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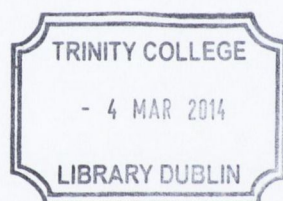
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Role of Membrane Lipids and Plasma Lipoproteins in the Regulation of Activated Protein C Function

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

September 2013

Jennifer Johnson



Thesis 10233

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Summary

Activated protein C (APC) is an important anticoagulant factor that serves to downregulate thrombin generation. APC also has several cytoprotective properties, including anti-apoptotic and endothelial barrier stabilizing signalling. This study aimed to investigate the role of lipids in mediating the APC pathway.

This study first evaluated the role of neutral membrane lipids in enhancing APC anticoagulant activity. Phosphatidylethanolamine (PE) and glucosylceramide (GlcCer) are neutral membrane lipids which stimulate APC anticoagulant activity. Previous studies have indicated that specific APC γ -carboxyglutamic acid (Gla) domain residues may mediate PE/GlcCer stimulation. In this study, APC Gla domain variants were assessed for sensitivity to PE/GlcCer. Interestingly, one APC variant (*APC-I18V*) exhibited similar anticoagulant activity to that of wild-type APC with PC/PS vesicles, but was not enhanced further by the presence of PE/GlcCer. In the presence of PC/PS/PE or PC/PS/GlcCer, thrombin generation was impaired by wild-type APC at 3-4-fold lower protein S concentration than that observed when PC/PS vesicles alone were used. *APC-I18V*, however, did not exhibit a similarly increased sensitivity to protein S in the presence of PE/GlcCer. Loss of PE or GlcCer enhancement of APC anticoagulant activity did not adversely affect EPCR/PAR-1 signalling, as EA.hy926 cells pre-treated with either wild type APC or *APC-I18V* exhibited significant attenuation of thrombin-induced permeability. Collectively, these results suggest PE and GlcCer enhancement of APC anticoagulant activity is mediated by increased sensitivity to protein S, and that isoleucine-18 in the APC Gla domain contributes to APC-specific functional enhancement by PE or GlcCer.

The interaction between high density lipoprotein (HDL) and APC was also examined. This study showed that HDL enhanced APC anticoagulant activity in a protein S-independent manner. Also, this study reported that HDL significantly enhanced the barrier stabilising properties of APC. HDL, at normal plasma levels, significantly enhanced APC ability to protect endothelial cells from thrombin-induced hyper-

permeability. This is the first report of a cofactor to APC cytoprotective signalling. The results of this study suggest that *in vivo* HDL may be an important cofactor to both APC's anticoagulant and cytoprotective signalling.

The results of this study emphasise the critical role of both neutral lipids and HDL in regulating protein C activity. It is hoped that the results of this study will significantly help in the development of second generation APC therapeutics that will be clinically beneficial in multiple disease settings.

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Abbreviations

Ala; A	Alanine
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
BSA	Bovine serum albumin
Ca ₂	Calcium
CaCl ₂	Calcium chloride
CAT	Calibrated automated thrombogram
cDNA	complementary deoxyribonucleic acid
CDP	Cytidine diphosphate
cm ²	Square centimetre
CO ₂	Carbon dioxide
CVD	Cardiovascular disease
Cys; C	Cysteine
DIPE	Dilinoleoyl-sn glycerol-3-phosphoethanolamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DOPC	dioleoylphosphatidylcholine
DOPE	dioleylethanolamine
DOPS	dioleoylphosphatidylserine
E. coli	Escherichia coli
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
Eki2	Ethanolamine kinase 2
ELISA	Enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase

EPCR	Endothelial protein c receptor
ETP	Endogenous thrombin potential
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FDPs	fibrin degradation products
FPLC	Fast protein liquid chromatography
FII	Prothrombin/Factor II
FIIa	Thrombin/Activated factor II
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FIX	Factor IX
FIXa	Activated IX
FX	Factor X
FXa	Activated factor X
FXII	Factor XII
FXIIa	Activated factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
G418	Geneticin
Gla	γ -carboxyglutamic acid
GlcCer	Glucosylceramide
Gln; Q	Glutamine
Glu; E	Glutamic acid
Gly; G	Glycine
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HAT	Hypoxanthine-aminopterin-thymidine
HBSS	Hank's Buffered Salt Solution
HCL	Hydrochloric acid
HDL	High Density Lipoprotein
HEK293	Human Embryonic Kidney-293 cell line

His; H	Histidine
HRP	Horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
Hya	erythro- β -hydroxyaspartic acid
IC ₅₀	Half maximal inhibitory concentration
IgG	Immunoglobulin G
Ile; I	Isoleucine
kb	Kilobases
kDA	Kilodaltons
K _m	Michaelis-Menten constant
L	Litre
Lac	Lactosylceramide
LB	Luria-Bertani
LDL	Low Density Lipoprotein
LDLR	Low-density lipoprotein receptor
Leu; L	Lecutine
Lys; K	Lysine
M	Molar
mAU	milli-absorbance unit
MEM	Minimal Essential Medium Eagle
Met; M	Methionine
mg	milligrams
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulphate
ml	Millilitre
mM	Millimolar
mm ²	Square millimetre
mRNA	messenger ribonucleic acid
MW	Molecular weight
NaCl	Sodium chloride
ng	Nanograms
NHS	H-hydroxysuccinimide
nm	Nanometer
nM	Nanomolar
OD	Optical density

PAI-1	Plaminogen activator inhibitor-1
PAR	Protease activated receptor
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCI	Protein C inhibitor
PE	Phosphatidylethanolamine
Phe; F	Phenylalanine
pM	Picomolar
PPP reagent	Platelet-poor plasma reagent
Pro; P	Proline
PS	Phosphatidylserine
PT	Prothrombin time
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
rpm	Revolutions per minute
RU	Response units
S1P	Sphingosine-1-phosphate
S1P1	Sphingosine 1-phosphate receptor-1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser; S	Serine
SHBG	Sex hormone binding globulin-like
SPR	Surface plasmon resonance
SR-BI	Scavenger receptor B type 1
sTF	Soluble tissue factor
TAFI	Thrombin activatable fibrinolysis inhibitor
TBE	Tris-borate-EDTA
TF	Tissue factor
TFF	Tangential flow filtration
TFPI	Tissue factor pathway inhibitor
Thr; T	Threonine
TM	Thrombomodulin
TMB	Tetramethylbenzidine
tPA	Tissue plasminogen activator

Trp; W	Tryptophan
Tyr; Y	Tyrosine
μg	Microgram
μl	Microliters
μM	Micromolar
μm	Micrometre
U	Units
UDP	Uridine diphosphate
V	Volts
Val; V	Valine
VKOR	Vitamin K epoxide reductase multiprotein complex
VKPD	Vitamin K dependent proteins
VLDL	Very low density lipoprotein
v/v	Volume per unit volume
vWF	von Willebrand factor
w/v	Weight per unit volume
°C	Degrees Celsius

CHAPTER 1 INTRODUCTION

1.1 Coagulation

Coagulation is the complex process by which blood loss is arrested through the formation of a fibrin clot. Coagulation is triggered by injury to the blood vessel wall and this process depends on a closely regulated balance of procoagulant and anticoagulant pathways. Thrombosis formation occurs by three steps: 1) vasoconstriction of the blood vessels, 2) formation of a platelet plug and 3) blood coagulation/secondary haemostasis. This third step is the focus of this study.

Originally, blood coagulation was believed to occur by two separate pathways, the extrinsic and intrinsic coagulation pathways, which merged to form the common pathway (Figure 1.1). Activation of this coagulation cascade consists of a system of inactive zymogens that are sequentially activated by proteolytic cleavage.

The extrinsic coagulation cascade (Figure 1.1) was described as being initiated when the vessel wall is damaged which exposes tissue factor (TF), which results in the formation of the TF/FVIIa complex. The TF/FVIIa complex cleaves and activates FX with the end result of prothrombin (FII) activation. Once some thrombin (FIIa) is formed it in turns activates FV and FVIII ensuring feedback amplification of the coagulation cascade.

Thrombin generation leads to the cleavage of fibrinogen to generate fibrin, which then polymerises and forms a stable platelet plug. This platelet plug is generated on exposure of the injured endothelium, which results in the exposure of von Willebrand

factor (vWF). Once fibrin is subsequently formed, fibrin polymerises and stabilises the platelet plug by incorporating the vWF and platelets into the fibrin clot¹. This fibrin clot is further stabilised by FXIII. These steps of the coagulation cascade occur in the presence of calcium ions and negatively charged phospholipids, which is required for these coagulation proteins to be functionally active².

Formation of fibrin seals the platelet plug at the site of injury, stabilising the clot to allow repair of the injured endothelium. Fibrinolysis limits fibrin deposition to the site of injury and a system of natural anticoagulants feedback and prevent further activation of the coagulant pathway.

The intrinsic pathway (or contact pathway) of coagulation (Figure 1.1) merges with the extrinsic pathway at the formation of FXa which is common to both pathways. The intrinsic pathway is thought to be initiated with the activation of FXII. FXIIa then activates the coagulation cascade which merges with the extrinsic pathway once FIXa activated FX (Figure 1.1). The precise mechanism of the initial activation of FXII is unclear but it involves components already present in the vasculature, including prekallikrein and high molecular weight kallikrein.

The fibrin clot is broken down in a process known as fibrinolysis, where plasminogen is cleaved by tissue plasminogen activator (tPA) to form plasmin. Plasmin degrades fibrin into fibrin degradation products (FDPs). Fibrinolysis is a tightly regulated process. tPA only converts plasminogen to plasmin in the presence of fibrin. tPA is controlled by plasminogen activator inhibitor-1 (PAI-1).

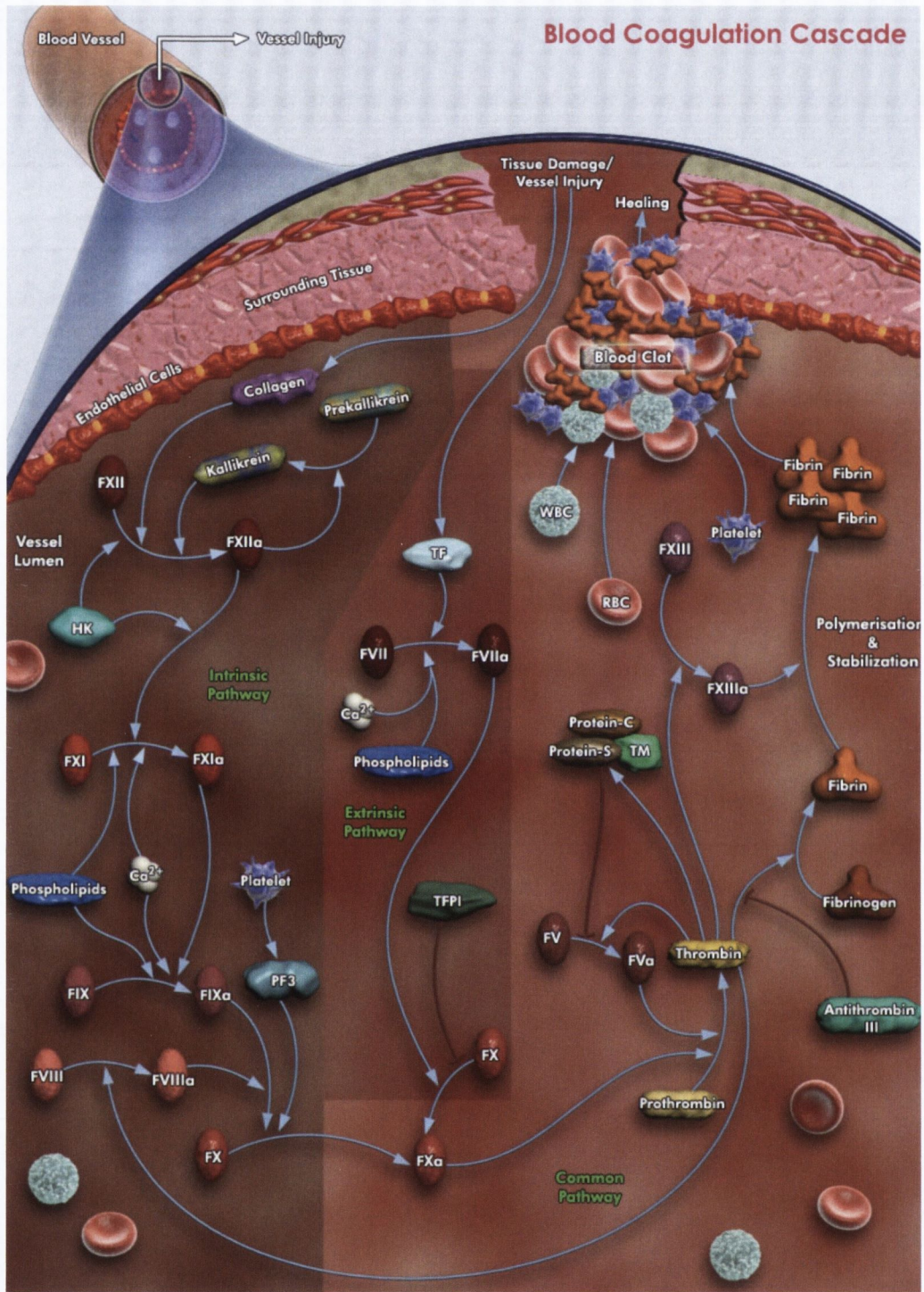


Figure 1-1: The classic pathway of coagulation.

The extrinsic coagulation pathway (or tissue factor pathway) is initiated upon vascular injury which results in exposure of tissue factor (TF). The intrinsic pathway (or contact factor pathway) is initiated by collagen and prekalikrein and kallikrein which results in the activation of FXI. The intrinsic and extrinsic pathway merge when FX is activated to form the common pathway. The procoagulant factors are then activated sequentially, resulting in the formation of a fibrin clot. This pathway requires the presence of Ca²⁺ ions and negatively charged phospholipids. Coagulation is downregulated through a series of negative feedback loops. Figure from qiagen.com

However, this model of coagulation consisting of intrinsic and extrinsic parts, has now largely been replaced by the cell based model of coagulation (Figure 1.2), developed using new coagulation assays. This describes coagulation as occurring in three steps: 1) initiation, 2) priming/amplification and 3) propagation. It is believed that this model more accurately represents what is occurring in vivo, and helps to explain the bleeding phenotype observed in haemophilia, which could not be explained by the classic coagulation cascade.

The cell based model describes coagulation in the following steps (all of which require the presence of negatively charged phospholipids and calcium ions)^{3,4}:

- 1) Initiation: TF is exposed on injured cells which activates FVII. TF-VIIa complex then activates FIX and FX. A small amount of thrombin is then produced when FXa activates FV and together they activate FII.
- 2) Priming: FIIa (thrombin) activates platelets, which causes the platelets to release their alpha granules containing von Willebrand factor (vWF), VIII and V. This results in the activation of the cofactors V and VIII. TFPI inhibits the TF-VIIa-FXa complex.
- 3) Propagation: FX is activated by FIXa/VIIIa complex on the surface of the activated platelet. Then the FXa-Va complex (which is protected from TFPI and antithrombin) activates multiple FII, resulting in large quantities of thrombin produced.

Similar to the common pathway, thrombin will activate the fibrinogen to form fibrin monomers that will polymerize to form a stable fibrin clot.

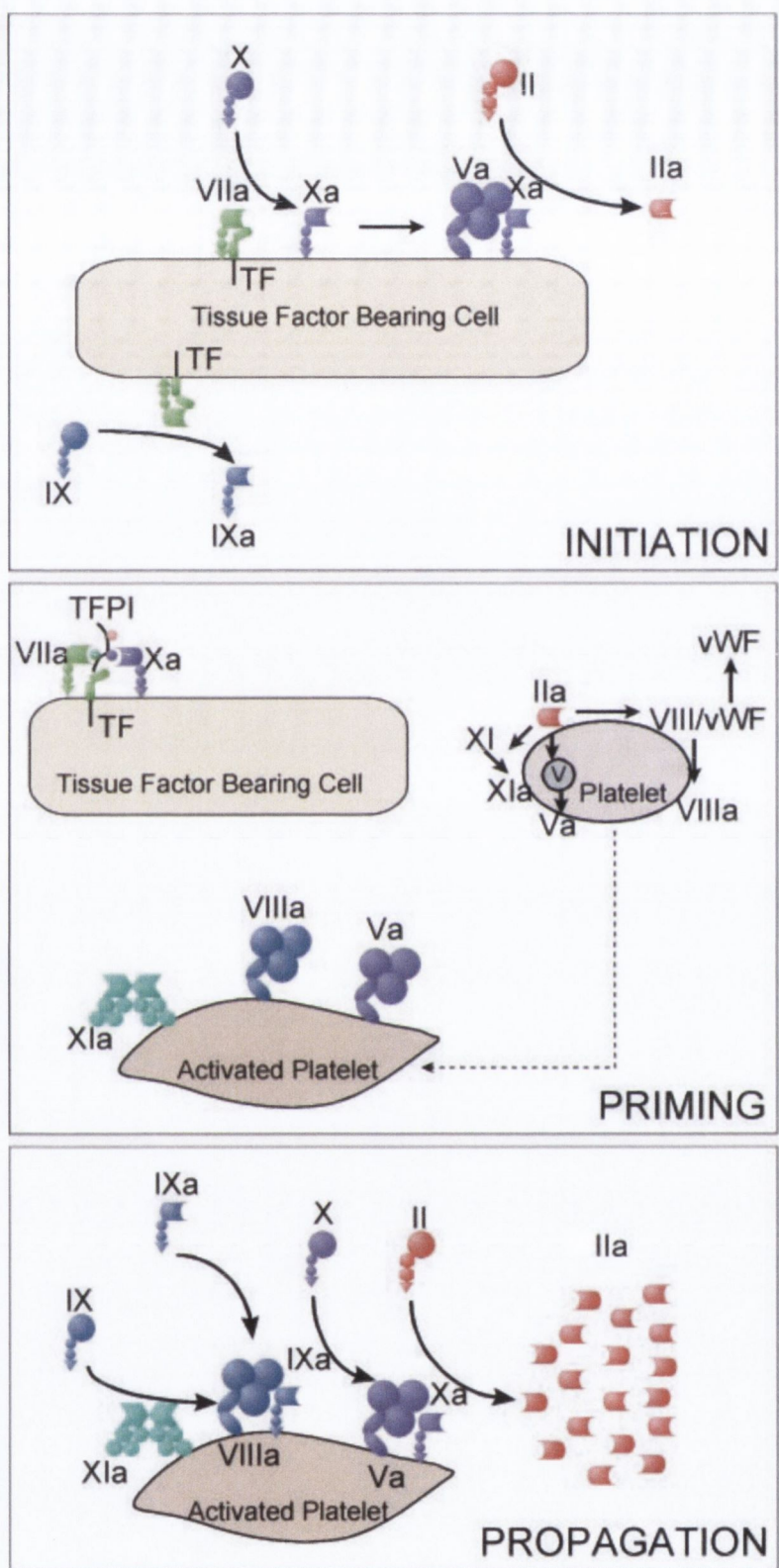


Figure 1-2: Cell based model of coagulation.

Cell based model describes coagulation as occurring in three steps: initiation, priming/amplification and propagation. These steps are shown here with their explanation in text. Picture from review on Platelets and Thrombin Generation³.

This cell based model suggests that the coagulation reactions are localized to the area of injury. Antithrombotic mechanisms on healthy endothelium prevent inappropriate activation of the cascade. Plasma protein inhibitors work more efficiently when the proteins have diffused away from the site of injury and are relatively inactive at the site of injury thereby insuring an adequate, stable clot is formed at the site of injury⁴. It has also been proposed that it may not be necessary to directly turn off the steps of coagulation e.g. by negative feedback loops, but instead the coagulation process may come to a natural end with the sequestration of available clotting factors and activated platelets in the clot⁴. The thrombin generated after the initial activation of fibrin is thought to activate tissue-activatable fibrinolysis inhibitor (TAFI) which limits proteolysis⁴. The presence of fibrin also triggers fibrinolysis.

1.2 Control of the coagulation cascade

The coagulation cascade has a series of positive feedback loops, i.e. thrombin activation of FV and FVIII, and negative feedback loops, i.e. thrombin in complex with thrombomodulin activates the anticoagulant, protein C. Coagulation is regulated at many of levels through the actions of certain inhibitors (Figure 1.1). As previously discussed plasminogen activation leads to plasmin degrading fibrin in a process known as fibrinolysis.

Tissue factor pathway inhibitor (TFPI) binds to the TF/FVIIa complex (see Figure 1.2; priming step) and inhibits the action of FXa. Antithrombin (AT) is a serine protease inhibitor (serpin) which inhibits the actions of the proteases FIIa, FIXa and FXa, in the absence of their cofactors⁵. Heparin, an anticoagulant which is also commonly used clinically and the naturally occurring heparan sulphate, which is found in most cells, acts by binding to and enhancing the activity of the serpin antithrombin⁶. Cofactors FVa and FVIIIa are inhibited by the actions of activated protein C (APC) and APC is itself inhibited by protein C inhibitor and α 1-antitrypsin (APC inhibition discussed below). APC role in coagulation and its inhibition will be discussed in more detail below.

1.3 Vitamin K dependent proteins

Vitamin K dependent proteins (VKDP) play an essential part in the coagulation cascade. VKDP consist of both procoagulant and anticoagulant proteins that are synthesized in the liver. These include prothrombin (FII), FVII, FIX and FX, protein C and protein S. In addition to VKDP involved in coagulation, VKDP are important for other physiological processes, e.g. in bone tissue (matrix Gla-protein (MGP) and osteocalcin) and cell signalling (growth arrest specific 6, Gas6)⁷.

The different VKDP involved in haemostasis all share a common and conserved structure which is also conserved between species⁸. The general structure of VKPD (Figure 1.3) consists of a Gla domain linked to a serine protease/catalytic domain by epidermal growth factor (EGF)-like domains. Exceptions to this general structure include protein S which possesses a sex hormone binding globulin-like (SHBG) domain in place of a catalytic domain and prothrombin where the EGF-like domains are replaced by kringle domain structure. The Gla domain is a highly conserved 45-amino acid region at the N-terminus end of VKDP.

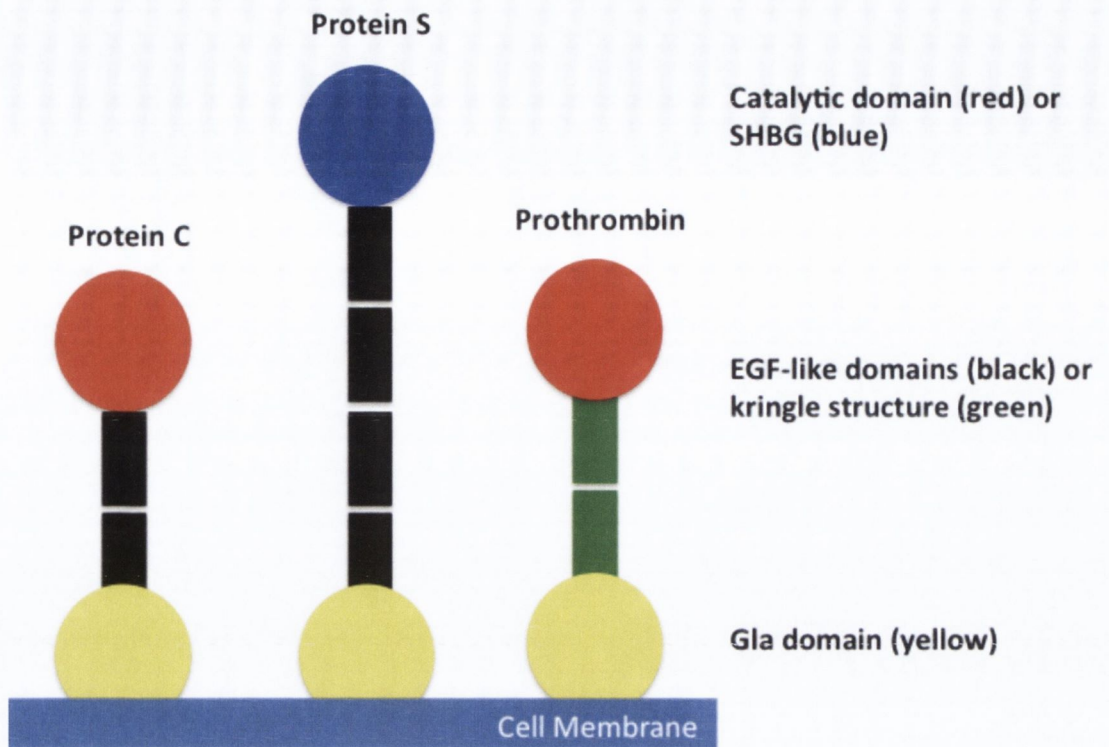


Figure 1-3: Structure of vitamin K dependent proteins.

Most VKDP are similar in structure with a Gla domain which interacts with the anionic phospholipid membrane, EGF-like domains (except prothrombin which contains two kringle structures) which link the Gla domain to the catalytic domain at the C-terminus, except protein S which has a sex hormone binding globulin (SHBG).

