Characterization of Prekallikrein Antibodies and Proteomic Identification of FXIa Substrates on Endothelial Cells

By

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A Thesis

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Abbreviations

APC – Activated Protein C

ARDS – Acute Respiratory Distress Syndrome

BBB – Blood–Brain Barrier

BK – Bradykinin

BMPR2 – Bone Morphogenetic Protein Receptor Type 2

cAMP / cGMP – Cyclic Adenosine / Guanosine Monophosphate

CAS – Contact Activation System

CTI – Corn Trypsin Inhibitor

DIC – Disseminated Intravascular Coagulation

DTT – Dithiothreitol

ECM – Extracellular Matrix

ECL – Enhanced Chemiluminescence

EGM-2 – Endothelial Growth Medium-2

FXI / FXIa – Coagulation Factor XI / Activated Factor XI

FXII / FXIIa – Coagulation Factor XII / Activated Factor XII

HAECs – Human Aortic Endothelial Cells

HK – High-Molecular-Weight Kininogen

HRP – Horseradish Peroxidase

HSP90 – Heat Shock Protein 90

HSPG2 – Heparan Sulfate Proteoglycan 2

HUVECs – Human Umbilical Vein Endothelial Cells

IgG – Immunoglobulin G

IL-1 / TNF-α – Interleukin-1 / Tumor Necrosis Factor Alpha

KKS – Kallikrein–Kinin System

Kal – Plasma Kallikrein

KLKB1 – Gene encoding Prekallikrein

LFQ – Label-Free Quantification

LTBR – Lymphotoxin Beta Receptor

MLC – Myosin Light Chain

MMP2 – Matrix Metalloproteinase 2

NEC / NECTIN1 – Nectin Cell Adhesion Molecule 1

NO – Nitric Oxide

NRP2 – Neuropilin 2

PAI-1 – Plasminogen Activator Inhibitor-1

PAR-1 – Protease-Activated Receptor-1

PBS – Phosphate Buffered Saline

PK – Prekallikrein

PLCG1 – Phospholipase C Gamma 1

PON2 – Paraoxonase 2

PPACK – D-phenylalanyl-prolyl-arginyl Chloromethyl Ketone

PRCP – Prolylcarboxypeptidase

PVDF – Polyvinylidene Fluoride

SEM – Standard Error of the Mean

SFM – Serum-Free Medium

SDS-PAGE – Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

STRAP – Serine/Threonine Kinase Receptor Associated Protein

TGF-β – Transforming Growth Factor Beta

TF – Tissue Factor

TFPI – Tissue Factor Pathway Inhibitor

TIE1 – Tyrosine Kinase with Immunoglobulin-Like and EGF-Like Domains 1

TJP1/2 – Tight Junction Proteins 1 and 2

TRAP-6 – Thrombin Receptor Activating Peptide-6

uPA / t-PA – Urokinase / Tissue-Type Plasminogen Activator

VLDLR – Very Low-Density Lipoprotein Receptor

VE-cadherin (CDH5) – Vascular Endothelial Cadherin

VEGFR2 – Vascular Endothelial Growth Factor Receptor 2

VIM – Vimentin

vWF – von Willebrand Factor

ZO-1 – Zonula Occludens-1

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Chapter 1

Introduction

The vascular endothelium is not merely a passive structural lining but a complex, highly active interface between circulating blood and the vessel wall. It plays essential roles in maintaining vascular integrity, regulating hemostasis, controlling permeability, modulating inflammatory responses, and orchestrating vessel remodeling. Of the many functions of endothelial cells, their role in preserving barrier function and modulating coagulation is central to both physiology and disease. Disruption of these roles underlies a wide range of pathological conditions, from inflammation to thrombosis and vascular leakage syndromes.

Endothelial Barrier Function

The endothelial barrier serves as a dynamic gatekeeper, regulating the bidirectional exchange of gases, solutes, and immune cells between the bloodstream and surrounding tissues[1,2]. This barrier function is structurally maintained by intercellular junctions—tight junctions, adherens junctions, and gap junctions—that form a cohesive and responsive monolayer across the vascular surface[3,4].

Tight junctions, composed of occludin, claudins, and associated scaffolding proteins like ZO-1, form a selective seal that limits paracellular diffusion[5]. Adherens junctions, primarily mediated by vascular endothelial (VE)-cadherin and cytoplasmic partners such as β-catenin, provide mechanical anchoring and are intimately linked to the actin cytoskeleton[6]. These structures are not static; they adapt to environmental stimuli such as cytokines, shear stress, and hypoxia.

During inflammation or exposure to mediators like thrombin or VEGF, intracellular signaling cascades (e.g., RhoA/ROCK pathway activation, calcium influx, MLC phosphorylation) lead to cytoskeletal contraction, destabilization of junctions, and increased endothelial permeability[7-9]. The resultant barrier breakdown facilitates leukocyte extravasation and plasma leakage—a protective process in acute

inflammation but one that contributes to pathology in chronic or dysregulated states such as acute respiratory distress syndrome (ARDS), sepsis, or tumor metastasis[10,11].

Importantly, barrier integrity is not uniform across the vascular bed. For example, the endothelium in the brain forms the highly selective blood–brain barrier (BBB), whereas the endothelium in liver sinusoids is fenestrated and more permissive[12]. This regional heterogeneity highlights the importance of context-specific regulation and has implications for targeted drug delivery and disease-specific therapies.

Endothelial Regulation of Coagulation

In its resting state, the endothelium maintains an antithrombotic surface, thereby ensuring blood fluidity and preventing intravascular coagulation[13]. This is achieved through several redundant and synergistic mechanisms:

Release of antiplatelet factors: Endothelial cells constitutively secrete prostacyclin (PGI_2) and nitric oxide (NO), which inhibit platelet adhesion and aggregation through elevation of intracellular cAMP and cGMP levels, respectively[14].

Expression of anticoagulant proteins: Thrombomodulin, present on the endothelial surface, binds thrombin and converts it from a procoagulant enzyme into an activator of protein C. Activated protein C (APC), together with its cofactor protein S (also produced by endothelium), inactivates coagulation factors Va and VIIIa[15]. Additionally, heparan sulfate proteoglycans on the endothelial surface accelerate the inactivation of thrombin via antithrombin III binding[13].

Fibrinolytic activity: Endothelial cells regulate clot breakdown by producing tissue-type plasminogen activator (t-PA) and urokinase (uPA), which catalyze the conversion of plasminogen into plasmin, the central fibrinolytic enzyme[16]. This process is modulated by the secretion of plasminogen activator inhibitor-1 (PAI-1), whose levels rise during inflammation and tilt the balance toward a prothrombotic state[17].

Upon vascular injury or activation by pro-inflammatory cytokines (e.g., IL-1, TNF-α), the endothelium undergoes a phenotypic switch to a procoagulant state: Expression of tissue factor (TF) on the endothelial surface initiates the extrinsic coagulation cascade[18].

Release of von Willebrand factor (vWF) from Weibel–Palade bodies facilitates platelet adhesion to exposed subendothelial collagen[19]. This functional shift is essential for effective hemostasis but can become pathological when dysregulated, leading to thrombosis, disseminated intravascular coagulation (DIC), or microvascular occlusion in diseases like atherosclerosis, cancer, and COVID-19-associated coagulopathy.

Recent studies have further emphasized the role of the protease-activated receptor-1 (PAR-1) on endothelial cells, which mediates many of thrombin's cellular effects, including barrier disruption, inflammatory gene expression, and promotion of a procoagulant phenotype[20]. Understanding the precise signaling mechanisms downstream of PAR-1 activation has become a major research focus for developing anti-inflammatory and antithrombotic therapies.

Coagulation Cascade

The coagulation system is an essential component of hemostasis, responsible for forming a stable fibrin clot to prevent blood loss following vascular injury. It operates through a highly coordinated sequence of enzymatic activations known as the coagulation cascade, which is traditionally divided into intrinsic, extrinsic, and common pathways (Figure 1).

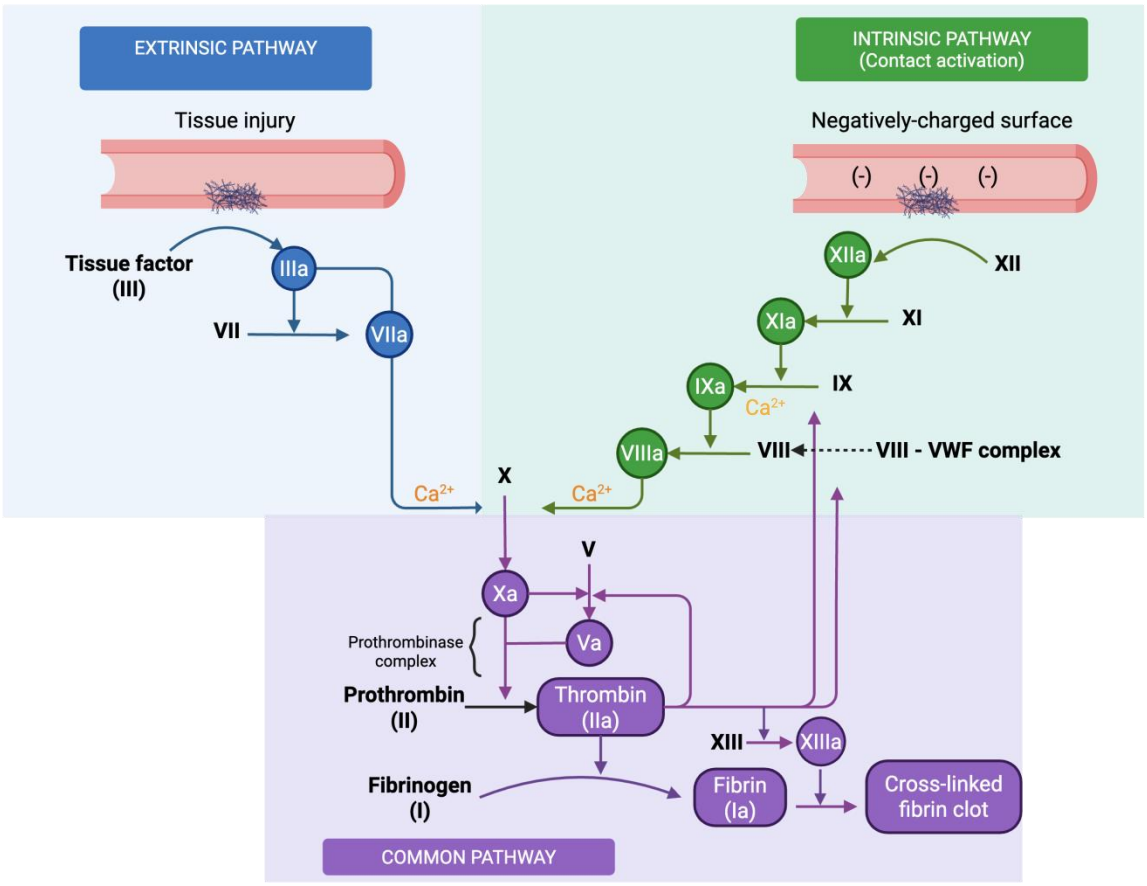

Figure 1. Overview of the coagulation cascade: intrinsic, extrinsic, and common pathways.

