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MOLECULAR DETERMINANTS OF VITAMIN K-DEPENDENT PROTEASE SIGNALLING

A Thesis Submitted to the University of Dublin, Trinity College, for the Degree of Doctor of Philosophy in the Faculty of Medicine

Submitted August 2013

Revised October 2013

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DECLARATION

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SUMMARY

In addition to facilitating fibrin clot formation, a growing body of evidence suggests that coagulation factors play an important role in regulating the innate immune response to infection. Vitamin K-dependent (VKD) proteases that control haemostasis also possess the ability to signal on cells via specific receptors to either exacerbate or inhibit inflammation. In this thesis, the capacity of activated protein C (APC), factor VIIa (FVIIa) and factor Xa (FXa), which share significant structural and receptor binding homology, to modulate inflammatory signalling pathways was investigated.

APC mediates cytoprotective effects on endothelial cells via interaction with the endothelial protein C receptor (EPCR) and activation of protease activated receptor 1 (PAR1). Despite binding to EPCR with similar affinity to APC, FVIIa failed to emulate APC-like cytoprotective signalling on endothelial cells and was unable activate PAR1. Further, substitution of the APC Gla and EGF1 domains with those of FVIIa ablated APC PAR1 signalling, indicating that the light chain of APC possesses features that are crucial to its cytoprotective activity on endothelial cells. FXa, which binds to EPCR with reduced affinity, mediated EPCR- and PAR1-dependent endothelial barrier protective signalling and anti-apoptotic effects on endothelial cells. Similarly, the presence of FX displayed endothelial barrier stabilising properties via interaction with annexin-2.

APC can also mediated anti-inflammatory signalling on monocytes, independently of PAR1. In this thesis, FXa is shown to limit pro-inflammatory cytokine production by monocytes and macrophages in response to inflammatory stimuli. FXa anti-inflammatory signalling on monocytes and macrophages also occurred independently of PAR1, via
apolipoprotein E receptor 2 (ApoER2)-dependent activation of PAR2. Thus, FXa displays cytoprotective and anti-inflammatory signalling properties on endothelial cells and myeloid cells which are comparable to those exerted by APC.

The anti-inflammatory efficacy of APC has led to its translation into a therapeutic agent for severe sepsis. Despite this, its clinical use has been limited by the risk of severe bleeding complications. Recently, our laboratory demonstrated that APC glycosylation crucially regulates APC signalling such that β-APC, a naturally occurring glycoform replete of a single N-linked glycan moiety, possesses significantly enhanced endothelial cell cytoprotective function. In this thesis, we show that β-APC displays enhanced PAR1-dependent anti-inflammatory activity on macrophages, but not ApoER2-dependent anti-inflammatory activity on monocytes. The enhanced activity mediated by removal of the Asn-329 glycan moiety occurs as a consequence of improved PAR1 proteolysis by APC. These results suggest that β-APC is the predominant glycoform mediating PAR1-dependent signalling by APC in vivo and may lead to APC preparations with enhanced signalling efficacy for use in the treatment of inflammatory disease.
PUBLICATIONS ARISING FROM THIS THESIS


In Preparation

- **Gleeson EM.** Kenny BA, O’Donnell JS, Preston RJS. Novel role of the APC light chain in enabling EPCR-dependent PAR1 proteolysis. *Manuscript in Preparation*

- **Gleeson EM,** Ní Áinle F, Kenny BA, O’Donnell JS, Coughlin SR, Preston RJS. Activated protein C glycosylation status dictates PAR1 proteolysis and anti-inflammatory signalling efficacy. *Manuscript in Preparation*
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ABBREVIATIONS

ADP  adenosine diphosphate
Ang  angiopoietin
Ala; A alanine
ALS  amyotrophic lateral sclerosis
AP-1 activator protein-1
APC  activated protein C
ApoER2 apolipoprotein E receptor 2
APTT activated partial thromboplastin time
Arg; R arginine
Asn; N asparagine
Asp; D aspartate
AT  antithrombin
ATP  adenosine triphosphate
BMDM bone marrow derived macrophages
BSA  bovine serum albumin
cDNA complementary deoxyribonucleic acid
C-terminal carboxy terminal
Cys; C cysteine
Dab1 disabled 1
DAMP danger associated molecular patterns
DIC  disseminated intravascular coagulation
DNA  deoxyribonucleic acid
DVT  deep vein thrombosis
E.coli  Escherichia coli
EDTA ethylenediaminetetraacetic acid
EGF  epidermal growth factor
ELISA enzyme linked immunosorbent assay
EPCR endothelial protein C receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPR-1</td>
<td>effector cell protease receptor 1</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETP</td>
<td>endogenous thrombin potential</td>
</tr>
<tr>
<td>FV(a)</td>
<td>(activated) factor V</td>
</tr>
<tr>
<td>FVII(a)</td>
<td>(activated) factor VII</td>
</tr>
<tr>
<td>FVIII(a)</td>
<td>(activated) factor VIII</td>
</tr>
<tr>
<td>FIX(a)</td>
<td>(activated) factor IX</td>
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<tr>
<td>FX(a)</td>
<td>(activated) factor X</td>
</tr>
<tr>
<td>FXI(a)</td>
<td>(activated) factor XI</td>
</tr>
<tr>
<td>FXII(a)</td>
<td>(activated) factor XII</td>
</tr>
<tr>
<td>FXIII(a)</td>
<td>(activated) factor XIII</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>Fuc</td>
<td>fucose</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin sulphate</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-carboxyglutamic acid</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
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<tr>
<td>GlcNAc</td>
<td>N-Acetyl-D-glucosamine</td>
</tr>
<tr>
<td>Gln; Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu; E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Gly; G</td>
<td>Glycine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>HEK293</td>
<td>hamster embryonic kidney-293 cell line</td>
</tr>
<tr>
<td>His; H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HMWK</td>
<td>high molecular weight kininogen</td>
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<tr>
<td>HRP</td>
<td>horse radish peroxidise</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td></td>
<td>xviii</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>K</td>
<td>kunitz</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Leu; L</td>
<td>Leucine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>Lys; K</td>
<td>Lysine</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MCP1</td>
<td>monocyte chemotactic protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium Eagle</td>
</tr>
<tr>
<td>MCH</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MPs</td>
<td>microparticles</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PAR</td>
<td>protease activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>protein C inhibitor</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerise</td>
</tr>
<tr>
<td>PECs</td>
<td>peritoneal macrophages</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
</tbody>
</table>
PK  
plasma kallikrein

Pro; P  
Proline

PRR  
pathogen recognition receptor

PZ  
protein Z

S1P  
sphingosine-1 phosphate

S1P1  
S1P receptor 1

SEM  
standard error of the mean

SDS-PAGE  
sodium dodecyl sulphate polyacrylamide gel electrophoresis

Ser; S  
Serine

SHBG  
sex hormone-binding globulin

SK  
sphingosine kinase-1

TF  
tissue factor

TFPI  
Tissue Factor Pathway Inhibitor

Thr; T  
Threonine

TLR  
toll-like receptor

TM  
thrombomodulin

TNFα  
tumour necrosis factor-α

tPA  
tissue plasminogen activator

TxA2  
thromboxane A2

Tyr; T  
Tyrosine

uPA  
urokinase plasminogen activator

VCAM-1  
vascular cell adhesion molecule-1

VKD  
vitamin K-dependent

VKOR  
vitamin K epoxide reductase multiprotein complex

v/v  
volume per unit volume

VWF  
von Willebrand factor

w/v  
weight per unit volume
CHAPTER 1: INTRODUCTION

1.1: Blood Coagulation

Haemostasis refers to the maintenance of a closed and fluid circulatory system. In the event of vascular injury, a series of events designed to rapidly halt blood loss from the site of damage occur that facilitate subsequent repair of the vasculature. This is achieved firstly by vasoconstriction of the damaged blood vessel to reduce blood flow to the site of injury and secondly via platelet aggregation and blood coagulation, which seals the breach in the vessel wall. Blood coagulation is therefore a cellular and biochemical response to vessel wall injury which involves formation of a temporary platelet plug and fibrin generation to reinforce platelet plug integrity\(^1\). These processes are followed by tissue repair and clot dissolution.
1.1.1: Primary haemostasis

Primary haemostasis refers to the process by which platelets adhere to the sub-endothelial surface to form aggregates in response to vascular injury\(^1\). Damage to the endothelium disrupts the barrier between plasma clotting components and the sub-endothelial matrix, which is composed of collagens, tissue factor (TF), fibronectin, laminin and other matrix proteins\(^2\). Initial platelet accumulation at the site of injury is facilitated by von Willebrand factor (VWF), a large multimeric plasma glycoprotein synthesized in endothelial cells and megakaryocytes\(^3\). Upon exposure of the sub-endothelial surface, VWF binds fibrillar collagen of the vessel wall via its A3 domain. Under shear forces present within the vasculature, collagen-tethered VWF unwinds to form strings, revealing multiple binding sites for the platelet surface receptor glycoprotein Ib alpha subunit (GPIb\(\alpha\)). This process enables platelet adherence via the GP1b/IX/V complex, a complex comprised of GPIb\(\alpha\), GPIb\(\beta\), GPIX and GPV subunits, and platelet “rolling” along VWF strings\(^4\).

Tethering to VWF strings facilitates activation of platelets by thrombin cleavage of protease activated receptor 1 (PAR1), a cell surface thrombin receptor, and other platelet agonists present in the injured vessel microenvironment. In addition, collagen interacts directly with immobilized platelets via the platelet receptor glycoprotein VI. Platelet activation leads to massive conformational change in platelet integrins \(\alpha_{26}\beta_3\) and \(\alpha_{26}\beta_1\), greatly enhancing their affinity for VWF, fibrinogen and collagen\(^5,6\). Activated platelets express soluble messengers such as ADP and thromboxane \(A_2\) (TXA\(_2\)) which recruit further platelets to the site of injury. Inter-platelet bridges are formed via \(\alpha_{26}\beta_3\) and adhesive proteins such as fibrinogen, fibrin and VWF\(^4\). Platelet activation induces scramblase-mediated transport of
negatively-charged phospholipids to the platelet surface, providing a catalytic surface for enzyme complexes of the coagulation cascade.

**Figure 1.1: Platelet aggregation:** Platelet aggregation is initiated by platelet adherence to exposed collagen or VWF at the site of vascular injury. In large blood vessels, where the rate of flow and shear stress is high, VWF is required to mediate high affinity interactions with platelet GPIbα in order to tether circulating platelets to the vessel wall. Reduced shear rate enables direct interactions between platelets and sub-endothelial proteins such as collagen. Platelet activation then occurs, resulting in structural transformation and release of soluble mediators ADP and TxA₂, which recruit and activate other platelets at the site of injury.
1.1.2: The coagulation cascade

Breach of the endothelial barrier simultaneously exposes sub-endothelial TF, the trigger for the coagulation cascade. TF is a 47kDa transmembrane glycoprotein that is constitutively expressed by cells of the sub-endothelial tissue, including vascular smooth muscle cells, pericytes and adventitial fibroblasts. Upon exposure to blood, sub-endothelial TF acts as the initiator of the extrinsic pathway of coagulation via formation of a complex with activated vitamin K-dependent (VKD) serine protease factor VII (FVIIa), which catalyzes the activation of VKD zymogens factor X (FX) and factor IX (FIX) to FXa and FIXa respectively.

The historical paradigm of haemostasis, in which haemostatic TF exists solely as a sub-endothelial receptor excluded from the vascular lumen by an intact endothelial layer, has been challenged by the observation that TF is expressed by cells circulating within the bloodstream. Exposure to bacterial endotoxin, lipopolysaccharide (LPS), can induce TF expression on peripheral blood monocytes both in vitro and in vivo and platelets have been reported to express TF in an activation-dependent manner, although this has been challenged by several groups. A soluble form of TF has been detected circulating in healthy volunteers and is increased in patients with cardiovascular disease, diabetes, sickle cell disease and endotoxemia. This soluble form of TF exists upon small cell fragments, or microparticles (MPs), released from activated or apoptotic leukocytes. Endothelial cells have also been reported to express TF in response to LPS and TF has been detected on circulating endothelial cells in a number of disease states including sickle cell disease, atherosclerosis, tuberculosis and inflammatory bowel disease. It remains unclear, however, whether this occurs as a result of endothelial cell TF expression or due to binding of...
haematopoetic cell derived TF-positive MPs to the endothelium, which is subsequently released\textsuperscript{10}.

A proportion of TF within the vasculature is alternatively spliced (asTF), generating a TF isoform lacking the transmembrane domain and possessing an altered C-terminus\textsuperscript{25}. asTF is unable to initiate coagulation\textsuperscript{26}. Further, the majority of circulating TF exists in a cryptic form which also lacks procoagulant activity due to the reduction of a disulphide bond, Cys-186-Cys-209, by the thioredoxin/thioredoxin reductase/NADPH system\textsuperscript{27,28}. Formation of a disulphide bond between these residues decrypts TF, restoring procoagulant function. The mechanism through which TF decryption is achieved \textit{in vivo} is incompletely understood but is thought to involve protein disulphide isomerase\textsuperscript{29}. The importance of circulating haematopoetic cell-derived TF in thrombus formation \textit{in vivo} has been established using wild type mice transplanted with the bone marrow of mice expressing only 1% normal levels of TF\textsuperscript{30}. Upon vessel injury in these mice fibrin deposition occurs predominantly adjacent to the vessel wall and thrombus size is significantly reduced, implicating a role for haematopoetic cell-derived TF in thrombus development. The role of haemopoetic cell-derived TF in thrombus formation was further underscored in a recently described murine model of deep vein thrombosis (DVT), in which pathological thrombus formation required monocytic TF\textsuperscript{31}.

The role of sub-endothelial TF in the initial phase of coagulation is well established. Once decrypted, TF binds to and activates FVII via induction of conformational changes in the serine protease domain\textsuperscript{32}. This interaction is enhanced by coincident FVII(a) Gla domain binding to the negatively charged phospholipid surface\textsuperscript{33}. Once activated, FVIIa remains associated with TF to form a ternary complex with either FX or FIX, converting them to FXa
and FIXa respectively\textsuperscript{34}. FXa generated by the ternary (or 'extrinsic tenase') complex activates tiny amounts of prothrombin to thrombin. Thrombin generated in this initial phase is incapable of supporting thrombus formation but is sufficient to activate procoagulant cofactors factor V (FV) and factor VIII (FVIII)\textsuperscript{35}. FVa and FVIIIa accelerate the enzymatic activity of the prothrombinase and intrinsic tenase complexes respectively and their generation results in rapid amplification of thrombin generation\textsuperscript{36}. FVIIIa associates with FIXa on the surface of negatively-charged phospholipids in the presence of Ca\textsuperscript{2+} to form the intrinsic tenase complex, which converts zymogen FX to FXa. Binding of FIXa to FVIIIa in the intrinsic tenase complex enhances the efficacy of FX activation by FIXa 200,000-fold\textsuperscript{37}. During the propagation phase of coagulation, the intrinsic tenase complex is the principal activator of FX, doing so 50-fold more efficiently than the extrinsic tenase\textsuperscript{38}.

An alternative process exists by which thrombin generation can be initiated that does not require TF. This process is referred to as the 'contact pathway' and is initiated by activation of factor XII (FXII) to FXIIa\textsuperscript{39}. Contact with the negatively charged phospholipid surface of activated platelets in the presence of high molecular weight kininogen (HMWK) and plasma kallikrein (PK) induces a conformational change in the FXII zymogen resulting in generation of small amounts of FXIIa. Numerous other physiological activators of FXII have also been proposed, including RNA, collagen and polyphosphate\textsuperscript{40}. FXIIa subsequently activates PK, which reciprocally generates additional FXIIa\textsuperscript{39}. FXIIa initiates coagulation via activation of factor XI (FXI) which in turn activates FIX to FIXa, a crucial component of the intrinsic tenase complex.
Interestingly, congenital deficiency of HMWK, PK or FXII is not associated with any bleeding phenotype. FXII-deficient mice are also protected in models of arterial thrombosis, pulmonary embolism and stroke and pharmacological inhibition of contact system activators can confer similar protection in these models. Moreover, blocking expression of contact system factors with antisense oligonucleotides provides protection in models of arterial and venous thrombosis. This suggests that FXIIa is not required for normal haemostasis, but plays an important role in thrombosis. In contrast, FXI deficiency is associated with a variable bleeding tendency, which demonstrates a key role for FXIa in the maintenance of normal haemostasis. The discrepancy in bleeding phenotypes associated with deficiency of FXII and FXI, 2 components of the contact pathway, is attributed to FXIIa-independent activation of FXI by thrombin.

Recently, polyphosphates, linear polymers of inorganic phosphates linked together via high energy phosphoanhydride bonds, have been identified as important stimulators of the coagulation cascade. Polyphosphates of about 60-100 units in length are present in dense platelet granules at a concentration of ~130 mM and are secreted upon activation. Microorganisms also contain polyphosphates that can range in length from hundreds up to thousands of phosphate units. Smith et al. (2006) demonstrated that polyphosphates stimulate initiation of the contact pathway via activation of FXII and also accelerate FV activation, resulting in an accelerated thrombin burst during plasma clotting reactions. Polyphosphate stimulation of coagulation is dependent on polymer length; the capacity of platelet-derived polyphosphates to trigger contact pathway activation is 1000-fold less potent than very long chain polyphosphates, such as those present in microorganisms.
contrast, short polyphosphates secreted by platelets are sufficient to enhance FVa generation and have also been implicated as inhibitors of TFPI function\textsuperscript{49,51,52}. Furthermore, platelet-derived polyphosphate accelerates activation of FXI by thrombin and FXI auto-activation\textsuperscript{53}.

The intrinsic and extrinsic pathways converge at the level of FX activation. FVa associates with FXa on the negatively charged phospholipid surface of activated platelets in the presence of Ca\textsuperscript{2+} to form the prothrombinase complex. The prothrombinase complex generates thrombin rapidly, 300,000 times more efficiently than FXa alone\textsuperscript{54}. Thrombin converts fibrinogen monomers to fibrin, via removal of fibrinopeptides, which spontaneously polymerize to form an insoluble fibrous mesh\textsuperscript{34}. The integrity of the fibrin mesh is stabilized by activated factor XIII (FXIIIa), a transglutaminase activated by thrombin\textsuperscript{55} that covalently cross-links fibrin at Gln and Lys residues\textsuperscript{56} increasing fibrin clot resistance to proteolytic degradation.

In this way primary haemostatic mechanisms in concert with fibrin formation initiate development of a haemostatic plug composed of activated platelets embedded within a fibrin mesh.
Figure 1.2: The coagulation cascade: During blood coagulation, protease-cofactor complexes assemble on negatively-charged membrane surfaces. The extrinsic tenase complex (TF/FVIIa), the intrinsic tenase complex (FVIIIa/FIXa) and the prothrombinase complex (FVa/FXa) are the 3 major enzyme complexes which assemble during coagulation. Each of these complexes requires the presence of Ca^{2+} and anionic phospholipid in order to function efficiently. The end result of blood coagulation is the formation of a fibrin clot.
1.1.3: Regulation of coagulation

Prevention of pathogenic thrombosis in the absence of vascular injury is integral to maintenance of the circulatory system. Unregulated haemostasis can lead to thromboembolic disease, vessel occlusion and ischaemic tissue injury. Thus, there exists a delicate balance between pro-thrombotic and anti-thrombotic regulators in vivo. The constitutively anti-thrombotic endothelial cell surface prevents initiation of coagulation in the absence of vascular injury. Furthermore, the coagulation cascade is regulated by 3 major endogenous anticoagulant mechanisms; antithrombin (AT)

1.1.3.1: Prevention of thrombus formation in the absence of vascular injury

Preclusion of thrombus formation in intact vessels is facilitated by a layer of endothelial cells that line the blood vessel and act as a barrier between quiescent blood clotting components present in plasma and coagulation initiators present in the sub-endothelial matrix. Endothelial cells also produce thromboregulatory components that actively inhibit blood clot formation. Nitric oxide (NO) is constitutively expressed by endothelial cells and acts upon circulating cells to inhibit thrombus formation via reduction of vascular smooth muscle cell contractility, platelet activation and endothelial cell surface adhesion molecule expression. Endothelial cells also express prostacyclin, which amplifies the effects of NO. In addition to these soluble mediators, endothelial cells express the ectonucleoside CD39, a membrane bound protease which degrades the potent platelet activators ATP and ADP.
1.1.3.2: Antithrombin

AT is a 58 kDa, 432 amino acid glycoprotein that inhibits thrombin, FXa, FIIa and FXIa\textsuperscript{57}. AT functions by forming an initial reversible complex with the target protease that causes disruption of the peptide bond between P1 and P1' residues contained within AT's reactive site loop. This disruption leads to formation of a covalent bond between the target protease active site amino acid Ser residue and the AT P1 residue, resulting in conformational changes in both AT and the target protease. Covalent association with AT renders the target protease inactive and the complex is cleared from the circulation\textsuperscript{64}.

The inhibitory efficacy of AT in vivo is dependent upon binding to heparin or heparin sulphate, negatively charged linear polysaccharide molecules present on the surface of vascular endothelial cells and secreted by mast cells\textsuperscript{64}. Heparin's core pentasaccharide binds to specific Lys residues present in AT initiating a conformational change that alters the reactive site loop, enhancing the efficacy of AT in protease inactivation\textsuperscript{65}. Binding of full-length heparin (> 26 saccharide units in length) to AT accelerates its interaction with thrombin 1000-fold and with FXa 10,000-fold\textsuperscript{66}. Clot bound thrombin and FXa are relatively protected from inhibition by AT, suggesting that AT functions to localize and prevent spreading of a growing thrombus\textsuperscript{67,68}.

1.1.3.3: Tissue Factor Pathway Inhibitor

FXa generation by the extrinsic tenase complex is regulated by TFPI. TFPI is a 43kDa, 276 amino acid glycoprotein comprised of 3 Kunitz domains (K1, K2 and K3), an acidic N-terminal region and a highly basic C-terminal region. TFPI is produced by endothelial cells and
platelets. Inhibition of the extrinsic tenase complex by TFPI is proposed to occur in 2 steps. Firstly, the P1 residue (Arg-107) of the K2 domain binds to the active site groove of FXa, inhibiting protease function. This is followed by K1 domain P1 residue (Lys-36) binding to the active site of TF-associated FVIIa resulting in the formation of the quaternary TFPI/FXa/TF/FVIIa complex, which has no catalytic activity.

Recent work by Hackeng et al. (2006) has demonstrated that the inhibitory activity of TFPI is enhanced by protein S. Interaction between protein S and the TFPI K3 domain enhances the affinity of TFPI for FXa. TFPI inhibition of TF-associated FVIIa does not occur in the absence of FXa. Once bound to FVa and prothrombin in the prothrombinase complex, FXa is protected from inhibition by TFPI. As such, TFPI regulates only the initial phase of coagulation.

1.1.3.4: The Protein C pathway

PC is a VKD serine protease which circulates in plasma as a zymogen. PC is converted to activated PC (APC) by the thrombin-thrombomodulin (TM) complex and attenuates coagulation by proteolytic inactivation of FVa and FVIIIa. The anticoagulant function of APC in plasma is dependent upon interaction with its cofactor protein S. As this pathway represents a central area of investigation in this thesis, its components and activity will be discussed later at greater length.
1.1.4: Fibrinolysis

Vascular repair at the site of injury is initiated once thrombus formation has occurred. Platelet derived growth factor (PDGF) is released from platelet α-granules and induces proliferation of vascular smooth muscle cells and fibroblasts. The stable clot is then dissolved via a tightly regulated process referred to as fibrinolysis. Fibrinolysis occurs upon proteolytic activation of plasminogen to plasmin by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Activated plasmin degrades the fibrin mesh into large soluble fragments that are then released into the circulation. tPA-stimulated plasminogen activation is strongly promoted by fibrin. tPA binds to both fibrin and plasminogen to form a ternary complex that results in (1000-fold) enhanced plasminogen activation by tPA. Fibrinolysis by plasmin is inhibited by plasminogen activator inhibitor (PAI)-1, PAI-2, α₂-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI).
1.2: VKD coagulation proteins

FVII, FIX, FX, prothrombin, PC, protein Z and protein S are VKD coagulation proteins characterised by the presence of a γ-carboxyglutamic acid (Gla) domain. FVII, FIX, FX and PC are further comprised of 2 epidermal growth factor (EGF)-like domains and a C-terminal serine protease domain. This domain structure is largely conserved in prothrombin, except for 2 Kringle domains that replace the aforementioned EGF-like domains, and protein Z, which contains a C-terminal pseudocatalytic domain in place of the serine protease domain.

Protein S differs from this template by the presence of 4 EGF-like domains, a thrombin-sensitive region and a sex hormone-binding globulin domain in place of the serine protease domain.

VKD coagulation proteins require post-translational modification by the VKD γ-glutamyl carboxylase in order for the Gla domain to adopt a function conformation. The VKD γ-glutamyl carboxylase converts reduced vitamin K to the vitamin K epoxide while simultaneously converting Glu residues present in the Gla domain of VKD proteins to negatively charged Gla residues via γ-carboxylation. This modification is essential for interaction of the Gla domain with Ca\(^{2+}\), a prerequisite for VKD coagulation protein folding and membrane binding function.
1.2.1: Glycosylation of VKD proteins

More than half of all known proteins possess covalently attached glycan structures and all of the VKD coagulation proteins are glycosylated. Glycosylation can be categorised into 2 types; N-linked and O-linked (Figure 1.3). N-linked glycosylation refers to transfer of a pre-assembled oligosaccharide core structure from a dolichol lipid donor onto specific Asn residues. This process occurs in the endoplasmic reticulum (ER) and is catalysed by the oligosaccharyltransferase enzyme complex. N-linked glycans typically possess a 14-sugar mannose-rich core (Glc$_3$Man$_9$GlcNAc$_2$) that subsequently undergoes remodelling by a series of ER glycosyltransferases and glycosidases.

For N-linked glycosylation to occur, Asn residues must exist as part of the consensus sequon Asn-X-Ser/Thr (where X is any amino acid except Pro). The hydroxy amino acid is postulated to relay a proton from the Asn residue to the active site of the oligosaccharyltransferase, which renders the b-amide group of the Asn residue more susceptible to nucleophilic attack on the dolichol-oligosaccharide complex. The free sulfhydryl group of a cysteine residue can similarly facilitate N-linked glycosylation, allowing glycosylation of Asn-X-Cys sequons, however this is believed to occur at a slower rate. N-linked glycosylation happens concurrently with other post-translational events including protein folding and as a result, not all viable Asn sequons are present at the luminal aspect of the ER membrane long enough for N-linked glycosylation to occur.

O-linked glycosylation refers to attachment of glycans to Ser or Thr residues. O-linked glycosylation differs from N-linked glycosylation in that it occurs in the Golgi and involves sequential addition of monosaccharide units rather than addition of a preassembled...
oligosaccharide core structure. Further, O-linked glycosylation does not require Ser or Thr residues to be present in a specific consensus sequence, although it occurs most commonly in Ser/Thr clusters which are also rich in proline or alanine.

Figure 1.3: Examples of typical N- and O-linked glycan structures expressed on human plasma glycoproteins: Glycans are composed of monosaccharide units, such as sialic acid, hexoses, hexosamines, deoxyhexoses, pentoses and uronic acid linked together. Most secreted and cell surface N-linked glycans are of the complex subtype and therefore are highly branched space-occupying structures, typically capped with negatively charged sialic acid residues. Reproduced from Preston et al., Blood 2013; May 9;121(19):3801-10.
1.3: Coagulation and Inflammation

Although classically believed to constitute independent physiological defence mechanisms, the idea that coagulation and inflammatory pathways are intrinsically linked has become an increasingly accepted paradigm. A large body of evidence demonstrates that activation of inflammation simultaneously triggers activation of the coagulation cascade and reciprocally, many mediators of blood clotting also initiate and modulate inflammatory signalling pathways.

1.3.1: Toll-like receptors

Toll-like receptors (TLRs) are a family of transmembrane signalling pathogen recognition receptors (PRRs) that are crucial for immune recognition of pathogen-associated molecular patterns (PAMPs) and certain endogenous danger-associated molecular patterns (DAMPs). TLR activation results in activation of nuclear factor κB (NF-κB) and activator protein 1 (AP-1) resulting in induction of important mediators of the immune response to infection, including pro-inflammatory cytokines, chemokines and anti-microbial peptides. 13 mammalian TLRs have been identified to date in humans all of which share significant structural homology with each other and with the IL-1 receptor. TLRs possess a ligand binding extracellular leucine-rich repeat (LRR) domain and a cytoplasmic Toll-IL-1 receptor (TIR) domain that upon ligand binding initiates intracellular signalling pathways via interaction with TIR-adaptor molecules such as myeloid differentiation factor 88 (MyD88), myeloid adapter-like protein (MAL), TIR-domain containing adaptor inducing protein inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM).
TLR signalling is mediated via formation of hetero- or homodimers comprised of 2 TLR receptors. Different TLRs recognize different microbial structures. LPS from the cell wall of gram-negative bacteria is recognized by TLR4\(^93\) while lipopeptides and lipoteichoic acid from gram-positive bacteria are recognized by TLR2 in conjunction with TLR1 or TLR6\(^94\). TLR5 is activated by flagellin from bacterial flagella\(^95\) and viral/bacterial nucleic acids are recognized by TLR3, TLR7, TLR8 and TLR9. TLR11 recognizes uropathogenic bacteria and a protozoan-derived profilin-like protein\(^96\). The ligands for TLR10, TLR12 and TLR13 are currently unknown.

TLRs are reported to stimulate coagulation via numerous mechanisms. Activated TLR4\(^97,98\), TLR2\(^99\) and TLR3\(^100\) have been reported to induce expression of TF on peripheral monocytes/endothelial cells upon stimulation. Further, TLR 1, 2, 4, 6, 8 and 9 have been identified on platelets\(^101\) and activation of TLR2 and 4 has been reported to facilitate platelet activation\(^102,103\). TLR-dependent activation of coagulation pathways has also been postulated to contribute to morbidity in septicemia. Experimental studies using porcine and murine models of LPS-induced sepsis demonstrate an increase in circulating TF\(^104\). TF-positive monocytes have also been identified in patients with severe sepsis and circulating TF correlates with unfavourable outcome in cases of meningococcal infection\(^105\). Recently, Kamimura et al. (2005) demonstrated that the anti-inflammatory cytokine IL-10 down-regulates TF expression on human monocytes, indicating the existence of common mechanisms that regulate both inflammation and coagulation\(^106\).
1.3.2: Protease-activated receptors

Coagulation proteases modulate inflammatory pathways via activation of protease-activated receptors (PARs), a subset of the G protein coupled receptor (GPCR) family. GPCRs are typically comprised of 7 transmembrane domains attached to an intracellular C terminus and extracellular N-terminal tail and represent the largest known receptor family. Activation of GPCRs is typically achieved via ligand binding to a site upon or between the transmembrane domains. This initiates conformational change in the intracellular portion of the GPCR that results in substitution of GDP with GTP from the α-subunit of the inactive heterotrimeric (αβγ) G protein, which in turn dissociates the G protein into Ga-GTP and Gβγ subunits. This process initiates activation of GPCR-mediated cytoplasmic signalling. GPCRs can couple to at least 16 heterotrimeric G protein subtypes that can be broadly characterised into Gs, Gi, Gq and G12/13 types. GPCRs can also signal in a G protein-independent manner, for example via β-arrestins. β-arrestin-mediated GPCR signalling leads to activation of Src family kinases and mitogen-activated protein kinases (MAPKs).

PARs possess a unique mode of receptor activation. Activation of PARs requires proteolytic cleavage of the extracellular N-terminal tail, resulting in generation of a ‘tethered ligand’ which binds to the 2nd extracellular loop of the receptor, initiating intracellular signalling (Figure 1.4). To date 4 PARs have been identified: PAR1, PAR2, PAR3 and PAR4. PAR1 was identified in 1991 as the prototypical thrombin receptor. APC, FXa and FVIIa have since been identified as activators of PAR1. PAR2 is activated by trypsin, FXa and FVIIa. PAR3 is activated by thrombin and APC and PAR4 by trypsin, thrombin, FXa and
FVIIa. PAR1, PAR2 and PAR4 can also be activated by synthetic peptides that mimic their respective tethered ligands.

The PAR domain structure is conserved and all 4 share ~25% sequence homology. The activating protease specificity of different PARs is therefore attributed to distinct N-terminal tail cleavage sequences and 2rd extracellular loop tethered ligand recognition sites. PAR activation is unlike other GPCRs in that it is irreversible. Once cleaved, the PAR tethered ligand that cannot diffuse away from the receptor; however the duration of PAR signalling is tightly regulated. PAR signalling can be shut off by phosphorylation of Ser and Thr residues present on the intracellular C-terminus, leading to uncoupling from G-protein signalling, or via β-arrestin-1 binding. Phosphorylation of C-terminal residues initiates receptor internalisation, preventing further protease exposure, which is followed either by lysosomal degradation or recycling of the receptor back to the cell surface.
Figure 1.4: PAR activation and signalling: PARs are comprised of 7 transmembrane domains attached to an intracellular C terminus and extracellular N-terminal tail. PAR activation is mediated by proteolytic cleavage of the extracellular N-terminal tail to reveal a 'tethered ligand' agonist which binds to the 2nd extracellular loop of the receptor. This results in substitution of GDP with GTP from the α-subunit of the inactive αβγ G protein, which dissociates the G protein into Gα-GTP and Gβγ subunits and initiates signalling.
1.3.2.1: PAR1 signalling by thrombin

Thrombin activation of PAR1 leads to activation of both platelets and endothelial cells. Human platelets express PAR1 and PAR4. At low concentrations of thrombin, blockade of PAR1 prevents platelet activation, while at high concentrations blockade of both PAR1 and PAR4 is required to prevent thrombin activation of platelets. Murine platelets express PAR3 and PAR4. Similarly, PAR3 is required for thrombin-mediated activation at low concentrations, while PAR3 and PAR4 appear to act in concert at higher concentrations.

Notably, PAR1 and PAR3, but not PAR4, possess hirudin-like sequences in the N-terminal tail. This sequence binds exosite I of thrombin to facilitate PAR activation and its absence may explain the reduced sensitivity of PAR4 to thrombin activation.

PAR1 constitutes the major mediator of thrombin-endothelial cell signalling in both humans and in mice, inducing expression of platelet activating factors, expression of pro-inflammatory cytokines and endothelial cell barrier permeability. Thrombin activates PAR1 by cleavage at Arg-41 within the N-terminal tail. PAR1 can couple to 3 α G protein subunits; Gαq, Gαi, and Gα12/13. Thrombin activation of PAR1 on cultured endothelial cells results in coupling to Gα12/13 and Gαq, which initiates activation of RAS homolog gene family member A (RhoA). This in turn causes disassembly of adherent junctions and reorganisation of the actin cytoskeleton, leading to transient disruption of the endothelial cell barrier.

Thrombin activation of PAR1 also initiates multiple intracellular kinase cascades leading to nuclear translocation of NF-κB, pro-inflammatory cytokine expression and induction of apoptosis.
1.3.2.2: PAR2 signalling by FVIIa and FXa

PAR2 is activated by trypsin, FXa and FVIIa and can couple to $\alpha_q$, $\alpha_i$, or $\alpha_{12/13}$ subunits. FVIIa activates PAR2 when in complex with TF. Non-coagulant, cryptic TF supports FVIIa-PAR2 activation and has been associated with activation of inflammatory signalling pathways. PAR2 activation induces activation of NF-κB, expression of pro-inflammatory cytokines and adhesion molecules and phosphatidylinositol 3-kinase (PI3K) signalling.

PAR2 activation also initiates G protein-independent signalling through recruitment of β-arrestin and induction of ERK1/2 and p38 MAPK signalling, which modulates cell migration and negatively regulates G protein-coupled receptor signalling pathways. TF-VIIa-PAR2 signalling on cancer cells is thought to play a role in angiogenesis and metastasis and in vivo studies using murine models of tumour metastasis have linked TF-VIIa-PAR2 signalling with tumour progression. FXa can also activate PAR2, as part of the extrinsic tenase complex and via interaction with alternative cell surface receptors.
1.4: Factor X

1.4.1: FX Structure

Human FX is a VKD coagulation zymogen synthesized in the liver that circulates at a concentration of approximately 170nM\(^1\).\(^{141}\),\(^{142}\). The gene encoding human FX spans 27kb on chromosome 13q34 and contains 8 exons and 7 introns\(^1\).\(^{143}\). The signal peptide, the propeptide and the Gla domain are encoded by exons 1 and 2 while exon 3 encodes a short aromatic stack. Exons 4 and 5 encode the EGF-like domains and exon 6 the activation peptide. The serine protease domain is coded for by exons 7 and 8. FX is structurally homologous to VKD coagulation proteins FVII, PC and prothrombin and the amino acid sequence of FX is highly conserved across mammalian species\(^1\).\(^{142}\).

FX is synthesized as a single-chain 59kDa precursor and converted to a heterodimer by excision of an Arg-Lys peptide bond between the second EGF-like domain and the activation peptide (Figure 1.5)\(^1\).\(^{144}\). The heavy chain is 303 amino acid residues in length and contains the activation peptide and the serine protease domain. The light chain, which is connected to the heavy chain by a single disulphide bond at Cys-132-Cys-302, is 139 amino acid residues in length and contains the Gla domain, the aromatic stack and both EGF-like domains. Prior to secretion, FX undergoes post-translational modification. 11 Glu residues in the N-terminal Gla domain of FX are converted to Gla by VKD gamma glutamyl carboxylase (1.2.1) and Asp-63 in the EGF1 domain undergoes \(\beta\)-hydroxylation, a modification required for Ca\(^{2+}\) binding in this domain\(^1\).\(^{145}\). FX possesses 2 N-linked and 2 O-linked glycosylation sites contained within the activation peptide of FX that regulate activation and plasma half life\(^1\).\(^{146}\),\(^{147}\).
1.4.2: FXa procoagulant activity and serpin inhibition

Proteolytic activation of FX by either the intrinsic or extrinsic tenase complex occurs via cleavage of a single Arg-195-Ile-196 peptide bond in the heavy chain to release a 12 kDa activation peptide\(^{142}\). Activation causes conformational rearrangement of a number of residues to facilitate formation of the catalytic triad and the S1 specificity pocket, a binding region that largely determines the substrate specificity of FXa\(^{148}\). Once activated, FXa converts prothrombin to thrombin in the prothrombinase complex. The predominant plasma inhibitors of FXa are AT \((1.1.4.1)\), TFPI \((1.1.4.2)\) and protein Z-dependent protease inhibitor (ZPI) which circulates in complex with protein Z (PZI-PZ). AT and TFPI can inhibit FXa only prior to incorporation into the prothrombinase complex, however interaction with FVa and prothrombin does not protect FXa from inhibition by ZPI-PZ\(^{149}\).
Figure 1.5: Structure of FX(a): The mature FX molecule is a 2 chain polypeptide consisting of a 17-kDa light chain joined to a 45-kDa heavy chain by a disulfide bond. The light chain contains the Gla domain and EGF-like domains separated by a short inter-EGF peptide sequence. The heavy chain contains the activation peptide (AP) and serine protease domain. [↑ - Gla residues; ⚫ - β-hydroxy amino acid residues; □ - N-linked glycans; ○ - O-linked glycans]
1.4.3: PAR signalling by FXa

FXa can activate PAR1, PAR2 and PAR4 with varying efficacy (i.e. PAR2 > PAR1 > PAR4)\textsuperscript{150}. FXa has been described to activate PAR1 and/or PAR2 on Hela cells\textsuperscript{151}, mesangial cells\textsuperscript{152}, osteoblasts\textsuperscript{153}, and endothelial cells\textsuperscript{140,150,154,155} and has been implicated in a wide variety of immunomodulatory roles. FXa induces expression of cytokines and chemokines MCP-1, IL-6 and IL-8 from HUVECs\textsuperscript{156,157} and IL-1 from macrophages\textsuperscript{158}. Additionally, FXa has a potent mitogenic effect upon human fetal lung fibroblasts\textsuperscript{159} and stimulates lymphocyte and human mesangial cell proliferation\textsuperscript{160}.

Conversely, recent studies have identified that FXa activation of PAR1/2 on endothelial cells can mediate downstream anti-inflammatory and cytoprotective signalling, reducing TNF-\alpha-induced activation of NF-\kappaB and endothelial cell barrier permeability\textsuperscript{139,154,161}. Variation in experimental conditions and cell types used may explain the discrepant pro- and anti-inflammatory effects of FXa reported in the literature. A study by Bachli et al. (2003) identified that the duration of exposure impacts hugely upon the nature of FXa signalling with prolongation of incubation periods increasing the pro-inflammatory potency of FXa\textsuperscript{162}. Alternatively, non-canonical PAR signalling, such as that observed in the case of PAR1, may facilitate diverse signalling pathways initiated by FXa activation of PAR2.

Various cell surface receptors have been implicated in FXa signalling on different cell types both in concert with and independently of PARs. A putative FXa receptor, effector cell protease receptor 1 (EPR-1), which binds to FXa via its inter-EGF sequence (residues Leu\textsuperscript{83} to Leu\textsuperscript{88}), was identified on lymphocytes by Altieri et al. (1994) and later on vascular endothelial cells and VSMCs by Nicholson and colleagues\textsuperscript{163,164}. EPR1 was reported to facilitate FXa
signalling leading to stimulation of lymphocyte proliferation\textsuperscript{160}, stimulation endothelial cell mitogenesis and nitric oxide release\textsuperscript{157,164}. Recently, the existence of EPR-1 has been questioned based on the inability to detect EPR-1-specific mRNA transcripts\textsuperscript{165}. Despite this, many reported FXa signalling activities require the 5 amino acid sequence contained within the FXa inter-EGF region postulated to represent the site through which FXa binds EPR-1\textsuperscript{139,140,166}. Bae and colleagues (2010) recently reported this sequence to be crucial for PAR2-mediated barrier protective and anti-inflammatory signalling by FXa on endothelial cells\textsuperscript{139}.

FXa has also been reported to mediate PAR1-dependent endothelial cell barrier protective effects\textsuperscript{139,154} via interaction with the endothelial cell protein C receptor (EPCR; 1.5.3)\textsuperscript{155}. Human FXa possesses a Met residue at position 8 within the EPCR binding motif of the Gla domain \(\omega\)-loop, a position occupied by Leu in EPCR-binding proteins (A)PC and FVII(a). It is currently unknown whether this substitution impacts significantly upon EPCR affinity.

Annexin-2, a member of the ubiquitously expressed annexin family, has also been implicated as a putative co-receptor for FXa-PAR1 signalling on endothelial cells\textsuperscript{167}. Annexins are phospholipid binding proteins thought to participate in the regulation of membrane organization and membrane trafficking of molecules, but can also act as cell surface receptors\textsuperscript{168}. Bhattacharjee \textit{et al.} (2005) reported that FXa binds to annexin-2 via the Gla domain to activate PAR1, however the physiological consequence of this signalling axis has not been further elucidated\textsuperscript{167}.
1.5: Protein C

1.5.1: PC structure

PC is a plasma VKD zymogen first identified by Stenflo et al. in 1976. PC is synthesised in the liver and circulates in plasma at a concentration of ~72nM (4-6µg/mL). The gene encoding human PC spans 11kb and consists of 8 exons and 7 introns. Exons 1 and 2 encode the prepropeptide, the propeptide and the Gla domain. Exons 3, 4 and 5 encode the aromatic stack and the EGF-like domains. The activation peptide and serine protease domain are coded for by exons 6, 7 and 8. PC is synthesised as a single chain polypeptide which is converted to a heterodimer via cleavage of an Arg-Lys peptide bond. Mature PC is comprised of a 41kDa heavy chain, containing the activation peptide and serine protease domain, and a 21kDa light chain containing the signal peptide, the Gla domain and 2 EGF-like domains (Figure 1.6). The protein C serine protease domain contains the Ser-195/His-57/Asp-102 active site as well as several prominent surface loops, the “37 loop” (residues 190-193), the basic “60 loop” (residues 214-222) and the autolysis (148) loop (residues 301-316), which play an important role in substrate interactions.

PC undergoes extensive posttranslational modification. 9 Glu residues present in the Gla domain are converted to Gla and Asp-71 in the EGF1 domain undergoes β-hydroxylation to facilitate Ca²⁺ binding that is required for anticoagulant function. PC contains 4 sites for putative N-linked glycosylation within the EGF1 (Asn-97) and protease domains (Asn-248, Asn-313 and Asn-329) that are highly conserved across mammalian species. Uniquely among VKD proteins, endogenous PC exists in 3 different glycoforms: ~70% of PC circulates fully glycosylated (α-PC), ~25% missing a glycan chain at Asn-329 (β-PC) and
the remaining ~5% missing glycans at both Asn-329 and Asn-248 (γ-PC)\textsuperscript{177}. The molecular basis for partial glycosylation of PC is currently unknown.

**Figure 1.6: Structure of (A)PC:** PC is a heterodimer comprised of a 21-kDa light chain, containing the Gla and EGF-like domains, and a 41-kDa heavy chain, containing activation peptide (AP) and the serine protease domain, joined by a disulfide bond. [\(\hat{\circ}\) - Gla residues; \(\circ\) - β-hydroxy amino acid residues; \(\Downarrow\) - N-linked glycans]
1.5.2: PC activation by the thrombin-TM complex

Zymogen PC is converted to APC by thrombin cleavage at Arg-169, which releases a 6kDa activation peptide to generate a new heavy chain N-terminus\(^{176}\). Thrombin activation of PC is enhanced ~2000-fold by association of thrombin with the endothelial cell glycoprotein receptor TM\(^{178}\). TM is an endothelial cell receptor comprised of a C-type lectin-like domain, 6 EGF-like domains, a Ser/Thr-rich region, a transmembrane domain and a short cytoplasmic tail. During PC activation, thrombin-TM binding occurs via interaction between thrombin exosite 1 and the EGF 5 and 6 domains of TM, a process that inhibits the procoagulant activity of thrombin\(^{179}\). PC-TM binding is mediated via interaction between the PC surface loops 37, 60 and the autolytic loop with the EGF4 domain of TM\(^{180-182}\).
1.5.3: The endothelial protein C receptor

Initially characterised by Fukudome et al. (1996), EPCR is a 46kDa, 238 amino acid transmembrane receptor which enhance the rate of PC activation by thrombin-TM. In vitro, the presence of EPCR results in a ≈3-fold reduction in K_m for protein C activation by thrombin-TM complex on endothelial cells, causing a ≈3-fold enhancement in PC activation. In baboons, antibody inhibition of EPCR reduced APC generation 20-fold, suggesting the role of EPCR in PC activation in vivo may be more prominent than observed using cultured endothelial cells.

EPCR is also expressed on vascular smooth muscle cells, eosinophils, neutrophils, monocytes, keratinocytes, hippocampal neurons and placental trophoblasts. Further, EPCR has recently been detected on the surface of bone marrow and fetal liver embryonic haematopoietic stem cells (HSCs). The gene encoding human EPCR is located on chromosome 20q11.2, spans 6 kb and is comprised of 4 exons and 3 introns. Exon 1 encodes the 5'-untranslated region and signal peptide. The majority of the extracellular region is coded for by exons 2 and 3, while exon 4 encodes the remaining extracellular domain, the transmembrane region, the cytoplasmic tail and the 3'-untranslated region.

EPCR shares both sequence and structural homology with the major histocompatibility class 1 (MHC)/CD1 family of proteins, in particular murine CD1d. The EPCR α-1 and α-2 domains consist of 2 antiparallel α-helices separated by a MHC-like hydrophobic lipid filled groove that sits upon an 8-stranded β-sheet platform. Unlike MHC class I/CD1 receptors, however, EPCR lacks the α-3 domain and therefore is unlikely to
associate with β-2 microglobulin. EPCR contains 4 putative N-linked glycan attachment sites (Asn-30, Asn-47, Asn-119, and Asn-155) which do not appear to affect ligand binding.

Zymogen PC and APC possess identical EPCR-binding motifs in the N-terminal Gla domain and bind EPCR with comparable affinity. (A)PC-EPCR binding is mediated via hydrophobic interactions between Phe-4 and Leu-8 located in the conserved ω-loop of the (A)PC Gla domain and Tyr-154 and Thr-157 residues at the distal end of EPCR α-1 and α-2 chains. Additionally, (A)PC Gla residues Gla-7, Gla-27 and Gla-29 partake in hydrogen bonding with EPCR residues Glu-86, Arg-87 and Gln-150. The Gla domain ω-loop amino acid residues which mediate EPCR binding are highly conserved across other VKD proteins, and are identical in FVII(a). Recent studies have confirmed that FVII(a) constitutes an additional EPCR ligand, binding to recombinant soluble and cell-bound EPCR with similar affinity to (A)PC. FX(a) has also been reported to interact with EPCR, although the affinity of FXa for EPCR in comparison to APC and FVIIa is not established.
1.5.4: APC anticoagulant pathway

APC anticoagulant activity is mediated via inactivation of cofactors FVa and FVIIIa\textsuperscript{205,206}. APC inactivation of FVa occurs via cleavage at Arg-306, Arg-506 and Arg-679\textsuperscript{179}. APC cleavage of FVa initially occurs at Arg-506 and results in generation of a FVa intermediate, with reduced cofactor activity due to diminished affinity for FXa. Subsequent cleavage at Arg-306 cause dissociation of the FVa A2 domain and complete loss of cofactor activity\textsuperscript{207}. Complete inactivation of FVa is greatly accelerated by the presence of negatively charged phospholipids\textsuperscript{208}. The function of APC cleavage of FVa at Arg-679 has not been established. Of note, FVa degradation by APC on the surface of activated platelets is markedly less efficient than upon the surface of endothelial cells\textsuperscript{209}. The molecular basis for this discrepancy is unclear; however it suggests that APC functions primarily as a regulator of lateral clot expansion by inhibiting thrombin generation at the endothelial perimeter of the clot, rather than extension of the clot upwards into the lumen of the blood vessel.

Efficient APC inactivation of FVa in plasma requires the VKD cofactor protein S. APC anticoagulant activity is enhanced ~50-fold by protein S using \textit{in vitro} purified protein assays, but is entirely dependent upon protein S for anticoagulant activity in plasma\textsuperscript{210}. Protein S binds negatively-charged phospholipids to form a membrane-bound complex with APC which stimulates APC cleavage of FVa at Arg-306, and to a lesser extent, at Arg-506\textsuperscript{179}.

APC also mediates anticoagulant activity via inactivation of FVIIIa, which follows a similar pattern to that of FVa. APC cleaves FVIIIa at Arg-336, Arg-562 and Arg-740 leading to loss of cofactor function\textsuperscript{206}. This process is enhanced by protein S and procofactor FV\textsuperscript{211}. The physiological significance of FVIIIa inactivation by APC is unclear, however, as circulating FVIII
is protected from APC cleavage when bound to VWF and in free form FVIIIa is extremely labile and prone to spontaneous disassembly. Further, incorporation into the intrinsic tenase complex prevents APC inactivation of FVIIIa.
1.5.5: APC cellular signalling

In addition to its anticoagulant function, APC can also modulate inflammation on various cell types via activation of PAR1-dependent and independent signalling pathways\textsuperscript{213}. APC signalling broadly induces anti-inflammatory, anti-apoptotic and endothelial barrier-stabilizing effects and is believed to contribute to the beneficial properties of exogenous APC administration in murine models of inflammatory disease and patients with severe sepsis.

1.5.5.1: APC cytoprotective signalling on endothelial cells

APC mediates cytoprotective signalling on endothelial cells via EPCR-dependent activation of PAR1\textsuperscript{184,214}. In contrast to thrombin-PAR1 signalling, APC activation of PAR1 enhances the integrity of the endothelial cell barrier and protects against thrombin disruption\textsuperscript{215}. Furthermore, APC inhibits endothelial cell NF-κB activation, cell surface adhesion molecule expression and apoptosis\textsuperscript{216-218}.

The molecular determinants of APC cytoprotective signalling are not fully characterised, however EPCR binding is an essential prerequisite to APC-PAR1 signalling on endothelial cells\textsuperscript{214,218}. In addition, Yang \textit{et al.} (2007) have demonstrated that mutation of protease domain residues Glu-330 and/or Glu-333 ablates APC cytoprotective endothelial cell signalling and proteolysis of PAR1, without altering EPCR binding or anticoagulant properties\textsuperscript{219}. This suggests that Glu-330/Glu-333 constitute an important PAR1 binding exosite on APC. The cytoprotective activity of APC on endothelial cells also requires co-localization of PAR1 with EPCR in caveolae, lipid-rich microdomains present on the cell membrane\textsuperscript{220}. It has been suggested that co-localization with EPCR in this manner may select:
PAR1 for APC-specific barrier protective/cytoprotective signalling. This hypothesis is supported by reports that occupancy of EPCR by the Gla domain of zymogen PC can recruit thrombin activation of PAR1 from a “barrier disruptive” to a “barrier protective” signalling pathway. Bae et al. (2007) demonstrated that a meizothrombin chimera possessing a PC Gla domain enhanced endothelial cell barrier integrity, while thrombin itself exerted a barrier-protective effect when co-incubated with an inactive PC mutant, PC$_{5360A}$. This suggests that when occupied by its canonical ligand, EPCR allosterically regulates the nature of PAR1 signalling via currently unknown mechanisms.