1.3.1 Vitamin K cycle

Vitamin K, in its reduced form, is required as a cofactor for γ -glutamyl carboxylase enzyme, which catalyses the γ -carboxylation of specific Glu residues to γ -carboxyglutamic acid (Gla) residues (Figure 1.4)⁹. γ -carboxylation of VKDP is required for their pro/anticoagulant function. Mammals have a very efficient system for extracting vitamin K from circulation which occurs primarily in the liver¹⁰, where most of the VKDP are synthesized. Evidence for the importance of carboxylation of coagulation factors is provided by mice deficient in γ -glutamyl carboxylase, which die either *in utero* or

shortly after birth from massive hemorrhage¹¹.

This vitamin K cycle is essential for the production of normal coagulation factors and this point is highlighted by the use of the anticoagulant warfarin. Warfarin inhibits the vitamin K epoxide reductase enzyme and results in the production of clotting factors with significantly reduced activity due to a lack of γ -carboxyglutamyl side chains.

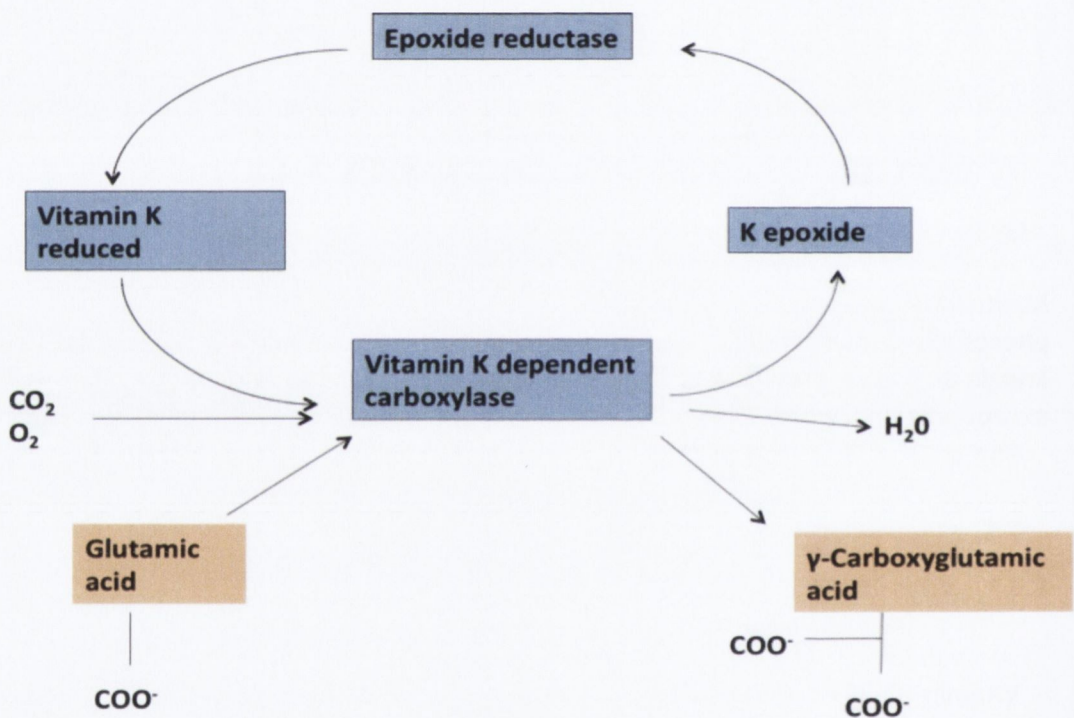


Figure 1-4:Vitamin K cycle.

Vitamin K dependent γ -carboxylase, in the presence of vitamin K, causes γ -carboxylation of glutamic acid (Glu) to γ -carboxyglutamic acid (Gla). The vitamin K is then recycled back to its reduced form by the action of epoxide reductase. In mammals this occurs mainly in the liver.

1.3.2 Gla domain

The 45 amino acid γ -carboxyglutamic acid (Gla) domain of VKDP's are required for binding to negatively charged phospholipids and therefore essential for their pro/anticoagulant function. The Gla domain undergoes two conformational transitions in the presence of Ca^{2+} before it can bind to phospholipids¹². The first transition is not cation-specific, while the second transition is metal-selective for Ca^{2+} . Upon Ca^{2+} binding 3 hydrophobic residues Phe4, Leu5 and Leu8 are exposed¹³, which are highly conserved between the Gla domains of various VKPD, including protein C. It is proposed that these conserved hydrophobic residues penetrate into the membrane and interact with a face of the membrane bilayer. The importance of these residues in binding to lipids was highlighted by mutating Leu-5 and Leu-8 to glutamine in protein C which abrogated functional protein binding to phospholipids^{13,14}.

1.4 Protein C: structure, activation and cofactors

Protein C is a vitamin K dependent glycoprotein. It is synthesised in the liver and secreted into the plasma in its inactive form. Activation of protein C by thrombin occurs on the surface of endothelial cells in the presence of membrane protein thrombomodulin¹⁵. Activated protein C (APC) then cleaves and inactivates cofactors FVa and FVIIIa resulting in down regulation of the coagulation cascade. This activity of APC is enhanced greatly in the presence of its cofactor protein S (discussed below).

The physiological importance of protein C was demonstrated when it was found that homozygous protein C deficiency in newborns results in life-threatening purpura fulminans¹⁶, while heterozygous protein C deficiency results in increased risk of venous thrombosis¹⁷. This importance was further supported by the generation of protein C deficient mice. Despite the normal embryonic development of protein C deficient mice, they experience bleeding and thrombosis and perinatal lethality within 24h¹⁸. These findings show the importance of protein C in life.

1.4.1 Structure and domains of Protein C

The human protein C gene is located on chromosome 2¹⁹ at position 2q13-14²⁰ and spans a region of approximately 11 kilobases of DNA²¹. The protein C gene encodes a 62kDa protein containing 419 amino acid in the mature protein and a leader sequence²² (Figure 1.5). The coding region consists of 8 exons and 7 introns which code for a leader sequence of 33 amino acids, a polypeptide linking sequence of 9 amino acids, the 45 amino acid Gla domain, 2 EGF-like domains, an activation region at amino

acids 144-169 and the serine proteins domain (170-419 amino acids)²¹.

Protein C consists of a heavy chain containing the serine protease domain and a light chain consisting of a Gla domain and 2 EGF-like domains (Figure 1.5). The serine protease domain is responsible for APC catalytic activity and is a trypsin-like serine protease domain. It consists of a conserved catalytic triad, containing a positively charged exosite which is homologous to the fibrinogen binding site of thrombin²³. As with other members of the trypsin class, APC cleaves preferentially at the C-terminal side of argininy residues and makes specific cleavages in each of its substrates (i.e. FVIIIa and FVa)^{24,25}. The exosite is critical for interaction with thrombomodulin during protein C activation by the thrombin-thrombomodulin complex²⁶ and this exosite is also shown to interact with FVa²⁷.

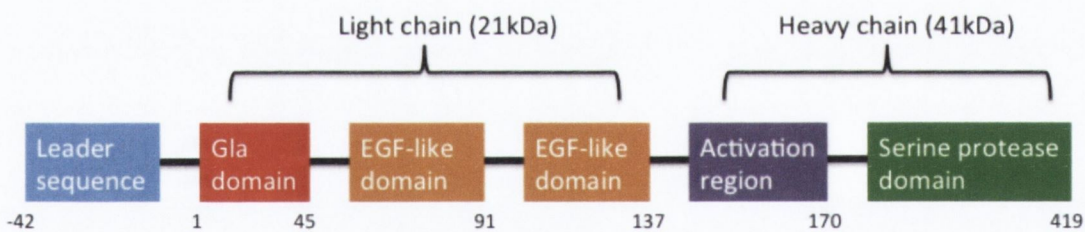


Figure 1-5:Protein C structure.

Protein C consists of a light chain (amino acids 1-137) and a heavy chain (amino acids 138-419). The light chain consists of a Gla domain and two EGF-like domains, while the heavy chain contains the serine protease domain and the region where protein C gets activated by thrombin. Protein C is expressed as a single chain which is linked by a Lys-Arg dipeptide which gets removed during processing. This results in a light and heavy chain which is linked by a disulphide bridge.

1.4.2 Post-translational modification of protein C

During protein processing of protein C the preproleader sequence and the dipeptide are removed resulting in a mature protein containing a light chain connect to the heavy chain by a disulfide bridge²¹ between Cys141 and Cys277. Protein C undergoes many post-translational modifications as summarised in Table 1.1.

γ -carboxylation is essential for the function of the Gla domain (see section 1.4.6). N-linked glycosylation is important for many different aspects of protein C function as defective N-linked glycosylation affects protein C secretion, activation by thrombin and anticoagulant activities²⁸. Lack of this N-linked glycosylation has been shown to increase APC anticoagulant and cytoprotective activities²⁹. Interestingly, while most of the circulating protein C in the plasma is fully glycosylated approximately 30% of circulating protein C is in a lower molecular weight form, termed β protein C which lacks glycosylation at position 329³⁰. Recently, it has been suggested that this β protein C, which would have an increased cytoprotective activity, might be important for mediating APC cytoprotective activities in vivo²⁹. The precise function of the β -hydroxylation modification in the EGF-like domain of protein C is uncertain.

The precise role of the two EGF-like domains in protein C is presently unclear. They are most likely to be important for protein C secretion as mutation of the Asn97 in human protein C results in a protein C variant which is poorly secreted in HEK293 cells²⁸. The EGF-like domains are also believed to be important for protein-protein interactions³¹. The EGF-like domain 1 has a calcium binding site³¹ and this also suggests that this region is of functional and/or structural importance. As the Gla domain is one of the main focus of this investigation it is discussed in more detail below.

Table 1-1 Posttranslational modifications of human protein C

Posttranslational modification	Result of modification	Domain involved	Reside/ Position	Function
N-linked glycosylation ²²	Attachment of N-linked glycans to Asn	EGF & Serine protease	Asn97 Asn248 Asn313 Asn 329	Secretion, anticoagulant activity and cytoprotective activity
γ -carboxylation ²⁸	Glu $\rightarrow$ Gla	Gla	Glu 6 Glu7 Glu14 Glu16 Glu19 Glu20 Glu25 Glu26 Glu29	Interaction with phospholipids, EPCR, protein S, accurate folding of Gla domain
β -hydroxylation ²²	Asp $\rightarrow$ erythro- β -hydroxyaspartic acid (Hya)	EGF	Asp 71	Uncertain role

1.4.3 Protein C activation

The anticoagulant and signalling properties of protein C require its activation. Protein C is activated by the thrombin/thrombomodulin complex on the surface of endothelial cells (Figure 1.6). Thrombin cleaves protein C at Arg169 in the N-terminal region leading to the removal of the activation peptide and exposure of the catalytic triad: His211, Asp257 and Ser360. Once the activation peptide is cleaved off, a disulfide bond is formed between EGF-2 domain and the serine protease domain (Figure 1.7).

In vivo, protein C activation is enhanced approximately 20-fold when bound to endothelial protein C receptor (EPCR)³², but approximately 4-fold *in vitro* and this enhancement is dependent of protein C Gla domain. Protein C circulates in plasma at 4µg/ml (approximately 70nM), however APC is found at very low levels of approximately 2ng/ml (approximately 40pM)³³.

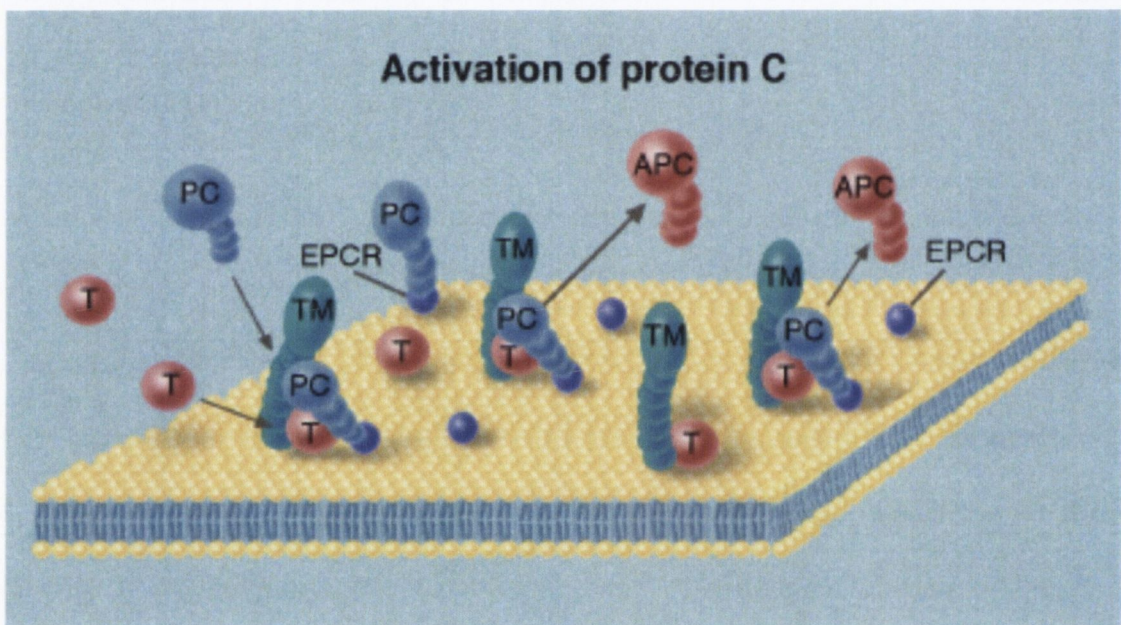


Figure 1-6: Activation of protein C.

Protein C activation occurs on the surface of the endothelial cell in the presence of thrombin and thrombomodulin (TM). Image from Blood review by Dahlbäck⁵.

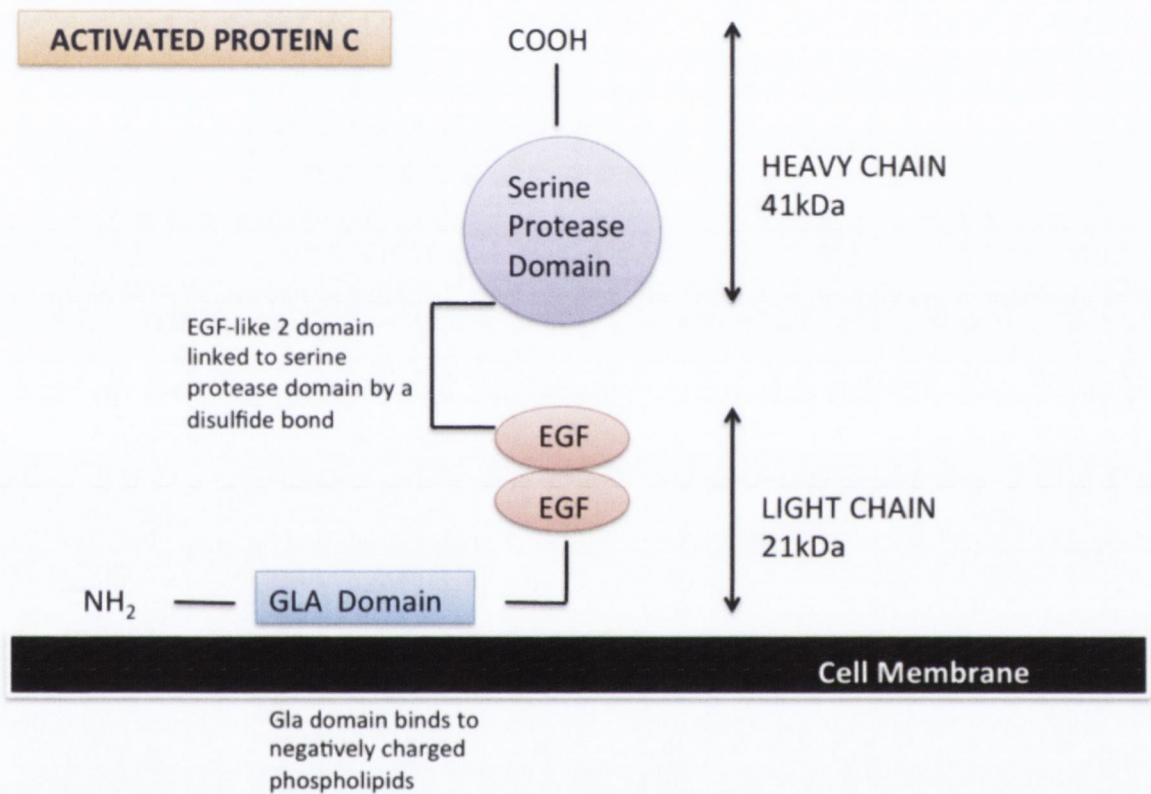


Figure 1-7: Structure of activated protein C.

Activated protein C consists of 4 domains: a Gla domain (blue), responsible for binding to the cell membrane, two EGF-like domains (pink) which link the Gla domain to the serine protease domain (purple) by a disulfide bond. The serine protease domain is responsible for APC catalytic activity.

1.4.4 Thrombomodulin

Thrombomodulin (TM) is a transmembrane glycoprotein expressed on all endothelial cells. TM consists of a lectin like domain, 6 EGF-like domains, a serine/threonine rich region, a transmembrane region and a short cytoplasmic tail. Thrombin binds to TM at EGF-like domains 5 & 6 at high affinity³⁴ and this TM-thrombin complex activates protein C (protein C binds to EGF-like domain 4). Therefore binding of thrombin to TM swaps its role from procoagulant to anticoagulant.

1.4.5 Endothelial protein C receptor (EPCR)

EPCR is a transmembrane glycoprotein (like TM) expressed on endothelial cells³⁵. EPCR binds protein C and is involved in both protein C activation and the cytoprotective signalling of APC. Protein C binds to EPCR with high affinity³⁶ and this interaction significantly enhances protein C activation by the thrombin-thrombomodulin complex. Interestingly, EPCR is found mainly on the macrovasculature with only a small expression in the microvasculature³⁵. Therefore, the precise mechanism of APC generation in the microvasculature is uncertain as EPCR is unlikely to play a significant role.

The importance of EPCR has been shown *in vivo* in baboons, where the infusion of thrombin in the presence of a monoclonal anti-APCR antibody significantly reduced the levels of APC generated, to around 10%³⁷. EPCR binds to protein C Gla domain, with Leu-8 in APC Gla domain critical for mediating this interaction³⁶. Surprisingly, it was

found that FVIIa binds to EPCR with similar affinity to protein C *in vitro*³⁶. However, the physiological role for this interaction is unclear at present.

1.4.6 APC Gla domain

The Gla domain is a 45 amino acid Ca^{2+} dependent N-terminal domain which is found in all vitamin K-dependent proteins. The Gla domain of APC, mediates its interaction with lipids, EPCR and protein S³⁶. The Gla domain of protein C has been shown to have a much lower affinity for negatively charged lipids compared with other vitamin K dependent proteins, including protein S⁸. It is possible to increase APC affinity for lipids, e.g. a human protein C Gla domain mutant, QGNSEDY-APC was found to have enhanced anticoagulant activity and increased phospholipid binding³⁸. The Gla domain is believed to undergo a conformational change upon binding to protein S, resulting in increased proteolytic activity of the serine protease domain³⁹. Leu-8 in APC Gla domain was found to be critical for interaction with soluble-EPCR³⁶. Leu-38 is necessary for APC to be sensitive to cofactor protein S enhancement of anticoagulant activity⁴⁰. Phe4, Leu5 and Leu8 cluster of amino acids are important for the interaction between APC and phospholipids^{13,41}. Residues specific to the APC Gla domain are critical for mediating the enhancement of APC anticoagulant activity by lipid phosphatidylethanolamine (PE) and cofactor protein S, as a protein C mutant containing the prothrombin Gla domain was found to be insensitive to both PE and protein S⁴².

The protein C Gla domain has been shown to bind to platelet factor 4 (PF4)⁴³. Interestingly PF4 has been found to increase the activation of protein C⁴⁴ whilst also impairing the anticoagulant activity of APC⁴⁵. One explanation for these seemingly

opposite effects is that PF4 increases the amount of APC for its cytoprotective/anti-inflammatory effects, while PF4 inhibits any harmful anticoagulant side effects by inhibiting the APC anticoagulant activity of the newly generated APC⁴⁵.

1.4.7 Recombinant APC variants

Previously studies have investigated amino acid substitutions in the APC Gla domain with the aim of localizing specific APC functions/interactions. One of the first studies substituted the whole prothrombin Gla domain into the APC Gla domain⁴². Prothrombin and APC Gla domain have about 40% sequence homology and have a conserved structure with both having 45 amino acids. When the APC Gla domain was mutated to that of prothrombin, it was found that the new chimera lack both protein S and PE sensitivity⁴². To localise the area more specific, small regions of the APC Gla domain were substituted with that of prothrombin³⁶. Using this approach, it was identified that Leu-8 in APC Gla domain was essential for EPCR binding, and that the region between amino acids 33-39 in APC Gla domain were essential for protein S sensitivity. Later, this region was further investigated and it was found the Leu-38 was critical for mediating protein S sensitivity²⁹. In this study APC/prothrombin Gla domain variants were utilized to investigate regions involved in lipid interactions, as will be discussed in Chapter 3.

Different APC variants have been constructed with altered anticoagulant and/or anti-inflammatory activities (Table 1.2). It is hoped that using these APC variants in various in vivo disease models we will be able to appreciate the involvement of APC anticoagulant or tective variants. For example, The 5-Ala-APC variant has minimal anticoagulant activity as this mutation effects the exosite for FVa inactivation, however, in murine models

of sepsis and stroke 5-Ala-APC retained comparable activity to wild type APC in protection of the mice from stroke⁴⁶.

Table 1-2 Anticoagulant and cytoprotective activities of APC variants

Protein variant	C	Defect of variant	Anticoagulant activity	Cytoprotective activity	In vivo results
APC-L38D ⁴⁰		Loss of protein S sensitivity	Minimal	Normal	Unknown
5-Ala APC ⁴⁶		Mutations in protease domain at exosite for FVa	Minimal	Normal	Protective against murine sepsis and stroke
E149A-APC ⁴⁶		Mutation in light chain	Increased	Defective	Poorer outcome from murine stroke as above
APC-70-80 loop mutant ⁴⁷		Defective Ca ²⁺ binding	Minimal	Normal	Unknown
APC-N329 ²⁹		Loss of glycosylation	Normal	Increased	Unknown
APC-QGNSEDY ³⁸		Increased phospholipid binding and increased proteolysis of FVa	Increased	Unknown	Unknown
APC-L38D/N329 ²⁹		Combination of previously defined mutants	Minimal	Increased	Unknown

1.4.8 Inhibitors of APC

APC has a slow rate of inhibition (compared to most other serine proteases) in plasma with a half life of 15min²⁶. APC is inhibited by protein C inhibitor (PCI), α 2-macroglobulin and α 1-antitrypsin⁴⁸. APC inhibition by PCI is enhanced around 400 fold in the presence of heparin⁴⁹. Two basic residues, Lys62 and Lys63, in APC serine protease domain have been found to be important for this heparin mediated PCI inhibition of APC⁴⁹. Unlike most coagulation enzymes, APC is not inhibited effectively by antithrombin, even in the presence of heparin⁵⁰.

1.4.9 Protein S

Protein S is a vitamin K dependent glycoprotein, which acts as a cofactor for APC. Newborns with homozygous protein S deficiency develop life-threatening purpura fulminans⁵¹, while individuals who are heterozygous for protein S deficiency are at increased risk of thrombotic disease^{52,53}. These phenotypes are similar to those of protein C deficiency demonstrating the requirement for both protein C and protein S expression. Protein S is synthesised in hepatocytes, endothelial and hematopoietic cells⁵⁴. Protein S circulates in plasma in 'free' form or in a complex bound to the complement protein, C4b-binding protein⁵⁵. Recent studies have reported that protein S, in addition to acting as a cofactor for APC, also possesses other anticoagulant properties independent of APC, including acting as a cofactor for tissue factor pathway inhibitor (TFPI)^{56,57}. Protein S may have other important non-anticoagulant functions. Protein S triggers receptor activation and proliferation in vascular smooth muscle cells (VSMCs)^{58,59}. Protein S has also been found to be neuroprotective and anti-

inflammatory in a murine model of stroke⁶⁰. The recent generation of protein S deficient mice has highlighted the importance of protein S in both coagulation and in vasculature development^{54,61}. Protein S deficient mice die *in utero* as a result of coagulopathy and vascular dysgenesis. The vascular defects in protein S deficient mice, which are not present in protein C knockout mice, highlight an important role for protein S which is independent of its APC dependent anticoagulant activity. Further, heterozygous protein S deficient mice were found to be prothrombogenic due to a deficiency in APC-independent protein S anticoagulant activity⁵⁴.