The extrinsic pathway is considered the principal physiological initiator of coagulation. It is triggered when tissue factor, a membrane-bound glycoprotein located in subendothelial tissues, is exposed to circulating blood. TF binds to factor VII, forming the TF–FVIIa complex, which catalyzes the activation of factor X to factor Xa and, to a lesser extent, factor IX to IXa. This event marks the start of thrombin generation and is tightly regulated by tissue factor pathway inhibitor (TFPI), which controls the activity of both FXa and the TF–FVIIa complex. Aberrant expression or regulation of TF is a key contributor to pathological thrombosis, particularly in cancer and inflammatory states[21].

In parallel, the intrinsic pathway, also referred to as the contact activation pathway, is activated by exposure to negatively charged surfaces and contributes to thrombin amplification. Factor XII is converted to XIIa, which activates prekallikrein (PK) to kallikrein and cleaves high molecular weight kininogen (HK) to generate bradykinin (BK),

a vasoactive peptide. Factor XIIa also activates factor XI to XIa, which then activates factor IX. Though its role in hemostasis is limited—as evidenced by the absence of bleeding in FXII-deficient individuals—the contact system is increasingly recognized for its involvement in inflammation and pathological thrombosis[22].

Beyond its historical association with coagulation, the contact activation system (CAS) closely interacts with the kallikrein–kinin system (KKS), a distinct yet overlapping proteolytic cascade primarily involved in inflammatory responses, regulation of vascular permeability, and vasodilation. Activation of FXII on biologic or artificial surfaces generates FXIIa, which subsequently converts PK to plasma kallikrein. Kallikrein, in turn, reciprocally activates FXII and cleaves HK, liberating BK. BK exerts its effects via bradykinin B2 and B1 receptors, promoting vasodilation, increased vascular permeability, and inflammatory signaling. While neither the CAS nor KKS is required for physiological hemostasis, both systems are implicated in pathological states such as hereditary angioedema, sepsis, and diseases involving aberrant inflammation. Their interconnection underscores the broader roles of these systems in linking coagulation, inflammation, and innate immune responses[22].

Both pathways converge at the common pathway with the generation of factor Xa, which, in complex with factor Va, calcium, and phospholipids, forms the prothrombinase complex. This complex catalyzes the conversion of prothrombin (factor II) to thrombin (factor IIa), a central enzyme in coagulation [23]. Thrombin cleaves soluble fibrinogen to insoluble fibrin monomers, which polymerize into fibrin strands. These strands are then cross-linked by activated factor XIII (XIIIa) to form a stable fibrin clot. Thrombin also activates multiple upstream factors, including factors V, VIII, and XI, creating a positive feedback loop that amplifies its own generation[24].

Thesis Overview

This thesis is organized into two main research projects that collectively advance the understanding of how components of the CAS and the interact with the vascular endothelium. Each project builds upon the mechanistic insights introduced in Chapter 1 and addresses distinct but complementary questions related to endothelial regulation of coagulation and inflammation.

Chapter 2 investigates the molecular mechanism of PK activation on endothelial cells. Using a combination of endothelial cell–based assays, monoclonal antibody mapping, and domain-swapping approaches, this study identifies the critical roles of the A1–A3 apple domains in facilitating PK activation. These findings refine our mechanistic understanding of the PK–HK–PRCP axis and highlight the potential of domain-specific PK antibodies as novel mechanistic probes and therapeutic candidates.

Chapter 3 explores the impact of activated coagulation FXIa on the endothelial proteome and secretome. Using cell surface biotinylation and label-free quantitative proteomics in human aortic endothelial cells, the study reveals that FXIa profoundly reprograms the endothelial landscape. FXIa downregulates molecules critical for barrier integrity, angiogenesis, and oxidative defense, while upregulating proteins involved in immune activation, extracellular matrix remodeling, and TGF-β signaling. These results demonstrate that FXIa functions not only as a coagulation protease but also as a potent modulator of endothelial dysfunction and vascular inflammation.

Together, these studies provide new insights into how PK and FXIa regulate endothelial biology through distinct mechanisms. By integrating mechanistic and proteomic approaches, this thesis establishes a framework for understanding the broader roles of CAS and KKS at the vascular interface and identifies potential therapeutic targets for thromboinflammatory diseases.

Chapter 2

Characterization of the Molecular Mechanism of Prekallikrein Activation on Endothelial Cells

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2.1 Abstract

Plasma PK is a multidomain glycoprotein comprising four apple domains (A1–A4) and one catalytic domain, playing a critical role in the activation of coagulation, fibrinolysis, kinin generation, and inflammation. On endothelial cells, PK can be activated either by factor XIIa or by prolylcarboxypeptidase (PRCP), but the structural requirements for this activation remain unclear. This study aimed to elucidate the specific apple domains of PK involved in its activation on the endothelial surface. Human umbilical vein endothelial cells (HUVECs) were incubated with PK, and its activation was assessed using chromogenic substrate hydrolysis. Monoclonal antibodies targeting each of the four apple domains were tested for their ability to inhibit PK activation, and the antibody binding sites were mapped using domain-swapped PK/FXI chimeras. PK activation occurred only in the presence of HUVECs and was inhibited by chloroquine, implicating PRCP. In the absence of HK, antibodies against all four apple domains significantly inhibited PK activation. With HK present, only antibodies against A1, A2, and A3 retained inhibitory activity, suggesting HK shields or alters A4's role. Notably, none of the antibodies affected plasma kallikrein activity. These findings reveal that the A1–A3 domains are critical for endothelial PK activation and that the identified monoclonal antibodies act independently of HK, likely via an allosteric mechanism. These antibodies represent valuable tools for dissecting PK function and hold therapeutic potential in vascular and inflammatory disorders.

2.2 Background

The kallikrein-kinin system is a complex proteolytic cascade involved in the regulation of vascular permeability, blood pressure, inflammation, and innate immunity. A key zymogen in this system is PK, a ~88 kDa glycoprotein encoded by the KLKB1 gene on chromosome 4q34–35, which circulates in plasma primarily bound to HK. Upon activation, PK is cleaved into a two-chain serine protease, kallikrein, composed of a ~50 kDa heavy chain and a ~28–31 kDa light chain. Kallikrein cleaves HK to release BK, a potent vasodilator and inflammatory mediator. PK also participates in the intrinsic pathway of coagulation, amplifying factor XII activation and linking coagulation with inflammation and fibrinolysis (Figure 2.1). Interestingly, while kallikrein accelerates FXII activation, it is not strictly required for its autoactivation, as shown in PK-deficient plasma, which self-corrects over time in in vitro assays[25].

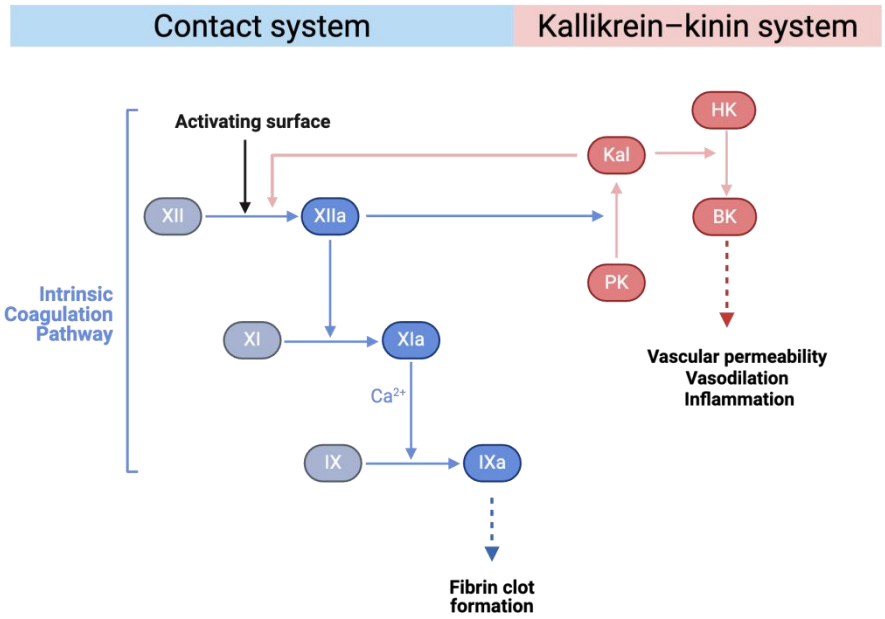


Figure 2.1 Role of PK at the interface of the contact system and the kallikrein–kinin system.
PK serves as a central zymogen linking coagulation and inflammation. Upon activation by FXIIa, PK is converted into plasma kallikrein (Kal), which in turn cleaves HK to release BK, a potent vasoactive and pro-inflammatory mediator. This activation loop is amplified through reciprocal activation between Kal and FXIIa. Within the intrinsic coagulation pathway, FXIIa further activates FXI to FXIa, propagating the coagulation cascade through subsequent activation of FIX to FIXa.