The paradoxical consequences of PAR1 activation by thrombin and APC occur due to induction of divergent signalling pathways by PAR1 in response to either activating protease. While thrombin activation of PAR1 results in coupling to $\alpha_{12/13}$ and $\alpha_4$ G protein subunits, APC activation of the same receptor does not induce canonical G protein signalling. Rather, APC-PAR1 activation induces $\beta$-arrestin signalling and recruitment of dishevelled-2, a scaffold protein, which regulates the actin cytoskeleton via activation of Rac. Russo and colleagues (2011) identified that PAR1 present in caveolar microdomains exists in a preassembled complex with $\beta$-arrestin, supporting the hypothesis that this cellular fraction of PAR1 is primed for cytoprotective signalling.

PAR1 signalling by APC, but not thrombin, has also been reported to induce transactivation of the sphingosine-1 phosphate 1 receptor (S1P1) and the angiopoietin (Ang) receptor, Tie2. EPCR-dependent activation of PAR1 by APC induces activation of sphingosine kinase-1 (SK1), an intracellular kinase responsible for generation of sphingosine-1 phosphate (S1P), the ligand for S1P1. S1P1 activation modulates Rac-1 signalling,
cytoskeletal rearrangement and PI3K/Akt signalling. The Ang/Tie2 axis regulates endothelial barrier permeability by activation of SK1. APC enhances expression of Tie2 and Ang1 in addition to inhibiting Ang2 expression in an EPCR- and PAR1-dependent manner.

Recent work has indicated that the site of proteolysis of PAR1 by thrombin and APC is distinct. Thrombin cleaves PAR1 at the canonical Arg-41 site, to generate a tethered ligand agonist beginning at Ser-42. EPCR-dependent cleavage of PAR1 by APC, however, occurs predominantly at Arg-46, generating an alternative tethered ligand agonist beginning at Asn-47. Mutation of the PAR1 Arg-46 cleavage site prevents APC anti-apoptotic effects on endothelial cells and a synthetic peptide mimicking the APC-derived tethered ligand induces APC-like activation of Rac1 and endothelial cell barrier protection both in vitro and in vivo. These studies suggest that PAR1-β-arrestin-mediated cytoprotective signaling activity is mediated by proteolysis of Arg-46 by APC, whereas proteolysis of Arg-41 results in G protein-dependent pro-inflammatory signalling by thrombin.
1.5.5.2: **Non-endothelial cell anti-inflammatory cell signalling by APC**

APC possesses anti-inflammatory and anti-apoptotic signalling properties on other cell types, including monocytes\textsuperscript{231-238}, macrophages\textsuperscript{239}, dendritic cells\textsuperscript{240}, and neuronal cells\textsuperscript{241,242}. Exposure to APC down regulates LPS-induced TNF-\(\alpha\) production on primary human blood monocytes\textsuperscript{234,236}, THP1 monocytic cells\textsuperscript{231} and U937 cells\textsuperscript{237}. APC is also reported to inhibit monocyte expression of other pro-inflammatory cytokines and chemokines such as IL-6, IL-8, IL-1\(\beta\)\textsuperscript{234,238}, monocyte chemotactic protein (MCP) and macrophage inflammatory protein 1 (MIP-1)\textsuperscript{235} in addition to increasing expression of the anti-inflammatory cytokine IL-10\textsuperscript{243}. APC reduces activation of NF-\(\kappa\)B and AP-1 in LPS stimulated human monocytes via inhibition of I\(\kappa\)B\(\alpha\) degradation\textsuperscript{236}.

The mechanism through which APC signals on monocytes is distinct from that observed on endothelial cells. The majority of studies report that APC's anti-inflammatory effects on monocytes occur independently of EPCR and PAR\textsuperscript{1}\textsuperscript{232,237,243}. Yang et al. (2009) recently demonstrated that the anti-inflammatory activity of APC on monocytes is mediated via interaction with ApoER2, a member of the low-density lipoprotein receptor (LDLR) family, which triggers activation of PI3K and GSK3\(\beta\) signalling pathways upon APC binding\textsuperscript{237}. This signalling axis is facilitated by interaction of the ApoER2 cytoplasmic domain NXPY motif with Fyn and Src kinases that initiate phosphorylation of disabled-1 (Dab1). Dab 1 in turn activates PI3K- and GSK3\(\beta\)-dependent signalling\textsuperscript{244}. APC binds to ApoER2 with high affinity\textsuperscript{245} however, the precise residues governing this interaction are currently unknown. Of note, mutation of Glu-149 in the EGF2 domain ablates APC inhibition of LPS-induced cytokine expression on
monocytes, without effecting EPCR-dependent PAR1 activation\(^{238}\). This residue represents a potential site through which APC-receptor interactions on monocytes may be mediated.

Conversely, APC anti-inflammatory signalling on murine macrophages requires PAR1. Cao et al. (2010) demonstrated that both human and murine APC inhibits LPS-induced pro-inflammatory cytokine expression on wild type, but not PAR1\(^{-/-}\) murine bone marrow-derived macrophages (BMDM)\(^{239}\). Interestingly, the same study reported that EPCR is not required for APC activation of PAR1 on this cell type. APC PAR1 signalling was maintained on EPCR\(^{/-}\) BMDMs and Gla-domainless APC mediated a similar anti-inflammatory effect as its wild type counterpart. Instead, APC was shown to bind to integrin CD11b/CD18 on the surface of macrophages and this interaction was essential for APC-PAR1 anti-inflammatory signalling on this cell type.
1.6: Therapeutic use of APC in inflammatory disease

1.6.1: APC administration in *in vivo* models of inflammatory disease

The concept that APC may be of therapeutic benefit in inflammatory disease was first proposed by Taylor *et al.* in 1987, following the observation that inhibition of PC activation was associated with increased mortality in a baboon model of *E.coli*-induced sepsis and furthermore, that APC infusion enhanced survival in the same sepsis model. Initially, the anti-inflammatory effect of APC *in vivo* was attributed solely to down regulation of thrombin generation, a potent pro-inflammatory mediator. *In vitro*, thrombin proteolysis of PAR1 is ~1000-fold more efficient than that of APC and, as thrombin must be present in order for APC generation to occur; the physiological relevance of APC-PAR1 signalling has in the past been questioned. However, experimental studies using PAR1 mice have confirmed that APC cytoprotective signalling via PAR1 occurs *in vivo* and is central to its conferred survival benefit, at least in murine models of inflammatory disease.

In a murine model of LPS-induced endotoxaemia, Kerschen *et al.* (2007) demonstrated that APC administration reduced lymphocyte apoptosis, vascular permeability and mortality in wild type, but not PAR1 mice, confirming an essential role for PAR1 signalling. Consistent with this, a non-proteolytic PC variant, PC<sub>S360A</sub>, did not protect against LPS-induced lethality. Further, the protective effect of APC was substantially reduced in mice expressing <10% normal EPCR levels, suggesting a key role for this receptor.

The precise receptor repertoire through which APC mediates its survival benefit *in vivo* remains controversial, however. Cao *et al.* (2010) demonstrated that Gla-domainless APC, which lacks the EPCR-binding domain, also protects against endotoxaemia-related
mortality albeit less effectively than wild type APC. In the same study, both APC and Gladomainless APC failed to protect against LPS-induced mortality in integrin CD11b^−/− mice indicating that CD11b-facilitated PAR1 signalling on macrophages, rather than EPCR-dependent endothelial cell signalling, may mediate APC protective effects in murine models of sepsis.

A recent study using bone marrow chimeric mice demonstrated expression of both EPCR and PAR1 on immune cells to be critical to the beneficial effects of APC in vivo. APC reduced LPS-induced mortality in mice deficient in endothelial cell PAR1, although to a lesser extent than wild type mice. This suggests that PAR1 signalling by exogenously administered APC on immune cells is of greater significance than PAR1 signalling on the vascular endothelium. Specifically, the protective effect of APC-PAR1 signalling on haemopoetic cells was found to be mediated by EPCR-expressing CD8^+ splenic dendritic cells. EPCR expression by immune cell subsets in mice varies greatly from that reported in humans however, thus the relative contribution of APC-PAR1 signalling on immune cell subsets in human sepsis is difficult to estimate. In contrast, Schuepbach and colleagues (2009) showed that administration of APC reduces endotoxaemia-induced vascular barrier disruption and pulmonary oedema in a PAR1-dependent manner, demonstrating APC EPCR/PAR1-dependent endothelial barrier stabilization occurs in vivo and can limit sepsis-related morbidity. As such, the relative contributions of APC signalling on vascular and immune cells to its therapeutic efficacy in vivo are not fully determined.

Administration of APC has also been shown to confer beneficial effects in other murine disease models. Cheng et al (2003) demonstrated that APC reduced brain infarction
volumes when administered to mice after ischaemic stroke$^{217}$. EPCR deficiency reduced the efficacy of APC in this model and co-administration of anti-PAR1 antibodies completely ablated the protective effect. Further work investigating the role of PARs in APC-mediated neuroprotection demonstrated the importance of both PAR1 and PAR3, with the beneficial effects of APC reduced in both PAR1$^{-/-}$ and PAR3$^{-/-}$ mice$^{242}$. Co-administration of APC with tPA (a clot-lysing agent used in stroke treatment) has been shown to reduce cerebral injury in a murine model of stroke$^{250}$ and prevent tPA-induced haemorrhage, a pathogenic side effect of tPA use$^{251}$. Importantly, administration of APC is effective post transient brain ischaemia$^{252}$ and administration of 3K3A-APC, a non-anticoagulant 'cytoprotective signalling selective' variant, also enhances the therapeutic efficacy of tPA in rodent models$^{253}$. The adjunctive use of recombinant APC with tPA in stroke in humans is currently being assessed in Phase II clinical trials.

In addition to reducing brain infarction volumes in stroke, APC has been shown to slow disease progression and extend survival in a murine model of amyotrophic lateral sclerosis (ALS)$^{254}$ and APC can improve functional outcome in mice subjected to traumatic brain injury$^{255,256}$. Administration of APC has also been demonstrated to be therapeutic in murine models of diabetic nephropathy$^{257}$, multiple sclerosis$^{258}$ and inflammatory bowel disease$^{259}$, but the molecular basis of the protective effect of recombinant APC is not fully characterised in these models.
1.6.2: APC therapy in severe sepsis

Sepsis is the leading cause of mortality in non-coronary intensive care units, and accounts for 200,000 deaths per annum in the USA incurring a financial burden of $17 billion annually. Plasma PC levels are reduced in up to 80% of patients with severe sepsis and are inversely associated with morbidity and mortality. Despite this, use of recombinant APC in the treatment of severe sepsis has produced confounding results. The PROWESS trial, carried out in 2001, reported that administration of recombinant APC reduced the risk of mortality by 19.4% in severe sepsis, leading to its approval by the FDA for use in this setting. The subsequent ADDRESS trial, evaluating the use of recombinant APC in patients with a low risk of death, demonstrated no enhancement in 28-day mortality, however. Similarly, in paediatric sepsis, the RESOLVE trial failed to observe any significant difference in 28-day mortality between APC and placebo groups. A systematic review published by the Cochrane Collaboration in 2011 advocated against the use of recombinant APC and recently, the PROWESS-SHOCK trial failed to identify any survival benefit associated with the use of APC in severe sepsis, leading to its withdrawal from the market.

Differences in inclusion criteria, dosing strategy and initiation of treatment may account for the discrepant outcomes of different clinical trials as the bleeding risk associated with APC administration discourages its use at dosages that are likely to be therapeutic. The ENHANCE trial confirmed the importance of APC initiation and dosing, demonstrating that early commencement of recombinant APC administration enhanced its ability to reduce mortality. In an effort to improve APC therapy in severe sepsis, APC variants with altered anticoagulant and cytoprotective signalling functions have been engineered. Mosnier et al.
2004) synthesized an APC variant (5A-APC) with impaired ability to bind FVa due to mutations in the positively charged surface loop that participate in APC-substrate interactions. This variant possessed normal EPCR-PAR1 cytoprotective function on endothelial cells but severely (>95%) reduced anticoagulant activity and reduced mortality in a murine model of endotoxaemia in a similar manner to wild type APC. Using an alternative approach, Bae et al. (2007) eliminated APC anticoagulant activity by engineering a disulfide bond in the Ca\textsuperscript{2+}-binding 70-80 loop, while maintaining EPCR-PAR1 barrier stabilizing function.

2 APC variants with altered properties have previously been generated in this laboratory. Preston et al. (2006) eliminated APC interaction with its anticoagulant cofactor, protein S, via a single point mutation at Leu-38, reducing APC anticoagulant efficacy in plasma ∼50-fold. Ni Áinle et al. provided the first description of an APC variant with enhanced cytoprotective signalling function. Mutation of the glycan attachment site at Asn-329, to generate the APC\textsubscript{N329Q} variant, increased APC barrier protective and anti-apoptotic signalling 5-fold compared to wild type APC. Combination of these point mutations to generate APC\textsuperscript{L38D/N329Q} produced a "non-anticoagulant" APC variant with enhanced cytoprotective signalling function. This variant yields significant therapeutic promise, potentially conferring enhanced anti-inflammatory effects without incurring any bleeding side effects. Notably, APC\textsubscript{N329Q} possesses a glycosylation profile identical to that of the β glycoform of PC. Thus, the generation of APC\textsubscript{N329Q} not only provides a novel therapeutic tool, but may inform the molecular mechanism regulating APC-PAR1 signalling in vivo.
**1.7: Project Aims**

A growing body of evidence illustrates the importance of VKD protease-mediated regulation of inflammation via PAR activation. APC cytoprotective signalling has been observed both *in vitro* and *in vivo* and has been translated into a therapeutic agent for severe sepsis. Numerous other VKD proteins share significant structural and receptor binding homology with APC, yet their capacity to modulate cytoprotective and anti-inflammatory signalling pathways is poorly defined. The clinical use of APC has been limited by the risk of severe bleeding complications. Recent work in our laboratory suggests that APC glycosylation may crucially regulate APC signalling, such that β-APC possesses significantly enhanced cytoprotective function and represents a potentially improved therapeutic tool. However the molecular basis for this enhanced activity is unknown. This project aims to:

- Investigate the molecular regulation of VKD protease EPCR-dependent cytoprotective signalling on endothelial cells

- Determine the capacity of VKD proteases, other than APC, to modulate TLR-induced myeloid cell pro-inflammatory signalling

- Determine the molecular basis of the enhanced PAR1-dependent cytoprotective signalling capacity of a recombinant APC variant mimicking the glycosylation profile of β-APC
CHAPTER 2: METHODOLOGY

2.1: Cell culture

All cell culture was carried out in a class II flow cabinet using standard aseptic techniques. Work surfaces and all materials used within the flow cabinet were sterilised with 70% (v/v) ethanol prior to use. All cells and cell lines used are outlined in Appendix I along with growth conditions. Cells were grown in humidified incubators at a temperature of 37°C with 5% CO₂.

2.1.1: Maintenance of immortalised cell lines

Adherent cell lines (HEK293, HEK293T, HEK Blue, EA.hy926 and RAW Blue) were passaged when 80-90% confluent. Media was decanted from the culture flasks and cells were washed with 5-10 mL sterile PBS buffer warmed to 37°C. In the case of HEK293, HEK293T, HEK Blue and EA.hy926 cells, trypsin was used to dissociate the cultured cells from the flask surface. Cells were incubated with 1-2 mL of 0.25% (v/v) trypsin/EDTA (Invitrogen, Life Technologies) for 5 minutes at 37°C, which was then inactivated by the addition of serum-containing medium. RAW Blue cells were detached using a cell scraper. Pre-warmed culture medium was added to create a cell suspension which was then used to reseed new tissue culture flasks at the density required. THP1 and THP1X-Blue-CD14 cell lines grow in suspension and were maintained between 1x10⁵ and 1x10⁶ cells per mL. Cell number was determined using a hemocytometer (Millipore) and passaged to the density required by addition of pre-warmed culture media. Long term storage of immortalised mammalian cell lines was achieved by cryo-preservation and storage in liquid nitrogen.
2.1.2: Isolation of peripheral blood mononuclear cells (PBMCs) from buffy coat whole blood component

Primary human PBMCs were isolated from buffy coat whole blood component obtained from healthy donors provided by the Irish Blood Transfusion Service. The buffy coat was mixed 1:4 with PBS warmed to 37°C and 10mL volumes of this mixture layered on top of 3mL of Ficoll-HistoPaque 1044 reagent (Sigma-Aldrich) in sterile 15mL Falcon tubes. Tubes were centrifuged at 2000rpm for 30 minutes at 18°C with no brake resulting in separation of the blood into a red cell pellet, white cell layer and plasma/platelet layer. The white cell layer, which resides between the plasma/platelet and the ficoll interface, was removed and washed extensively with PBS. The cells were re-suspended in RPMI 1640 medium supplemented with 1U/mL penicillin/0.1mg/mL streptomycin solution and 10% (v/v) FBS and counted using a hemocytometer. Cells were seeded in 96-well microtitre plates at a density of 2 x10^6 cells/mL, incubated at 37°C/5% CO₂ for 3 hours and subsequently washed twice with PBS to remove any non-adherent cells. Culture medium was replaced and cells were incubated for a further 24 hours prior to use.

2.1.3: Mice

Primary murine macrophages were obtained from wild type BALB/c mice and PAR2⁻/⁻ mice on a BALB/c background (originally from Jackson Laboratories) which were maintained in house by the Translational Immunology Group, Trinity College Dublin. Animals were kept in individually ventilated and filtered cages under positive pressure (Teniplast, Northants, UK) and Specific Pathogen-Free conditions. Mice were fed an irradiated diet and housed on irradiated bedding. Food and water were supplied ad libitum. All animal experiments were
performed in compliance with Irish Department of Health and Children regulations and were approved by the Trinity College Dublin Bio Resources ethical review board.

2.1.4: Culture of murine bone marrow-derived macrophages (BMDMs)

BMDMs, obtained from the femurs and tibia of mice, were isolated using standard techniques\(^{271}\). BMDMs were plated in 6-well plates and cultured in RPMI 1640 medium supplemented with 1U/mL penicillin/0.1mg/mL streptomycin solution, 10\% (v/v) FBS and 10ng/mL granulocyte macrophage colony-stimulating factor (R&D Systems) for 7 days prior to use.

2.1.5: Culture of murine peritoneal macrophages (PECs)

PECs were obtained via lavage of the murine peritoneal cavity with 5mL ice-cold PBS. The resulting cells were washed in PBS and re-suspended in RPMI 1640 medium supplemented with 1U/mL penicillin/0.1mg/mL streptomycin solution and 10\% (v/v) FBS\(^{272}\). Cells were seeded in 24-well microtitre plates at a density of 2x10\(^6\) cells/mL, incubated at 37\(^\circ\)C/5\% CO\(_2\) for 3 hours and subsequently washed twice with PBS to remove any non-adherent cells. Culture medium was replaced and cells were incubated for a further 24 hours prior to use.
2.2: Recombinant protein expression

Human wild type PC and variants (PC\textsubscript{N248Q}, PC\textsubscript{N313Q}, and PC\textsubscript{N329Q}) were generated previously in the lab. Recombinant murine wild type PC and murine PC\textsubscript{N330Q} cDNA plasmids previously produced in the laboratory, using a pRc/CMV/PC plasmid template (a kind gift from Professor Bjorn Dahlback, University of Lund, Sweden; Appendix III) using standard cloning techniques\textsuperscript{273}. A recombinant PC/FVII hybrid (PC\textsubscript{FVII-Gla/EGF1}) consisting the FVII Gla and EGF1 domains attached to the EGF2 and protease domains of PC was also expressed using a pCMV6 plasmid (Appendix III). Large-scale expression of recombinant PC preparations was achieved by stable transfection of a HEK293 cells. Transfected cells were isolated using selective antibiotics and highly expressing colonies expanded. The growth medium containing recombinant PC was collected and isolated using a modified anion-exchange 'pseudo-affinity' chromatography protocol.

2.2.1: Stable transfection of HEK293 cells

HEK293 cells were selected for expression due to their capacity to express recombinant PC with correct post-translational modifications\textsuperscript{274}. Culture medium was supplemented with 10mg/mL vitamin K to facilitate \(\gamma\)-carboxylation of recombinant PC. HEK293 cells were trypsinised and seeded in 6-well microtitre plates at a density of 2 x 10\(^5\) cells/mL. The plate was incubated at 37\(^\circ\)C/5% CO\(_2\) until ~50-70% confluence was reached (approximately 24 hours later).

Plasmids were prepared for transfection by diluting 4\(\mu\)g plasmid cDNA in 250\(\mu\)L OptiMEM reduced serum medium (Invitrogen, Life Technologies). 10\(\mu\)L of lipofectamine 2000
(at 2mg/mL; Invitrogen, Life Technologies), a liposomal transfection reagent, was simultaneously diluted in a separate 250μL volume of OptiMEM. The 2 mixtures were incubated separately for 5 minutes at room temperature then combined and left to stand at room temperature for a further 20 minutes. HEK293 cells grown to confluence in 6-well tissue culture plates were washed twice with 1mL sterile PBS. The plasmid/lipofectamine mixture was added to cells and the plate incubated for 4 hours, after which the cells were washed again with PBS and growth media applied. The cells were incubated at 37°C/5% CO₂ until full confluence was reached (approximately 24-48 hours post transfection). After this, cells were trypsinized and re-suspended in growth medium containing the selective agent G418 at a final concentration of 50μg/mL. Cells were plated in 10cm² dishes and grown in the presence of G418-containing media (replaced every 24 hours to maintain selective pressure) until untransfected HEK293 cells died, leaving behind colonies of transfected cells (approximately 10 days post transfection). Individual transfected colonies were picked and seeded into wells of a 96-well microtitre plate. Once confluent, colonies were assessed for PC expression using an ELISA.

2.2.2: Expression and concentration of recombinant PC

Transfected cell colonies expressing PC were selected, expanded and stored by cryopreservation. Large scale production was achieved by expansion of selected colonies to full confluence in a multi-surface cell vessel (500mL; HYPERflask; Corning). At this point growth medium was replaced with 500mL serum-free OptiMEM containing vitamin K (10μg/mL) and the flask was incubated for 4-5 days. The medium was collected and
concentrated to ~50mL by filtration using a Pellicon XL tangential flow filter (TFF) device (Millipore).

2.2.3: Isolation of fully γ-carboxylated recombinant PC

A Fast Performance Liquid Chromatography (FPLC) system (GE Healthcare) was used to purify recombinant PC. Samples were first desalted into a running buffer (50mM Tris/150mM NaCl, pH 7.4; Appendix II) using a HiTrap HiPrep 26/10 53mL desalting column (GE Healthcare) packed with Sephadex™ G25 superfine. During recombinant expression, PC synthesis often overwhelms the cell’s endogenous capacity to fully γ-carboxylated the PC Gla domain. This posttranslational modification is essential for PC function\(^\text{275}\). Recombinant PC was therefore subjected to “pseudo-affinity” chromatography to isolate the correctly γ-carboxylated fraction using a HiTrap Q HP 5mL sepharose anion exchange column (GE Healthcare).

The HiTrap™ Q HP column was washed with deionised water and then equilibrated with 5 column volumes of running buffer. The sample for purification was passed across the column via super loop injection and PC binds to and retained in the column. The column was then washed with running buffer to remove loosely bound proteins and recombinant PC was then eluted by passing over a Ca\(^{2+}\) containing elution buffer (50mM Tris/150mM NaCl/10mM CaCl\(_2\), pH 7.4; Appendix II). Positively charged Ca\(^{2+}\) ions bind γ-carboxylated PC with high affinity, and thus release it from the column by competing for negatively charged Gla residue binding. Eluted PC was collected in 1mL fractions, pooled and desalted back into running buffer using a 5mL HiTrap™ desalting column (as outlined above). Remaining uncarboxylated PC was subsequently dissociated from the column by 1M NaCl elution.
2.3: Activation of human/murine PC

Activation of recombinant human/murine PC was carried out in 2 ways, depending on the assay in which the APC preparation was to be assessed; using Protac, a thrombin-like snake venom activator isolated from Akigstrodon Contirtrix (Immuno, Sweden) or using the Thrombin CleanCleave kit (Sigma-Aldrich).

2.3.1: Activation of PC using PROTAC

PC (5µg/mL) was incubated with 0.25U Protac in a buffer containing 50mM Tris-HCl/100mM NaCl, pH 7.4 (Appendix II) in a total volume of 1mL, for 1 hour at 37°C followed by overnight incubation at 4°C.

2.3.2: Activation of PC using Thrombin CleanCleave kit

100µl Thrombin agarose gel from the Thrombin CleanCleave kit was re-suspended by gentle agitation and pelleted by centrifugation at 2500rpm for 2 minutes. The agarose gel was washed twice by re-suspension in Tris Buffered Saline (TBS) containing 10mM CaCl₂ (Appendix II) followed by centrifugation as outlined above. PC was desalted into TBS containing 10mM CaCl₂ using Zeba Spin desalting columns (Pierce) and 900µl was mixed with 100µl of the thrombin agarose gel and incubated on a rotating platform for 3 hours. The thrombin agarose was pelleted by centrifugation at 7500rpm for 5 minutes and the APC preparation decanted. This process was repeated twice to ensure full activation of the PC sample. 1IU hirudin was added to all APC preparations to inactivate any remaining trace of thrombin.
2.4: Human and murine PC ELISAs

Human and murine PC sandwich ELISAs were developed in-house to assess the concentration of all human and murine PC variants utilized.

2.4.1: Human PC ELISA

Maxisorp 96-well plates (Nunc, UK) were coated with a polyclonal sheep anti-human PC antibody (Haematological Technologies Inc.) diluted to a final concentration of 25μg/ml in a carbonate buffer (50mM Na₂CO₃/NaHCO₃, pH 9.6; Appendix II) and incubated overnight at 4°C. Wells were washed 3 times with 400μL TBS/0.05% (v/v) Tween 20 (TBS-T) and incubated with 2% (w/v) bovine serum albumin (BSA) in TBS for 2 hours at room temperature to block non-specific binding sites. A standard curve consisting of serial dilutions of human PC (62.5nM-1000nM) in TBS containing 0.2% (w/v) BSA was used. The standard curve and test samples were applied to the plate in duplicate and incubated for 1 hour at room temperature. The plate was washed and PC bound to each well was detected using horseradish peroxidise (HRP)-conjugated polyclonal sheep anti-human PC antibody (Haematological Technologies Inc.) diluted in TBS-T containing 0.2% (w/v) BSA to a final concentration of 10μg/ml.

HRP-conjugation of the antibody for detection was carried out using the Lightning Link HRP Conjugation Kit (InnovaBiosciences) according to the manufacturer’s instructions. HRP-conjugated polyclonal sheep anti-human PC antibody was incubated with the plate for 1 hour at room temperature. The plate was washed once again and the HRP-conjugated antibody detected using 3,3′,5,5′-Tetramethylbenzidine (TMB). TMB can act as a hydrogen
donor for the reduction of hydrogen peroxide to water by HRP and the resulting diimine causes the solution to take on a blue colour. The reaction was halted by addition of H₂SO₄, turning the solution yellow. The colour change was measured at an optical density (OD) of 450nm at room temperature using a spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader, Molecular Devices, CA, USA).

2.4.2: Murine PC ELISA

Maxisorp 96-well plates were coated with a polyclonal sheep anti-murine PC antibody (Haematological Technologies Inc.) diluted to a final concentration of 15µg/ml in carbonate buffer and incubated overnight at 4°C. Wells were washed 3 times with 400µL TBS/0.05% (v/v) Tween 20 (TBS-T) and incubated with 2% (w/v) bovine serum albumin (BSA) in TBS for 2 hours at room temperature to block non-specific binding sites. A standard curve was developed using serial dilutions of murine plasma in TBS containing 0.2% (w/v) BSA. The PC concentration of murine plasma is ~ 5µg/ml. Murine plasma dilutions ranging from 1/5 to 1/80 (~1000-62.5ng/ml) were used. Serial dilutions of test samples were also carried out in TBS containing 0.2% (w/v) BSA. The standard curve and test samples were applied to the plate in duplicate and incubated for 1 hour at room temperature. The plate was washed as outlined above and murine PC bound to each well was detected using HRP-conjugated polyclonal sheep anti-murine PC antibody (Haematological Technologies Inc.) diluted in TBS-T containing 0.2% (w/v) BSA to a final concentration of 5µg/ml. The plate was incubated for 1 hour at room temperature, washed and the HRP-conjugated antibody detected using TMB as outlined in 2.4.1.
2.5: Assessment of APC amidolytic activity

The proteolytic activities of human/murine/bovine APC and variants thereof was determined by steady-state hydrolysis of the APC-specific chromogenic substrate CS-21(66) (Biophen). Serial dilutions of each APC preparation were incubated with CS-21(66) in 100mM NaCl/20mM Tris-HCl/2.5mM CaCl₂/0.1mg/mL BSA, pH 7.5 (Appendix II). The rate of CS-21(66) hydrolysis was measured at an OD of 405nm at room temperature using a spectrophotometer. The rate of substrate hydrolysis for each APC preparation tested was determined and then compared to a standard curve.
2.6: SDS-PAGE, comassie staining and western blot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) followed by either comassie staining or western blot analysis was used to confirm the molecular weight and glycosylation status of human, murine and bovine PC/APC variants.

2.6.1: Comassie staining

1μg of PC/APC was diluted in NuPAGE loading buffer (Invitrogen, Life Technologies) and 1μl of Reducing Agent (Invitrogen, Life Technologies) was added for reduced samples. Each sample was incubated at 70°C for 10 minutes to denature the protein and separated by SDS-PAGE on a precast NuPAGE 10% polyacrylamide BisTris gel (Invitrogen, Life Technologies) for 35 minutes at 200V. The gel was then washed with 10mL of water 3 times and agitated in 20mL of SimLy Blue Stain (Invitrogen, Life Technologies) for 1 hour. The stain was decanted and the gel washed repeatedly prior to examination.

2.6.2: Western blotting

10-50ng of protein was diluted in NuPAGE loading buffer and 1μL of Reducing Agent, incubated at 70°C for 10 minutes and separated by SDS-PAGE on a precast NuPAGE 10% polyacrylamide BisTris gel (Invitrogen, Life Technologies) for 35 minutes at 200V. Following electrophoresis, the protein samples were transferred on to a polyvinylidene fluoride (PVDF) membrane using the iBlot semi-dry transfer system (Invitrogen, Life Technologies). The membrane was washed 3 times in 10mL PBS-0.02% (v/v) Tween (PBS-T) for 5 minutes prior to incubation with 20mL 5% (w/v) dried milk in PBS-T (5% milk) for 1 hour to block non-
specific membrane binding sites and subsequently washed 3 more times, as outlined above. Detection of human, murine and bovine PC/APC variants was achieved by incubation of the membrane with species-specific primary anti-bodies (Table 2.1) diluted in 5% milk. The membrane was washed 3 more times and incubated with a HRP-conjugated secondary antibody (Table 2.1) diluted in 5% milk for 1 hour, followed by 3 further washes. Protein bands were detected by incubation of the membrane with ECL Plus detection reagents (GE Healthcare) for 1 minute prior to exposure to photographic film for 1-30 seconds, which was subsequently developed to allow visualization of the protein bands.

Table 2.1 Human, murine and bovine PC western blotting antibodies

<table>
<thead>
<tr>
<th>Primary anti-body</th>
<th>Secondary anti-body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PC</td>
<td></td>
</tr>
</tbody>
</table>
| Polyclonal sheep anti-human PC (HTI)  
Dilution: 1:2000 | Anti-sheep HRP (SantaCruz Biotech)  
Dilution: 1:10000 |
| Murine PC         |                     |
| Monoclonal rat anti-murine PC (HTI)  
Dilution: 1:1000 | Anti-rat HRP (SantaCruz Biotech)  
Dilution: 1:10000 |
| Bovine PC         |                     |
| Polyclonal sheep anti-murine PC (HTI)  
Dilution: 1:1000 | Anti-sheep HRP (SantaCruz Biotech)  
Dilution: 1:10000 |
| HIS-tagged protein |                     |
| HRP-conjugated anti-HIS antibody (Sigma-Aldrich) |                     |
| FVII              |                     |
| Polyclonal sheep anti-human FVII (HTI)  
Dilution: 1:1000 | Anti-sheep HRP (SantaCruz Biotech)  
Dilution: 1:10000 |
2.7: Activation of PC on the surface of an endothelial cell line

Activation of human PC variants by thrombin on the surface of EA.hy926 cells was measured. EA.hy926 cells were seeded 96-well microtitre plates at a density of $2 \times 10^5$ cells/ml and grown to confluence over 24 hours. Cells were washed 3 times in Hank’s buffered salt solution (HBSS; Appendix II) and incubated with PC in HBSS supplemented with 3mM CaCl$_2$ and 0.6mM MgCl$_2$, 1% (w/v) BSA and 0.1% (w/v) sodium azide. Activation was initiated by addition of thrombin (5nM) to each well followed by incubation at 37°C for 30 minutes. The reaction was then stopped with 135nM hirudin (Sigma). APC generation was determined by assessment of APC amidolytic activity in the cell supernatant. 50µl of the supernatant was added to 50µl CS-21(66) (Biophen) (2mg/ml). The rate of absorbance change was measured at 405nm. The kinetic parameters were determined according to the Michaelis-Menten equation using Prism® software.
2.8: Assessment of coagulation protease binding to soluble EPCR

The binding affinity of coagulation proteases for sEPCR was characterised by Surface Plasmon Resonance (SPR) using a dual flowcell BIAcore X biosensor system (BIAcore, Sweden). BIAcore sensor chips were used as a surface upon which coagulation protein binding to sEPCR was determined. BIAcore sensor chips are comprised of glass coated with a thin layer of gold modified with a carboxymethylated dextran (CM5) layer. The dextran layer provides a hydrophilic environment to facilitate molecule attachment and prevent denaturation.

10μg/mL monoclonal anti-EPCR antibody, RCR-2 (kind gift of Dr. K. Fukudome, Saga Medical School) was covalently immobilized on a CM5 sensor chip using amine coupling chemistry. RCR-2 was selected as a capture antibody because it does not inhibit APC binding to EPCR. The sensor chip surface carboxymethyl groups modified using N-hydroxysuccinimide (NHS) and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) to facilitate covalent attachment. EDC reacts with carboxyl groups to form amine-reactive intermediates which in the presence of NHS, form stable amine-reactive NHS esters that react with RCR-2 amines and other nucleophilic groups to form covalent bonds. This technique allowed permanent immobilisation of RCR-2 onto the surface of the 2 flow cells of the sensor chip.

Soluble EPCR was desalted into HBS-P buffer (100mM HEPES/150mM NaCl, pH7.4) containing 3mM CaCl₂, 0.6mM MgCl₂ and bound to the test flow cell via RCR-2. Coagulation proteases were individually injected over both flow cells (the RCR-2 only flow cell was used to identify non-specific binding) at a rate of 10μL/min for 60 seconds and binding kinetics recorded. Samples were then dissociated using HBS-EP buffer (containing 3mM EDTA) and the RCR-2 immobilised CM5 chip was regenerated with 10μL of 10mM glycine-HCl (pH 2).
2.9: Glycosidase removal of N-linked glycans from VKD coagulation proteases

Removal of N-linked glycan structures was achieved by incubation of VKD coagulation proteases (1μg) with N-glycosidase from Flavobacterium meningosepticum (PNGase F; 1000IU; New England Biolabs Inc., Ipswich, MA) in 50mM sodium phosphate buffer at a pH of 7.5 for 1 hour at 37°C. PNGase F cleaves between the innermost GlcNAc and asparagines residues of complex oligosaccharides from N-linked glycoproteins, enzymatically removing entire N-linked carbohydrate chains.
2.10: Assessment of endothelial barrier permeability

2.10.1: Coagulation protease mediated endothelial barrier protection

APC can mediate endothelial barrier protection via EPCR and PAR1 dependent cellular signalling. In order to assess the barrier protective capacity of other VKD coagulation proteases, a protease barrier permeability assay was developed. EA.hy926 cells were trypsinised and plated on polycarbonate membrane transwell inserts (Costar, 3 μM pore size, 12-mm diameter) contained within a 12-well microtitre plate. Plates were incubated at 37°C/5%CO2 until full confluence was achieved (approximately 48 hours). The transwell inserts were drained and the cells washed with sterile PBS then treated with serum-free DMEM supplemented with 3mM CaCl2 and 0.6mM MgCl2 (assay buffer 2; Appendix II). Cells were incubated with VKD coagulation proteases and activate site inhibited VKD proteases (Haematological Technologies, USA) for 3 hours, and then treated with 5nM thrombin for 10 minutes to induce endothelial permeability. The transwell inserts were drained and the cells were washed with sterile PBS and incubated with 200μL FITC dextran (Sigma-Aldrich) (250μg/mL) in PBS. Endothelial barrier permeability was determined by assessment of the increase in fluorescence at 490nM Excitation (Ex)/ 525nM Emission (Em) in the outer chamber beneath the transwell insert over time due to transmigration of FITC dextran.

Endothelial permeability relative to thrombin only-treated cells was determined using the following equation:

\[
\text{Permeability (\%) = \left( \frac{X-N}{P-N} \right) \times 100}
\]

Where X is the test sample, N is the PBS-treated negative control sample and P is the thrombin-treated positive control sample.
Zymogen PC occupancy of EPCR can recruit PAR1 to a barrier protective signalling pathway irrespective of the activating protease. In order to assess the barrier protective capacity of other VKD zymogens, a zymogen barrier permeability assay was developed. EA.hy926 cells were grown to confluence on polycarbonate membrane transwell inserts and prepared for assay as outlined in 2.10.1. Cells were incubated with PC/ FX/ FVII for 1 hour, after which 10nM thrombin was added to each well and incubated for a further 3 hours to induce endothelial permeability. Endothelial barrier permeability was determined using FITC dextran as previously described.
2.11: Assessment of endothelial cell apoptosis

Endothelial cell apoptosis was assessed using the APO Percentage\textsuperscript{®} kit (Biocolor). During apoptosis phosphatidylserine, which is usually confined to the inner cell membrane leaflet, transfers to the outer cell membrane leaflet. This process permits intracellular uptake of APO Percentage dye by apoptotic cells, enabling visible differentiation from healthy cells.

EA.hy926 cells were trypsinised, plated on a 96-well microtitre plate and incubated at 37\textdegree C/5\% CO\textsubscript{2} until full confluence was achieved (approximately 24 hours). Medium was decanted and cells were washed with PBS and then treated with serum-free DMEM supplemented with 3mM CaCl\textsubscript{2} and 0.6mM MgCl\textsubscript{2}. Cells were incubated with APC/FXa for 3 hours, after which 20\textmu M staurosporine was added to each well and incubated for a period of 3 hours to induce apoptosis. Supernatant was decanted and cells were then treated with 100\textmu l serum-free DMEM containing 10\textmu l APO Percentage dye and 20 \textmu M staurosporine for 45 minutes.

Finally, cells were washed to remove non-bound dye and digital microscopic images were taken. Cellular APO Percentage dye uptake was quantified by conversion of digital images into pixel counts with Adobe\textsuperscript{™} Photoshop\textsuperscript{™} software. Average pixel counts were based on analysis of at least 3 images per well. Endothelial apoptosis relative to staurosporine only-treated cells was determined using the following equation:

\[ \text{Apoptosis} \% = \frac{(X-N)}{(P-N)} \times 100 \]

Where X is the test sample, N is the PBS-treated negative control sample and P is the staurosporine-treated positive control sample.
2.12: THP1 cell viability

THP1 cell viability was assessed using the CellTiter-Fluor™ Cell Viability Assay (Promega) which measures the relative number of viable cells in a population (NB REF). The assay uses constitutive protease activity within live cells as a biomarker of cell viability. A fluorogenic, cell-permeable, peptide substrate (Gly-Phe-AFC) enters intact cells and is cleaved by a “live-cell” protease to generate a fluorescent signal proportional to the number of living cells. Activity of this “live-cell” protease is lost upon loss of membrane integrity consistent with apoptosis.

THP1 cells were pelleted via centrifugation at 1500rpm for 5 minutes. Culture medium was discarded and cells were re-suspended in serum-free RPMI 1640 medium supplemented with 0.6mM CaCl\(_2\) and 0.2mM MgCl\(_2\) (assay buffer 3; Appendix II). Cells were seeded in 96-well microtitre plates at a density of 2 x10\(^5\) cells/mL, treated with FXa (20nM)/PBS for 3 hours and subsequently stimulated with 500ng/mL LPS for 4 hours. 10\(\mu\)L Gly-Phe-AFC Viability Substrate was diluted in 2mL of the assay buffer as per the manufacturer’s instructions. 20\(\mu\)L of the mixture was added to each test well and the plate was mixed by orbital shaking (300–500rpm) for 30 seconds. The plate was incubated for at 30 minutes at 37°C. Fluorescence was measured at 400nM Ex/ 505nM Em.
2.13: TNFα secretion from THP1 cells using HEK Blue TNFα/IL1β reporter cells

HEK Blue TNFα/IL-1β cells are a HEK293 reporter cell line expressing TNFα and IL-1β receptors as well as a NF-κB/AP-1 inducible secreted ALP reporter (Appendix I). Exposure of the HEK Blue TNFα/IL-1β cells to TNFα results in dose-dependent activation of the NF-κB/AP-1 pathways and expression of the secreted ALP reporter gene. These cells can thus be used to detect TNFα secretion. HEK Blue TNFα/IL-1β cells were trypsinised (outlined in section 2.1.1), re-suspended in fresh culture medium and plated in 96-well microtitre plates at a density of 5 x 10⁵ cells/mL. Cells were incubated at 37°C for approximately 8 hours.

THP1 cells were pelleted via centrifugation at 1500rpm for 5 minutes. Culture medium was discarded and cells were resuspended in serum-free RPMI 1640 medium supplemented with 0.6mM CaCl₂ and 0.2mM MgCl₂. Cells were seeded in 96-well microtitre plates at a density of 3 x 10⁶ cells/mL and treated with VKD coagulation proteases for 3 hours followed by stimulation with PRR agonists. Supernatants were collected thereafter and incubated with HEK Blue TNFα/IL-1β reporter cells (plated as outlined above) for 18 hours. ALP activity in the HEK Blue cell supernatant was detected using QUANTI-Blue (Invivogen) detection medium that contains a colorimetric ALP substrate. Colorimetric measurements were taken using a spectrophotometer at 650nm.

ALP activity relative to LPS only-treated cells was determined using the following equation:

\[
\text{ALP activity (\%) } = \left( \frac{(X-N)}{(P-N)} \right) \times 100
\]

Where X is the test sample, N is the PBS-treated negative control sample and P is the LPS-treated positive control sample.
Figure 2.1: Assessment of TNFα secretion by THP-1 cells using HEK Blue TNFα/IL-1β reporter cells: THP1 cells were stimulated with PRR agonists for 4 to 6 hours to induce secretion of pro-inflammatory cytokines. THP-1 cell supernatant was then applied to HEK Blue TNFα/IL-1β reporter cells and incubated overnight. TNFα present in the THP1 cell supernatant binds to the TNF receptor (TNFR) and initiates activation of NFκB resulting in expression of ALP. The amount of ALP present in the HEK Blue supernatant, measured using a colorimetric ALP substrate, is therefore relative to TNFα expression by THP1 cells.
2.14: Quantification of NF-κB/AP-1 activation by THP1-XBlue-CD14

NF-κB activation was measured using the THP1-XBlue-CD14 cell line (Appendix I). THP1-XBlue-CD14 cells are THP1 cells stably expressing a NF-κB/AP-1-inducible secreted ALP reporter and co-expressing CD14 to enhance sensitivity to LPS. THP1-XBlue-CD14 were re-suspended in serum-free RPMI 1640 medium supplemented with 0.6mM CaCl₂ and 0.2mM MgCl₂ at a density of 3x10⁶ cells/mL, then seeded in 96-well microtitre plates. Cells were treated with FXa (0-20nM) for 3 hours and stimulated overnight with LPS (500ng/ml). ALP activity in the supernatant was detected using QUANTI-Blue medium containing a colorimetric ALP substrate. Colorimetric measurements were taken at 650nm.
2.15: Assessment of cytokine secretion by PBMCs and murine macrophages

Sandwich ELISAs purchased from R&D Systems were used to measure TNFα, IL-6 and IL-10 expression from human PBMCs, murine RAW cells and primary macrophages, according to the manufacturer’s instructions.
2.16: Quantification of NF-κB/AP-1 activation using RAW Blue

Macrophage NF-κB activation was measured using the RAW Blue cell line. RAW Blue cells are a mouse leukaemic macrophage cell line stably expressing an NF-κB/AP-1 inducible secreted ALP reporter. RAW Blue cells were grown to confluence in 24-well microtitre plates. Cells were washed with PBS and treated with proteases in serum free RPMI 1640 medium supplemented with 0.6mM CaCl$_2$ and 0.2mM MgCl$_2$ for 3 hours prior to stimulation with LPS for 6 hours. ALP activity in the supernatant was detected using QUANTI-Blue medium containing a colorimetric ALP substrate. Colorimetric measurements were taken at 650nm.
2.17: Preparation of cDNA plasmids for assessment of PAR1 proteolysis

Assessment of PAR1 proteolysis was carried out using a PAR1 construct fused to secreted human placental ALP at Ala-36 with an intervening FLAG epitope (DYKDDDD) contained with the mammalian expression vector pRc/CMV\textsuperscript{117} (AP-PAR1; a generous gift from S. Coughlin, University of California, San Francisco). In this case, the pRc/CMV vector contains the AP-PAR1 cDNA sequence cloned between Hind III and NotI restriction endonuclease sites.

Proteolysis of this construct by either thrombin or APC resulted in liberation of the ALP tag into the cell supernatant, which was then quantified using a colorimetric ALP substrate. Two variants of the AP-PAR1 cDNA construct, generated by Genewiz (Boston), were used to identify the specific site at which human APC variants cleaved PAR1. Individual mutation of the thrombin (Arg-41) and APC (Arg-46) PAR1 cleavage sites to alanine residues produced AP-PAR1 variants that could exclusively be cleaved at the Arg-45 and Arg-41 cleavage sites, respectively. The experiments were carried out on HEK293T cells co-transfected with a human EPCR construct containing an N-terminal green fluorescent protein (GFP) tag (previously produced in the laboratory) or Mac1 integrin subunits CD11b\textsuperscript{277} and CD18\textsuperscript{278} obtained from Addgene (Plasmids 8631 and 8638) (Appendix III). Standard subcloning techniques were used to increase the quantity of AP-PAR1, variant AP-PAR1, EPCR-GFP and TM-GFP cDNA plasmids.
2.18: Assessment of PAR1 proteolysis by VKD proteases

2.18.1: Transient transfection of HEK293T cells

HEK293T cells were trypsinised and seeded in 24-well microtitre plates at a density of $1 \times 10^5$ cells/mL. The plate was incubated at 37°C/5% CO₂ until ~50% confluence was reached (approximately 24 hours later).

AP-PAR1/AP-PAR1 variants, EPCR-GFP, CD11b and CD18 plasmids were prepared for transfection by diluting 0.5μg plasmid cDNA in 50μL OptiMEM reduced serum medium in the presence of 1.5μL of TransIT 20/20 (Mirus), a liposomal transfection reagent, per well to be transfected. The mixture was incubated for 20 minutes at room temperature. Supernatant was decanted from HEK293 cells that had been plated for transfection. Cells were washed with 1mL sterile PBS and treated with 450μL fresh culture medium. The plasmid/TransIT 20/20 mixture was added to each well and the plate was incubated at 37°C for 24 hours.

2.18.2: Assessment of PAR1 proteolysis using AP-PAR1 assay

Cells were drained of culture medium, washed with sterile PBS and treated with serum-free DMEM supplemented with 0.6mM CaCl₂ and 0.2mM MgCl₂ (assay buffer 1; Appendix II). Cells were incubated with VKD coagulation proteases as outlined for 3 hours after which the supernatant was removed and ALP activity was measured using QUANTI-Blue detection medium that contains a colorimetric ALP substrate. Colorimetric measurements were taken using a spectrophotometer at 650nm.
Figure 2.2: AP-PAR1 proteolysis assay: HEK293T cells were transfected with a PAR1 construct fused to secreted human placental ALP at Ala-36 and cotransfected with EPCR. APC treatment of the cells resulted in EPCR binding, proteolysis of the AP-PAR1 construct and liberation of the ALP tag into the cell supernatant. ALP activity in the supernatant was then quantified using a colorimetric ALP substrate.
2.19: Data analysis

Data are presented as mean +/- SD of at least 3 separate experiments. Statistical analysis of experimental data was performed using the 2-tailed Student's t test and the level of significance was set at a probability of <0.05.
CHAPTER 3: ROLE OF EPCR-BINDING VITAMIN K-DEPENDENT COAGULATION PROTEINS IN MAINTAINING ENDOTHELIAL CELL INTEGRITY

3.1: EPCR-PAR1 signalling properties of APC and FVIIa

3.1.1: Activation of PAR1 by APC and FVIIa

To investigate EPCR-dependent activation of PAR1 by VKD proteases, an assay that directly measures the rate of PAR1 proteolysis was established. HEK293T cells were transfected with plasmid constructs expressing human EPCR, possessing a C-terminal GFP tag to facilitate assessment of transfection efficacy (EPCR-cGFP), and a modified human PAR1 mutant, AP-PAR1. AP-PAR1 contains an alkaline phosphatase (AP) tag N-terminal to the canonical PAR1 cleavage site. Proteolysis of this construct results in liberation of the AP tag into the cell supernatant, which is then quantified using a colorimetric substrate (Chapter 2.18)\(^\text{247}\).

HEK293T cells expressing EPCR-cGFP and AP-PAR1 (293T\(^{\text{EPCR/AP-PAR1}}\)) were generated. Expression of EPCR-cGFP was determined by light microscopy and was observed in >90% of transfected cells (Figure 3.1.1). AP-PAR1 expression was determined by treatment with thrombin, the canonical activator of PAR1. Thrombin induced substantial release of AP into the cell supernatant such that all AP was released by incubation with 100pM thrombin (Figure 3.1.1 a). In contrast, no AP was released in the absence of thrombin. Treatment of 293T\(^{\text{EPCR/AP-PAR1}}\) cells with thrombin initiated dose-dependent release of AP (Figure 3.1.1 c), indicating that EPCR-cGFP expression does not hinder AP-PAR1 proteolysis.
Figure 3.1.1: Thrombin activation of PAR1 on 293T cells transfected with EPCR/AP-PAR1: HEK293T cells transfected with (a) AP-PAR1 (b) EPCR-cGFP or (c) AP-PAR1 and EPCR-cGFP were treated with thrombin (●; 0.1-1nM) for 3 hours in assay buffer 1. EPCR-cGFP and AP-PAR1 expression were assessed using light microscopy and by measurement of AP activity in the cell supernatant using QUANTI Blue, respectively (Chapter 2.18). Experiments were performed in triplicate and data are presented as mean ± S.D.
To examine whether co-expression of EPCR is required for APC activation of AP-PAR1 on 293T cells, APC was incubated with both $293^{\text{AP-PAR1}}$ cells and $293^{\text{EPCR/AP-PAR1}}$ cells and PAR1 proteolysis was measured. Incubation of APC with $293^{\text{AP-PAR1}}$ failed to induce significant release of AP into the cell supernatant (Figure 3.1.2 a, black bars). In contrast, incubation with thrombin resulted in robust proteolysis of AP-PAR1 in the absence of EPCR (Figure 3.1.2 a, black bars). Incubation of either thrombin or APC with $293^{\text{EPCR/AP-PAR1}}$ cells induced significant liberation of AP (Figure 3.1.2 a, white bars). AP-PAR1 proteolysis by APC was dose-dependent (Figure 3.1.2 b, closed circles) and 3-4 orders of magnitude less efficient than that of thrombin (Figure 3.1.2 b, triangles), in keeping with previous results. An APC variant rendered proteolytically inactive due to activate site blockade using a tripeptide chloromethyl ketone inhibitor (APC_{DEGR}), which is incapable of cleaving a short APC-specific chromogenic substrate (data not shown), was also tested. As expected, incubation of $293^{\text{EPCR/AP-PAR1}}$ cells with APC_{DEGR} did not result in AP release (Figure 3.1.2 b, open circles).

Thus, APC activation of PAR1 on $293^{\text{EPCR/AP-PAR1}}$ is EPCR-dependent and requires a functional APC active site. This expression system can therefore replicate the receptor requirements for APC proteolysis of PAR1 on endothelial cells.
Figure 3.1.2: EPCR-dependent activation of PAR1 by APC using 293T<sup>AP-Par1</sup> cells (black bars) or 293T<sup>AP-Par1</sup> cells (white bars) were treated with thrombin (1nM) or APC (100nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was assessed using QUANTI Blue. (b) 293T<sup>AP-Par1</sup> cells were treated with thrombin (▲; 1nM), APC (●; 25-100nM) or APC<sub>degr</sub> (O; 100nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
FVIIa was incubated with 293T	extsuperscript{EPCR/AP-PAR1} cells to examine whether co-expression of EPCR can facilitate activation of PAR1 by FVIIa on this cell line. Treatment with FVIIa failed to induce release of AP from 293T	extsuperscript{EPCR/AP-PAR1} cells (Figure 3.1.3) demonstrating that although FVIIa binds to EPCR in an identical manner to APC, it is unable to emulate APC-like EPCR-dependent activation of AP-PAR1 on HEK293T cells.