1.5 Anticoagulant activity of APC

APC anticoagulant activity is mediated by binding to negatively-charged phospholipids, where it becomes associated with its cofactor, protein S (Figure 1.8). The APC/protein S complex then cleaves and inactivates procoagulant FVa and FVIIIa, resulting in down-regulation of thrombin generation. Despite the well-established role of protein S cofactor function in APC anticoagulant activity, the precise mechanism behind protein S enhancement of APC is still unclear. Indeed, the direct interaction between APC and protein S has yet to be solved. Recently it has been found that Leu-38 in APC Gla domain is critical for protein S cofactor function, and when this residue was mutated APC was found to be totally insensitive to protein S⁴⁰.

APC cleaves FVa at positions Arg306 and Arg506 resulting in loss of cofactor function. In the absence of protein S, APC cleaves FVa in a biphasic process, where Arg506 and Arg306 are cleaved simultaneously but at different rates; Arg506 cleavage occurs at a much faster rate than Arg306. Once Arg306 is cleaved it results in complete loss of cofactor FVa function⁶². In the presence of protein S, APC cleavage of Arg306 is increased approximately 20-fold⁶³, resulting in a much more rapid loss of FVa activity. The enhancement of APC anticoagulant activity by protein S has been suggested to be caused by relocation of APC active site in the presence of protein S³⁹. This relocation leads to a more exposed APC active site on the membrane surface, which may then allow for increased cleavage at Arg306. APC resistance is a condition which is associated with venous thrombosis and is characterized by lack of sensitivity to APC⁶⁴.

A common cause of APC resistance is a FV gene mutation referred to as Factor V Leiden where Arg506Gln substitution occurs resulting in a FV protein which is less sensitive to

APC/protein S proteolysis⁶⁴. Factor V Leiden is a common mutation, occurring in 5-10% of the population. Factor V Leiden was believed to be an important risk factor for venous thrombosis, however recent studies have found that it is not a risk factor⁶⁵.

APC resistance can occur in the absence of FV Leiden. This acquired APC resistance is associated with oral contraceptive use, hormone replacement therapy, antiphospholipid antibodies, pregnancy and other conditions⁶⁶. The precise reason for the acquired APC resistance in these patients is not fully understood, but is thought to be due to alterations in the levels of coagulation factors. For example, oral contraceptive use is associated with a decrease in protein S levels⁶⁶.

As seen in factor V Leiden inadequate inactivation of FVa results in a thrombophilic phenotype. However, factor V deficiency, a rare deficiency referred to as parahæmophilia, results in a bleeding disorder which varies between mild and severe in affected individuals⁶⁷. This highlights just how important it is to maintain the balance between procoagulant and anticoagulant *in vivo*.

APC cleavage of FVIIIa occurs at Arg562 and Arg336. Interestingly, protein S only increases this APC cleavage of FVIIIa in the presence of FV, which can act as a cofactor for APC cleavage of FVIIIa⁶⁸. Physiologically APC/protein S/FV cleavage of FVIIIa is probably insignificant as FVIIIa spontaneously dissociates⁶⁸ leading to loss of FVIIIa cofactor function.

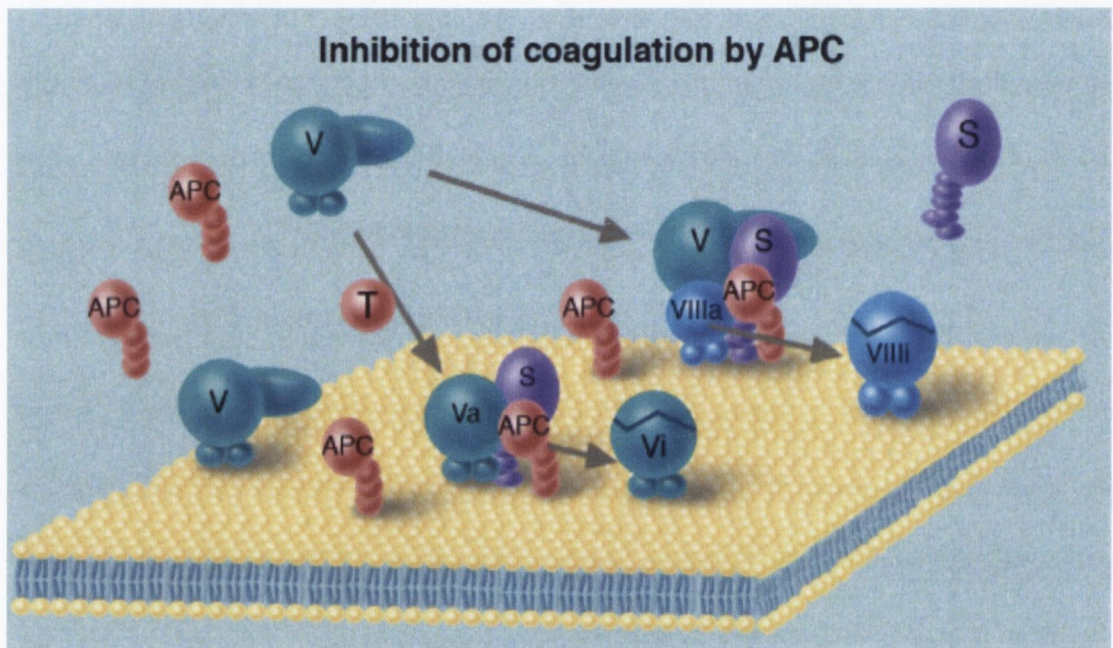


Figure 1-8: Anticoagulant action of APC.

APC inactivates FVa and FVIIIa resulting in a downregulation of the coagulant cascade. Protein S significantly enhances APC anticoagulant activity of FVa. When protein S and FV are present APC cleavage of FVIIIa is increased⁵.

1.6 Lipid enhancement of the protein C anticoagulant pathway

The coagulation cascade occurs on the endothelial cell surface and on activated platelets, when activated coagulation proteins bind to phospholipids. In order for normal function of the anticoagulant protein C pathway critical negatively charged and neutral membrane lipids must be present; the most important ones are phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and glucosylceramide (GlcCer). As well as these membrane bound lipids, certain circulating lipids have been shown to enhance the APC anticoagulant pathway, including the lipoprotein high-density lipoprotein (HDL), cardiolipin and cholesterol⁶⁹.

1.6.1 Phosphatidylcholine

Phosphatidylcholine is a neutral phospholipid that normally resides on the outside of the plasma membrane. It is one of the most abundant plasma membrane lipids. Phosphatidylcholine is an important membrane lipid for coagulation and it has been shown to suppress the activation of prothrombin by FXa⁷⁰.

1.6.2 Phosphatidylserine

PS is a negatively charged phospholipid which represents up to 2-10% of total phospholipid in membranes⁷¹ and is normally found on the inner leaflet of the membrane. However, PS exposure on the outer leaflet occurs in cells at an early stage

of apoptosis and is a recognition signal for phagocytes⁷². Also, PS is exposed on the surface of activated platelets where it plays an important role in coagulation⁷³. The importance of this exposure of PS on normal coagulation can be seen in the case of Scott syndrome, a moderate to severe bleeding disorder. Scott syndrome is characterised by a defective scramblase which results in inadequate exposure of PS on the platelet surface.

1.6.3 Phosphatidylethanolamine

PE is an important neutral phospholipid and is the second most common phospholipid in mammalian cell membranes after PC⁷¹. PE is normally confined to the inner leaflet of the membrane⁷¹. However, interestingly PE is an abundant phospholipid on the outer leaflet of activated platelets⁷⁴. PE is synthesised via two distinct pathways: the PS decarboxylation pathway and the cytidine diphosphate (CDP)-ethanolamine (Kennedy) pathway. The PS decarboxylation pathway occurs in the mitochondria where PS is decarboxylated by the enzyme phosphatidylserine decarboxylase to produce PE. The CDP-ethanolamine pathway occurs in a series of sequential steps where ethanolamine is converted to PE by a series of different enzymes.

Mice deficient in Ethanolamine kinase 2 (*Eki2*), one of the enzymes required for PE synthesis, experience maternally derived placental thrombosis and increased perinatal mortality and a reduced litter size⁷⁵. However, these results have been disputed in a subsequent study⁷⁶, in which no effect upon litter size was observed. Both studies found no difference in cell lipid expression, most likely due to the fact that PE can be synthesised by two separate pathways. Possible differences between these studies

could be explained by different mechanisms of gene knockout⁷⁶. The knockout mechanism used by Tian *et al.*⁷⁵ may have resulted in expression of a variant/alternatively spliced *Eki2* protein which had aberrant function.

PE has been found to enhance the anticoagulant activity of protein C upon incorporation into PC/PS phospholipid vesicles⁷⁴. When present in the phospholipid mixture, PE increased APC inactivation of FVa in the presence protein S, and may increase APC affinity for negatively-charged (PS containing) phospholipids. The precise mechanism underlying this enhancement remains unclear but is dependent upon the APC Gla domain⁴². Despite the fact that the prothrombin Gla domain shares significant homology to the protein C Gla domain, the prothrombin Gla domain is not affected by the presence of PE⁴².

1.6.4 Glucosylceramide

GlcCer, a simple neutral glycosphingolipid, is an important component of the cell membrane. GlcCer is produced when glucose is transferred from uridine diphosphate (UDP)-glucose to ceramide by the enzyme glucosylceramide synthase. GlcCer tends to be located within lipid rafts or caveolae⁷⁷ within the cell membrane and is involved in cell proliferation, survival and signal transduction. GlcCer is the precursor to the formation of most glycosphingolipids. The importance of glycosphingolipids and glucosylceramide *in vivo* is demonstrated by the embryonic lethality of homozygous knockout mice for the glucosylceramide synthase gene⁷⁸.

GlcCer has been found to enhance the anticoagulant activity of APC when incorporated

into PC/PS vesicles⁷⁹. The importance of this enhancement by GlcCer *in vivo* was demonstrated in a case-control study where low levels of GlcCer were found to be correlated with risk of venous thrombosis⁸⁰. It has been suggested that the APC resistance observed during oral contraceptive treatment may be, in part at least, due to decreases in plasma levels of GlcCer⁸¹. GlcCer has been suggested to harbour specific APC binding sites that increase APC affinity for the phospholipid layer. Despite the physiological importance of GlcCer enhancement of APC anticoagulant activity the mechanism behind this enhancement remains unclear.

In addition to GlcCer other neutral glycosphingolipids have been found to increase APC anticoagulant activity, including lactosylceramide (LacCer) and globotriaosylceramide⁷⁹. Short chain fatty acid-containing analogs of GlcCer have also been shown to increase APC anticoagulant activity, for example, C8-GlcCer⁸².

1.6.5 Cardiolipin

Cardiolipin, an anionic phospholipid found in human plasma, enhances APC anticoagulant activity in plasma and in FVa inactivation⁸³. This enhancement of anticoagulant activity was partially dependent on the presence of protein S. Cardiolipin increased APC/protein S affinity for phospholipid surfaces⁸³. Cardiolipin mediated enhancement of APC anticoagulant activity may be of importance physiologically as anticardiolipin antibodies are known to be associated with risk of thrombosis⁸⁴.

1.6.6 Cholesterol

Cholesterol is a steroid fat produced in the liver and is found in different forms in the body, examples include incorporated into lipoproteins and as membrane cholesterol. Cholesterol incorporated into PC/PS phospholipid vesicles also enhanced APC cleavage of FVa, where the Arg306 cleavage in the presence of protein S is particularly affected⁸⁵. Cholesterol was found to only affect the anticoagulant APC pathway, as in the absence of APC cholesterol had no effect on a FXa-dependent-1-stage clotting time⁸⁵.

1.7 Lipoproteins and coagulation

Circulating lipoproteins have been found to affect various elements of coagulation. Lipoproteins are complexes of proteins (named apolipoprotein) and lipids that play an important role in the transport of cholesterol and triglycerides, which are insoluble in plasma. Lipoproteins are classified by their size and density into four subtypes: chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Cardiovascular disease (CVD) is associated with abnormal levels of lipoproteins. CVD is directly related to the plasma level of LDL and is inversely related to the plasma level of HDL⁸⁶.

Lipoproteins have been found to support various coagulation activities. VLDL stimulates prothrombin activation by FXa with cofactor FVa⁸⁷. Oxidation of LDL supported enhanced thrombin generation by the prothrombinase complex⁸⁸. LDL, particularly oxidized LDL, enhances FX activation⁸⁹. VLDL and LDL enhances FVII activation by FXa with and without FVa⁹⁰.

Interestingly, while most of the lipoproteins exhibit a procoagulant role, HDL enhances anticoagulant activity, which may be one of the reasons HDL is considered a 'good' lipoprotein. Plasma HDL, but not LDL, has an enhancement effect on the APC/protein S anticoagulant pathway⁶⁹. HDL significantly enhanced the activity of the APC/Protein S complex in the inactivation of FVa, and this was seen with normal plasma levels of HDL⁶⁹, suggesting that this may be an important in vivo cofactor of APC.

1.7.1 High Density Lipoprotein

HDL is an important lipoprotein with antiatherogenic, antithrombotic and anti-inflammatory functions⁹¹. HDL circulates in plasma and is comprised of approximately 50% protein and is enriched in apo-AI and apo-AII apolipoprotein. Despite the well-recognised beneficial properties of HDL, the mechanism underlying these beneficial effects remains unclear. HDL cholesterol levels are inversely related to the incidence of coronary heart disease⁹².

HDL are so called due to their high density and small size as compared to the other lipoproteins. HDL particles can be subdivided into different classes which vary significantly in size, density and function⁹³. These HDL classes have different lipid composition, apolipoproteins and different charges which result in different functions⁹⁴. The major forms of HDL are pre- β -HDL, HDL2, HDL3, with pre- β -HDL having a very important antiatherogenic function⁹³.

The best-known role of HDL is in reverse cholesterol transport (RCT). HDL helps in RCT by transporting cholesterol found in foam cells in peripheral tissues to the liver, where the liver catabolises the cholesterol or it is excreted in the bile⁹³. Importantly, this transfer of cholesterol to HDL does not occur in the plasma but instead occurs in the extracellular spaces, for example in the intima of the blood vessels⁹³.

HDL possess anti-inflammatory activity⁹¹. However, the precise mechanism occurring in vivo is not clear at present. Many mechanisms for these anti-inflammatory actions have been proposed. HDL is known to inhibit the expression of adhesion markers on endothelial cells, inhibit the oxidation of LDL and has antiapoptotic properties⁹¹.

Sphingosine 1-phosphate (S1P) associated with HDL (HDL is the major plasma carrier of S1P) promotes endothelial barrier enhancement and function⁹⁵.

HDL antithrombotic role may contribute to its protective role in cardiovascular disease. The precise natures of HDL antithrombotic actions are unclear⁹¹. One mechanism might be through scavenger receptor B type I (SR-BI) which is the main HDL receptor expressed on cell surfaces, including platelets⁹⁶. Once HDL is oxidized it can exert antiplatelet activity through SR-BI⁹⁶. Another mechanism is through nitric oxide production which occurs when HDL binds to SR-BI on endothelial cells which leads to upregulation of endothelial nitric oxide synthase (eNOS)⁹⁶. Production of nitric oxide results in vasodilation which increases blood flow⁹¹. HDL also enhances prostacyclin production which can inhibit platelets⁹¹.

The antithrombotic effects of HDL could also be due to its ability to enhance the protein C anticoagulant pathway⁶⁹. However, these results have been disputed in a recent study which found that the enhancement effect seen by HDL was in fact due to contaminating negatively charged phospholipids⁹⁷. Interestingly, HDL infusion leads to an increase expression of thrombomodulin in rabbits⁹⁸, therefore suggesting that HDL may indirectly result in an increase in the level of APC. Further evidence of HDL anticoagulant activity was provided by an *ex vivo* platelet aggregation study which found that platelet aggregation was inversely related to HDL levels⁹⁹.

Although the importance of HDL has been known for decades, there is still much to be understood about its biology. For instance, although HDL levels are known to be inversely related to coronary heart disease, there is only modest evidence that raising HDL by pharmacological strategies, for example nicotinic acid, is of any benefit¹⁰⁰. To

further complicate the role of lipoproteins in CVD, recently it has been found that there may be a subfraction of HDL containing apolipoprotein C-III which may be proatherogenic and be directly associated with risk of coronary heart disease¹⁰¹.

1.8 APC cytoprotective signalling

Recombinant APC is beneficial in the treatment of severe sepsis¹⁰² and was FDA approved for the treatment of late stage severe sepsis. However, its use was limited due to its side effect of serious bleeding. Recently its licence has been withdrawn. Interestingly it is thought that the beneficial effects of APC in sepsis are not due to APC anticoagulant activity. Two other anticoagulants, TFPI and antithrombin, failed to improve survival in severe sepsis^{103,104}. Rather, APC's beneficial effects in sepsis are the result of APC cytoprotective signalling. This has led to different approaches to limit anticoagulant activity by disruption of APC substrate and cofactor molecular interactions, whilst preserving anti-inflammatory function^{40,47,105,106}.

There are many proposed mechanisms of APC cytoprotective activity which are summarised in Figure 1.9. The best described mechanism of APC protective signalling is mediated by APC bound to EPCR, which cleaves and activates protease activated receptor-1 (PAR1). The Gla domain of APC is vital for this PAR1-EPCR cytoprotective signalling. This results in broad anti-inflammatory, barrier stabilization and anti-apoptotic signalling on endothelial cells^{107,108}. PAR1 is a transmembrane G-coupled receptor and originally described as a thrombin receptor. Interestingly, thrombin signalling through PAR1 results in a pro-inflammatory and barrier destabilization effect. Thrombin cleaves PAR1 three to four orders of magnitude more rapidly than APC¹⁰⁹. Noteworthy, endogenously generated APC was found to mediate more effective barrier protection than exogenous APC¹¹⁰.

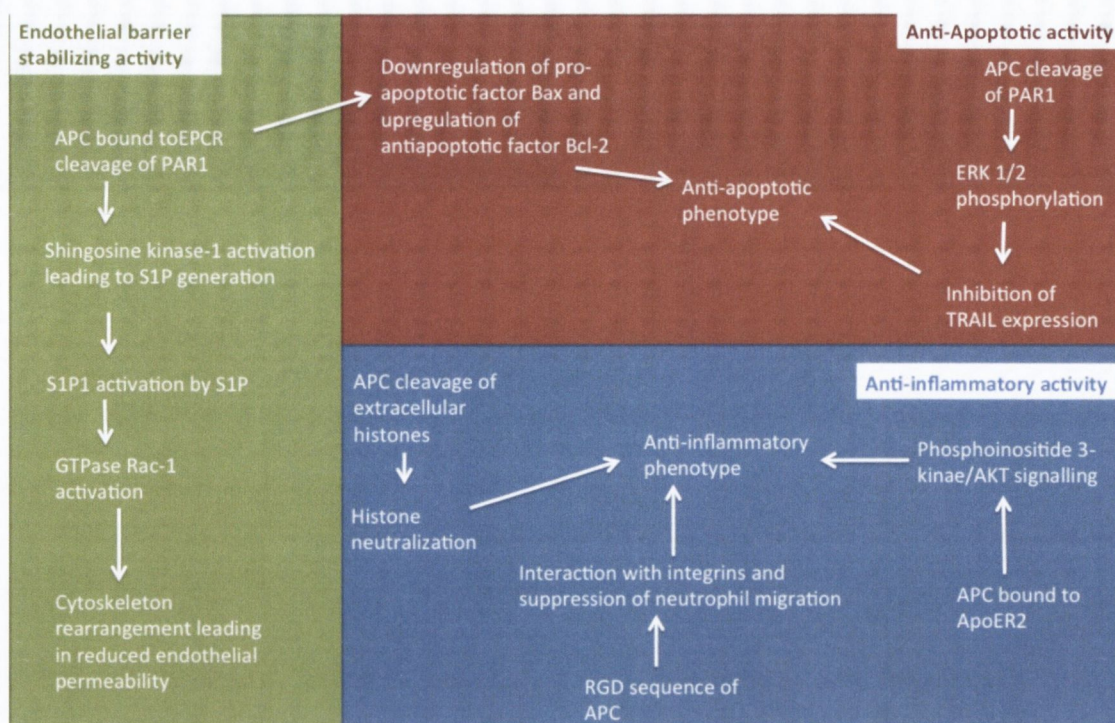


Figure 1-9: Summary of proposed cytoprotective signalling activity of APC.

The precise mechanism(s) through which APC exerts its cytoprotective activity *in vivo* is unclear at present. Multiple proposed mechanisms have been suggested and this figure summarises some of the best described *in vitro* anti-inflammatory, anti-apoptotic and endothelial barrier stabilizing activities of APC.

APC has also been found to be beneficial in animal models in a wide range of inflammatory diseases including inflammatory bowel disease¹¹¹, multiple sclerosis¹¹², and ischemic stroke¹¹³ (Table 1.3). In contrast to severe sepsis, certain disease settings require both anticoagulant and cytoprotective signalling for full beneficial effects of APC, as demonstrated in a murine model of experimental autoimmune encephalomyelitis¹¹⁴ and in acute kidney injury¹¹⁵.

1.8.1 Lipids rafts and APC signalling

All the receptors required for activation of protein C and for APC signalling are collocated within specific lipid rafts, caveolae with caveolin-1¹¹⁶. Lipid rafts are

microdomains that exist in the lipid bilayer of our cell membranes. It is thought that lipid rafts are constantly been formed and unformed in the lipid bilayer with cholesterol been key to their development¹¹⁷. Lipid rafts are places where certain receptors and lipids co-localise and it is believed that these rafts function in cell signalling and cell trafficking¹¹⁷. There are different subset of lipid rafts and one key subset lipid raft in relation to APC signalling is caveolae¹¹⁸. Caveolae are formed by caveolin proteins, which are membrane bound proteins¹¹⁸. Caveolin protein tightly binds cholesterol¹¹⁸.

APC protective signalling through the PAR1/EPCR axis has been shown to require both caveolin-1 and the presence of lipid rafts^{116,119} (Figure 1.10). APC cleavage of PAR1 in the absence of caveolin-1 leads to a similar pro-inflammatory cell phenotype to thrombin cleavage of PAR1¹¹⁹. Very high concentrations of APC lead to a barrier destabilization effect, similar to that seen following treatment with thrombin. Conversely, very low dose treatment with thrombin is barrier stabilizing¹⁰⁹. The exact reason for this is unclear at present, but it has been suggested that APC recruits PAR1 to protective signalling pathway through coupling of PAR1 to pertussis toxin-sensitive Gi-protein¹²⁰.

APC activation of PAR1 results in cross-activation of sphingosine 1-phosphate receptor 1 (S1P1) which leads to further barrier-stabilization effects¹²¹. Cross-activation of the S1P1 receptor was found to be critical for APC endothelial cell barrier stabilization¹²¹. APC treatment may result in S1P1 transport into the lipid rafts within the cell membrane.

Table 1-3 Beneficial effect of APC *in vivo* in different disease settings

Disease Model	Animal	APC effect	Comments	Ref
Recombinant APC				
Sepsis (PROWESS trial)	Human	Increased survival (recently disputed see below)	Side-effect of severe bleeding	102
Sepsis	Mouse-LPS	Increased survival; wild type APC and signalling only APC (5A-APC) equally effective	APC has no effect in PAR ^{7/-} mice or mice with reduced EPCR function	122
Sepsis	Baboon (<i>E.coli</i>)	APC cleave extracellular histones; APC rescued baboons for lethal dose of <i>E.coli</i>		123
Experimental autoimmune encephalomyelitis	Mouse	significant improvement of disease severity; however no effect on disease course or relapse rate	Required BOTH anticoagulant and signalling properties	114
Ischemia stroke	Mouse	Anti-inflammatory, neuroprotective and increased survival	Required PAR1 and PAR3	124
Amyotrophic lateral sclerosis (ALS)	Mouse	Inhibited nuclear transport of Sp1; slowed disease progression	EPCR required to cross blood brain barrier; PAR1 + PAR3 required for protective signalling	125
Inflammatory bowel disease	Mouse	Improvement of disease		111
Acute kidney injury	Rat (LPS)	APC reversed LPS-induced systemic hypotension and decreases in renal blood flow and volume	Required BOTH anticoagulant and signalling properties	115
Pancreatic islet transplantation (Diabetes)	Mouse	Prevention of endothelial dysfunction and improved outcome		126
B16-F10 metastasis model	Mouse	Reduction by 44% in lung metastases	Reduced associated with decrease in lung P-selectin + TNF α mRNA levels	127
Endogenous APC				
Diabetic nephropathy	Mouse	APC-mediated inhibition of glomerular apoptosis protects renal function	Thrombomodulin-dependent protein C activation required	128
Cancer cell extravasation	Mouse	Endogenous APC limits cancer cell extravasation	Protective effect of APC depends on the S ₁ P ₁ pathway	129

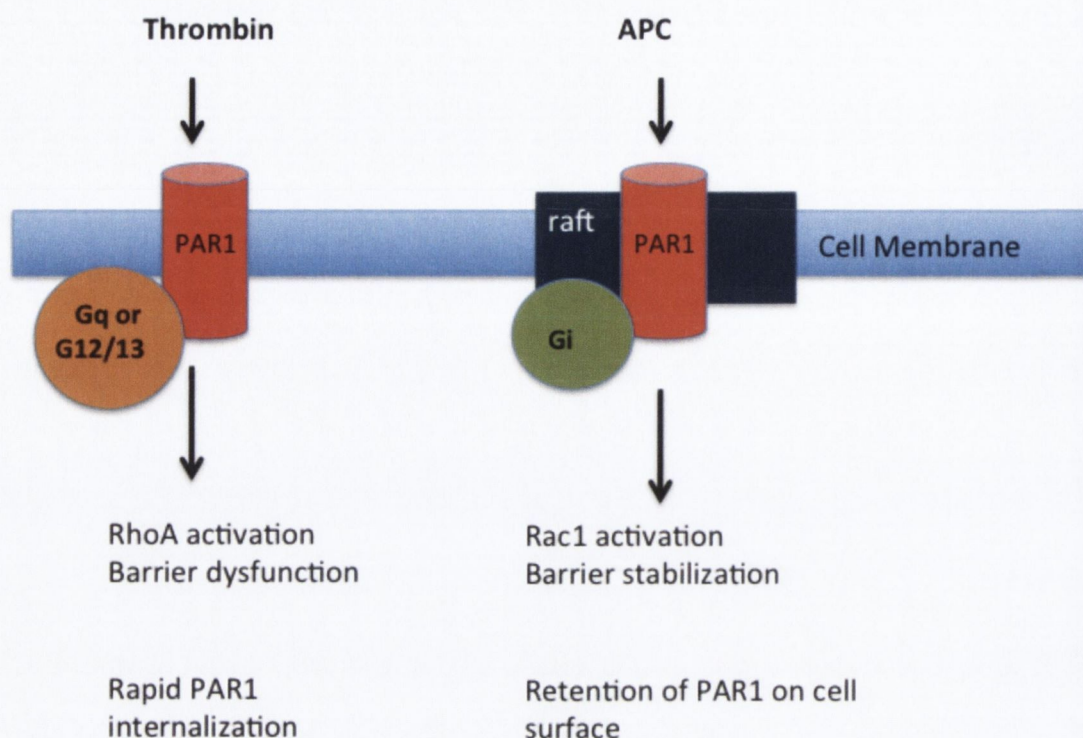


Figure 1-10: Pro-inflammatory and anti-inflammatory signalling via cleavage of PAR1.