Structurally, PK comprises four apple domains (A1–A4) at the N-terminus and a C-terminal catalytic domain. These apple domains are believed to mediate protein-protein interactions, including those required for assembly on cell surfaces and binding to cofactors such as HK. HK binds PK discontinuously, primarily interacting with Apple domain 2, but also with domains 1 and 4[26]. Notably, the structural organization of PK is highly homologous to FXI, which shares the apple domain architecture. However, unlike FXI, which circulates as a disulfide-linked dimer, PK lacks the cysteine in Apple domain 4 necessary for dimerization and thus functions as a monomer within the KKS[27].

PK activation can occur via two major pathways (Figure 2.2): (1) by activated FXIIa on negatively charged surfaces, such as polyphosphates or extracellular nucleic acids, and (2) independently of FXIIa, through cleavage by PRCP, a membrane-associated serine protease expressed on endothelial cells. PRCP, traditionally classified as an exopeptidase of the S28 family, uniquely functions as an endoproteinase in this context, with multiple regions of PRCP and PK mediating high-affinity binding (Km ~ 5 nM)[28.] This pathway appears to be the dominant mode of PK activation in the vascular compartment and contributes significantly to basal BK production and endothelial homeostasis. Additional activators such as heat shock protein 90 (HSP90) have also been implicated in promoting PK autoactivation on endothelial surfaces[29].

Dysregulation of PK activation has been linked to a variety of vascular pathologies, including hereditary and acquired angioedema, hypertension, diabetic nephropathy with proteinuria, and retinal vascular leakage[30,31]. Interestingly, Klkb1$^{-/-}$ mice display delayed thrombosis not due to reduced contact activation per se, but because of reduced vessel-wall tissue factor expression, suggesting non-canonical roles for PK in vascular biology beyond the contact system[32].

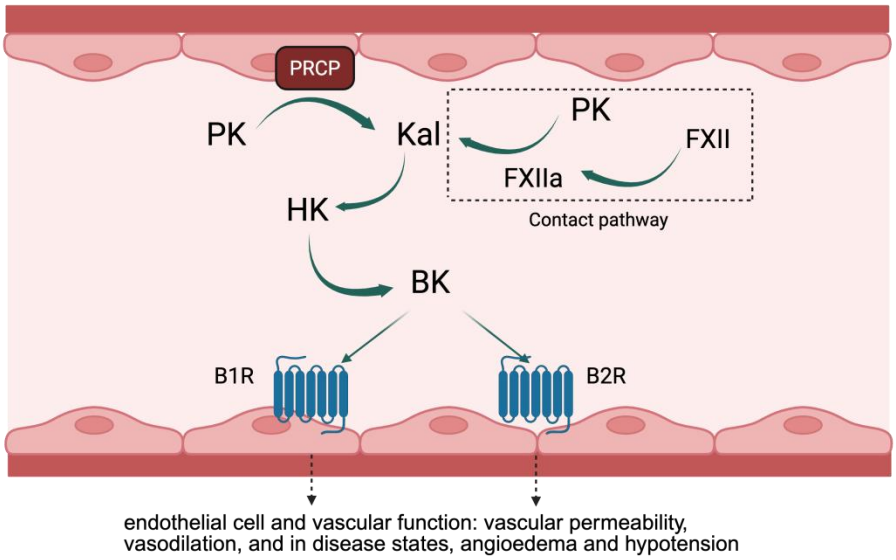


Figure 2.2 Activation of PK via contact pathways and ECs.
PK is activated to plasma kallikrein (Kal) either through the classical contact pathway—initiated by activated Factor XII (FXIIa) —or independently via PRCP expressed on endothelial cells. Active kallikrein cleaves HK to release BK, which then binds to bradykinin receptors B1R and B2R on endothelial cells. This signaling cascade regulates vascular permeability and vasodilation, and contributes to pathological conditions such as angioedema and hypotension.

Despite the known physiological importance of endothelial-mediated PK activation, the precise molecular mechanism by which PK interacts with the endothelial surface, especially with PRCP, remains poorly characterized. In particular, the contribution of individual apple domains of PK to its binding, orientation, and cleavage on endothelial cells is not well understood. Previous studies have largely focused on fluid-phase activation or artificial surface models, while relatively little is known about the structural determinants guiding PK activation in a cellular context.

Understanding how PK is recognized and activated by PRCP on vascular endothelium has important implications for diseases involving vascular inflammation, thrombosis, and bradykinin-mediated angioedema. It may also help identify novel therapeutic targets that can selectively modulate the contact system without impairing hemostasis. In this study, we aim to dissect the role of each apple domain in PK during its activation on endothelial cells, using a combination of monoclonal antibody mapping and domain-swapping chimeric proteins. This will help elucidate the structural requirements for PK

activation in a biologically relevant setting and provide new insights into contact system regulation at the endothelial interface.

2.3 Materials and Methods

2.3.1 Reagents

For endothelial cell-based PK activation assays, Human Umbilical Vein Endothelial Cells were obtained from Lonza (Walkersville, MD, USA) and cultured in endothelial growth medium (EGM-2). Human plasma PK and HK were purchased from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant human HK was kindly provided by Dr. Tom E. Gailani (Vanderbilt University, Nashville, TN, USA). PK activation was measured using the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA; Diapharma Group, West Chester, OH, USA).

Corn trypsin inhibitor (CTI), a serine protease inhibitor of FXIIa, was purchased from Haematologic Technologies (Essex Junction, VT, USA), and a small-molecule FXIIa inhibitor was obtained from KalVista Pharmaceuticals (Salisbury, UK). Chloroquine (Sigma-Aldrich, St. Louis, MO, USA) was used as a PRCP inhibitor.

Monoclonal antibodies against human and mouse PK were generated in-house. Anti-human PK antibodies included 6G10 (A1 domain), 3B8 and 6B9 (A2 domain) and 4G7, 5E2, and 1G5 (A4 domain). Anti-mouse antibodies included 5H1 (A1 domain) and 4H11 (A3 domain). 6G10, 4H11, and 5H1 antibodies are cross-reactive with both mouse and human PK (Figure 2.3).

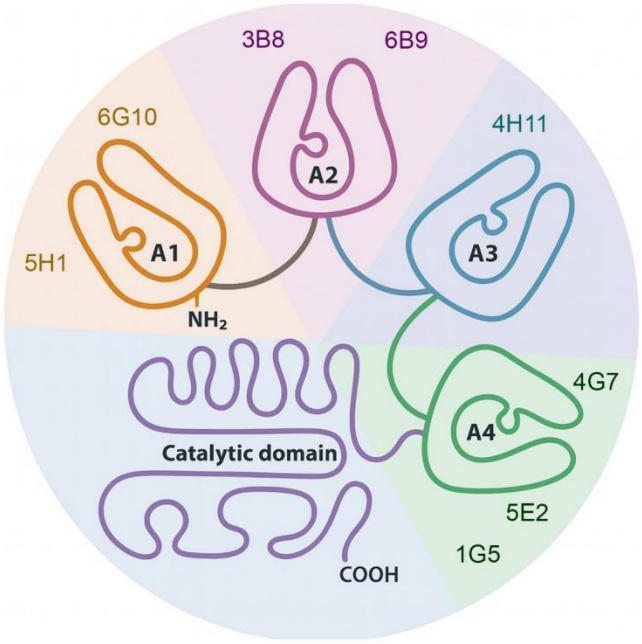


Figure 2.3 Domain structure of PK and epitope mapping of monoclonal antibodies. Schematic representation of PK showing its modular domain organization (A1–A4 and catalytic domain). Monoclonal antibodies were generated against distinct PK domains.

3.3.2 PK Activation Assay

PK activation on endothelial cells was assessed using a chromogenic substrate assay. HUVECs were seeded in 96-well flat-bottom plates (2×10^4 cells/well) and cultured to confluence. For starvation conditions, cells were incubated overnight in serum-free media. For the activation assay, reagents were preincubated in HEPES-buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4) containing 10 µM $ZnCl_2$ for 10 min at room temperature. Depending on the condition, we either (i) preincubated human plasma PK (20–50 nM) with/without HK (20 nM) and the indicated anti-PK monoclonal antibodies (500 nM), or (ii) preincubated PK with 5C12 (anti-FXIIa Ab; 0.267 µM) or with the small-molecule FXIIa inhibitor (1 µM) prior to addition to cells. The preincubated mixture was then added to the HUVEC monolayers and incubated at 37°C for 1 hour. After incubation, PK activation was measured by adding the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA) at a final concentration of 1 mM. The reaction was allowed to proceed at 37°C for 1h, and absorbance was measured at 405 nm using a

microplate reader. The increase in absorbance was used as an indicator of kallikrein activity.

3.3.3 FXIIa inhibition assay

In flat-bottom 96-well plates, 80 µL of purified human FXIIa (80 nM) was dispensed per well. Where indicated, CTI (40 µg/mL), Chloroquine (3, 6, or 12 mM), or the selective FXIIa inhibitor (1 µM) was added; mixtures were pre-incubated for 30 min at 37 °C. Reactions were initiated by adding 20 µL of the chromogenic substrate S-2302 (1 mM) to a total volume of 100 µL. The release of p-nitroaniline was monitored at 405 nm at 37 °C on a plate reader. Initial rates (ΔA405/min) were converted to apparent FXIIa concentrations using a standard curve generated with parallel wells containing FXIIa at 0, 12.5, 25, 50, and 100 nM under identical conditions.

3.3.4 Analysis of data

Data are presented as mean ± SEM; each dot represents an independent biological replicate (technical duplicates/ triplicates were averaged per replicate). All tests were two-tailed and performed in GraphPad Prism (v9.x). A p value < 0.05 was considered significant. Significance is indicated as: ns ($p \geq 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). For all comparisons, we used unpaired Student's t-tests; Welch's correction was applied when variances were unequal.

2.4 Results

To investigate the mechanisms underlying PK activation on HUVECs, HUVECs were incubated with PK (50 nM) for 60 minutes (Figure 2.4A). This incubation resulted in the generation of 6517 ± 833 pM of active kallikrein, indicating that HUVECs support efficient activation of PK on their surface. In contrast, incubation of PK alone—without HUVECs—did not result in detectable kallikrein activity, suggesting that specific cellular factors or surface molecules on HUVECs are required to initiate PK activation.