Figure 3.1.3: Comparative rate of PAR1 activation by APC and FVIIa using 293T	extsuperscript{EPCR/AP-PAR1} cells: 293T	extsuperscript{EPCR/AP-PAR1} cells were treated with thrombin (1nM), APC (100nM) or FVIIa (100nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. Experiment was performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01; * = p ≤ 0.05]
3.1.2: APC mediates endothelial cell barrier protective signalling

An endothelial cell barrier permeability assay was used to investigate whether EPCR-dependent PAR1 activation potential correlated with the cytoprotective signalling capacity of EPCR-binding VKD proteases. Endothelial cell barrier permeability was determined by measuring migration of FITC-dextran through a layer of confluent EA.hy926 cells (Chapter 2.10). To establish thrombin-mediated endothelial cell barrier permeability, EA.hy926 cells were treated with thrombin. Thrombin, as expected, induced a significant increase in endothelial cell barrier permeability which was proportional to the length of incubation ($p \leq 0.001$) (Figure 3.1.4 a). To assess the ability of APC to counteract thrombin-induced endothelial cell barrier permeability, APC (2.5-20nM) was incubated with EA.hy926 cells prior to thrombin treatment. Treatment with APC mediated dose-dependent protection from thrombin-induced barrier disruption such that 20nM APC inhibited barrier permeability induced by thrombin by 89±2% and half maximal inhibition ($IC_{50}$) was observed at ~ 3.8nM APC ($p \leq 0.001$) (Figure 3.1.4 b).
Figure 3.1.4: APC protection against thrombin-induced endothelial cell barrier permeability: (a) EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with PBS (O) or thrombin (●; 5nM) in assay buffer 2 for 10 minutes. The cells were then washed with PBS and incubated with 400μl FITC dextran (250μg/mL). Migration of FITC dextran through the EA.hy926 cell layer into the outer chamber is dependent upon endothelial cell barrier permeability and was assessed over time (2-20 minutes) by measuring fluorescence in the outer chamber at OD_{490nm/525nm} (Chapter 2.10). (b) Confluent EA.hy926 cells were treated with APC (●; 2.5-20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10 minutes, after which and endothelial cell barrier permeability was assessed. Endothelial permeability is presented as a percentage of maximum permeability induced by thrombin (i.e. 100% permeability). Experiments were performed in triplicate and data are presented as mean ± S.D..
Protection of the endothelial cell barrier by APC against thrombin-induced permeability requires EPCR binding to facilitate PAR1 activation\textsuperscript{214}. To determine whether the observed protection mediated by APC in this assay was EPCR-dependent, 3 approaches were used. Firstly, a truncated APC variant missing the EPCR-binding Gla domain, Gla-domainless APC (APC\textsubscript{GD}), was tested for its ability to maintain endothelial cell barrier integrity. Unlike APC, APC\textsubscript{GD} failed to confer any protection against thrombin-induced permeability (Figure 3.1.5 a).

Secondly, a monoclonal anti-EPCR antibody (RCR-252) that prevents APC-EPCR binding was added to the cells prior to incubation with APC. The presence of RCR-252 also completely blocked the barrier protective effect of APC (Figure 3.1.5 b). Finally, EA.hy926 cells were incubated with APC\textsubscript{L8V}, an APC variant with defective EPCR binding\textsuperscript{202}, and endothelial cell barrier permeability was induced by thrombin and assessed. APC\textsubscript{L8V} failed to protect the endothelial cell barrier against thrombin-induced permeability (Figure 3.1.5 c). Collectively, these data indicate that the ability of APC to enhance endothelial barrier integrity within this assay is entirely dependent on its ability to bind to EPCR.
Thrombin:
Permeability (% Thrombin treated cells)

- + + +
- + - -
- - + -
- - - -

+ + + +
- + - -
- - + -
- - - -

+ + + +
- + - -
- - + -
- - - -

Apc:
- + + +
- + - -
- - + -
- - - -

RCR: 2.52
- + - -
- - + -
- - - -

Apc:
- + + +
- + - -
- - + -
- - - -

Apc:
- + + +
- + - -
- - + -
- - - -

Apc:
- + + +
- + - -
- - + -
- - - -

Permeability (% Thrombin treated cells)
Figure 3.1.5: APC protection against thrombin-induced endothelial cell barrier permeability is dependent upon EPCR binding: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with (a) APC or APC_{G0} (both 10nM), (b) APC (10nM) in the presence and absence of RCR-252 (25μg/ml) or (c) APC or APC_{LV} (both 10nM) for 3 hours in assay buffer 2. Cells were subsequently treated with thrombin (5nM) for 10 minutes, after which endothelial cell barrier permeability was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
To confirm the role of PAR1 in APC-mediated endothelial cell barrier protection in this assay, EA.hy926 cells were incubated with APC in the presence of a monoclonal anti-PAR1 antibody (ATAP2), which prevents PAR1 activation by APC\(^{214}\). ATAP2 blocked the barrier protective effect of APC (Figure 3.1.6) demonstrating that APC enhancement of endothelial cell barrier integrity requires PAR1 activation. Together, these results confirm that the endothelial cell barrier permeability assay is a sensitive and accurate tool with which to investigate EPCR-PAR1-dependent endothelial cell barrier protective signalling.
Figure 3.1.6: APC protection against thrombin-induced endothelial cell barrier permeability is dependent upon activation of PAR1: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with APC (10nM) for 3 hours in the presence and absence of ATAP2 (10μg/ml), then treated with thrombin (5nM) for 10 minutes and endothelial cell barrier permeability was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001]
3.1.3: Effect of FVIIa on endothelial cell barrier permeability

The ability of FVIIa to confer APC-like PAR1-dependent barrier protection was investigated. FVIIa can bind to EPCR with similar affinity to APC\(^{202}\), however its capacity to mediate PAR1-dependent endothelial cell barrier protective effects is controversial. FVIIa failed to activate AP-PAR1 on 293T cells co-expressing EPCR (Figure 3.1.3). Therefore we sought to compare FVIIa protection of the endothelial cell barrier to that of APC. APC and FVIIa (1.25-20nM) were incubated with EA.hy926 cells prior to thrombin treatment. As expected, APC mediated dose-dependent protection from thrombin-induced barrier disruption (Figure 3.1.7, circles). FVIIa, however, failed to mediate any protection at all concentrations tested (Figure 3.1.7, triangles) indicating that despite binding to EPCR in a similar manner to APC, FVIIa is unable to mediate APC-like PAR1-dependent signalling to induce protection of endothelial cell barrier integrity.
Figure 3.1.7: FVIIa does not mediate protection against thrombin-induced endothelial cell barrier permeability: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with APC (●) or FVIIa (▲) (both 1.25-20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10 minutes after which endothelial cell barrier permeability was assessed. Experiment was performed in triplicate and data are presented as mean ± S.D..
3.2: Characterisation of a recombinant PC_{FVII-Gla/EGF1} chimeric protein

3.2.1: Expression and characterisation of PC_{FVII-Gla/EGF1}

To investigate the discrepancy between APC and FVIIa EPCR-dependent PAR1 signalling, a hybrid protein consisting of the FVII Gla and EGF1 domains attached to the EGF2 and protease domains of PC, PC_{FVII-Gla/EGF1}, was generated (Figure 3.2.1 a). A pCMV6 vector containing cDNA encoding the FVII Gla and EGF1 domains attached to the EGF2 and serine protease domain of APC was used to stably transfect HEK293 cells. This vector also contained a HHHHHH (HIS) tag to enable simple identification of the hybrid protease. Colonies expressing PC_{FVII-Gla/EGF1} were expanded and incubated with serum-free conditioned media for 3-5 days in the presence of Vitamin K1, to ensure proper γ-carboxylation. The resulting protein was partially purified (Chapter 2.2) and activated by thrombin (Chapter 2.3). Amidolytic activity was determined by measurement of steady-state hydrolysis of the APC-specific chromogenic substrate CS-21(66) over time (Chapter 2.5) and used to determine the concentration of APC_{FVII-Gla/EGF1}.

To determine whether the structural integrity was retained, recombinant PC_{FVII-Gla/EGF1} was subjected to SDS-PAGE analysis followed by Western blotting (Chapter 2.6) using anti-HIS, anti-PC and anti-FVII antibodies. Under reducing conditions, the heavy chain of recombinant PC_{FVII-Gla/EGF1} migrated with a molecular weight of ~41kDa, corresponding to that of PC (Figure 3.2.1 b and c). Interestingly, PC_{FVII-Gla/EGF1} migrated as a doublet, which is indicative of 2 distinct glycoforms (α and β) similar to that observed with wild type PC. To investigate the glycosylation status of PC_{FVII-Gla/EGF1}, PC_{FVII-Gla/EGF1} was treated with PNGase F to enzymatically remove putative N-linked glycans. PNGase F treatment reduced the molecular
mass of PC$_{FVII\text{-Gla/EGF1}}$ by ~10kDa (Figure 3.2.1 c) corresponding with the loss of N-linked glycan moieties and confirming that PC$_{FVII\text{-Gla/EGF1}}$ is glycosylated. Under non-reducing conditions, PC$_{FVII\text{-Gla/EGF1}}$ migrated with a molecular weight of ~60kDa as detected using a polyclonal antibody directed against FVII (Figure 3.2.1 d), indicating that the presence of FVII epitope(s) derived from the FVII Gla and EGF1 domains in PC$_{FVII\text{-Gla/EGF1}}$. FVIIa, as expected migrated with a molecular weight of ~50kDa. The molecular weight of PC$_{FVII\text{-Gla/EGF1}}$ (~60kDa) was close to that predicted based on the combined molecular weight of the FVII light chain (~20kDa) and the heavy chain of PC (~41kDa). Collectively, these data confirm that PC$_{FVII\text{-Gla/EGF1}}$ is structurally intact and normally glycosylated.
Figure 3.2.1: Characterization of PC_{FVII-Gla/EGF1} by western blotting: (a) Schematic diagram of the domain structure of PC, FVII and PC_{FVII-Gla/EGF1}. (b, c and d) Recombinantly expressed PC_{FVII-Gla/EGF1} or PNGase F treated PC_{FVII-Gla/EGF1} was subjected to SDS-PAGE under reducing (b and c) and non-reducing (d) conditions and Western blot analysis using (b) an anti-HIS antibody (c) and an anti-PC polyclonal antibody or (d) an anti-FVII polyclonal antibody.
3.2.2: The anticoagulant function of APC<sub>FVII-Gla/EGF1</sub>

To assess the role of the APC Gla and EGF1 domains in APC anticoagulant function, APC<sub>FVII-Gla/EGF1</sub> inhibition of TF-initiated thrombin generation in PC-deficient plasma was determined. Initiation of coagulation with soluble TF (1pM) and CaCl<sub>2</sub> (16.67mM) resulted in rapid generation of thrombin (Figure 3.2.2). As expected, APC dose-dependently attenuated TF-initiated thrombin generation such that peak thrombin generated was reduced ~3.5-fold by 2.5nM APC (Figure 3.2.2 a and c). In contrast, APC<sub>FVII-Gla/EGF1</sub> exhibited significantly impaired ability to reduce thrombin generation (Figure 3.2.2 b and c) such that 10nM APC<sub>FVII-Gla/EGF1</sub> reduced total thrombin generation by less than 20%, indicative of reduced anticoagulant activity of the chimeric enzyme.
a

Time (minutes)

Fila (nM·min⁻¹)

- No APC
- 2.5nM APC
- 5nM APC
- 10nM APC

b

Time (minutes)

Fila (nM·min⁻¹)

- No APC
- 2.5nM APC
- 5nM APC
- 10nM APC

C

ETP

nM

- APC
- APC

93
Figure 3.2.2: APC_{APC-Gia/EGF1} displays minimal anticoagulant function in plasma: The anticoagulant activity of (a) APC and (b) APC_{APC-Gia/EGF1} was assessed in PC-deficient plasma using a thrombin generation assay. Plasma was incubated with phospholipid vesicles (4μM; 60% phosphatidylcholine, 20% phosphatidyserine and 20% phosphatidylethanolamine) and TF alongside APC or APC_{APC-Gia/EGF1}. Thrombin generation was initiated with 1pM sTF and CaCl₂ and measured by comparing rate of fluorogenic substrate hydrolysis to a thrombin calibrated standard. Thrombin generated in the absence of APC is represented by the red line. (c) Endogenous thrombin potential (ETP) (which represents area under a graph of thrombin generation) was calculated. Experiments were performed in duplicate and are presented as mean ± S.D..
3.2.3: EPCR binding and endothelial cell activation of PC_{FVII-Gla/EGF1}

To investigate whether PC_{FVII-Gla/EGF1} possesses an intact Gla domain that can bind EPCR, EPCR-dependent activation of PC_{FVII-Gla/EGF1} on endothelial cells by the thrombin-TM complex was assessed. Ea.hy926 cells were treated with PC (20nM)/PC_{FVII-Gla/EGF1} (18nM) and thrombin (5nM) for 3 hours in the presence and absence of RCR-252. APC generation was measured using the APC-specific chromogenic substrate CS-21(66). As expected, incubation of PC with thrombin on the surface of endothelial cells resulted in generation of APC (Figure 3.2.3) and was significantly reduced in the presence of RCR-252. Activation of PC_{FVII-Gla/EGF1} by thrombin on endothelial cells was also observed (Figure 3.2.3) and was reduced ~3-fold by the presence of RCR-252. This demonstrates that PC_{FVII-Gla/EGF1} possesses an intact Gla domain that binds EPCR to enhance the rate of activation by the thrombin-TM complex on endothelial cells.
Figure 3.2.3: EPCR-dependent activation of $\text{PC}_{\text{FVII-Gla/EGF1}}$ by thrombin: EA.hy926 cells were treated with $\text{PC}_{\text{FVII-Gla/EGF1}}$ (18nM) and thrombin (1nM) the presence of RCR-252 (25$\mu$g/mL) for 3 hours in assay buffer 2. APC generation was measured by incubation of cell supernatant with the APC-specific chromogenic substrate CS-21(66). Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
3.2.4: EPCR-dependent PAR1 signalling by APC_{FVII-Gla/EGF1}

To measure EPCR-dependent proteolysis of PAR1, APC, FVIIa and APC_{FVII-Gla/EGF1} were incubated with 293T_{EPCR/AP-PAR1} cells and AP-PAR1 proteolysis measured. As expected, APC but not FVIIa activated AP-PAR1 in this system (Figure 3.2.4 a). Despite retaining EPCR-binding capacity, APC_{FVII-Gla/EGF1} displayed severely diminished ability to cleave AP-PAR1. The ability of APC_{FVII-Gla/EGF1} to mediate endothelial cell barrier protection was also compared to that of APC and FVIIa. APC, FVIIa or APC_{FVII-Gla/EGF1} was incubated with EA.hy926 cells prior to thrombin treatment. Similar to FVIIa, APC_{FVII-Gla/EGF1} failed to mediate protection against thrombin-induced barrier permeability (Figure 3.2.4 b) indicating that despite possessing the EGF2 and protease domains of APC and normal EPCR binding capacity, APC_{FVII-Gla/EGF1} cannot mediate APC-like PAR1-dependent signalling to induce protection of endothelial cell barrier integrity. Thus, features of APC Gla and EGF1 domains of APC, in addition to EPCR binding, are necessary for EPCR-dependent activation of PAR1 by APC.
Figure 3.2.4: PAR1-dependent signalling by APC_{FVII-Gla/EGF1}: (a) 293_EPC/AP-PAR1 cells were treated with thrombin (1nM), APC (50nM), FVIIa (50nM) or APC_{FVII-Gla/EGF1} (50nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. (b) EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with APC or APC_{FVII-Gla/EGF1} (both 20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10 minutes after which endothelial cell barrier permeability was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001]
3.2.5: PAR1-independent signalling by APC<sub>FVII-Gla/EGF1</sub>

APC-mediated cellular signalling on monocytic cells inhibits pro-inflammatory cytokine release in response to LPS<sup>231</sup>. Unlike APC cytoprotective signalling on endothelial cells, the anti-inflammatory effects of APC on monocytes occur independently of PAR1 activation via ApoER2<sup>237</sup>. To confirm that PAR1 is not required for APC signalling on monocytes, PBMCs were treated with a PAR1 antagonist (FR131117) prior to incubation with APC. The cells were then stimulated with LPS and TNFα secretion measured by ELISA. PAR1 antagonism did not induce TNFα expression from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 3.2.5 a, white and black bars). Treatment with 20nM APC inhibited LPS-induced TNFα secretion by 64±13% (Figure 3.2.5 a, grey bars) and PAR1 antagonism failed to prevent APC impairment of pro-inflammatory cytokine release in response to LPS. This data indicates that APC anti-inflammatory signalling on monocytes, in contrast to APC cytoprotective signalling on endothelial cells, does not require PAR1 activation.

To investigate the domain requirements for APC-mediated PAR1-independent inhibition of pro-inflammatory cytokine expression from monocytes, PBMCs were incubated with APC<sub>FVII-Gla/EGF1</sub> and stimulated with LPS (Figure 3.2.5 b). APC<sub>FVII-Gla/EGF1</sub> exhibited anti-inflammatory activity on LPS-treated monocytes similar in efficacy to that of APC, reducing TNFα production by ~40%. Collectively, these results show that the presence of APC EGF2 and serine protease domains in APC<sub>FVII-Gla/EGF1</sub> is sufficient to enable APC-like, PAR1-independent anti-inflammatory activity on monocytes, but precludes EPCR/PAR1-dependent signalling.
Figure 3.2.5: PAR1-independent signalling by APC_{FVII-Gla/EGF1}: (a) PBMCs were isolated from buffy coat whole blood component and treated with FR171113 (1.25μM) for 30 minutes, prior to incubation with PBS (black bars) or APC (grey bars) for 3 hours in assay buffer 3, then stimulated with LPS (50ng/mL) for 18 hours. TNFα secretion was measured by ELISA. PBS treatment (white bars) was used as a negative control. (b) PBMCs were isolated from buffy coat whole blood component and treated APC or APC_{FVII-Gla/EGF1} (both 20nM) for 3 hours in assay buffer 3, then stimulated with LPS (50ng/mL) for 18 hours. TNFα secretion was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [* = p ≤ 0.05]
3.3: EPCR-dependent PAR1 signalling properties of FXa

3.3.1: FXa mediates endothelial cell barrier protective signalling

Human FXa has been reported to bind to EPCR and activate both PAR1 and PAR2 on endothelial cells\textsuperscript{154} and mediates EPCR-PAR1 dependent enhancement of endothelial cell barrier integrity\textsuperscript{155}. To compare the endothelial cell barrier protective function of FXa with APC, EA.hy926 cells were incubated with APC or FXa (1.25-20nM) for 3 hours prior to induction of endothelial cell barrier permeability using thrombin. As expected, APC treatment protected the endothelial cell barrier from thrombin-induced disruption (Figure 3.3.1). FXa similarly mediated dose-dependent protection from thrombin-induced barrier disruption, such that 20nM FXa reduced thrombin-induced permeability by 59±4% (p < 0.01) (Figure 3.3.1).
Figure 3.3.1: FXa mediates protection against thrombin-induced endothelial cell barrier permeability: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with APC (●) or FXa (▲) (both 1.25-20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10 minutes. Experiment was performed in triplicate and data are presented as mean ± S.D..
To determine whether EPCR is important for FXa protection of the endothelial cell barrier, 2 approaches were used. Firstly, EA.hy926 cell were treated with a truncated FXa variant missing the EPCR-binding Gla domain, Gla-domainless FXa (FXa$_{GD}$), and its ability to maintain endothelial cell barrier integrity in response to thrombin was tested. FXa$_{GD}$ failed to confer any protection against thrombin-induced permeability (Figure 3.3.2 a). Secondly, EA.hy926 cells were treated with FXa in the presence of the monoclonal anti-EPCR antibody RCR-252 prior to induction of endothelial cell barrier permeability with thrombin. RCR-252 completely blocked the FXa-mediated barrier protective effect (Figure 3.3.2 b) confirming that, like APC, the ability of FXa to enhance endothelial barrier integrity is dependent on EPCR.
Figure 3.3.2: FXa protection against thrombin-induced endothelial cell barrier permeability is dependent upon EPCR binding: (a) EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with FXa or FXa<sub>GD</sub> (20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10. (b) Confluent EA.hy926 cells were treated with FXa (20nM) for 3 hours in the presence and absence of RCR-252 (25μg/ml) then treated with thrombin (5nM) for 10 minutes. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
To determine the role of PAR1 in FXa-mediated endothelial cell barrier protection, EA.hy926 cells were incubated with FXa in the presence of the cleavage blocking monoclonal anti-PAR1 antibody ATAP2 and endothelial cell barrier permeability was induced using thrombin. ATAP2 reduced the protective effect of FXa by ~ 70% (Figure 3.3.3). However small, but significant, PAR1 independent reduction in thrombin-induced barrier permeability was also observed.

Figure 3.3.3: FXa protection against thrombin-induced endothelial cell barrier permeability is dependent upon activation of PAR1: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with FXa (20nM) for 3 hours in the presence and absence of ATAP (25µg/ml) then treated with thrombin (5nM) for 10 minutes Experiment was performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
Bovine FXa is a close structural homolog of its human counterpart, with > 70% of the amino acid sequence conserved between both species. To investigate the ability of bovine FXa to confer barrier protective effects on human endothelial cells, EA.hy926 cells were incubated with bovine FXa for 3 hours prior to induction of endothelial barrier permeability with thrombin. Treatment of cells with bovine FXa failed to mediate any significant protection against thrombin-induced permeability (Figure 3.3.4) suggesting that specific structural features of the human FXa molecule mediate EPCR-dependent PAR1 signalling that result in protection of human endothelial cell barrier integrity.

![Graph](image)

**Figure 3.3.4: Bovine FXa does not mediate protection against thrombin-induced endothelial cell barrier permeability:** EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with FXa or FXa\textsubscript{BOVINE} (20nM) for 3 hours in assay buffer 2 prior to treatment with thrombin (5nM) for 10 minutes Experiment was performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
3.3.2: FXa inhibition of endothelial cell apoptosis

In addition to enhancing barrier integrity, activation of the EPCR-PAR1 signalling axis on endothelial cells by APC can confer protection against cellular apoptosis\textsuperscript{216}. The anti-apoptotic activity of APC was assessed by measuring the effect of APC on staurosporine-induced Ea.hy926 cell apoptosis using the Apo \textit{Percentage} kit. During apoptosis phosphatidylserine, which is usually confined to the inner cell membrane leaflet, transfers to the outer cell membrane leaflet. This process permits intracellular uptake of the pink APO \textit{Percentage} dye by apoptotic cells, enabling visualization of apoptotic cells using light microscopy. APO \textit{Percentage} dye uptake was then quantified by conversion of digital images into pixel counts with Adobe\textsuperscript{™} Photoshop\textsuperscript{™} software (Chapter 2.11).

To establish staurosporine-induced endothelial cell apoptosis, EA.hy926 cells were treated with staurosporine for 3.5 hours and apoptotic cells quantified by treatment with the APO \textit{Percentage} pink dye. Minimal dye accumulation was observed the control PBS-treated cells (Figure 3.3.5). As expected, staurosporine treatment induced dye accumulation in almost all cells visualized (Figure 3.3.5). To establish APC efficacy in mediating anti-apoptotic signalling on endothelial cells, APC was incubated with EA.hy926 cells for 3 hours prior to staurosporine treatment. Prior incubation with 20nM APC mediated an 80±10% reduction in staurosporine-induced apoptosis (Figure 3.3.5).
Dye Accumulation
(Pixel count)

0 4000 8000 12000

APC
Staurosporine

200uM APC + 20uM Staurosporine
20uM Staurosporine
PBS
Figure 3.3.5: APC mediates protection against staurosporine-induced apoptosis on endothelial cells: EA.hy926 cells were grown to confluence in a 96-well plate and treated with control PBS or APC (20nM) in assay buffer 2. Medium was aspirated and cells were then incubated with staurosporine (20μM) for 3.5 hours after which 100μl fresh assay buffer containing 10μl APO Percentage dye and staurosporine (20μM) was added to each well. Cells were incubated for 45 minutes and washed twice using PBS (Chapter 2.11). (a) Cells were visualized by light microscopy and photographed. (b) Uptake of APO Percentage dye was quantified by converting digital photograph images into pixel counts using Adobe™ Photoshop™ software. Average pixel counts were based on analysis of at least 3 images per well. Results presented in panel (b) represent mean ± S.D. of 3 separate experiments. [** = p ≤ 0.01]
To investigate the ability of FXa to mediate protection against apoptosis, EA.hy926 cells were incubated with FXa (1.25-20nM) for 3 hours prior to treatment with staurosporine. FXa significantly inhibited staurosporine-induced endothelial cell apoptosis with similar efficacy to APC (Figure 3.3.6). The effect of FXa was dose-dependent such that 20nM FXa reduced staurosporine-induced apoptosis by 89±6% (p < 0.001) and half maximal protection (IC₅₀) was observed at ~ 5.6nM FXa (Figure 3.3.6 b).
Dye Accumulation (Pixel count)

![Graph showing dye accumulation with pixel count on the y-axis and various concentrations on the x-axis.]

![Images comparing dye accumulation under different conditions: 20nM + 20µM Staurosporine, 20µM Staurosporine, and PBS.]
Figure 3.3.6: FXa mediates protection against staurosporine-induced apoptosis on endothelial cells: EA.hy926 cells were grown to confluence in a 96-well plate and treated with control PBS (O) or FXa (●; 1.25-20nM) in assay buffer 2. Medium was aspirated and cells were then incubated with staurosporine (20μM) to induce apoptosis. (a) Apoptotic cells were visualized by light microscopy and photographed. (b) Uptake of APO percentage dye was quantified by converting digital photograph images into pixel counts using Adobe™ Photoshop™ software. Average pixel counts were based on analysis of at least 3 images per well. Results presented in panel (b) represent mean ± S.D. of 3 separate experiments. [** = p ≤ 0.01]
3.4: Proteolysis-independent endothelial cell cytoprotective activity of PC and FX(a)

3.4.1: EPCR-dependent protection of endothelial cell barrier integrity by PC

Occupancy of EPCR by PC reverses thrombin-induced barrier disruption by recruiting thrombin activated PAR1 to a protective signalling pathway\(^{222}\). To investigate this, a modified endothelial cell barrier permeability assay was used to assess the barrier protective effect of thrombin when EPCR was occupied by PC (Chapter 2.11). To prevent the confounding effect of PC activation by thrombin during the experiment, a PC variant rendered inactive by substitution of the active site serine to alanine (PC\(_{360A}\)) was used\(^{279}\). EA.hy926 cells were grown to confluence upon 12-well membrane permeable transwell inserts and treated with PC\(_{360A}\) (2.5-20nM) for 1 hour, after which thrombin (5nM) was added and incubated for 3 hours. Endothelial cell barrier permeability was determined by measuring migration of FITC dextran from the insert into the outer chamber, as before (Chapter 2.11). PC\(_{360A}\) mediated significant and dose-dependent protection from thrombin-induced barrier disruption such that 20nM PC\(_{360A}\) reduced thrombin-induced permeability by 65±3% (p < 0.01) (Figure 3.4.1).
Figure 3.4.1: PC$_{S360A}$-mediated protection against thrombin-induced endothelial cell barrier permeability: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with PC$_{S360A}$ (●; 2.5-20nM) for 1 hour in assay buffer 2 prior to treatment with thrombin (5nM) for 3 hours after which endothelial barrier permeability was assessed. Experiment was performed in triplicate and data are presented as mean ± S.D..
The role of EPCR binding in enabling PC\textsubscript{S360A} protection of the endothelial cell barrier was determined using the monoclonal anti-EPCR antibody RCR-252. EA.hy926 cells were incubated with PC\textsubscript{S360A} and RCR-252 prior to induction of endothelial cell barrier permeability using thrombin. Blockade of EPCR completely prevented PC\textsubscript{S360A} reversal of thrombin-mediated barrier disruption (Figure 3.4.2), confirming the protective ability of thrombin in the presence of PC\textsubscript{S360A} is mediated via PC\textsubscript{S360A}-EPCR occupancy.

Figure 3.4.2: PC\textsubscript{S360A} protection against thrombin-induced endothelial cell barrier permeability is mediated via occupancy of EPCR: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with PC\textsubscript{S360A} (20nM) in the presence and absence of RCR-252 (25\textmu g/ml) for 1 hour in assay buffer 2. Cells were then treated with thrombin (5nM) for 3 hours and endothelial barrier permeability was assessed. Experiment was performed in triplicate and data are presented as mean ± S.D.. [*** = p \leq 0.001]
3.4.2: FX_{DEGR} and FX protection against endothelial cell barrier permeability

FX has been described to recruit both PAR1 and PAR2 to an endothelial cell barrier protective pathway\(^{139}\). To determine whether FX(a) can reverse thrombin-induced endothelial cell barrier disruption independently of PAR1 proteolysis, 2 approaches were used.

Firstly, a variant of FXa in which the active site was blocked using a tripeptide chloromethyl ketone inhibitor (FX_{DEGR}) was used. Blockade of the active site prevents the confounding effects of FXa proteolysis of PAR1, allowing independent analysis of the effect of FXa-EPCR interaction on thrombin-PAR1 barrier-disruptive signalling. FX_{DEGR} was unable to mediate cleavage of a short chromogenic substrate, confirming the lack of FXa enzymatic activity (data not shown). EA.hy926 cells were treated with FX_{DEGR} (2.5-20nM) for 1 hour followed by treatment with thrombin (5nM) for 3 hours. Treatment of cells with FX_{DEGR} prior to thrombin resulted in significant and dose-dependent reduction in endothelial cell barrier permeability. 20nM FX_{DEGR} reduced thrombin-induced barrier disruption by 40±9% (p ≤ 0.05) (Figure 3.4.3).
Figure 3.4.3: FXa\textsubscript{DEGR} mediates protection against thrombin-induced endothelial cell barrier permeability. EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with FXa\textsubscript{DEGR} (\bullet; 2.5-20nM) for 1 hour in assay buffer 2 prior to treatment with thrombin (5nM) for 3 hours after which endothelial barrier permeability was assessed. Experiment was performed in triplicate and data are presented as mean ± S.D..
Secondly, to compare the protective effect of FX to PC₅₃₆₀₄, EA.hy926 cells were incubated with PC₅₃₆₀₄ or FX at plasma concentration (72nM and 170nM, respectively) for 1 hour followed by treatment with thrombin (5nM) for 3 hours. As expected, PC₅₃₆₀₄ significantly reduced thrombin-induced endothelial barrier disruption (Figure 3.4.4 a). Prior incubation with FX (170nM) resulted in a similar reduction in endothelial cell barrier permeability (79±8%) (Figure 3.4.4 a). The protective effect of FX was dose-dependent and half maximal reduction in barrier permeability was observed at ~85nM FX (Figure 3.4.4 b).
Figure 3.4.4: FX mediates protection against thrombin-induced endothelial cell barrier permeability: (a) EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with PC$_{S360A}$ (72nM) or FX (170nM) for 1 hour in assay buffer 2 prior to treatment with thrombin (5nM) for 3 hours after which endothelial barrier permeability was assessed. (b) Confluent EA.hy926 cells were treated with FX (○; 12.5-200nM) for 1 hour in assay buffer 2, then treated with thrombin (5nM) for 3 hours and endothelial permeability was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
3.5: Cellular receptors required for FX-mediated protection of endothelial cell barrier integrity

3.5.1: The role of EPCR in FXaDEGR and FX protection of endothelial cell barrier integrity

To investigate the mechanism through which FXaDEGR/ FX regulation of endothelial barrier integrity is mediated, monoclonal antibodies directed against putative FX cell surface receptors were utilized. Firstly, to determine the role of EPCR, RCR-252 was used. EA.hy926 cells were incubated with FXaDEGR or FX in the presence of RCR-252 prior to induction of endothelial cell barrier permeability with thrombin. Similar to PC, blockade of EPCR completely prevented the barrier protective effect of FXaDEGR (Figure 3.5.1 a) signifying the requirement for EPCR in FXaDEGR-mediated endothelial cell barrier protection. Interestingly, the effect of FX was largely maintained in the presence of RCR-252 (Figure 3.5.1 b), indicating FX-mediated barrier protection occurs via an alternative cellular signalling mechanism.
Figure 3.5.1: FXαDEGR and FX mediate protection against thrombin-induced endothelial cell barrier permeability via distinct cellular signalling mechanisms: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with (a) FXαDEGR (20nM) or (b) FX (170nM) in the presence and absence of RCR-252 (25µg/ml) for 1 hour in assay buffer 2. Cells were treated with thrombin (5nM) for 3 hours and endothelial barrier permeability was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p < 0.001; ** = p < 0.01]
To investigate the discrepancy in EPCR requirement FXαDEGR and FX, the binding affinity of FX and FXa for sEPCR was assessed by surface plasmon resonance (Chapter 2.8). FXa or FX (62.5–1000nM) was injected over both flow cells at a flow rate of 10μl/min for 60 seconds and sEPCR binding was recorded. FX displayed minimal binding affinity for sEPCR at all concentrations tested, resulting in a $K_D < 2 \mu M$ (Figure 3.5.2 a). FXa, however, bound to sEPCR with a reduced affinity compared to PC ($K_D \sim 350nM$ compared to $\sim 120nM$ in the case of PC$^{202}$) (Figure 3.5.2 b). Differences in EPCR binding properties between FX and FXa were unexpected, as both zymogen and activated FX possess identical Gla-domain ω-loop sequences. The discrepancy suggests that although FX(a) lacks the ω-loop sequence required for canonical EPCR binding, the active form of the protease possesses features, unavailable in the zymogen form, which can facilitate a unique interaction with EPCR
Figure 3.5.2: FX and FXa bind to hsEPCR with discrepant affinities: Monoclonal anti-EPCR antibody, RCR-2 (10μg/ml) was immobilized by amine coupling to both flow cells of a CM5 sensor chip. sEPCR was bound to the test flow cell via RCR-2 (RCR-2 immobilized to the reference flow cell served to detect non-specific binding). Serial dilutions of (a) human FX (b) FXa (both 62.5–1000nM) were individually injected over both flow cells at a flow rate of 10μl/min for 60 seconds.
3.5.2: The role of Annexin-2 in FX protection of endothelial cell barrier integrity

Annexin-2 is a putative endothelial cell FXa receptor\textsuperscript{167}. To determine the role of Annexin-2 in FX reversal of thrombin-induced endothelial cell barrier disruption an anti-Annexin-2 monoclonal antibody was used. EA.hy926 cells were treated with FX in the presence of the anti-Annexin-2 antibody for 1 hour followed by treatment with thrombin (5nM) for 3 hours. As previously described, FX significantly reduced thrombin-induced barrier disruption (Figure 3.5.3 a). Inhibition of Annexin-2 completely prevented the endothelial cell barrier protective effect of FX (Figure 3.5.3 a). The effect of the antibody was dose-dependent with half maximal inhibition observed at \(~3\mu g/ml\) (Figure 3.5.3 b).
Figure 3.5.3: FX protection against thrombin-induced endothelial cell barrier permeability is prevented by antibody inhibition of Annexin-2: EA.hy926 cells were grown to confluence on polycarbonate membrane permeable transwell inserts and treated with FX (170nM) for 1 hour in assay buffer 2 in the presence and absence of a monoclonal anti-annexin-2 antibody (a) (10µg/ml) (b) (●; 2.5-10µg/ml), then treated with thrombin (5nM) for 3 hours. Experiments were performed in triplicate and data are presented as mean ± S.D.. [***= p ≤ 0.001]
To verify that inhibition of annexin-2 specifically modulates FX-mediated endothelial cell barrier protection, rather than independently regulating barrier integrity, EA.hy926 cells were treated with control PBS, PC5360A or FX in the presence of the monoclonal anti-Annexin-2 antibody for 1 hour followed by treatment with thrombin (5nM) for 3 hours. Importantly, inhibition of annexin-2 had no effect on thrombin-induced barrier permeability alone and similarly, PC5360A protection of the endothelial cell barrier was maintained in the presence of the same anti-annexin-2 antibody (Figure 3.5.4, black bars). These results demonstrate that annexin-2 specifically mediates FX enhancement of endothelial barrier integrity.
Figure 3.5.4: Annexin-2 specifically regulates FX protection against thrombin-induced endothelial cell barrier permeability: Confluent EA.hy926 cells were treated with control PBS, PC₅₃₆₀ₐ (72nM) or FX (170nM) for 1 hour in the presence (black bars) and absence (white bars) of an anti-annexin-2 antibody (10μg/ml), then treated with thrombin (5nM) for 3 hours and endothelial permeability was assessed. Experiment was performed in triplicate and data are presented as mean ± S.D.. [*** = p < 0.001]
3.6: Discussion

EPCR is a crucial modulator of APC cytoprotective effects on in vitro cultured cells and in vivo inflammatory disease models. PC and APC contain identical Gla domain EPCR binding motifs that interact with EPCR with comparable affinity. The EPCR binding motif of the (A)PC Gla domain ω-loop is highly conserved among other human VKD proteases (Figure 3.6.1).

This chapter aimed to define the role of other VKD proteases with putative EPCR binding affinity, human FVIIa and FX(a), in mediating cytoprotective signalling on endothelial cells.

3.6.1: APC, but not FVIIa, mediates EPCR-dependent PAR1 cytoprotective signalling

APC signalling on endothelial cells requires EPCR-dependent activation of PAR1. HEK293T cells transfected with EPCR and AP-PAR1 were used to investigate the capacity of homologous VKD proteases to mediate PAR1 proteolysis. Thrombin, as previously described, activated AP-PAR1 in a highly efficient manner (Figure 3.1.1). In contrast, APC proteolysis of AP-PAR1 occurred only on 293T<sup>AP-PAR1</sup> cells co-transfected with EPCR (Figure 3.1.2). These results are in keeping with previous studies that have identified EPCR as an essential co-receptor for APC activation of AP-PAR1 on transfected HEK293T cells, CHO cells and HUVECs. In the presence of EPCR, APC activation of PAR1 was 3-4 orders of magnitude less efficient than that of thrombin, consistent with the relative efficacy of thrombin and APC activation of PAR1 on HUVECs. Collectively, these results demonstrate that the receptor requirements and efficacy of coagulation protease PAR1 activation on transfected HEK293T cells closely mimic those reported on endothelial cells.
Figure 3.6.1: Gla domain ω loop residues Phe-4 and Leu-8 specifically regulate PC binding to EPCR: (a) Molecular model depicting interaction between the PC Gla domain (blue) and sEPCR (green). Residues Phe-4 and Leu-8 of the protein C ω-loop (red) and as well as Gla residues 7, 25 and 29 interact with residues in sEPCR. (b) Amino acid sequences of Gla domain residues 1 - 9 of human VKD coagulation proteins. The canonical EPCR binding motif (FLEEL) is highlighted in white.
Human FVII(a) possesses a Gla domain Ω loop EPCR-binding motif which is identical to that of (A)PC (Figure 3.6.1 b). FVII(a) binds to EPCR with similar affinity to APC\textsuperscript{202,204} and prevents (A)PC binding to endothelial cells via competitive inhibition of EPCR binding\textsuperscript{203,204,280,281}. A study published by Sen \textit{et al.} (2011) reported EPCR-dependent activation of PAR1 by FVIIa on HUVECs\textsuperscript{281}. Further, FVIIa activation of PAR1 in this manner conferred protection against thrombin-induced HUVEC barrier permeability via activation of Rac1 intracellular signalling. In our hands, despite its capacity to bind to EPCR, FVIIa failed to activate AP-PAR1 on 293T\textsuperscript{AP-PAR1} cells co-expressing EPCR (Figure 3.1.3), indicating EPCR-binding cannot facilitate FVIIa activation of PAR1.

Ghosh \textit{et al.} (2007) similarly reported that FVIIa failed to activate AP-PAR1 on CHO\textsuperscript{EPCR}\textsuperscript{203}. In the same study, FVIIa also failed to activate AP-PAR2 on CHO\textsuperscript{EPCR} which may be explained by the absence of TF\textsuperscript{282}. In the same way, the absence of additional, as yet unidentified, cellular components/receptors present on HUVECs from the 293T\textsuperscript{AP-PAR1/EPCR} model may explain this discrepancy. In our study, FVIIa also failed to mediate EPCR-PAR1 barrier protective signalling on EA.hy926 cells (Figure 3.1.7). The lack of efficacy of FVIIa in EA.hy926 cell barrier protection has also been reported by Bae \textit{et al.}\textsuperscript{139,222}. Sen \textit{et al.} (2011) attributed the discrepancy between FVIIa barrier protection on EA.hy926 cells and HUVECs to differences in receptor expression levels\textsuperscript{281}. TF is a high affinity receptor for FVIIa and under quiescent conditions EA.hy926 cells express TF whereas HUVECs do not. It is possible that in the presence of TF, FVIIa is prevented from engaging in EPCR-PAR1 cellular signalling.

This is contradicted however, by reports that the presence of TF does not inhibit FVIIa-EPCR binding in SPR experiments\textsuperscript{204}. Additionally, FVIIa binds to EA.hy926 cells with
high affinity in an EPCR-dependent manner, demonstrating that FVII(a)-EPCR binding occurs on EA.hy926 cells\textsuperscript{204}. Of note, EPCR binding has been reported to result in FVII(a) endocytosis on endothelial cells, a process which would prevent further interaction with additional cell surface receptors, such as PARs\textsuperscript{280}. Our results indicate that FVIIa is unable to emulate APC-like EPCR-PAR1 signalling on 293T\textsuperscript{AP-PAR1/EPCR} or EA.hy926 cells. The PAR1-dependent barrier protective effect of FVIIa on HUVECs must, in contrast to APC, require cellular components additional to EPCR, which are not present on EA.hy926 cells, to facilitate efficient activation of PAR1.

To identify the molecular determinants of the discrepant EPCR-dependent PAR1 signalling capacities of APC and FVIIa on EA.hy926 cells, we generated a recombinant PC/FVII hybrid variant, PC\textsubscript{FVII-Gla/EGF1}, consisting of the FVII Gla and EGF1 domains attached to the EGF2 and protease domains of PC (Figure 3.2.1 a). Chimeric proteins characterized by domain exchanges often exhibit misfolding, limiting subsequent functional analysis. SDS PAGE and Western blotting demonstrated however, that PC\textsubscript{FVII-Gla/EGF1} migrated with a molecular weight similar to that predicted and possessed both PC and FVII epitope(s) (Figure 3.2.1). Further, under reducing conditions, the heavy chain of PC\textsubscript{FVII-Gla/EGF1} migrated as a doublet, suggesting that, similar to PC, the heavy chain of PC\textsubscript{FVII-Gla/EGF1} is expressed in distinct glycoforms, α and β. These data confirm that PC\textsubscript{FVII-Gla/EGF1} is structurally intact and normally glycosylated.

APC\textsubscript{FVII-Gla/EGF1} displayed minimal anticoagulant activity in an in vitro assay of thrombin generation (Figure 3.2.2). The domain requirements for APC anticoagulant activity are not fully understood. In a study by Geng and colleagues (1997), an APC/FVII chimera in which the APC Gla domain was replaced with that of FVII demonstrated normal anticoagulant
activity\textsuperscript{281}, suggesting the Gla domain of FVII can support phospholipid and protein binding requirements necessary for APC anticoagulant activity. Coupled with our results, this suggests that unidentified amino acid residues in the EGF1 domain of APC, not present in FVII, are crucial for enabling APC anticoagulant activity. The specific residues not conserved between the EGF1 domains of APC and FVII are currently being assessed for their role in APC anticoagulant activity.

In contrast to its lack of anticoagulant activity, PC\textsubscript{FVII-Gla/EGF1} bound to EPCR on endothelial cells (Figure 3.2.3) and endothelial cell activation of PC\textsubscript{FVII-Gla/EGF1} by the thrombin-TM complex was observed. Antibody inhibition of EPCR reduced PC\textsubscript{FVII-Gla/EGF1} activation by thrombin-TM on the endothelial cell surface 3-fold, similar to that reported in the case of PC\textsubscript{186}. This data suggests that PC\textsubscript{FVII-Gla/EGF1} has an intact Gla domain that binds EPCR and that the Gla domain residues required to support EPCR enhancement of PC activation are conserved in FVII.

Despite its ability to bind EPCR, APC\textsubscript{FVII-Gla/EGF1} exhibited severely diminished PAR1 activation on 293T\textsuperscript{EPCR/AP-PAR1} cells and failed to protect the endothelial cell barrier against thrombin-induced disruption (Figure 3.2.4) indicating that the EGF2 and serine protease domains of APC are insufficient to enable APC-like PAR1 signalling when connected to the Gla and EGF1 domains of FVII. This data highlights for the first time that APC-specific EGF1 domain amino acid residues, not present in FVIIa, are required for the EPCR-dependent PAR1 barrier protective signalling. Further, the molecular requirements for EPCR enhancement of PC activation differ from those required for EPCR facilitation of PAR1 signalling by APC.
Interestingly, APC_{FVIIa/EGF1} mediated normal reduction in LPS-induced TNFα expression from PBMCs (Figure 3.2.5). APC anti-inflammatory activity on monocytes is PAR1-independent and requires interaction with ApoER2 rather than EPCR^{237}. The domain requirements for APC signalling on monocytes have not been characterised, however Mosnier et al. (2009) reported that mutation of Glu-149 ablates APC's anti-inflammatory effect of this cell type, indicating that the EGF2 domain is likely important in this function^{238}. The APC EGF2 domain is preserved in APC_{FVIIa/EGF1}, which may explain the retention of APC-like anti-inflammatory effects on monocytes coupled with the loss of EPCR-dependent PAR1 signalling properties.
3.6.2 FX(a) mediates cytoprotective signalling on endothelial cells

Human FXa can initiate cellular signalling via activation of both PAR1 and PAR2 on endothelial cells.\cite{139,140,154}. Despite this, FXa failed to activate AP-PAR1 on HEK293 cells, but successfully activated AP-PAR2 on the same cell model.\cite{140} Activation of AP-PAR1 by FXa has been observed on HEK293T cells co-transfected with EPCR\cite{155} suggesting that, similar to APC, FXa activation of PAR1 is dependent upon the presence of EPCR.

Human FX(a) contains a Met residue at position 8 within the EPCR binding motif, in contrast to PC which contains a Leu at this site. FXa inhibited thrombin-induced endothelial cell barrier permeability in a similar manner to APC (Figure 3.3.1). The FXa Gla domain was required and the effect was prevented by an anti-EPCR antibody (Figure 3.3.2), in keeping with previous reports that FXa protection of endothelial cell barrier integrity is EPCR dependent.\cite{155} Interestingly, while the effect of FXa was largely inhibited by a function-blocking anti-PAR1 antibody, a small but consistent protective effect was maintained in its presence (Figure 3.3.3). Unlike APC, FXa is also reported to mediate endothelial cell barrier protection via PAR2 activation, a process which is facilitated by interaction of the FXa inter-EGF sequence Leu\textsuperscript{83} - Leu\textsuperscript{88} with a putative FXa receptor.\cite{139,154,161} The identity of this endothelial cell receptor for FXa, however, remains unknown. These results suggest that in addition to mediating APC-like EPCR-PAR1 signalling, FXa can also confer PAR1-independent barrier protection possibly via activation of PAR2.

Bovine FXa, a close structural homolog of human FXa, was unable to reverse thrombin-induced barrier disruption (Figure 3.3.4) indicating that the molecular requirements for barrier protective signalling are not conserved between both species. Bovine FXa differs from
human FXa in a number of structural features. The Gla domain Q loop of bovine FXa possesses a Val residue at position 8, which is not compatible with EPCR binding in PC or APC. Deficient EPCR-binding may contribute to the absence of PAR1-dependent barrier protective function from bovine FXa. Additionally, the FXa inter-EGF sequence Leu\textsubscript{83} - Leu\textsubscript{88}, known to be important for human FXa barrier protective function, is not conserved.

FXa dose-dependently inhibited staurosporine-induced apoptosis on endothelial cells (Figure 3.3.6) with similar efficacy to that of APC, indicating FXa can mediate APC-like pleiotropic signalling on endothelial cells. This result is in keeping with a previous study which demonstrated that FXa enhances cellular viability on BHK cells\textsuperscript{284}. Contrastingly, Borensztajn et al. (2007) observed no effect of FXa treatment on HUVEC apoptosis\textsuperscript{285}. In this study, FXa was incubated with cells for 24 hours, however, compared with just 3 hours in Figure 3.3.6, which, due to the transient nature of GPCR signalling, may account for the discrepant results.

PC occupancy of EPCR can recruit thrombin activated PAR1 to a barrier protective signalling pathway\textsuperscript{222}. In Figure 3.4.1, co-incubation of thrombin with a PC active site mutant, PC\textsubscript{S360A}, dose-dependently reversed thrombin-induced endothelial cell barrier disruption in an EPCR dependent manner (Figure 3.4.2). Co-incubation of FXa\textsubscript{DEGR} with thrombin dose-dependently inhibited endothelial cell barrier disruption (Figure 3.4.3). FXa\textsubscript{DEGR} has previously been shown to bind to EA.hy926 cells in an EPCR-dependent manner\textsuperscript{155} and the barrier protective effect of FXa\textsubscript{DEGR} was reversed by an anti-EPCR antibody (Figure 3.5.1).

FX also dose-dependently reversed thrombin-induced barrier disruption (Figure 3.4.4). Surprisingly, the receptor requirements for FXa\textsubscript{DEGR} and FX regulation of endothelial cell barrier integrity were not conserved. While the effect of FXa\textsubscript{DEGR} was completely
prevented by inhibition of EPCR, the effect of FX was maintained in the presence of the same function blocking anti-EPCR antibody (Figure 3.5.1). During the course of this study, the EPCR independent nature of FX endothelial cell barrier protection has also been reported by Bae and colleagues. Of note, the interaction between FX and its activated form with EPCR may not be conserved, despite possessing identical Ω loop EPCR-binding motifs. FXa bound to sEPCR with a K_d of ~350nM, 2-3-fold that of (A)PC (Figure 3.5.2). The affinity of FX for sEPCR was much lesser, however. This result is consistent with a previous study that reported poor binding affinity between FX and sEPCR using SPR, in which a K_d in the micromolar range was reported. Further, FX failed to compete with FVIIa for EPCR binding on CHO^EPCR and did not interact with HUVECs in an EPCR-dependent manner. Interestingly, human FX is reported to bind to murine sEPCR with high affinity (K_d ~ 292nM). The same methionine instead of leucine replacement present at position 8 in the Ω loop of FX occurs in murine PC, the canonical murine EPCR ligand, indicating that this Ω loop sequence is likely optimal for murine, rather than human, EPCR binding.

A second study by Puy et al. (2010) also reported poor binding affinity of FXa for human sEPCR using SPR. The concentration of FXa used in this study (100nM) is too low to observe appreciable interaction between FXa and sEPCR using this method, as we observed a K_d of ~ 350nM (Figure 3.5.2). Furthermore, Schuepbach et al. (2010) observed that FXa binds to CHO^EPCR in an EPCR-dependent manner. Contrastingly, two groups have reported that FXa fails to compete with either APC or FVIIa for HUVEC or human aortic endothelial cell binding. Interestingly, Schuepbach et al. (2010) identified that FXa binding to endothelial cells is mediated via interactions with cell surface receptors including, but not...
exclusive to, EPCR. Thus, the discrepancy between FXa-EPCR binding on HUVECs and EA.hy926 cells may occur due to the presence of additional putative FXa endothelial cell receptors on the surface of HUVECs which prevent FXa from disrupting the interaction between EPCR and its higher affinity ligands. Importantly, numerous studies have confirmed that FXa PAR1 and PAR2 signalling requires EPCR, strongly indicating a functional interaction between FXa and EPCR.

The notion that an alternative co-receptor facilitates PAR-dependent signalling by FXa has also been postulated. Bhattacharjee et al. (2008) reported that FXa activation of PAR1 on HUVECs is mediated via interaction with Annexin-2. The possibility that the EPCR-dependent barrier protective effects of both FXa and FXaDEGR might also require or be enhanced by interaction with Annexin-2 cannot be eliminated. Interestingly, the barrier protective effect of FX, which was independent of EPCR, was completely prevented by an anti-annexin-2 antibody (Figure 3.5.3). Annexin-2 is a ubiquitously expressed cell surface receptor which participates in membrane regulation and trafficking of molecules.

Bhattacharjee and colleagues (2008) did not observe any interaction between annexin-2 and FX on the surface of HUVECs. Of note, the experiments carried out in this study were performed in the absence of Mg²⁺, which has previously been reported to be essential for FX Gla domain-mediated receptor interactions. The presence of Mg²⁺ in Figure 3.4.3 may facilitate FX interaction with annexin-2 and explain this discrepancy. Inhibition of annexin-2 antibody did not prevent PC endothelial cell barrier protection (Figure 3.5.4) confirming the specific role of annexin-2 in modulating FX barrier-protective activity.
Figure 3.6.2: FX(a) endothelial cell barrier protective signalling: The results presented in this chapter indicate that zymogen FX can interact with Annexin-2 on endothelial cells to protect against endothelial cell barrier disruption. Upon activation, FXa can also enhance endothelial cell barrier integrity via EPCR-dependent activation of PAR1. Furthermore, when bound to EPCR FXa regulates thrombin PAR1 signalling such that barrier protective effects are observed.
3.6.3 Conclusion

The results presented in Chapter 3 offer new insights into the molecular requirements for VKD coagulation protein cytoprotective signalling on endothelial cells. The discrepant PAR1 signalling capacities of APC and FVIIa demonstrate that EPCR binding does not necessarily confer cytoprotective signalling properties to VKD coagulation proteins and that features of the APC Gla and EGF1 domains, not present in FVIIa, are crucial for PAR1-dependent, but not PAR1-independent signalling. Further, the molecular features required for EPCR-dependent PAR1 activation and signalling differ from those required for EPCR enhancement of PC activation by the thrombin-TM complex. FXa, which interacts with EPCR with a different Gla domain Ω loop sequence and lower binding affinity than (A)PC/FVII(a), can mediate barrier protective and anti-apoptotic signalling via EPCR-dependent activation of PAR1. FXa\textsubscript{DEGR} mediated proteolysis-independent barrier protection via EPCR. FX, which interacts poorly with EPCR for reasons not yet fully understood, conferred unique endothelial barrier protective effects via interaction with annexin-2.