Both APC and thrombin cleave and activate PAR1 however they set up different signalling pathways; with thrombin leading to proinflammatory signalling while APC leads to anti-inflammatory signalling. Not alone does this resultant signalling diverge, they also have differing effect on the PAR1 receptor with thrombin leading to rapid PAR1 internalization while APC resulting in retention of PAR1 on the cell surface. For APC to have these effects on cleaving PAR1 APC must be bound to EPCR located in caveolin-1 enriched lipid rafts (dark blue square) on the cell membrane. Once thrombin cleaves PAR1 it signals through Gq or G12/13 protein (brown), however when APC/EPCR cleaves PAR1 it signals through Gi protein (green).

APC signalling through PAR1 leads to Rac1 activation while thrombin leads to RhoA signalling (Figure 1.10). APC protective signalling is ablated in cells lacking caveolin-1 while this has no effect upon thrombin signalling¹¹⁹. This suggests that for APC mediated Rac1 activation to occur, it is necessary for PAR1 to be within caveolae in the cell membrane. Interesting, when EPCR is occupied by either protein C or APC, thrombin cleavage of PAR1 is barrier stabilizing (this is known as occupancy effect)¹⁰⁹. This suggests that in the presence of protein C, active or inactive, PAR1 cleavage results

in the protective Rac1 activation. Interestingly, FVIIa, which binds to EPCR with similar affinity to protein C³⁶, had no effect on thrombin signalling through PAR1¹⁰⁹ and therefore exhibits no EPCR occupancy effect. The molecular basis for this discrepancy is unknown.

As well as APC barrier stabilizing effects on endothelial cell, APC also mediates antiapoptotic signalling. APC antiapoptotic signalling has been shown to be mediated through increased expression of the antiapoptotic genes, e.g. Bcl-2 and decreased expression of the proapoptotic genes, e.g. p53 and TRMP-2^{113,130}. APC downregulation of p53 is mediated through PAR1 and EPCR¹¹³. Some studies have shown that APC can mediate antiapoptotic effects in an EPCR-independent manner, for example, APC can inhibit TRAIL expression without EPCR¹³¹.

APC ability to mediate PAR1 signalling was found to require two specific glutamic acid residues in the serine protease domain, E167 and E170¹³². Interestingly, when these residues were mutated only the cytoprotective properties of APC were lost, while the variants retained normal anticoagulant activity¹³². Therefore the APC-PAR1 interaction is most likely occurring in this region of the APC serine protease domain.

1.8.2 Emerging roles of APC in cytoprotection and disease

There is now emerging evidence that the cytoprotection of APC is mediated not just by EPCR/PAR1 signalling axis but that APC can bind to and signal through other receptors on more cells (Figure 1.11) resulting in diverse cytoprotection¹³³.

One possibly mechanism is through PAR3 mediated signalling¹³⁴. PAR3, while it cannot

signal directly, can heterodimerize with either PAR1 or PAR2 leading to cytoprotective signalling which is caveolin-1 dependent¹³⁴. For example, in a podocyte cell line, which does not express EPCR or PAR1, APC was found to have antiapoptotic signalling properties through cleavage of PAR3¹³⁴.

APC has been shown to inhibit neutrophil migration *in vitro* and *in vivo*. This inhibition is mediated by APC binding to β -integrins on the neutrophil¹³³. The RGD sequence of APC, a motif common to proteins that interact with integrins, was found to be critical for this inhibition. An APC variant (RGE-APC) was still capable of barrier stabilization signalling through EPCR/PAR1. APC may simultaneously bind to EPCR and integrins on the neutrophil surface; however, they found that EPCR was not critical for mediating APC inhibition of neutrophil migration.

APC binds to ApoER2 on the surface of U937 monocytic cells¹³⁵. ApoER2 is a member of the low-density lipoprotein receptor (LDLR) family and is important in Reelin signalling within the brain¹³⁵. APC binding to ApoER2 resulted in Dab1 phosphorylation and Akt activation that would result in cell survival and proliferation and this activity was found to be independent of PAR1 and EPCR. APC binds to ApoER2 with higher affinity than sEPCR when assessed by surface plasma resonance. The molecular requirements of APC signalling via ApoER2 are not known but Gla domainless APC had similar effect to normal APC.

APC seems to play an important role in cancer cell metastasis and extravasation. Endogenous APC prevents cancer cell extravasation in a murine model as a result of APC activation of the S1P1 and not due to APC anticoagulant activity¹²⁹. Administration of APC to a murine model of metastatic cancer reduced the number of lung cancer

metastases by over 40%¹²⁷. However, contrary to these reports APC had been shown to promote breast cancer cell invasion and chemotaxis by signalling through EPCR/PAR1 signalling¹³⁶.

APC protection in sepsis may not be due to the cytoprotective signalling alone. A novel role of APC in severe sepsis has been identified¹²³. The beneficial effect of APC in sepsis may be due to APC effects on extracellular histones. Histones, which are normally confined to the cell nucleus, were found to be released during sepsis leading to endothelial dysfunction and organ damage. However, in this report administration of APC was shown to lead to cleavage of these extracellular histones resulting in increased survival¹²³.

Recently, the use of APC in the treatment of severe sepsis has been put in serious doubt. A recent study, the PROWESS-SHOCK trial, showed that Xigris (drotrecogin alfa (activated)), the recombinant APC which had been licensed for the use in severe sepsis, was no more effective at treating severe sepsis than a placebo¹³⁷. One interesting outcome of this trial was that there was no significant increase in bleeding between the treated and control group, despite the treated group receiving APC. However, it is clear now that APC is more than just an anticoagulant and it is important to fully understand the cytoprotective effects of APC *in vivo* to see if it is possible to manipulate these effects for therapeutic benefit.

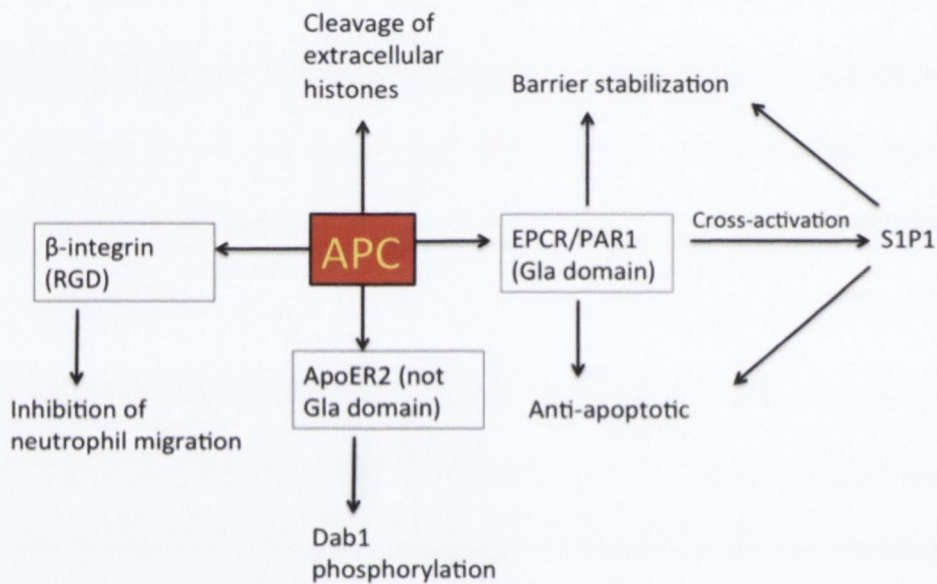


Figure 1-11: Cytoprotective signalling by APC.

Some of the key signalling pathways involved in the protective effects of APC. APC binding to EPCR is mediated via the Gla domain, however ApoER2 binding site residues outside the Gla domain. B-integrin binding to APC is at the RGD sequence.

1.9 Aims of project

The anticoagulant activity of APC is enhanced significantly by the presence of its cofactor protein S, however, it is now clear that other factors, especially lipids, play an important role in mediating APC anticoagulant activity *in vivo*. The aims of this present study will be to investigate the role of different lipids, both membrane lipids and lipoproteins on the protein C pathway.

Firstly the role the neutral lipids PE and GlcCer play in APC anticoagulant activity will be investigated. Although this neutral lipid enhancement is clinically significant^{80,81} the precise nature of the molecular interaction is unclear at present. It is hoped that by gaining insight into the nature of this molecular enhancement by PE and GlcCer, a more complete understanding of APC anticoagulant activity *in vivo* will be achieved.

APC Gla domain is known to be important for phospholipid binding. Currently, it is also believed that the Gla domain of APC plays a key role in mediating the specific neutral lipid enhancement of APC anticoagulant activity⁴². Therefore, this study hopes to identify the residues of APC Gla domain involved in mediating this interaction.

Next, lipoprotein enhancement of APC anticoagulant activity will be examined. It has previously been reported that HDL significantly enhances APC anticoagulant activity leading HDL to be called a cofactor for APC by some authors⁶⁹. Since the precise functions and role of HDL in disease is becoming less clear with the recent finding that a subfraction of HDL may in fact be proatherogenic, it is important to try and understand the interaction HDL makes with plasma proteins. Therefore, in this study the

interaction between HDL and APC will be investigated.

The nature of the lipoprotein HDL enhancement of APC has been reported to be distinct from the phospholipid enhancement⁶⁹ and therefore this study will try and gain insight into the molecular mechanisms behind this activity. Recently, there has been conflicting evidence about whether HDL in fact has an ability to enhance APC anticoagulant activity⁹⁷. Also, as it is unknown if HDL has any other effects on the protein C pathway, including protein C activation or APC cytoprotective activity, these will also be investigated.

As well as APC anticoagulant activity, APC also has important anti-inflammatory and anti-apoptotic properties. However, the precise molecular mechanisms behind these activities are still unclear. Recombinant APC was licensed for the treatment of late-stage sepsis and may also be therapeutic in a number of other disease states. However, recently recombinant APC was been withdrawn from the market for the treatment of severe sepsis, and there is still vast uncertainty over the nature of APC cytoprotective properties. Despite this, the beneficial effect of recombinant APC in murine models of sepsis is not believed to be associated with its anticoagulant activity, but instead arises from EPCR-dependent PAR1 signalling¹²². APC signalling via EPCR-PAR1 exerts broad anti-inflammatory and anti-apoptotic signalling on endothelial cells¹⁰⁷. Several approaches to limit anticoagulant activity by disruption of APC substrate and cofactor molecular interactions, whilst preserving anti-inflammatory function, have been described^{40,47,105,106}. This study will investigate whether loss of neutral lipid enhancement of APC anticoagulant activity, has an effect on APC cytoprotective activities. It will also investigate whether HDL has any effect on APC cytoprotective

signalling.

This study hopes to provide a deeper understanding of lipid enhancement of the protein C pathway. This should provide a valuable insight into the functions of protein C pathway *in vivo*, which should further our understanding of the mechanisms of both APC anticoagulant and cytoprotective activities. This should help determine the role of APC in the treatment of various diseases especially severe sepsis.

1.9.1 Summary of aims

The aims of this study can be summarized as:

- 1) Characterization of the molecular basis of the PE and GlcCer enhancement of APC anticoagulant activity
- 2) Investigate if PE and GlcCer lipid enhancement is necessary for the cytoprotective signalling activity of APC
- 3) Investigate the effects of the lipoprotein HDL on APC anticoagulant and cytoprotective activity

CHAPTER 2 Materials and Methods

2.1 Generation of protein C variants

Protein C Gla domain variants were constructed using previously described methods³⁶. The protein C Gla domain variants used in these study consist of substitutions of prothrombin non-converted Gla domain amino acids into protein C Gla domain. These substitutions were used to investigate neutral lipid enhancement of APC anticoagulant activity as previously it had been determined that replacing the entire protein C Gla domain with that of prothrombin resulted in the loss of PE neutral lipid enhancement of APC anticoagulant activity⁴². Site directed mutagenesis was used to generate recombinant protein C variants. After mutagenesis, the mutated plasmid was amplified by transformation into bacterial cells. Large quantities of bacterial cells were grown and DNA plasmid isolated. This plasmid DNA was then sequenced to ensure the required mutation was accurately incorporated into the protein C cDNA sequence.

2.1.1 Recombinant protein vectors

The recombinant human protein C variants used in this study were generated using a human protein C mammalian expression vector. The amino acid substitutions of these recombinant protein C variants are shown in Figure 2.1. This is a double-stranded DNA plasmid, containing full-length human protein C cDNA sequence, a CMV and SV40

promoter for enhanced expression of the inserted protein C gene, ampicillin resistance gene for selection in bacterial cell culture and neomycin resistance gene for selection mammalian cell culture (Figure 2.2: vector was a kind gift of Professor Björn Dahläck, University of Lund, Sweden). The protein C cDNA sequence was cloned between Hind III and Xba I restriction endonuclease sites. This plasmid was used as a template for site directed mutagenesis of the protein C gene.

1	6	11	16	21	26	31	36	41	45	Gla domain
ANSFL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DTLAF	WSKHV		APC-WT
AN <u>T</u> FL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DTLAF	WSKHV		APC-S3T
ANSFL	EE <u>VRK</u>	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DTLAF	WSKHV		APC-L8V/H10K
ANSFL	EELRH	<u>GN</u> LER	ECIEE	ICDFE	EAKEI	FQNVD	DTLAF	WSKHV		APC-S11G/S12N
ANSFL	EELRH	SSLER	EC <u>V</u> EE	ICDFE	EAKEI	FQNVD	DTLAF	WSKHV		APC-I18V
ANSFL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	<u>T</u> TLAF	WSKHV		APC-D36T
ANSFL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DT <u>D</u> AF	WSKHV		APC-L38D
ANSFL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DTL <u>V</u> F	WSKHV		APC-A39V
ANSFL	EELRH	SSLER	ECIEE	ICDFE	EAKEI	FQNVD	DTLAF	<u>WAKYT</u>		APC-S42A/H44Y/V45T
AN <u>T</u> FL	EE <u>VRK</u>	<u>GN</u> LER	EC <u>V</u> EE	<u>TC</u> <u>SYE</u>	EA <u>FEA</u>	<u>LESST</u>	<u>ATDVF</u>	<u>WAKYT</u>		<u>PROTHROMBIN</u>

Figure 2-1: Sequence of the APC Gla domain of the recombinant protein C variants used in this study.

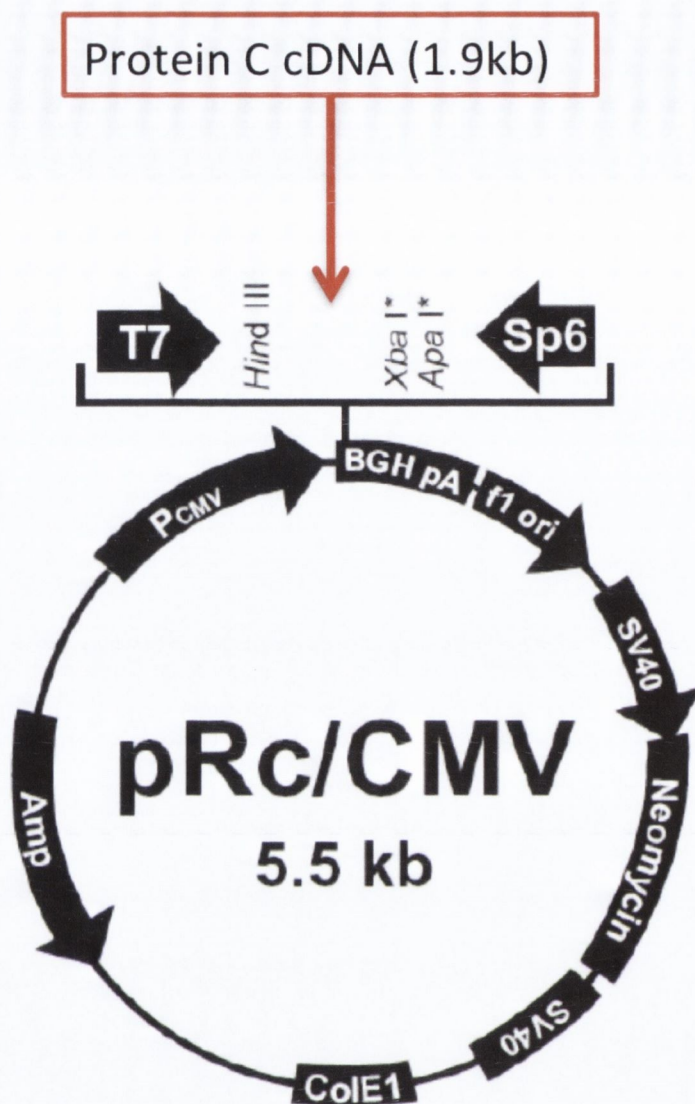


Figure 2-2: The pRc/CMV/Protein C cDNA vector.

2.1.2 Summary of the generation of the recombinant protein C variants

The mutant protein C variants used in this study had been previously generated by Dr. Roger Preston^{40,138}. Therefore, only a brief outline of this process is given here. QuickChange Site Direct Mutagenesis Kit (Stratagene, USA) was used to create amino acid substitutions within the protein C cDNA vector. This is a PCR based assay optimised for introducing the specific nucleotide substitutions. Transformation using

NovaBlue cells. Then NovaBlue competent *E. coli* cells (Merck Biosciences) were used to transform the mutated plasmid DNA. The plasmid DNA was purified from the bacteria cell pellet using the Plasmid Miniprep system (Qiagen).

To confirm presence of the desired mutation in the protein C cDNA, DNA samples was sent to the DNA sequencing service at the University of Dundee (www.dnaseq.co.uk/contact.html). The sequencing was performed using multiple sequencing primers to sequence the entire protein C cDNA using Applied Biosystems capillary DNA sequencers.

2.2 Production of recombinant protein variants

Each recombinant protein C variant was expressed in HEK293 cells. Selection of transfected cells were achieved by using G418 (Geneticin, Sigma) which is toxic to normal HEK293 cells. However, cells that expression the protein C vector are resistant to G418 due to the presence of a neomycin resistant gene. Once expressed the recombinant protein variant was purified and concentrated, prior to analysis by western blotting to fully characterise the protein variant.

2.2.1 Cell culture

All cells were maintained at 37°C with 5% CO₂ in vented tissue culture flasks (Fischer Scientific, Ireland). Cell culture work was performed using aseptic techniques in a laminar flow class II hood.

Human Embryonic Kidney (HEK) 293 cells (European Collection of Cell Cultures, Wiltshire, UK) were used for the expression of recombinant protein C variants. HEK293 cells were maintained in Minimal Essential Medium Eagle-alpha (MEM-α: Biosciences, Ireland) supplemented with 2mM L-glutamine (Biosciences, Ireland), 100 units/ml penicillin-0.1mg/ml streptomycin (Sigma), and 10% foetal bovine serum (FBS: Sigma).

The endothelial cell line, EA.hy926 cells (kind gift of Dr. C. Edgell, University of North Carolina, Chapel Hill, NC) were used to study APC signalling and activation of protein C, as this cell line expresses thrombomodulin (TM), EPCR and PAR1. EA.hy926 cell line is an immortalized cell line derived by fusion of the human umbilical vascular endothelial

cells (HUVEC) with the human lung epithelial carcinoma cell line A549. While no endothelial cell model can replicate precisely what is occur *in vivo*, Ea.hy926 is the best-described and studied cell model for human vascular endothelial cells¹³⁹. Ea.hy926 cells have the advantages of been cheap, easy to culture and have been found to maintain their characteristics during multiple passages¹⁴⁰. Ea.hy926 cells exhibit contact inhibition similar to endothelial¹⁴⁰. Ea.hy926 cells cell surface expression is similar to that of endothelial, expression tPA, thrombomodulin, PAI-I, tissue factor, vWF, selectins and adhesion models, e.g. ICAM-1¹⁴¹. In this study Ea.hy926 cells have the benefit of been a good model for endothelial cells that give reproducible results. All the experiments in this study uses Ea.hy926 cells were done statically, while *in vivo* the endothelial is under constant stress caused by the constant flow of blood over it. Therefore, despite the fact that Ea.hy926 cells are a excellent model of endothelial cells, they cannot fully replicate *in vivo* conditions.

EA.hy926 cells were maintained in Dulbecco's Modified Eagle Medium: High Glucose (DMEM: Gibco, Biosciences Ireland) containing L-Glutamine, 4500 mg/L D-Glucose and Sodium Pyruvate which was supplemented with sodium hypoxanthine-aminopterin-thymidine (HAT: Biosciences, Ireland), 100units/ml penicillin-0.1mg/ml streptomycin (Sigma) and 10% FBS (Sigma). Penicillin/streptomycin was used to prevent bacterial contamination of the cell lines and HAT was used to maintain selection for hybrid EA.hy926 cells.

2.2.2 Cell passage

EA.hy926 cells and HEK293 cells were passaged when they reached confluence on the surface of the tissue culture flask. First, the media was removed and the cells washed in PBS (Sigma). It was important to remove all the cell media as the serum inhibits the activity of trypsin. Then, 1ml of 0.25% Trypsin, 1mM EDTA/tetrasodium salt (Biosciences, Ireland) was added to the flask and left at 37°C, for approximately 3min, until the cells became visually detached from surface of flask. The trypsin was then neutralised with the addition of media containing serum. The HEK293 cells were then seeded at a concentration of approximately $2 \times 10^5/35\text{mm}^2$ (in a 6 well plate Flask Sigma). EA.hy926 cells were passaged up to 5 times before new stock was used. When the cells were passaged they were made up to a suspension of approximately 2×10^5 cells/ml.

2.2.3 Cryopreservation and recovery of cell lines/ transfected HEK293 cells

Stocks of the cell lines and stably transfected HEK293 cells expressing recombinant protein C variants were cryopreserved. To cryopreserve cells, after the cells were trypsinized (Section 2.2.2.), the cells were centrifuged at 1000rpm for 5min at room temperature and the supernatant discarded. Then the cell pellet was resuspended in the appropriate media (MEM or DMEM), without antibiotic and with 10% (v/v) dimethyl sulfoxide (DMSO). DMSO is used as a cryoprotectant as it prevents the formation of ice crystals that would lyse the cells. This cell suspension was then aliquoted in 1ml volumes in cyro-vials (Fisher Scientific) and immediately placed in an isopropanol box at -80°C for between 6-24h. The vials were then transferred to liquid

nitrogen. To recover these cells, the cells were removed from the liquid nitrogen, warmed to 37°C for about 1 minute (until the cell solution was just defrosted) and the 1ml solution added to 15ml of media (MEM or DMEM). The cells were then centrifuged at 1000rpm for 5min at room temperature. The supernatant was then removed, and the cells resuspended in 12ml of fresh media and transferred to a T75 flask and incubated as per usual. The media of these cells was replaced 24h later, and the cells were then allowed to grow to confluence.

