¹-plasminogen to Glu¹-plasmin activation by apo(a), in an attempt to further understand the factors that can modify this inhibitory mechanism. We investigated a possible role for apo(a) isoform size in this process because it has been identified as an independent risk factor for cardiovascular disease [56,57] although the mechanistic basis for this increased risk remains undefined. Also, Lp(a) isoform size has been reported to affect fibrin binding, such that small isoforms bind more avidly to fibrin [12,61]. This was a notable finding because the inhibitory mechanism of plasminogen activation as described by Hancock and colleagues [67] outlines the interaction between apo(a) and fibrin as an important component of the overall mechanism.

We found that apo(a) isoform size is not a primary determinant of the ability of apo(a) to inhibit plasminogen activation. Specifically, small apo(a) isoforms were not observed to inhibit plasminogen activation to a greater extent than large apo(a) isoforms, as postulated from the aforementioned observed difference in fibrin binding. These findings therefore coincide with those of the inhibition of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion by apo(a), wherein similarly, apo(a) isoform size was not an important effector of the inhibitory mechanism [152].

However we did find that individual preparations of both r-apo(a) and r-plasminogen had variable behaviours in our fluorescent plasminogen activation assay. It has recently been reported that both proteins are modified by addition of oxidized phospholipid, oxidized phosphotidylcholine [111,164]. Furthermore, it has been reported that such oxidized phospholipid-protein adducts have functional consequences [165,183].

In Chapter 4, we present evidence that the extent of to which apo(a) reacts with an monoclonal-antibody against the oxidized phosphotidylcholine (OxPtdC)-modification correlates reasonably well with the ability of r-apo(a) to inhibit tPA-mediated plasminogen activation. However, this was used as only a qualitative assessment and we therefore quantified the phosphorus content of the r-apo(a) protein and determined the correlation between the phosphorus content of individual preparations with their initial rates of plasminogen activation. It was found that phosphorus content correlated ($r = -0.66$) with the extent to which tPA was able to activate plasminogen in the presence of individual preparations of r-apo(a). Alternatively stated, the phosphorus content of the apo(a) preparations was directly associated with their ability to inhibit plasminogen activation.

It was similarly determined that the phosphorus content of plasminogen was strongly correlated ($r = 0.95$) with its ability to be activated by tPA but not with its ability to be inhibited by apo(a). This correlation was again reasonably reflected in the qualitative immunoblot assessment of r-plasminogen using the anti-OxPtdC antibody. Plasminogen, however, is phosphorylated [168,169], whereas no such reports exist for apo(a). Our attempts at distinguishing between the effects of phosphorylation and phospholipid modification by means of enzyme digestion were inconclusive. This is because treatments of r-plasminogen with either phospholipase A₂, to remove the phospholipid or alkaline phosphatase, to remove phosphate

groups were quantified to have reduced phosphorus content while contradictorily, no difference was observed in the anti-OxPtdC antibody reactivity with either treatment. This therefore represents one aspect of this investigation that remains incomplete. Despite this, we have evidence of a number of novel findings. In particular, apo(a) isoform size was not found to have a significant effect on tPA-mediated plasminogen activation. However, we have reported the novel findings that the phosphorus content of apo(a) is associated with its ability to inhibit plasminogen activation and that the phosphorus content of plasminogen is associated with its ability to be activated by tPA. These findings suggest that protein modifications are materially important to the activation of plasminogen by tPA and to the inhibition of plasminogen activation by apo(a).

5.2 Pathophysiological Implications

The results herein present a picture in which Lp(a)/apo(a) can affect plasminogen activation on the fibrin/degraded fibrin surface with obvious implications for thrombolysis *in vivo*. However, these results also have broader implications, as outlined below.

5.2.1 Glu¹-Plasminogen to Lys⁷⁸-Plasminogen Conversion

The inhibition of plasminogen activation also occurs on the surface of vascular cells and platelets, where it may not only affect fibrinolysis but also key vessel wall events in the atherosclerotic process, including smooth muscle cell migration and proliferation. There is evidence to suggest that the mechanism of plasminogen activation attenuation by Lp(a)/apo(a) differs in the fibrin milieu and on the surface of vascular cells and platelets based on the observations that the domains/sequences in apo(a) responsible for inhibiting plasminogen activation may differ in the fibrin and pericellular settings [181]. Interestingly, it has been suggested that stimulation of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion on the endothelial and monocyte cell surfaces is the principal mechanism by which plasmin formation is

enhanced in this milieu [142,184]. Thus, inhibition of this conversion may constitute the *primary* mechanism by which Lp(a)/apo(a) inhibits plasminogen activation on the surface of at least some vascular cell types.