The continuation of this study will involve further characterisation of the co-receptors involved in FXa PAR1-dependent and PAR1-independent cytoprotective signalling on endothelial cells. While the results presented in Chapter 3 indicate that EPCR binding is an essential prerequisite to FXa barrier protection, the role of additional receptor interactions (e.g. Annexin-2, PAR2) in facilitating/enhancing FXa signalling in this manner will be investigated. Finally, the novel interaction between FX and annexin-2 will be further characterised and the molecular mechanism through which annexin-2-mediated FX endothelial cell barrier protection is conferred will be probed.
CHAPTER 4: FXa REGULATION OF TLR-INDUCED PRO-INFLAMMATORY SIGNALLING ON MYELOID CELLS

4.1: VKD protease regulation of LPS-induced cytokine secretion on myeloid cells

In addition to mediating cytoprotective effects on endothelial cells, APC signalling inhibits pro-inflammatory responses on monocytes and macrophages. The capacity of other VKD coagulation proteases, which share significant structural homology with APC, to modulate monocyte and macrophage pro-inflammatory function is poorly understood.

The secretion of TNFα from THP1 cells stimulated with LPS was measured as a representative marker of monocytic inflammation. To achieve this, a HEK293 reporter cell line expressing TNFα and IL-1β receptors and an NF-κB/AP-1 inducible secreted AP reporter (HEK Blue; Appendix I) was used to detect the presence of TNFα in the supernatant of stimulated monocytes. TNFα present in the THP1 cell supernatant bound to the TNFα receptor to initiate activation of NF-κB/AP-1, resulting in AP expression. AP activity in the HEK Blue cell supernatant increased significantly and proportionately when treated with supernatant from THP1 cells exposed to increasing concentrations of LPS (p ≤ 0.001) (Figure 4.1.1, closed circles). Minimal AP activity was observed when HEK Blue cells were incubated with the cell supernatant from unstimulated THP1 cells (Figure 4.1.1, open circles).
Figure 4.1.1: HEK Blue TNF-α/IL-1β reporter cell measurement of the THP1 cell response to LPS: (a) THP1 cells were stimulated with LPS (●; 6.25-100ng/mL) for 4 hours in assay buffer 3 after which the cell supernatant was aspirated and incubated with HEK Blue TNF-α/IL-1β reporter cells for 18 hours. TNFα present in the THP1 supernatant induced activation of NF-κB and secretion of AP, which was quantified using QUANTI Blue (Chapter 2.13). PBS-treated THP1 cell supernatant was used as a negative control (O). Experiment was performed in triplicate and data are presented as mean ± S.D..
LPS induces cytokine expression from myeloid cells by TLR4 ligation, which initiates a pro-inflammatory cellular response. To verify that AP activity in the HEK Blue cell supernatant was a result of LPS stimulation, THP1 cells were treated with an inhibitor of TLR4 pathway signalling (Polymixin B) prior to LPS treatment. Polymixin B completely prevented THP1 cell supernatant induction of AP secretion by the reporter cells, demonstrating that LPS-induced TNFα release is responsible for AP expression by HEK Blue cells (Figure 4.1.2 a). To confirm that the HEK Blue cells were insensitive to LPS and therefore not stimulating AP expression in an autocrine manner, HEK Blue reporter cells were incubated with LPS for 18 hours and AP activity in the supernatant was assessed. As expected, direct stimulation with LPS failed to induce secretion of AP from HEK Blue cells (Figure 4.1.2 b).

Finally, to confirm that AP activity in the HEK Blue cell supernatant occurs as a result of TNFα stimulation, LPS-stimulated THP1 cell supernatant was incubated with HEK Blue cells in the presence of a monoclonal anti-TNFα antibody. The anti-TNFα antibody completely prevented LPS-stimulated THP1 cell supernatant induction of AP secretion, demonstrating that the HEK Blue cell response is solely mediated by TNFα present in the THP1 cell supernatant (Figure 4.1.2 c). Collectively these data demonstrate that HEK Blue TNFα/IL-1β reporter cells are an accurate and specific tool with which to measure LPS-induced TNFα secretion from THP1 cells.
Figure 4.1.2: HEK Blue TNF-α/IL-1β reporter cells measure LPS-induced secretion of TNFα from THP1 cells: (a) THP1 cells were treated with the TLR4 signalling inhibitor polymixin B (10μg/mL) prior to stimulation with LPS (500ng/mL) for 4 hours in assay buffer 3. THP1 cell supernatant was incubated with HEK Blue reporter cells and AP secretion was measured using QUANTI Blue. (b) HEK Blue reporter cells were stimulated with LPS (500ng/mL) for 18 hours in assay buffer 3 after which secretion of AP was quantified using QUANTI Blue. (c) THP1 cells were stimulated with LPS (500ng/mL) for 4 hours in assay buffer 3 after which cell supernatant was aspirated and incubated with HEK Blue reporter cells in the presence of a monoclonal anti-TNFα antibody. Activation of NF-κB and secretion of AP was quantified using QUANTI Blue. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p < 0.001]
To investigate the ability of VKD coagulation proteases to modulate monocyte and macrophage pro-inflammatory function, THP1 cells were incubated with APC, FVIIa, FIXa or FXa for 3 hours followed by exposure to LPS or PBS. TNFα secretion was measured using HEK Blue cells. Incubation of THP1 cells with APC, FVIIa, FIXa or FXa alone induced negligible TNFα expression (Figure 4.1.3a, white bars). As expected, LPS treatment of THP1 cells induced robust secretion of TNFα (Figure 4.1.3a, black bars). Prior incubation with APC (20nM) inhibited the THP1 cell response to LPS stimulation resulting in a 50±9% reduction in TNFα secretion (Figure 4.1.3a, black bars). In contrast, activated coagulation factors VIIa and IXa neither diminished nor enhanced the LPS response (Figure 4.1.3a, black bars). Similar to the anti-inflammatory activity of APC, FXa (20nM) significantly inhibited TNFα secretion in response to LPS, by 66±7% (Figure 4.1.3a, black bars). Treatment of THP1 cells with increasing FXa (0.625-20nM) demonstrated that the attenuation of TNFα secretion mediated by FXa was dose-dependent, with half maximal inhibition (IC50) observed at ~3nM FXa, and similar in efficacy to that mediated by APC (Figure 4.1.3b).
AP activity (% LPS-treated cells)

AP activity

Protease (nM)

APC
FXa

APC
FXa
FXIIa
FXIa

AP activity (% LPS-treated cells)
Figure 4.1.3: FXa reduces LPS-induced TNFα secretion from THP1 cells: (a) THP1 cells were incubated with PBS/APC/FVIIa/FIXa/FXa (all 20nM) for 3 hours prior to stimulation with control PBS (white bars) /LPS (black bars; 500ng/mL) for 4 hours in assay buffer 3. TNFα secretion was subsequently measured using HEK Blue reporter cells. AP activity in the cell supernatant is presented as a percentage of maximal AP activity induced by LPS (i.e. 100%).

(b) THP1 cells were incubated with APC (O)/ FXa (●) (both 0.625-20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p < 0.001]
To investigate whether FXa inhibition of LPS-induced TNFα secretion was dependent upon the length of FXa pre-incubation, THP1 cells were pre-incubated with FXa for up to 6 hours, followed by stimulation with LPS and TNFα secretion was measured using HEK Blue cells. The efficacy of FXa anti-inflammatory signalling increased with increasing pre-incubation period with maximal inhibition observed after 3 hours pre-incubation (Figure 4.1.4). Incubation of THP1 cells with FXa for less than 1 hour prior to LPS treatment failed to significantly reduce secretion of TNFα (Figure 4.1.4), demonstrating that in order to significantly affect cytokine production, FXa anti-inflammatory signalling must be initiated prior to LPS stimulation.
Figure 4.1.4: FXa reduction of LPS-induced TNFα secretion from THP1 cells is dependent upon the length of FXa pre-incubation: THP1 cells were incubated with PBS (O)/ FXa (●; 20nM) for up to 6 hours prior to stimulation with LPS (500ng/mL) for 4 hours in assay buffer. TNFα secretion was subsequently measured using HEK Blue reporter cells. Experiments were performed in triplicate and data are presented as mean ± S.D.
FXa has previously been implicated as both a driver and inhibitor of cellular apoptosis on various cell types however does not affect monocyte viability\textsuperscript{284,285}. Nevertheless, alterations in cellular viability could impact upon TNF\(\alpha\) expression. In order to verify that the observed effect of FXa on LPS-induced cytokine secretion is not a consequence of altered monocyte viability, THP1 cell viability after FXa treatment was assessed using the CellTiter-Flour Viability assay (Chapter 2.12). This assay uses constitutive protease activity within live cells, lost upon degradation of membrane integrity during apoptosis, as a marker of cell viability. Cells were treated with a fluorogenic, cell-permeable, peptide substrate cleaved only in viable cells to generate a fluorescent signal that is proportional to the number of non-apoptotic cells\textsuperscript{276}.

To verify the sensitivity of the CellTiter-Flour Viability assay system, THP1 cells were treated with staurosporine after which cell viability was assessed. Staurosporine treatment induced a dose-dependent decrease in cellular viability \((p \leq 0.001)\) (Figure 4.1.5 a). Next, THP1 cells were treated with FXa for 3 hours and stimulated with LPS, after which viability was assessed. Viability of cells treated with LPS alone or in combination with FXa did not differ significantly from that of untreated cells (Figure 4.1.5 b). This demonstrates that THP1 cell viability is not affected during the course of the assay system used.
Figure 4.1.5: The anti-inflammatory activity of FXa on LPS-induced TNFα secretion does not occur as a consequence of altered THP1 cell viability: (a) THP1 cells were incubated with staurosporine (●; 0.625-10μM) for 5 hours in assay buffer 3. (b) THP1 cells were incubated with PBS/ FXa (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (500ng/mL) for 4 hours. Cell viability was assessed using the CellTiter-Flour viability assay (Chapter 2.12). Experiments were performed in triplicate and data are presented as mean ± S.D..
To investigate whether FXα regulation of LPS-induced TNFα secretion was conserved on primary human monocytes, PBMCs were isolated from buffy coat whole blood component (Chapter 2.1). PBMCs were treated with APC or FXα for 3 hours. The cells were stimulated with LPS for 18 hours and TNFα secretion was measured using ELISA. Similar to THP1 cells, APC/FXα alone did not induce TNFα secretion (Figure 4.1.6, open symbols) while LPS stimulation induced a robust TNFα response. Exposure to APC or FXα (both 20nM) prior to stimulation with LPS however, resulted in significant reduction in LPS-induced TNFα secretion by 63±7% and 69±11%, respectively (p ≤ 0.001) (Figure 4.1.6 a, closed symbols). The effect of FXα was dose-dependent with half maximal inhibition observed at < 3nM FXα, similar to the anti-inflammatory efficacy of FXα on the surface of THP1 monocytes (Figure 4.1.3 b).
Figure 4.1.6: FXa reduces LPS-induced TNFα secretion from PBMCs: PBMCs were isolated from buffy coat whole blood component (Chapter 2.1) and incubated with APC (▲; 20nM) or FXa (●; 0.313-20nM) for 3 hours in assay buffer 3 prior to stimulation with control PBS (open symbols)/ LPS (closed symbols; 50ng/mL) for 18 hours. TNFα secretion was determined using ELISA. Experiment was performed in triplicate and data are presented as mean ± S.D..
To assess the ability of FXa to regulate inflammatory cytokine production from macrophages, BMDMs were obtained from the femurs and tibia of wild type BALB/c mice (Chapter 2.1.3 and 2.1.4). The effect of FXa on both pro-inflammatory (TNFα and IL-6) and anti-inflammatory (IL-10) cytokine secretion in response to LPS was assessed. BMDMs were treated with FXa for 3 hours. The cells were stimulated with LPS for 18 hours and TNFα, IL-6 and IL-10 were measured by ELISA. As observed for both THP1 cells and PBMCs, FXa was unable to induce production of TNFα, IL-6 or IL-10 (Figure 4.1.7 a, b and c, white bars). LPS stimulation induced robust expression of TNFα, IL-6 and IL-10 (Figure 4.1.7 a, b and c, black bars). Prior-exposure to FXa (20nM) significantly inhibited LPS-induced secretion of both TNFα and IL-6 from BMDMs, by 47±17% and 71±20%, respectively (Figure 4.1.7 a and b, black bars). Exposure to FXa did not alter IL-10 levels compared to LPS treatment alone (Figure 4.1.7 c, black bars).
PBS L n mL-10 (pg/ml) 0 50 100 150

PBS 2000 mL-6 (pg/ml) mTNFα (pg/ml)

PBS FXα

PBS LPS

PBS FXα

PBS LPS

PBS FXα

PBS LPS
Figure 4.1.7: FXa reduces LPS-induced TNFα and IL-6 secretion from murine BMDMs:
Murine BMDMs were obtained from the femurs and tibia of wild type BALB/c mice and incubated with FXa (20nM) for 3 hours prior to stimulation with PBS (white bars) or LPS (black bars; 20ng/mL) for 6 hours after which secretion of TNFα, IL-6 and IL-10 was determined using ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
4.2: FXa regulation of cytokine secretion induced by activation of extracellular TLRs

4.2.1: THP1 cell TNFα secretion induced by activation of extracellular TLRs

NF-κB activation and pro-inflammatory cytokine expression on monocytes and macrophages can be initiated upon detection of TLR ligands other than LPS by TLR family members with different ligand specificity. Coagulation protease modulation of cytokine production induced by activation of TLRs other than TLR4 has not, however, been determined.

THP1 cell TNFα secretion was measured as a common marker of monocytic inflammation induced by stimulation of extracellular TLRs using HEK Blue reporter cells. THP1 cells were stimulated with activating ligands directed against specific TLR hetero- and homodimers. The TLR1/2 heterodimer was activated using a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial lipopeptides (Pam3CSK4) (Figure 4.2.1 a). The TLR2/2 homodimer was activated using heat-killed *Listeria monocytogenes* (HKLM) (Figure 4.2.1 b). The TLR5/5 homodimer was activated using bacterial flagellin (Figure 4.2.1 c) and the TLR2/6 heterodimer was activated using a synthetic lipoprotein derived from *Mycoplasma salivarium* (FLS1) (Figure 4.2.1 a). THP1 cells were incubated with TLR activating ligands for 4 hours after which the supernatant was aspirated and incubated with HEK Blue cells. AP activity in the HEK Blue cell supernatant increased proportionately with increasing TLR activating ligand concentration (p < 0.001) (Figure 4.2.1).
Figure 4.2.1: HEK Blue TNF-α/IL-1β reporter cell measurement of THP1 cell TNFα secretion induced by activation of extracellular TLRs: THP1 cells were stimulated with TLR agonists (a) Pam3CSK4 (14.7-500ng/mL), (b) HKLM (1.25x10^6 - 1x10^7 cells), (c) flagellin (3.13-25ng/mL) or (d) FLS1 (3.13-25ng/mL) for 4 hours in assay buffer 3 after which cell supernatant was incubated with HEK Blue reporter cells. AP activity in the HEK Blue cell supernatant was measured using QUANTI Blue, as before. Experiments were performed in triplicate and data are presented as mean ± S.D..
4.2.2: FXa regulation of TNFα secretion induced by activation of extracellular TLRs on THP1 cells

To investigate the ability of FXa to regulate TNFα secretion in response to activation of extracellular TLRs other than TLR4, THP1 cells were treated with FXa prior to stimulation with activating ligands for the TLR2/2 homodimer (HKLM), TLR1/2 heterodimer (Pam3CSK4), TLR5/5 homodimer (flagellin) and TLR2/6 heterodimer (FLS1) or LPS for 4 hours and TNFα secretion was measured. As observed in Figure 4.2.1, exposure to all TLR activating ligands induced secretion of TNFα (Figure 4.2.2, black bars). Prior exposure to FXa significantly inhibited TNFα secretion induced by all TLR activating ligands tested (Figure 4.2.2, white bars). Incubation with FXa reduced THP1 cell TNFα secretion by 34±9% upon TLR2/2 homodimer activation by HKLM, 45±6% upon TLR1/2 heterodimer activation by Pam3CSK4, 59±10% upon TLR5/5 homodimer activation by flagellin and by 19±3% upon TLR2/2 homodimer activation by FLS1. These results indicate that FXa inhibits the pro-inflammatory response induced by multiple TLR ligands, presumably by acting on shared signalling components downstream of TLR activation.
Figure 4.2.2: FXa reduces TNFα secretion induced by activation of various extracellular TLRs on THP1 cells: THP1 cells were incubated with PBS (black bars)/ FXa (white bars; 20nM) for 1 hour in assay buffer 3 prior to stimulation with TLR agonists LPS (500ng/mL), HKLM (5x10^5 cells), Pam3CSK4 (500ng/mL), flagellin (50ng/mL) or FLS1 (50ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiment was performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01; * = p ≤ 0.05]
4.3: Molecular requirements for FXa regulation of LPS-induced cytokine secretion on monocytes

To characterize the molecular mechanism that mediates FXa regulation of myeloid cell cytokine response to LPS stimulation, FXa isoforms and truncations were assessed and compared to full length FXa. FXa-β is an isoform of FXa generated in vivo as a result of autoproteolytic excision of a 4kDa peptide from its archetypal α form. Both α and β FXa have identical procoagulant function and enzymatic activity, but has been suggested to possess discrepant cell surface receptor affinities. To investigate whether FXa anti-inflammatory signalling efficacy on monocytes is similar between α and β FXa isoforms, THP1 cells were treated with either FXa-α or FXa-β for 3 hours prior to stimulation with LPS. FXa-β inhibited LPS-induced TNFα secretion from THP1 cells to a similar degree as FXa-α, demonstrating that both isoforms are equally effective in regulating the cytokine response to LPS stimulation on monocytes (Figure 4.3.1 a).

A truncated variant of FXa missing the N-terminal Gla domain, FXaGD, was also tested. In contrast to full length FXa, FXaGD failed to significantly impair LPS-induced TNFα secretion (Figure 4.3.1 b), highlighting a requirement for the Gla domain for FXa anti-inflammatory activity on myeloid cells. To determine the capacity of bovine FXa, a close structural homolog of human FXa, to mediate anti-inflammatory signalling on monocytes, THP1 cells were treated with bovine FXa prior to LPS stimulation. Despite their structural homology, bovine FXa was completely ineffective in reducing LPS-induced TNFα expression compared to its human counterpart, suggesting that the molecular requirements for FXa anti-inflammatory activity on THP1 cells are not present in bovine FXa (Figure 4.3.1 c).
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- Graph A: Fxα: - Fxβ: +
- Graph B: Fxα: + Fxβ: +
- Graph C: Fxα: + Fxβ: -

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Figure 4.3.1: LPS-induced TNFα secretion from THP1 cells is reduced by α and β isoforms of FXa but not Gla-domainless or bovine FXa: THP1 cells were incubated with FXa or (a) FXa-β (b) FXa<sub>GD</sub> or (c) FXa<sub>BOVINE</sub> (all 20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (500ng/mL) for 4 hours. TNFα secretion was measured using HEK Blue reporter cells. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = p < 0.01]
To assess whether proteolytic activity is required for FXa regulation of LPS-induced TNFα secretion, a FXa variant rendered proteolytically inactive by activate site blockade with a tripeptide chloromethyl ketone inhibitor (FXaDEGR) was used. THP1 cells were incubated with FXaDEGR prior to stimulation with LPS and TNFα secretion was measured using HEK Blue cells. In contrast to fully active FXa, FXaDEGR failed to significantly reduce LPS-induced TNFα secretion indicating proteolytic activity of FXa is necessary for regulation of LPS-induced pro-inflammatory cytokine release (Figure 4.3.2).

**Figure 4.3.2: Proteolytic activity is required for FXa reduction of LPS-induced TNFα secretion from THP1 cells:** THP1 cells were incubated with FXa/ FXaDEGR (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiment was performed in triplicate and data are presented as mean ± S.D. [*** = p < 0.001]
During thrombin generation, FXa associates with FVa upon exposed negatively-charged phospholipids on the plasma membrane to activate prothrombin in vivo. The presence of FVa could therefore feasibly modulate FXa anti-inflammatory activity. The effect of FXa interaction with FVa on FXa regulation of the cytokine response to LPS was investigated. THP1 cells were treated with FXa in the presence of FVa for 3 hours prior to stimulation with LPS and TNFα secretion was measured using HEK Blue cells. The presence of FVa had no effect upon the ability of FXa to suppress LPS-induced TNFα secretion (Figure 4.3.3) indicating that despite acting as a procoagulant cofactor for FXa, FVa does not affect its anti-inflammatory activity on myeloid cells.

Figure 4.3.3: FXa impairment of LPS-induced TNFα secretion from THP1 cells is maintained in the presence of FVa: THP1 cells were incubated with FXa (20nM) in the presence of FVa (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiment was performed in triplicate and data are presented as mean ± S.D. [** = p ≤ 0.01]
The FXa EGF-like domains are connected by a short peptide sequence containing amino acid residues 83-88. This inter-EGF sequence has previously been identified as a region required for FXa binding to the surface of monocytes and macrophages. To investigate whether the FXa inter-EGF region modulates FXa inhibition of the cytokine response to LPS stimulation on monocytes, a short peptide replicating the inter-EGF sequence of FXa (FX\textsubscript{83-88}) was used. The FX\textsubscript{83-88} peptide competitively inhibits FXa-monocyte interactions which are mediated by the FXa inter-EGF region. THP1 cells were incubated with FXa for 3 hours in the presence of FX\textsubscript{83-88} prior to stimulation with LPS for 4 hours. The peptide alone did not induce cytokine secretion and had no effect on LPS-induced TNF\textalpha{} production (Figure 4.3.4 a, white and black bars). Despite this, FX\textsubscript{83-88} added with FXa completely attenuated FXa inhibition of TNF\textalpha{} secretion in response to LPS (Figure 4.3.4 a, grey bars). To ensure the specificity of the inhibitory effect of FX\textsubscript{83-88}, a scrambled version of the same peptide, FX\textsubscript{83-88SCR}, was tested. FX\textsubscript{83-88SCR} similarly did not induce cytokine secretion alone nor affect LPS-induced TNF\textalpha{} production (Figure 4.3.4 a). Furthermore, FX\textsubscript{83-88SCR} did not affect FXa reduction of the cytokine response to LPS stimulation, confirming the specificity of FX\textsubscript{83-88} inhibition (Figure 4.3.4 a). The effect of FX\textsubscript{83-88} was dose-dependent (IC\textsubscript{50} ~ 1.3\mu{}g/mL FX\textsubscript{83-88} peptide) (Figure 4.3.4 b) underscoring the importance of the FXa inter-EGF amino acid sequence 83-88 in mediating regulation of the cytokine response to LPS stimulation.
Figure 4.3.4: FXa reduction of LPS-induced TNFα secretion from THP1 cells requires the inter-EGF domain sequence FX$_{83-88}$: THP1 cells were treated with (a) FX$_{a_{83-88}}$/FX$_{a_{83-88}SCR}$ (100μg/mL) (b) FX$_{a_{83-88}}$ (3.13-100μg/mL) for 30 minutes prior to incubation with PBS (black bars) and FXa (a, grey bars; b; 20nM) for 3 hours in assay buffer 3 and stimulated with LPS (500ng/mL) for 4 hours. TNFα secretion was measured using HEK Blue reporter cells. PBS treatment (white bars) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D. [*** = p < 0.001]
To investigate whether FXa regulation of TNFα secretion induced by activation of different TLRs occurs via a conserved molecular mechanism, the molecular requirements for FXa inhibition of cytokine response to TLR 1/2 activation by Pam3CSK4 were characterized. As with LPS, FXa isoforms and truncations FXa-β, FXaG0 and bovine FXa were used to investigate FXa regulation of Pam3CSK4-induced cytokine secretion (Figure 4.3.1).

THP1 cells were incubated with either FXa or FXa-β/FXaG0/bovine FXa for 3 hours prior to stimulation with Pam3CSK4 for 4 hours and TNFα secretion measured using HEK Blue cells. As observed upon LPS stimulation, FXa-β was equally effective as the α isoform in reducing Pam3CSK4-induced TNFα secretion on THP1 cells (Figure 4.3.5 a) while incubation with FXaG0, in contrast to full length FXa, failed to inhibit Pam3CSK4-induced pro-inflammatory cytokine expression (Figure 4.3.5 b). Bovine FXa was similarly ineffective in reducing Pam3CSK4-induced TNFα secretion (Figure 4.3.5 c).
Figure 4.3.5: Pam3CSK4-induced TNFα secretion from THP1 cells is reduced by α and β isoforms of FXa but not Gla-domainless or bovine FXa: THP1 cells were incubated with FXa/ (a) FXa-β (b) FXa GD (c) bovine FXa (all 20nM) for 3 hours in assay buffer 3 prior to stimulation with Pam3CSK4 (Pam; 500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiments were performed in triplicate and data are presented as mean ± S.D. [** = p < 0.01]
To assess whether proteolytic activity is required for FXa regulation of TLR2-induced TNFα secretion, FXaDEGR was used. THP1 cells were incubated with FXaDEGR for 3 hours prior to stimulation with Pam3CSK4 for 4 hours and TNFα secretion measured using HEK Blue cells. FXaDEGR failed to significantly reduce Pam3CSK4-induced TNFα secretion indicating that blockade of the FXa active-site also ablates the ability of FXa to inhibit TLR2-induced pro-inflammatory cytokine production (Figure 4.3.6).

Figure 4.3.6: Proteolytic activity is required for FXa reduction of Pam3CSK4-induced TNFα secretion from THP1 cells: THP1 cells were incubated with FXa/ FXaDEGR (20nM) for 3 hours in assay buffer 3 prior to stimulation with Pam3CSK4 (Pam; 500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. Experiment was performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
Finally, FX_{83-88} was used to investigate whether the FXa inter-EGF region is required for FXa regulation of the response to TLR2 stimulation. THP1 cells were incubated with FXa for 3 hours in the presence of FX_{83-88} prior to stimulation with Pam3CSK4 for 4 hours. The presence of FX_{83-88} did not alter Pam3CSK4-induced cytokine production alone, but completely attenuated FXa inhibition of TNFα secretion in response to Pam3CSK4 (Figure 4.3.7). A scrambled version of the same peptide (FX_{83-88}SCR) had no such inhibitory effect (Figure 4.3.7).

Collectively, these results demonstrate that the molecular requirements for FXa regulation of TNFα secretion induced by Pam3CSK4 activation of TLR2 are consistent with those observed in the case of LPS activation of TLR4 (section 4.2) and suggest that FXa regulation of TLR-induced cytokine secretion likely occurs due to regulation of conserved downstream components of TLR activation pathways.
Figure 4.3.7: FXa reduction of Pam3CSK4-induced TNFα secretion from THP1 cells requires the inter-EGF domain sequence FX\textsubscript{83-88}: THP1 cells were treated with FXa\textsubscript{83-88}/FXa\textsubscript{83-88SCR} (100µg/mL) for 30 minutes prior to incubation with PBS (black bars) and FXa (grey bars, 20nM) for 3 hours in assay buffer 3 and stimulated with Pam3CSK4 (Pam; 500ng/mL) for 4 hours. TNFα secretion was measured using HEK Blue reporter cells. Experiment was performed in triplicate and data are presented as mean ± S.D. [* = p ≤ 0.5; ** = p ≤ 0.001]
4.4: Signalling mechanism mediating FXa regulation of LPS-induced cytokine secretion on monocytes

4.4.1: FXa regulation of LPS-induced NF-κB activation on THP1 cells

Activation of the transcription NF-κB controls the expression of an array of pro-inflammatory cytokine genes. We sought to examine whether NF-κB activation by LPS was impaired by FXa-mediated cellular signalling. To enable this, a THP1 cell line stably transfected with an NF-κB-dependent secreted AP reporter construct (THP1-XBlue-CD14 cells) was used. Exposure of THP1-XBlue-CD14 cells to inflammatory stimuli results in activation of the NF-κB/AP-1 pathways and expression of the secreted AP reporter gene. AP can then be measured using a colorimetric AP substrate, QUANTI Blue.

THP1-XBlue-CD14 cells were treated with LPS for 6 hours to induce activation of NF-κB/AP-1 and expression of AP. AP activity in the THP1-XBlue-CD14 cell supernatant increased significantly and proportionately with increasing LPS stimulation (p ≤ 0.001) (Figure 4.4.1 a). Minimal AP activity was observed in the supernatant of unstimulated THP1-XBlue-CD14 cells (Figure 4.4.1 a). To investigate the effect of FXa on LPS-induced NF-κB activation, THP1-XBlue-CD14 cells were incubated with FXa for 3 hours and subsequently stimulated with LPS for 6 hours. LPS stimulation resulted in NF-κB activation (Figure 4.4.1 b). Prior incubation with FXa significantly inhibited NF-κB activation (p ≤ 0.001) (Figure 4.4.1 b).
Figure 4.4.1: FXa reduces LPS-induced NF-κB activation on THP1 cells: THP1-XBlue-CD14 NF-κB reporter cells were used to investigate the effect of FXa on LPS-induced NF-κB activation (Chapter 2.14). (a) THP1-XBlue-CD14 cells were stimulated with LPS (●; 12.5-200ng/mL) for 6 hours in assay buffer 3. Activation of NF-κB by LPS results in secretion of AP by the THP1-XBlue-CD14 cells which was quantified using QUANT! Blue. (b) THP1-XBlue-CD14 cells were incubated with PBS (●)/ FXa (O; 20nM) for 1 hour in assay buffer 3 prior to stimulation with LPS (31.3-500ng/mL) for 6 hours. Secretion of AP by the THP1-XBlue-CD14 cells was quantified using QUANT! Blue. Experiments were performed in triplicate and data are presented as mean ± S.D..
4.4.2: Role of PI3K signalling in FXa regulation of LPS-induced TNFα secretion from THP1 cells

Numerous negative regulatory mechanisms exist in order to control the magnitude of NF-κB activation upon TLR activation. Pertinently, APC, a structural homolog of FXa, induces PI3K activation to negatively regulate the cytokine response to LPS stimulation on U937 monocytes. To investigate whether PI3K pathway activation is also required for FXa regulation of LPS-induced NF-κB activation and cytokine production, a PI3K inhibitor (wortmannin) was utilised. THP1 cells were treated with FXa for 3 hours in the presence of wortmannin prior to stimulation with LPS for 4 hours. TNFα secretion was measured using HEK Blue cells.

Wortmannin alone did not induce TNFα expression from THP1 cells and but resulted in a small increase in LPS-induced TNFα secretion (Figure 4.4.2 a, white and black bars). The presence of wortmannin with FXa completely attenuated FXa inhibition of TNFα secretion in response to LPS (Figure 4.4.2 a, grey bars). The effect of wortmannin was dose-dependent (IC$_{50}$ ~ 0.5μM) (Figure 4.4.2 b). To verify that PI3K pathway activation is required for FXa regulation of the inflammatory response to LPS on monocytes, PBMCs were treated with wortmannin prior to incubation with FXa for 3 hours. The cells were then treated with LPS for 18 hours and secretion of TNFα was measured by ELISA. As was observed on THP1 cells, wortmannin did not induce TNFα expression from unstimulated cells but caused a small increase in LPS-induced TNFα secretion (Figure 4.4.2 c, white and black bars). Similarly, wortmannin completely prevented FXa impairment of LPS-induced TNFα secretion (Figure 4.4.2 c, grey bars).
Figure 4.4.2: FXa reduction of LPS-induced TNFα secretion from monocytes is inhibited by wortmannin: THP1 cells were treated with wortmannin ((a) 1.25µM and (b) 0.313-2.5 µM) for 30 minutes prior to incubation with PBS (a, black bars; b, O) / FXa (a, grey bars; b, •; 20nM) for 3 hours in assay buffer 3 then stimulated with LPS (500ng/mL) for 4 hours. TNFα secretion was measured using HEK Blue reporter cells. (c) PBMCs were isolated from buffy coat whole blood component and treated with wortmannin (1.25µM) for 30 minutes prior to incubation with PBS (a, black bars) / FXa (a, grey bars; 20nM) for 3 hours in assay buffer 3 and stimulated with LPS (50ng/mL) for 18 hours. TNFα secretion was measured using ELISA. PBS treatment (white bars) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001]
The PI3K pathway modulates the magnitude of the cytokine response to primary activation of multiple TLRs\textsuperscript{290}. To investigate whether PI3K pathway activation is also required for FXa regulation of Pam3CSK4-induced TNF\(\alpha\) secretion, the PI3K inhibitor wortmannin was used. THP1 cells were treated with FXa in the presence of wortmannin prior to stimulation with Pam3CSK4 and secretion of TNF\(\alpha\) was measured using HEK Blue cells. Wortmannin treatment alone did not induce TNF\(\alpha\) expression from THP1 cells but resulted in a small increase in Pam3CSK4-induced TNF\(\alpha\) production (Figure 4.4.3, white and black bars). Its presence alongside FXa completely attenuated FXa inhibition of TNF\(\alpha\) secretion in response to TLR2 activation (Figure 4.4.3, grey bars) demonstrating that FXa regulation of both TLR4 and TLR2 activation requires the PI3K signalling pathway.
Figure 4.4.3: FXa reduction of Pam3CSK4-induced TNFα secretion from THP1 cells is inhibited by wortmannin: THP1 cells were treated with wortmannin (1.25μM) for 30 minutes prior to incubation with PBS (black bars) / FXa (grey bars; 20nM) for 3 hours in assay buffer 3 and stimulated with Pam3CSK4 (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. PBS treatment (white bars) was used as a negative control. Experiment was performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
4.5: Cell surface receptor requirements for FXa regulation of LPS-induced cytokine secretion on myeloid cells

4.5.1: Role of EPCR in FXa regulation of LPS-induced cytokine secretion on myeloid cells

FXa binds to EPCR on endothelial cells to mediate protection from thrombin-induced endothelial cell barrier disruption. To determine whether EPCR might also mediate FXa anti-inflammatory activity on monocytes, we used a monoclonal anti-EPCR antibody (RCR-252). THP1 cells were treated with FXa in the presence of RCR-252 prior to stimulation with LPS and TNFα secretion was measured. RCR-252 did not induce TNFα secretion from unstimulated cells however its presence caused reduction in LPS-induced cytokine production in the absence of FXa (Figure 4.5.1, white and black bars). Despite this, the relative efficacy of FXa anti-inflammatory signalling was similar in the presence and absence of RCR-252 (Figure 4.5.1, grey bars). This suggests that FXa regulation of the cytokine response to LPS on monocytes occurs independently of EPCR.
Figure 4.5.1: FXa reduction of LPS-induced TNFα secretion from THP1 cells does not require interaction with EPCR: THP1 cells were incubated with RCR-252 (25μg/ml) for 30 minutes, prior to incubation with PBS (black bars)/ FXa (grey bars, 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. PBS treatment (white bars) was used as a negative control. Experiment was performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
4.5.2: Role of LDLR family members in FXa regulation of LPS-induced cytokine secretion on myeloid cells

VKD coagulation proteases have recently been identified as novel ligands for LDLR family members that can mediate diverse functional activities. ApoER2 is a crucial regulator of anti-inflammatory signalling by the FXa homolog APC on U937 monocytic cells. Furthermore, another LDLR, lipoprotein receptor protein 1 (LRP1) can bind to FXa in complex with tissue factor pathway inhibitor (TFPI) to facilitate FXa catabolism.

In order to determine whether an LDLR interaction might also modulate FXa anti-inflammatory activity on monocytes, we used receptor-associated protein (RAP), a protein which binds to the ligand binding region of all LDLRs. THP1 cells were treated with FXa in the presence of RAP prior to stimulation with LPS and TNFα secretion was measured. RAP did not induce TNFα secretion from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 4.5.2, white and black bars). The presence of RAP, however, completely ablated FXa inhibition of LPS-induced TNFα secretion (Figure 4.5.2, grey bars) indicating a prominent role for an LDLR family member as a mediator of FXa anti-inflammatory signalling on monocytes.
Figure 4.5.2: FXa reduction of LPS-induced TNFα secretion from THP1 cells requires LDLR family member interaction: THP1 cells were incubated with RAP (80μM) for 30 minutes, prior to incubation with PBS (black bars)/ FXa (grey bars, 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. PBS treatment (white bars) was used as a negative control. Experiment was performed in triplicate and data are presented as mean ± S.D. [*** = p ≤ 0.001]
To examine the role of LDLRs in FXa regulation of pro-inflammatory cytokine secretion on macrophages, BMDMs in were obtained from wild type BALB/c mice. BMDMs were treated with FXa in the presence of RAP. The cells were stimulated with LPS and secretion of TNFα and IL-6 was measured by ELISA. As observed on THP1 cells, FXa reduction of LPS-induced secretion of both TNFα (Figure 4.5.3 a) and IL-6 (Figure 4.5.3 b) was prevented by the presence of RAP, indicating a crucial role for an LDLR family member in FXa inhibition of LPS inflammatory signalling on both monocytes and macrophages.
Figure 4.5.3: FXa reduction of LPS-induced cytokine secretion from macrophages requires LDLR family member interaction: Murine BMDMs were treated with RAP (80μM) for 30 minutes prior to incubation with FXa (20nM) for 3 hours in assay buffer 3 and stimulated with LPS (50ng/mL) for 18 hours. (a) TNFα and (b) IL-6 secretion was determined by ELISA (Chapter 2.15). Experiments were performed in triplicate and data are presented as mean ± S.D.. [* = p ≤ 0.001]
In order to determine which LDLR was the target for RAP, THP1 cells were incubated with FXa in the presence of mouse monoclonal antibodies directed against the intracellular C-terminus and the extracellular N-terminus of ApoER2 prior to LPS treatment. In addition, mouse isotype IgG was added at an identical concentration to both anti-ApoER2 antibodies. Neither the anti-ApoER2 antibodies nor the mouse isotype IgG induced TNFα secretion from untreated cells or had any effect upon LPS-induced pro-inflammatory cytokine production (Figure 4.5.4 a, white and black bars). The presence of the antibody directed against the intracellular ApoER2 C-terminus had no effect on FXa anti-inflammatory signalling (Figure 4.5.4 a, grey bars). Similarly, mouse isotype IgG did not inhibit the effect of FXa. The presence of the antibody directed against the extracellular ApoER2 N-terminus, however, completely prevented FXa impairment of LPS-induced TNFα secretion (Figure 4.5.4 a, grey bars). The inhibitory effect of ApoER2 blockade was dose-dependent (Figure 4.5.4 b), indicating a novel role for ApoER2 in enabling FXa anti-inflammatory activity on THP1 cells.
Figure 4.5.4: FXa reduction of LPS-induced TNFα secretion from monocytes requires ApoER2: (a) C-/N-terminal targeted anti-ApoER2 monoclonal antibody or control mouse IgG (10μg/mL) (b) N-terminal targeted anti-ApoER2 monoclonal antibody (0.625-10μg/mL) was incubated with THP1 cells for 30 minutes, prior to incubation with PBS (a, black bars; b, O)/FXa (a, grey bars; b, ●; 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. PBS treatment (white bars) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p < 0.001]
4.6: The role of PARs in FXa regulation of LPS-induced cytokine secretion on myeloid cells

FXa can activate both PAR1 and PAR2 to mediate anti-inflammatory activity on endothelial cells\(^\text{139}\). Blockade of the FXa active site prevented inhibition of LPS- and Pam3CSK4-induced cytokine expression from monocytes suggesting that activation of PAR1 and/or PAR2 by FXa might contribute to its regulation of the TLR-induced pro-inflammatory response.

To investigate a possible role for PAR1, THP1 cells were incubated with FXa in the presence of the function blocking anti-PAR1 antibody, ATAP2, prior to stimulation with LPS, followed by assessment of TNF\(\alpha\) secretion. PAR1 blockade did not induce TNF\(\alpha\) expression from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 4.6.1 a, white and black bars). Similarly the presence of ATAP2 did not inhibit FXa impairment of LPS-induced TNF\(\alpha\) secretion (Figure 4.6.1 a, grey bars).

To verify that FXa anti-inflammatory signalling on monocytes occurs independently of PAR1, PBMCs were treated with a PAR1 antagonist (FR131117) prior to incubation with FXa. The cells were then stimulated with LPS and TNF\(\alpha\) secretion was measured. PAR1 antagonism did not induce TNF\(\alpha\) expression from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 4.6.1 b, white and black bars). As was observed on THP1 cells, blockade of PAR1 failed to prevent FXa impairment of LPS-induced TNF\(\alpha\) secretion (Figure 4.6.1 b, grey bars) demonstrating that FXa regulation of TLR activation on monocytes does not require PAR1 activation.
Figure 4.6.1: FXa reduction of LPS-induced TNFα secretion from monocytes does not require PAR1: (a) THP1 cells were treated with ATAP2 (10μg/mL) for 30 minutes, prior to incubation with PBS (black bars)/ FXa (grey bars, 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (500ng/mL) for 4 hours after which TNFα secretion was measured using HEK Blue reporter cells. (b) PBMCs were treated with FR171113 (1.25μM) for 30 minutes, prior to incubation with PBS (black bars)/ FXa (grey bars) for 3 hours in assay buffer 3, then stimulated with LPS (50ng/mL) for 18 hours. TNFα secretion was measured using ELISA. PBS treatment (white bars) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001]
To investigate whether PAR2 activation is required for FXa regulation of LPS-induced cytokine expression, THP1 cells were incubated with FXa for 3 hours in the presence of a PAR2 antagonist (GB83), prior to stimulation with LPS. PAR2 antagonism did not induce TNFα expression from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 4.6.2 a and b). Blockade of PAR2, however, dose-dependently prevented FXa inhibition of LPS-induced TNFα secretion on THP1 cells (p ≤ 0.001) (Figure 4.6.2 a). Similarly, treatment of PBMCs with GB83 prior to FXa exposure and LPS stimulation resulted in a dose-dependent reduction in the anti-inflammatory activity of FXa (p ≤ 0.001) (Figure 4.6.2 b). A higher concentration of the PAR2 antagonist was required to achieved half maximal inhibition of the FXa anti-inflammatory effect on PBMCs compared to THP1 cells (IC₅₀ ~ 1.4µM compared to 0.3µM on PBMCs and THP1 cells, respectively) likely due to altered expression levels of PAR2 between THP1 cells and primary monocytes. These results demonstrate that FXa regulation LPS-induced cytokine expression on monocytes is mediated via activation of PAR2.
Figure 4.6.2: FXa reduces LPS-induced TNFα expression from monocytes via proteolytic activation of PAR2: (a) THP1 cells or (b) PBMCs were treated with GB83 (0.0625-6.25μM) prior to incubation with PBS (○) or FXa (●; 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (a, 500ng/mL; 4 hours) (b, 50ng/mL; 18 hours). TNFα secretion was determined (a) using HEK Blue TNFα reporter cells (b) by ELISA. PBS treatment (□) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D..
To investigate the role of PAR2 in regulation of macrophage cytokine expression by FXa, peritoneal macrophages (PECs) were isolated from wild type and PAR2−/− BALB/c mice and exposed to FXa/ FXaDEGR prior to LPS stimulation and secretion of pro-inflammatory cytokines was measured. LPS treatment induced similar levels of TNFα and IL-6 expression from both wild type and PAR2−/− macrophages and treatment of wild type PECs with FXa significantly inhibited PEC pro-inflammatory cytokine expression upon LPS stimulation. TNFα and IL-6 secretion was impaired by 76±13% and 76±16%, respectively (Figure 4.6.3 a and b, black bars). In keeping with the impaired anti-inflammatory activity of FXaDEGR on monocytes, FXaDEGR suppression of TNFα and IL-6 production from PECs was 2-3 fold impaired compared to active FXa, reducing TNFα and IL-6 secretion expression by 35±16% and 34±16%, respectively, although this failed to reach statistical significance.

Incubation of PAR2−/− macrophages with FXa prior to LPS stimulation mediated only a 36±12% and 39±14% reduction in TNFα and IL-6 production, respectively (Figure 4.6.3 a and b, grey bars). The anti-inflammatory effect of FXa on PAR2−/− macrophages was half that of FXa on wild type macrophages and failed to reach statistical significance in the case of TNFα. Prior exposure to FXaDEGR reduced pro-inflammatory cytokine secretion to a similar degree on both wild type and PAR2−/− macrophages (Figure 4.6.3 a and b). Furthermore the efficacy of FXaDEGR was similar to that of fully active FXa on PAR2−/− macrophages. Collectively, these data indicate that FXa proteolytic activation of PAR2 is required for FXa regulation of TLR-induced cytokine secretion on primary macrophages.
Figure 4.6.3: FXa reduces LPS-induced cytokine secretion from macrophages via proteolytic activation of PAR2: Murine PECs were isolated from wild type (black bars) and PAR2^−/− (grey bars) BALB/c mice and exposed to PBS/FXa/FXa_DGR (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (20ng/mL) for 18 hours. (a) TNFα and (b) IL-6 secretion was determined by ELISA. PBS treatment was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01; * = p ≤ 0.05]
4.7: Discussion

4.7.1: FXa regulates TLR-induced pro-inflammatory signalling on myeloid cells

Numerous studies have shown that APC can initiate anti-inflammatory signalling on myeloid cells. The aim of this chapter was to define the role of homologous VKD proteases in regulating monocyte and macrophage inflammatory signalling pathways. FVIIa and FXa have been described to drive the response to pro-inflammatory stimuli. FVIIa increases secretion of pro-inflammatory cytokines IL-6 and IL-8 and production of reactive oxygen species from monocyte derived macrophages upon LPS stimulation. Similarly, exposure to FXa has been reported to induce pro-inflammatory cytokine expression from HUVECs and macrophages.

In contrast, incubation of THP-1 cells with APC, FVIIa, FIXa or FXa induced negligible TNFα expression in the current study and similarly, exposure to FXa failed to stimulate a pro-inflammatory response on BMDMs. The paradoxical effects of FXa and FVIIa reported may occur as a result of divergent experimental conditions. In each of the above studies, cells were exposed to FVIIa or FXa for extended time periods ranging from 12 to 48 hours to illicit pro-inflammatory effects. Interestingly, a study by Bachli et al. (2003) identified that the duration of exposure impacts hugely upon the nature of FVIIa and FXa signalling. In this study, incubation of murine fibroblasts with FVIIa or FXa for 12-24 hours significantly increased expression and secretion of MCP-1, IL-6 and IL-8 while incubation of the same cells with the same concentration of FVIIa or FXa for 1-9 hours failed to induce pro-inflammatory cytokine production, in keeping with our observations.
Moreover, the FVIIa or FXa concentrations used varied greatly between studies and in all cases far exceeded those utilized in the data described in this thesis. Notably, at high concentrations (200nM) APC can also mediate pro-inflammatory effects and disrupt endothelial cell barrier integrity\textsuperscript{222}. Conversely, at very low concentrations (50pM) thrombin can elicit both barrier protective and anti-apoptotic effects on endothelial cells\textsuperscript{222}. Recent work investigating the effect of FXa activation of PARs on endothelial cells has indicated that anti-inflammatory and cytoprotective effects are observed when cells are exposed to lower concentrations of FXa (5-20nM) for shorter durations (3hrs)\textsuperscript{139,154,155,161}. Incubation of myeloid cells with similar concentrations of FXa for a similar duration as described in this chapter also produced anti-inflammatory effects. Collectively, these studies suggest that coagulation proteases can differentially regulate inflammatory signalling pathways depending on the experimental conditions used.

As previously described, pre-incubation with APC inhibited the THP-1 cell and PBMC inflammatory response to LPS stimulation (Figure 4.1.3)\textsuperscript{232,234-236}. Coagulation FVIIa and FIXa neither diminished nor enhanced TNFα secretion however, pre-incubation with FXa significantly inhibited the response to LPS on THP1 cells and PBMCs (Figures 4.1.3 and 4.1.6). The effect of FXa was dose-dependent with a greater than 50% reduction in TNFα expression observed as a result incubation with <3nM FXa. Of note, the anti-inflammatory effect was only observed upon cells pre-incubated with FXa for at least 1 hour before LPS stimulation (Figure 4.1.4). This suggests that FXa signalling must be initiated prior to the application of an inflammatory stimulus and indicates that that the discrepant reported pro- and anti-inflammatory effects of FXa may be further explained by differences in experimental design.
Incubation of THP1 cells with FXa did not alter cell viability (Figure 4.1.5), consistent with a study published by Borensztajn et al. (2007), which reported that while capable of inducing apoptosis on various cancer cell lines, FXa signalling has no effect on monocyte viability\(^\text{285}\). FVa acts as a cofactor for FXa procoagulant function in the prothrombinase complex. The presence of FVa had no effect on FXa anti-inflammatory signalling on monocytes (Figure 4.3.3). This is in keeping with a study published by Riewald et al. (2001), which reported that the presence of FVa did not alter FXa PAR-mediated activation of MAPK on HeLa cells\(^\text{151}\). FVa is therefore an important cofactor for procoagulant activity, but cannot modulate FXa signalling functions.

The ability of FXa to regulate cytokine expression was not limited to that induced by TLR4 activation. Monocytes and macrophages express multiple TLRs which individually recognize specific PAMPs\(^\text{290}\). Experiments in which synthetic agonists directed against specific TLRs were co-incubated with FXa demonstrated that FXa regulates the cytokine response to activation of multiple TLR homo- and hetero-dimers (including TLR 1/2, TLR2/2, TLR5/5 and TLR2/6) (Figure 4.2.2). Despite the specificity of activating PAMPs, there is substantial overlap within the network of intracellular mediators which co-ordinates and regulates the signalling pathways induced by different TLRs\(^\text{290}\). All TLRs with the exception of TLR3 signal via MyD88-dependent activation of IRAKs that, following activation of further downstream kinases, leads to NF-κB activation and up-regulation of pro-inflammatory cytokine expression\(^\text{289}\).

Similarly, negative regulators of TLR signalling are highly conserved. IRAKM and SOCS1 are reported to reduce cytokine expression by TLR4 and TLR9 and are speculated to partake
in regulation of inflammatory signalling mediated by activation of other TLRs\textsuperscript{290}. Activation of PI3K has also been described to inhibit TLR2-, TLR4- and TLR-9 induced inflammation \textit{in vivo}. The broad anti-inflammatory effect of FXa in response to activation of multiple TLRs suggests that FXa functions to modulate shared downstream components of TLR signalling pathways.

NF-κB is the major transcription factor that regulates genes responsible for the cytokine response to TLR activation on monocytes and macrophages. Upon stimulation, TLRs initiate a cascade of phosphorylation events through which NF-κB becomes activated enabling it to enter the nucleus and up regulate expression of specific genes including those encoding pro-inflammatory cytokines\textsuperscript{290}. Prior exposure to FXa directly inhibited LPS-induced activation of NF-κB on THP1 cells (\textbf{Figure 4.4.1}). This result is in keeping with reports that FXa reduces TNFα-induced NF-κB activation on endothelial cells\textsuperscript{139} and indicates that the anti-inflammatory effect of FXa is achieved primarily by regulation of a component of the pathway controlling TLR induced NF-κB activation, rather than by impairment of receptor activation or inflammatory cytokine release.

Transcriptional regulation of pro-inflammatory gene expression is achieved through modulation of positive and negative signal transduction pathways. The PI3K pathway has been implicated in both negative\textsuperscript{294,295} and positive\textsuperscript{296} regulation of TLR induced pro-inflammatory signalling. Importantly, activation of the PI3K pathway in human monocytes and THP-1 cells is demonstrated to limit LPS-induction of TNFα expression\textsuperscript{297}. Interestingly, regulation of PI3K signalling by other homologous VKD proteases has been described previously. APC phosphorylation of the adaptor protein disabled-1 (Dab1) activates PI3K signalling resulting in AKT phosphorylation and reduced TNFα expression by U937
monocytes. Consistent with this, the ability of FXa to regulate TLR2 and TLR4-induced TNFα expression from PBMCs and THP1 cells was completely and dose-dependently abolished by the PI3K inhibitor Wortmannin (Figures 4.4.2 and 4.4.3), suggesting FXa inhibition of pro-inflammatory cytokine production on monocytes is achieved via PI3K-dependent negative regulation of NF-κB activation.
4.7.2: FXa regulation of TLR-induced pro-inflammatory signalling requires ApoER2 and proteolytic activation of PAR2

FXa cytoprotective signalling on endothelial cells is mediated via interaction with EPCR\textsuperscript{155} and a FXa truncation missing the EPCR-binding Gla domain failed to reduce LPS- and Pam3CSK4-induced TNFα expression from THP1 cells (Figures 4.3.1 and 4.3.5). The presence of a function blocking anti-EPCR antibody did not prevent the anti-inflammatory effect of FXa on monocytes however, (Figure 4.5.1) demonstrating that FXa signalling on myeloid cells occurs independently of EPCR. Interestingly, the anti-inflammatory function of APC on monocytes also occurs independently of EPCR via a RAP sensitive mechanism, which specifically requires APC interaction with ApoER2\textsuperscript{232,237,243}.