2.2.4 Stable transfection of HEK293 cells with recombinant protein C variants

Recombinant protein C variants were expressed by stable transfection of HEK293 cells. HEK293 cells were grown to approximately 70% confluence in a 6-well plate. The transfection mix was prepared by diluting 4µg of purified plasmid DNA encoding the protein C variant to be expressed into 250µl Opti-MEM I Reduced Serum Medium (Biosciences, Ireland). Separately 10µl of lipofectamine 2000 reagent at 1mg/ml (Invitrogen) was added to 240µl OptiMEM and both these dilutions left at room temperature for 5min. After this incubation, they were mixed and incubated for 20min. During this time, the media was removed from the cells and washed twice in PBS and then 1.5ml Opti-MEM was added to each well of the 6-well plate. Then the 500µl mix of DNA/lipofectamine was added to the well. The cells were left at 37°C for 4h and then the media changed to standard MEM-alpha. The cells were then left to grow to confluency before being transferred into a 10cm² dish. When confluent the selective agent G418 (Sigma) was added to the cells at a final concentration of 50µg/ml. To retain the selective pressure the media containing G418 was replaced every 48h. After

10-14 days the untransfected cells died and the remaining transfected cells formed colonies. Approximately 10 of the surviving colonies were then picked and plated in a 96-well plate. The cells were then allowed to grow to confluency. The highest expressing protein C colony was then determined by performing a protein C ELISA (Section 2.2.8) on the media from the cells. This colony was then grown to fill a 1L hyperflask™(Fischer Scientific), a multisurface cell vessel containing a total growth area of 1750cm². Upon confluency, the media was changed to 1L Opti-MEM I Reduced Serum Medium (Biosciences, Ireland) supplemented with 20µg/ml Vitamin K₁ (Roche) and then the stably transfected cells were left to express the protein into the media for 4 days. Vitamin K was used to ensure the proper γ-carboxylation of recombinant protein C.

2.2.5 Concentration of recombinant protein variants

The process of purification and concentration of the recombinant proteins is summarised in Figure 2.3.

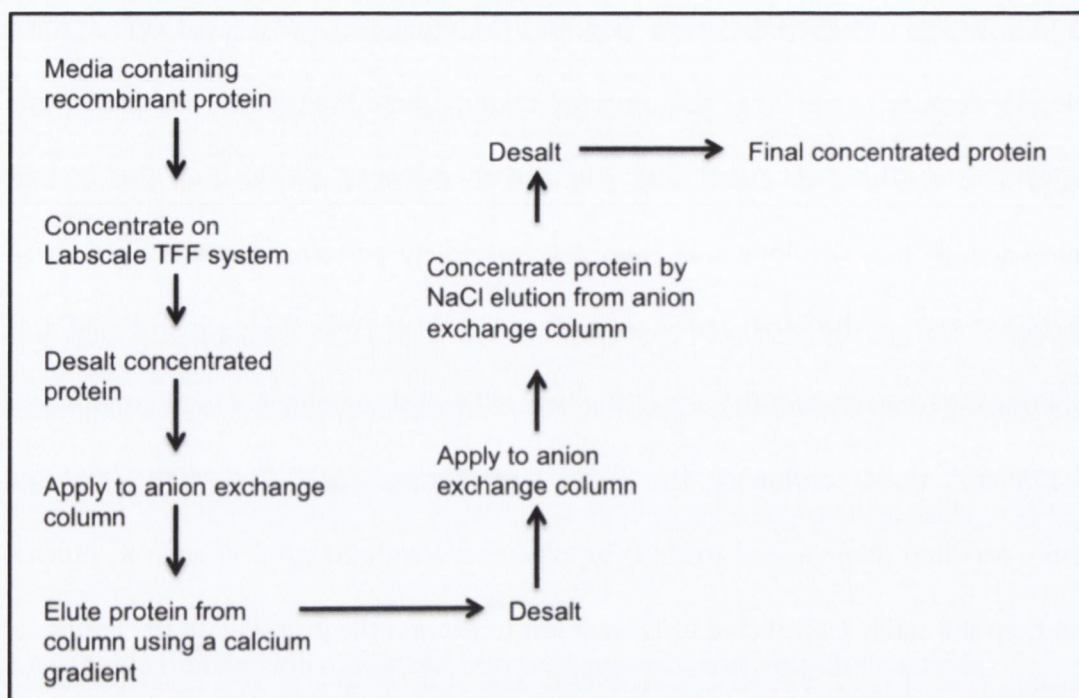


Figure 2-3: Flowchart showing the process of purification and concentration of the protein C recombinant proteins.

Prior to purification, the media containing the expressed protein C was concentrated 10 fold using a Labscale tangential flow filtration (TFF) system with the Pellicon XL50 Ultrafiltration device (Millipore). After 3 days the media containing the expressed protein C was removed (500ml in total) and was subjected in TFF to produce a final concentration 50ml.

Filtration is a pressure drive separation process that utilises membrane to separate out components in a liquid solution. In TFF (Figure 2.4) the fluid is pumped tangentially along the surface of the membrane and pressure applied at 90° serves to force a portion of the fluid through the membrane to the filtrate side. The retained components in TFF do not build up on the surface of the membrane, but instead are swept along by the tangential flow. This feature of TFF makes it very good at size-based separation. Concentration is achieved as the excess fluid travels across the

membrane into the waste tube, while the protein C is prevented crossing the membrane, as it is too big in size to cross the membrane.

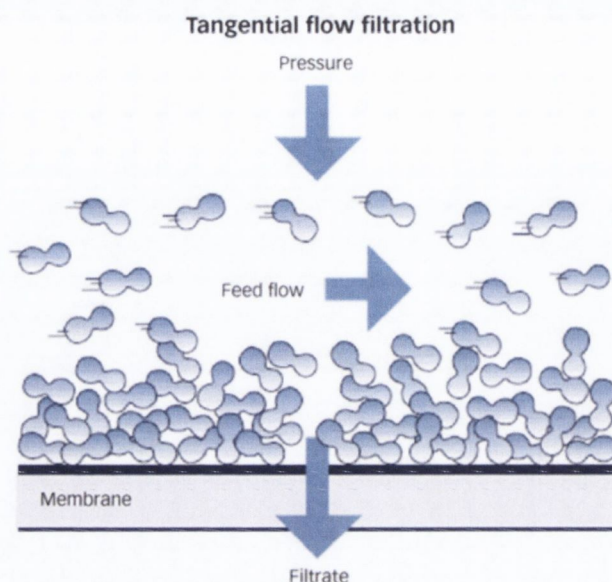


Figure 2-4: Tangential Flow Filtration system

This image (from millipore.com) demonstrates tangential flow filtration (TFF).

2.2.6 Purification of recombinant protein variants

Each protein C variant was partially purified using a Fast Performance Liquid Chromatography (FPLC) system (AKTA FPLC: GE Healthcare). FPLC is a form of liquid chromatography, which can be used to purify proteins. FPLC can sort molecules based on size or charge. Prior to this thesis work commencing some wild-type protein C and protein C-I18V had already been purified³⁶. However, due to the quantity of these proteins required for this study I purified more.

The concentrated medium was first desalted using a 5ml HiTrap desalting column (GE Healthcare), to establish buffer-exchanged into 50mM Tris-HCl (ph 7.4), 150mM NaCl

(termed running buffer). The HiTrap desalting column is packed with the size exclusion matrix Sephadex G-25 Superfine and allows for rapid buffer exchange of proteins.

To partially purify the recombinant protein C, the fact that a correctly γ -carboxylated protein C is negatively charged was utilised. This negative charged protein will bind to a positively charged resin, like Q sepharose. A 5ml HiTrap Q HP column, which is prepacked with Q Sepharose, which is an anion exchanger was used. In the column, stable ether linkages form between the gel matrix of the column and anionic functional groups of the protein. This HiTrap column was first equilibrated with 5 column volumes (or 25ml) running buffer to remove any preservatives in the column. Next 25ml of elution buffer (50mM Tris, 150mM NaCl, 1M NaCl (pH 7.4)) was run through the column to remove any bound proteins to the column, then to prepare the column for the sample injection, 25ml of running buffer was passed through the column. The buffer exchanged protein C was then injected manually into the FPLC superloop. From here it was automatically injected onto the column at a flow rate of 1ml/min. The protein C bound to the column during this phase. To elute the protein, a 50mM Tris-HCl (pH7.4), 150mM NaCl and 30mM CaCl_2 buffer was passed through the column over a concentration gradient of Ca^{2+} (0-30mM). This buffer is used as it contains calcium, which binds to a γ -carboxylated protein C causing the protein C to dissociate from the column (Figure 2.5). At about 10mM Ca^{2+} the protein suddenly came off the column as can be seen in Figure 2.5. Where between fraction A11 and B7 there is a peak in the absorbance values. This subfractions were collected into 1ml volumes which were subsequently pooled.

Following this calcium elution the sample was buffer-exchanged again to remove the CaCl_2 from the protein sample.

Then the sample was applied to a 5ml Hi Trap Q HP column and a 1M NaCl elution was performed to concentrate the protein (from 3ml to 1.5ml).

Eluted protein was then buffer-exchanged into 20mM Tris-HCl (pH 7.4), 150mM NaCl and the recombinant protein C characterised by SDS-PAGE/western blotting.

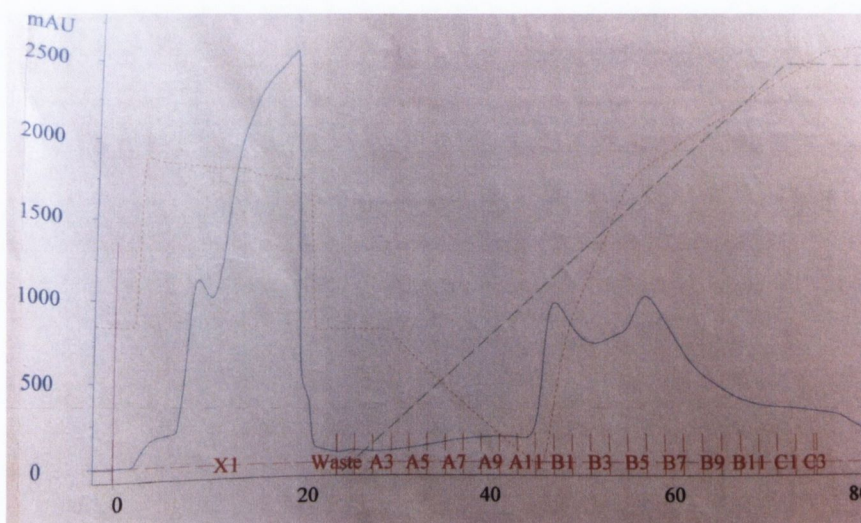


Figure 2-5: Anion Exchange CaCl₂ gradient for protein C-l18V.

FPLC chromatogram from the purification step for protein C-l18V. Time (min) is shown on the X-axis and absorbance (mAU) on the Y-axis. The dotted green line represents the increasing calcium gradient. The purified protein is first eluted at subfraction A11 where there is an increase in the absorbance reading.

2.2.7 SDS-PAGE/ Western Blotting analysis of recombinant protein variants

The recombinant protein C was characterised using SDS-PAGE and western blot analysis to detect protein C. 30ng protein was mixed with non reducing loading buffer (final concentration: 75mM Tris-HCL, pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl

sulphate (SDS) and 0.07% (w/v) bromophenol blue) and boiled for 10min at 100°C to denature the protein. This solution was then loaded and separated on a precast 10% polyacrylamide Tris-HCL gel (Bio-Rad, UK) at 100V for 60min in running buffer (final concentration: 25mM Tris base, pH 8.3, 192mM glycine and 0.1% (w/v) SDS). Standards of known molecular weights were included (ColourBurst Electrophoresis Markers, Sigma, UK).

Proteins were transferred to a nitrocellulose membrane at 100V for 1 hour at 4°C in transfer buffer (final concentration: 25mM Tris base, pH 8.3, 192mM glycine and 20% (v/v) methanol). The nitrocellulose membrane was then washed in PBS-Tween (Sigma: 0.01M PBS and 0.1% (v/v) Tween). Non-specific binding sites on the nitrocellulose membrane were blocked using blocking buffer (5% (w/v) non-fat dried milk (Marvel) in PBS-Tween) at room temperature for 1 hour. The membrane was washed 3 times in PBS-Tween for 10min at room temperature before the antibody was applied. This blocked membrane was then incubated with a sheep polyclonal anti-protein C antibody (Abcam, USA) diluted in blocking buffer for 1 hour at room temperature. The membrane was then washed 3 times for 10min in PBS-Tween. A horseradish peroxidase (HRP)-conjugated anti-sheep IgG polyclonal secondary antibody (Abcam, USA) was diluted in blocking buffer was added to the membrane and incubated for 45min at room temperature. The membrane was then washed again as before.

To detect the protein bands the membrane was incubated for 2min with *Pierce Super Signal West Pico Chemiluminescent Substrate* reagent (Thermo Scientific) to detect the HRP conjugated secondary antibody. The excess reagent was removed and the membrane developed using photographic film (Sigma). A marker containing proteins of known size (Sigma) was included to enable protein mass determination. A typical

western blot is shown in Figure 2.6 which shows that the purified protein C was detected as expected by both protein C antibodies and Gla antibodies. To ensure that any functional differences observed by the protein C mutants were the results of that mutation and not an artifact of the purification process or contamination, all experiments compared the results to protein C-WT.

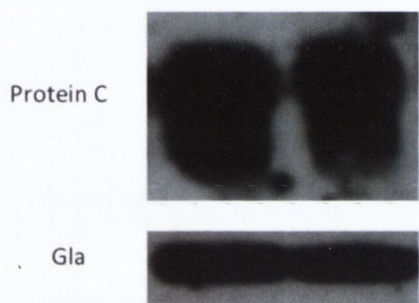


Figure 2-6: Western blot of recombinant protein C variants.

Western blot showing wild type protein C on the left and protein C-I18V on the right, detected using either a polyclonal anti-protein C antibody (top panel) or a polyclonal antibody specific to protein C Gla residues (bottom panels).

2.2.8 Determination of protein C concentration by ELISA

A commercially available protein C ELISA kit (Zymutest, Hyphen-Biomed, Neuville-Sur-Oise, France) was used to determine the concentration of recombinant protein C to select the highest expressing colony (Section 2.2.4) and after purification (Section 2.2.6). This is a highly sensitive two-step immune-assay for the detection of protein C in plasma; however, it can also be used to determine the concentration of recombinant protein C in the cell culture media and after purification.

This protein C ELISA kit uses a standard curve derived from plasma of known protein C concentration to determine the concentration of the unknown samples. The protocol is shown in Table 2.1. The 96-well ELISA plate was provided precoated with a rabbit polyclonal antibody specific for human protein C. Sample diluent, conjugate diluent,

TMB substrate and stop solution/Sulfuric acid were provided ready to use. Wash solution was warmed to 37°C to completely dissolve the solution then it was diluted 1:20 with distilled water. The Anti-(H)-PC-HRP immunoconjugate was restored with 7.5ml of the conjugate diluent and this was allowed to fully dissolve and was shaken gently to homogenize the contents prior to use.

Table 2-1 Protein C ELISA protocol

Reagent	Volume	Procedure
Test samples and standards	200µl	Introduce the solutions in to the corresponding micro ELISA wells
Incubate for 1 hour at room temperature		
Wash Solution (20 fold diluted in distilled water)	300µl	Proceed to 5 successive washing steps
Conjugate (anti PC polyclonal antibody coupled with peroxidase). Restored with 7.5ml of conjugate diluent	200µl	Introduce the Anti-(H)-PC-HRP immunoconjugate into each well
Incubate for 1 hour at room temperature		
Wash solution (20 fold diluted in distilled water)	300µl	Proceed to 5 successive washing
TMB/H2O2 substrate	200µl	Immediately after washing, introduce the substrate into the wells. Note: the substrate distribution, row by row, must be accurate and at exact time intervals
Incubate for <u>exactly</u> 5min at room temperature		
0.45M Sulfuric acid/stop solution	50µl	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the sulphuric acid
Wait for 10min in order to allow the colour to stabilise and measure the absorbance at 450nm. Subtract the blank values		

The kit came with plasma protein C standards (Plasma PC Calibrator) which were restored with 2ml of sample diluent and this provided plasma with known protein C

concentration, “C” (the exact concentration was provided with each kit). The standards were then diluted, as shown in Figure 2.7, to provide a standard curve. Samples of the recombinant protein were diluted in the sample diluent (1:10 to 1:20) and tested in duplicate. If the samples were outside the standard curve, the dilutions were increased or decreased as required and retested.

Protein C concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma PC calibrator at C	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05ml	0 ml
Vol. of Sample diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95ml	1 ml

Mix gently for a complete homogenisation.

Figure 2-7: Preparation of standards for protein C ELISA.

The sample and controls were added to the 96-well ELISA plate which is pre-coated with rabbit polyclonal antibody specific for human protein C, which the test samples bind to. This is then detected by using anti-hPC-HRP-immunoconjugate which is a rabbit polyclonal antibody coupled to HRP specific for human protein C. The amount of this conjugate is then detected by TMB/H₂O₂. This reaction is then neutralised by sulphuric acid. The colour is then allowed to stabilise before the absorbance of the plate is read using a Versa max, microplate reader (Molecular Devices) at 450nm. A standard curve was constructed using the plasma PC calibrator samples (Figure 2.8) and the concentrations of the unknown samples then determined.

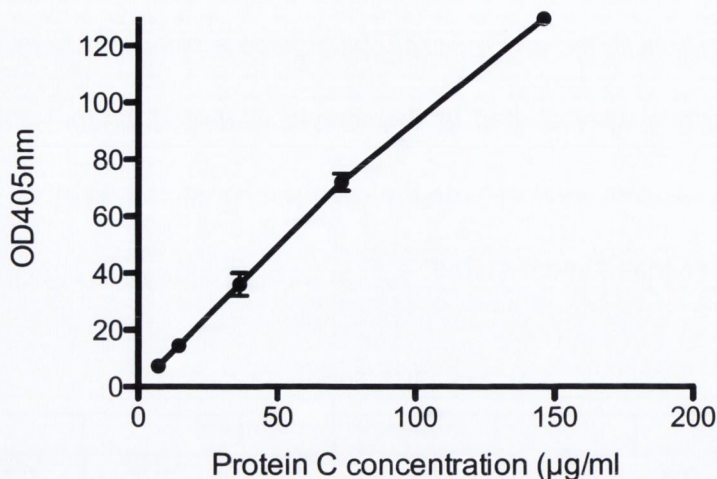


Figure 2-8: Typical standard curve for protein C ELISA using the plasma protein C calibrator.

2.2.9 Activation of recombinant protein C variants

Recombinant protein C was activated using Protac (Hyphen-Biomed, Neuville-Sur-Oise, France), using a previously described method³⁶. Protac is a protein C activator purified from the venom of copperhead snake, *Agkistrodon Contortrix*. Protac is a serine proteinase of approximately 40kDa, which rapidly activates protein C independent of thrombin and thrombomodulin¹⁴². Recombinant protein C was incubated in 50mM Tris-HCL (pH 7.4), 100mM NaCl and 0.25 units Protac (HYPHEN BioMed, Neuville-Sur-Oise, France), in a total volume of 1ml, for 1 hour at 37°C. This APC was then stored in small aliquots at -80°C.

2.2.10 Chromogenic determination of APC concentration

To determine the catalytic activity of each protein C variant activated using Protac, APC

steady state hydrolysis of the APC specific chromogenic substrate, BIOPHEN CS21 (66) (Hyphen-Biomed, Neuville-Sur-Oise, France) was measured. The cleavage of this substrate was determined by reading the change in absorbance over time at 405nm of the sample in a 96well plate. APC of unknown concentration was incubated in 100mM NaCl, 2.5mM CaCl₂, 20mM Tris-HCL (pH 7.5) and 0.1mg/ml bovine serum albumin (BSA) with 0.3mg/ml BIOPHEN CS21 (66). BIOPHEN CS21 (66) hydrolysis was then assessed at 405nm for 10min using a VersaMax® plate reader (Molecular Devices). The chromogenic substrate was added immediately before the plate was read and the final volume in the well was 50µl. The rate of the unknown APC concentration was then compared to a standard curve of known APC concentrations (Figure 2.9) and this allowed the concentration of the unknown to be determined. APC of known concentrations were made up over a range of 0-20nM using hAPC from Haematologic Technologies Inc. The concentration of the stored recombinant APC (which ranged from 19nM to 110nM APC) was checked prior to use, to ensure accuracy. The benefit of checking the accuracy of the APC concentration in this manner was that it ensured that the APC was catalytically active, as opposed to an ELISA which would just show the actual concentration of the protein.

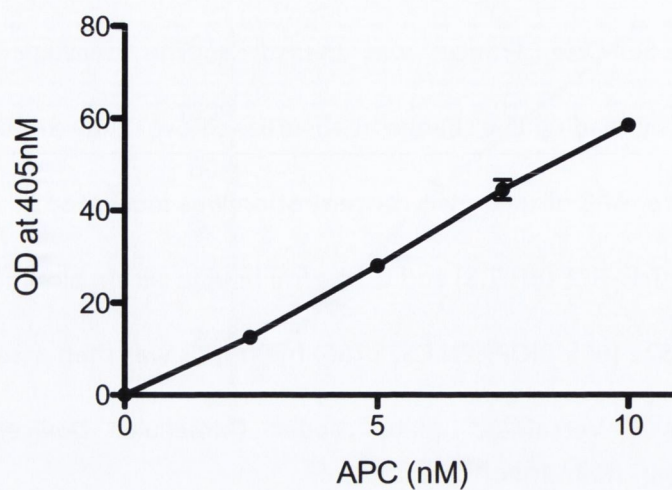


Figure 2-9: Typical standard curve using APC of known concentration

2.3 Functional analysis of recombinant protein C variants

The anticoagulant activity of the recombinant protein C variants were assessed for their sensitivity to neutral lipid enhancement. Phospholipid vesicles of different compositions were prepared and the effect of each lipid preparation upon APC anticoagulant activity determined using a thrombin generation assay (Section 2.3.3). Then, the effect of different phospholipid vesicles on APC proteolysis of FVa, either protein S dependent or independent inactivation, was assessed using the purified FVa inactivation assay (Section 2.3.5 and 2.3.6). Recombinant protein C binding to EPCR was assessed using surface plasmon resonance (Section 2.3.7). Separately, the effect of plasma lipoproteins on APC cytoprotective activity was investigated. The thrombin induced endothelial cell permeability assay was used as a model of APC-EPCR/PAR1 protective signalling (Section 2.3.8). The effect of HDL on APC protective signalling was assessed using this assay. To assess whether HDL has any effect on protein C activation, protein C activation on the surface of endothelial cells was investigated (Section 2.3.9).

2.3.1 Preparation of phospholipid vesicles

Lyophilised pre-formulated phospholipids (dioleoylphosphatidylcholine (DOPC)/dioleoylphosphatidylserine (DOPS), 80%:20% w/w, DOPC/DOPS/dioleylethanolamine (DOPE) 60%:20%:20% w/w and DOPC/DOPS/GlcCer 60%:20%:20% w/w) were purchased from Avanti Polar Lipids, AL, USA. These concentrations were used as they are believed to represent cell membranes and have

been used in others investigating the role of PE and GlcCer on APC anticoagulant activity^{42,79}. These lyophilised phospholipids were supplied in 10mg aliquot as dried lyophilized lipids and stored at -20°C. These lipids were prepared by hydration using 1ml H₂O, final concentration of lipids in glass vial to 10mg/ml. The phospholipid vesicles were then mixed vigorously, using a vortex, until a homogenized, cloudy suspension of the lipids was observed. To achieve small unilamellar vesicles, the phospholipid vesicles were extruded 11 times through a 0.1µm polycarbonate membrane (Avanti Polar Lipids, AL, USA) using an Avanti Mini-Extruder with glass syringes (Avanti Polar Lipids, AL, USA).

Dilinoleoyl-sn glycerol-3-phosphoethanolamine (DIPE), DOPS, DOPC, C8 β-D-glucosylceramide (C8-GlcCer), C12 β-D lactosylceramide (C12-LacCer) and C12 β-D Glucosylceramide (C12-GlcCer) were obtained in chloroform solutions from Avanti Lipids (AL, USA) and stored at -20°C. Phospholipid mixtures were prepared in 4mg amounts in brown glass vials and the chloroform evaporated using a slow flow of nitrogen gas and unilamellar vesicles were then prepared as above.

Phospholipid vesicles were kept at 4°C and discarded after 4 days of storage.

2.3.2 Lipoproteins

HDL and LDL derived from human plasma were purchased from Merck Biosciences. These were native HDL or LDL isolated from human plasma. Composition of the HDL was 45-55% lipid, 45-55% protein. HDL came in 10mg vials, which contained about 5mg

of protein. This was reconstituted with 0.5-1ml of water, depending on the concentration required. LDL had a composition of 78-81% lipid, 19-22% protein. LDL came in 10mg vials, which contained about 2mg of protein. This was reconstituted with 0.5-1ml of water, depending on the concentration required. Both preparations of lipoproteins were stored at 4°C, for up to 3 days.

2.3.3 Determination of APC anticoagulant activity in protein C-deficient plasma

To investigate APC anticoagulant activity and the effects of different phospholipid preparations, the Calibrated Automated Thrombogram (CAT) assay was used with thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). The thrombogram describes the formation of thrombin in plasma in real time as the plasma is clotting. It is therefore a useful tool to assay both procoagulant and anticoagulant activities of coagulation factors. The endogenous thrombin potential (ETP) is beneficial when investigating APC anticoagulant activity as it examines thrombin formation, as opposed to the activated partial thromboplastin time (APTT) and prothrombin time (PT) which only measures the time for the blood to clot.