As such, our findings that apo(a) can inhibit Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion on fibrin suggest the possibility that on the pericellular surface apo(a) may also be able to inhibit plasminogen activation. Furthermore, as mentioned, the inhibition of plasminogen activation on the vascular cell surface has implications beyond fibrinolysis through to events involved in atherogenesis and atherosclerosis. Therefore, apo(a) could potentially affect the atherogenic/atherosclerotic process through a mechanism of inhibition of plasminogen activation on the cell surface, with a primary emphasis on Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion.

5.2.2 Fibrin Clot Structure

Clinical studies have identified Lp(a) as a risk factor for venous thromboembolism in adults [33-35] and children [36-38], and for spontaneous [39-41] and recurrent [39,42] ischemic stroke in children. Similarly, epidemiological studies have also identified an association between altered clot structure and thromboembolic diseases, including ischemic stroke and venous thromboembolism (reviewed in [85]). As demonstrated in Chapter 3, apo(a) modulates fibrin clot structure generating dense clots composed of thin fibrin fibers and reduced permeability. This clot structure has been designated as ‘prothrombotic’ because it is most frequently reported to be resistant to fibrinolysis and simultaneously, it is associated with thrombotic diseases [85,87].

In addition to its effects on fibrinolysis, which lead to the persistence of thrombi in the vasculature and thus thrombosis, it has also been reported that clots formed with thin fibrin fibers and reduced permeability have decreased elasticity and therefore increased rigidity [156,157],

which impairs the ability of the clot to deform elastically and thereby compromises its stability. Furthermore, these clots can be brittle and break off easily, resulting in a high incidence of embolic events [185].

These findings together suggest a possible mechanism by which Lp(a)/apo(a) can promote the incidence of both thrombotic and embolic events and thus can partly explain the association of elevated levels of Lp(a) with an increased risk of thromboembolic events.

5.2.3 Oxidative Modification of Lp(a) & Apo(a)

Oxidized Lp(a) (OxLp(a)) has been reported to be associated with additional specific biological effects compared with native Lp(a). OxLp(a) has been reported to increase Lp(a)-mediated plasminogen activator inhibitor-1 (PAI-1) production in vascular endothelial cells [96], which would down-regulate fibrinolysis by inhibiting the action of tPA. Also, OxLp(a) has been demonstrated to be taken up by macrophages through scavenger receptors [98], and thereby can contribute to atherogenesis through the formation of foam cells. The increased expression of the monocyte cell adhesion molecule Mac-1, has also been attributed to OxLp(a), which induces the adhesion of monocytic cells to endothelial cells [99] and thereby promotes the inflammatory response characteristic of atherosclerosis. These effects of OxLp(a) suggest possible pathogenic mechanisms by which Lp(a) can promote atherogenesis or the acceleration of atherosclerosis.

Conventionally, proatherogenic effects have been attributable to the low density lipoprotein-component of Lp(a). However oxidized phospholipids have been reported to be equally distributed among the lipid and protein fractions of OxLp(a) [106]. It is therefore possible that at least part of the observed effects of OxLp(a) can be attributable to OxPL-modifications on apo(a). This is supported by the findings presented herein, which provide evidence that the OxPL-modification on apo(a) is functionally significant. As such, both proatherogenic and

profibrinolytic mechanisms can be ascribed to apo(a) in contradiction to the conventional belief that the apo(a) component of Lp(a) is only responsible for profibrinolytic mechanisms.

This idea is further supported by the fact that a number of the effects attributable to Lp(a)/apo(a) have also been attributed to OxPL. Of particular interest is in the case of smooth muscle cells (SMCs), wherein it has been reported that OxPLs stimulate both their differentiation and proliferation [176,177]. Similarly, Lp(a)/apo(a) has been reported to enhance the proliferation [179] and migration [180] of human vascular SMCs. Interestingly, Lp(a)/apo(a) exerts its effect by inhibiting the generation of the endogenous inhibitor of SMC migration, transforming growth factor- β (TGF- β) [178-180], through a mechanism that involves the ability of apo(a) to inhibit plasmin generation [181]. Plasmin cleaves and activates latent TGF- β , and by inhibiting plasminogen activation and thereby decreasing the amount of plasmin that is formed, Lp(a)/apo(a) suppresses the activation of the inhibitor TGF- β and promotes human vascular SMC proliferation [186] and migration [187]. It is thus possible that these two sets of seemingly separate mechanisms are actually directly related to OxPtdC-modified apo(a), and possibly even OxPtdC-modified plasminogen, as central components.

Separately, it has been postulated that OxLp(a) might attenuate the fibrinolytic activity to a greater extent than native Lp(a) [173] based on the finding that OxLp(a) inhibits plasminogen binding to U937 monocyte/macrophage cell surfaces [174]—an important component of cell-surface plasminogen activation [175]—to a greater extent than native Lp(a). The present study contributes to this growing body of evidence that OxPL may play a role in the antifibrinolytic effects associated with Lp(a)/apo(a) with the finding that the association of r-apo(a) with OxPtdC correlates with the extent to which r-apo(a) is able to inhibit tPA-mediated plasminogen activation.