It is well established that virtually all plasma PK circulates in complex with HK. HK binds PK in a discontinuous manner, primarily involving the A2 domain of PK, but also engaging the A1 and A4 domains[26]. Despite this tight association, PK is capable of

binding to HUVECs both in the presence and absence of HK[33], indicating that multiple modes of interaction may facilitate PK localization to the cell surface.

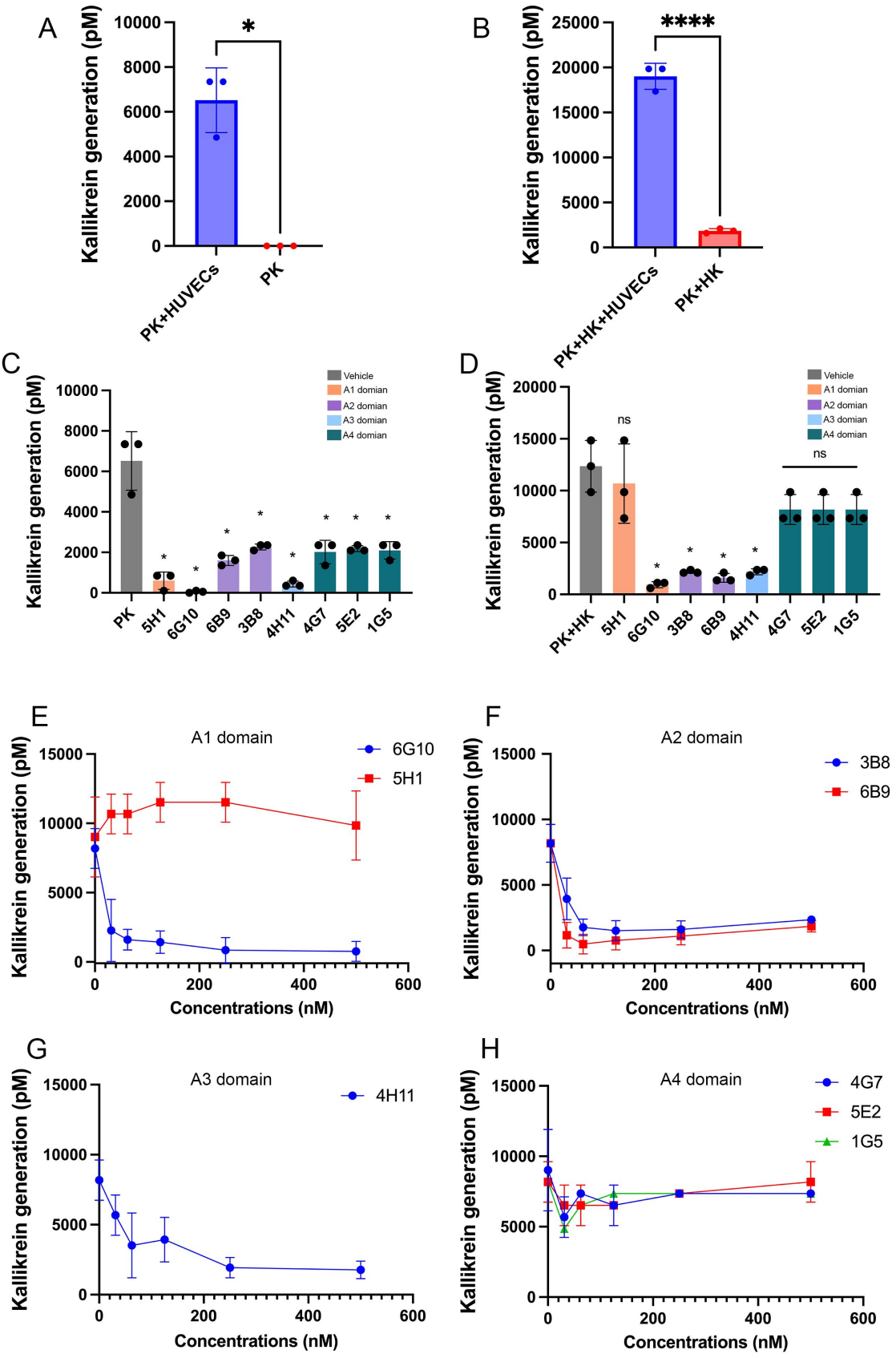
Consistent with this, when PK (50 nM) was co-incubated with HK (20 nM) and applied to HUVECs, a substantially higher level of kallikrein (17167 ± 845 pM) was generated compared to PK alone (Figure 2.4B). This dramatic increase highlights the critical synergistic role of HK in enhancing PK activation on the endothelial surface. These findings suggest that HK not only stabilizes PK binding but may also facilitate its optimal positioning for activation by endothelial enzymes.

To further define the molecular determinants required for endothelial PK activation, we assessed the effect of monoclonal antibodies targeting individual apple domains of PK on kallikrein generation. As shown in figure 2.4C, antibodies specific to the A1 domain (5H1, 6G10), A2 domain (6B9, 3B8), A3 domain (4H11), and A4 domain (4G7, 5E2, 1G5) significantly inhibited kallikrein generation, though to differing extents. Notably, 6G10 (A1) almost completely abolished PK activation, while 5H1 (A1), 6B9 and 3B8 (A2), and 4H11 (A3) showed strong inhibition. A4-targeting antibodies (4G7, 5E2, 1G5) led to moderate but significant reductions. These results suggest that multiple apple domains contribute to PK's activation on HUVECs, with the A1 and A2 domains being particularly critical for functional interaction with the endothelial surface.

To determine whether the presence of HK alters the contribution of each apple domain to PK activation, similar antibody-blocking experiments were conducted under conditions where PK was pre-complexed with HK. As shown in figure 2.4D, A1-targeting antibody 5H1 no longer significantly inhibited PK activation in the presence of HK, while 6G10 (also A1) still showed strong inhibition. Importantly, antibodies against the A2 (3B8, 6B9) and A3 (4H11) domains remained effective inhibitors, suggesting that these domains are essential for activation even in the context of PK-HK complex formation. In contrast, A4-targeting antibodies (4G7, 5E2, 1G5) had no significant inhibitory effect under these conditions. These results imply that although HK enhances PK activation, it does not fully shield critical functional domains such as A2 and A3, which remain accessible and necessary for endothelial-mediated activation.

To determine the relative inhibitory potency of antibodies targeting individual apple domains, we performed dose–response experiments with monoclonal antibodies specific to the A1, A2, A3, and A4 domains of PK, across concentrations ranging from 0 to 500 nM. As shown in figure 2.4E, both A1-targeting antibodies (5H1 and 6G10) were evaluated. While 6G10 exhibited potent, dose-dependent inhibition of kallikrein generation, achieving near-complete suppression at concentrations as low as 62.5 nM, 5H1 failed to reduce kallikrein levels even at high concentrations, suggesting that its epitope within A1 is either non-functional or occluded under experimental conditions. In figure 2.4F, two A2 domain-targeting antibodies (3B8 and 6B9) were tested. Both antibodies markedly suppressed PK activation in a concentration-dependent manner, with 6B9 showing slightly greater potency at lower concentrations. These data confirm that the A2 domain is essential for PK activation on endothelial cells and that blockade at this site effectively interferes with its function.

In figure 2.4G, we assessed the A3 domain-specific antibody 4H11. This antibody demonstrated a clear concentration-dependent inhibition of kallikrein generation, consistent with earlier endpoint data. Its inhibitory profile closely mirrors that of the most potent A1 and A2 antibodies, further emphasizing the functional relevance of the A3 domain. In contrast, as shown in figure 2.4H, antibodies targeting the A4 domain (4G7, 5E2, 1G5) exhibited minimal inhibition across all tested concentrations. kallikrein levels remained largely unchanged, suggesting that the A4 domain may be less involved in endothelial-mediated activation of PK or that its conformation limits accessibility to these antibodies. Together, these results define a domain-specific hierarchy of antibody-mediated inhibition, with A1, A2, and A3 domains representing key functional regions required for efficient activation of PK on endothelial surfaces.


A
Kallikrein generation (pM)
10000
8000
6000
4000
2000
0
*
PK+HUVECs
PK
B
Kallikrein generation (pM)
25000
20000
15000
10000
5000
0
****
PK+HK+HUVECs
PK+HK
C
Kallikrein generation (pM)
Vehicle
A1 domian
A2 domian
A3 domian
A4 domian
PK
5H1
6G10
6B9
3B8
4H11
4G7
5E2
1G5
D
Kallikrein generation (pM)
ns
ns
PK+HK
5H1
6G10
3B8
6B9
4H11
4G7
5E2
1G5
E
A1 domain
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Kallikrein generation (pM)
Concentrations (nM)
F
A2 domain
3B8
6B9
Kallikrein generation (pM)
Concentrations (nM)
G
A3 domain
4H11
Kallikrein generation (pM)
Concentrations (nM)
H
A4 domain
4G7
5E2
1G5
Kallikrein generation (pM)
Concentrations (nM)

Figure 2.4 Domain-specific antibodies block PK activation by endothelial cells.

(A) Kallikrein generation was significantly enhanced in the presence of PK and HUVECs compared to PK alone. (B) Addition of HK further increased kallikrein generation in the presence of HUVECs compared to PK+HK without HUVECs. (C) Domain-specific monoclonal antibodies targeting different regions of the target protein were tested for their ability to inhibit kallikrein generation. Antibodies against the A1, A2, A3, and A4 domains showed significant inhibition compared to the vehicle control. (D) In the presence of HK, A2 domain antibodies (6G10, 6B9, 4H11) significantly inhibited kallikrein generation, while A1 and A4 domain antibodies showed no significant effect. (E–H) Dose-response curves of selected antibodies targeting different domains. (E) A1 domain antibody (5H1) did not inhibit kallikrein generation across concentrations, while A2 domain antibody (6G10) showed strong dose-dependent inhibition. (F) A2 domain antibodies 3B8 and 6B9 both reduced kallikrein generation in a dose-dependent manner. (G) 4H11 (A2 domain) also demonstrated dose-dependent inhibition. (H) A4 domain antibodies (4G7, 5E2, and 1G5) showed modest or no dose-dependent inhibition.