The benefit of using the CAT assay in our study is that the phospholipid vesicles can be altered in each sample so that we can examine the effect of different phospholipid vesicles. Thrombin generation was measured using the Fluoroskan Ascent Plate Reader (Thermo Lab System, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). The rate of thrombin generation in plasma is measured by cleavage of the fluorescence thrombin substrate, Z-Gly-Gly-Arg-AMC.HCL. A thrombin calibrator (Tcal) standard (Thrombinoscope BV, Maastricht, the

Netherlands) is included on every plate as this ensures the accurate determination of the molar concentration of the thrombin generated.

In the normal CAT assay, 20 μ l of a PPP reagent (Thrombinscope BV, Maastricht, the Netherlands), which contains a mixture of phospholipids and TF, is added to the 80 μ l platelet poor plasma in a round bottom 96-well plate. FluCa is made up using warmed Fluo-Buffer, which contains Hepes (pH 7.4) and CaCl₂, and Fluo-substrate, which contains the fluorogenic substrate solubilized in DMSO (Thrombinscope BV, Maastricht, the Netherlands) and 20 μ l of FluCa is used to initiate thrombin generation (total volume in each well is 120 μ l).

This assay was altered for this study by using a mixture of sTF and the reconstituted phospholipid vesicles e.g. PC/PS, and APC to a total volume of 20 μ l instead of the PPP reagent.

For the measurement of APC anticoagulant activity, 80 μ l protein C deficient plasma (Hyphen-Biomed, Neuville-Sur-Oise, France) was incubated with 10 μ M of the indicated phospholipid vesicles and 5pM soluble tissue factor (sTF, Dade-Innovin) in the presence or absence of APC (final volume in each well of 100 μ l). The APC was added to the plasma immediately before FluCa to prevent its inactivation by plasma inhibitors. Thrombin generation was initiated with 20 μ l FluCa (total volume in each well 120 μ l). The ETP and various other parameters for thrombin generation, including lag time, time to peak, peak thrombin (Figure 2.10) were then measured using the Thrombinscope software in real time over the course of 60min. Experiments were performed in triplicate and results recorded as ETP.

Bovine APC (bAPC: Haematologic Technologies) anticoagulant activity was assessed using the CAT assay with the PPP reagent in bovine plasma (Sigma). 80 μ l of bovine

plasma was incubated with 20µl PPP reagent in the presence of 10nM bAPC to a total volume of 110µl and thrombin generation initiated with FluCa.

Normal Thrombogram in platelet poor plasma

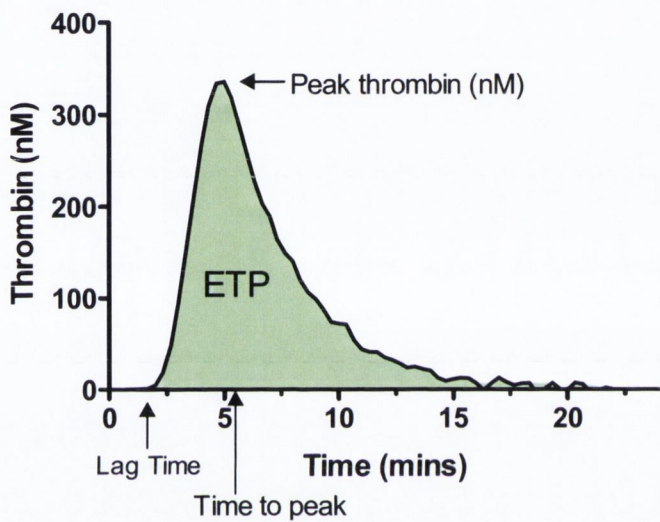


Figure 2-10: Thrombin generation curve in platelet poor plasma.

Normal calibrated automated thrombogram (CAT) showing some of the important parameters of thrombin generation: lag time, time to peak, and peak height and endogenous thrombin potential (ETP). ETP is defined as the area under the thrombin generation curve.

2.3.4 Protein S enhancement of APC anticoagulant activity in protein S-deficient plasma

The effect of PE and GlcCer in the phospholipid vesicles upon protein S cofactor enhancement of APC anticoagulant function was assessed using a modified thrombin generation assay to that described above. 80µl protein S-deficient plasma (Hyphen-

Biomed, Neuville-Sur-Oise, France) in the presence of 10 μ M of the indicated phospholipid vesicles and 5pM soluble tissue factor, was incubated with APC (10nM) in the presence or absence of plasma-purified protein S (9-150nM), to a final volume in each well of 110 μ l. Again to prevent inactivation by plasma inhibitors, the protein S and APC were added to the plasma immediately before the addition of the fluorogenic substrate. Thrombin generation was then initiated with the fluorogenic substrate and the ETP determined as before (Section 2.3.3). All experiments were performed in triplicate and results recorded as the mean ETP.

2.3.5 Determination of the effects of phospholipid composition on protein S mediated proteolysis of FVa by APC

The effect of PE and GlcCer on protein S mediated proteolysis of FVa by APC was investigated using a purified FVa inactivation assay as previously described³⁶. Human protein S (3.25-50nM) was incubated with 8nM APC, 28nM FVa and 80 μ M phospholipid vesicles in 40mM Tris-HCl (pH 7.4), 150mM NaCl, 3mM CaCl₂, 0.3% (w/v) BSA (2nM APC, 7nM FVa, and 20 μ M phospholipid vesicles, final concentrations) for 2min at 37°C. Following this incubation, a 2 μ l aliquot was added to 1nM FXa, 0.5 μ M prothrombin and 25 μ M phospholipid vesicles (final concentrations) at 37°C for 3min, and the reaction stopped by addition of 5 μ l of ice cold 0.5M EDTA. 40 μ l of the reaction mixture was then removed and incubated with 40 μ l of 2mM thrombin chromogenic substrate BIOPHEN CS-01(38) (Hyphen-Biomed, Neuville-Sur-Oise, France) to assess thrombin generation, as the rate of thrombin generation is proportional to FVa activity. The rate of cleavage was measured on plate reader at 405nm for 10min at room temperature. Experiments

were performed in triplicate and data plotted as mean residual FVa cofactor activity $\pm$ SEM, with no APC samples defined as 100% FVa cofactor activity. A typical result is shown in Figure 2.11..

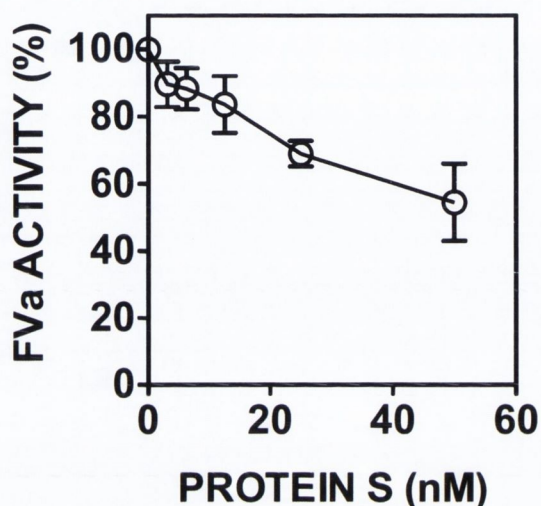


Figure 2-11: FVa inactivation by APC in the presence of protein S.

A typical graph of protein S dependent FVa inactivation by APC in the presence of PC/PS. Samples that contained no APC were defined as 100% FVa cofactor activity.

2.3.6 Determination of the effects of phospholipid composition on Protein S

Independent APC-Mediated Factor Va Proteolysis

To investigate the effect of different phospholipid vesicles on the protein S independent APC-mediated FVa inactivation, FVa degradation by APC was assessed in a purified system, similarly to previously described methods. 8nM APC was incubated at 37°C with 80μM phospholipid vesicles and 4nM FVa (Hematologic Technologies Inc.) in 40mM Tris-HCl, 140mM NaCl, 3mM CaCl₂ and 0.3% w/v BSA (2nM APC, 20μM phospholipids and 1nM FVa, final concentrations). At specified time points over 20min,

2 μ l aliquots were removed and added to a prothrombinase mixture (25 μ M phospholipid vesicles, 1nM factor Xa and 0.5 μ M prothrombin, final concentrations, Hematologic Technologies Inc.) for 3min at 37°C. Each reaction was then stopped using 5 μ l of ice-cold 0.5M EDTA. 40 μ l of the reaction mixture was removed and incubated with 40 μ l of 2mM thrombin chromogenic substrate BIOPHEN CS-01(38) (Hyphen-Biomed, Neuville-Sur-Oise, France) to assess thrombin generation, as the rate of thrombin generation is proportional to the FVa activity. The rate of chromogenic substrate cleavage was measured at 405nm for 10min at room temperature using a plate reader. Samples that contained no APC were defined as 100% FVa cofactor activity. All experiments were performed in triplicate and data represented as the mean residual FVa cofactor activity $\pm$ SEM. A typical result is shown in Figure 2.12.

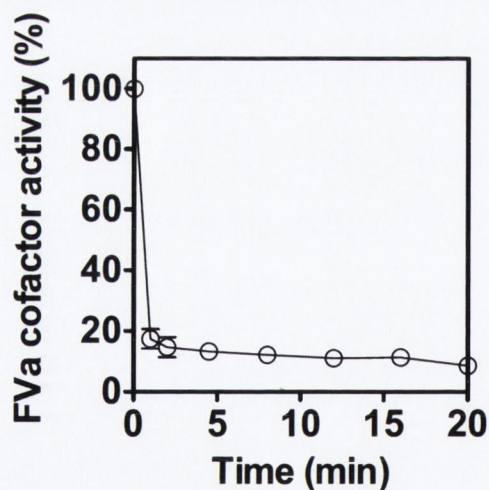


Figure 2-12: FVa inactivation by APC in the absence of protein S.

A typical graph of protein S independent FVa inactivation by APC in the presence of PC/PS phospholipids. Samples that contained no APC were defined as 100% FVa cofactor activity.

2.3.7 Protein C binding to EPCR

To assess the binding affinities of the recombinant protein C variants to soluble EPCR (sEPCR) surface plasmon resonance (SPR) analysis using the BIAcore X100 biosensor system (GE Life Sciences) was used (Figure 2.13). SPR is a process whereby upon binding to the surface the molecule alters the refractive index of the light, and this change can be monitored in real time.

10µg/ml of the anti-EPCR monoclonal antibody, RCR-2 (kind gift of Dr. K. Fukudome, Saga Medical School) was permanently covalently immobilised onto a carboxymethylated dextran (CM5) sensor chip (GE healthcare) using amine coupling chemistry. RCR-2 was chosen as it does not inhibit protein C binding to EPCR, so allowing the protein C-EPCR protein interaction to be studied. Amine coupling results in a random orientation of the receptors on the surface. Amine coupling is achieved by activation of the carboxymethyl dextran surface of the sensory chip using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). This creates amino-reactive intermediates, which are unstable and react with the primary amine groups of the RCR-2 ligand. This reaction creates covalent bonds between the sensor chip and RCR-2 (Figure 2.13). This technique was used to covalent attach RCR-2 to the two flow cells of the CM5 sensor chip (control and test cell).

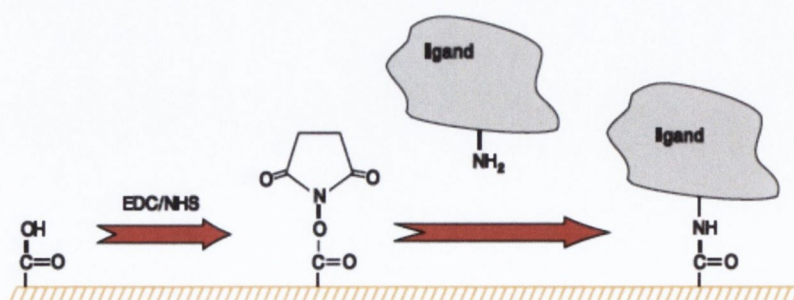
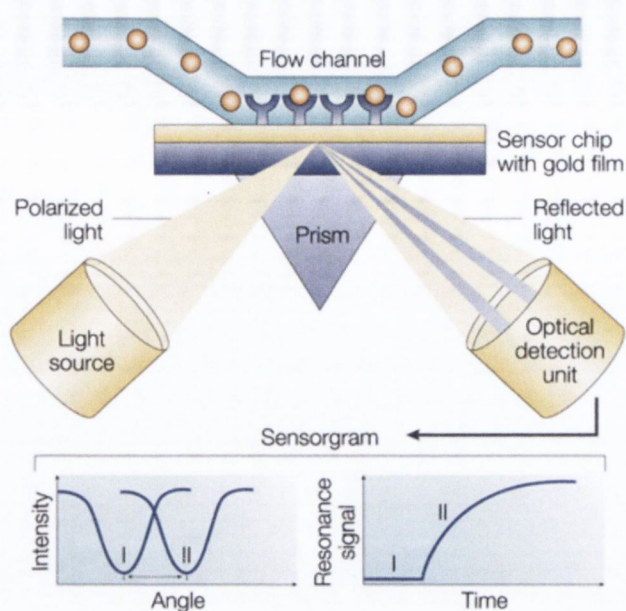


Figure 2-13: Amino coupling of ligand (RCR-2) to sensory chip (CM5).

sEPCR in HBS-P buffer (100mM HEPES, pH7.4, 150mM NaCl, 0.005% v/v surfactant P20) was then bound to RCR-2 on the test flow cell (flow cell 2) until a response of approximately 1000 response units (RU) was achieved. Flow cell 1, with no sEPCR present, was used as a control to detect any non-specific binding.

Wild type or variant protein C (30-1000nM) was then injected over both flow cells at flow rate of 5 μ l/min for 90s. The binding between protein C and sEPCR was recorded in real time. The protein C-sEPCR binding was dissociated using HBS-EP buffer (HBS-P buffer containing 3mM EDTA: Biacore). A typical association and dissociation graph is shown in Figure 2.15.

The RCR-2 surface was regenerated using 10 μ l of 10mM glycine-HCL (pH 2: Biacore) after each experiment and the CM5 chip stored at 4°C.



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Figure 2-14: Surface Plasmon Resonance (SPR) set up.

SPR principles: Changes in the refractive index in the vicinity of sensor ship is detected and seen as a sharp decrease in the reflected light (sensorgram). This angle shifts (from I to II) when molecules bind to the surface and change the mass. This shifting of the angle is recorded in real time and plotted as resonance signal (which is proportional to mass change) versus time¹⁴³.

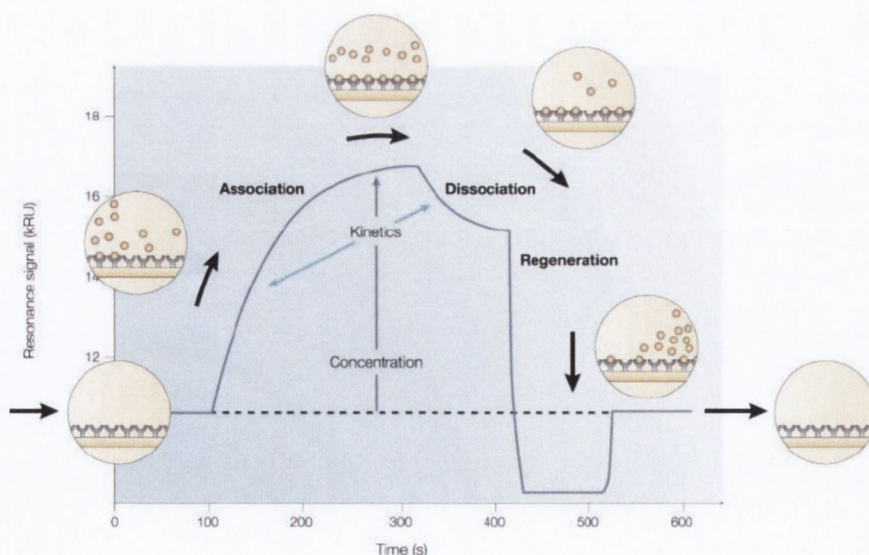


Figure 2-15: Typical Biacore graph showing the association and dissociation of a molecule with the receptor on chip¹⁴³.

In this study sEPCR was bound to the chip and protein C was the passed over the surface¹⁴³.

2.3.8 Measurement of endothelial cell barrier protection by APC

The endothelial cell barrier permeability assay was used to measure APC ability to protect endothelial cells from thrombin-induced permeability. EA.hy926 cells were grown to confluency on either 6-well or 12-well 3µm transwells inserts (Milipore). When confluent the cells were rinsed carefully in PBS (Sigma, UK) and media changed to serum-free DMEM media in the presence of 3mM CaCl₂ and 0.6mM MgCl₂, and the indicated concentrations of APC and/or HDL (0-20nM APC and/or 0-1.5mg/ml HDL). If blocking antibodies (e.g. RCR-252) were used, the cells were first incubated at 37°C in the presence of each antibody for 10min prior to the addition of APC. The cells were then incubated for 3h at 37°C. 5nM thrombin (Sigma, UK) was then added to the cells for 10min at 37°C. After which the cells were washed with PBS. The inner chamber (insert) was then incubated with 0.67mg/ml Evans Blue with 4% BSA (Sigma) in DMEM and the outer chamber incubated with DMEM at 37°C for 20min. Changes in endothelial cell barrier permeability were determined by taking 100µl (6-well inserts) or 50µl (12-well inserts) aliquots from the outer chamber and measuring the increase in absorbance at 650nm. Any increase in absorbance is due to the transmigration of Evans Blue-BSA into the outer chamber with increasing transmigration signifying an increase in the barrier permeability. If 50µl aliquot was taken this was added to 50µl of normal DMEM media prior to absorbance being read. The total 100 µl of each sample was placed in a 96 well plate and the absorbance read on the VersaMax® plate reader (Molecular Devices) at 650nm. Experiments were performed in triplicate and plotted as the mean OD₆₅₀ ± SEM. A typical result is shown in Figure 2.16.

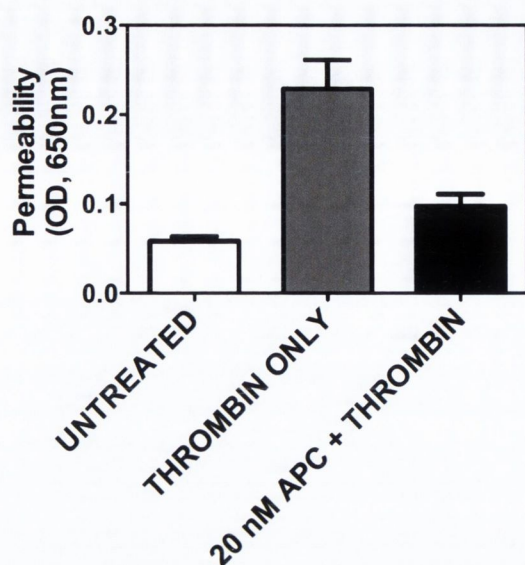


Figure 2-16: A typical endothelial permeability assay.

This graph shows the increased permeability of the EA.hy926 cells in the presence of thrombin (grey column), shown by the increase in the OD_{650nm} versus the untreated/control cells (white column). The protective effects of APC can be seen by the decrease in OD_{650nm} of the APC treated cells (black column). There is a statistically significant difference between thrombin only (grey) and those cells pretreated with APC before thrombin treatment (black), $p < 0.05$, showing that APC protect the cells from thrombin induced permeability.

2.3.9 Protein C activation on endothelial cells

To measure the rate of protein C activation on the surface of endothelial cells, EA.hy926 cells were plated in 96-well flat bottom sterile plates and grown to confluency. Then the cells were washed twice in HBSS (Sigma). Serial dilutions of protein C (500-62.5nM) were made in HBSS with 3mM $CaCl_2$ and 0.6mM $MgCl_2$ and placed on the cells, final volume of 100 μ l. 10nM thrombin was added to each well for 30min at 37°C, final volume of 110 μ l in each well. The activation of protein C was stopped by the addition of 5 μ l of 10 units/ml of hirudin, a direct thrombin inhibitor. The amount of APC generated was then determined by removing 50 μ l of the reaction

mixture and adding 50µl BIOPHEN CS 21(66) APC chromogenic substrate (Figure 2.17). The rate of cleavage was determined by assessment of absorbance at 405nm on the VersaMax® plate reader (Molecular Devices). The K_m (Michaelis-Menten constant: which is the substance concentration at which half maximal velocity occurs) was then determined by plotting the V_{max} for substrate cleavage against protein C concentration using Michaelis-Menten nonlinear regression method using GraphPad Prism.

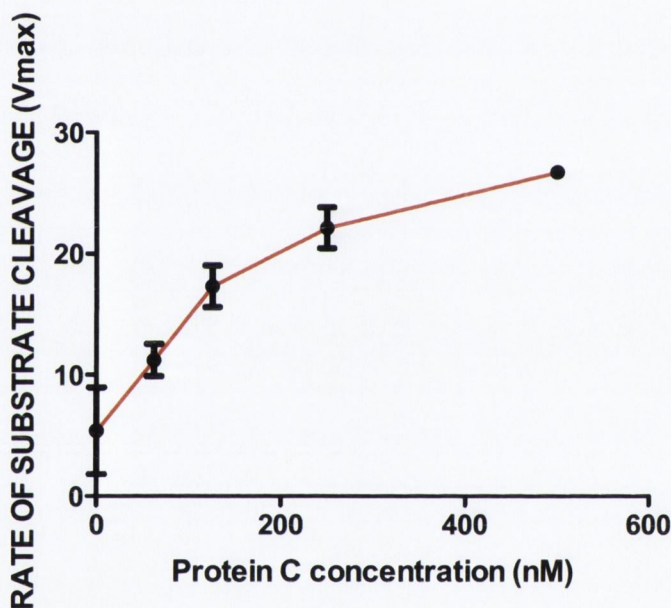


Figure 2-17: Protein C activation on the surface of EA.hy926 cells.

This is a typical graph obtained for protein C activation by thrombin on the surface of EA.hy926 cells. Protein C (62.5-500nM) was incubated on the surface of the cells in the presence of thrombin and the amount of APC generated determined by the rate of substrate cleavage. This plot shows the V_{max} for each protein C concentration.

2.3.10 Inhibition of APC in plasma

To assess the inhibition of protein C in plasma both variant and wild type APC (APC-WT)

was inhibited in plasma and the loss of APC activity determined. APC-WT and/or recombinant APC variant at 20nM was incubated in normal pooled plasma (NPP) at 37°C (total volume 0.5ml). At designated time points, over the course of 60min, aliquots (50µl) were removed and the residual APC amidolytic activity was determined, as before, using the chromogenic substrate for APC (BIOPHEN CS2166). 100% amidolytic activity was defined as the Vmax for the sample removed immediately i.e. at the 0 minute time point. Results were present as mean APC amidolytic activity $\pm$ SEM.

2.3.11 PyMOL software imaging

PyMOL molecular visualization system (www.pymol.org) was used to generate a space-occupying model of the APC Gla domain, using structure of APC bound to EPCR as the template. Residue isoleucine-18 of APC Gla domain was then highlighted.

2.3.12 Data and statistics

Data was analysed by Graph Pad prism version 5.0 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com). All assay results were reported in triplicate and presented as the mean $\pm$ SEM. To assess statistical differences unpaired two-tailed student *t* test was used and values of $p < 0.05$ were reported as statistically significant.

CHAPTER 3 Identification of amino acids in protein C Gla domain involved in lipid mediated enhancement of APC anticoagulant activity

3.1 Importance of PE and GlcCer enhancement of APC anticoagulant activity

In this chapter the enhancement effects of the neutral lipids PE and GlcCer on APC anticoagulant activity is investigated. Despite the fact that it is well established that PE and GlcCer enhance APC anticoagulant activity^{74,82}, the molecular basis for this activity remains unclear. As this neutral lipid enhancement is clinically significant^{80,81} it was decided to investigate the mechanism behind this enhancement, in the hope of increasing the understanding of the anticoagulant activity of APC *In vivo*.

It is well established that the APC Gla domain plays a vital role in phospholipid binding⁴². Currently, it is also believed that the Gla domain of APC plays a key role in mediating the specific neutral lipid enhancement of APC anticoagulant activity⁴². Therefore, in this chapter a series of APC Gla domain variants are examined in the hopes of identifying the region of the APC Gla domain that is involved in mediating this effect. The fact that despite the significant homology between the prothrombin Gla domain and the protein C Gla domain, prothrombin is not responsive to the presence of neutral lipids was utilised⁴². Therefore, substitutions between non-conserved Gla domain residues of the prothrombin Gla domain were inserted into the protein C Gla domain¹³⁸. The APC variants tested are shown in Table 3.1. These variants had been previously expressed and characterized in the laboratory (Table 3.1)^{36,40}.

In order to investigate the APC Gla domain residue responsible for this neutral lipid enhancement, a thrombin generation assay was developed which is sensitive to the presence of phospholipid and APC, and then using this tool the series of APC variants were examined for their sensitivity to PE and GlcCer. In the next chapter the role of protein S in this lipid enhancement will be investigated.