FXa reduction of LPS-induced TNFα expression on monocytes was abolished in the presence of RAP and also by a monoclonal antibody directed against the ligand binding domain of ApoER2 (Figures 4.5.2 and 4.5.4). These results identify ApoER2 as a critical receptor not only in APC, but also in FXa mediated anti-inflammatory signalling on monocytes. ApoER2 is an LDLR comprised of 5 domains; a ligand binding domain, an EGF-like domain, an O-glycosylation domain, a transmembrane domain and a cytoplasmic domain\textsuperscript{298}. Similar to other LDLR family members, the cytoplasmic domain of ApoER2 contains an NXPY motif. Upon interaction with a signalling ligand, the NXPY motif of ApoER2 facilitates phosphorylation of Dab1 by Fyn and Src kinases which in turn induces activation of PI3K and GSK3β signalling pathways\textsuperscript{299}. This model is in keeping with the wortmannin sensitivity of FXa anti-inflammatory signalling on monocytes. White \textit{et al.} (2008) identified that APC directly binds to ApoER2 with high affinity\textsuperscript{245}. Whether the anti-inflammatory effect of FXa occurs as
a result of interaction between FXa and ApoER2 or participation of ApoER2, in an FXa-receptor signalling complex remains to be elucidated. The anti-inflammatory effect of FXa on murine macrophages was also inhibited by the presence of RAP (Figure 4.5.3). A previous study failed to identify the presence of ApoER2 on murine BMDM suggesting that an alternative, as yet unidentified, LDLR distinct from ApoER2 facilitates the RAP sensitive anti-inflammatory effect of FXa on murine macrophages\(^\text{239}\).

Active site blockade substantially diminished the effect of FXa on LPS- and Pam3CSK4-induced TNF\(\alpha\) expression on both monocytes (Figures 4.3.2 and 4.4.6) and macrophages (Figure 4.6.3), indicating a prominent role for FXa proteolytic activity in mediating anti-inflammatory activity and strongly suggesting the involvement of 1 or more PARs. Consistent with this finding are recent studies which demonstrate that FXa signalling is primarily mediated via proteolytic activation of PAR1 and/or PAR2\(^\text{139,151,154}\). FXa can mediate cellular signalling via activation of both PAR1 and PAR2 on different cell types but what dictates FXa's preferred target PAR is unknown. Neither a function-blocking anti-PAR1 antibody nor a PAR1 antagonist had any effect on FXa reduction of LPS-induced cytokine production from THP1 cells or PBMCs (Figure 4.6.1). The anti-inflammatory effect of FXa on monocytes was completely prevented by the presence of a PAR2 antagonist however (Figure 4.6.2). In addition, truncation of FXa's Gla domain, which reportedly reduces FXa proteolytic cleavage of PAR2\(^\text{161}\), completely prevented the anti-inflammatory effect of FXa.

FXa exhibited a significantly reduced ability to inhibit LPS-induced cytokine production on PAR2\(^{-/-}\) macrophages compared to wild type macrophages (Figure 4.6.2) further confirming that FXa anti-inflammatory activity on myeloid cells is mediated by PAR2.
activation. This is in agreement with previous studies, which indicate that anti-PAR2 antibodies block FXa-dependent reduction of NF-κB activation in response to TNFα treatment\textsuperscript{139,161}. Of note, PAR2 activation facilitates Gα\textsubscript{i}-dependent PI3K activation\textsuperscript{133}. Given the wortmannin-sensitivity of FXa anti-inflammatory activity on monocytes, PAR2-dependent activation of PI3K signalling represents a potential mechanism by which FXa suppression of LPS-induced cytokine production is mediated.

Notably, the FXa inter-EGF sequence Leu\textsuperscript{83} – Leu\textsuperscript{88} has previously been identified as crucial for FXa activation of PAR2\textsuperscript{140,161} and is postulated to mediate binding to a putative FXa myeloid cells receptor, EPR-1\textsuperscript{300}. The existence of a FXa receptor corresponding to EPR-1 has been questioned based on the inability to detect EPR-1-specific mRNA transcripts\textsuperscript{165} however, many reported FXa signalling activities require the 5 amino acid sequence contained within the FXa inter-EGF region postulated to represent the site through which FXa binds EPR-1\textsuperscript{139,166}. Furthermore, EPR-1 binding has been reported to localize FXa proximal to PAR2, supporting its preferential activation\textsuperscript{140}.

A peptide mimic of the FX inter-EGF sequence, FX\textsubscript{83-88}, produced a dose-dependent decrease in the ability of FXa to regulate LPS-stimulated cytokine production by THP-1 cells such that FXa anti-inflammatory activity could be completely blocked (Figures 4.3.4 and 4.3.7). This suggests that FXa interaction with a putative cell surface receptor via Leu\textsuperscript{83}-Leu\textsuperscript{88} is required in this signalling function. Of note, bovine FXa was unable to mount a comparable anti-inflammatory response to that of human FXa (Figures 4.3.1 and 4.3.5). The inter-EGF sequence of bovine FXa in not conserved with that of its human counterpart, providing a possible explanation for species-specific loss of function.
4.7.3 Conclusion

Our results indicate that exogenous FXa is comparable to APC in limiting pro-inflammatory cytokine production on monocytes and macrophages using in vitro cellular assays of inflammation. The global impact of FXa regulation of TLR responsiveness in vivo remains to be investigated.

The continuation of this project aims to ascertain whether FXa signalling on monocytes and macrophages is likely to be of physiological or pathological significance in vivo. Myeloid cell-specific APC anti-inflammatory signalling is crucial in protecting mice from LPS-induced lethality. Assessment of the efficacy of FXa in this setting, however, is complicated by multiple confounding factors. FXa has potent procoagulant activity and therefore administration of exogenous FXa may induce thrombotic complications in vivo. Additionally, once generated FXa is rapidly inhibited by serpins in plasma limiting the half life of exogenously administered FXa and therefore the duration of potential anti-inflammatory signalling in vivo. Prior studies that have utilized modified recombinant APC variants with signalling-selective activity to regulate murine endotoxemic responses provide useful insight as to how the anti-inflammatory activity of exogenous FXa could be investigated in vivo.

We intend to design and generate recombinant FXa variants with impaired ability to assemble into the prothrombinase complex and/or associate with inhibitors, but which retain the ability to associate with newly-identified myeloid cell surface receptors. Specific residues which are crucial for FXa interaction with its cofactor FVa have been identified. Mutation of such residues prevents FXa incorporation into the prothrombinase complex and
reduces FXa procoagulant potency > 5-fold. Similarly, the molecular regions within FXa which
are recognised by the inhibitory serpins anti-thrombin and protein Z have been
described\textsuperscript{304,305}. Mutation of residues contained within these regions could potentially extend
the half life of exogenously administered recombinant FXa in vivo.

Combination of these functional mutations will provide recombinant FXa variants
with reduced procoagulant potency and simultaneously reduced affinity for inhibitory
serpins. The anti-inflammatory signalling capabilities of these variants in response to TLR
activation upon monocytes and macrophages will be assessed using \textit{in vitro} cellular assays of
inflammation and ultimately used to investigate the effect of FXa anti-inflammatory activity
in murine models of inflammatory disease. This future work will yield further insight as to
whether the unanticipated anti-inflammatory activity of FXa can be converted into novel
therapeutic approaches for acute and/or chronic inflammatory disorders, similar to APC.
CHAPTER 5: THE ROLE OF APC GLYCOSYLATION IN PAR1-DEPENDENT CYTOPROTECTIVE SIGNALLING

5.1: Effect of enzymatic removal of N-linked glycans on APC anti-inflammatory signalling on monocytes

PAR1-dependent cytoprotective signalling by APC on endothelial cells is regulated by an N-linked carbohydrate moiety at Asn-329\(^{270}\). To determine the mechanism through which N-linked glycans on APC modulate cellular signalling and also whether APC glycosylation regulates PAR1-independent signalling, APC and APC N-linked sequon variants were investigated for their ability to mediate anti-inflammatory activity on LPS-stimulated monocytes.

5.1.1: Enzymatic removal of APC N-linked glycans

To investigate the role of APC glycosylation in mediating PAR1-independent anti-inflammatory signalling on monocytes, APC N-linked glycans were digested with an N-glycosidase (PNGase F). PNGase F fully removes N-linked glycans by cleaving between glycosylated Asn residues and the innermost GlcNAc of the oligosaccharides chain (Chapter 2.9). APC was incubated with PNGase F for 1 hour at 37°C. APC and PNGase F-treated APC (APC\(_{PNG}\)) were then subjected to SDS-PAGE and Comassie staining under non-reducing conditions (Chapter 2.6).
Untreated APC migrated as a double band representing α and β glycoforms of APC (Figure 5.1.1). The doublet appeared just below the 60kDa marker indicating a molecular weight of between ~50 and 60kDa. APC_{PNG} migrated further suggesting a molecular weight of between ~45 and 50kDa (Figure 5.1.1). The reduced molecular weight of APC_{PNG} demonstrates the successful removal of N-linked glycans via treatment with PNGaseF.

Figure 5.1.1: Enzymatic removal of N-linked glycans from APC using PNGase F: APC (1μg) was incubated with PNGase F (1000 U) at 37°C for 1 hour. APC and APC_{PNG} (both 500ng) were subjected to 4-20% SDS-PAGE under non-reducing conditions. Comassie staining was performed (Chapter 2.6).
To determine whether removal of N-linked glycan moieties influences APC active site function, the rate of hydrolysis of an APC-specific synthetic tripeptide chromogenic substrate (p-Glu-Pro-Arg-pNA.HCl) (CS-21(66)) by APC and APC\textsubscript{PNG} was compared (Chapter 2.5). The rate of CS-21(66) hydrolysis by APC\textsubscript{PNG} did not differ significantly from that of untreated APC, demonstrating that enzymatic removal of APC N-linked glycans does not effect amidolytic activity (Figure 5.1.2 a).

APC\textsubscript{PNG} inhibition of TF-initiated thrombin generation in PC-deficient plasma was determined to assess the effect of N-linked glycans on APC anticoagulant function. Initiation of coagulation with soluble TF (1pM) and CaCl\textsubscript{2} (16.67mM) resulted in rapid generation of thrombin (Figure 5.1.2 b, red line). As expected, APC attenuated TF-initiated thrombin generation such that peak thrombin generated was reduced ~3.5-fold by 3nM APC (Figure 5.1.2 b, blue line). Enzymatic removal of N-linked glycans enhanced APC attenuation of TF-initiated thrombin generation with 3nM APC\textsubscript{PNG} causing a ~7-fold reduction in peak thrombin generated (Figure 5.1.2 b, green line), corresponding to an approximately 2-fold increase in anticoagulant activity upon PNGase digestion.
Figure 5.1.2: APC\textsubscript{PNG} hydrolyses a chromogenic substrate with similar efficacy to APC, but displays enhanced anticoagulant function in plasma

(a) Amidolytic activity of APC (●) and APC\textsubscript{PNG} (O) (both 1000nM) was determined by measuring the rate of hydrolysis of the APC-specific synthetic chromogenic substrate CS-21(66) (2mg/ml) over time. The reaction was carried out at room temperature in a buffer containing 100mM NaCl and 20mM Tris-HCl (pH 7.5) with 2.5mM CaCl\textsubscript{2} and 0.1mg/ml BSA. APC amidolytic activity is expressed in terms of substrate cleaved, measured at OD\textsubscript{405nm}. The rate of CS-21(66) hydrolysis between samples was compared using linear regression, with statistical significance set at p ≤ 0.05. Experiments were performed in duplicate and are presented as mean ± S.D.. (b) The anticoagulant activity of APC and APC\textsubscript{PNG} was assessed in PC-deficient plasma using a thrombin generation assay. Plasma was incubated with sTF and phospholipid vesicles (4μM; 60% phosphatidylcholine, 20% phosphatidylycerine and 20% phosphatidylethanolamine) and APC (blue line; 3nM) or APC\textsubscript{PNG} (green line; 3nM) was added. Thrombin generation was initiated with CaCl\textsubscript{2} and measured by comparing rate of fluorogenic substrate hydrolysis to a thrombin standard. Thrombin generated in the absence of APC is represented by the red line.
5.1.2: APC anti-inflammatory signalling on monocytes

APC-mediated cellular signalling on monocytic cells inhibits pro-inflammatory cytokine release in response to LPS\(^{237}\). The anti-inflammatory activity of APC on monocytes is PAR1 independent. Instead, APC interacts with ApoER2 to initiate a poorly understood signalling pathway that involves PI3K, reelin-dependent signalling.

To confirm the absence of a role for PAR1 in APC signalling on monocytes, THP1 cells were incubated with APC in the presence and absence of the function blocking anti-PAR1 antibody, ATAP2, then stimulated with LPS. LPS-induced TNFα secretion from THP1 cells was then measured using HEK Blue TNF-α/IL-1β reporter cells (Chapter 4.1.1). Treatment of cells with LPS induced robust secretion of TNFα (Figure 5.1.3 a, black bars). As expected, blockade of PAR1 did not induce TNFα expression from unstimulated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 5.1.3 a, white and black bars). Incubation with APC significantly inhibited the cytokine response to LPS stimulation resulting in a 55±11% reduction in TNFα secretion for LPS-treated THP1 cells (Figure 5.1.3 a, grey bars). As was observed on PBMCs (Figure 3.2.5), inhibition of PAR1 did not impair the anti-inflammatory effect of APC.

To confirm the role of ApoER2 in APC PAR1-independent signalling on monocytes, THP1 cells were incubated with APC in the presence of a mouse monoclonal antibody directed against ApoER2 prior to stimulation with LPS. The anti-ApoER2 antibody did not induce TNFα secretion from untreated cells and had no effect upon LPS-induced pro-inflammatory cytokine production (Figure 5.1.3 b, white and black bars). However, its
presence completely prevented APC impairment of LPS-induced TNFα secretion (Figure 5.1.3 b, grey bars).

Finally, wortmannin was used to confirm the requirement for PI3K pathway activation to facilitate APC anti-inflammatory activity on monocytes. THP1 cells were treated with APC in the presence of wortmannin prior to stimulation with LPS and secretion of TNFα was measured using HEK Blue cells. Wortmannin treatment did not induce TNFα expression from THP1 cells, but did result in a small increase in LPS-induced TNFα secretion (Figure 5.1.3 b, white and black bars). Its presence alongside APC completely prevented APC inhibition of TNFα secretion in response to LPS (Figure 5.1.3 b, grey bars). Collectively, these data confirm that APC anti-inflammatory signalling on monocytes occurs via ApoER2-mediated PI3K pathway signalling and does not require PAR1 activation.
Figure 5.1.3: APC reduction of LPS-induced TNFα secretion from THP1 cells does not require PAR1: THP1 cells were treated with (a) ATAP2 (10µg/mL) (b) an anti-ApoER2 monoclonal antibody (10µg/mL) (c) wortmannin (1.25µM) for 30 minutes, prior to incubation with PBS (black bars) or APC (grey bars, 20nM) for 3 hours in assay buffer 3. Cells were then stimulated with LPS (500ng/mL) for 4 hours, after which TNFα release was measured using HEK Blue reporter cells, as before. AP activity in the cell supernatant is presented as a percentage of maximal AP activity induced by LPS (i.e. 100%). Experiments were performed in triplicate and data are presented as mean ± S.D.. [* = p < 0.05]
To investigate the role of glycosylation in APC PAR1-independent signalling on monocytes, THP1 cells were incubated with APC or APC\(_{\text{PNG}}\) prior to stimulation with. As expected, incubation of THP1 cells with APC resulted in a dose-dependent reduction in LPS-induced TNF\(\alpha\) production (Figure 5.1.4, circles). Incubation with APC\(_{\text{PNG}}\) resulted in similar a reduction in TNF\(\alpha\) secretion. The anti-inflammatory efficacy of APC\(_{\text{PNG}}\) did not differ significantly from that of untreated APC at all concentrations tested (Figure 5.1.4, triangles), demonstrating that N-linked glycan modification does not significantly influence ApoER2-dependent APC anti-inflammatory signalling on THP1 cells.

![Figure 5.1.4: APC and APC\(_{\text{PNG}}\) reduce LPS-induced TNF\(\alpha\) secretion from THP1 cells with similar efficacy](image)

**Figure 5.1.4: APC and APC\(_{\text{PNG}}\) reduce LPS-induced TNF\(\alpha\) secretion from THP1 cells with similar efficacy:** THP1 cells were incubated with APC (●) or APC\(_{\text{PNG}}\) (▲) (both 1.25-20nM) for 3 hours in assay buffer 3 prior to stimulation with control PBS (open symbols) or LPS (closed symbols; 500ng/mL) for 4 hours. TNF\(\alpha\) secretion was measured using HEK Blue reporter cells. The experiment was performed in triplicate and data are presented as mean ± S.D.. Statistical significance was set at \(p \leq 0.05\).
5.1.3: APC anti-inflammatory signalling on macrophages

Unlike monocytes, APC inhibition of pro-inflammatory cytokine release in response to LPS on murine macrophages is PAR1-dependent. APC anti-inflammatory signalling on macrophages occurs via interaction with CD11b/CD18 integrin followed by activation of PAR1\(^{239}\). To establish as assay to investigate the anti-inflammatory activity of APC on macrophages, immortalised murine macrophage cells (RAW264.7) were treated with APC for 3 hours followed by stimulation with LPS for 18 hours. Secretion of TNF\(\alpha\) and IL-6 was subsequently measured by ELISA. APC significantly inhibited pro-inflammatory cytokine release in response to LPS stimulation, reducing TNF\(\alpha\) and IL-6 secretion by 31\(\pm\)4% and 38\(\pm\)5%, respectively (20nM; \(p \leq 0.05\)) (Figure 5.1.5 a and b).
Figure 5.1.5: APC reduces LPS-induced pro-inflammatory cytokine secretion from RAW264.7 macrophages: RAW264.7 macrophages were incubated with APC (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of (a) TNFα and (b) IL-6 was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D. [* = p ≤ 0.05]
To confirm the requirement for PAR1 in APC anti-inflammatory signalling on LPS-stimulated macrophages, 2 approaches were used. Firstly, APC treated with a tripeptide chloromethyl ketone inhibitor (APCDEGR) to block enzymatic activity was used to prevent APC proteolysis of PAR1 (Chapter 3.1.1). RAW264.7 macrophages were treated with APCDEGR then stimulated with LPS. In contrast to APC, APCDEGR failed to significantly reduce LPS-induced TNFα secretion (Figure 5.1.6 a), confirming the need for proteolytic activity to enable APC anti-inflammatory signalling on macrophages.

Secondly, RAW264.7 macrophages were treated with a PAR1 antagonist (FR131117) prior to incubation with APC. Cells were then stimulated with LPS and secretion of TNFα was measured. FR131117 treatment alone did not induce cytokine expression from the RAW264.7 cells and did not affect in LPS-induced cytokine production (Figure 5.1.6 b, white and black bars). In combination with APC, FR131117 prevented inhibition of TNFα secretion in response to LPS stimulation (Figure 5.1.6 b, grey bars). Collectively, these results demonstrate that APC anti-inflammatory signalling on RAW246.7 macrophages requires proteolytic activation of PAR1.
Figure 5.1.6: APC-mediated reduction of LPS-induced pro-inflammatory cytokine secretion from RAW264.7 macrophages requires proteolytic activation of PAR1: (a) RAW264.7 macrophages were incubated with APC or APC$_{DEGR}$ (20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of TNFα was measured by ELISA. (b) RAW264.7 macrophages were treated with FR171113 (1.25µM) for 30 minutes, prior to incubation with PBS (black bars) or APC (grey bars) for 3 hours in assay buffer 3, then stimulated with LPS (50ng/mL) for 18 hours. Secretion of TNFα was measured by ELISA. PBS treatment (white bars) was used as a negative control. Experiments were performed in triplicate and data are presented as mean ± S.D.. [* = p ≤ 0.05]
To determine whether, similar to endothelial cells, APC de-glycosylation enhances PAR1-dependent signalling on macrophages, RAW264.7 cells were incubated with APC\textsubscript{PNG} prior to stimulation with LPS. As expected, incubation of RAW264.7 cells with APC resulted in a dose-dependent reduction in LPS-induced TNF\textalpha and IL-6 expression (p \leq 0.01) (Figure 5.1.7 a and b). Enzymatic removal of N-linked glycans enhanced APC attenuation of the cytokine response to LPS stimulation \sim 2-fold. 20nM APC\textsubscript{PNG} reduced secretion of TNF\textalpha and IL-6 by 49\pm10\% and 66\pm7\%, respectively, compared with 27\pm9\% and 36\pm7\% in the case of untreated APC (p \leq 0.01) (Figure 5.1.7 a and b).
Figure 5.1.7: PNGase F treatment enhances APC reduction of LPS-induced pro-inflammatory cytokine secretion from RAW264.7 macrophages: RAW264.7 macrophages were incubated with APC (black bars) or APC\textsubscript{PNG} (grey bars) (both 5-20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of (a) TNF\textalpha and (b) IL-6 was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = \( p < 0.01 \)]
APC<sub>PNG</sub> enhanced anti-inflammatory signalling on primary murine macrophages (BMDMs) was also examined. BMDMs were exposed to APC<sub>PNG</sub> followed by stimulation with LPS. As observed on RAW264.7 cells, APC significantly inhibited LPS-induced production of TNFα and IL-6 by 28±1% and 27±2%, respectively (Figure 5.1.8 a and b). Enzymatic removal of N-linked glycans significantly enhanced APC inhibition of LPS-induced cytokine expression. APC<sub>PNG</sub> treatment reduced secretion of TNFα and IL-6 by 58±3% and 52±3%, respectively (Figure 5.1.8 a and b). This represents a 2-fold enhancement in APC anti-inflammatory signalling efficacy, similar to that observed on RAW264.7 cells, and confirms that N-linked glycan structures also regulate PAR1-dependent anti-inflammatory signalling by APC on murine macrophages.
Figure 5.1.8: PNGase F treatment enhances APC reduction of LPS-induced pro-inflammatory cytokine secretion from BMDMs: Murine BMDMs were obtained from the femurs and tibia of wild type BALB/c mice (Chapter 2.1.3 and 2.1.4) and incubated with APC or APC_{PNG} (50nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of (a) TNFα and (b) IL-6 was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01]
5.1.4: Effect of enzymatic removal of N-linked glycans on APC proteolysis of PAR1

293T<sup>epcr/par1</sup> cells were used to investigate the mechanism of glycosylation-regulated APC proteolysis of PAR1. As PNGase F was not removed from the deglycosylated APC samples, 2 approaches were used to ensure that PNGase F treatment did not independently affect PAR1 proteolysis in this assay system. Firstly, the efficacy of thrombin and APC proteolysis of PAR1 on 293T<sup>epcr/par1</sup> cells pre-treated with PNGase F was compared to that of untreated cells.

Cells were incubated with PNGase F (24 U/mL; a concentration consistent with that present after addition of PNGase F to protein samples) for 3 hours. Cells were then washed with PBS and treated with thrombin or APC for a further 3. As expected, treatment with thrombin or APC induced release of AP into the cell supernatant from untreated cells (Figure 5.1.9 a, white bars). No difference in proteolysis of AP-PAR1 by thrombin or APC treatment was observed between PNGase F-treated and untreated 293T<sup>epcr/par1</sup> cells (Figure 5.1.9 a, white and black bars). Secondly, thrombin was incubated with PNGase F (24U/mL) at 37°C for 1 hour and incubated with 293T<sup>epcr/par1</sup> cells for 3 hours. PNGase F treatment did not alter thrombin proteolysis of PAR1 (Figure 5.1.9 b). These results demonstrate that PNGase F does not independently affect proteolysis of AP-PAR1 on 293T<sup>epcr/par1</sup> cells.
Figure 5.1.9: Effect of PNGase F treatment on activation of AP-PAR1 on 293T<sup>EP<sub>CR/AP-PAR1</sub></sup> cells: (a) 293T<sup>EP<sub>CR/AP-PAR1</sub></sup> cells were treated with control PBS (white bars) or PNGase F (black bars; 1000 U) for 3 hours followed by incubation with thrombin (1nM) or APC (100nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. (b) Thrombin (1μg) was incubated with PNGase F (1000 U) at 37°C for 1 hour then incubated with 293T<sup>EP<sub>CR/AP-PAR1</sub></sup> cells for 3 hours in assay buffer 1 and AP activity in the cell supernatant was assessed. Experiments were performed in triplicate and data are presented as mean ± S.D.. Statistical significance was set at p < 0.05.
To investigate the role of glycosylation in APC proteolysis of PAR1, 293T^{	ext{EPCR/AP-PAR1}} cells were incubated with APC or APC_{PNG} for 3 hours. As expected, treatment with APC resulted in dose-dependent liberation of AP into the cell supernatant (Figure 5.1.10, closed circles). Enzymatic removal of N-linked glycans significantly increased APC activation of PAR1. The rate of proteolysis of PAR1 by APC_{PNG} was enhanced 2-3-fold compared with untreated APC (p < 0.001) (Figure 5.1.10, open circles) demonstrating that N-linked glycans modulate PAR1 activation by APC. This novel role for APC glycosylation as a negative regulator of PAR1 activation is consistent with the improved capacity of APC_{PNG} to activate PAR1 dependent anti-inflammatory signalling on macrophages but not ApoER2-mediated signalling pathways on monocytes.
Figure 5.1.10: PNGase F treatment enhances APC activation of PAR1 on 293T<sup>EPCR/AP-PAR1</sup> cells: 293T<sup>EPCR/AP-PAR1</sup> cells were incubated with APC (●) or APC<sub>PNG</sub> (○) (both 12.5-50nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D.
5.2: Role of individual N-linked glycan attachment sites upon APC anti-inflammatory signalling on myeloid cells

5.2.1: Characterization of recombinant wild type PC and variants with altered glycosylation profiles

Recombinant PC variants rendered incapable of carbohydrate moiety attachment at specific N-linked glycosylation sequons were used to investigate the role of site-specific glycosylation in regulating APC anti-inflammatory signalling on macrophages. Recombinant PC variants containing asparagine (N) to glutamine (Q) substitutions at each of the 4 N-linked glycosylation sites (Asn-97, Asn-248, Asn-313 and Asn-329) were previously prepared in the laboratory 270. Recombinant wild type PC, PC_{PNG} and variant PC was characterised by 4-20% SDS-PAGE analysis followed by Western blotting. Recombinant PC was detected using a sheep anti-PC polyclonal antibody. The wild type PC heavy chain migrated at ~35 kDa as a doublet, representing PC heavy chain glycoforms α and β (Figure 5.2.1, lane 1). PNGase F-treated PC migrated ~10kDa further due to the reduction in molecular weight caused by removal of N-linked glycans (Figure 5.2.1, lane 2). The molecular weight of each recombinant PC N-linked glycan variant was similarly reduced, by ~5-10 kDa compared with wild-type PC. The PC_{N329Q} variant mimics the glycosylation profile of the naturally occurring β glycoform of PC. PC_{N329Q} was represented by a single band demonstrating the presence of a single, β PC-like, glycoform (Figure 5.2.1, lane 3). A second faint band was observed in both the PC_{N248Q} and PC_{N313Q} variant preparations demonstrating that the naturally occurring α and β glycoforms are present in addition to the absence of glycosylation at the mutated Asn residue (Figure 5.2.1, lane 4 and 5).
Figure 5.2.1: Characterization of recombinant PC N-linked glycan variants by SDS-PAGE/Western blotting: Wild type (lane 1) and PNGase F-treated recombinant PC (lane 2) and PC_{N329Q} (lane 3), PC_{N248Q} (lane 4) and PC_{N313Q} (lane 5) recombinant variants (all 10ng) were subjected to 4-20% SDS-PAGE under reducing conditions followed by Western blotting using a anti-PC sheep polyclonal antibody.
To create activated forms of each recombinant PC variant, type PC and variants were activated by incubation with the Thrombin CleanCleave Kit (Chapter 2.3). Amidolytic activity was then compared to ensure that active site function was conserved between variants. Amidolytic activity was assessed by incubation with CS-21(66). Hydrolysis of CS-21(66) over time (0-12 minutes) was quantified by measurement of OD405nm. No significant difference was observed in the rate of CS-21(66) hydrolysis between thrombin-activated recombinant wild type PC and PC variants (Figure 5.2.2).
Figure 5.2.2: Chromogenic substrate hydrolysis of APC N-linked glycan variants: Amidolytic activity of APC (●), APC_{N329Q} (O), APC_{N248Q} (▲), APC_{N313Q} (■) (all 250nM) was determined by measuring the rate of hydrolysis of the APC-specific synthetic chromogenic substrate CS-21(66) (2mg/ml) over time. The reaction was carried out at room temperature in a buffer containing 100mM NaCl and 20mM Tris-HCl (pH 7.5) with 2.5mM CaCl\textsubscript{2} and 0.1mg/ml BSA. APC amidolytic activity is expressed in terms of substrate cleaved, measured at OD\textsubscript{405nm}. The rate of CS-21(66) hydrolysis between samples was compared using linear regression, with statistical significance set at p ≤ 0.05. Experiment was performed in duplicate and is presented as mean ± S.D.
5.2.2: Role of individual N-linked glycan moieties in APC anti-inflammatory signalling on monocytes

To compare the efficacy of APC glycan variants in ApoER2-dependent signalling on monocytes, PBMCs were incubated with APC, APC_{N248Q}, APC_{N313Q} or APC_{N329Q} for 3 hours prior to stimulation with LPS. As expected, APC reduced LPS-induced TNFα expression (Figure 5.2.3), as did APC_{N248Q}, APC_{N313Q} and APC_{N329Q}. The anti-inflammatory signalling efficacy of APC glycan variants on monocytes did not differ significantly from wild type APC (Figure 5.2.3). These results mirror those previously observed using APC_{PNG} and confirm that glycosylation does not regulate ApoER2 signalling by APC on monocytes.
Figure 5.2.3: Site-directed mutagenesis of N-linked glycosylation sequons does not significantly alter APC inhibition of LPS-induced TNFα secretion from PBMCs

PBMCs were isolated from buffy coat whole blood component and treated with APC, APC_{N329Q}, APC_{N313Q}, or APC_{N329Q} (all 20nM) for 3 hours in assay buffer 3, then stimulated with LPS (50ng/mL) for 18 hours. TNFα secretion was measured by ELISA. The experiment was performed in triplicate and data are presented as mean ± S.D.. [* = p ≤ 0.05]
5.2.3: Role of individual N-linked glycan moieties in APC proteolysis of PAR1

To investigate the role of individual glycan moieties in regulating APC activation of PAR1, 293T\textsuperscript{EPCR/AP-PAR1} cells were used. 293T\textsuperscript{EPCR/AP-PAR1} cells were incubated with APC, APC\textsubscript{N248Q}, APC\textsubscript{N313Q} or APC\textsubscript{N329Q} for 3 hours and AP activity in the cell supernatant was assessed. Treatment with APC liberated AP from PAR1 into the cell supernatant, as before (Figure 5.2.4). APC\textsubscript{N313Q} also resulted in AP release, which was similar in magnitude to that wild type APC. APC\textsubscript{N248Q} activation of AP-PAR1 was modestly (~1.4-fold) enhanced compared to wild type APC. Proteolysis of AP-PAR1 by APC\textsubscript{N329Q}, however, was markedly enhanced (~2.5-fold) compared with its wild type counterpart. These results demonstrate that regulation of PAR1 proteolysis by APC N-linked glycan moieties is mediated by the glycan chain at Asn-329.
Figure 5.2.4: Mutation of N-linked glycosylation site at Asn-329 enhances APC activation of PAR1 on 293T<sup>EPCR/AP-PAR1</sup> cells: 293T<sup>EPCR/AP-PAR1</sup> cells were incubated with APC, APC<sub>N248Q</sub>, APC<sub>N313Q</sub> or APC<sub>N329Q</sub> (all 50nM) for 3 hours assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D.. [* = p < 0.05]
5.2.4: Role of Asn-329 glycan moiety in APC anti-inflammatory signalling on macrophages

To investigate whether regulation of APC PAR1-dependent signalling on macrophages by N-linked glycan moieties is also mediated by the glycan chain at Asn-329, RAW264.7 cells were used. RAW264.7 cells were incubated with APC or APC\\textsubscript{N329Q} followed by stimulation with LPS. APC dose-dependently reduced TNF\(\alpha\) and IL-6 expression from LPS-stimulated RAW264.7 cells (Figure 5.2.5 a and b). APC\textsubscript{N329Q} was significantly (>2-fold) more effective in attenuating the cytokine response to LPS stimulation (\(p \leq 0.05\)). Treatment with 20nM APC\textsubscript{N329Q} reduced TNF\(\alpha\) and IL-6 secretion by 54±4% and 41±4%, respectively, compared with 25±7% and 21±3% in the case of wild type APC (Figure 5.2.5 a and b).
Figure 5.2.5: APC\textsubscript{N329Q} displays enhanced ability to inhibit LPS-induced pro-inflammatory cytokine secretion from RAW264.7 macrophages compared to wild type APC: RAW264.7 macrophages were incubated with APC (black bars) or APC\textsubscript{N329Q} (grey bars) (both 5-20nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of (a) TNF\alpha and (b) IL-6 was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [** = p ≤ 0.01; * = p ≤ 0.01]
The anti-inflammatory effect of APC\textsubscript{N329Q} on primary murine macrophages (BMDMs) was also investigated. Murine BMDMs were treated with either APC or APC\textsubscript{N329Q} for 3 hours prior to stimulation with LPS. As expected, APC significantly inhibited LPS-induced TNF\(\alpha\) expression by 29±2% (Figure 5.2.6). Similar to that observed on RAW264.7 cells, APC\textsubscript{N329Q} inhibition of LPS-induced cytokine production from BMDMs was enhanced 2-fold compared with wild type APC. 20nM APC\textsubscript{N329Q} reduced TNF\(\alpha\) secretion by 51±4% (Figure 5.2.6). Collectively, these data suggest that the glycan moiety at Asn-329 specifically regulates APC activation of PAR1 and therefore PAR1-dependent anti-inflammatory signalling on macrophages.
Figure 5.2.6: Mutation of the N-linked glycosylation site at Asn-329 enhances APC reduction of LPS-induced pro-inflammatory cytokine secretion from BMDMs: Murine BMDMs were obtained from the femurs and tibia of wild type BALB/c mice and incubated with APC or APC\textsubscript{N329Q} (50nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of TNF\textalpha was measured by ELISA. Experiment was performed in triplicate and data are presented as mean ± S.D. [* * = p < 0.01; * = p < 0.05]
5.2.5: Role of Asn-329 glycan moiety in integrin CD11b/CD18-mediated proteolysis of PAR1 by APC

In contrast to EPCR-dependent PAR1 signalling on endothelial cells, APC PAR1-dependent anti-inflammatory signalling on macrophages requires the presence of CD11b/CD18. To assess whether modification of APC glycosylation can accelerate CD11b/CD18-mediated proteolysis of PAR1, HEK293T cells were transfected with AP-PAR1 only (293T<sup>AP-PAR1</sup>) or co-transfected with either EPCR (293T<sup>AP-PAR1/EPCR</sup>) or CD11b/CD18 (293T<sup>AP-PAR1/CD11b/CD18</sup>). 293T<sup>AP-PAR1</sup> cells, 293T<sup>AP-PAR1/EPCR</sup> cells and 293T<sup>AP-PAR1/CD11b/CD18</sup> cells were treated with either thrombin or APC. As previously shown (Chapter 3.1), thrombin did not require co-receptor binding to liberate AP from PAR1 on 293T<sup>AP-PAR1</sup> cells (Figure 5.2.7; white bars). Incubation of 293T<sup>AP-PAR1</sup> cells with APC failed to induce significant release of AP into the cell supernatant while co-transfection with EPCR facilitated APC proteolysis of PAR1 and liberation of AP (Figure 5.2.7, white and black bars). Incubation of 293T<sup>AP-PAR1/CD11b/CD18</sup> cells with APC also resulted in proteolysis of PAR1 and AP release but with reduced affinity compared to when EPCR was present. This demonstrates that CD11b/CD18 can similarly act as a co-receptor for APC activation of PAR1 (Figure 5.2.7; grey bars) but with reduced efficacy compared with EPCR.
Figure 5.2.7: AP-PAR1 proteolysis on 293T cells co-transfected with EPCR or integrin CD11b/CD18: 293T<sup>AP-PAR1</sup> cells (white bars) and 293T<sup>AP-PAR1</sup> cells co-transfected with EPCR (black bars) or integrin CD11b/CD18 (grey bars) were treated with thrombin (1nM) or APC (100nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D.
To investigate CD11b/CD18-mediated PAR1 activation by APC\textsubscript{N329Q}, 293\textsuperscript{T}\textsuperscript{AP-PAR1} cells and 293\textsuperscript{T}\textsuperscript{AP-PAR1/CD11b/CD18} cells were treated with APC, APC\textsubscript{PNG} or APC\textsubscript{N329Q}. As expected, APC proteolysis of AP-PAR1 occurred only in cells co-transfected with CD11b/CD18 (Figure 5.2.8, black bars). APC\textsubscript{PNG} and APC\textsubscript{N329Q} similarly failed to activate AP-PAR1 in the absence of a co-receptor (Figure 5.2.8, black bars). Proteolysis of AP-PAR1 by APC\textsubscript{PNG} and APC\textsubscript{N329Q} was significantly enhanced compared to wild type APC on cells co-expressing CD11b/CD18 (Figure 5.2.8, grey bars). This result demonstrates that elimination of the glycan chain at Asn-329 accelerates not only EPCR-dependent, but also CD11b/CD18-mediated activation of PAR1 by APC.
Figure 5.2.8: APC_{PNG} and APC_{N329Q} display enhanced proteolysis of AP-PAR1 on 293T cells co-transfected with integrin CD11b/CD18: 293T^{AP-PAR1} cells (black bars) and 293T^{AP-PAR1} cells co-transfected with integrin CD11b/CD18 (black bars) were treated with APC, APC_{PNG} or APC_{N329Q} (all 50nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D. [*** = p ≤ 0.001]
5.3: The molecular basis for glycan regulation of PAR1 activation by APC

Site-directed mutagenesis was used to generate thrombin-specific and APC-specific PAR1 cleavage site variants (AP-PAR1_{R41A} and AP-PAR1_{R46A}). These variants were used to investigate whether enhanced activation of PAR1 by APC_{PNG} or APC_{N329Q} occurs as a result of accelerated proteolysis at the recently identified APC Arg-46 cleavage site or due to proteolysis at the canonical thrombin cleavage site, Arg-41.

HEK293T cells transfected with EPCR (293T^{EPCR}) were co-transfected with either wild type AP-PAR1, AP-PAR1_{R41A} or AP-PAR1_{R46A} and treated with thrombin or APC after which AP activity in the cell supernatant was assessed. As expected, treatment with thrombin or APC resulted in PAR1 proteolysis and liberation of AP from 293T^{EPCR} co-transfected with wild type AP-PAR1 (Figure 5.3.1 a). Thrombin similarly mediated robust liberation of AP on 293T^{EPCR} cells co-transfected with AP-PAR1_{R46A} (Figure 5.3.1 b). APC proteolysis of AP-PAR1_{R46A} on 293T cells was substantially reduced compared with wild type AP-PAR1; however a small but significant effect was observed. Thrombin failed to liberate AP from 293T^{EPCR} cells co-transfected with AP-PAR1_{R41A} (Figure 5.3.1 c). In contrast, APC treatment resulted in significant proteolysis of AP-PAR1_{R41A}, however the efficacy of APC was reduced more than 2-fold compared with wild type AP-PAR1.

These results demonstrate that the Arg-41 cleavage site is necessary for thrombin activation of PAR1. Additionally, the Arg-46 cleavage site is required for optimal APC activation of PAR1. APC proteolysis can also occur at Arg-41, but with markedly reduced efficacy.
Figure 5.3.1: PAR1 proteolysis on 293T<sup>ΔPCR</sup> cells co-transfected with cleavage site-specific AP-PAR1 variants: 293T<sup>ΔPCR</sup> cells were co-transfected with (a) AP-PAR1<sup>WT</sup> (b) AP-PAR1<sup>R46A</sup> (c) AP-PAR1<sup>R41A</sup> and treated with thrombin (1nM) or APC (50nM) for 3 hours in assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. Experiments were performed in triplicate and data are presented as mean ± S.D.. [*** = p ≤ 0.001; ** = p ≤ 0.01]
To determine at which site PAR1 cleavage is enhanced by APC\textsubscript{PNG} or APC\textsubscript{N329Q}, 293T\textsuperscript{EPCR} cells co-transfected with either wild type AP-PAR1, AP-PAR1\textsubscript{R41A} or AP-PAR1\textsubscript{R46A} were treated with either APC, APC\textsubscript{PNG} or APC\textsubscript{N329Q}. As expected, the rate of wild type AP-PAR1 proteolysis by APC\textsubscript{PNG} and APC\textsubscript{N329Q} was significantly greater than that of untreated wild type APC (p < 0.001) (Figure 5.3.2 a). Similarly, proteolysis of AP-PAR1\textsubscript{R41A} by APC\textsubscript{PNG} and APC\textsubscript{N329Q} was enhanced ~2-3-fold compared with untreated wild type APC (p < 0.01) (Figure 5.3.2 b). In contrast, APC, APC\textsubscript{PNG} and APC\textsubscript{N329Q} activated AP-PAR1\textsubscript{R46A} with similarly modest efficacy (Figure 5.3.2 c). APC\textsubscript{N329Q} activation of AP-PAR1\textsubscript{WT} and AP-PAR1\textsubscript{R41A} occurred marginally more quickly than APC\textsubscript{PNG}, possibly due to incomplete removal of the glycan moiety at Asn-329 by PNGase F digestion. Collectively, these results demonstrate that enhanced activation of PAR1 and subsequent signalling by APC\textsubscript{PNG} or APC\textsubscript{N329Q} occurs due to accelerated proteolysis at the Arg-46 cleavage site on PAR1.
Figure 5.3.2: APC N-linked glycans specifically regulate APC proteolysis of PAR1 at Arg-46: 293T cells were co-transfected with (a) AP-PAR1WT (b) AP-PAR1R41A (c) AP-PAR1R46A and treated with thrombin (■; 1nM) or APC (●), APC$_{PNG}$ (O) or APC$_{329Q}$ (▲) (all 6.25-50nM) for 3 hours in assay buffer 1. AP activity in the cell supernatant was measured using QUANTI Blue. Experiments were performed in triplicate and data are presented as mean ± S.D..
5.4: Role of N-linked glycan moieties in murine APC PAR1-dependent anti-inflammatory signalling

5.4.1: Characterization of glycosylation profile of murine PC

Murine PC is a close structural homolog of its human counterpart, in which 3 of the 4 N-linked glycosylation sites are conserved. Similarly, murine PC possesses the same unusual Asn-X-Cys consensus sequence at Asn-330. To investigate whether murine protein C mirrored the same glycoform pattern of human PC, murine APC was treated with PNGase F at 37°C for 1 hour and characterised by reducing 10% SDS-PAGE. Western blotting was performed using a sheep anti-murine PC polyclonal antibody. The murine PC heavy chain migrated as a diffuse band at ~35 kDa (Figure 5.4.1, lane 1) suggesting the presence of heavy chain glycosylation. PNGase F-treated murine PC migrated further, suggesting a reduction in molecular weight of approximately 10kDa (Figure 5.4.1, lane 2). As in human PC, PNGase F can enzymatically remove N-linked glycans from murine PC.
Figure 5.4.1: Characterization of wild type and PNGase F treated recombinant murine PC by SDS-PAGE/Western blotting: Wild type and PNGase-F treated recombinant murine PC (10ng) was subjected to 10% SDS-PAGE under reducing conditions. Western blotting was performed using a polyclonal sheep anti-murine PC antibody.
To further investigate the possibility of different glycoforms, murine PC and a variant incapable of carbohydrate moiety attachment at Asn-330 (murine PC$_{N330Q}$) was recombinantly expressed. Plasmids encoding wild-type murine PC and murine PC$_{N330Q}$ that had been previously prepared in the laboratory were used to transfect HEK293 cells. Recombinant murine PC was expressed and partially purified (Chapter 2.2).

Upon isolation, murine PC and murine PC$_{N330Q}$ were characterised by reducing 10% SDS-PAGE followed by Western blotting using a sheep anti-murine PC polyclonal antibody. As previously observed, the wild murine PC heavy chain migrated as a diffuse band of ~40 kDa (Figure 5.4.2, lane 1) suggesting heavy chain glycosylation. The molecular weight of murine PC$_{N330Q}$ was reduced in comparison to wild-type murine PC and was represented by a single band, as has previously been observed in its human PC equivalent (Figure 5.4.2, lane 2). Murine PC, similar to its human counterpart, is therefore predicted to exist as α and β glycoforms due to partial glycosylation as the unusual Asn-X-Cys consensus sequence at Asn-330.
Figure 5.4.2: Characterization of recombinant wild type and mutant murine PC by SDS-PAGE/Western blotting: Recombinant wild type murine PC and murine PC_{N330Q} (both 10ng) were subjected to 10% SDS-PAGE under reducing conditions. Western blotting was performed using a polyclonal sheep anti-murine PC antibody.
Wild-type murine PC and variant murine PC\textsubscript{N330Q} colonies expressing between 0.7-1.2 µg/ml per day were expanded, incubated with serum-free conditioned media for 3-5 days in the presence of Vitamin K\textsubscript{1}. The resulting protein was partially purified and quantified using ELISA (Chapter 2.4). Recombinant wild type and variant murine PC was activated by incubation with the Thrombin CleanCleave Kit (Chapter 2.5). To ensure that active site function was conserved, amidolytic activity of recombinant wild type murine APC and PNGase F-treated murine APC (Figure 5.4.3 a) or murine APC\textsubscript{N330Q} (Figure 5.4.3 b) was compared. Hydrolysis of CS-21(66) over time (0-12 minutes) was quantified by measurement of OD\textsubscript{405nm}. No significant difference was in the rate of hydrolysis of CS-21(66) was observed as a result of PNGase F treatment (Figure 5.4.3 a) or between wild type murine APC and murine APC\textsubscript{N330Q} (Figure 5.4.3 b).
Figure 5.4.3: Murine APC, murine APC\textsubscript{PNG} and murine APC\textsubscript{N330Q} hydrolyse a chromogenic substrate with similar efficacy: Amidolytic activity of (a) murine APC (●) or murine APC\textsubscript{PNG} (○) (both 250nM) (b) murine APC (●) or murine APC\textsubscript{N330Q} (○) (both 125nM) was determined by measuring the rate of hydrolysis of the APC-specific synthetic chromogenic substrate CS-21(66) (2mg/ml) over time. The reaction was carried out at room temperature in a buffer containing 100mM NaCl and 20mM Tris-HCl (pH 7.5) with 2.5mM CaCl\textsubscript{2} and 0.1mg/ml BSA. APC amidolytic activity is expressed in terms of substrate cleaved, measured at OD\textsubscript{405nm}. The rate of CS-21(66) hydrolysis between samples was compared using linear regression, with statistical significance set at \( p \leq 0.05 \). Experiments were performed in duplicate and are presented as mean ± S.D..
5.4.2: Role of N-linked glycan moieties in murine APC proteolysis of PAR1

To investigate the role of glycosylation in murine APC proteolysis of PAR1, 293T<sup>EpCR/AP-PAR1</sup> cells were used as previously described. 293T<sup>EpCR/AP-PAR1</sup> cells were incubated with murine APC or murine APC<sub>PNG</sub> for 3 hours and AP activity in the cell supernatant was assessed. Treatment with murine APC resulted in activation of PAR1 and liberation of AP into the cell supernatant (Figure 5.4.4). Enzymatic removal of N-linked glycans significantly enhanced murine APC proteolysis of AP-PAR1 by ~2-fold compared with untreated murine APC (Figure 5.4.4) demonstrating that, similar to its human counterpart, N-linked glycosylation modulates murine APC activation of PAR1.
Figure 5.4.4: PNGase F treatment enhances murine APC activation of PAR1 on 293T<sub>EPCR/APC-PAR1</sub> cells: 293T<sub>EPCR/APC-PAR1</sub> cells were incubated with murine APC or murine APC<sub>PNG</sub> (both 50nM) for 3 hours assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D.. [* = p < 0.05]
To investigate the efficacy of murine APC$_{N330Q}$ activation of PAR1, 293T$^{EPCR/AP-PAR1}$ cells were incubated with murine APC or murine APC$_{N330Q}$ and AP activity in the cell supernatant was assessed. As expected, treatment with murine APC resulted in dose-dependent liberation of AP into the cell supernatant (Figure 5.4.5 a, closed circles). Murine APC$_{N330Q}$ proteolysis of AP-PAR1, however, was enhanced almost 2-fold compared with its wild type counterpart (p < 0.05) (Figure 5.4.5 a, open circles). To verify that the enhanced proteolysis of PAR1 observed upon PNGase F digestion occurred as a result of glycan moiety removal at Asn-330, murine APC$_{N330Q}$ was incubated with PNGase F at 37°C for 1 hour and incubated with 293T$^{EPCR/AP-PAR1}$ cells. PNGase F-treatment did not alter murine APC$_{N330Q}$ efficacy in AP-PAR1 proteolysis (Figure 5.4.5 b) confirming that, like human APC, N-linked glycan regulation of murine APC proteolysis of PAR1 is primarily mediated by the glycan chain at Asn-330.
Figure 5.4.5: Mutation of the N-linked glycosylation site at Asn-330 enhances murine APC activation of PAR1 on 293T_EPCR/AP-PAR1 cells: (a) 293T_EPCR/AP-PAR1 cells were incubated with murine APC or murine APC_N330Q (both 6.25-50nM) for 3 hours assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. (b) 293T_EPCR/AP-PAR1 cells were incubated with murine APC_N330Q or murine APC_N330Q-PNG (both 50nM) for 3 hours assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. Experiments were performed in triplicate and data are presented as mean ± S.D.. Statistical significance was set at p ≤ 0.05.
5.4.3: Role of Asn-330 glycan moiety in murine APC PAR1-dependent anti-inflammatory signalling on macrophages

To investigate whether the glycan chain at Asn-330 regulates the ability of murine APC to initiate PAR1-dependent signalling on macrophages, RAW264.7 cells were incubated with either murine APC or murine APC<sub>N330Q</sub>, then stimulated with LPS. As previously demonstrated, incubation of RAW264.7 cells with murine APC resulted in a dose-dependent reduction in LPS-induced TNFα and IL-6 expression (p < 0.05) (Figure 5.4.6 a and b). Murine APC<sub>N330Q</sub> was significantly more effective in attenuation of the cytokine response to LPS stimulation however (p < 0.01). Treatment with 50nM murine APC<sub>N330Q</sub> reduced LPS-induced TNFα and IL-6 secretion by 59±13% and 65±7%, respectively, compared with 37±13% and 41±6% in the case of wild type murine APC (Figure 5.4.6 a and b).
Figure 5.4.6: Mutation of N-linked sequon at Asn-330 enhances murine APC reduction of LPS-induced pro-inflammatory cytokine secretion from RAW264.7 macrophages: RAW264.7 macrophages were incubated with murine APC (●) or murine APC<sub>N330Q</sub> (○) (both 6.25-50nM) for 3 hours in assay buffer 3 prior to stimulation with LPS (50ng/mL) for 18 hours. Secretion of (a) TNFα and (b) IL-6 was measured by ELISA. Experiments were performed in triplicate and data are presented as mean ± S.D.. [* = p ≤ 0.05]
5.5: Role of N-linked glycan moieties in bovine APC PAR1-dependent anti-inflammatory signalling

5.5.1: Characterization of glycosylation profile of bovine APC

Despite similarly possessing an unusual Asn-X-Cys sequon at Asn-327 bovine APC has been shown to be fully glycosylated at all 4 N-linked sequons, and therefore exists solely in the α-PC form. To investigate the glycosylation profile of bovine APC, bovine APC was treated with PNGase F and characterised by 10% SDS-PAGE analysis. Western blotting was performed using a sheep anti-murine PC polyclonal antibody which can also detect bovine protein C. The bovine PC heavy chain migrated as a single band at ~40 kDa (Figure 5.5.1, lane 1) indicating that bovine PC exists as a single glycoform. PNGase F treatment reduced the molecular weight of bovine PC by ~10kDa, consistent with the loss of attached N-linked glycans (Figure 5.5.1, lane 2).
Figure 5.5.1: Characterization of wild type and PNGase F treated recombinant bovine PC by SDS-PAGE/Western blotting: Wild type and PNGase F treated bovine APC (10ng) was subjected to 10% SDS-PAGE under reducing conditions. Western blotting was performed using a polyclonal sheep anti-murine PC antibody (Chapter 2.6).
5.5.2: Role of N-linked glycan moieties in bovine APC proteolysis of PAR1

To investigate the role of glycosylation in bovine APC proteolysis of PAR1, 293T<sup>EPOR/AP-PAR1</sup> cells were used. 293T<sup>EPOR/AP-PAR1</sup> cells were incubated with bovine APC or bovine APC<sub>PNG</sub> and AP activity in the cell supernatant was assessed. Bovine APC was markedly less efficient in activation of AP-PAR1 compared to its human and murine counterparts despite retaining the capacity to cleave AP-PAR1 (p < 0.01) (Figure 5.5.2). Enzymatic removal of N-linked glycans, however, significantly enhanced bovine APC proteolysis of PAR1 ~3-fold (p < 0.001) (Figure 5.4.2) demonstrating that glycosylation modulates the rate of bovine APC proteolysis of PAR1.
Figure 5.5.2: PNGase F treatment of bovine APC enhances activation of PAR1 on 293TEPCR/AP-
PAR1 cells: (a) 293TEPCR/AP-PAR1 cells were incubated with bovine APC or bovine APCPNG (both 6.25-50nM) for 3 hours assay buffer 1 and AP activity in the cell supernatant was measured using QUANTI Blue. The experiment was performed in triplicate and data are presented as mean ± S.D..
Chapter 5.6: Discussion

5.6.1: Molecular basis of partial glycosylation of PC at Asn-329

Glycosylation can modulate the function of coagulation proteases in numerous ways, including the rate of activation, cofactor function and susceptibility to degradation. PC possesses 4 N-linked glycosylation sequons many of which are conserved across mammalian species (Figure 5.6.1). Understanding the functional significance of PC glycosylation is particularly important due to the presence of endogenous plasma glycoforms, α, β and γ (Figure 5.6.1), which occur due to partial glycosylation at Asn-329, in the case of β-PC, and both Asn-329 and Asn-248, in the case of γ-PC. Partial glycosylation at Asn-329 is thought to occur due to the presence of the unusual consensus sequence, Asn-X-Cys, which is less efficiently glycosylated than Ser/Thr containing N-linked sequons.