Although previous studies have shown that the endothelial cell surface can activate PK, whether PRCP directly participates in this process remains controversial. Some have suggested that this activation might be due to contamination with FXIIa in the cell culture medium or in the HK reagent, thereby indirectly triggering PK activation. To exclude this possibility, we first subjected HUVECs to serum starvation (overnight incubation without serum) and then added PK or PK+HK. The results showed that serum starvation did not significantly alter kallikrein generation (Figure 2.5A,B, ns), indicating that the serum-containing culture medium did not contain FXIIa contamination capable of activating PK.

Next, to test whether PRCP was responsible for PK activation, we treated the cells with chloroquine, which has been reported as a PRCP inhibitor. Chloroquine markedly inhibited PK activation (Figure 2.5C). However, to our surprise, the control FXIIa inhibitor CTI also almost completely blocked PK activation (Figure 2.5C), raising doubts about the specificity of chloroquine toward PRCP. To address this, we co-incubated chloroquine with FXIIa and measured its enzymatic activity. The results revealed that chloroquine also inhibited FXIIa activity, with an effect almost identical to that of CTI (Figure 2.5D). We also have some concerns that CTI may affect other proteases.

Next, to address the concerns regarding the non-specific effects of CTI, we introduced a highly specific small-molecule inhibitor of FXIIa from KalVista. This inhibitor does not inhibit kallikrein even at micromolar concentrations, and since it is not a protein, it eliminates the risk of any potential artifacts. As shown in Figure 2.5E, this small-molecule inhibitor also inhibited PK activation on endothelial cells. However, we concluded that this inhibitor might not be as highly specific as initially thought, and we cannot exclude the possibility that it could also inhibit PRCP. To further investigate, we used 5C12, a highly specific antibody against FXIIa. This antibody binds exclusively to FXIIa, making it more specific than any inhibitor. The results showed that 5C12 did not inhibit PK activation on endothelial cells, suggesting that we can confidently rule out FXIIa contamination as a contributing factor in endothelial cell-mediated PK activation.

It is known that plasma-derived HK may contain trace amounts of FXII/FXIIa, which can be detected in specific substrate assays and even by Western blotting. In our previous experiments, we used human plasma-derived HK, so we cannot entirely exclude the possibility of FXIIa contamination. To address this, we next compared the effects of recombinant HK and plasma-derived HK on PK activation. As shown in Figure 2.5F, there was no significant difference between recombinant HK and plasma-derived HK in their capacity to activate PK on endothelial cells. This suggests that FXIIa contamination in plasma-derived HK does not significantly contribute to PK activation on endothelial cells.

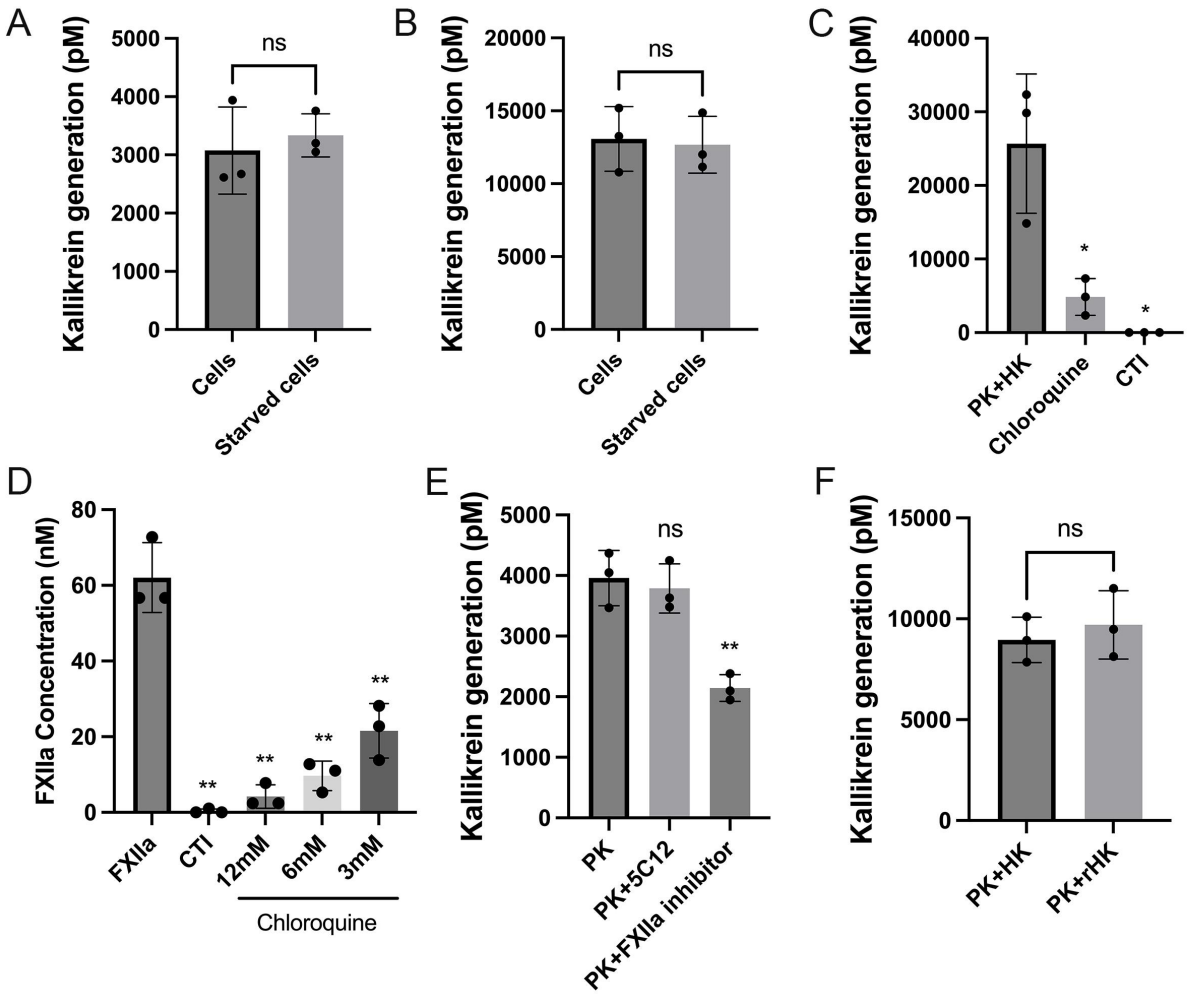

Figure 2.5 Excluding FXIIa contamination and evaluating inhibitor specificity in endothelial PK activation.

(A–B) Serum starvation (overnight, serum-free) did not alter kallikrein generation by PK (A) or PK+HK (B) on HUVECs, arguing against FXIIa contamination in culture medium. (C) Chloroquine (3 mM) markedly reduced PK+HK–dependent kallikrein generation; the FXIIa inhibitor CTI likewise strongly suppressed the signal, prompting specificity concerns. (D) In a purified FXIIa assay (80 nM), CTI completely suppressed FXIIa formation, while chloroquine, an inhibitor of PRCP, reduced FXIIa generation in a dose-dependent manner (12 mM, 6 mM, and 3 mM). (E) In the cell assay, the FXIIa-specific antibody 5C12 did not inhibit PK activation on endothelial cells, whereas the selective FXIIa inhibitor reduced kallikrein generation. (F) Recombinant HK (rHK) and plasma-derived HK (pHK) supported comparable PK activation (ns), indicating that trace FXII/FXIIa in pHK does not account for the endothelial signal.

2.5 Discussion

This study characterizes the structural and mechanistic basis of PK activation on endothelial cells and clarifies the relative roles of PRCP and FXIIa in this process. The main findings can be summarized as follows: (i) endothelial cells efficiently activate PK, and this activity is markedly potentiated by high-molecular-weight kininogen (HK); (ii) inhibition with domain-specific monoclonal antibodies reveals a hierarchy among PK's apple domains, with A1, A2, and A3 being indispensable for cell-mediated activation; (iii) multiple lines of evidence exclude FXIIa contamination as an explanation for PK activation on endothelial cells, including the ineffectiveness of a highly selective anti-FXIIa antibody (5C12) and the comparable activity of recombinant and plasma-derived HK; and (iv) small-molecule inhibitors classified as PRCP or FXIIa antagonists (chloroquine and a KalVista FXIIa inhibitor) suppressed activation readouts, but these effects likely reflect off-target or pathway-level influences when used in isolation. Taken together, the results support a model in which the endothelial surface, through PRCP and HK, positions PK in an orientation dependent on A1–A3 domains that enables its productive conversion to kallikrein.

Our experiments demonstrate that the endothelial cell surface provides a functional platform for PK activation. PK alone in buffer produced no detectable kallikrein, whereas incubation with endothelial monolayers yielded measurable activity (Figure 2.4A). The presence of HK strongly amplified this effect (Figure 2.34). Since PK circulates predominantly in complex with HK, this amplification is physiologically consistent, suggesting that HK not only co-localizes PK with its activators but also acts as a scaffold to increase local PK concentration and/or orient it properly for cleavage. The observation that PK still binds to cells in the absence of HK, albeit with reduced efficiency, suggests that multiple binding interactions mediate its association with the endothelial surface.

Antibody-blocking experiments further defined the PK regions required for endothelial activation. Without HK, antibodies targeting A1, A2, A3, and to a lesser extent A4 reduced kallikrein generation (Figure 2.4C), indicating contributions from several apple domain surfaces. With HK present, inhibition by A4 antibodies disappeared, while the

effects of A1 antibodies became epitope-dependent (5H1 inactive, 6G10 strongly inhibitory), and A2/A3 inhibition persisted (Figure 2.4D). Dose–response experiments confirmed these trends: 6G10 (A1), 6B9/3B8 (A2), and 4H11 (A3) potently blocked activation in a concentration-dependent manner, while A4 antibodies were largely ineffective (Figure 2.4E–H). These results suggest that HK masks or renders A4 dispensable by reorienting PK, while A1, A2, and A3 remain exposed and essential. The contrasting effects of the two A1 antibodies further indicate that not all epitopes within a domain are functionally equivalent: the 6G10 epitope likely maps to a critical interface, such as a docking site for PRCP/HK or a region regulating conformational change, whereas the 5H1 epitope may be inaccessible or non-essential under HK-bound conditions. Collectively, the findings outline an activation-competent orientation of PK on endothelium that depends on discrete apple domain surfaces rather than the catalytic domain alone.