Each recombinant protein C variant was activated with Protac and the APC concentration determined (Section 2.3.3). Prior to each and every experiment the APC concentration was checked to ensure that any changes observed were due to the mutation in the protein C Gla domain and not the result of concentration differences.

Table 3-1 Properties of recombinant protein C Gla domain variants

Properties of protein C variants which have previously been established^{36,40}. Activity is indicated relative to wild type protein C (unknown activity is left blank).

APC Gla domain variant	EPCR affinity	Anticoagulant activity	Cytoprotective activity
APC-S3T	Normal	Normal	
APC-L8V/H10K	No affinity	Normal	
APC-S11G/S12M	Normal	Increased mildly (increased phospholipid binding)	
APC-I18V	Normal	Normal	
APC-D36T		Decreased mildly	
APC-L38D	Normal	Decreased substantially (not sensitive to protein S)	Normal
APC-A39V		Decreased mildly	
APC-S42A/H44Y/V45T	Normal	Normal	

3.2 Phospholipid composition and the thrombin generation assay

To determine the anticoagulant activity of APC in the presence of each phospholipid

preparation, a thrombin generation assay was developed. Protein C deficient plasma was used so that it could be established that any anticoagulant effect observed was due to the recombinant protein C variants that were added to the plasma. A concentration of 5pM sTF was used in all experiments, as at this concentration there was a good, reliable thrombin generation; with a lag time of less than 3min showing that the thrombin generated was due to the normal physiological coagulation process and not due as a result of the contact pathway.

Another important issue was to ensure that the presence of PE or GlcCer in the phospholipid vesicle did not affect the amount of thrombin generated, as this ensured that any results observed were due to an enhancement effect of these lipids on APC anticoagulant activity alone, and not due to any direct effect on thrombin generation. Therefore the role of phospholipid vesicles containing PE or GlcCer in mediating TF-initiated thrombin generation was assessed in the presence of PC/PS (80%:20%), PC/PS/PE (60%/20%/20%) or PC/PS/GlcCer (60%/20%/20%) lipid vesicles in protein C deficient plasma. All three phospholipid mixtures resulted in a similar amount of thrombin generated (Figure 3.1). The ETP for PC/PS was $1307.5 \pm 11.5 \text{ nM/min}$, PC/PS/PE was $1351 \pm 19 \text{ nM/min}$ and PC/PS/GlcCer $1282.5 \pm 21.5 \text{ nM/min}$ with no statistically significant difference between these groups (with $p > 0.07$ for PC/PS/PE or PC/PS/GlcCer compared to PC/PS).

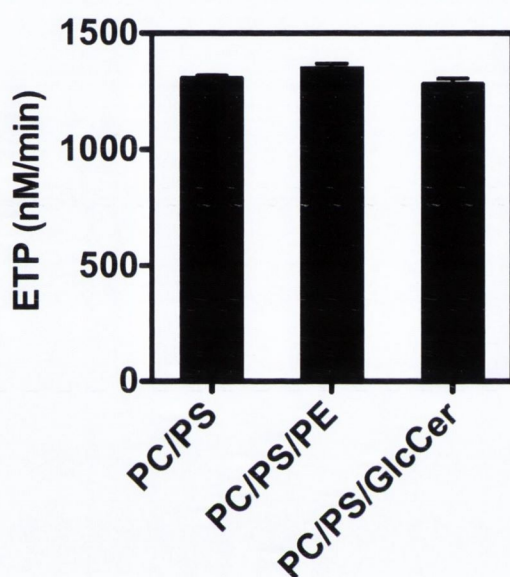


Figure 3-1: Amount of thrombin generation in the presence of different phospholipid vesicles is similar.

Thrombin generation in protein C-deficient plasma in the presence of each lipid preparation (80%/20% PC/PS, 60%/20%/20% PC/PS/PE and 60%/20%/20% PC/PS/GlcCer) was determined. The results represent the mean $\pm$ SEM for 3 independent experiments.

3.3 PE and GlcCer enhancement of APC anticoagulant activity

Next this ETP based assay was examined to ensure that it was sensitive to PE and GlcCer enhancement of APC anticoagulant activity. To characterize APC anticoagulant activity in this setting, recombinant APC-WT (1.5-6nM) was added to the plasma and the ability of APC to inhibit thrombin generation in the presence of each phospholipid preparation was determined (Figure 3.2).

In the presence of PC/PS APC-WT had little ability to decrease thrombin generation (Figure 3.2; top panel). Even at the highest concentration of APC-WT (6nM: Figure 3.2; top panel black circles), the thrombin generation is not significantly decreased compared to in the absence of APC (Figure 3.2; top panel white circles).

Similar levels of thrombin generation were found in the presence of either PC/PS/PE or PC/PS in the absence of APC-WT (Figure 3.2). However in the presence of increasing APC-WT concentration there was a corresponding decrease in the thrombin generated, showing APC increased anticoagulant activity. In the presence of 6nM APC-WT and PC/PS/PE (Figure 3.2; middle panel black circles) there is a dramatically reduced amount of thrombin generated compared to that in the absence of APC, $p < 0.007$ (Figure 3.2; middle panel white circles).

The behaviour of APC in the presence of PC/PS/GlcCer was similar to that of seen in the presence of PC/PS/PE. In the presence of 6nM APC-WT and PC/PS/GlcCer there is a much lower amount of thrombin generated (Figure 3.2; bottom panel black circles) compared to no APC, $p < 0.003$ (Figure 3.3; bottom panel white circles).

While the amount of thrombin generated in the presence of PC/PS, PC/PS/PE or PC/PS/GlcCer was determined to be similar in the absence of APC (Figure 3.2) the rate of the thrombin generated (as seen by the shape of the curves in Figure 3.2; white circles) appears to be slightly altered for PC/PS/PE compared to those of PC/PS or PC/PS/GlcCer. This can be observed by the higher peak height for PC/PS/PE which is about 240nM while for the other lipids it remains below 200nM thrombin. Also, in the presence of PE there is a shift of the graph to the right representing the fact the reaction occurs at a faster rate in presence of PE, compared to PC/PS and PC/PS/GlcCer (Figure 3.2). The precise mechanism for this observation is unclear. However, this most likely represents a faster rate of proteolysis by APC in the presence of PE within phospholipid mixture compared with PC/PS/GlcCer.

Figure 3.3 shows the amount of thrombin generated with either PC/PS, PC/PS/PE or PC/PS/GlcCer in the presence of a range of APC concentrations. 100% ETP was set at the amount of thrombin generated in the absence of APC. 6nM APC reduced total thrombin generation (ETP) by $75\pm3\%$ and $88\pm2\%$ with PC/PS/PE and PC/PS/GlcCer vesicles respectively, but only reduced thrombin generation by $37\pm3\%$ in the presence of PC/PS vesicles alone (Figure 3.3). This represents a statistically significant difference (with $p<0.008$) in the amount of thrombin generated in the presence APC and PC/PS/PE or PC/PS/GlcCer compared to PC/PS.

This data confirms that the presence of PE or GlcCer enhances APC anticoagulant activity in plasma. These results demonstrate that the thrombin generation assay used in this project is sensitive to a) APC anticoagulant effect and b) that the presence of PE/GlcCer impacts upon the protein C pathway and not the procoagulant thrombin

generation pathway. Therefore, we can use this assay to screen the recombinant protein C variants for their sensitivity to enhancement of the specific lipids PE or GlcCer.

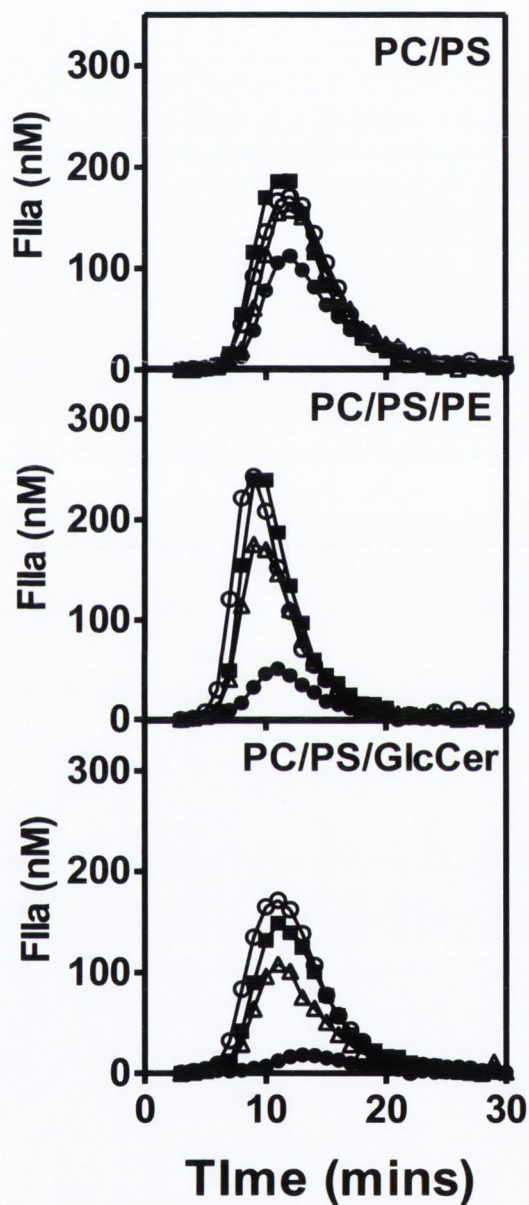


Figure 3-2: PE and GlcCer-containing phospholipid vesicles enhance the anticoagulant activity of APC-WT.

APC anticoagulant activity was assessed in the presence of 10 μ M lipid vesicles containing 80%/20% PC/PS (top panel), 60%/20%/20% PC/PS/PE (middle panel) or 60%/20%/20% PC/PS/GlcCer (bottom panel) (○, no APC; ■, 1.5nM APC; 3nM APC, △; 6nM APC, ●). The results represent the mean $\pm$ SEM for 3 independent experiments.

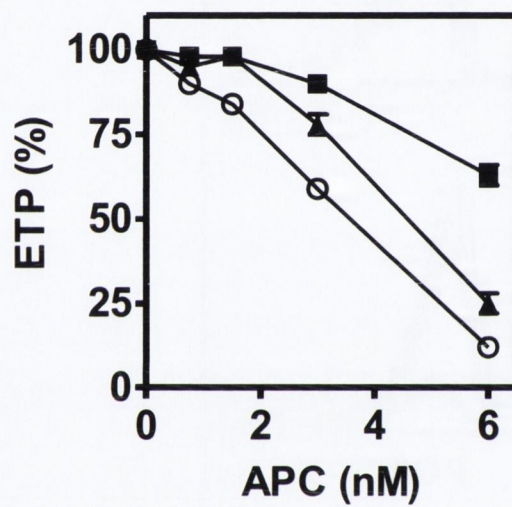


Figure 3-3: PE and GlcCer-containing phospholipid vesicles enhance the anticoagulant activity of APC-WT.

The endogenous thrombin potential (ETP, % of thrombin generated in the absence of APC) was determined with each lipid preparation (80%/20% PC/PS, ■; 60%/20%/20% PC/PS/PE, ▲; 60%/20%/20% PC/PS/GlcCer, ○) in the presence of APC (0.75-6nM). The results represent the mean $\pm$ SEM for 3 independent experiments.

3.4 Identification of APC Gla domain amino acid residues that mediate PE and GlcCer enhancement of APC anticoagulant activity

As the APC Gla domain mediates binding to specific membrane phospholipids, APC Gla domain variants (Table 3.1) were utilised to determine which specific amino acid residues within this region can mediate sensitivity to the enhancement effect of PE and GlcCer on APC anticoagulant activity. The anticoagulant activity of each APC variant was determined in the presence of lipid vesicles containing PC/PS, PC/PS/PE or PC/PS/GlcCer. The thrombin generated in the presence of PC/PS and 10nM of each APC was defined as 'normal'. Fold changes were calculated based on the enhancement of PC/PS/PE or PC/PS/GlcCer. As observed in the previous experiments, APC-WT anticoagulant activity was found to be enhanced approximately 3-fold by the presence of lipids containing either PE or GlcCer compared to PC/PS vesicles alone (Figure 3.4). This represent a statistically significant difference in APC activity between PC/PS and either PC/PS/PE or PC/PS/GlcCer with $P < 0.002$ and $P < 0.03$, respectively

The majority of the APC variants tested were found to enhance APC anticoagulant activity 2-4 fold in the presence of PC/PS/PE and PC/PS/GlcCer (Figure 3.4). The effect of lipid composition on the variant APC-L38D could not be determined, as this variant has little anticoagulant activity due a lack of protein S affinity⁴⁰.

APC-S3T exhibited mildly impaired GlcCer-mediated anticoagulant activity (Figure 3.4B), but was enhanced normally by PE (Figure 3.4A).

APC-L8V/H10K was found to have similar enhancement by both PE and GlcCer

compared to APC-WT. This is interesting as it is known that L8 in APC Gla domain has been previously shown to be critical for EPCR binding³⁶.

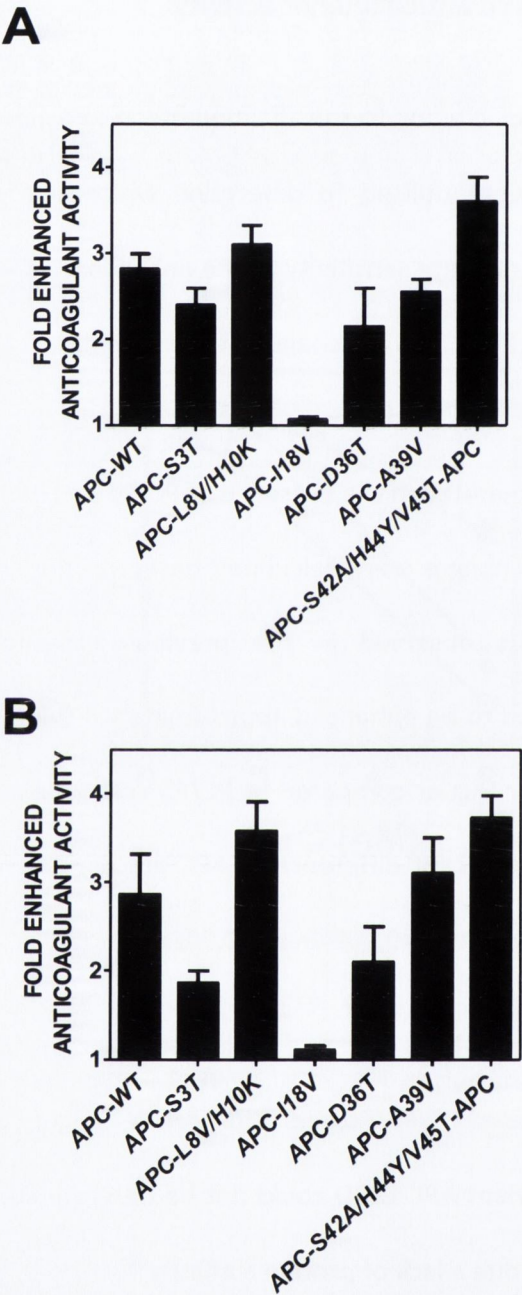


Figure 3-4: Ile-18 is critical for mediating both PE and GlcCer stimulation of APC anticoagulant activity.

Thrombin generation was determined in protein C-deficient plasma in the presence of 10 μ M of each lipid vesicle preparation (PC/PS, PC/PS/PE or PC/PS/GlcCer, ratios as before) and 10nM APC-WT or variant APC. The fold-enhancement of APC anticoagulant activity observed with **A**, PC/PS/PE or **B**, PC/PS/GlcCer compared to PC/PS was determined. The results represent the mean $\pm$ SEM for 3 independent experiments.

However, the anticoagulant activity of APC-I18V was not increased by the presence of PC/PS/PE or PC/PS/GlcCer vesicles (Figure 3.4). There represent a significant difference between APC-WT and APC-I18V in the presence of PC/PS/PE ($p<0.05$) or PC/PS/GlcCer ($p<0.05$). APC-I18V while behaving similarly to that of APC-WT in the presence of PC/PS, was not effected by the presence of PE or GlcCer and therefore showed no enhancement (Figure 3.4). This data indicates that APC Gla domain amino acid residue Ile-18 plays a critical role in mediating the effect of specific membrane lipid cofactor stimulation of APC anticoagulant function.

Interestingly, *APC-S11G/S12N* exhibited dramatically enhanced anticoagulant activity compared to APC-WT in the presence of all lipid vesicle compositions, including PC/PS (Figure 3.5). Due to this dramatic increase in anticoagulant activity, a much lower APC concentration of 1.25nM was used in order to observe the effect of lipid composition. It was observed that the enhancement effect observed for APC-S11G/S12N was approximately 7 fold in the presence of either PE or GlcCer compared with that for PC/PS. This shows that this region in the APC Gla domain is very important for mediating lipid affinity for anticoagulant activity.

Due to its lack of sensitivity to both PE and GlcCer, APC-I18V was chosen for further study.

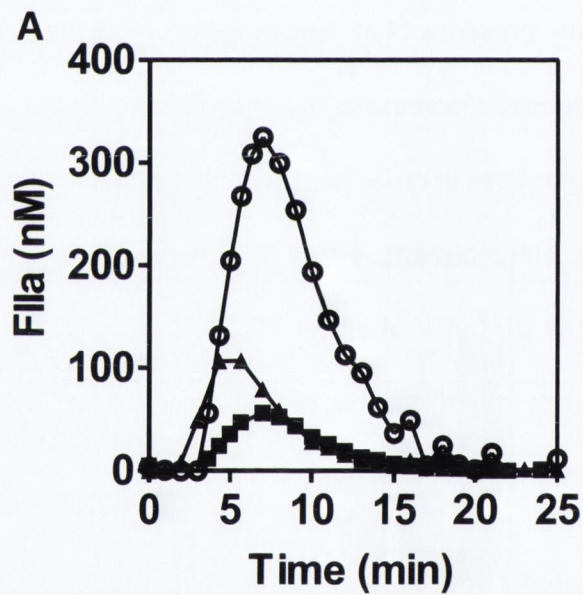


Figure 3-5: Altered thrombin generation in the presence some protein C Gla domain variants. Inhibition of thrombin generation in protein C-deficient plasma in the presence of the APC variant APC-S11G/S12N at 1.25nM and 10 μ M of each lipid vesicle preparation tested (PC/PS, ○; PC/PS/PE, ▲; PC/PS/GlcCer, ■). The results represent the mean $\pm$ SEM for 3 independent experiments.

3.5 APC-I18V is insensitive to other membrane lipids which enhance APC anticoagulant activity

In addition to PE and GlcCer, several other membrane lipids have been described which can affect APC anticoagulant activity⁸². These include dilinoleoyl-sn glycerol-3-phosphoethanolamine (DIPE), C8 β -D-glucosylceramide (C8-GlcCer), C12 β -D-lactosylceramide (C12-LacCer) and C12 β -D glucosylceramide (C12-GlcCer)⁸². The effect of these lipids on the enhancement of APC anticoagulant activity was determined (Figure 3.6, black bars). APC-WT anticoagulant activity was enhanced just over 2-fold in the presence of DIPE, C8-GlcCer, C12-GlcCer and LacCer (Figure 3.6) which was less than the 3-fold enhancement found with PE and GlcCer. The enhancement of anticoagulant activity of APC-WT in the presence of either DIPE, C8-GlcCer, C12-GlcCer or LacCer compared to PCPS was statistically significant with $p < 0.0005$.

APC-I18V anticoagulant activity was tested with DIPE, C8-GlcCer, C12-LacCer and C12-GlcCer lipids to determine if APC-I18V was also insensitive to enhancement of APC anticoagulant activity by these membrane lipids. However, unlike APC-WT, APC-I18V was not affected by the presence of these lipids (Figure 3.6; white bars). This lack of sensitivity of APC-I18V to DIPE, C8-GlcCer, C12-GlcCer and LacCer was statistically significant compared to the response of APC-WT in the presence of these lipids with $P < 0.002$. Therefore, *APC-I18V* exhibits complete loss of sensitivity to different membrane lipid cofactors that normally enhance APC anticoagulant activity.

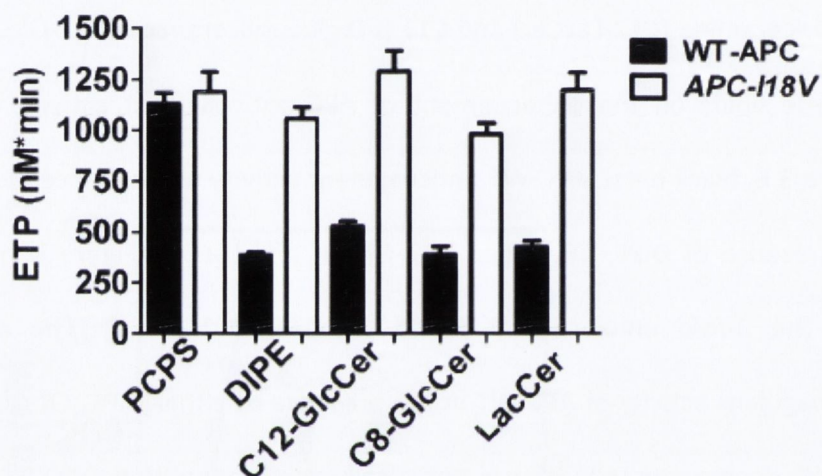


Figure 3-6: APC-I18V anticoagulant activity is not enhanced by neutral membrane lipids which stimulate APC anticoagulant activity.

Thrombin generation was initiated in protein C deficient plasma with 5pM sTF, 100mM CaCl₂ and 10μM lipid vesicle preparation (PC/PS, 80%/20%; PC/PS/DIPE, 60%/20%/20%; PC/PS/C12-GlcCer, 60%/20%/20%; PC/PS/C8-GlcCer, 60%/20%/20%; PC/PS/LacCer, 60%/20%/20%) in the presence of APC-WT (*black bars*) or APC-I18V (*white bars*) (both 10nM). Each experiment was performed in triplicate and values represent the mean ETP ± SEM.

3.6 PE and GlcCer enhancement of APC-WT, but not APC-I18V, anticoagulant activity increases with phospholipid vesicle concentration

To see if APC-I18V insensitivity to both PE and GlcCer could be overcome by increasing the phospholipid concentration, the thrombin generation assay was performed in the presence of increasing phospholipid vesicle concentration (2.5-20 μ M) while keeping all other conditions constant.

APC-WT anticoagulant activity was largely independent of PC/PS vesicle concentration (Figure 3.7A; white circles). However, increasing APC-WT anticoagulant activity, as seen by a significant decrease in the amount of thrombin generation (ETP), was seen with increasing PC/PS/PE and PC/PS/GlcCer vesicle concentration (Figure 3.7B&C; white circles).

APC-I18V anticoagulant activity exhibited similar limited lipid concentration dependence to APC-WT in PC/PS vesicles (Figure 3.7A). In contrast to APC-WT, APC-I18V was independent of PC/PS/PE (Figure 3.7B; triangles) and PC/PS/GlcCer (Figure 3.7C; triangles) vesicle concentration.

Taken together, these results show that APC-I18V is completely insensitive to PE and GlcCer, and that this insensitivity cannot be overcome by increasing the phospholipid vesicle concentration.

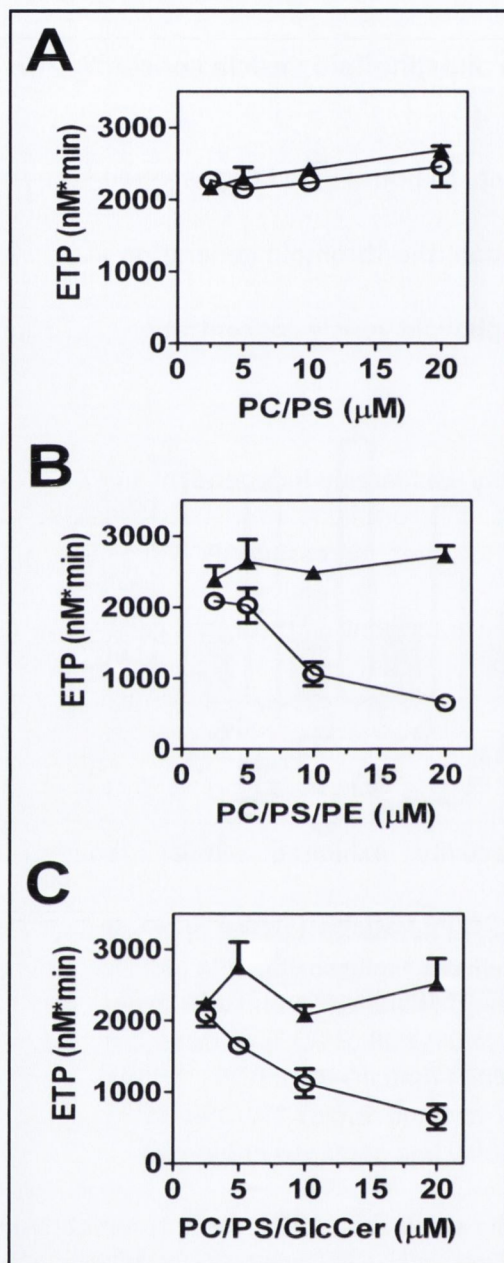


Figure 3-7: APC-I18V anticoagulant activity is insensitive to lipid vesicle concentration.