5.3 Future Directions

In order to move forward with the findings presented herein, first and foremost the source of the phosphorus content of both apo(a) and plasminogen must be identified as deriving from either phosphorylation and/or oxidized phospholipid modification. The results presented in Chapter 4 were inconclusive in this regard and therefore need to be further examined. The enzymatic digestion by both phospholipase A₂ and phosphatase requires optimization with subsequent definitive assessment of the remaining protein-modifications associated with the digested proteins. Furthermore, tPA-mediated plasminogen activation experiments should be repeated using the enzymatically-digested proteins in order to definitively link the protein modification with the observed increase in inhibition of plasminogen activation by high phosphorus-containing apo(a) proteins and the observed increase in activation by tPA of the high-phosphorus containing plasminogen proteins.

Building on the novel discoveries we have presented, the effect of apo(a) on Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion should be investigated on the cell surface, because of its fundamental role in plasminogen activation in this milieu [184]. Interference with this reaction on the cell surface would provide apo(a) with an additional mechanism by which to not only affect fibrinolysis but also possibly atherogenesis. Furthermore, the OxPL-modification on both apo(a) and plasminogen should be investigated in the context of Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion. This should be examined not only on the cell-surface but perhaps more importantly on the smooth muscle cell surface. This is because (i) smooth muscle cell migration and proliferation involves cell-surface associated plasminogen activation, (ii) cell-associated plasminogen activation has been demonstrated to be reliant on Glu¹-plasminogen to Lys⁷⁸-

plasminogen conversion for efficient plasminogen activation [142,184], and (iii) OxPL have been demonstrated to stimulate smooth muscle cell proliferation [176,177].

With respect to fibrin clot structure, [184] identifying specific domains in apo(a) responsible for the modification of fibrin structure would be useful (i) in understanding the mechanistic basis of the effect, (ii) for the integration of this apo(a)-dependent effect with those of the inhibition of both Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion and tPA-mediated plasminogen activation, and (iii) for potentially designing therapeutic targets to attenuate the effect of apo(a) on fibrinolysis, as mentioned previously. Additionally, despite our efforts, a mechanistic basis for the increased risk associated with small apo(a) isoforms remains unknown. Hence, apo(a) isoform size should also be investigated in the context of the modification of fibrin clot structure. This is once again warranted because fibrin binding is a critical component of the mechanism by which apo(a) can modify fibrin structure. This is particularly evident given that the effect of apo(a) on clot structure can be observed using purified solutions of apo(a) and fibrin.

As described in Chapter 3, the α C-domain of fibrin is the identified high-affinity binding site for apo(a) [159]. The effect of apo(a) on fibrin clot structure should therefore be examined in the context of this domain. However, fibrin structures lacking the α C-domain are reported to be impaired with respect to lateral aggregation, and also form clots with thin fibrin fibers and a dense clot structure [188]. It is possible that if apo(a) did not mediate its effects on fibrin structure through the α C-domain, clots generated in the presence of both apo(a) with fibrinogen lacking the α C-domain would produce an additive effect, generating even denser clots with thinner fibers than evident in fibrin structures generated by either factor alone. There is likely a limit as to how thin fibers can become and how densely they can pack and therefore, this method may be limited in its ability to definitively determine the effect of apo(a) on fibrin clot structure in the absence of

the α C-domain. Alternatively, the clots generated using fibrinogen lacking the α C-domain could be compared with clots generated in the presence of apo(a) and the two conditions compared in order to assess their similarity and thereby draw conclusions as to whether apo(a) may interfere with the appropriate functioning of the α C-domain.

Once again, the OxPL modification on apo(a) should be investigated in the context of fibrin clot structure modification, in order to facilitate the integration of this effect with those seen in the inhibition of tPA-mediated plasminogen activation and Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion. If the OxPL-apo(a) modification affects all three of these mechanisms, conclusions can then potentially be drawn as to function of the OxPL-apo(a) modification with respect to its interaction with fibrin, as this is the one binary interaction that is common to all three mechanisms.

By using our understanding of the role of apo(a) in a specific reaction of the plasminogen activation process (e.g. Glu¹-plasminogen to Glu¹-plasmin activation) to modify a second reaction (e.g. Glu¹-plasminogen to Lys⁷⁸-plasminogen conversion), a better understanding of how the individual reactions interrelate and influence each other can be obtained to provide a better picture of the pathophysiological situation *in vivo*, wherein these reactions are interdependent. In doing so, the integrated model of the effects of Lp(a)/apo(a) on the plasminogen activation system on the surface of fibrin/degraded fibrin will be a nearer to becoming a reality. The data presented in this thesis advance our understanding of the inhibition of plasminogen activation on the fibrin/degraded fibrin surface and bring us a few steps closer to understanding the mechanisms and pathophysiological consequences of the interaction of apo(a) with the plasminogen activation system.

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