A long-standing issue in this field is distinguishing genuine PRCP-dependent PK activation from artifacts caused by trace FXIIa contamination. Our data address this challenge through several complementary approaches. Serum depletion did not affect kallikrein generation in PK-only or PK+HK conditions (Figure 2.5A, B), arguing against exogenous FXIIa contamination. Chloroquine, often cited as a PRCP inhibitor, reduced PK activation (Figure 2.5C) but also inhibited purified FXIIa comparably to CTI (Figure 2.5D), demonstrating its lack of selectivity. Similarly, a highly selective KalVista FXIIa inhibitor suppressed PK activation on cells (Figure 2.5E), which could suggest FXIIa involvement, but this interpretation remains uncertain because the inhibitor is active in purified FXIIa assays while the cell assay reports downstream kallikrein activity, and off-target effects cannot be ruled out. By contrast, the FXIIa-specific monoclonal antibody 5C12 failed to block PK activation on endothelial cells (Figure 2.5E), providing direct evidence against a requirement for FXIIa. Finally, both recombinant HK and plasma-derived HK supported equivalent activation (Figure 2.5F), excluding plasma FXII/FXIIa contamination as a confounding factor. Taken together, these findings indicate that the observed PK activation on endothelial cells is not attributable to FXIIa contamination but is instead mediated predominantly by PRCP in cooperation with HK.

2.6 Conclusions

Our results provide a coherent picture of how PK is activated by endothelial cells. HK substantially enhances activation, and domain mapping indicates that A1, A2, and A3 form a functional interface necessary for productive cleavage. A battery of orthogonal controls argues that the activity detected on endothelium is not an artifact of FXIIa contamination: serum starvation had no effect, recombinant HK behaved like plasma HK, and an FXIIa-exclusive antibody failed to inhibit activation, even though chloroquine and a small-molecule FXIIa inhibitor could suppress the assay. The most parsimonious explanation is that PRCP is the predominant activator of PK on endothelial cells under our conditions, and that HK organizes PK via specific apple-domain contacts to enable activation. These insights refine the mechanistic understanding of the kallikrein–kinin system at the vascular interface and open avenues for targeted modulation of bradykinin generation in disease.

Chapter 3

Cell Surface Proteome Alterations in Aortic Endothelial Cells Induced by Coagulation Factor XI

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3.1 Abstract

Coagulation factor XIa is increasingly recognized as more than a coagulation protease, as it interacts with diverse endothelial substrates and ligands to influence vascular integrity, immune responses, and inflammatory processes implicated in atherosclerosis. Yet, its precise impact on endothelial signaling, extracellular matrix remodeling, and immune regulation remains unclear. To investigate these roles, human aortic endothelial cells were incubated with FXIa for six hours, followed by cell surface biotinylation and proteomic analysis, alongside secretome profiling using biotin-labeling controls. FXIa treatment markedly altered the endothelial proteomic landscape, downregulating proteins associated with barrier function (TJP1/2, NECTIN1), angiogenesis (NRP2, TIE1, NOTCH4), and oxidative stress defense (PON2), while upregulating factors in TGF-β signaling (TGFB1, BMPR2, STRAP), immune activation and antigen presentation (HLA-A/B, LTBR, NCR3LG1). Secretome analysis revealed pronounced increases in extracellular matrix–related proteins, including VIM, COL18A1, TGM2, ADAMTS4, MMP2, LOXL2, and HSPG2. Together, these findings demonstrate that FXIa reprograms the endothelial proteome and secretome, promoting immune activation and extracellular matrix remodeling while suppressing angiogenesis and oxidative stress responses, thereby underscoring its potential role in endothelial dysfunction and thromboinflammatory disease.

3.2 Background

Coagulation factor XI is a plasma serine protease zymogen whose activated form, factor XIa, plays a pivotal role in the classical intrinsic coagulation cascade. By activating factor IX and promoting thrombin generation, FXIa contributes critically to thrombus formation and hemostasis, and has long been regarded as a coagulation-specific protease[34,35]. However, increasing evidence indicates that the biological functions of FXIa extend far beyond coagulation. It has been implicated in vascular homeostasis, inflammation, and immune regulation[36], suggesting that FXIa may serve as a molecular bridge linking the coagulation system to vascular pathophysiology.

Vascular endothelial cells form the inner lining of blood vessels and act as a dynamic interface between circulating blood and the vessel wall. In addition to providing a physical barrier, they regulate vascular tone, hemodynamics, leukocyte adhesion, and inflammatory signaling through the release of bioactive molecules[13,37]. Under physiological conditions, endothelial cells maintain an anti-thrombotic, anti-inflammatory, and selectively permeable state, which preserves vascular homeostasis[38]. However, in pathological settings such as atherosclerosis, diabetes, and hypertension, endothelial dysfunction arises[39]. This is characterized by increased permeability, upregulation of adhesion molecules, and excessive secretion of inflammatory mediators, thereby facilitating monocyte and lymphocyte adhesion and infiltration, amplifying local inflammation, and promoting chronic vascular injury and remodeling[40]. Endothelial dysfunction is thus considered an early and central event in the development of atherosclerotic cardiovascular disease.

Recent studies have revealed that FXIa may directly contribute to endothelial dysfunction. At the endothelial level, FXIa has been shown to directly compromise vascular barrier function. Specifically, FXIa forms a complex with PAI-1 and signals through very-low-density lipoprotein receptor (VLDLR), thereby activating downstream VEGFR2–PLCγ1–ERK and Src pathways. This process drives ADAM10 translocation to the plasma membrane, where it cleaves the extracellular domain of VE-cadherin, resulting in the release of soluble VE-cadherin fragments and a concomitant increase in

endothelial permeability. Notably, inhibition of FXI activation, blockade of FXIa catalytic activity, or suppression of ADAM10 function attenuates this effect. Importantly, in a nonhuman primate model of sepsis, inhibition of FXI activation significantly reduced circulating soluble VE-cadherin levels, highlighting the pathological relevance of FXIa-mediated endothelial barrier disruption[14]. These findings suggest that FXIa may play a crucial role in shaping the vascular inflammatory microenvironment.

This hypothesis has been partially supported by animal and clinical studies. In murine models of atherosclerosis, inhibition of FXIa markedly reduced vascular wall inflammation, attenuated plaque burden, and improved endothelial barrier function[41]. Clinically, FXIa inhibitors have emerged as promising next-generation anticoagulants. Compared with conventional agents such as warfarin or direct oral anticoagulants, FXIa inhibitors demonstrate comparable efficacy in preventing thrombosis but with a significantly lower risk of major bleeding. Several clinical trials have shown encouraging results in preventing atrial fibrillation–related stroke and venous thromboembolism[42,43]. These findings not only underscore the critical role of FXIa in cardiovascular disease but also highlight its translational therapeutic potential beyond traditional anticoagulation.

Although accumulating evidence links FXIa to vascular pathology, the underlying molecular mechanisms remain poorly defined. In particular, how FXIa modulates endothelial signaling pathways, alters the composition of the surface proteome, or reshapes the secretome to influence extracellular matrix remodeling and immune cell recruitment has not been systematically addressed. This gap in knowledge limits our understanding of the broader physiological and pathological roles of FXIa beyond coagulation.

Therefore, investigating the interaction between FXIa and endothelial cells is of considerable importance. Determining whether FXIa induces changes in the endothelial cell surface proteome and secretome may provide novel insights into its role in vascular signaling, barrier regulation, and immune modulation. Such findings could not only clarify the contribution of FXIa to vascular disease mechanisms but also identify

potential therapeutic targets for the prevention and treatment of atherosclerosis and related cardiovascular disorders.

3.3 Materials and Methods

3.3.1 Reagents

HAECs and endothelial growth medium (EGM-2) were purchased from Lonza (Walkersville, MD, USA). Phosphate-buffered saline (PBS) was obtained from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). Human coagulation FXIa was purchased from Enzyme Research Laboratories (South Bend, IN, USA), and D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) from Haematologic Technologies (Essex Junction, VT, USA). Zinc chloride ($ZnCl_2$), glycine, ammonium bicarbonate, and sodium deoxycholate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sulfo-NHS-LC-LC-Biotin and NeutrAvidin agarose were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and protease inhibitor cocktail from Roche (Basel, Switzerland). Sequencing-grade trypsin was obtained from Promega (Madison, WI, USA). RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA), protease inhibitor cocktail (Roche, Basel, Switzerland), BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), 4× Laemmli loading buffer (Bio-Rad, Hercules, CA, USA), dithiothreitol (Bio-Rad, Hercules, CA, USA), SDS-PAGE gel and system (Bio-Rad, Hercules, CA, USA), PVDF membranes (Millipore, Billerica, MA, USA), non-fat dry milk (Bio-Rad, Hercules, CA, USA), Tween-20 (Sigma-Aldrich, St. Louis, MO, USA), HRP-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA), anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA), HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), and ECL chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA).

3.3.2 Endothelial Cell Culture, Treatment, and Surface Biotinylation

HAECs were cultured in 150 mm dishes containing 30 mL endothelial growth medium under standard conditions (37 °C, 5% CO_2). Prior to experiments, cells were starved overnight in SFM and washed three times with SFM. For stimulation, cells were

incubated with either vehicle control or coagulation FXIa (30 nM) in SFM supplemented with 10 μM $ZnCl_2$ for 6 h at 37 °C. To terminate enzymatic activity, 100 μM PPACK was added for 10 min. Surface biotinylation was performed using 0.5 mg/mL Sulfo-NHS-LC-LC-biotin at 4 °C for 30 min with gentle shaking. Cells were washed three times with ice-cold quenching buffer (50 mM glycine in PBS containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4), with 5 min incubation at each wash to neutralize unreacted biotin.

For surface proteome analysis (Figure 3.1A), cells were lysed in 2.5 mL lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail. Lysates were incubated on ice for 30 min, scraped, sonicated (30 s), and centrifuged at 16,000 g for 5 min at 4 °C. Supernatants were collected for subsequent NeutrAvidin affinity purification.