In addition to facilitating glycosylation at Asn-329, Cys-331 is postulated to be involved in disulphide bonding with Cys-345. It has been speculated that Cys-331 can only interact with the glycosyltransferase enzyme prior to formation of the Cys-331-Cys-345 disulphide bond, thus resulting in partial glycosylation at Asn-329. Consistent with this, expression of recombinant PC in HEK293 cells, in which the rate of protein synthesis is increased, yields a higher proportion of β-PC compared to plasma PC (40-50% versus 20-30%, respectively). Conversely, Gil et al. (2009) demonstrated that in transgenic porcine models, the ratio of α:β PC did not differ in animals expressing 10-fold reduced PC levels. Thus, the molecular basis for partial glycosylation of PC remains controversial.

Western blotting analysis suggests that recombinantly expressed murine PC also exists in various glycoforms (Figure 5.4.1), in keeping with previously published reports.
The murine PC glycosylation sequon at Asn-330 is identical to that at Asn-329 on its human counterpart (Figure 5.6.1 b) and, similar to human PC_{N329Q}, mutation of this residue resulted in a murine PC_{N330Q} mutant that migrated as a single band, suggestive of the loss of the fully glycosylated glycoform (Figure 5.4.2). Human and murine PC glycoforms therefore likely occur as a result of partial glycosylation at the same sequon, although further analysis is required to confirm this.

In contrast, bovine (A)PC migrates on SDS-PAGE as a single band, indicating that bovine PC exists as a single glycoform (Figure 5.5.1). The equivalent sequon in bovine PC (Asn-327) also possesses an unusual Asn-X-Cys sequon but differs from human and murine PC with respect to the amino acid residue preceding the Cys. In bovine PC the ‘X’ residue is Ala, while in both human and murine PC the same position is occupied by a negatively-charged Glu (Figure 5.6.1). Kasturi et al. (1997) and Shakin-Eshelmann et al. (1996) previously reported that the presence of a Glu residue at position X in Asn-X-Ser sequons is unfavourable for glycosylation, whereas efficient oligosaccharide transfer occurs at Asn-Ala-Ser sequons. Thus, it is possible that the presence of a negatively charged amino acid residue at the ‘X’ position could impair oligosaccharyltransferase binding to the sequon and the negatively charged dolichol-PP-oligosaccharide precursor. The apparent absence of bovine PC glycoforms suggests that partial glycosylation of human and murine PC at Asn-329 and Asn-330, respectively, may in fact occur due the presence of negatively charged Glu at the X position, rather than the conserved ‘Cys’ at the C-terminus of this sequon. Again, further studies are required to test this hypothesis.
Figure 5.6.1: Protein C glycosylation

(a) α, β and γ PC glycoforms. (b) Alignment of known PC amino acid sequences demonstrates that the usual Asn-X-Cys sequon at Asn-329 (yellow) is conserved across mammalian species.
5.6.2: Role of N-linked glycosylation in modulating APC function

Removal of APC N-linked glycans enabled characterisation of the role of N-linked glycosylation upon APC function. APC amidolytic activity was not altered by enzymatic removal of human or murine APC N-linked glycans or by mutation of individual human or murine APC N-linked glycosylation sites (Figures 5.1.2 and 5.2.2), in keeping with previous results from our laboratory\(^{270}\). Contrastingly, Grinnell et al. (1991) reported that mutation of individual protease domain N-linked sequons resulted in a small increase in amidolytic activity. The reason for this discrepancy remains unknown, but may be accounted for by differences in the chromogenic substrate used by Grinnell et al. and that used in this thesis. Notably, none of the APC protease domain N-linked glycan attachment sites are proximal to the APC catalytic triad\(^ {174}\).

Consistent with previous reports\(^ {270,312}\), glycosidase treatment of human APC mildly enhanced its anticoagulant function (Figure 5.1.2). The effect of individual APC glycans on anticoagulant function is controversial. Grinnell et al. reported that mutagenesis of individual protease domain N-linked sequons increases activity of the protease 2-3 fold in an APTT\(^ {306}\). In contrast, a naturally occurring APC mutant (APC\(_{N329T}\)) was found to exhibit marginally reduced anticoagulant activity as a consequence of reduced FVa inactivation at both the Arg-306 and Arg-506 cleavage sites\(^ {313}\). Similarly, individual mutation of all 4 APC glycan attachment sites resulted in a limited increase in APC anticoagulant function in an \textit{in vitro} assay of thrombin generation\(^ {270}\).

We have previously demonstrated that APC\(_{N329Q}\) displays enhanced PAR1-dependent endothelial cell barrier protective and anti-apoptotic signalling properties compared to wild
type APC\textsuperscript{270}. Contrastingly, PNGase F treatment did not alter APC anti-inflammatory signalling on THP1 cells (Figure 5.1.4) and similarly, all 3 protease domain sequon variants reduced LPS-induced TNFα secretion from PBMCs with similar efficacy to wild type APC (Figure 5.2.3). The presence of an anti-PAR1 antibody or a PAR1 antagonist did not alter APC anti-inflammatory activity on either THP1 cells or PBMCs, indicating that APC signalling on monocytic cells occurs independently of PAR1 (Figures 3.2.5 and 5.1.3). Consistent with this, a study published by Yang et al. (2009) reported that the anti-inflammatory effect of APC on monocytes was mediated via ApoER2-dependent activation of PI3K signalling (Figure 5.1.3)\textsuperscript{237}. Other studies have similarly reported that APC inhibition of LPS-induced IFN-γ and TNFα production and IκBα degradation on monocytic cells does not require activation PAR1\textsuperscript{232,243,314}. As such, APC N-linked glycosylation specifically regulates PAR1-dependent, but not PAR1-independent anti-inflammatory signalling.

Yang et al. (2007) recently identified that the 162-helix in the protease domain of APC, in particular the Glu-330/Glu-333 site, is critical for APC interaction with PAR1\textsuperscript{219}. Mutation of either or both of these residues ablates PAR1-dependent cytoprotective signalling by APC on endothelial cells, whereas EPCR binding is unchanged. Furthermore, APC\textsubscript{E330A} and APC\textsubscript{E333A} mutants were unable to activate PAR1 suggesting these residues may constitute a PAR1 binding exosite. The crystal structure of the APC protease domain (Figure 5.6.2) demonstrates that this PAR1 binding exosite is located proximally to the Asn-329 glycosylation sequon. We predict that the presence of a bulky glycan chain at Asn-329 may sterically hinder the interaction between APC and PAR1. Thus, the enhanced PAR1-dependent cytoprotective signalling capacity of APC\textsubscript{N329Q} on endothelial cells occurs due to
increased PAR1 activation as a consequence of increased access to this PAR1 binding exosite. The failure of glycosylation modification to alter PAR1-independent signalling activities of APC on monocytes is consistent with this hypothesis.

Figure 5.6.2: APC Asn-329 sequon occurs proximal to Glu-330/Glu-333 PAR1 binding exosite: Molecular model of APC serine protease domain, generated based upon Gla domainless APC crystal structure using PYMOL molecular visualization software. APC’s serine protease domain contains the catalytic triad (His-211, Asp-257, Ser-360; yellow), 3 N-linked sequons (turquoise) and the Glu-330/Glu-333 PAR1 binding exosite (red). The Asn-329 sequon, which regulates APC cytoprotective signalling, is situated next to a binding exosite essential for APC interaction with PAR1.
5.6.3: Molecular basis for modulation of APC cytoprotective signalling by the glycan moiety at Asn-329

To investigate the hypothesis that the glycan moiety at Asn-329 modulates APC activation of PAR1, HEK293T cells co-expressing AP-PAR1 and EPCR were used to assess PAR1 cleavage efficacy. Glycosidase treatment increased the rate of APC activation of AP-PAR1 2-3-fold (Figure 5.1.10). When APC variants containing individual proteases domain sequon mutations were tested, APC_{N329Q} activation of AP-PAR1 was similarly enhanced ~3-fold (Figure 5.2.4). These results demonstrate that the glycan moiety at Asn-329 regulates APC proteolysis of PAR1 and its elimination results in an enhancement in the rate of activation. APC_{N248Q} also displayed mildly enhanced proteolysis of AP-PAR1 compared with wild type APC whereas no enhancement was observed in the case of APC_{N313Q}. Notably, Asn-248 is also located on the right hand side of the active site, significantly closer to the 162-helix than Asn-313, which is located below that active site proximal to the 148-loop (Figure 5.6.2). As such it is possible that the glycan moiety at Asn-248 could also sterically effect APC interaction with PAR1, albeit to a lesser extent than that at Asn-329. Notably, this suggests that the glycosylation profile of γ-APC facilitates further enhancement of PAR1 activation, although this remains to be tested.

PAR1 activation by either APC or thrombin initiates divergent cellular signalling. APC-PAR1 signalling mediates broadly cytoprotective and anti-inflammatory effects, whereas thrombin activation of PAR1 results in pro-inflammatory and endothelial cell barrier-disruptive signal transduction. Activation of PAR1 by thrombin promotes coupling to G protein α subunits 12 and 13 as well as Gαq, which leads to RhoA activation and disassembly of adherens
junctions causing disruption of the endothelial cell barrier. Conversely, APC activates a subset of PAR1 present in caveolae which exists in a preassembled complex with β-arrestins to activate Rac1 and the dishevelled-2 scaffold, promoting endothelial cell barrier integrity. APC proteolysis of PAR1 occurs primarily at Arg-46, a site distinct from the Arg-41 cleavage site at which canonical PAR1 cleavage by thrombin occurs. The generation of distinct tethered ligands via specific cleavage sites represents a viable mechanism through which divergent signalling pathways are initiated by APC and thrombin.

In our study, AP-PAR1 mutants containing Ala substitutions at the Arg-41 and Arg-46 cleavage sites were used to confirm that APC proteolysis of PAR1 occurs primarily at Arg-46. APC activation at Arg-41 was also observed at a much lower rate, similar to previous published reports. Notably, APC was less effective in activation of AP-PAR1 compared to wild type AP-PAR1. The reduced efficacy of APC in activation of AP-PAR1 has similarly been observed by Mosnier et al. (2012) who reported an EC50 for APC activation of AP-PAR1 approximately 5-fold higher than that of its wild type counterpart. The reason for this discrepancy is poorly understood at present but may indicate that Ala substitution at Arg-41 disrupts cleavage at Arg-46 or possibly conformationally alters the resultant N-terminal ligand.

Activation of AP-PAR1 by PNGase F-treated APC and APCN329Q was enhanced 3-fold compared to wild type APC, whereas APCPNG and APCN329Q proteolysis of AP-PAR1R46A was minimal and occurred at similar rate to that of the wild type protein. This result demonstrates that the enhanced efficiency of APCPNG and APCN329Q occurs exclusively due to increased proteolysis at Arg-46. These results correlate well with the enhanced
cytoprotective signalling function of APC_{PNG} and APC_{N329Q} on endothelial cells we have reported previously. Comparison of the rate of AP-PAR1 activation by APC_{N329Q} and APC_{PNG} revealed a marginally enhanced rate by APC_{N329Q}. This is likely a result of incomplete removal of the glycan moiety at Asn-329 by enzymatic digestion, a prerequisite to maintaining functional competence. Murine APC_{PNG} and murine APC_{N330Q} also activated AP-PAR1 ~2-fold faster than wild type murine APC (Figure 5.4.4). PNGase F treatment of murine APC_{N330Q} demonstrated that, as in the case of its human counterpart, the glycan moiety at Asn-330 primarily modulates murine APC activation of PAR1 (Figure 5.4.5). The above data obtained using human and murine APC indicate that β and γ PC glycoforms are likely the predominant mediators of APC-PAR1 signalling in vivo.

In addition to its cytoprotective role on endothelial cells, APC mediates anti-inflammatory signalling on macrophages. Human and murine APC reduced LPS-induced production of TNFα and IL-6 from murine macrophages, which was abated by APC active site blockade or PAR1 antagonism (Figure 5.1.6). Interestingly, APC anti-inflammatory signalling on macrophages is reported to occur via a distinct molecular mechanism to that described on endothelial cells, in which APC PAR1-dependent anti-inflammatory activity is dependent on the presence of CD11b/CD18^{239}. Further, CD11b/CD18 has been described as a crucial regulator of the beneficial effects of APC therapy in murine models^{239}, highlighting the importance of APC anti-inflammatory activity on macrophages in vivo.

To investigate CD11b/CD18-facilitated APC proteolysis of 293T^{AP-PAR1} cells were co-transfected with the CD11b/CD18, in place of EPCR. Substitution of EPCR with CD11b/CD18 resulted in APC activation of AP-PAR1 (Figure 5.2.7). Furthermore, APC_{N329Q} activated of AP-
PAR1 in HEK293T cells co-tranfected with CD11b/CD18 was 2-fold more efficient than wild type APC (Figure 5.2.8). This confirms that the glycan moiety at Asn-329 increases APC activation of PAR1 regardless of the APC capture receptor. Similarly, the anti-inflammatory effect of both APC<sub>PNG</sub> (Figures 5.1.7 and 5.1.8) and APC<sub>N329Q</sub> (Figures 5.2.5 and 5.2.6) on RAW264.7 murine macrophages and BMDMs was enhanced ~3-fold compared with wild type APC. In addition, murine APC<sub>N330Q</sub> was significantly more efficient in reducing LPS-induced TNFα and IL-6 production by RAW264.7 cells (Figure 5.4.6) confirming that the glycan moiety at Asn-329 (or Asn-330 in the case of murine APC) also specifically regulates PAR1-dependent anti-inflammatory signalling by APC on macrophages.

Interestingly, activation of AP-PAR1 by bovine APC, which exists exclusively in fully glycosylated form, was minimal, while PNGase F treated bovine APC cleaved AP-PAR1 efficiently (Figure 5.5.2). The inability of untreated bovine APC to activate AP-PAR1 suggests that the presence of a glycan moiety at Asn-329 may not simply reduce but in fact largely prevent APC interaction with PAR1. As such, PAR1 activation by wild type human and murine APC may be mediated exclusively by the ~30% β and ~5% γ APC present. Wild type recombinant APC, which contains approximately 30-40% of the β glycoform, was consistently ~3-fold less effective than APC<sub>N329Q</sub> in PAR1 proteolysis and PAR1-dependent signalling functions on endothelial cells and macrophages. The relative efficacies of wild type APC versus APC<sub>N329Q</sub> suggest that PAR1 proteolysis and signalling is mediated predominantly by APC glycoforms missing the glycan moiety at Asn-329.
5.6.4: Recombinant β-APC as a therapeutic agent

The relative efficacy of α and β APC in PAR1 signalling is relevant to the use of recombinant APC (Xigris) in the treatment of severe sepsis. The use of recombinant APC in the treatment of severe sepsis has produced confounding results. The PROWESS trial, carried out in 2001, reported that administration of recombinant APC reduced the risk of mortality by 19.4% in severe sepsis\(^{262}\). The PROWESS-SHOCK trial, however, failed to identify any survival benefit associated the use of APC in severe sepsis, leading to its withdrawal from the market\(^{266}\).

Kerschen et al. (2007) demonstrated that administration of APC failed to confer any survival benefit in PAR1 deficient mice, indicating a crucial role for PAR1 signalling in APC protection against sepsis morality\(^{240}\). The results of this study suggest that only the 30-40% β-APC present in wild type APC efficiently activates and signals through PAR1, however the ratio of α:β APC present in Xigris has not been defined. Therefore, APC\(_{N329Q}\), a variant which exists exclusively in the β glycoform, potentially represents an improved therapeutic agent.

The continuation of this study will further investigate the impact of glycosylation at Asn-329 on APC PAR1-dependent signalling function by generation an α-APC variant which is fully glycosylated at all 4 sequons. In the case of AT, mutation of the naturally occurring Asn-X-Ser sequon at the 135 site to Asn-X-Thr results in full glycosylation at this site\(^{85}\). Similar approaches will be used to generate α-APC which will facilitate direct comparison of PAR1 proteolysis and signalling efficacies of the 2 predominant endogenous APC glycoforms. Further, the efficacy of murine APC\(_{N330Q}\) will be compared with that of wild type murine APC in murine models of severe sepsis and other inflammatory disease models. These studies will provide valuable insight into the therapeutic potential of recombinant β APC.
References


85. Picard, V., Ersdal-Badju, E. & Bock, S.C. Partial glycosylation of antithrombin III asparagine-135 is caused by the serine in the third position of its N-glycosylation consensus sequence and is


# Appendix I

## Immortalised cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Culture medium and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Embryonic Kidney (HEK) 293 (American Type Culture Collection (ATCC))</td>
<td>Minimal Essential Medium Eagle (MEM alpha; Invitrogen, Life Technologies, Life Technologies) supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin (Invitrogen, Life Technologies), 2mM L-glutamine (Invitrogen, Life Technologies) and 10% (v/v) Foetal Bovine Serum (FBS; Invitrogen, Life Technologies)</td>
</tr>
<tr>
<td>HEK293T (ATCC)</td>
<td>MEM alpha supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin, 2mM L-glutamine and 10% (v/v) FBS</td>
</tr>
<tr>
<td>HEK Blue TNFα/IL1β (HEK293 cell line stably expressing TNFα and IL-1β receptors and an NF-κB/AP-1 inducible secreted alkaline phosphatase (ALP) reporter; Invivogen)</td>
<td>Dulbecco Modified Eagles Medium (DMEM) (Invitrogen, Life Technologies, Life Technologies), supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin solution, 2mM L-glutamine, 10% (v/v) FBS and 100 μg/mL Zeocin (Invivogen)</td>
</tr>
<tr>
<td>THP1 (Human acute monocytic leukemia cell line; ATCC)</td>
<td>RPMI 1640 Medium (2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate; Invitrogen, Life Technologies) supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin solution and 10% (v/v) FBS</td>
</tr>
<tr>
<td>THP1X-Blue-CD14 (THP1 cell line stably expressing CD14 and an NF-κB/AP-1 inducible secreted ALP reporter; Invivogen)</td>
<td>RPMI 1640 Medium supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin solution, 10% (v/v) FBS, 200 μg/mL Zeocin and 250 μg/mL geneticin sulphate (G418; Sigma-Aldrich)</td>
</tr>
<tr>
<td>RAW Blue (Mouse leukaemic macrophage cell line stably expressing an NF-kB/AP-1 inducible secreted ALP reporter; Invivogen)</td>
<td>DMEM supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin solution, 10% (v/v) FBS, 2mM L-glutamine and 200 μg/mL Zeocin</td>
</tr>
<tr>
<td>EA.hy926 (Immortalised endothelial cell line produced by hybridisation of human umbilical vascular endothelial cells (HUVEC) with the lung epithelial cell line A549; J. Edgell, University of Carolina, Chapel Hill)</td>
<td>DMEM supplemented with 1U/mL penicillin and 0.1mg/mL streptomycin solution, 2mM L-glutamine, 10% (v/v) FBS and 1% (v/v) hypoxanthine-aminopterin-thymidine (HAT) solution (Sigma-Aldrich).</td>
</tr>
</tbody>
</table>
Appendix II

FPLC running buffer
50mM Tris (2.54 g/L Trizma HCl; 0.47 g/L Trizma base)
150mM NaCl (8.76 g/L)
pH 7.4

FPLC elution buffer
50mM Tris (2.54 g/L Trizma HCl; 0.47 g/L Trizma base)
150mM NaCl (8.76 g/L)
30nM CaCl₂ (3.33 g/L)
pH 7.4

10 X G7 buffer
500mM sodium phosphate (pH 7.5)

10 X APC amidolytic activity assay buffer
1M NaCl
0.2M Tris-HCl (TBS, pH 7.5)
25mM CaCl₂

ELISA carbonate buffer
50mM Na₂CO₃ (1.7 g/L)
50mM NaHCO₃ (2.86 g/L)

Hank’s buffered salt solution (HBSS) buffer composition
0.137 M NaCl
5.4 mM KCl
0.25 mM Na₂HPO₄
0.44 mM KH₂PO₄
1.3 mM CaCl₂
1.0 mM MgSO₄
4.2 mM NaHCO₃

LB broth
1% (w/v) tryptone
0.5% (w/v) yeast extract
1% (w/v) NaCl

LB Agar
1% (w/v) tryptone
0.5% (w/v) yeast extract
1% (w/v) NaCl
1% (w/v) bacto-agar
**SOC medium**
0.5% w/v yeast extract
2% tryptone
10 mM NaCl
2.5 mM KCl
10 mM MgCl₂
10 mM MgSO₄
20 mM Glucose

**Assay buffer 1**
Serum-free DMEM supplemented with 0.6mM CaCl₂ and 0.2mM MgCl₂

**Assay buffer 2**
Serum-free DMEM supplemented with 3mM CaCl₂ and 0.6mM MgCl₂

**Assay buffer 3**
Serum-free RPMI 1640 medium supplemented with 0.6mM CaCl₂ and 0.2mM MgCl₂
# Appendix III

cDNA Plasmids

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vector</th>
<th>Selection (bacterial)</th>
<th>Selection (cellular expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine PC</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>Murine PC&lt;sub&gt;N330Q&lt;/sub&gt;</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>PC&lt;sub&gt;FVIIa/EGF1&lt;/sub&gt;</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>AP-PAR1</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>AP-PAR1&lt;sub&gt;R41A&lt;/sub&gt;</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>AP-PAR1&lt;sub&gt;R56A&lt;/sub&gt;</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>Neomycin</td>
</tr>
<tr>
<td>EPCR-cGFP</td>
<td>pRc/CMV</td>
<td>Ampicillin</td>
<td>GFP</td>
</tr>
<tr>
<td>CD11b</td>
<td>pCDM8</td>
<td>Ampicillin and Tetracycline</td>
<td></td>
</tr>
<tr>
<td>CD18</td>
<td>pcDNA3.1</td>
<td>Ampicillin</td>
<td></td>
</tr>
</tbody>
</table>

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PUBLICATIONS
Activated factor X signaling via protease-activated receptor 2 suppresses pro-inflammatory cytokine production from LPS-stimulated myeloid cells

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Activated factor X signaling via protease-activated receptor 2 suppresses pro-inflammatory cytokine production from LPS-stimulated myeloid cells

Running Head: FXa anti-inflammatory activity on myeloid cells

Eimear M. Gleeson¹⁻³, James S. O'Donnell¹⁻⁴, Emily Hams⁵, Fionnuala Ni Áinle³, Bridget-Ann Kenny³, Padraic G. Fallon⁵, and Roger J.S. Preston¹⁻³

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Abstract

Vitamin K-dependent proteases generated in response to vascular injury and infection enable fibrin clot formation, but also trigger distinct immuno-regulatory signaling pathways on myeloid cells. Factor Xa, a protease crucial for blood coagulation, also induces protease-activated receptor-dependent cell signaling. Factor Xa can bind both monocytes and macrophages, but whether factor Xa-dependent signaling stimulates or suppresses myeloid cell cytokine production in response to Toll-like receptor activation is not known. In this study, exposure to factor Xa significantly impaired pro-inflammatory cytokine production from lipopolysaccharide-treated peripheral blood mononuclear cells, THP-1 monocytic cells and murine macrophages. Furthermore, factor Xa inhibited nuclear factor-kappa B activation in THP-1 reporter cells, requiring phosphatidylinositol 3-kinase activity for its anti-inflammatory effect. Active-site blockade, γ-carboxyglutamic acid domain truncation and a peptide mimic of the factor Xa inter-epidermal growth factor-like region prevented factor Xa inhibition of lipopolysaccharide-induced tumour necrosis factor-α release. In addition, factor Xa anti-inflammatory activity was markedly attenuated by the presence of an antagonist of protease-activated receptor 2, but not protease-activated receptor 1. The key role of protease-activated receptor 2 in eliciting factor Xa-dependent anti-inflammatory signaling on macrophages was further underscored by the inability of factor Xa to mediate inhibition of tumour necrosis factor-α and interleukin-6 release from murine bone marrow-derived protease-activated receptor 2-deficient macrophages. We also show for the first time that, in addition to protease-activated receptor 2, factor Xa requires a receptor-associated protein-sensitive low-density lipoprotein receptor to inhibit lipopolysaccharide-induced cytokine production. Collectively, this study supports a novel function for factor Xa as an endogenous, receptor-associated protein-sensitive, protease-activated receptor 2-dependent regulator of myeloid cell pro-inflammatory cytokine production.
Introduction

During sepsis, invading pathogens activate pattern recognition receptors (PRRs) expressed on a variety of cell types using specific pathogen-association molecular patterns (PAMPs) present in bacteria, viruses, fungi and parasites (1). Toll-like receptors (TLRs) are the most studied PRR family, and their activation triggers signal transduction pathways that up-regulate pro-inflammatory cytokine expression vital for the resolution of infection (2). Lipopolysaccharide (LPS) from gram-negative bacteria activates TLR4 to induce pro-inflammatory cytokine generation and leads to rapid induction of tissue factor (TF) expression on leukocytes (3), triggering blood coagulation in the absence of blood vessel damage (4). In sepsis, LPS-induced aberrant TF expression, depletion of anticoagulant plasma proteins (5) and down-regulation of vascular cell surface receptors (6) leads to unregulated coagulation protease activation and disseminated intravascular coagulopathy (DIC), often causing multiorgan failure and death (7).

Coagulation proteases generated as a consequence of infection can interact with vascular and leukocyte surface receptors to either promote, or inhibit, pro-inflammatory signaling pathways. Inhibition of TF (8) and thrombin (9) is protective in murine endotoxemia. In contrast, the anticoagulant protease activated protein C (APC) suppresses LPS or cytokine-induced inflammation on monocytes (10) macrophages (11, 12) and vascular endothelial cells (13). Deficiency (14), or impaired generation (15, 16) of APC increases sensitivity to LPS challenge in mice and recombinant APC has been used in the treatment of individuals with severe sepsis (17).

Activated factor X (FXa) is a vitamin K-dependent protease generated rapidly upon TF exposure. FXa as part of the prothrombinase complex catalyzes thrombin generation, leading to fibrin deposition. FXa is critical for effective blood coagulation, as evidenced by the severe bleeding phenotype of FX-deficient individuals (18) and the embryonic or perinatal lethality exhibited by FX⁻/⁻ mice (19).
Like other coagulation proteases, FXa cell signaling is transduced by protease-activated receptors (PARs). Although structurally homologous to APC, FXa has been described both as a driver (20, 21) and inhibitor (22, 23) of TLR- and cytokine-induced inflammation depending on the cell type and signaling receptors activated. FXa can activate both PAR1, PAR2 and to a lesser extent, PAR4 (24). Co-receptors for FXa activation of PARs appear crucial in dictating FXa signaling specificity and multiple non-PAR cell receptors for FXa have been identified. Effector protease receptor 1 (EPR-1) was originally characterized as a high-affinity FXa receptor on platelets, endothelial cells and various leukocyte subsets (25-27). However, the molecular mechanism through which EPR-1-bound FXa exerts these cellular effects has not been described, and the identity of EPR-1 is itself controversial (28). FXa also has affinity for the endothelial cell protein C receptor (EPCR) (29). Blockade of EPCR-FXa interaction with an anti-EPCR monoclonal antibody prevents PAR1 activation by FXa and inhibits FXa cytoprotective signaling on endothelial cells (29). Furthermore, annexin-2 has been shown to bind specifically to an FXa isoform (FXa-β) and facilitate PAR1 activation on endothelial cells, but its role in response to inflammatory stimuli is unknown (30). Therefore, the receptor signaling requirements and downstream cellular consequences of FXa signal transduction are complex, cell-type dependent and often divergent, and the determinants of this signaling diversity are not fully understood.

FX(a) binds both monocytes and macrophages with high affinity (31) and is rapidly activated upon LPS activation and TF decryption, playing a crucial role in thrombin generation and subsequently fibrin deposition. However, whether FXa generated on the surface of myeloid cells contributes to the innate immune response beyond catalysing coagulation is not known, as PAR-dependent signaling by FXa on myeloid cells and its role in response to TLR activation has not been characterised. In this study, we show that FXa inhibits pro-inflammatory cytokine response to TLR stimulation in monocytes and macrophages via PAR2 activation and downstream activation and suppression of PI3K and NF-κB signaling, respectively.
Furthermore, FXa structural determinants and cell surface receptor(s) required to facilitate FXa-mediated hyposensitivity to TLR stimulation are identified, thus delineating a novel role for FXa in regulating the inflammatory response to TLR activation on myeloid cells.
Methods

Materials

See detailed Materials section in ‘Supplementary Methods’ for information relating to plasma-derived purified proteins, synthetic peptides and antibodies used in this study.

Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from buffy coat whole blood component obtained from healthy donor pools provided by the Irish Blood Transfusion Service. PBMCs were isolated by centrifugation at 2000rpm in Ficoll-Hypaque density gradient using the Boyum method (32) then cultured RPMI containing 10% FBS. See ‘Supplementary Methods’ for detailed description of PBMC isolation and culture.

Quantification of TNFα secretion from THP-1 monocytic cells

THP-1 cells were re-suspended in serum-free RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 3mM CaCl₂ and 0.6mM MgCl₂ at a density of 3x10⁶ cells/mL and seeded in 96-well microtitre plates. Cells were incubated with vitamin K-dependent proteases (FVIIa, FIXa, FXa, FXaDEGR, FXαDESGLA or APC; 0.313-20nM) for 1-3 hours as described for each assay, then stimulated with TLR agonists for 4 hours. Cell viability was not compromised by FXa incubation (Supplementary Figure 2). Supernatants were collected thereafter and TNFα generation determined using human TNFα DuoSet ELISA (R&D Systems, MN, USA) or using a HEK Blue TNFα/IL-1β cell line (Invivogen, Toulouse, France). Exposure of the HEK Blue TNFα/IL-1β cells to TNFα resulted in dose-dependent activation of the NF-κB/AP-1 pathways and expression of the secreted alkaline phosphatase (ALP) reporter gene (Supplementary Figure 1). ALP activity in the supernatant was detected using QUANTI-Blue medium (Invivogen, Toulouse, France) containing a colorimetric ALP substrate. Colorimetric
measurements were taken at 650nm. ALP activity relative to LPS only-treated cells was determined using the following equation:

\[
ALP \text{ activity (\%)} = \frac{(X-N)}{(P-N)} \times 100
\]

Where \(X\) is the test sample, \(N\) is the untreated PBS sample and \(P\) is the LPS-treated positive control sample.

**Quantification of NF-\(\kappa B\) activation by THP1-XBlueCD14 cells**

NF-\(\kappa B\) activation was measured using THP1-XBlueCD14 cells (Invivogen, Toulouse, France). THP1-XBlueCD14 cells stably co-express CD14 and an NF-\(\kappa B\)/AP-1-inducible secreted ALP reporter. THP1-XBlueCD14 were re-suspended in serum-free RPMI 1640 medium supplemented with 3mM CaCl\(_2\) and 0.6mM MgCl\(_2\) at a density of 3\times10^6 cells/mL and seeded in 96-well plates. Cells were incubated with FXa (20nM) for 1 hour and subsequently stimulated with LPS (31-500ng/ml) for 6 hours. ALP activity in the supernatant was detected with QUANTI-Blue, as described above.

**Mice**

PAR2\(^+\) mice, originally from Jackson Laboratories, were on a BALB/c background and maintained in-house. For additional details, see ‘Supplementary Methods’ section.

**Isolation and culture of murine bone marrow-derived macrophages**

Bone marrow-derived macrophages were prepared from mice by standard techniques (33). For additional details, see ‘Supplementary Methods’ section.
Quantification of cytokine secretion from human PBMCs and murine bone marrow-derived macrophages

Human PBMCs or murine bone marrow-derived macrophages were washed with PBS and incubated with FXa/APC/FXaDEGR (20nM) in serum-free RPMI 1640 medium supplemented with 1mM CaCl$_2$ and 0.2mM MgCl$_2$ for 3 hours prior to stimulation with LPS (PBMCs; 50ng/ml and macrophages; 20ng/ml) for 18 hours. Supernatants were collected and TNFα and IL-6 detected using DuoSet ELISAs (R&D Systems, MN, USA) for human and murine cytokines.
Results

FXa attenuates pro-inflammatory cytokine production from myeloid cells in response to LPS

To examine whether FXa exposure promotes or suppresses the pro-inflammatory cytokine response of myeloid cells to LPS, the effect of FXa on TNFα production from LPS-treated primary monocytes (PBMCs) was assessed. FXa inhibited LPS-induced TNFα secretion from PBMCs in a significant and concentration-dependent manner (IC₅₀~3nM FXa; Figure 1a). FXa alone, however, did not induce TNFα production, or negatively impact cell viability (Supplementary Figure 2). The extent of FXa-mediated inhibition of TNFα release was similar to that observed in the presence of the homologous anti-inflammatory enzyme APC (Figure 1a). When monocytic THP-1 cells were used in place of PBMCs in the same assay, prior exposure to FXa resulted in a significant (60±9% at 20nM FXa; p<0.001) and dose dependent (IC₅₀~3nM) reduction in LPS-induced TNFα secretion, that was again comparable to that observed with APC in the same assay (Figure 1b). Comparison of FXa anti-inflammatory activity with other homologous vitamin K-dependent proteases showed that, of the procoagulant vitamin K-dependent proteases tested, only FXa could replicate APC anti-inflammatory activity on monocytes (Figure 1c).

Pro-inflammatory gene activation and cytokine expression can be initiated upon detection of TLR ligands other than LPS, by activation of TLR family members with different ligand specificity. Coagulation protease modulation of cytokine production induced by activation of TLRs other than TLR4 has not, however, been determined. To investigate this, FXa was added to THP-1 cells prior to stimulation with activating ligands for the TLR2/2 homodimer (HKLM), TLR1/2 heterodimer (Pam3CSK4), TLR5/5 homodimer (flagellin) and TLR2/6 heterodimer (FLS-1). TNFα generation in response to each TLR agonist was significantly inhibited by FXa (p<0.05, Figure 1d). Importantly, co-incubation of FXa with a chloromethyl ketone inhibitor (DEGR), essentially eliminated FXa inhibition of LPS-induced cytokine release.
from THP-1 cells (Figure 1e). This demonstrated that FXa proteolytic activity was required for attenuation of cytokine release in response to LPS and furthermore, that LPS contamination of plasma-derived FXa was not responsible for the reduced cytokine response induced by FXa.

**FXa attenuation of LPS-induced pro-inflammatory cytokine secretion on monocytes and macrophages requires PAR2 and is RAP-sensitive**

FXa can activate either PAR1 or PAR2 to mediate FXa-dependent anti-inflammatory or cytoprotective activity on endothelial cells. Active-site inhibition diminished FXa ability to inhibit cytokine release upon TLR4 activation by LPS from THP-1 cells (Figure 1e), suggestive of a key role for PAR proteolysis in mediating FXa anti-inflammatory signaling on myeloid cells. To determine which PAR was required to mediate this phenomenon, PBMCs were treated with either a PAR1 or PAR2 antagonist (FR131117 and GB83, respectively) in conjunction with FXa and then challenged with LPS. PAR1 antagonism did not affect FXa impairment of LPS-induced TNFα production at any concentration tested, however, PAR2 antagonism dose-dependently inhibited FXa anti-inflammatory function until complete inhibition was observed at approximately 1μM GB83 (Figure 2a). As anticipated, GB83 had no effect upon APC-mediated TNFα inhibition. Replacement of PBMCs with THP-1 cells in the same assay illustrated a similar dependence on PAR2, rather than PAR1, to enable FXa anti-inflammatory signaling on this cell type (Figure 2b). To further characterize the role of PAR2 in FXa anti-inflammatory signaling on myeloid cells, macrophages isolated from wild type and PAR2<sup>−/−</sup> BALB/c mice were exposed to FXa, FXa<sub>DEGR</sub> or APC prior to LPS stimulation. The inhibitory activity of FXa upon TNFα and IL-6 production from both LPS-treated wild type macrophages was approximately double that observed when the same macrophages were incubated with FXa<sub>DEGR</sub> (Figure 2c and 2d). Accordingly, when the same assay was performed in the presence of PAR2<sup>−/−</sup> macrophages, both FXa and FXa<sub>DEGR</sub> failed to significantly inhibit TNFα and IL-6 production upon LPS challenge (Figure 2e and 2f). In contrast, APC significantly restricted TNFα and IL-6 release
from LPS-stimulated wild type and PAR2−/− macrophages (Figure 2c-f), in agreement with previous studies highlighting the importance of PAR1, rather than PAR2, in mediating APC anti-inflammatory activity on this cell type (12).

FXa can utilize a number of co-receptors to elicit PAR-dependent signaling, but those required for suppression of the pro-inflammatory cytokine response to TLR activation are not known. LDL receptor family members have been identified as potential mediators of vitamin K-dependent protease signaling on monocytic cells (34). In order to determine whether an LDL family receptor interaction might also modulate PAR2-dependent FXa anti-inflammatory activity on myeloid cells, receptor-associated protein (RAP; which binds to the ligand binding region of all LDL family receptors and prevents ligand interaction), was added in combination with FXa. RAP sensitivity of FXa-mediated attenuation of cytokine production from LPS-treated THP-1 monocytes and murine macrophages was then determined. RAP alone had no effect upon LPS-induced pro-inflammatory cytokine production in the absence of FXa, but completely ablated FXa anti-inflammatory activity on THP-1 monocytes (Figure 3a). Similarly, incubation solely with RAP did not alter LPS-induced cytokine production from LPS-treated macrophages, but completely inhibited FXa inhibition of TNF and IL-6 release (Figure 3b & 3c), highlighting a novel role of RAP-sensitive LDL receptors in mediating FXa anti-inflammatory cell signaling. Given the important role of ApoER2 in mediating APC anti-inflammatory signaling on monocytes, we sought to determine whether ApoER2 was the target for RAP inhibition of FXa anti-inflammatory activity on THP-1 cells. To achieve this, FXa was incubated with THP-1 cells in the presence of a mouse anti-ApoER2 monoclonal antibody directed against the extracellular region of human ApoER2 (anti-ApoER2 mAb 1). Anti-ApoER2 mAb 1 dose-dependently attenuated FXa impairment of LPS-induced TNFα production (Figure 5d and inset). In contrast, anti-ApoER2 mAb 2 (directed against the ApoER2 intracellular region) and a mouse IgG1 antibody isotype control had no effect upon FXa anti-inflammatory activity, suggesting a novel role for the extracellular region of ApoER2 in enabling FXa anti-inflammatory activity on THP-1 monocytes.
These studies indicate that PAR2, in conjunction with a RAP-sensitive membrane receptor, is necessary for optimal FXa-mediated suppression of LPS-induced pro-inflammatory cytokine production from myeloid cells.

**FXa anti-inflammatory signaling causes inhibition of LPS-induced NF-κB activation and is sensitive to wortmannin**

Activation of the transcription factor nuclear factor-κB (NF-κB) controls the expression of an array of pro-inflammatory cytokine genes and is a shared downstream effector of TLR-activated signaling pathways. To examine whether NF-κB activation by LPS was impaired by prior exposure to FXa, THP-1 cells stably transfected with an NF-κB-dependent secreted ALP reporter construct were incubated with FXa and then stimulated with LPS. FXa was found to significantly inhibit NF-κB activation at all LPS concentrations tested (p<0.05; Figure 4a).

Numerous negative regulatory mechanisms exist in order to control the magnitude of the pro-inflammatory response upon TLR-mediated NF-κB activation. Pertinently, FXa homolog APC induces PI3K activation and Akt phosphorylation to negatively regulate LPS-TLR4 signaling on U937 monocytes(34). To investigate whether PI3K/Akt pathway activation is similarly required for FXa regulation of LPS-dependent cytokine production, THP-1 cells were treated with FXa and LPS in the presence of the PI3K inhibitor wortmannin and the production of TNFα measured. Wortmannin completely inhibited FXa-mediated suppression of TNFα production in response to LPS, with half-maximal inhibition of FXa activity observed at 0.5μM (Figure 4b and 4c). Similarly, PI3K inhibition by wortmannin ablated the negative regulation of TNFα production by FXa following stimulation with Pam3CSK4 as a ligand for TLR1/2 (data not shown). To further characterize the receptor requirements for FXa-mediated inhibition of NF-κB activation in THP-1 cells, the assay was repeated in the presence of GB83 and RAP. Similar to the effect of wortmannin, both GB83 and RAP completely prevented inhibition of NF-κB activation by FXa in THP-1 cells (Figure 4d). FXa_{DEGR} and FXa truncated at the N-terminal Gla domain (FXa_{DESGLA})
were unable to inhibit NF-κB activation in a similar manner to FXa (Figure 4e), highlighting the importance of FXa enzymatic activity and Gla domain in mediating FXa anti-inflammatory activity.

**Structural determinants of FXa anti-inflammatory activity on myeloid cells**

To characterise the molecular requirements for FXa inhibition of pro-inflammatory cytokine release upon TLR activation, a range of FXa isoforms and truncations were utilised. The ability of FXaDESGLA to prevent LPS-induced TNFα production from THP-1 cells was significantly impaired compared to full-length FXa (Figure 5a). A similar response was observed when FXaDESGLA was used to prime cells prior to Pam3CSK4 treatment (data not shown). Bovine FXa, a close structural homolog of human FXa possessing approximately 70% amino acid sequence similarity to its human counterpart, was surprisingly ineffective in reducing LPS and Pam3CSK4-induced TNFα expression, suggesting that the molecular requirements for FXa anti-inflammatory activity on THP-1 monocytes are not conserved between these species (Figure 5a).

Once activated, FXa associates with activated factor V (FVa) on plasma membrane negatively charged phospholipids to activate prothrombin. FVa could theoretically inhibit FXa interaction with cell surface receptors necessary for anti-inflammatory signaling. We found that FVa, however, had no effect upon the ability of FXa to suppress LPS-induced cytokine production from THP-1 cells (Figure 5b); indicating that in the presence of its procoagulant cofactor, the ability of FXa to negatively regulate LPS-stimulated cytokine production is maintained.

An intermediary amino acid sequence connecting the two FXa EGF-like domains (amino acid residues 83-88) has been previously shown to be important for FXa cell signaling (35). To investigate the contribution of the inter-EGF region to FXa inhibition of LPS-induced cytokine production on THP-1 monocytes, a short synthetic peptide mimicking this region (FX83-88) was
co-incubated with FXa and LPS-induced TNFα production measured (Figure 5c). The peptide alone had no effect on TNFα production, but its presence alongside FXa dose-dependently attenuated FXa-mediated inhibition of TNFα secretion in response to LPS (IC$_{50}$ = 1.3μg/mL FX$_{83-88}$ peptide, Figure 5c; inset). To ensure specificity, a scrambled version of this peptide was tested, but unlike FX$_{83-88}$ peptide, it was unable to inhibit FXa anti-inflammatory activity (Figure 5c).
Discussion

FXa signaling can induce disparate downstream outcomes depending upon the PAR activated, the cell type assessed or the cellular output measured. Consequently, precise determination of the role of FXa cell signaling \textit{in vivo} has been difficult to ascertain. Moreover, despite the observation of robust FXa signaling \textit{in vitro}, rapid inhibition of FXa by plasma serpins may limit the role of FXa signaling in its free form under normal physiological conditions. However, there are a number of pathological states, particularly DIC, where procoagulant proteases such as FXa are excessively generated and not subject to the same strict regulatory mechanisms that exist in the absence of infection. In such instances, FXa generated as a consequence of persistent coagulation activation has increased potential to bind leukocytes and trigger cell signaling.

Recent studies have highlighted the role of proteases associated with haemostasis in modulating innate immunity, either by simultaneously promoting both coagulation and inflammation (9), or by regulating inflammation and thus limiting mortality in preclinical animal models of endotoxemia or in patients with severe sepsis (15). The role of FXa in this context is currently not well understood, and has been described to induce cell signaling conducive to both the promotion and attenuation of inflammation. Therefore, in this study, we sought to determine how FXa signals on innate immune cells whose primary physiological role is to initiate a pro-inflammatory response upon pathogen detection. In doing so, we demonstrate that FXa induces a refractory state to TLR stimulation via PAR2 activation, and thereby impairs pro-inflammatory cytokine production from TLR-stimulated myeloid cells.

FXa did not induce the generation of pro-inflammatory cytokines in any of the cell types tested when administered alone, or when co-administered simultaneously with TLR ligands. In contrast, prior exposure to FXa produced significant and dose-dependent inhibition of LPS-induced cytokine production in THP-1 cells, PBMCs and primary murine macrophages. A half-maximal reduction of TNFα production was elicited at ~3nM FXa in all myeloid cell types tested.
The ability of FXa to regulate cytokine expression was not limited to that initiated by TLR4 activation and experiments utilising synthetic agonists directed against specific TLRs demonstrated that FXa regulates the pro-inflammatory cytokine response upon activation of multiple TLRs (including TLR 1, TLR2, TLR5 and TLR6), with varying degrees of efficacy. Given the enzymatic activity and broad range of identified substrates for FXa, we considered whether reduced sensitivity to LPS on FXa-treated myeloid cells was a consequence of FXa shedding of TLRs from the cell surface. However, conserved FXa cleavage sites were not identified in the extracellular portions of any of the TLRs under investigation, indicating that receptor proteolysis from the cell surface is unlikely to contribute to FXa anti-inflammatory activity.

Proteolytic activation of either PAR1 or PAR2 by FXa confers cytoprotective effects on endothelial cells, but it is not known if they mediate FXa signaling on myeloid cells and if so, which PAR is required to mediate anti-inflammatory FXa activity. A likely role for PARs in enabling FXa suppression of pro-inflammatory production was demonstrated by the diminished capacity of FXaDEGR to attenuate LPS-induced TNFα expression on both monocytes and macrophages. Furthermore, we found that FXa exhibited a significantly reduced ability to inhibit LPS-induced cytokine production from THP-1 monocytes in the presence of PAR2, but not PAR1, antagonists. Also, in contrast to murine wild type macrophages, LPS-induced TNFα/IL-6 production from PAR2⁻/⁻ macrophages was largely impervious to the anti-inflammatory activity of FXa. This is consistent with the proposed molecular mechanism of action of FXa cytoprotective activity on endothelial cells, where previous studies have shown that anti-PAR2 antibodies block FXa-dependent maintenance of endothelial cell barrier integrity, down-regulation of cell surface adhesion protein expression upon TNFα treatment and diminished NF-κB activation (23). Interestingly, PAR2 activation by non-physiological synthetic peptides also results in diminished LPS-induced TNFα and IL-6 expression from primary murine macrophages (36). Our study thus identifies a novel role for FXa as an endogenous PAR2 activator with similar anti-inflammatory activity on myeloid cells.
The downstream signaling induced upon PAR2 activation by FXa to limit inflammatory cytokine generation is not known and the intracellular pathways that mediate decreased sensitivity to LPS have not yet been fully delineated. However, we have observed that FXa significantly inhibited NF-κB activation in LPS-activated THP-1 reporter cells. Furthermore, FXa anti-inflammatory signaling requires functional PI3K and is inhibited by the presence of wortmannin. The PI3K-Akt pathway has a well-characterised role in mediating cell survival, and has been implicated in both negative and positive regulation of TLR-induced pro-inflammatory cell signaling (37, 38). Activation of the PI3K-Akt pathway in human monocytes and THP-1 cells has been demonstrated to limit LPS-induced up-regulated NF-κB, AP-1 and TNFα expression (37). Furthermore, pharmacological inhibition of PI3K/Akt increases LPS-induced coagulation and inflammation in murine models of endotoxemia (39). Our data implies FXa activation of PI3K/Akt-dependent pathways in THP-1 cells is an important step in enabling FXa anti-inflammatory signaling on myeloid cells. Furthermore, this suggests that FXa utilises a similar downstream signaling mechanism to its homolog APC on monocytes, which also induces PI3K and Akt phosphorylation to attenuate LPS-TLR4 pro-inflammatory signaling (34).

The molecular determinants of specific PAR cleavage by FXa are incompletely understood, but appear to be regulated by FXa cell surface co-receptor interactions. For example, interaction between FXαβ and annexin 2 facilitates activation of PAR1, but not PAR2, on endothelial cells, whereas EPCR binding is proposed to support FXa activation of both PAR1 and PAR2 on the same cell type (29, 30, 40). Given the ability of RAP to inhibit APC anti-inflammatory signaling on U937 monocytic cells, we sought to identify whether FXa anti-inflammatory signaling on THP-1 monocytes was similarly RAP-sensitive. FXa anti-inflammatory signaling was strongly inhibited by the presence of RAP on both monocytes and macrophages, indicating a crucial role for an LDL receptor family member in enabling FXa signaling activity on these cells. In addition, a mouse monoclonal antibody directed against the extracellular region of ApoER2 blocked FXa inhibition of TNFα release from THP-1 monocytes. Extensive studies to
characterise the role of ApoER2 and other potential LDL receptor family members that may contribute to FXa anti-inflammatory activity on myeloid cells are required. This is particularly true given FXa anti-inflammatory activity on bone marrow-derived murine macrophages derived from BALB/c mice was also found to be sensitive to RAP inhibition despite previous studies that indicate that ApoER2 is not expressed on bone marrow-derived murine macrophages (12). This suggests an as-yet-unidentified LDL receptor family member expressed on bone-marrow-derived macrophages, distinct from ApoER2, may represent an alternative target for the potent RAP-mediated inhibition of FXa anti-inflammatory activity observed on LPS-treated bone marrow-derived macrophages.

In this study, a peptide mimicking this FXa amino acid sequence (FX$_{33-58}$) shown previously to be crucial for PAR2-dependent barrier protective and anti-inflammatory activity of FXa on endothelial cells (22, 23), produced a dose-dependent decrease in FXa regulation of LPS-stimulated cytokine production from THP-1 cells, such that FXa anti-inflammatory activity could be completely blocked by the presence of this peptide. Interestingly, bovine FXa, whose inter-EGF region amino acid sequence is not similar to its human counterpart, was unable to mount an anti-inflammatory response similar to that of human FXa, providing a possible explanation for the observed species-specific loss of function.

The anti-inflammatory activity of FXa on myeloid cells was inhibited by N-terminal Gla domain truncation. In contrast, the FXa Gla domain may not be required for PAR2-dependent FXa signaling on endothelial cells (22). It is not clear at this stage whether FXa Gla domain truncation confers long-range structural changes that disrupt FXa myeloid receptor binding sites on the Gla domainless protease, or itself represents a crucial binding site for FXa myeloid cell surface receptors.

Collectively, this study suggests that FXa acts in a similar manner to APC in limiting pro-inflammatory cytokine production on monocytes and macrophages. Unlike APC, FXa utilises
PAR2, rather than PAR1, to initiate downstream anti-inflammatory signaling on macrophages, but similarly, attenuates NF-κB activation and activates PI3K signaling pathways to inhibit inflammation in LPS-treated monocytes. Myeloid cell-specific APC anti-inflammatory signaling is crucial in protecting mice from LPS-induced lethality (12, 41). Assessment of the efficacy of FXa in this setting however, is complicated by multiple confounding factors, including the potent procoagulant activity of FXa, its rapid inhibition by serpins in plasma (42) and its pleiotropic cell-dependent signaling properties. However, the success of prior studies utilizing modified recombinant APC variants (15) with signaling-selective activity to regulate murine endotoxemic response provides a useful insight as to how the anti-inflammatory activity of exogenous FXa could be investigated in vivo. Recombinant FXa variants with impaired ability to assemble into the prothrombinase complex and/or associate with inhibitors, but retained ability to interact with myeloid cell surface signaling receptors, may yield important insights into the role of FXa anti-inflammatory signaling when applied to pre-clinical animal models of acute inflammatory disease.

**Authorship & Disclosures:**

EMG. designed and performed experiments, analyzed data, wrote the manuscript. EH, FNA and BAK performed experiments, JSO'D and PGF analyzed data, RJSP designed experiments, analyzed data and wrote manuscript. The authors have no conflicts of interest to declare.

**References**


Figure Legends

Figure 1: FXa inhibits LPS-induced monocyte TNFα secretion. (a) PBMCs were exposed to FXa (0.313-20nM; ■) or APC (20nM; ▲) for 3 hours prior to stimulation with LPS (50ng/mL; 18 hours). TNFα production was determined by ELISA. No TNFα was detected in the absence of LPS (□). (b) The relative anti-inflammatory activity of APC (0.625-20nM; □) and FXa (0.313-20nM; ■) was measured by incubation with THP-1 cells for 3 hours, prior to stimulation with LPS (500ng/mL; 4 hours). TNFα secretion was detected using HEK Blue TNFα reporter cells (ALP activity) as described in Methods. (c) THP-1 cells were exposed to vitamin K-dependent proteases (APC, FXa, FVIIa, FIXa, all 20nM) and then treated with PBS or LPS (500ng/mL; 4 hours) before TNFα release was determined using HEK Blue TNFα reporter cells (d) THP-1 cells were exposed to FXa or FXa_DGR (20nM) for 1 hour prior to stimulation with LPS (500ng/mL) for 4 hours and then released TNFα detected using HEK Blue TNFα reporter cells. All results represent the mean of at least 3 independent experiments ± S.D..

Figure 2: FXa inhibits LPS-induced pro-inflammatory cytokine production from myeloid cells via PAR2 activation. (a) PBMCs were exposed to PAR1 (FR131117; O) or PAR2 (GB83; ■) antagonists for 30 minutes prior to FXa (20nM) co-incubation for 3 hours, followed by LPS treatment (50ng/mL; 18 hours). TNFα was then measured by ELISA. LPS-induced TNFα in the absence of FXa (▲) and FXa- and PAR2-dependent FXa suppression of LPS-induced TNFα in the presence of APC/GB83 is also shown (◆). (b) THP-1 cells were exposed to GB83 (0.125-1μM) for 30 minutes prior to incubation with FXa (20nM; ■) or APC (20nM; ◆) for 3 hours. Similarly, THP-1 cells were exposed to FR131117 (1.25μM) for 30 minutes prior to incubation with FXa (20nM, O) for 3 hours. Cells were treated with LPS (500ng/mL; 4 hours) and TNFα secretion was detected using HEK Blue TNFα reporter cells. LPS-induced TNFα in the absence of FXa or PAR antagonists is shown (▲). GB83 did not induce TNFα production in the absence of LPS (□). Murine bone marrow-derived macrophages were isolated from wild type (c & d) and PAR2−/− (e & f) BALB/c mice and exposed to APC/FXa/FXa_DGR (all 20nM) for 3 hours prior to addition of PBS or LPS (20ng/mL) for 18 hours. Murine TNFα (c & e) and IL-6 (d & f) were determined by ELISA and the mean ± S.D. from 3 independent experiments is shown.

Figure 3: PAR2-dependent FXa suppression of LPS-induced cytokine production from myeloid cells is sensitive to RAP. (a) THP-1 cells were incubated with RAP (80μM) for 30 minutes, prior to PBS (black bars) or FXa (grey bars, 20nM) incubation for 3 hours. Cells were then treated with LPS (500ng/mL; 4 hours) and TNFα secretion was detected using HEK Blue TNFα reporter cells. Treatment with PBS alone (white bars) was used as a negative control. (b...
and c) Murine bone marrow-derived macrophages were treated with RAP (80μM) for 30 minutes prior to FXa incubation (20nM) for 3 hours and stimulated with LPS (20ng/mL; 18 hours). Murine (b) TNFα and (c) IL-6 production was determined by ELISA. (d) THP-1 cells were incubated with two anti-ApoER2 (10μg/mL; inset 0-10μg/mL) or mouse IgG (10μg/mL) antibodies for 30 minutes, prior to PBS (black bars; inset - ▲) or FXa (grey bars; inset - ■, 20nM) incubation for 3 hours. Cells were then treated with LPS (500ng/mL; 4 hours) and TNFα secretion was detected using HEK Blue TNFα reporter cells. Treatment with PBS alone (white bars) was used as a negative control.

**Figure 4:** FXa anti-inflammatory PAR2-dependent signaling results in inhibition of LPS-induced NF-κB activation and is sensitive to wortmannin. (a) THP1-Blue CD14 NF-κB reporter cells were incubated with FXa (□; 20nM) or PBS (■) for 1 hour prior to stimulation with LPS (31.3-500ng/mL; 6 hours) and measurement of ALP activity. (b, c) THP-1 cells were treated with wortmannin (b – 1.25μM, c – 0.313-2.5μM) for 30 minutes prior to co-incubation with PBS (black bars) or FXa (grey bars, 20nM; 1 hour), and then stimulated with LPS (500ng/mL) for 4 hours. PBS treatment (white bars, b) did not elicit TNFα secretion. (d) THP1-Blue CD14 NF-κB reporter cells were incubated with GB83 (1μM), wortmannin (1.25μM) or RAP (80μM) for 30 minutes prior to exposure to co-incubation with PBS (black bars) or FXa (grey bars, 20nM; 1 hour), and then stimulated with LPS (500ng/mL) for 4 hours. PBS treatment (white bars) did not elicit NF-κB activation. (e) THP1-Blue CD14 NF-κB reporter cells were incubated with FXa, FXaDEGR or FXaDESLA for 3 hours prior to stimulation with LPS (500ng/mL) for 4 hours then ALP activity was detected. Results represent the mean ± S.D. of at least 3 independent experiments.

**Figure 5:** Structural and domain requirements for FXa anti-inflammatory activity on myeloid cells. (a) THP-1 cells were exposed to FXa, FXaDESLA or bovine FXa (bFXa; all 20nM) for 3 hours prior to stimulation with LPS (500ng/mL) for 4 hours then TNFα in the THP-1 supernatant measured. (b) THP-1 cells were exposed to FXa (20nM) in the presence or absence of FVα (20nM) for 3 hours prior to stimulation with LPS (500ng/mL) for 4 hours. (c) THP-1 cells were treated with FXa83-88 or FXa83-88SCR (100μg/mL) for 30 minutes prior to either PBS (black bars) or FXa (grey bars, 20nM) for 3 hours and then with LPS (500ng/mL) for 4 hours. PBS in the absence of FXa/LPS (white bars) had no effect on cytokine production when assessed using HEK Blue TNFα reporter cells. The dose-dependent inhibition of FXa83-88 on FXa anti-inflammatory activity on THP-1 cells is also shown (inset).
Supplementary Methods

Materials
Plasma-purified human FVIIa, FIXa, FXa, FXaDEGR, FXaDEGLA, bovine FXa, FVa and APC were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Synthetic peptides mimicking FX sequence L^{83}FTRKL^{88}G and a scrambled version of the same FX sequence (KFTGRLL) were synthesized on request by Genscript Inc. (Piscataway, NJ, USA). TLR agonists (Pam3CSK4, Heat-killed *Listeria monocytogenes* (HKLM), bacterial flagellin, FSL-1) and wortmannin were purchased from Invivogen (Toulouse, France). Endotoxin-free receptor-associated protein (RAP) was purchased from Innovative Research, Inc. (Novi, MI, USA). Histopaque 1077 was purchased from Sigma-Aldrich Life Sciences (Cambridge, UK). Anti-ApoER2 monoclonal antibodies and mouse IgG1 isotype control antibody were obtained from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Boston, MA, USA).

Isolation of human peripheral blood mononuclear cells (PBMCs)
PBMCs were isolated from buffy coat whole blood component obtained from a healthy donor pool provided by the Irish Blood Transfusion Service. PBMCs were isolated by centrifugation at 2000 rpm in Ficoll-Hypaque density gradient using the Boyum method and maintained in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies, Paisley, UK). Cell viability before each assay was assessed by Trypan Blue staining. Cells were seeded in 96-well microtitre plates at a density of 2 x 10^6 cells/ml and incubated at 37°C and 5% CO_2 for 3 hours (after which any non-adherent cells were removed) then cultured for a further 24 hours.

Mice
PAR2^{−/−} mice, originally from Jackson Laboratories, were on a BALB/c background and maintained in house. Animals were kept in individually ventilated and filtered cages under positive pressure (Teniplast, Northants, UK) and Specific Pathogen-Free (SPF) conditions. Mice were fed an irradiated diet and housed on irradiated bedding. Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and were approved by the Trinity College Dublin BioResources ethical review board.
Isolation and culture of murine bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared from mice by standard techniques. Briefly, bone marrow cells obtained from the femurs and tibia of PAR2\textsuperscript{-/-} and BALB/c wild type mice. Bone marrow cells were plated in 6-well plates and cultured in RPMI 1640 medium supplemented with 10\% fetal calf serum, 100U/ml penicillin, 100\mu g/ml streptomycin and 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) for 7 days. Purity of macrophages was analysed using flow cytometry with data collection on a CyAn (Beckman Coulter). Data were analyzed using FlowJo software (Tree Star). Cultured cells were stained with BD Biosciences mAb; F4/80-APC (BM8) and eBiosciences mAb; CD11b-PerCP (M1/70). Flow buffers used contained 2mM EDTA to exclude doublets. Using appropriate isotype-controls, quadrants were drawn and data were plotted on logarithmic scale density-plots. Cultured cells expressed both CD11b and F4/80 markers for macrophages.

Statistical analysis

All experiments were performed in triplicate as a minimum and plotted as the mean ± standard deviation (S.D.). Unpaired Student t-tests were used to determine statistical significance, and p values < 0.05 were deemed significant.
Supplementary Figure 1: Exposure of THP-1 cells to LPS and Pam3CSK4 results in expression of TNFα that can be measured using HEK Blue™ TNF-α/IL-1β reporter cells. THP-1 cells were treated with (a and c) LPS (6.25-100ng/ml) (b and d) Pam3CSK4 (31.3-500ng/ml) for 4 hours. TNFα secretion was determined by incubating treated THP-1 cell supernatant with HEK Blue™ TNF-α/IL-1β reporter cells, resulting in a dose-dependent increase in secreted ALP expression. THP-1 cells were treated with the LPS inhibitor polymixin (PolyB), prior to exposure to (c) LPS (200ng/ml; 4hrs) or (d) Pam3CSK4 (500ng/ml; 4hrs). PolyB completely prevented LPS-induced TNFα secretion but had no effect on Pam3CSK4-induced TNFα secretion. The presence of an anti-TNFα monoclonal antibody prevented induction of secreted ALP expression by both LPS/Pam3CSK4 treated THP-1 supernatant, confirming ALP activity was a consequence of TNFα in the supernatant.
Supplementary Figure 2: The effect of FXa on LPS-induced TNFα secretion does not occur as a consequence of altered THP-1 cell viability. To verify that the observed effect of FXa on LPS-induced cytokine secretion is not a consequence of altered monocyte viability, THP1 cell viability after FXa treatment was assessed using the CellTiter-Flour Viability assay. This assay uses constitutive protease activity within live cells, lost upon degradation of membrane integrity during apoptosis, as a marker of cell viability. Cells were treated with a fluorogenic, cell-permeable, peptide substrate cleaved only in viable cells to generate a fluorescent signal that is proportional to the number of non-apoptotic cells [Niles, A.L. et al. Anal Biochem. 2007 Jul 15;366(2):197-206]. THP-1 cells were incubated with PBS or FXa (20nM) for 3 hours prior to stimulation with LPS (500ng/mL) for 4 hours. Cell viability was assessed using the CellTiter-Flour viability assay. Viability of cells treated with LPS alone or in combination with FXa did not differ significantly from that of untreated cells.
Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities

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Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities

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Recent improvement in modern analytical technologies has stimulated an explosive growth in the study of glycobiology. In turn, this has lead to a richer understanding of the crucial role of N- and O-linked carbohydrates in dictating the properties of the proteins to which they are attached and, in particular, their centrality in the control of protein synthesis, longevity, and activity. Given their importance, it is unsurprising that both gross and subtle defects in glycosylation often contribute to human disease pathology. In this review, we discuss the accumulating evidence for the significance of glycosylation in mediating the functions of the plasma glycoproteins involved in hemostasis and thrombosis. In particular, the role of naturally occurring coagulation protein glycoforms and inherited defects in carbohydrate attachment in modulating coagulation is considered. Finally, we describe the therapeutic opportunities presented by new insights into the role of attached carbohydrates in shaping coagulation protein function and the promise of carbohydrate modification in the delivery of novel therapeutic biologics with enhanced functional properties for the treatment of hemostatic disorders. (Blood. 2013;121(19);3801-3810)

Introduction

Glycan structures are attached to more than half of all known proteins,1 and genes encoding the molecular apparatus required for glycosylation constitute 1% to 2% of the human genome.2 Despite the prevalence of glycan attachment to human proteins and lipids, the field of glycobiology has traditionally represented something of a Cinderella subject. However, recent advances in synthetic, and particularly analytic, methodologies have led to heightened awareness regarding the structural and functional significance of carbohydrate structures on proteins. Accumulating data make it clear that the glycan structures expressed on many glycoproteins play critical roles in modulating functional activity. In addition, variation in carbohydrate structures has been implicated in the pathogenesis of a number of human diseases. Moreover, it seems inevitable that evidence regarding the physiological and pathological importance of carbohydrate expression will continue to emerge in the coming years. In this context, it is perhaps unsurprising that regulation of glycan expression on novel recombinant therapeutic glycoproteins is already established as a key quality parameter within the pharmaceutical industry. In this review, we provide an overview of the critical roles played by carbohydrate determinants in regulating human hemostasis and thrombosis. In particular, using exemplar coagulation glycoproteins, we have sought to highlight some of the different molecular mechanisms through which glycan variation can influence glycoprotein biology. Although we have selected specific examples and focused on plasma coagulation glycoproteins, these concepts can nevertheless be considered a paradigm equally applicable to other human secretory glycoproteins.

Protein glycosylation

N-linked glycosylation

N-linked glycans on human glycoproteins are attached to the amide nitrogens of asparagine (Asn) side chains. N-linked glycosylation begins in the endoplasmic reticulum (ER),1,4 where a preassembled oligosaccharide core structure is transferred from a dolichol lipid donor onto specific Asn residues within nascent polypeptide chains.5 This reaction is catalyzed by the enzyme complex oligosaccharyltransferase, which targets Asn residues located in the consensus sequence Asn-X-serine (Ser)/threonine (Thr) (where X can be any amino acid except proline).6 Importantly, N-linked glycosylation within the ER is actually a cotranslational event occurring on the luminal aspect of the ER membrane. As a consequence, depending on polypeptide folding and conformation, not all Asn residues in a consensus sequence will necessarily be glycosylated. The net effect, therefore, is that polar N-linked glycans are typically found on the surfaces of glycoproteins, rather than being buried deep within the protein interior.

The initial 14-sugar core N-linked structure attached during protein synthesis in the ER is mannose-rich (Glc3Man9GlcNAc2). This core glycan is subsequently remodeled by a series of glycosyltransferases and glycosidases as the protein passes through the ER and onto the Golgi.7 This process commences in the ER with the removal of 2 terminal glucose moieties by the exoglycosidases glucosidase 1 and 2. Glucose cleavage enables the protein to interact with 2 homologous ER lectins, calnexin (Cnx) and calreticulin (Crt), and thereby engage in a folding cycle.8 Once properly folded, glycoproteins are subsequently transported to the Golgi, where the
O-linked Glycan Structures | N-linked Glycan Structures

**Figure 1.** Examples of typical N- and O-linked glycan structures expressed on human plasma glycoproteins.

![Diagram of glycan structures](image)

N-linked glycans are further modified. Unsurprisingly, given that more than a hundred different glycosyltransferases are encoded by the human genome, the final N-linked carbohydrate structures can be complex and heterogeneous in nature. Nevertheless, N-linked glycans can be classified into 1 of 3 subgroups: high-mannose, hybrid, or complex (Figure 1). This process is distinct from that of glycation, which refers to the nonenzymatic irreversible attachment of reducing sugars to proteins, and in contrast to glycosylation, it is not enzyme-controlled or dependent on predefined attachment sites.

**O-linked glycosylation**

O-linked glycans on human glycoproteins are attached to Ser or Thr residues. O-linked glycosylation differs from N-linked glycosylation in a number of important regards. First, O-linked glycosylation is a true posttranslational modification, as O-linked carbohydrate structures are only synthesized on proteins as they transit through the Golgi. Second, there is no preassembled O-linked oligosaccharide core structure. Rather, O-linked carbohydrate synthesis involves sequential addition of monosaccharide units in a stepwise manner. These reactions are catalyzed by a series of specific glycosyltransferases analogous to those required for N-linked glycan synthesis. Finally, for O-linked glycosylation to occur, Ser or Thr residues do not need to exist as part of a specific consensus sequence. Nevertheless, previous studies have shown that O-linked glycosylation of Ser or Thr is more common if Ser/Thr residues are present in clusters or are located in areas rich in proline or alanine residues. Final O-linked glycan structures are simpler than complex N-linked sugars (Figure 1).

**Heterogeneity of glycosylation**

In view of the number of distinct human glycosyltransferases and glycosidases already described, it is perhaps not surprising that glycan databases include descriptions of more than 500 different N-linked carbohydrate structures. The marked heterogeneity of N-linked glycans structures has proven one of the major obstacles to the investigation of the potential physiological and pathological significance of carbohydrate structures. A further level of complexity is added by virtue of the fact that many glycoproteins contain multiple individual N- and O-linked glycosylation sites. In addition, different types of glycan structures can be expressed on each of these specific Asn residues within the same protein. Importantly, expression levels for the individual glycosyltransferase and glycosidase enzymes vary significantly between different tissues and can also be influenced by disease state or normal aging. As a consequence, a given individual can express various glycoforms of a particular glycoprotein that differ only with respect to their carbohydrate profiles.

Many of the human proteins involved in regulating normal hemostasis circulate as soluble glycoproteins in plasma. Before their secretion, these proteins often undergo complex posttranslational modification, including significant glycosylation. As a result, complex branching carbohydrate structures can account for up to 25% of their final molecular mass. These carbohydrate structures play critical, but often underappreciated, roles in modulating many of the key biological properties of these coagulation proteins.

**Role of glycans in modulating intracellular trafficking**

Carbohydrate determinants regulate transit through the ER

Secretory glycoproteins, including coagulation factors, are synthesized by ER-bound ribosomes. After processing within the ER, proteins follow an intracellular pathway through the ER–Golgi intermediate compartment (ERGIC) to the Golgi before finally being secreted into the plasma. Within the lumen of the ER, significant folding and modification of newly synthesized proteins occurs. This process is regulated by a series of enzymes and molecular chaperones, including immunoglobulin-binding protein (BiP), Cxx, and Crr. Only correctly folded proteins are allowed to exit the ER. Misfolded proteins either are retained within the ER or are subject to degradation by the ER-associated protein degradation pathway.
Previous studies have clearly defined the critical role played by carbohydrate structures in regulating glycoprotein interaction with ER-resident molecular chaperones. In particular, the 14-sugar core N-linked structure on nascent polypeptide chains is a key regulator of these interactions. Cnx and Crt are homologous lectins that bind monoglycosylated polypeptides in concert with the thiol oxidoreductase ER p57, facilitating correct folding and preventing protein aggregation. The fate of the Cnx/Crt-bound polypeptide is ultimately determined by uridine diphosphate-glucose:glycoprotein glucosyltransferase (GT), which acts as a folding sensor that detects characteristic biophysical properties of misfolded proteins. If misfolding is detected, the protein is reglucosylated by GT and re-enters the Cnx/Crt cycle, where it can either continue until correctly folded or be transferred for degradation. If properly folded, GT does not reglucosylate high mannoses, and the protein is ready for transport to the Golgi apparatus.

The physiological relevance of carbohydrate determinants in regulating transit through the ER has been highlighted in a series of elegant studies examining biosynthesis of the homologous coagulation glycoproteins factor V (FV) and factor VIII (FVIII), respectively. Once activated, these glycoproteins play crucial roles in the coagulation cascade, acting as cofactors in the prothrombinase and intrinsic tenase complexes. FV and FVIII share identical domain structures (A1-A2-B-A3-C1-C2), and significant structural homology exists between their A and C domains. In contrast, the B domains of FV and FVIII exhibit limited sequence similarity. Nevertheless, both domains are extensively glycosylated, containing 25 (FV) and 18 (FVIII) potential N-linked glycosylation sites, respectively. Moreover, although the amino acid sequence encoding the FVIII B domain has diverged widely between human, porcine, and murine species, the majority of the N-linked glycosylation sites has remained strikingly conserved. In spite of their homologous structures, expression studies have demonstrated that FV is secreted from mammalian cells significantly more efficiently than FVIII. Limiting steps in FVIII secretion have been identified and include extended interactions with ER chaperone molecules, which in turn limit its progress to the Golgi and onward to secretion from the cell. In particular, residues within the A1 domain of FVIII have been shown to mediate stable interaction with BIP. In contrast, FV does not associate with BIP. Furthermore, FVIII has also been shown to bind both Crt and Cnx, which also slows its secretion. Unsurprisingly, these interactions are mediated in large part through N-linked glycan structures expressed within the FVIII B domain. Although FV can also interact with the chaperone Crt, it does not appear to bind to Cnx.

Carbohydrate determinants regulate transit from ER to Golgi

On successful folding and packaging, new proteins travel from the ER to the Golgi for additional posttranslational modifications before secretion. This is achieved by formation of coat protein complex II (COPII) vesicles, which bud from the ER lumen and migrate to the Golgi apparatus via the ERGIC. Despite their different ER processing, FV and FVIII have a shared prerequisite for specialized ER-to-Golgi transport machinery. In particular, lectin mannose-Bip 1 (LMAN1; also known as ERGIC-53) and multiple coagulation factor deficiency protein 2 (MCFD2) are cargo transporters for ER-to-Golgi traffic of FV and FVIII.

N-linked oligosaccharides are key to FV/FVIII interactions with the LMAN1/MCFD2 complex. LMAN1 association with FV/FVIII is enhanced by the presence of fully glucose trimmed mannose 9 structures on B domain–located carbohydrates, as demonstrated using an LMAN1 mutant with defective mannose binding ability and, consequently, severely diminished FVIII-LMAN1 interaction. LMAN1/MCFD2 gene mutations that prevent interaction with FV/FVIII (or each other) have been shown to be the cause of combined FV/FVIII deficiency, an autosomal recessive disorder associated with a mild to moderate bleeding tendency caused by reduced FV and FVIII plasma levels of 5% to 30%. Cumulatively, these data serve to emphasize the critical importance of N-linked glycan structures in regulating the intracellular trafficking of secretory glycoproteins.

Role of glycans in modulating susceptibility to proteolysis

In addition to modulating intracellular processing, carbohydrate expression on plasma glycoproteins influences a number of their biological properties. In particular, glycans have been shown to modulate susceptibility to proteolysis. The critical importance of sugar structures in this regard has been highlighted through a series of recent studies on the proteolysis of the large, multimeric sialoglycoprotein, von Willebrand Factor (VWF). Before secretion, VWF undergoes extensive posttranslational modification, including significant glycosylation. As a result, each VWF monomer contains 12 potential N-linked and 10 potential O-linked glycosylation sites with carbohydrate structures. The glycan structures of VWF have been characterized and shown to be highly complex and heterogeneous in nature. The most common N-linked structure is a monosialylated biantennary complex. In contrast, the majority of the O-linked glycans of VWF are composed of the tumor-associated T-antigen. Thus, both the N- and O-linked VWF glycans are commonly capped by terminal negatively charged sialic acid moieties. Unusually, covalently linked ABO(H) blood group carbohydrate determinants have also been described as terminal sugar residues on a proportion of both the N-linked (13%) and O-linked (1%) glycans of VWF.

Plasma VWF multimer composition is a critical determinant of functional activity. High–molecular weight multimers bind both subendothelial collagen and platelet glycoprotein Ibα, with significantly higher affinities than lower–molecular weight forms.

Interestingly, the O-linked glycans of VWF have recently been reported to modulate the critical interaction with glycoprotein Ibα. In normal plasma, the multimeric composition of circulating VWF is tightly controlled by a disintegrin and metalloproteinase with thrombospondin type repeat 13 (ADAMTS13). The physiologic importance of regulating VWF multimer composition is highlighted in type 2A VWD disease and thrombotic thrombocytopenic purpura. In type 2A VWD, increased proteolysis is associated with concomitant loss of High–molecular weight multimers and a bleeding phenotype. Conversely, inherited or acquired ADAMTS13 deficiency or dysfunction results in thrombotic thrombocytopenic purpura. However, type 2A VWD, increased proteolysis is associated with concomitant loss of High–molecular weight multimers and a bleeding phenotype.
however, recent data from our laboratory have shown that depletion of terminal α-2-6 linked sialic acid from the N-linked glycans of VWF specifically inhibits susceptibility to ADAMTS13 proteolysis.32 Furthermore, terminal expression of ABO(H) blood group determinants on VWF glycans has also been shown to influence VWF permissiveness to ADAMTS13-mediated proteolysis (in the order O > B > A > AB).33,34 Site-directed mutagenesis studies have suggested that glycan expression at Asn-1574 in the VWF A2 domain adjacent to the ADAMTS13 cleavage site may be of particular importance in this context.35 Mutation of this Asn residue with subsequent elimination of the associated glycan chain resulted in markedly enhanced susceptibility of VWF to ADAMTS13 proteolysis. In contrast, mutation of neighboring glycan Asn-1515 had no such effect. On the basis of these findings, therefore, it is clear that variations in carbohydrate expression profiles can critically regulate plasma glycoprotein susceptibility to proteolysis, and thereby influence normal physiology. Moreover, these alterations in carbohydrate structure may involve only subtle changes in terminal glycan determinant expression. In addition, glycan variation at particular N-linked sites may also be of specific importance in this regard.

Role of glycans in modulating biological activity

Previous in vitro studies have demonstrated that modification of carbohydrate determinants on plasma glycoproteins, by either exoglycosidase digestion or site-directed mutagenesis, can significantly influence key aspects of biological function. It is important to note, however, that even in normal individuals, some plasma glycoproteins naturally circulate as different glycoforms (Table 1). Although these protein isoforms contain identical amino acid compositions, they differ with respect to the number and/or type of their attached glycan structures. As a consequence of altered glycosylation profiles, individual glycoforms may demonstrate clinically relevant differences in their functional properties. Within the coagulation cascade, there are several notable examples of naturally occurring, partially glycosylated plasma glycoforms that exhibit differential functional properties compared with their fully glycosylated counterparts.

Coagulation is initiated in vivo by the exposure of tissue factor (TF) on extravascular cells on vascular injury, which then interacts with activated factor VII (FVIIa) to activate factor X (FX). TF has N-linked glycosylation consensus sequences at 3 positions (Asn-11, Asn-124, and Asn-137); however, their contribution to TF procoagulant activity is subject to debate. TF possesses different glycan structures depending on whether it was derived from human placenta or generated via recombinant expression in bacterial or insect cells.36-38 Deglycosylation of placenta-derived TF resulted in a significant (fourfold) reduction in catalytic rate (kcat) for extrinsic FXase activity, indicating an important role for N-linked glycosylation in modulating TF procoagulant function.38 In contrast, previous studies have reported that recombinant TF expressed in Escherichia coli possessed similar procoagulant activity to that expressed in mammalian cells.39 In addition, recent studies have shown that recombinant TF mutants lacking specific individual N-linked glycan consensus sequences also exhibit functional activity similar to that of wild-type TF.40

Human coagulation FX is activated in vivo by FIXa and FVIIa in the presence of cofactors FVIIa and TF, respectively. FX possesses 2 N-linked (Asn-39 and Asn-49) and 2 O-linked (Thr-17 and Thr-29) glycosylation sites, all of which are contained within the activation peptide of the zymogen protein. Various reports have suggested that FX carbohydrate moieties can modulate FX activation.41-43 Mutagenesis of FX N- and O-linked glycan attachment sites significantly increased FX activation by FVIIa or FIXa but exhibited a limited effect on the catalytic efficiency of either the intrinsic (FIXa/FVIIa) or extrinsic (TF/FVIIa) FXase complexes.42 Enzymatic desialylation of FX attenuates the rate of activation by either the intrinsic or extrinsic FXase complex, implying an important role for terminal sialic acids in enhancing FXase complex formation.43 Further to their role in FX activation, N-linked (but not O-linked) glycans on the FX activation peptide have been proposed to protect FX from rapid clearance via glycan-dependent interactions with macrophages in mice, accounting for its prolonged plasma half-life in comparison with related vitamin K-dependent zymogens.44-45

The serine proteinase inhibitor antithrombin constitutes the major plasma inhibitor of thrombin and circulates as a single-chain glycoprotein that possesses 4 N-linked glycosylation sites at Asn-96, Asn-135, Asn-155, and Asn-192, respectively. These glycan structures exist predominantly in the form of disialylated biantennary complex chains.46 Two different plasma glycoforms of antithrombin (α- and β-antithrombin) have been described.47 Fully glycosylated α-antithrombin accounts for the majority of total plasma antithrombin. In addition, a minor glycoform (β-antithrombin) contributes 10% to 15% of total plasma antithrombin. This glycoform is identical to α-antithrombin but for the absence of any N-linked oligosaccharide expression at Asn-135.48 The loss of this specific glycan chain results in markedly enhanced protease inhibitor activity.49,50 As a consequence, despite representing only a small minority of plasma antithrombin, β-antithrombin has been suggested to be the principal mediator of antithrombin protease inhibitor activity in vivo.50 Kinetic and crystallographic analyses of the molecular basis underlying the enhanced activity of β-antithrombin have demonstrated that the presence of the oligosaccharide structure at Asn-135 sterically impedes a conformational change required to activate antithrombin on heparin/heparan binding.51 Thus, absence of this steric hindrance at Asn-135 in β-antithrombin enables rapid adoption of an active conformation once bound to heparin, thereby enhancing its inhibitory activity.52

Protein C (PC), similar to antithrombin, is crucial for the regulation of thrombin generation in vivo. PC circulates in zymogen form and is activated by the thrombin–thrombomodulin complex. After activation by the thrombin–thrombomodulin complex, activated PC (APC) inhibits further thrombin generation by proteolytic degradation of procoagulant-activated cofactors FVa and FVIIIa. PC possesses 4 N-linked glycosylation sequons: 1 located within its first epidermal growth factor (Asn-97) and the remaining 3 located in its protease domain (Asn-248, Asn-313, and Asn-329).53

In addition, different glycoforms of human PC have been described in normal human plasma: α-PC accounts for 70% of total plasma PC and is characterized by the presence of complex biantennary oligosaccharide chains at all 4 N-linked glycosylation sites, β-PC accounts for approximately 25% of total plasma PC and differs from α-PC in that it is not glycosylated at Asn-329,53 and γ-PC represents only 5% of total plasma PC and lacks oligosaccharide chains attached at both Asn-729 and Asn-248. Several lines of evidence support the hypothesis that these different glycoforms of PC have important biological differences. For example, site-directed mutagenesis studies have suggested that PC activation by the
Table 1. Coagulation glycoproteins—carbohydrate composition and biological relevance

<table>
<thead>
<tr>
<th>Hemostatic glycoprotein</th>
<th>N-linked sites</th>
<th>O-linked sites</th>
<th>Functional significance of glycan structures</th>
<th>Physiological and pathological glycoforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>4</td>
<td>0</td>
<td>Promotes fibrinogen solubility</td>
<td>Pathological fibrinogens</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lima, Caracas, Niigata, Pontose, Asahi, and Kaiserslautern</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>3</td>
<td>0</td>
<td>None described</td>
<td>None described</td>
</tr>
<tr>
<td>TF</td>
<td>3</td>
<td>ND</td>
<td>Glycans modulate procoagulant activity</td>
<td>None described</td>
</tr>
<tr>
<td>Factor V</td>
<td>26</td>
<td>ND</td>
<td>Glycans modulate intracellular trafficking</td>
<td>Physiological: FVα and FVα2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from ER to Golgi. N-linked glycans also influence FVα phospholipid binding affinity and FVα susceptibility to APC mediated proteolysis</td>
<td></td>
</tr>
<tr>
<td>Factor VII</td>
<td>2</td>
<td>2</td>
<td>Influence hepatic clearance and plasma half-life of recombinant FVIIa. Loss of O-linked glycans impairs procoagulant activity of FVIIa in plasma.</td>
<td>None described</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>24</td>
<td>7</td>
<td>Glycans influence FVIII folding and intracellular trafficking during biosynthesis. N-linked glycans also regulate FVIII uptake by dendritic cells and clearance by the ASGPR.</td>
<td>Pathological factor VIII (Met1772Thr) and (Ile566Thr)</td>
</tr>
<tr>
<td>Factor IX</td>
<td>2</td>
<td>5</td>
<td>None described</td>
<td>Pathological factor IX (Arg94Ser)</td>
</tr>
<tr>
<td>Factor X</td>
<td>2</td>
<td>2</td>
<td>Both N- and O-linked glycans are negative modulators of FX zymogen activation. N-linked glycans also regulate FX clearance.</td>
<td>None described</td>
</tr>
<tr>
<td>Factor XI</td>
<td>5</td>
<td>ND</td>
<td>None described</td>
<td>None described</td>
</tr>
<tr>
<td>Factor XII</td>
<td>2</td>
<td>7</td>
<td>None described</td>
<td>None described</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>3</td>
<td>ND</td>
<td>None described</td>
<td>None described</td>
</tr>
<tr>
<td>VWF</td>
<td>12</td>
<td>10</td>
<td>N-linked and O-linked glycans influence VWF synthesis, secretion, and biological activity. Glycan expression also regulates susceptibility to ADAMTS13 proteolysis and is a critical determinant of VWF clearance.</td>
<td>Physiological: ABO blood group-specific glycoforms</td>
</tr>
<tr>
<td>PC</td>
<td>4</td>
<td>0</td>
<td>Modulate PC zymogen activation by thrombin thrombomodulin complex. Glysals also influence the anticoagulant and antiinflammatory properties of APC.</td>
<td>Physiological: α-protein C, β-protein C, γ-protein C</td>
</tr>
<tr>
<td>Protein S</td>
<td>3</td>
<td>ND</td>
<td>None described</td>
<td>Pathological protein S Heerlen (Ser460Pro)</td>
</tr>
<tr>
<td>Antithrombin</td>
<td>4</td>
<td>0</td>
<td>Glycans influence conformational changes in antithrombin after heparin binding, and thereby regulate serpin activity.</td>
<td>Physiological: α-antithrombin and β-antithrombin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathological: antithrombin (Ile7Aaa), antithrombin (Ser72Asn)</td>
</tr>
</tbody>
</table>

ND, not determined.

Thrombin–thrombomodulin complex is modulated by the presence of N-linked oligosaccharides at Asn-313. APC anticoagulant activity may also be subject to modulation by its glycan structures, but reports on its importance have been conflicting. Specifically, a naturally occurring PC mutation encoding only β-PC (N329T) exhibited mildly reduced anticoagulant activity when purified from plasma, activated, and assayed for its ability to degrade FVα.

In contrast, a recombinant version of β-APC in which glycosylation at Asn-329 was eliminated exhibited approximately twofold increased anticoagulant activity compared with wild-type recombinant APC. In addition to its anticoagulant role, APC also possesses potent anti-inflammatory and antiapoptotic activity that is mediated at least in part by activation of protease-activated receptor 1 (PAR1).

We have recently demonstrated that a recombinant APC mutant that mimics the glycosylation pattern of β-APC (APC-N329Q) exhibits an increased capacity to maintain endothelial cell barrier integrity and inhibit endothelial cell apoptosis compared with wild-type APC. Interestingly, Asn-329 is located proximal to a putative PAR-1 binding exosite on the surface of the APC protease domain, and recent work has indicated that elimination of the oligosaccharide chain at this position accelerates the rate of PAR1 cleavage by APC, possibly by facilitating increased PAR1 access to the binding...
exo site on APC (E. M. Gleeson, J. S. O’Donnell, and R. J. Preston, unpublished data). On the basis of these findings, therefore, it is clear that the different APC glycoforms present in normal human plasma exhibit important differences in their biological activities that are likely to be of physiological and pathological relevance.

Partial N-linked glycosylation resulting in the synthesis of heterogeneous glycoforms with distinct biological properties has also been reported for a number of important procoagulant plasma glycoproteins. Human FV is abundantly glycosylated, with both N- and O-linked carbohydrate structures accounting for 15% to 25% of the total molecular mass. FV is activated by limited specific proteolysis by either thrombin or FXa and then serves as a critical cofactor in the prothrombinase complex. Subsequently, FVa is inactivated by APC-catalyzed proteolysis at Arg-306 and Arg-506. Inactivation of FVa by APC plays a critical role in down-regulating thrombin formation. Two different glycoforms of FV are present in the normal human circulation. As a consequence, activation by thrombin results in the generation of 2 distinct forms of FV (FVα and FVα2) that differ only with respect to their glycosylation profiles. Site-directed mutagenesis studies have established that unlike FVa1, the FVa2 glycoform appears to result from partial glycosylation at Asn-2181 in the C-terminal C2 domain. Importantly, several reports have shown that this variation in the N-linked glycan component of FVa significantly modulates its functional properties. For example, the affinity of the human FVa2 glycoform binding to anionic phospholipids was approximately threefold higher than that of FVa3. Moreover, FVa1 and FVa2 also displayed differential susceptibilities to APC-mediated proteolysis. In particular, at low phospholipid concentrations, FVa3 was inactivated at a 15-fold slower rate compared with FVa2. These distinct biological differences serve as a further example of how the relative concentrations of naturally occurring coagulation protein glycoforms have the potential to markedly influence overall thrombin generation at sites of vascular injury.

Role of glycans in modulating clearance

N- and O-linked carbohydrate structures play major roles in determining the rate of clearance of many human glycoproteins from plasma. Terminal sialic acids are of critical importance in this regard. The removal of capping sialic acid residues leading to exposure of penultimate Gal and GalNAc moieties typically results in markedly enhanced glycoprotein clearance. In mammals, desialylation is achieved by a family of 4 sialidases (also known as neuraminidases; Neu1-Neu4) that catalytically remove α-glycosidase-linked sialic acid groups from carbohydrate structures. This cleavage is mediated primarily via the hepatic lectin asialoglycoprotein receptor (ASGPR or Ashwell receptor). A member of the calcium-dependent (C-type) lectin receptor family abundantly expressed in the liver, ASGPR is composed of 2 homologous trans-membrane polypeptides (Asgr-1 and Asgr-2) that assemble into a hetero-oligomer on the cell surface. The C-terminal extracellular domains of Asgr-1 and Asgr-2 form a carbohydrate recognition domain that selectively binds glycoproteins expressing either β-3-galactose (βGal) or N-acetyl-d-galactosamine (GalNAc) terminal sugar determinants in a calcium-dependent manner. However, these βGal and GalNAc residues are more typically expressed on plasma glycoproteins as subterminal moieties on oligosaccharide chains capped by sialic acid. If the terminal sialic acid residue is lost, the ASGPR can bind the exposed βGal or GalNAc and mediate endocytosis.

The critical importance of sialic acid expression in determining plasma half-life has been observed for several different coagulation glycoproteins. Enzymatic removal of terminal sialic acid residues from the abundantly sialylated VW F ex vivo markedly reduces plasma half-life in rabbits (240 vs 5 minutes for normal and desialylated VW F, respectively). In keeping with this observation, genetic inactivation of a specific sialyltransferase (ST3Gal-IV) in a transgenic mouse also resulted in significantly reduced plasma VW F levels as a consequence of a twofold increased rate of clearance. The importance of the ASGPR in modulating physiological VW F clearance is further underlined by recent data demonstrating that VW F half-life is significantly increased in ASGPR-1 knockout mice.

In addition to its role in regulating VW F plasma clearance, the ASGPR may also modulate the clearance of a number of other coagulation glycoproteins, including FVIII. As previously described, FVIII is heavily glycosylated, and the N-linked glycans of human FVIII are commonly capped by negatively charged sialic acid residues. Surface plasmon resonance studies have demonstrated that FVIII also binds the ASGPR with high affinity (KD ≈ 2 nM). This interaction is mediated through the N-linked carbohydrate structures clustered within the B domain of FVIII. Furthermore, administration of an ASGPR antagonist significantly inhibited FVIII clearance in mice, suggesting that the ASGPR may contribute to normal physiological clearance of FVIII from plasma.

Similar to sialic acid, ABO(H) blood group determinants are also expressed as terminal sugar residues on the carbohydrate structures of both VW F and FVIII. This ABO(H) expression has direct clinical relevance, as ABO blood group is major determinant of plasma VW F levels. Group O individuals have 25% less circulating VW F compared with non-O individuals (group A, B, or AB). Moreover, plasma VW F levels are even lower in individuals with the rare Bombay blood group phenotype, in which H antigens are not expressed. The effect of ABO(H) blood group antigens on VW F levels is explained by differences in clearance rates between each blood group. As such, the VW F plasma half-life is significantly shorter in normal group O vs non-O individuals (10.0 vs 25.5 hours, respectively). Nevertheless, the molecular mechanism underlying the enhanced clearance of group O VW F remains unknown. However, given that the ASGPR selectively binds either GalNAc or Gal residues, it seems likely that another clearance receptor is responsible for modulating this phenomenon. A weak effect of the Secretor blood group locus on plasma VW F levels has also been reported. Interestingly, this blood group system is similar to ABO, in that it is characterized by the presence or absence of specific terminal carbohydrate determinants on oligosaccharide structures. To date, it remains unclear whether this Secretor influence is also modulated through an effect on VW F clearance.

In addition to the ASGPR, a variety of other lectin receptors has been characterized. These lectins typically contain a carbohydrate recognition domain that has binding specificity for particular terminal glycans moieties expressed on N- and/or O-linked carbohydrate structures. Examples of other lectins that have been implicated in modulating glycoprotein clearance include Mac-1 (a2mβ3), the macrophage galactose lectin, and the scavenger receptor C-type lectin. The relative contribution of these individual receptors in mediating clearance of individual plasma coagulation glycoproteins has not yet been defined. However, recent data have demonstrated that macrophage-mediated endocytosis may be important in the physiological clearance of both VW F and FVIII. Furthermore, data from our laboratory have shown that the rate of VW F clearance by
macrofages is markedly influenced by VWF glycan expression. Recent data have also demonstrated that gaelcisin 1, gaelcisin 3, and siglec 5 can also all bind to human VWF. In addition, other putative lectin-like receptors that may be involved in determining plasma levels of the VWF-FVIII complex have been identified through genome-wide association studies and include C-type lectin domain family 4 member M and stabulin. Thus, although the molecular mechanisms responsible for modulating the clearance of glycoproteins from plasma remain poorly understood, carbohydrate expression is of critical importance in regulating the rate of clearance.

Aberrant glycosylation can cause human disease

Although rare, almost 50 different congenital disorders of glycosylation have been identified. These disorders typically involve defects in N-linked glycosylation and are associated with severe multigorgan clinical phenotypes including skeletal and neurological abnormalities. Significant coagulopathies have also been observed in children with congenital disorders of glycosylation. In particular, factor XI, PC, antithrombin, and protein S are commonly deficient. The molecular mechanism or mechanisms responsible for the reduced plasma levels of these specific coagulation glycoproteins remains unclear. Nevertheless, significant thrombotic and bleeding complications are well recognized as constituting important clinical features of these conditions.

Aberrant glycosylation of specific proteins, including coagulation factors, has also been implicated in the etiology of human pathology. Point mutations that result in the introduction of novel N-linked glycosylation sites are of particular importance. For example, the amino acid substitution Ile359Thr within the heavy chain of FV (FV Liverpool) creates a new N-linked glycosylation consensus sequence, such that an additional glycan chain is expressed at Asn-357. As a result, the FVα-Ile359Thr molecule is resistant to APC-mediated proteolysis, and consequently, FV Liverpool is associated with a prothrombotic phenotype. A number of different amino acid substitutions that introduce additional N-linked glycosylation sites have also been described in patients with congenital dysfibrinogenemia. These include fibrinogens Lima (Aa Arg141Ser), Caracas II (Aa Ser434Asn), Ashai (γ Met310Thr), and Kaiserslauten (γ Lys380Asn). In each of these cases, the attachment of an extra N-linked glycan causes impaired functional activity and a consequent bleeding tendency. Similarly, an FIX gene mutation that results in an extra glycosylation site has been identified in a family with hemophilia B. Interestingly, the Arg94Ser substitution actually leads to the introduction of a new O-linked glycosylation site in the second epidermal growth factor-like domain of FIX, which in turn markedly attenuates activation by FXIa.

In contrast, mutations leading to the loss of a single specific N-linked glycosylation site have also been implicated in disease pathology. Protein S is a plasma glycoprotein that is important in regulating thrombin generation in vivo. First, protein S functions as a nonenzymatic cofactor for APC inactivation of FVa and FVIIa. In addition, protein S may also regulate hemostasis by APC-independent inhibition mechanisms. Protein S Heerlen is found in approximately 0.5% of the population and is characterized by a Ser to Pro substitution at position 460. This change results in the loss of N-linked glycosylation at Asn-458 and has been associated with an increased risk for venous thromboembolism. The clinical phenotype relates in part to the fact that the Ser460Pro substitution results in reduced plasma protein S levels because of an enhanced clearance. In addition, protein S Heerlen demonstrates reduced cofactor activity for APC-inactivation of FVIIa.

Glycan modification: therapeutic implications and opportunities

As summarized in this review, carbohydrate structures on human coagulation proteins play essential roles in determining stability, circulatory half-life, and biological activity. As a consequence, in the production of biopharmaceuticals, glycosylation is of critical importance. In particular, for the synthesis of recombinant glycoprotein therapeutics, it is well established that glycosylation profiles can vary significantly, depending on the cell line chosen for expression. Moreover, recombinant proteins generated in vitro can also demonstrate significant heterogeneity in terms of their glycan profiles. This obviously has major implications, given that many studies of coagulation protein structure and function have used recombinant proteins that may express carbohydrate determinants that differ markedly to those expressed on the native human proteins. Unsurprisingly, these glycan variations also can have important therapeutic sequelae. For example, recombinant FVIII (rFVIII; NovoSeven) used for the treatment of patients with hemophilia with inhibitors contains 2 N-linked and 2 O-linked glycans and is expressed in baby hamster kidney (BHK) cells. Although all 4 sites are glycosylated in the purified rFVIII molecule, approximately 10% of rFVIII molecules from BHK cells have N-linked glycans lacking terminal sialylation. Moreover, a further 30% of the rFVIII possesses significantly reduced N-linked sialic acid expression. This variable sialylation has important consequences in determining the plasma half-life of therapeutic rFVIII, as hyposialylated rFVIII is rapidly cleared from the circulation through the hepatic ASGPR.

Patients with hemophilia A can be treated using either plasma-derived or recombinant FVIII products. Unsurprisingly, glycan expression differences significantly between plasma-derived and recombinant FVIII. Moreover, glycosylation variation has also been described between different commercial recombinant FVIII products that have been synthesized in different mammalian cell lines (including Chinese hamster ovary and BHK). For example, rFVIII from Chinese hamster ovary cells express the NeuGc epitope, which accounts for 0.5% of total sialic acid. In contrast, Gal-α(1,3)Gal structures have been identified on ~3% of BHK-expressed rFVIII. Importantly, high levels of antibodies against NeuGc and Gal-α(1,3)Gal both occur naturally in most humans. Interestingly, recent studies have also demonstrated that specific glycan chains on FVIII may influence dendritic cell uptake mediated through the macrophage mannose receptor (CD206). Thus, removal of the mannosylated sugars at Asn-239 (A1 domain) or Asn-2118 (C1 domain) abrogated dendritic cell endocytosis of FVIII and presentation to CD4 T-cells. Importantly, these data raise the possibility that variations in glycan expression on recombinant FVIII products may influence immunogenicity and, consequently, risk for inhibitor development in patients with hemophilia.

As our understanding of the critical role played by glycan structures in regulating the biological activity and half-life of plasma glycoproteins continues to further develop, it seems likely that significant opportunities for the development of novel therapeutic
agents will arise. In particular, glycoengineering involving targeted selective carbohydrate modification may enable the development of recombinant glycoprotein therapies with improved clinical efficacy. From the data presented in this review, the wide spectrum of coagulation factor properties that can be influenced by protein glycosylation are readily apparent. Manipulation of carbohydrate structures may be useful in prolonging half-life of recombinant clotting factor concentrates; for example, through hyper-sialylation. Alternatively, glycoengineering may be useful in reducing the immunogenicity of recombinant therapeutic glycoproteins. Clearly, even minor glycan modification of terminal sugar moieties, or indeed the loss or introduction of a specific glycosylation site, may be enough to develop a glycoform with enhanced therapeutic properties.

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The endothelial cell protein C receptor: cell surface conductor of cytoprotective coagulation factor signaling

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Abstract Increasing evidence links blood coagulation proteins with the regulation of acute and chronic inflammatory disease. Of particular interest are vitamin K-dependent proteases, which are generated as a hemostatic response to vascular injury, but can also initiate signal transduction via interactions with vascular receptors. The endothelial cell protein C receptor (EPCR) is a multi-ligand vitamin K-dependent protein receptor for zymogen and activated forms of plasma protein C and factor VII. Although the physiological role of the EPCR-FVII(a) interaction is not well-understood, protein C binding to EPCR facilitates rapid generation of APC in response to excessive thrombin generation, and is a central requirement for the multiple signal-transduction cascades initiated by APC on both vascular endothelial and innate immune cells. Exciting recent studies have highlighted the emerging role of EPCR in modulating the cytoprotective properties of APC in a number of diverse inflammatory disorders. In this review, we describe the structure-function relationships, signal transduction pathways, and cellular interactions that enable EPCR to modulate the anticoagulant and anti-inflammatory properties of its vitamin K-dependent protein ligands, and examine the relevance of EPCR to both thrombotic and inflammation-associated disease.

Keywords Endothelial cell protein C receptor • Coagulation proteases • Protein C • Factor VII • Sepsis

Background In addition to their well-established hemostatic functions, vitamin K-dependent (VKD) coagulation proteins can also modulate immune response upon infection or injury [1]. These immuno-regulatory effects require initiation of intracellular signaling networks in vascular and non-vascular cells. To trigger signaling, VKD coagulation proteases must first interact with extracellular receptors. In some instances, this interaction alone is sufficient to trigger signal transduction [2]. More commonly, however, extracellular receptor interaction positions VKD proteases such that protease-sensitive receptor activation and intracellular signal transduction can occur. These 'effector' receptors constitute a family of cleavage-sensitive G-protein coupled receptors (GPCRs) called protease-activated receptors (PARs) [3, 4]. PAR activation commonly occurs upon presentation of the VKD protease by a proximal coagulation protein co-receptor [5–8]. The endothelial cell protein C receptor (EPCR) fulfils this role for several VKD coagulation factors. VKD protein interaction with EPCR does not, however, serve solely to localize VKD proteases for PAR activation. Rather, EPCR binding can directly influence the subsequent nature of downstream PAR-activated signaling outcomes [9]. EPCR-dependent signaling by VKD proteins is implicated in a myriad of 'cytoprotective' responses to injury or infection. This review highlights recent advances in our understanding of EPCR physiological function, biochemistry and expression, and further explores the central position of EPCR in the regulation of coagulation and immune cell function.
EPCR expression, structure, and post-translational modification

EPCR was initially characterized by Esmon and colleagues as a high-affinity endothelial cell surface receptor for zymogen and activated protein C [10]. Although originally identified as an endothelial cell receptor, EPCR has since been detected in a number of cell types, including vascular smooth muscle cells [11], eosinophils [12], neutrophils [13], monocytes [14], keratinocytes [15], hippocampal neurons [16], cardiomyocytes [17], and placental trophoblasts [18]. EPCR was recently shown to be expressed on the surface of bone marrow [19] and fetal liver embryonic hematopoietic stem cells (HSCs) [20]. EPCR+ HSCs exhibit potent hematopoietic reconstitution activity and demonstrate durable and equivalent differentiation for all blood lineages [19]. Consequently, EPCR expression is now an established HSC phenotypic marker.

The human EPCR gene (PROCR) is located on chromosome 20q11.2, spanning approximately 8 kilobases (kb) [21]. PROCR, consisting of four exons and three introns, encodes a protein of 238 amino acids [10, 21] to yield a mature protein of approximately 46 kDa [22]. Exon 1 encodes amino acid residues 1–24, which comprise the 5'-untranslated region and signal peptide. Exons 2 and 3 encode amino acids 24–108 and 108–231, respectively, which constitute the majority of the extracellular region. Exon 4 encodes amino acids 201–238, which includes the remaining extracellular domain, the transmembrane region (comprising 21 amino acid residues; 211–231), a short cytoplasmic tail (amino acid residues 232–238), and 3'-untranslated region [21]. Two major transcription initiation sites have been identified and are located 79 and 82 bp upstream of the transcription start site [23]. PROCR expression is tightly regulated by multiple Sp1 transcription sites contained within the 5' flanking region [24].

EPCR shares both sequence and three-dimensional structural homology with the major histocompatibility class I (MHC)/CD1 family of proteins, in particular murine CD1d [10]. The EPCR z1 and z2 domains consist of two antiparallel z-helices that sit upon an eight-stranded z-sheet platform. An MHC-like hydrophobic lipid-filled groove is formed between the two helices [25] (Fig. 1). Unlike MHC class I/CD1 receptors, however, EPCR lacks the z-3 domain and is therefore unlikely to associate with z-2 microglobulin.

EPCR undergoes significant post-translational modification prior to extracellular expression. EPCR contains four putative N-linked glycan attachment sites (Asn-30, Asn-47, Asn-119, and Asn-155), although the precise function of each individual glycan chain is unknown. Recombinant soluble EPCR variants in which each individual N-linked glycan attachment site was deleted by site-directed mutagenesis did not exhibit reduced ligand binding [26]. EPCR contains an intracellular C-terminal unpaired cysteine (Cys-221), which constitutes a putative palmitoylation site [10]. EPCR palmitoylation may contribute to the membrane localization and intracellular trafficking of EPCR, although a precise definition of its contribution to EPCR function is unresolved.