For secretome analysis (Figure 3.1B), biotinylated cells were incubated with FXIa (30 nM) in serum-free medium containing 10 μM $ZnCl_2$ for 6 h. Culture supernatants were collected, pooled with cell washes, centrifuged at 2000 g for 5 min, and concentrated using Amicon filters (3,000 Da cutoff) prior to affinity purification. Experimental controls included non-biotinylated conditions.

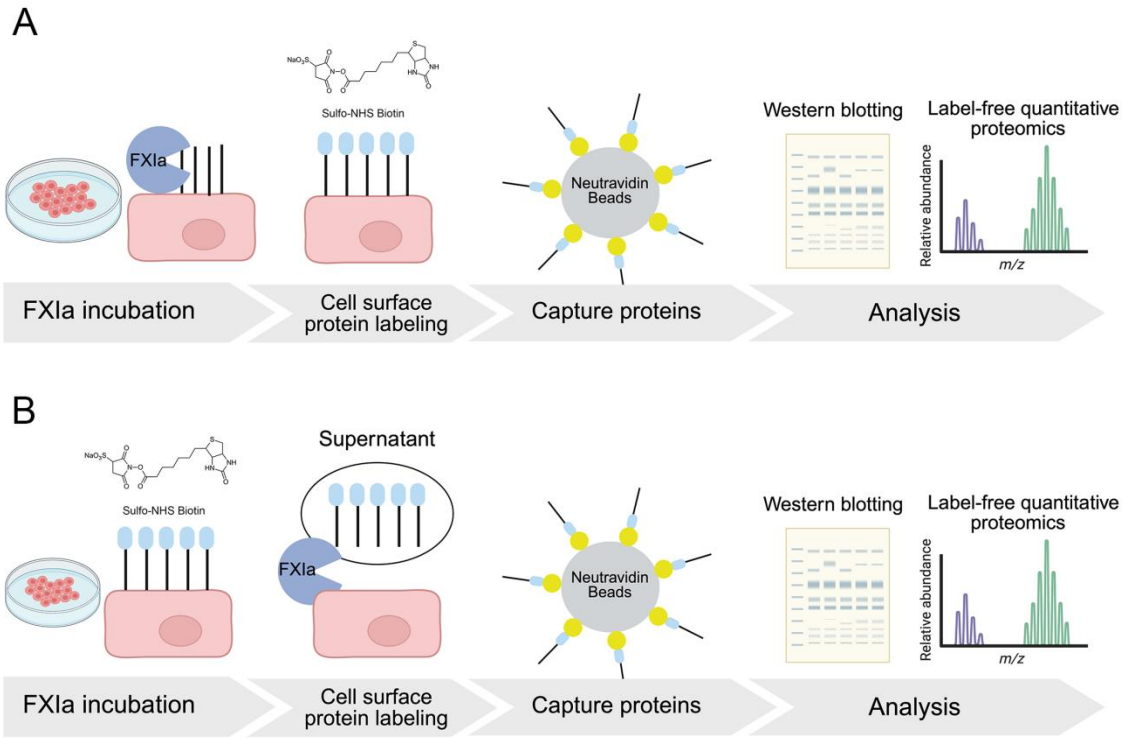

Figure 3.1. Experimental workflow for analysis of FXIa-induced changes in endothelial cell membrane and secretome proteins.
(A) Schematic representation of the cell surface proteomics approach. HAECs were incubated with FXIa, followed by cell surface biotinylation. Biotinylated membrane proteins were enriched using NeutrAvidin beads and analyzed by Western blotting and label-free quantitative proteomics.
(B) Schematic representation of the cell secretome proteomics approach. HAECs were first labeled with sulfo-NHS-biotin and then incubated with FXIa. Biotinylated proteins released into the supernatant were captured with NeutrAvidin beads and subjected to Western blotting and label-free quantitative proteomics.

3.3.3 NeutrAvidin Affinity Purification and Protein Digestion

NeutrAvidin resin (250 µL) was equilibrated with lysis buffer and incubated with either cell lysates or concentrated culture media at 4 °C for 2 h with rotation. Beads were pelleted (1,000 g, 3 min) and washed five times each with lysis buffer, PBS, and 250 mM ammonium bicarbonate. Beads were then resuspended in 200 µL ammonium bicarbonate buffer. An aliquot (50 µL) was reserved for Western blotting, while the remaining fraction was stored at –20 °C until proteomic analysis.

3.3.4 Western blotting

For Western blot analysis, bead-bound proteins were denatured in 4× SDS loading buffer (200 mM Tris-HCl, 8% SDS, 0.4% bromophenol blue, 40% glycerol) supplemented with 100 mM DTT. Samples were boiled at 95 °C for 5 min, centrifuged briefly, and resolved by SDS–PAGE. Proteins were transferred to PVDF membranes, blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), and incubated with primary antibodies overnight at 4 °C. Membranes were washed and incubated with HRP-conjugated secondary antibodies, followed by visualization using enhanced chemiluminescence (ECL).

3.3.5 Proteomic Data Processing and Analysis

Proteomic datasets derived from cell surface and secretome preparations were analyzed using a label-free quantitative (LFQ) proteomics workflow. Raw MS/MS spectra were processed with standard bioinformatics pipelines to identify and quantify proteins across experimental conditions. Protein intensities were normalized across samples to correct for technical variation and batch effects. Relative protein abundance was then determined by calculating the $\log_2$ fold change (FXIa-treated vs. vehicle control). Proteins were subsequently grouped into functional categories based on their annotated biological roles, including extracellular matrix organization, cell adhesion, angiogenesis, oxidative stress response, cytokine signaling, and immune regulation. Data were visualized as bar plots, with the y-axis representing $\log_2$ fold changes and the x-axis listing individual proteins. No inferential statistical tests were applied; the results are presented descriptively to highlight protein abundance trends following FXIa stimulation.

3.4 Results

3.4.1 FXIa Restructures the Endothelial Cell Surface Proteome

After 6 hours of stimulation with FXIa, the surface proteome of HAECs underwent profound changes (Figure 3.2). Label-free quantitative proteomic analysis revealed significant alterations in several key molecular groups. Compared with controls, tight

junction proteins TJP1/2 and the adhesion molecule NECTIN1 were markedly downregulated, suggesting disruption of intercellular junctions and impaired barrier integrity. Similarly, receptors and regulators associated with angiogenesis and vascular development, such as NRP2, TIE1, and NOTCH4, showed significant reductions, indicating suppression of endothelial repair and vessel formation. The antioxidant enzyme PON2 was also decreased, suggesting heightened susceptibility to oxidative stress. In contrast, immune- and inflammation-related molecules were upregulated. HLA-A/B were increased, indicating enhanced antigen-presenting capacity, while LTBR upregulation suggested activation of lymphotoxin-related signaling, and NCR3LG1 induction may promote natural killer (NK) cell interaction. Furthermore, FXIa markedly elevated TGFB1, BMPR2, and STRAP, all components of the TGF-β signaling axis, suggesting a shift of endothelial cells toward an immune-activated and pro-fibrotic state. Taken together, FXIa induced a proteomic signature characterized by the downregulation of protective factors and upregulation of pro-inflammatory mediators, highlighting its disruptive impact on endothelial homeostasis.

3.4.2 FXIa Induces Extensive Remodeling of the Endothelial Secretome

Secretome analysis revealed that FXIa profoundly altered extracellular protein composition in HAECs (Figure 3.3). Following FXIa stimulation, proteins associated with extracellular matrix (ECM) remodeling were strongly enriched, including VIM, COL18A1, TGM2, ADAMTS4, MMP2, LOXL2, and HSPG2. These proteins are involved in matrix degradation (MMP2, ADAMTS4), fiber crosslinking (LOXL2, TGM2), basement membrane components (HSPG2), and cytoskeletal remodeling (VIM). This pattern suggests that FXIa drives a dynamic “degradation–reconstruction” process within the ECM. In particular, elevated levels of MMP2 and ADAMTS4 indicated enhanced proteolytic remodeling, while increased LOXL2 and TGM2 suggested reinforcement of crosslinking and stiffening processes. Release of COL18A1 and HSPG2 further indicated disruption of basement membrane integrity. In addition, proteins associated with cell adhesion, cytokine signaling, and stress responses were also altered, underscoring the role of FXIa in reshaping the extracellular environment toward an inflammatory and remodeling phenotype.

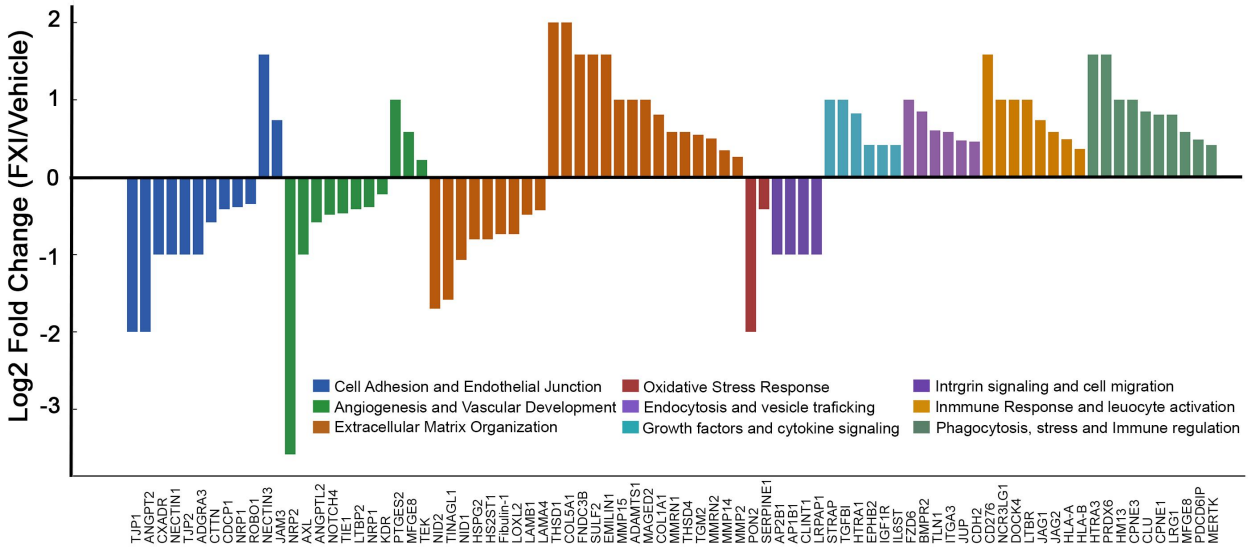

Figure 3.2 Log_2 Fold Change in Cell Surface Protein Expression Induced by FXIa Treatment. Differential expression of surface-associated proteins in HAECs following 6 h FXIa stimulation compared to vehicle control. Proteins are grouped by biological function, including cell adhesion and endothelial junctions, angiogenesis and vascular development, extracellular matrix organization, oxidative stress response, endocytosis and vesicle trafficking, growth factor and cytokine signaling, integrin signaling and cell migration, immune response and leukocyte activation, and phagocytosis/immune regulation.