EPCR is a multi-ligand coagulation protein receptor

Zymogen protein C and activated protein C (APC) bind EPCR with comparable affinity [10, 22]. Both share an identical EPCR-binding motif, which is located within the N-terminal γ-carboxyglutamic acid rich (Gla) domain [27]. Resolution of the crystal structure of extracellular soluble EPCR (sEPCR) bound to a protein C Gla domain fragment identified a number of protein C amino acid residues that are crucial for EPCR interaction [25]. This interactive surface is
surprisingly small: Phe-4 and Leu-8 located in the conserved 'ω-loop' of the protein C/ APC Gla domain mediate hydrophobic interactions with Tyr-154 and Thr-157 at the distal end of EPCR x1 and x2 chains. Additional hydrogen bonding between protein C Gla residues Gla-7, Gla-27, and Gla-29 and EPCR residues Glu-86, Arg-87, and Gln-150 were also identified. Site-directed mutagenesis studies have also confirmed that additional EPCR amino acids proximal to the site of ligand binding may also contribute to protein C-EPCR interaction [26]. The amino acid residues of the protein C/ APC Gla domain involved in modulating EPCR binding are highly conserved across other VKD proteins, and are completely identical in factor X and its activated form, activated factor VII (FVIIa) [28]. Recent studies have confirmed that FVII(a) constitutes an additional ligand for both recombinant soluble [28] and cell-bound EPCR [29, 30]. Activated factor X (Fxa) has also been reported to require EPCR to initiate PAR-dependent signaling [7], although the affinity of human Fxa for EPCR in comparison to APC and FVIIa is unknown.

In addition to differences between the ability of individual VKD proteins to bind to EPCR, sequence and functional binding analyses suggest species-specific EPCR binding. Rat APC, for example, possesses a valine at position 8, which has been shown to be incompatible with EPCR binding in the human APC Gla domain [28]. Interestingly, the murine APC-EPCR binding motif differs slightly to that of human PC/ APC/ FVII(a) in that Leu-8 is replaced by methionine at this position. This EPCR binding motif is also present in human FX, which interacts with murine [31], but not human EPCR [32]. On this basis, the presence of Met at position 8 in the murine protein C ω-loop would preclude binding to human EPCR. The specific requirements for EPCR ligand interaction are, therefore, incompletely understood. Due to the almost complete conservation of the VKD protein binding region in EPCR from species characterized thus far [33], amino acid residues proximal to the VKD protein binding site must ultimately contribute to the affinity of EPCR-ligand interactions [26]. For example, in the crystal structure of sEPCR bound to the protein C ω-loop, direct interactions between Leu-8 of protein C with Leu-86 of sEPCR were observed [25]. Leu-86 is conserved in murine EPCR, but is surrounded by multiple amino acid residues which are not shared between human and murine EPCR, including a 2-amino acid insertion in the murine EPCR sequence at positions 88 and 89 [26, 33]. Consequently, this subtle alteration may provide an optimal binding surface for Met-8 (rather than Leu-8)-containing VKD protein ω-loops and thereby enable interaction with murine protein C/ APC and non-murine VKD proteins that possess a Met-8 ω-loop motif, such as human FX. The specificity of the VKD protein ω-loop binding pocket is further highlighted by the failure of murine FVII to bind either human or murine sEPCR [32]. Murine FVII possesses a Leu at position 4 of its ω-loop, whereas Phe is present in all other VKD protein EPCR ligands. This indicates that substitution of Phe-4 is not compatible with EPCR binding, irrespective of species origin.

EPCR has been reported to also bind non-VKD protein ligands, although the functional significance of these interactions is not fully understood. A phosphatidyethanolamine moiety bound with high affinity to the central groove of the EPCR molecule was identified upon sEPCR crystallization [25]. Interestingly, the EPCR homolog CD1d utilizes the same groove to bind lipid antigens and present them to natural killer T (NKT) cells, resulting in their activation [34]. However, no role for EPCR in lipid presentation to NKT cells has been ascribed to date. The bound lipid may contribute to EPCR structural integrity, as detergent removal of lipid from EPCR resulted in significant loss of protein C/ APC binding affinity [25]. Cell surface and recombinant sEPCR also bind to the neutrophil serine protease proteinase-3 (PR3) [35], which is secreted from neutrophils upon activation or expressed on the activated neutrophil cell surface. Optimal sEPCR binding to PR3 on PMA-activated neutrophils requires Mac-1, and may presage cell-bound EPCR cleavage by PR3 [36].

**Regulation of coagulation by EPCR–ligand interactions**

Protein C-EPCR complex formation on endothelial cells accelerates APC generation by the thrombin–thrombomodulin complex, and thus enhances the anticoagulant response upon thrombin generation [37]. EPCR binding results in approximately a fourfold reduction in kₐ for protein C activation by thrombin–thrombomodulin complex on endothelial cells [37, 38]. The molecular basis of EPCR-dependent increased protein C activation is unknown, but current evidence suggests that EPCR positions protein C for optimal thrombin cleavage of the protein C activation peptide. Surprisingly, no direct interaction between thrombomodulin and EPCR has been described, and their requisite proximity on the surface of endothelial cells may be a consequence of co-localization in caveolin-rich lipid microdomains [39]. In baboons, co-infusion of thrombin with an anti-EPCR monoclonal antibody reduced APC generation 20-fold [40], suggesting the role of EPCR in protein C activation in vivo may be more prominent than is observed using cultured endothelial cells. Normal protein C activation upon thrombin challenge was observed upon EPCR⁻/⁻ mouse bone marrow transplantation into EPCR⁻/⁻ mice, demonstrating that non-hematopoietic cell (presumably endothelial) EPCR is the main site of protein C activation in vivo [41].

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In contrast to protein C-EPCR binding, the physiological significance of FVIIa and/or FXa interactions with EPCR are less well understood. EPCR has been shown to attenuate FXa generation by the FVIIa-TF complex [30, 31] via EPCR interactions with FVIIa or FX. FXII activation by FXa is also reduced by the presence of EPCR, which is presumably achieved by reduction in FVII/FXa interaction with cell-surface phospholipids upon EPCR complex formation [42]. Additionally, FVII binding to EPCR may have an indirect procoagulant role [29, 30]. The apparent affinity of FVII for EPCR suggests it is likely to remain bound to EPCR in vivo [28]. However, given the normal FVII plasma concentration (10nM) compared to protein C (70nM) it is unlikely to represent an effective inhibitor of protein C activation. Nonetheless, administration of pharmacological doses of recombinant FVIIa may serve to inhibit APC generation on endothelial cells [29, 30].

The importance of EPCR in the regulation of coagulation in vivo has been characterized using transgenic mice. Mice completely deficient in EPCR (EPCR<sup>−/−</sup>) die in utero by embryonic day 10. In contrast, mice with heterozygous EPCR deficiency (EPCR<sup>+/−</sup>) survive to birth and are phenotypically similar to EPCR<sup>++</sup> mice [43]. Absence of EPCR expression on giant trophoblast cells, rather than embryonic cells, is responsible for the early embryonic loss associated with EPCR<sup>−/−</sup> mice [44]. However, the role played by EPCR in regulating hemostasis at the fetomaternal interface is not fully understood. Transgenic mice expressing approximately 1% of normal EPCR expression (EPCR<sub>LOW</sub>) are viable and exhibit no thrombotic tendency, indicative of a requirement for only limited EPCR expression for embryonic viability [45, 46]. Conversely, a transgenic mouse line (EPCR<sub>HIGH</sub>) with at least eightfold increased EPCR expression, exhibits higher circulating APC levels and as a result, rapidly reduced thrombin generation and fibrin deposition upon procoagulant challenge with FXa and phospholipid vesicle infusion [47].

**Soluble EPCR**

sEPCR, comprising the EPCR extracellular domain, is detectable in plasma [48]. sEPCR retains the ability to bind protein C/APC with similar affinity to membrane-bound EPCR [49], and is released from the endothelial surface by metalloproteinase (tumor necrosis factor-α converting enzyme (TACE)/ADAM17)-dependent shedding [50, 51]. sEPCR plasma concentration is bimodally distributed in the general population, with significantly elevated levels in approximately 10% of adults and 20% of children [52]. Unlike the membrane bound receptor, sEPCR acts to inhibit APC anticoagulant function. First, sEPCR competes with membrane-bound EPCR to bind zymogen protein C and thereby inhibits APC generation. Second, sEPCR also blocks the interaction of APC with cell surface phospholipids [49, 53], preventing membrane complex formation with cofactor protein S and substrate activated factor V. Pro-inflammatory cytokines IL-1β and TNF-α, phorbol esters and thrombin enhance EPCR shedding from endothelial cell surfaces via activation of MAP kinase signaling pathways [54, 55]. Accordingly, higher levels of sEPCR have been reported in patients with systemic inflammatory diseases and conditions associated with increased thrombin production [56].

As EPCR is crucial for effective APC generation, genetic aberrations leading to diminished membrane EPCR expression or function may be expected to contribute to an increased risk of thrombosis. A 23-bp insertion in PROC1 was identified in individuals with early myocardial infarction and deep vein thrombosis. In vitro expression studies showed that a severely truncated EPCR molecule with diminished function [57] is generated as a consequence of this insertion, although its broader contribution to thrombotic risk is difficult to establish due to its rarity.

Of the four defined PROC1 haplotypes, the A3 haplotype is most associated with an elevated risk of thrombosis [58, 59]. This haplotype results in elevated sEPCR levels compared to other haplotypes, and is characterized by a glycine substitution at Ser-219 in the EPCR transmembrane region [58]. This substitution has been shown to result in increased sensitivity to EPCR cell surface shedding by ADAM17 [60]. Recent evidence indicates that an additional means by which the A3 haplotype contributes to elevated sEPCR levels is via expression of an alternatively spliced truncated PROC1 mRNA [61, 62]. This alternatively spliced mRNA isoform is truncated at the 3' end of exon 3, and therefore does not encode exon 4. The expressed EPCR therefore does not contain the EPCR transmembrane and intracellular domains, but instead possesses a C-terminal 56-amino acid tail. Transfection of a vector encoding alternatively-spliced EPCR cDNA into HEK 293 cells resulted in direct EPCR secretion and no membrane retention, highlighting the mechanism by which this EPCR mRNA transcript could lead to elevated sEPCR levels in individuals carrying the A3 haplotype [61]. Interestingly, the presence of the Ser219Gly substitution has been shown to result in significantly increased plasma levels of both protein C and factor VII in healthy middle-aged men, which is likely a consequence of reduced vascular membrane localization of these EPCR ligands [63].

**Molecular basis of EPCR-dependent signaling by coagulation proteases**

Important recent studies have suggested that APC administration may have therapeutic applications in a range of
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diverse settings, including sepsis, post-ischemic stroke, diabetic nephropathy, multiple sclerosis, cancer, inflammatory bowel disease, and neurodegenerative disease [64–70]. EPCR binding is required for the beneficial effects of APC in each of these disease models. APC binding enables PAR1 activation and APC-specific PAR1/G-protein coupling [5]. EPCR blockade prevents APC activation of PAR1 therefore EPCR is an obligate receptor for APC signaling via PAR1 on endothelial cells [5]. APC can cleave and activate PAR1, but not PAR2 on endothelial cells. However, APC has been shown to activate PAR2 on EPCR/PAR2 transfected cells, and PAR1 cleavage by APC may initiate PAR2 activation by an unknown trans-activation mechanism [5, 71, 72]. APC induces multiple cytoprotective benefits, including down-regulation of injury-induced vascular barrier permeability both in vitro [6] and in vivo [73], down-regulation of inflammatory cytokines (IL-6, TNF-α) [74, 75] and up-regulation of IL-10 [76]. Furthermore, APC-EPCR-dependent PAR1 activation attenuates apoptosis in both endothelial cells [77] and neurons [78, 79].

The cellular microenvironment is crucial for EPCR-dependent PAR1 signaling by APC. Chemical disruption of lipid rafts disables EPCR-dependent protective PAR1 signaling by APC against thrombin-induced endothelial cell barrier permeability and TNF-α-induced apoptosis in endothelial cells [39]. PAR1 recruitment with EPCR to caveolin-rich microdomains alters G-protein coupling specificity, and thus the downstream signaling response upon PAR1 activation [9]. Specifically, APC-EPCR binding preceding PAR1 proteolysis alters PAR1 activation-dependent G protein coupling from Gq/12/13 to Gi [9]. As such, caveolae formation is crucial for EPCR-dependent APC signaling via PAR1 [80]. The physical means by which EPCR associates with lipid rafts in endothelial cells is unknown, but may be linked to interaction with caveolin-1 [39]. C-terminal EPCR palmitoylation is also expected to contribute to EPCR raft localization.

Prior studies utilizing PAR1 and PAR2 reporter constructs in heterologous cell expression systems indicated that FVII(a) binding to EPCR does not facilitate PAR1 cleavage and fails to prevent thrombin-induced endothelial cell barrier permeability [29]. Furthermore, FVIIa activation of PAR1 did not take place on EPCR-expressing immortalized endothelial cells [9]. However, recent studies indicate that EPCR-bound FVIIa can activate PAR1 on primary endothelial cells (HUVECs) and results in similar downstream protective signaling to that of APC [8]. EPCR-dependent FVIIa-PAR1 signaling on endothelial cells results in endothelial barrier stabilization, MAPK phosphorylation and Rac1 activation. Furthermore, EPCR has recently been described to interact with the extrinsic tensin complex (FX-TF-FVIIa) to modulate PAR activation in this setting [31].

FXa can also elicit cytoprotective signaling via PAR1/2 cleavage that is dependent upon EPCR [7, 81]. FXa binds endothelial cells and EPCR-transfected Chinese Hamster Ovary (CHO) cells. The presence of a monoclonal anti-EPCR antibody inhibits FXa activation of PAR1 and consequently ERK1/2 phosphorylation and endothelial cell barrier protection. These data suggest that many of the PAR-dependent signaling properties of FXa on endothelial cells require interaction with EPCR as a co-receptor.

EPCR-bound APC initiates alternative signal transduction pathways independent of PAR1 activation. Apolipoprotein E receptor 2 (ApoER2)-bound APC induces disabled 1 and glycogen synthase 3β phosphorylation via PI3 K/Akt signaling pathway on monocyte U937 cells. An antibody that inhibited APC-EPCR interaction attenuated APC inhibition of TF-mediated procoagulant activity via this pathway on LPS-treated monocytes. PAR1 blockade, however, had no effect [2]. This indicates that the APC-EPCR complex can stimulate anti-inflammatory signal transduction in a PAR1-independent manner on myeloid cells.

**Ligand-bound EPCR determines PAR1 signaling outcome**

In addition to EPCR-dependent PAR1 signaling by APC, recent research by the Rezaie laboratory has shown that zymogen protein C-bound EPCR is also important in the control of PAR1 proteolysis-dependent signaling [9]. EPCR bound to an enzymatically inactive APC variant containing an active site mutation that disrupts the APC active site, exhibits protection from thrombin-induced endothelial cell barrier permeability and stauroporine-induced apoptosis [9], amongst other cytoprotective benefits [82–85]. Similarly, PAR1 activation by PAR1 peptide agonists and thrombin in the presence of protein C-bound EPCR inhibits P-selectin secretion from Weibel-Palade bodies in endothelial cells, limiting leukocyte adhesion upon cytokine stimulation [86]. The molecular basis for thrombin-PAR1 signaling in the presence of protein C-bound EPCR on endothelial cells has been further defined. EPCR occupancy by protein C induces EPCR dissociation from caveolin-1-rich lipid rafts on the endothelial cell surface, altering the G-protein coupling specificity of PAR1 from Gq/11 to Gs [9]. This in turn leads to sphingosine-1 phosphate receptor 1 (S1P1) phosphorylation via the PI3 K/Akt pathway [87]. Rac1 becomes activated and NF-κB down-regulated. Recently, a positive feedback loop has been identified whereby Rac1 activation up-regulates Ang1/Tie2 expression, amplifying PI3 K/Akt signaling and S1P1 phosphorylation. The importance of the PI3 K/Akt signaling pathway was evidenced using a
The prototypal EPCR ligand APC exhibits pleiotropic signaling functions in vivo, regulating inflammatory, barrier-function, and apoptosis pathways. Baboon challenged with a sublethal dose of LPS in conjunction with an anti-EPCR antibody that blocks EPCR ligand interaction died more rapidly than those treated with a non-blocking anti-EPCR antibody [82]. As such, ligand binding to EPCR was established as a crucial step in host response to sepsis. Later studies in which EPCR ligands were challenged with LPS showed reduced mortality compared to wild-type mice [47]. As EPCR is expressed in both vascular endothelial cells and leukocytes, mice solely expressing hematopoietic or non-hematopoietic EPCR were utilized to define the cellular source of EPCR required for protective benefit from sepsis [41]. Interestingly, mice deficient in hematopoietic cell EPCR exhibited a similar response to LPS challenge as wild-type mice, indicating non-hematopoietic-derived EPCR is crucial for maintaining endogenous immune response to LPS challenge [41]. Further information on the role of EPCR in host response to sepsis is provided by transgenic EPCRlow mice using the same endotoxemia model. As expected, EPCRlow mice exhibited reduced survival compared to wild-type mice, and administration of APC had limited protective effect in these mice [89].

Efforts to characterize the cellular source of EPCR required to mediate the protective effects of exogenously administered APC in murine models of sepsis identified EPCR+CD8−CD11c− dendritic cells and EPCR+ hematopoietic cellular precursors as important for APC-induced mortality [93]. This suggests that the cellular target for pharmacological APC is distinct from that utilized by the endogenous protein C-EPCR pathway to dampen the inflammatory response to LPS. Further clinical studies are necessary to elucidate the role of EPCR+ dendritic cells in recombinant APC protection from sepsis-induced mortality.

**Crucial role of EPCR interaction with APC beyond sepsis**

There is increasing evidence that diminished EPCR expression and/or function can be a contributory factor in conditions characterized by chronic inflammation and autoimmune disease. For example, individuals with active inflammatory bowel disease (IBD) exhibit depleted EPCR expression and increased EPCR shedding on their colonic mucosal microvasculature, caused by local generation of TNF-z and IL-1β in the inflamed local environment [69]. The deleterious effect of vascular EPCR depletion in vivo was highlighted by mice with dextran-sodium stibiate-induced colitis, which lost the ability to effectively generate APC. 'Replacement' of missing APC by recombinant APC administration reduced disease activity, weight loss.

**The role of EPCR in APC therapy of severe sepsis**

The prototypal EPCR ligand APC exhibits pleiotropic signaling functions in vivo, regulating inflammatory, extravascular environment.
and mucosal inflammation by inhibition of chemokine production and leukocyte adhesion to the colonic microvascular endothelium [69].

The presence of anti-EPCR auto-antibodies has been associated with an increased risk of deep vein thrombosis [94], myocardial infarction [95], and fetal death [96]. Anti-EPCR antibodies could feasibly inhibit APC anticoagulant activity by limiting protein C-EPCR binding and slowing protein C activation. Indeed, an anti-EPCR IgM antibody isolated from an individual with anti-phospholipid syndrome effectively inhibited protein C activation on endothelial cells [96]. Furthermore, given the established link between chronic inflammation and vascular disease, impairment of EPCR-dependent APC cytoprotective function may also be expected to contribute to the deleterious effect of anti-EPCR auto-antibodies in vascular disease.

APC can also mediate multiple neuroprotective benefits, which are dependent upon its interaction with EPCR on central nervous system (CNS) cells and the blood–brain barrier (BBB) [97]. EPCR mediated APC-PAR1 signaling enhances brain endothelial barrier integrity [6, 98] and stimulates angiogenesis [99–101]. APC–EPCR interaction promotes BBB integrity, and in doing so, reduces the passage of neurotoxic circulatory proteins into the CNS [70] and leukocyte migration across the BBB [102]. APC also directly inhibits microglial inflammation via activation of PAR1 [70] and neuronal cell apoptosis via PAR1 and PAR3 activation [78, 79, 103]. This neuroprotective effect of APC on CNS cells is indirectly facilitated by EPCR, as APC–EPCR complex formation is required for APC transcytosis across the BBB and delivery into the cerebrospinal fluid [104]. Recombinant APC administration is anti-inflammatory, neuroprotective, and increases neurological function and survival in murine models of ischemic stroke [64, 102]. Interestingly, this therapeutic benefit was not observed in transgenic EPCR<sub>low</sub> mice, highlighting the importance of EPCR in the neuroprotective effect of APC in vivo [64]. APC administration is similarly neuroprotective in murine models of embolic stroke [105], hemorrhagic brain injury [65], and amyotrophic lateral sclerosis (ALS) [70]. Recombinant non-anticoagulant APC administration to mice with a superoxide dismutase (SOD) mutation causing ALS-like symptoms enhanced BBB integrity and enabled cytoprotective neuronal signaling. This was evidenced by diminished SOD mutant expression and proinflammatory cytokine markers, reduced disease severity and increased lifespan. APC was unable to modulate BBB integrity or neuroprotective signaling in EPCR<sub>low</sub> mice, demonstrating the crucial role of EPCR interaction in the protective effects of APC in this mouse model. These studies highlight a potential role for non-anticoagulant APC as a drug target for multiple neurological diseases.

EPCR expression has been detected in a number of cancer cell lines, including monoblastic leukemia, glioblastoma, osteosarcoma, erythroleukemia, and prostate cancer cells [106]. Interestingly, EPCR can dictate divergent cancer cell behaviors: EPCR-dependent APC cleavage of PAR1 increases invasion and chemotaxis of breast cancer cells, without alteration of cell proliferation [107]. In contrast, EPCR<sub>low</sub> mice with melanoma metastasis are less prone to metastatic infiltration into both the lungs and liver compared to wild-type mice. In vitro analysis of APC-treated cultured B16-F10 melanoma cells indicate that this function is a consequence of APC-EPCR complex-dependent down-regulation of tumor adhesion and transendothelial migratory functions [108].

Summary

Since its identification as a co-receptor for protein C activation by the thrombin–thrombomodulin complex on endothelial cells, discovery of additional roles in regulating VKD protease signal transduction has established EPCR as a central player in the convergent pathways of hemostasis and inflammation. EPCR binding unlocks the signaling potential of APC and potentially other ligands on numerous cell types, initiating down-regulation of multiple inflammatory processes. EPCR interaction is central to many of the potential therapeutic applications proposed for recombinant APC, and its prominent role in regulating endogenous response to infection has been unveiled in numerous animal models of inflammatory disease. Significant advances have been made in identifying novel EPCR ligands, but further efforts are required to establish what role, if any, these interactions may have in modulating hemostasis and/or immunity. Future studies will aid our understanding of the part played by EPCR in recently identified PAR-dependent and -independent signaling pathways, and further cement EPCR’s position as the conductor of the complex symphony of VKD protease cytoprotective signaling.

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Activated Protein C N-Linked Glycans Modulate Cytoprotective Signaling Function on Endothelial Cells

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Activated protein C (APC) has potent anticoagulant and anti-inflammatory properties that limit clot formation, inhibit apoptosis, and protect vascular endothelial cell barrier integrity. In this study, the role of N-linked glycans in modulating APC endothelial cytoprotective signaling via endothelial cell protein C receptor/protease-activated receptor 1 (PAR1) was investigated. Enzymatic digestion of APC N-linked glycans (PNG-APC) decreased the APC concentration required to achieve half-maximal inhibition of thrombin-induced endothelial cell barrier permeability by 6-fold. Furthermore, PNG-APC exhibited increased protection against stauroporine-induced endothelial cell apoptosis when compared with untreated APC. To investigate the specific N-linked glycans responsible, recombinant APC variants were generated in which each N-linked glycan attachment site was eliminated. Of these, APC-N329Q was up to 5-fold more efficient in protecting endothelial barrier function when compared with wild type APC. Based on these findings, an APC variant (APC-L38D/N329Q) was generated with minimal anticoagulant activity, but 5-fold enhanced endothelial barrier protective function and 30-fold improved anti-apoptotic function when compared with wild type APC. These data highlight the previously unidentified role of APC N-linked glycosylation in modulating endothelial cell protein C receptor-dependent cytoprotective signaling via PAR1. Furthermore, our data suggest that plasma β-protein C, characterized by aberrant N-linked glycosylation at Asn-329, may be particularly important for maintenance of APC cytoprotective functions in vivo.

In response to thrombin generation, zymogen plasma protein C is converted to activated protein C (APC) by the thrombin-thrombomodulin complex on endothelial cells and serves to limit clot development (1). APC attenuates coagulation by proteolytic inactivation of procoagulant-activated cofactors factor Va (FVa) and factor VIIIa (FVIIa) (2, 3). APC also has important non-anticoagulant properties. APC activates the G protein–coupled thrombin receptor, protease-activated receptor 1 (PAR1), when bound to its prototypic receptor, the endothelial cell protein C receptor (EPCR) (4). PAR1 activation by EPCR-bound APC mediates broad protective cellular benefits. APC inhibits endothelin-induced secretion of TNF-α by macrophages (5), reduces cellular NFκB activation in endothelial cells (5), and prevents leukocyte adhesion to activated endothelial cells (6). APC also reduces apoptosis by blocking the pro-apoptotic activity of p38 in human brain endothelium (7). Moreover, APC signaling induces stabilization of endothelial cell barrier integrity via sishngosine-1-phosphate release and sphingosine-1-phosphate receptor 1 (S1P1) activation (8, 9). Prevention of vascular leakage by EPCR-bound PAR1-S1P1 activation is a contributory factor in rescuing mice from lipopolysaccharide-induced lethality (10).

The anti-inflammatory and anti-apoptotic properties of APC are of proven therapeutic benefit. APC reduces the relative risk of mortality in individuals with severe sepsis when compared with placebo, and recombinant APC (Xigris®) is licensed for the treatment of severe sepsis (11). Recent evidence suggests that APC anticoagulant activity is no required to reduce mortality in murine models of sepsis (12). Consequently, removal or alteration of APC anticoagulant activity, but retention of the anti-inflammatory activity, has been postulated as a potential method to improve APC therapy (13).

Protein C is secreted from the liver into plasma as glycoprotein and possesses four N-linked glycosylation attachment sites. Of these, three attachment sites (Asn-248, Asn-313, and Asn-329) are located in the protein C/APC serine protease domain, and one is present in the C-terminal EGF domain (Asn-97). The N-linked glycans attached to protein C/APC are sialylated bi- or tri-antennary complex structure (14). Interestingly, the Asn-329 glycan attachment site does not possess a typical N-linked glycosylation consensus sequence (NX(S/T)) but instead utilizes a cysteine residue in place of the serine/threonine amino acid residue (NA'C) (15). Further, the Asn-329 glycan attachment site is deficient in the protein C/APC serine protease domain, and possesses four N-linked glycosylation attachment sites. Of these, three attachment sites (Asn-248, Asn-313, and Asn-329) are located in the protein C/APC serine protease domain, and one is present in the C-terminal EGF domain (Asn-97). The N-linked glycans attached to protein C/APC are sialylated bi- or tri-antennary complex structure (14). Interestingly, the Asn-329 glycan attachment site does not possess a typical N-linked glycosylation consensus sequence (NX(S/T)) but instead utilizes a cysteine residue in place of the serine/threonine amino acid residue (NXC) (15). Furthermore, an N-linked glycan chain is only attached at Asn-329 in 70–80% plasma protein C (14, 16). The role of each N-linked glycan chain in protein C activation and APC anticoagulant
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activity has been previously investigated. Amino acid substitution of the N-linked glycan attachment site at Asn-97 was found to impair in vitro protein C secretion from mammalian cells. Furthermore, N-linked glycosylation at Asn-248 was found to be important for generation of the protein C disulfide-linked heterodimer (17). The same study showed that individual mutagenesis of each N-linked glycosylation attachment site on the APC serum protease domain increases APC generation by the thrombin-thrombomodulin complex and improves anticoagulant activity in a modified activated partial thromboplastin time (APTT) assay (17).

In this study, the role of APC N-linked glycans in regulating EPCR-dependent PAR1 signaling on endothelial cells was assessed. Enzymatic removal of N-linked glycans significantly enhanced APC endothelial barrier protective and anti-apoptotic functions. N-Linked glycosylation at amino acid position Asn-329 was identified as a key regulator of APC-EPCR-dependent PAR1 signaling on endothelial cells. These data indicate that deglycosylated, non-anticoagulant recombinant APC variants may represent a novel tool for treatment of sepsis and other inflammatory diseases.

EXPERIMENTAL PROCEDURES

Materials—Plasma-purified human protein C and thrombin were purchased from Hematologic Technologies Inc. (Essex Junction, VT). PNGase F was purchased from New England Biolabs Inc. (Ipswich, MA). Sheep anti-protein C polyclonal antibody was from Abcam (Cambridge, UK). APC chromogenic substrate BIOPHEN CS-21(66), Protac, and protein C deficient plasma were from HYPHEN BioMed (Neuville-Sur-Oise, France). Thrombin generation assay reagents (platelet-poor plasma reagent, fluorogenic substrate, thrombin calibration standard) were purchased from Thrombinoscope BV, Maastricht, The Netherlands. EA.hy926 cells were a kind gift from Dr. C. Edgell, University of North Carolina, Chapel Hill, NC (18). Polycarbonate membrane Transwell permeable supports (Costar, 12-mm diameter) were from Millipore (Billerica, MA). Anti-EPCR monoclonal antibody RCR-252 and staurosporine were purchased from Applied Biosystems Inc. (Foster City, CA). APOPercentage apoptosis kit was purchased from Biocolor (Belfast, Northern Ireland, UK).

Deglycosylation of Protein C by PNGase F Digestion—To remove N-linked glycans, plasma-purified and recombinant protein C/APC were incubated with PNGase F and G7 buffer for 4 h at 37°C, as per manufacturer’s instructions. PNGase F-treated protein C was activated with Protac, as described previously (19). Briefly, protein C (5 μg/ml) was incubated with 0.25 units of Protac in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl for 1 h at 37°C. PNGase F-treated APC was characterized by SDS-PAGE analysis and Western blotting.

Generation of Recombinant Protein C Variants—Recombinant PC variants PC-N97Q, PC-N248Q, PC-N313Q, and PC-N329Q were generated by site-directed mutagenesis, expressed, purified, and characterized as described previously (19). Wild type protein C and protein C variants were activated with Protac as outlined above (19, 20). The concentration of each recombinant APC concentration was determined by active-site titration against a calibration curve generated from the amidolytic activity of APC of known concentration. APC chromogenic substrate cleavage by each recombinant APC preparation was determined as described previously (19).

Assessment of APC Anticoagulant Activity in Protein C deficient Plasma—APC anticoagulant function in protein C deficient plasma was assessed using a Fluoroskan Ascent plate reader (Thermo Lab System, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV). Briefly, 80 μl of plasma were incubated with 20 μl of platelet-poor plasma reagent containing 5 μM soluble tissue factor and 4 μM phospholipids (60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine) in the presence or absence of APC (2.5–20 nm). Thrombin generation was initiated by automatic dispensation of fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC-HCl) and 100 mM CaCl2 into each well (final concentrations, Z-Gly-Gly-Arg-AMC-HCl, 0.42 mM and CaCl2, 16.67 mM). Thrombin generation was determined using a thrombin calibration standard. The area under the thrombin generation curve (endogenous thrombin potential, ETP) was measured. Experiments were performed in triplicate, and data were reported as mean ± S.E.

Measurement of Endothelial Cell Barrier Protection by APC—Endothelial cell barrier permeability was determined as described previously, with minor modifications (20, 21). Briefly, EA.hy926 cells were grown to confluence on polycarbonate membrane Transwell permeable supports (Costar, 3-μm pore size, 12-mm diameter) and incubated with APC (0.63–20 nm; all final concentrations). After 3 h, EA.hy926 cells were treated with 5 nm thrombin in serum-free medium for 10 min. The cells were then washed and incubated with 0.67 mg/ml Evans Blue with 4% bovine serum albumin (BSA; Sigma). Changes in endothelial cell barrier permeability were determined by assessment of the increase in absorbance at 650 nm in the outer chamber over time due to transmigration of Evans Blue-BSA. To assess the role of APC-EPCR binding, supernatant was removed, and an anti-EPCR monoclonal antibody (RCR-252) was incubated with the cells for 30 min (25 μg/ml, final concentration) followed by standard permeability measurement (see above). The same assay was used to evaluate the effect of thrombin (5 nm for 3 h) on permeability of EA.hy926 cells pretreated with a non-enzymatic protein C variant PC-S360A (2.5–10 nm) for 15 min. Experiments were performed in triplicate and plotted as the mean ± S.E. Permeability (percentage) was determined using the following equation

\[
P = \frac{(X - C)}{(F - C)} \times 100\% \quad \text{(Eq. 1)}
\]

where P is permeability (percentage), X is the APC-treated OD650, C is the OD650 of untreated EAhy926 cells, and F is the OD650 for thrombin-treated EA.hy926 cells.
Regulation of Apoptosis-related Gene Expression in Endothelial Cells by APC—Confluent EA.hy926 cells in 6-well plates were pretreated with APC for 4 h. EA.hy926 cell apoptosis was then induced by incubation with staurosporine (20 μM) for 4 h. Staurosporine-treated cells were trypsinized and RNA-extracted using the RNeasy mini kit (Qiagen). Reverse transcription (high capacity cDNA reverse transcription kit, Applied Biosystems) followed by RT-PCR was performed using Bax (Hs00180269 м1), Bcl-2 (Hs00153350 м1), and β-actin (Hs99999903 м1) TaqMan® gene expression assays (Applied Biosystems) in an Applied Biosystems 7500 real time PCR system. Experiments were performed in triplicate and plotted as a percentage of the mean staurosporine-treated bax/bcl-2 ratio (100% apoptosis).

Determination of APC-mediated Protection of Apoptotic Endothelial Cells—EA.hy926 cells were grown to confluence in a 96-well plate and then treated for 3 h with wild type or variant APC (0.625–20 μM). Apoptosis was then induced by incubation with staurosporine (20 μM) for 4 h. 30 min prior to the end of this incubation period, an apoptosis-specific purple dye (Biocolor) was added to each well. The cells were then washed twice with phosphate-buffered saline and photographed. Apoptosis was quantified by converting digital photographs into pixel counts using Adobe® Photoshop® software, according to the manufacturer’s instructions. Average pixel counts calculated were based on analysis of at least three images per well. Three independent experiments were performed, and data are reported as the "pixel ratio" calculated from the pixel count from each APC-treated well relative to pixels calculated from staurosporine-treated wells.

RESULTS

Deglycosylation of APC by PNGase F—To enable assessment of the role of APC N-linked glycans in mediating APC cytoprotective signaling, N-linked carbohydrate moieties were removed from APC by enzymatic digestion with PNGase F. Untreated and PNGase F-treated APC (PNG-APC) were characterized by reducing SDS-PAGE analysis and Western blotting using an anti-protein C monoclonal antibody that detects the protein C/APC heavy chain (HC, shown). The anticoagulant activity of APC (DIC) and PNG-APC (2.5–20 nM) was assessed in protein C-deficient plasma by a thrombin generation assay. Thrombin generation was initiated with 5 pm soluble tissue factor and 100 nm CaCl₂ and assessed as described under "Experimental Procedures." The percentage of ETP was determined for thrombin generation in the presence and absence of APC, with 100% ETP defined as ETP in the absence of any APC species.

PNG-APC hydrolisis of a short APC-specific chromogenic substrate (data not shown).

Enzymatic Removal of N-Linked Glycans Improves APC Protection against Thrombin-induced Endothelial Cell Barrier Permeability and Staurosporine-induced Apoptosis—APC protects the endothelium from thrombin-induced permeability in an EPCR- and PAR-1-dependent manner (23). "To determine the functional consequences of APC deglycosylation on EPCR-dependent PAR1 signaling, PNG-APC cytoprotective activity (2.5–20 nM) was assessed in a thrombin-induced endothelial cell permeability assay. As expected, preincubation with untreated APC induced a dose-dependent improvement in endothelial cell barrier integrity (Fig. 2A). Interestingly, preincubation with PNG-APC maintained barrier integrity at ~6-fold lower concentration than wild type APC. Half-maximal endothelial barrier protection was achieved at 9.5 nM for wild type APC, yet only 1.71 nM PNG-APC was required to achieve the same level of endothelial barrier protection (Fig. 2A). The presence of Protac and PNGase F alone had no effect upon thrombin-induced endothelial cell barrier permeability (supplemental Fig. 1).

EPCR occupancy by protein C causes thrombin to exert barrier-protective, rather than barrier-disruptive, intracellular signaling (24). A protein C variant containing an amino acid substitution in the serine protease catalytic triad (PC.S360A) that renders the variant enzymatically inactive was preincubated with EA.hy926 cells in the presence of 5 nM thrombin. As shown previously (24), EPCR occupancy by PC.S60A induced a barrier-protective response upon PAR1 activation by thrombin. PNGase F-treatment of PC-S360A did not affect EPCR occupancy-mediated reversal of thrombin signaling specificity upon PAR1 proteolysis. (data not shown). PNG-APC signaling via PAR1 was completely abolished in the presence of a monoclonal antibody that prevents APC-EPCR binding (RCR-252; Fig. 2B). These data indicate that enhanced PNG-APC cytoprotective signaling is dependent on both APC proteolytic activity and EPCR binding.
Removal of N-Linked Glycans at Asn-329 Mediates Enhanced Protective APC Signaling—PNGase F treatment of APC enhances the EPCR-PAR1-dependent barrier-protective and anti-apoptotic functions of endothelial cells. However, PNGase F does not completely deglycosylate APC under conditions required to maintain APC function and does not provide information as to the specific N-linked glycans that regulate the role of APC N-linked glycans in mediating APC-mediated cell signaling inhibits endothelial cell apoptosis in an EPCR-PAR1-dependent manner (7, 23). To examine the role of APC N-linked glycans in APC regulation of pro/anti-apoptotic gene expression, the ability of PNG-APC to modulate EA.hy926 cell pro/anti-apoptotic gene expression was assessed by RT-PCR analysis of the Bax/Bcl-2 ratio in the presence of staurosporine. At identical concentrations (10 nM), PNG-APC was almost twice as effective in reducing staurosporine-induced apoptosis as wild type APC (p < 0.05; Fig. 2C). Removal of N-Linked Glycans at Asn-329 Mediates Enhanced Protective APC Signaling—PNGase F treatment of APC enhances the EPCR-PAR1-dependent barrier-protective and anti-apoptotic functions of endothelial cells. However, PNGase F does not completely deglycosylate APC under conditions required to maintain APC function and does not provide information as to the specific N-linked glycans that regulate the role of APC N-linked glycans in mediating APC-mediated cell signaling inhibition of endothelial cell apoptosis in an EPCR-PAR1-dependent manner (7, 23). To examine the role of APC N-linked glycans in APC regulation of pro/anti-apoptotic gene expression, the ability of PNG-APC to modulate EA.hy926 cell pro/anti-apoptotic gene expression was assessed by RT-PCR analysis of the Bax/Bcl-2 ratio in the presence of staurosporine. At identical concentrations (10 nM), PNG-APC was almost twice as effective in reducing staurosporine-induced apoptosis as wild type APC (p < 0.05; Fig. 2C).

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APC (supplemental Fig. 2). In contrast, APC-N329Q was significantly more barrier-protective than wild type APC and achieved maximum protection (~90%) from thrombin-induced endothelial barrier permeability at 1.25 nM APC. The same concentration of wild type APC reduced endothelial barrier leakage by only 21% (Fig. 3C). The enhanced activity of APC-N329Q was not due to improved affinity for EPCR as surface plasmon resonance analysis revealed a comparable soluble EPCR affinity as wild type APC (Kₐ ~193 nM, supplemental Fig. 3) (19, 20, 25, 26). Similarly, 5 nM wild type APC was ineffective in reducing staurosporine-induced apoptotic cell death but has limited anticoagulant function (25). To examine the anticoagulant and barrier-protective signaling properties of APC but has limited anticoagulant function (25). To examine the anticoagulant and barrier-protective signaling properties of APC-N329Q, we treated EA.hy926 cells with staurosporine for 12 h, and uptake was determined by accumulation of an apoptosis-specific dye in staurosporine-treated EA.hy926 cells. Untreated EA.hy926 cells were largely impermeable to the dye, as expected (Fig. 4A). In comparison, staurosporine treatment led to widespread purple dye accumulation in EA.hy926 cells (Fig. 4A). Wild type and variant APC reduced dye accumulation and therefore endothelial cell apoptosis in a concentration-dependent manner (Fig. 4, A and B). Interestingly, variants APC-N248Q and APC-N313Q were more protective than wild type APC at all concentrations tested, but this enhanced anti-apoptotic function failed to reach statistical significance (Fig. 4B). At 1.25 nM, wild type APC reduced endothelial cell apoptosis by ~30%, as determined by analytical digital photomicroscopy (Fig. 4, A and B). Remarkably, however, APC-N329Q virtually ablated apoptotic cell dye accumulation at the lowest APC-N329Q concentration tested (0.625 nM). A similar rate of endothelial cell apoptosis inhibition was only observed at 20 nM wild type APC (Fig. 4B).

Enzymatic Deglycosylation of Non-anticoagulant Recombinant APC Causes Enhanced PAR1-mediated Protection of Endothelial Cell Barrier Integrity—Non-anticoagulant recombinant APC variants represent a potentially improved recombinant APC therapy for severe sepsis. APC-L38D has similar anti-inflammatory and anti-apoptotic properties to wild type APC but has limited anticoagulant function (25). To examine the functional consequences of APC-L38D deglycosylation, the anticoagulant and barrier-protective signaling properties of PNGase F-treated APC-L38D (PNG-APC-L38D) were examined. Like APC-L38D, PNG-APC-L38D was unable to inhibit thrombin generation in protein C-deficient plasma (data not shown). However, PNG-APC-L38D exhibited improved EPCR-PAR1-dependent barrier-protective function when compared with its glycosylated counterpart at each APC concentration tested (Fig. 5). Enzymatic deglycosylation of non-anticoagulant recombinant APC therefore leads to improved APC signaling function via EPCR-PAR1 on endothelial cells. APC-L38D/N329Q Has Minimal Anticoagulant Activity but Exhibits Enhanced Barrier-protective and Anti-apoptotic Activity on Endothelial Cells—To generate a non-anticoagulant APC variant with improved EPCR-dependant signaling function without glycosidase treatment, a recombinant APC variant was prepared containing the L38D amino acid substitution (to limit anticoagulant function) combined with the N329Q amino acid substitution (to enhance APC-protective signaling). When assessed by SDS-PAGE and Western blotting, this variant migrated as a single band similar to that of APC-N329Q. APC-L38D/N329Q exhibited limited anticoagulant activity in a thrombin

FIGURE 4. Inhibition of endothelial cell apoptosis by recombinant APC N-linked glycan variants. A, endothelial cell apoptosis was measured by accumulation of apoptosis-specific dye (pink-purple) in staurosporine-treated EA.hy926 cells following incubation with wild type/variant APC. Untreated/staurosporine-treated EA.hy926 cells (top panels) and wild type/variant APC (1.25 nM, middle and bottom panels) are shown. Images are representative of three independent experiments. B, APC concentration-dependent reduction in endothelial cell apoptosis (•, wild type APC; ○, APC-N248Q; △, APC-N313Q; □, APC-N329Q; 0.625–20 nM). Uptake of apoptosis-specific dye was quantified by converting digital photographs into pixel counts using Adobe* Photoshop* software according to manufacturer’s instructions. Average pixel counts calculated were based on analysis of at least three images per well.
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Endothelial cell barrier protection. Endothelial cell barrier permeability was assessed in the presence of wild type APC (○) and PNG-APC-L38D (■) (1.25–10 nM) for 3 h prior to incubation with 5 nM thrombin. Endothelial barrier permeability was assessed by leakage of Evans Blue-BSA through the endothelial cell barrier, as described above.

FIGURE 5. Enzymatic deglycosylation of non-anticoagulant APC variant APC-L38D with PNGase F enhances APC-L38D-mediated endothelial cell barrier protection. Endothelial cell barrier permeability was assessed in the presence of wild type APC (○) and PNG-APC-L38D (■) (1.25–10 nM) for 3 h prior to incubation with 5 nM thrombin. Endothelial barrier permeability was assessed by leakage of Evans Blue-BSA through the endothelial cell barrier, as described above.

FIGURE 6. APC-L38D/N329Q possesses no anticoagulant activity in plasma but demonstrates enhanced cytoprotective PAR-1 signaling. A. Thrombin generation in protein C-deficient plasma was assessed in the presence of wild type APC and APC-L38D/N329Q. Thrombin generation (mm X min) was initiated with platelet-poor plasma reagent and CaCl₂ as before, and the percentage of TFP (thrombin generation in the absence of APC) was determined. (○) no APC; (□) 5 nM wild type APC; (●) 10 nM wild type APC; (♦) 20 nM APC-L38D/N329Q; (△) EPCR-PAR1-dependent endothelial cell barrier protection by APC-L38D/N329Q is more potent than wild type APC. Barrier permeability assays using EA.hy926 cells were performed in the presence of wild type APC (○) or APC-L38D/N329Q (■) (1.25–10 nM) prior to thrombin treatment. Permeability is expressed as a percentage of total thrombin-induced endothelial cell barrier permeability. C. Endothelial cell apoptosis was measured by accumulation of apoptosis-specific dye (pink-purple) in staurosporine-treated EA.hy926 cells following incubation with wild type APC or APC-L38D/N329Q (1.25 nM). D. APC concentration-dependent reduction in endothelial cell apoptosis (○), wild type APC; (■), APC-L38D/N329Q; 0.3125–20 nM.

generation assay using protein C-deficient plasma such that no anticoagulant activity was observed at 20 nm APC-L38D/N329Q (Fig. 6A). However, 1.25 nm APC-L38D/N329Q reduced endothelial barrier permeability by 72%, whereas wild type APC only reduced thrombin-induced permeability by 15% at the same APC concentration (Fig. 6B). Therefore, despite possessing virtually no anticoagulant activity, APC-L38D/N329Q possesses up to 5-fold enhanced endothelial cell barrier-protective function when compared with wild type APC. Furthermore, when assessed in an endothelial cell apoptosis assay, APC-L38D/N329Q was a significantly better inhibitor of endothelial cell apoptosis than wild type APC (Fig. 6, C and D). Apoptotic specific dye accumulation in EA.hy926 cells was only completely inhibited at 20 nm wild type APC, whereas the same protective effect was observed at 0.625 nm APC-L38D/N329Q. These data suggest that APC-L38D/N329Q is ~30-fold more effective than wild type APC in preventing staurosporine-induced endothelial cell apoptosis.

DISCUSSION

Reduced protein C plasma concentration is observed in the majority of cases of severe sepsis and is strongly associated with high morbidity and mortality in this setting (11, 27). The anticoagulant activity of APC means that its administration is associated with an increased risk of severe bleeding. To address this, "second generation" recombinant APC variants have been designed that possess limited anticoagulant activity but retain full cytoprotective signaling properties. This was originally demonstrated using an APC variant with defective factor Va substrate recognition and anticoagulant activity but normal cytoprotective function (13). Recombinant APC variants with similar divergent functional activities have since been described in which APC cofactor sensitivity has been abrogated (25) or where engineered disulfide bonds were incorporated into the protease domain to specifically prevent anticoagulant activity (28). To date, no recombinant non-anticoagulant APC variant has been described that also exhibits improved cytoprotective signaling function.

Glycosylation of membrane signaling receptors and soluble ligands is a common mechanism by which signaling networks can be regulated and can influence multiple aspects of signal transduction, including ligand recognition and affinity (29), intracellular trafficking, and receptor activation (30, 31). In this study, the role of N-linked glycosylation of APC in modulating EPCR-dependent APC endothelial cell signaling via PAR1 was examined. Enzymatic deglycosylation of APC significantly reduced the APC concentration required to achieve maximum protection against thrombin-induced endothelial cell barrier permeability and apoptosis (Fig. 2). Assessment of recombinant APC variants in which individual N-linked glycan attachment sites were removed identified the A/-linked glycan at Asn-329 as a critical modulator of APC endothelial cell signaling. APC-N329Q impaired thrombin-induced endothelial cell barrier permeability up to 6-fold more efficiently than wild type APC and completely inhibited staurosporine-induced endothelial cell apoptosis at ~30-fold lower APC concentration than wild type APC (Figs. 3 and 4). Similarly, a PNGase F-treated non-anticoagulant APC variant (PNG-L38D-APC) and an APC variant with no anticoagulant activity but specific substitution of the Asn-329 glycan attachment sites was only completely inhibited at 20 nM wild type APC, whereas the same protective effect was observed at 0.625 nm APC-L38D/N329Q. These data suggest that APC-L38D/N329Q is ~30-fold more effective than wild type APC in preventing staurosporine-induced endothelial cell apoptosis.
Plasma protein C is composed of three distinct glycoforms. 55–78% of plasma protein C possesses N-linked glycans at each of the four potential N-linked glycan attachment sites (α-protein C) (14, 16, 17). 22–45% of plasma protein C is aberrantly glycosylated at Asn-329 and therefore possesses only three N-linked glycan chains (β-protein C). The final glycoform (γ-protein C) is N-linked-glycosylated at two positions (Asn-97 and Asn-313) and constitutes ~5% of total plasma protein C (16, 17). The molecular basis for partial glycosylation at the Asn-329 N-linked glycan attachment site is unknown but is not linked to level of protein C expression as transgenic pigs that display both high and low protein C expression levels exhibit similar protein C glycosylation patterns (14). One possible explanation is that the unusual NXC glycan attachment site at Asn-329 is a less favorable substrate for glycosyltransferase processing than the typical NX(S/T) site (16).

The N-linked glycan attachment site at position Asn-329 is located in the protein C/APC serine protease domain (32) and is conserved across mammalian species (Fig. 6A). The importance of N-linked glycosylation at this site in regulating APC anticoagulant activity is controversial. In this study, we observed a small increase (up to 2-fold) in anticoagulant activity of PNGase F-treated APC and APC-N329Q variant when added to protein C-deficient plasma and anticoagulant activity determined by attenuation of thrombin generation (Figs. 1 and 3). In agreement with our findings, previous characterization of the APC-N329Q variant found that removal of the Asn-329 N-linked glycan enhanced protein C activity by thrombin in the presence of soluble thrombomodulin and increased APC anticoagulant function 2-fold when assessed by APTT assay (17). In contrast, a naturally occurring protein C variant containing the N329T substitution was associated with reduced anticoagulant activity. This variant protein was immunopurified from plasma, and its rate of protein C activation and anticoagulant activity was determined. PC-N329T was found to be activated by thrombin to the same extent as normal protein C, but in its activated form, it exhibited a reduced rate of FVa proteolysis when compared with normal APC (33).

The molecular mechanism(s) for the increased cytoprotective signaling functions of APC-N329Q observed in this study is currently unknown. However, Asn-329 is in close proximity to two amino acids (Glu-330/Glu-333) that form a putative PAR1-binding exosite on the APC protease domain surface (Fig. 7B). A previous study found that mutagenesis of this region did not alter APC anticoagulant activity but completely prevented EPCR-dependent APC signaling via PAR1 on endothelial cells (34). Consequently, we hypothesize that the presence of a complex N-linked glycan chain at Asn-329 regulates PAR1 access to its exosite-binding region on APC and subsequently inhibits the rate at which EPCR-dependent, APC-mediated PAR1 activation can occur. As such, increased APC-N329Q access to the PAR1-binding exosite facilitates increased PAR1 proteolysis. However, an increased rate of PAR1 activation by its prototypal ligand thrombin is associated with pro-inflammatory, rather than increased anti-inflammatory, downstream endothelial cell signaling. PAR1 activation by thrombin (10,000-fold more rapid than EPCR-bound APC on endothelial cells (35)) initiates distinct G protein coupling to that of APC-activated PAR1 (G12 and G13) rather than Gq (24). Caveolar compartmentalization of PAR1 and/or EPCR occupancy by APC have been proposed as potential mechanisms by which the divergent downstream consequences of PAR1 activation are mediated (24, 36). Interestingly, a meizothrombin chimeric variant containing a protein C/APC Glα domain has also been shown to exhibit significantly faster PAR1 cleavage than APC yet still possesses APC-like barrier-protective functions as a consequence of its ability to bind EPCR (24). This suggests that EPCR binding of PNGase F-treated APC or APC-N329Q is critical for determining protective signaling transduction induced by PAR1 proteolysis on endothelial cells, irrespective of PAR1 activation rate.

The physiological relevance of different plasma protein C glycoforms is not well understood. Interestingly, the zymogen form of the APC-N329Q variant used in this study is predicted to possess a similar glycosylation pattern to that of plasma β-protein C. Our study suggests that once activated, β-protein C may be particularly important for APC cytoprotective function in vivo. Furthermore, this implies that β-protein C plasma concentration may be a more reliable marker of endogenous APC anti-inflammatory potential than total plasma protein C concentration. Further studies are underway to assess the influence of β-protein C in mediating cytoprotective activity in the setting of acute inflammatory disease.

In summary, APC N-linked glycosylation plays a significant role in the regulation of APC cytoprotective function. In particular, we have identified the N-linked glycan moiety attached at amino acid Asn-329 in the APC serine protease domain as a key modulator of N-linked glycan-enhanced APC cytoprotective signaling function on endothelial cells. Furthermore, this observation has led to the generation of recombinant anti-coagulant APC variants in which the pro-C protein complexes.
APC Glycosylation and Cytoprotective Signaling

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