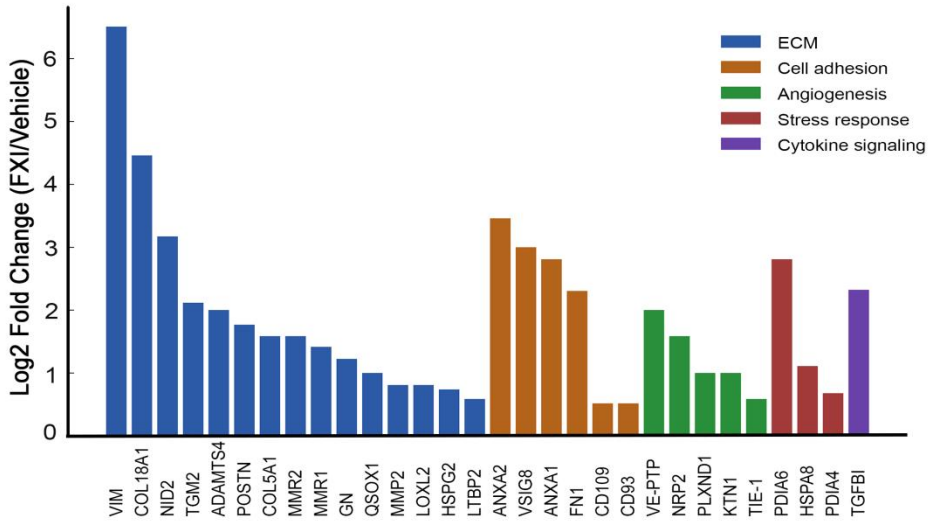

Figure 3.3 Log_2 fold changes of secreted proteins in FXIa-treated cells. Proteomic analysis of HAEC secretome after 6 h stimulation with FXIa. Proteins are grouped by functional categories, indicated by different colors: extracellular matrix (blue), cell adhesion (brown), angiogenesis (green), stress response (red), and cytokine signaling (purple).

3.4.3 Functional Pathway Enrichment and Network Analysis

Integration of surface proteome and secretome data revealed that FXIa exerts a dual regulatory effect on endothelial cells On the one hand, it strongly suppressed pathways associated with barrier function, intercellular adhesion, angiogenesis, and oxidative defense, reflected by the downregulation of TJP1/2, NECTIN1, NRP2, TIE1, NOTCH4, and PON2. On the other hand, FXIa activated immune-related and extracellular remodeling pathways, as indicated by the upregulation of HLA-A/B, LTBR, NCR3LG1, and the induction of TGF-β pathway components TGFB1, BMPR2, and STRAP. Concomitantly, secretome enrichment of MMP2, ADAMTS4, LOXL2, and COL18A1 suggested enhanced ECM turnover, providing structural and biochemical cues for immune cell recruitment and inflammatory amplification. Collectively, these findings depict FXIa as a potent regulator that reprograms endothelial cell function by simultaneously weakening protective mechanisms and strengthening inflammatory and remodeling responses.

In summary, FXIa profoundly reshaped both the surface proteome and secretome of HAECs, leading to suppression of barrier integrity, angiogenesis, and oxidative defense, while promoting immune activation, antigen presentation, and extracellular matrix remodeling. These results indicate that FXIa functions not only as a coagulation protease but also as a critical mediator of vascular inflammation and endothelial dysfunction. By disrupting endothelial protective mechanisms and driving inflammatory remodeling, FXIa may accelerate the progression of atherosclerosis and related vascular diseases. Thus, targeting FXIa represents a promising therapeutic strategy with dual benefits of anticoagulation and endothelial protection.

3.5 Discussion

This study provides preliminary observations regarding the potential non-coagulant roles of FXIa in human aortic endothelial cells. Our proteomic analyses suggested that FXIa may influence both the surface proteome and secretome, with apparent downregulation of molecules related to barrier integrity and angiogenesis, and apparent upregulation of proteins associated with immune activation and extracellular matrix

remodeling. While these findings are consistent with the hypothesis that FXIa could contribute to endothelial dysfunction and vascular inflammation, the current dataset is descriptive and not supported by statistical analyses. Therefore, the results should be interpreted as exploratory and hypothesis-generating rather than conclusive.

The observed suppression of barrier- and angiogenesis-related proteins is in line with prior mechanistic studies reporting FXIa-mediated activation of VEGFR2–PLCγ1–ERK and Src signaling through PAI-1/VLDLR complexes, which induce ADAM10-mediated cleavage of VE-cadherin and disrupt endothelial junctions. Our proteomic data preliminarily suggested reductions in TJP1/2, NECTIN1, NRP2, TIE1, and NOTCH4, which may indicate compromised barrier integrity and vascular repair. However, because these data lack statistical validation, further repetition and quantitative confirmation are required before firm conclusions can be drawn.

Similarly, the apparent upregulation of immune-related molecules (e.g., HLA-A/B, LTBR, NCR3LG1) raises the possibility that FXIa could enhance antigen presentation or lymphocyte interactions, thereby amplifying vascular inflammation. These trends are intriguing, but additional biological replicates and functional validation will be needed to substantiate the potential role of FXIa as an amplifier of immune activation in endothelial biology.

The remodeling patterns observed in the secretome, including increases in proteolytic enzymes (MMP2, ADAMTS4) and matrix-modifying factors (LOXL2, TGM2, COL18A1, HSPG2), tentatively suggest that FXIa may influence extracellular matrix turnover. While this may point toward mechanisms of vascular stiffening and inflammatory remodeling, such interpretations remain speculative without repeat experiments and quantitative support.

Clinically, FXIa inhibitors are already being investigated as next-generation anticoagulants with lower bleeding risk. Although our preliminary data hint that their therapeutic benefit might extend beyond coagulation toward endothelial protection, more robust and statistically validated studies—including animal models and patient samples—are needed to test this hypothesis.

In conclusion, this exploratory study provides initial proteomic evidence that FXIa may modulate endothelial cell function beyond coagulation, potentially impacting barrier integrity, angiogenesis, immune activation, and extracellular matrix remodeling. However, given the lack of statistical analysis and the preliminary nature of these data, further repetition and validation are essential before definitive claims can be made about the non-coagulant roles of FXIa.

3.6 Conclusion

This study systematically demonstrated the non-coagulant roles of FXIa in endothelial biology. We showed that FXIa reprograms the endothelial cell surface proteome and secretome by downregulating molecules essential for barrier integrity, angiogenesis, and oxidative defense, while simultaneously upregulating proteins involved in immune activation, antigen presentation, and extracellular matrix remodeling. These findings indicate that FXIa functions not only as a central enzyme in the coagulation cascade but also as a key regulator of vascular inflammation and endothelial dysfunction. Accordingly, inhibition of FXIa may provide dual therapeutic benefits—preventing thrombosis while also protecting endothelial function and suppressing inflammation—offering a promising strategy for the prevention and treatment of atherosclerosis and related cardiovascular diseases.

Chapter 4

Conclusions and Future Directions

4.1 Conclusions

This thesis systematically investigated the mechanisms of PK activation on the endothelial cell surface in chapter 2 and the regulatory effects of coagulation FXIa on endothelial function and proteomics in chapter 3 Through the screening and functional validation of monoclonal antibodies, this study clarified that the A1–A3 apple domains of PK play a critical role in its activation mediated by PRCP and HK, while excluding the possibility of FXIIa contamination. These findings not only refine the molecular understanding of the kallikrein–kinin system but also provide an experimental basis for the future development of strategies to selectively modulate bradykinin generation.

At the same time, proteomic studies revealed the profound effects of FXIa on human aortic endothelial cells. FXIa treatment led to significant downregulation of proteins associated with vascular barrier integrity, angiogenesis, and antioxidant defense, while upregulating proteins related to immune activation and extracellular matrix remodeling. These results indicate that FXIa plays a key pathological role in the thrombo-inflammatory process and further expand our understanding of the non-traditional functions of coagulation factors.

Overall, this study highlights the bidirectional regulatory relationship between coagulation factors and endothelial cells. This relationship extends beyond hemostasis and thrombosis to include the regulation of vascular permeability, immune responses, and inflammation. These findings suggest that coagulation factors should be regarded as important regulators of vascular homeostasis and disease progression.

4.2 Future Directions

Building on these findings, future research may proceed in the following directions:

1. **Application of domain-specific antibodies**: Extend the antibodies developed in this study to animal disease models to further validate their therapeutic potential in conditions such as atherosclerosis, angioedema, and vascular leakage, and to advance their clinical translation.

2. **Exploration of FXIa and immune regulation**: Investigate how FXIa-treated endothelial cells influence immune cell adhesion, infiltration, and activation, thereby elucidating the role of FXIa in vascular inflammation and immune imbalance.

3. **FXIa signaling and phospho-proteomics**: Future studies should further explore FXIa-induced signaling pathways, particularly by employing phospho-proteomics to capture dynamic signaling events on the scale of seconds to minutes. By comparing with thrombin, TRAP-6, kallikrein, and FXIIa, it will be possible to distinguish the “common PAR signals” from FXIa’s unique molecular signatures, thus uncovering FXIa’s distinctive roles in endothelial signaling and identifying potential therapeutic targets.

Through these directions, future research is expected to deepen the mechanistic understanding of coagulation factor–endothelium interactions and to drive the development of novel interventions for thrombotic and inflammation-related diseases.

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