Thrombin generation was measured in protein C deficient plasma in the presence of APC-WT (○) or APC-I18V (▲). To determine the dependence of lipid vesicle concentration on APC anticoagulant activity, lipid vesicles (2.5-20 μM) containing either (A) PC/PS (B) PC/PS/PE or (C) PC/PS/GlcCer were used in each experiment. No thrombin generation was observed in the absence of lipid vesicles. The mean ETP (nM^*min) $\pm$ SEM was determined at each lipid vesicle concentration. Each experiment was performed in triplicate.

3.7 Bovine APC is enhanced by the presence of PE and GlcCer

Bovine APC (bAPC) does not have isoleucine at position 18, and instead has serine¹⁴⁴, making it a useful tool to study PE or GlcCer APC anticoagulant enhancement. Using the thrombin generation assay, bAPC anticoagulant activity was tested in bovine plasma in the presence of PC/PS, PC/PS/PE or PC/PS/GlcCer (Figure 3.8). bAPC was found to be enhanced in the presence of both PE and GlcCer; however PE was more effective at enhancing the anticoagulant activity of bAPC, as seen by the reduced amount of thrombin generation in the presence of PC/PS/PE (Figure 3.8). This suggests that the lipid enhancement of anticoagulant activity of bovine protein C does not occur by the same mechanism as human APC.

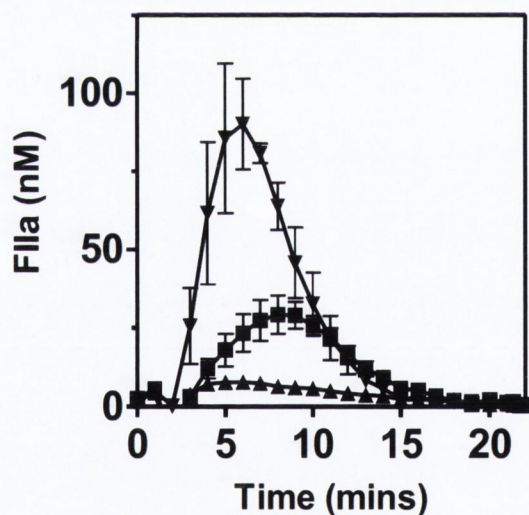


Figure 3-8: Presence of PE/GlcCer enhances anticoagulant activity of bovine APC.

Thrombin generation was assessed in bovine plasma in the presence of PC/PS (▼), PC/PS/PE (▲) or PC/PS/GlcCer (■) in the presence of bAPC (10nM). The data represents the mean of three independent determinations $\pm$ SEM.

3.8 Location of isoleucine 18 in APC Gla domain

The APC Gla domain has been previously reported to interact directly with PE⁴². Fluorescence resonance energy transfer (FRET) analysis was used to look at the interaction between APC Gla domain and GlcCer⁸². FRET analysis is used to investigate the distance between two molecules and works on the principle that FRET only occurs when the two molecules in question are very close together. This FRET analysis indicated that the APC Gla domain has specific GlcCer binding sites which could enhance APC binding to negatively charged phospholipids⁸². The results in this chapter show isoleucine 18 is critical for specific lipid enhancement of APC anticoagulant activity. To further understand this the location of this residue within the APC Gla domain was determined. Using PYMOL molecular visualisation software we observed that isoleucine 18 is located on the outer surface of the APC Gla domain (Figure 3.9). This is in keeping with these previous reports as it suggests that isoleucine is in a position where it could directly bind/interact with either PE or GlcCer, resulting in altered anticoagulant activity.

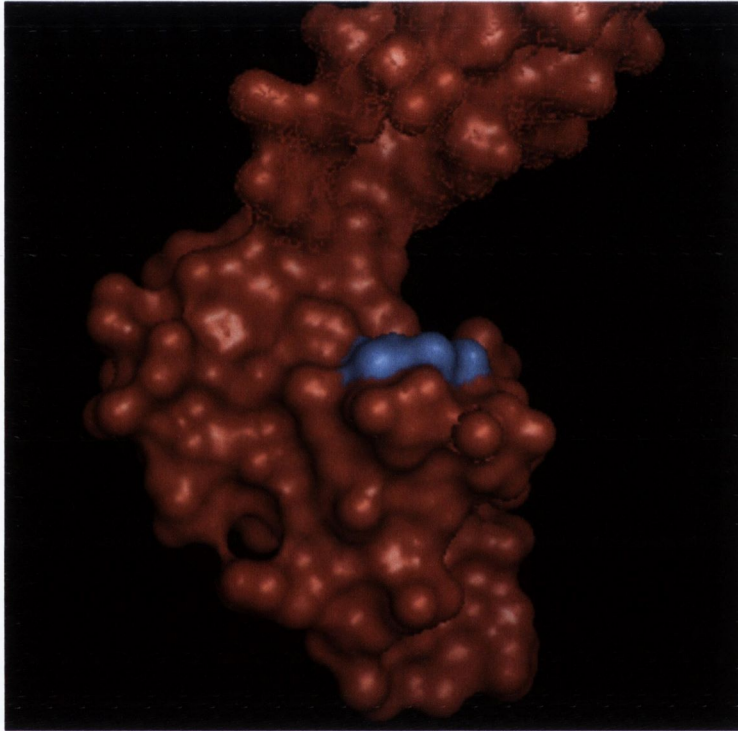


Figure 3-9: Location of isoleucine-18 in APC Gla domain.

Residue I18 in APC shown in blue located on the outside of the Gla domain. Model generated using PYMOL molecular visualization software.

3.9 Discussion

3.9.1 PE and GlcCer enhancement of APC anticoagulant activity

Phosphatidylserine (PS) exposure on the surface of activated cells close to the site of vascular injury is critical for thrombus formation and regulation, and is required for binding of coagulation serine proteases to the cell membrane. In the case of APC, *in vitro* studies have highlighted the requirement of additional membrane lipid moieties for optimal APC anticoagulant function^{79,145,146}. In particular, the phospholipid PE and the glycolipid GlcCer have been shown to enhance APC affinity for PS-containing phospholipid vesicles, and lead to a corresponding increased ability to inactivate FVa.

PE may also exert an important role in the regulation of APC anticoagulant function *in vivo*. Deletion of the ethanolamine kinase 2 (*Etnk2*) gene to disrupt PE synthesis results in placental thrombosis and spontaneous late-stage foetal death in *Etnk2*-deficient mice⁷⁵. Furthermore, the presence of PE increases anti-phospholipid antibody-mediated APC inhibition¹⁴⁷. This implies a key role for PE in APC anticoagulant function and the possible thrombotic consequences of blocking PE anticoagulant stimulation. Certain lipid molecule determinants of PE enhancement of APC function have been identified. The PE head group and degree of polyunsaturation are important mediators of the APC cofactor function of PE². PE can also create hexagonal phases⁷³ that may induce PS clustering, thereby stimulating APC-PS interaction and anticoagulant activity. PE may also minimize the steric hindrance created by bulky PC moieties, or induce

direct interactions with PS which result in a structural rearrangement of the PS head group¹⁴⁸.

The physiological importance for GlcCer in regulating APC anticoagulant activity is implied by a case-control study in which plasma GlcCer levels were found to be significantly lower in patients with venous thromboembolism (VTE) compared to normal controls¹⁴⁹. Furthermore, plasma levels of other glycolipids did not correlate with risk of VTE. However, the mechanism for glycolipid enhancement of APC anticoagulant function is not fully understood¹⁵⁰.

The Gla domain of APC is believed to play a key role in mediating the specific neutral lipid enhancement of APC anticoagulant activity⁴². Therefore, in this study we utilised the fact that despite the significant homology between the prothrombin Gla domain and the protein C Gla domain, prothrombin is not responsive to the presence of neutral lipids⁴². Therefore, substitutions between non-conserved Gla domain residues of the prothrombin Gla domain were inserted into the protein C Gla domain¹³⁸ and we examined these recombinant APC variants for their ability to be enhanced by either PE or GlcCer.

3.9.2 Isoleucine-18 in APC Gla domain critical for PE and GlcCer enhancement of APC anticoagulant activity

In the results described in this chapter the residues of APC Gla domain involved in the PE and GlcCer enhancement of APC anticoagulant activity have been determined. Interestingly, a single amino acid substitution, I18V, resulted in a complete lack of PE

and GlcCer enhancement of APC anticoagulant activity.

APC-I18V was not enhanced by PE- or GlcCer-containing phospholipid vesicles in the plasma-based thrombin generation assays (Figure 3.5) and increasing the phospholipid concentration did not result in any enhancement effects by PE and GlcCer demonstrating a complete lack of sensitivity of APC-I18V to neutral lipid enhancement (Figure 3.8).

APC-I18V variant has previously been shown to exhibit normal phospholipid vesicle binding to PC/PS in the absence of PE/GlcCer¹³⁸. This suggests that this variant, APC-I18V, lacks the ability to interact with PE or GlcCer, despite having normal phospholipid binding to PC/PS.

Other membrane lipids have been described that have similar ability to enhance APC anticoagulant activity to PE and GlcCer. These lipids were investigated to see if APC-I18V was sensitive to their effects. The lipids examined were dilinoleoyl-sn glycerol-3-phosphoethanolamine (DIPE), C8 β -D-glucosylceramide (C8-GlcCer), C12 β -D-lactosylceramide (C12-LacCer) and C12 β -D glucosylceramide (C12-GlcCer). In the thrombin generation assay, APC-I18V was not sensitive to any enhancement effect of these lipids, unlike APC-WT. This demonstrates that APC-I18V is completely insensitive to neutral lipid enhancement, regardless of which neutral membrane lipid is involved. It is notable that the observed enhancement of these lipids on APC-WT anticoagulant activity was found to be lower than the enhancement effects of PE and GlcCer, suggesting that there may be slightly different mechanisms behind the lipid enhancement of APC anticoagulant activity.

APC-I18V consists of a substitution between isoleucine and valine, which are both small neutral non-polar amino acid, and therefore it is surprising that this relatively minor mutation results in an APC variant that exhibits a total lack of sensitivity to neutral lipid enhancement. However, the fact that this seemingly small charge has such a profound effect on APC anticoagulant activity highlights the importance of isoleucine 18 in APC Gla domain.

3.9.3 Additional APC Gla domain residues involved in PE and GlcCer enhancement of APC anticoagulant activity

The recombinant variant APC-L38D could not be assessed for PE and GlcCer enhancement of APC anticoagulant activity as this recombinant APC variant lacks protein S sensitivity and therefore has significantly reduced anticoagulant activity⁴⁰. Therefore, leucine-38 in APC Gla domain could be involved in neutral lipid enhancement of APC anticoagulant activity. As APC-L38D variant will not be able to be assessed using coagulation assays, a lipid binding assay should be developed to assess the involvement of this residue in neutral lipid enhancement of APC anticoagulant activity.

A minor reduction in APC sensitivity to GlcCer was observed in the *APC-S3T* variant, despite normal enhancement by PE. This suggests that although the I18V substitution ablated both PE and GlcCer stimulation, the amino acid residues required for APC stimulation by either lipid may not be entirely identical. Again, this suggests that there may be slightly different mechanisms behind the lipid enhancement of APC

anticoagulant activity.

In contrast to *APC-S3T* and *APC-I18V*, *APC* variant *APC-S11G/S12N* down-regulated thrombin generation with drastically improved efficiency compared to *APC-WT* in a thrombin generation assay (Figure 3.5). Interestingly, enhanced *APC-S11G/S12N* anticoagulant activity occurred irrespective of the presence of PE/GlcCer in phospholipid vesicles. This variant contains residue substitutions similar to previous *APC* variants (e.g. *APC-QGNSEDY*) that were shown to exhibit increased affinity for PS-containing phospholipid vesicles and enhanced anticoagulant activity^{38,151,152}. Increased *APC-S11G/S12N* anticoagulant activity may contribute to the enhanced activity of the *APC* chimera containing the N-terminal 22 amino acid residues of prothrombin, which despite also possessing the I18V substitution, is significantly more active independent of the presence of PE¹⁵³.

Therefore, although isoleucine 18 in *APC* Gla domain has been found to be critical for mediating the enhancement effects of PE and GlcCer, other residues may play a part and there may well be slightly different mechanisms behind the different lipids ability to enhance *APC* anticoagulant activity.

3.9.4 PE and GlcCer enhancement of bovine *APC* anticoagulant activity

A role for additional Gla domain residues is suggested by previous reports of GlcCer enhancement of bovine *APC*, which has been shown to bind GlcCer, despite possessing serine at position 18¹⁴⁴. Results presented here shown that bovine *APC* was enhanced by PE and GlcCer in the thrombin generation assay. Unlike human *APC*, bovine *APC* was

found to be more sensitive to the enhancement effects of PE compared to GlcCer.

The reason that bovine APC is enhanced by PE and GlcCer despite lacking isoleucine could be due to the utilisation of alternative Gla domain amino acid residues for the bovine APC-lipid interaction. Of note, APC exhibits significant species-specific phospholipid membrane affinity, with human APC exhibiting 10-fold higher affinity for PS-containing phospholipid vesicles, suggestive of alternative modes of phospholipid interaction between different APC species¹⁵⁴.

The fact that bovine APC is enhanced by the presence of PE and GlcCer is highly suggestive of the fact that there are different mechanisms of lipid enhancement of APC anticoagulant activity in the different species. While isoleucine at position 18 in APC Gla domain is essential for lipid enhancement in humans, the presence of serine in bovine APC is significant for lipid enhancement. Interestingly, a direct comparison of bovine protein C and human protein C found that bovine protein C bound to PC/PS vesicles much more weakly than human protein C⁸. Further work is required to investigate if a APC-I18S substitution in human APC Gla domain leads to a APC variant that is responsive to either PE or GlcCer.

3.9.5 APC Gla domain and lipid binding

At present the precise nature of the APC-and indeed the other coagulant factors-interaction with lipids on cell membranes is unclear. Disruption of the Ca²⁺ bound hydrophobic ω -loop region (amino acid residues 1-7) diminishes vitamin K dependent protein affinity for phospholipids, but does not explain the specific requirement of PS

for membrane binding to occur¹⁵⁵. A crystal structure complex of Ca^{2+} -bound bovine prothrombin fragment to a PS analogue, lysophosphatidylserine (lysoPS), indicates Gla domain-bound Ca^{2+} ions and exposed Gla residues 17 and 21 make important contacts with the serine head group of lysoPS. Each Gla residue and corresponding Ca^{2+} ion that take place in this binding event are present in each vitamin K dependent protein, suggesting a conserved mode of specific PS binding¹⁵⁵.

No information as to the structural basis of PE/GlcCer interaction with vitamin K dependent proteins is available. However, an atomic model of anionic lipid membrane-bound FVII Gla domain suggested deep insertion of the ω -loop into the membrane bilayer, facilitated by direct ionic interactions between Ca^{2+} ions bound to the outer surface of the FVII Gla domain and the PS head group¹⁵⁶. In this model (Figure. 3.10), the corresponding FVII amino acid residue to APC isoleucine-18 (Lys-18) is located close to the membrane surface and can make interactions with PS during molecular dynamic simulation. If the APC ω -loop inserts into the membrane bilayer in a similar manner to FVIIa, the expected localization of isoleucine 18 at the membrane surface provides a possible rationale for how isoleucine 18 could mediate putative lipid head group interactions and consequently, lipid cofactor enhancement of APC anticoagulant activity. Alternatively, rather than disrupt direct interactions with PE/GlcCer, the I18V amino acid substitution could disrupt the spatial arrangement of bound Ca^{2+} ion or Gla domain residues that may contribute to PE/GlcCer binding. As shown in Figure 3.9, isoleucine 18 resides on the outer surface of the APC Gla domain in a location where it could be the cause of these interactions.

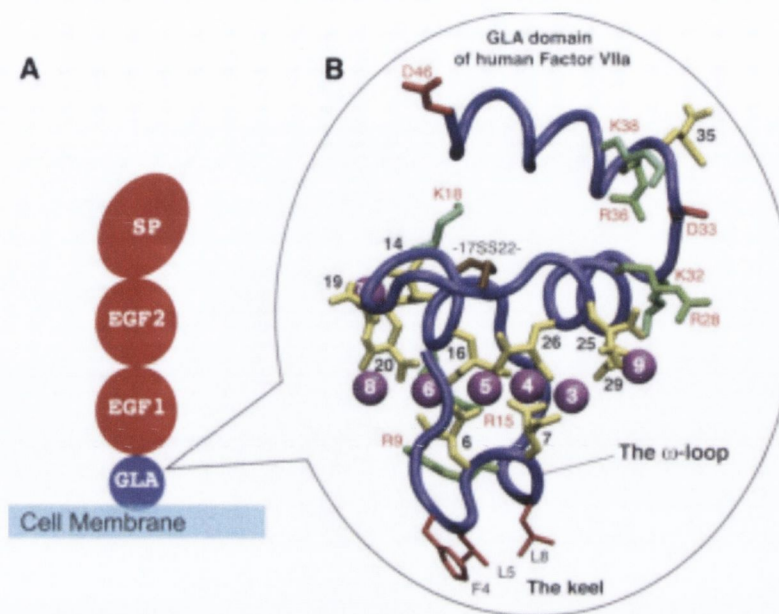


Figure 3-10: Structure of the Gla domain of human FVIIa.

This figure is taken from the Ohkubo and Tajkhorshid paper¹⁵⁶. In this model you can see that residue 19 of FVIIa is located away from the ω -loop which would insert into the cell membrane¹⁵⁶. Therefore, this would suggest that the location of isoleucine-18 in APC Gla domain would be remote from the ω -loop and would be at a location free to interact with other lipids and proteins. (The purple balls in this figure represent the Ca²⁺ ions).

3.9.6 Summary

This study has identified critical amino acid residues that contribute to the regulation of APC enhancement by membrane lipid cofactors. This study has identified that isoleucine-18 in APC Gla domain is critical for mediating both PE and GlcCer lipid enhancement of APC anticoagulant activity. When isoleucine 18 is mutated to valine in APC Gla domain, the neutral lipid enhancement by PE and GlcCer is totally abolished.

The APC-I18V variant therefore represents an effective tool to probe the role of membrane lipid cofactors in protein C anticoagulant pathway. Further experiments are required to determine the precise mechanism behind the specific lipid enhancement of APC. This aspect will be investigated in the subsequent chapter.

CHAPTER 4 Investigation of the anticoagulant and cytoprotective activities of APC-I18V

4.1 Mechanism of PE and GlcCer enhancement of APC anticoagulant activity

The mechanism behind PE and GlcCer enhancement of APC anticoagulant activity is unknown^{74,82}. From the results in Chapter 3, APC-I18V has been identified as an APC recombinant variant which lacks sensitivity to the PE and GlcCer enhancement of APC anticoagulant activity. Therefore, in this chapter this variant is utilised to further understand the mechanism behind the neutral lipid enhancement of APC anticoagulant activity. First the role of PE and GlcCer in mediating protein S independent and dependent FVa inactivation by APC anticoagulant activity was investigated. Subsequent experiments investigated if loss of PE and GlcCer sensitivity affects APC-I18V ability to bind to EPCR and mediate cytoprotective effects.

4.2 Effect of different phospholipid compositions on protein S independent APC anticoagulant activity

As the inactivation of FV by APC is strongly dependent on binding to negatively charged phospholipids¹⁵⁷, the effect of PE and GlcCer in the phospholipid mix on FVa inactivation was investigated. An assay was developed to examine FVa inactivation in

the absence of protein S (Section 2.3.6). FVa was incubated in the presence of APC with PC/PS, PC/PS/PE or PC/PS/GlcCer over the course of 12min. Aliquots of FVa was sampled over the time course and the amount of FVa was determined by adding this sample to a prothrombinase assay, as the amount of thrombin generated in this assay is directly proportionally to the amount of FVa cofactor activity.

Under these conditions it was found that FVa cofactor activity decreases rapidly over the course of the 12min (Figure 4.1). We found that the IC_{50} for FVa inactivation for PC/PS was 120s, for PC/PS/PE was 110s and for PC/PS/GlcCer was 120s. Therefore, in the absence of protein S, the presence of PE or GlcCer has little effect on APC anticoagulant activity. Therefore, this shows that for the enhancement effect of PE and GlcCer on APC anticoagulant activity is dependent on protein S.

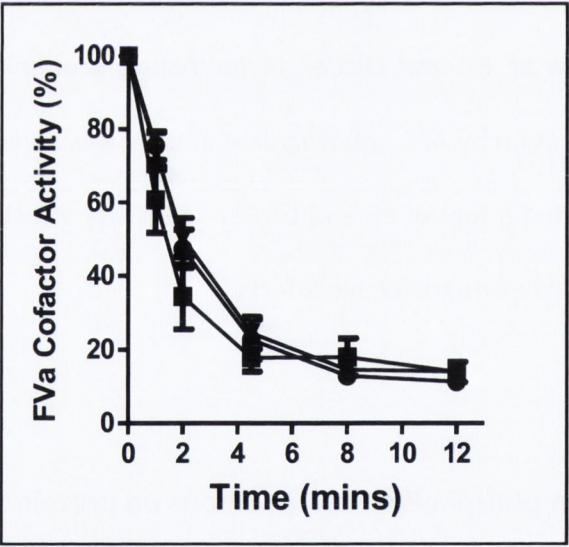


Figure 4-1: Different phospholipid compositions do not affect protein S independent APC anticoagulant activity.

FVa protein S independent assay performed using phospholipid vesicles of PS/PS (●), PSCPE (■) or PCPSGlcCer (▲). All experiments were performed in triplicate and data represented as mean residual FVa cofactor activity $\pm$ SEM.

4.3 APC-I18V displays a diminished sensitivity to protein S in the thrombin generation assay

PE- or GlcCer-containing phospholipid vesicles have been previously reported to enhance APC-dependent protein S cofactor function^{74,79,158}. The ability of APC-WT and APC-I18V to be enhanced by protein S in the presence of lipid vesicles containing PC/PS, PC/PS/PE or PC/PS/GlcCer was assessed using a protein S-sensitive thrombin generation assay as described in Section 2.3.4. APC-WT and APC-I18V exhibited similar weak sensitivity to protein S in the presence of PC/PS vesicles (Figure 4.2:circles). However, when either PE or GlcCer were added to the PC/PS lipid vesicle preparation, APC-WT attenuated thrombin generation at a 3-4-fold lower protein S concentration than that observed with PC/PS vesicles (Figure 4.2A). The IC_{50} for PC/PS was 131nM, PC/PS/PE was 35nM, PC/PS/GlcCer was 52nM, (Figure 4.2A).

APC-I18V anticoagulant activity was not similarly increased in the presence of PE or GlcCer-containing lipid vesicles. Instead APC-I18V displayed a similar response in the presence of the different lipid mixture, with similar IC_{50} 's for each lipid. (IC_{50} for PC/PS/PE was 142nM, PC/PS and PC/PS/GlcCer was ~150nM: Figure 4.2B). These results show that protein S cofactor function is dependent on APC sensitivity to the enhancement by PE or GlcCer.

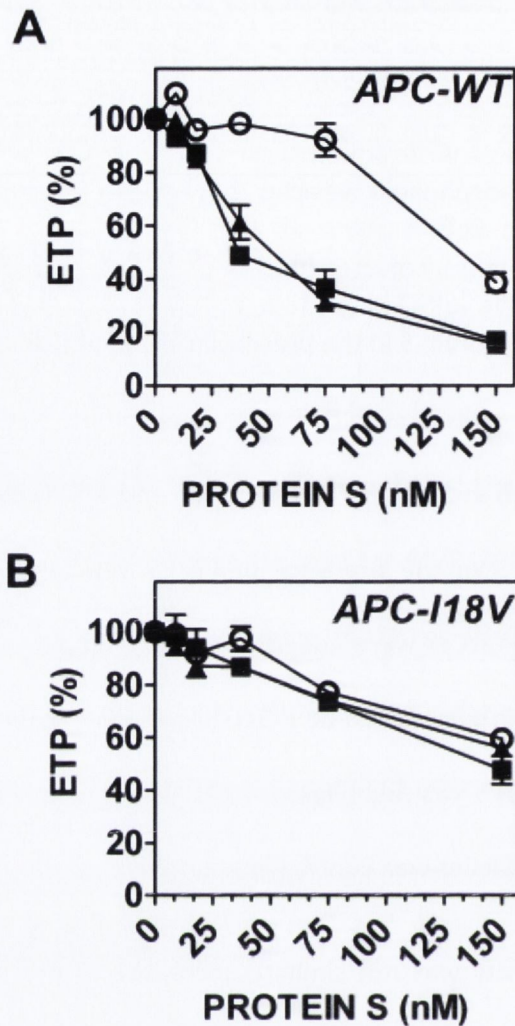


Figure 4-2: APC-I18V is insensitive to protein S cofactor activity in protein S-deficient plasma. The ability of **(A)** APC-WT or **(B)** APC-I18V (both 10nM) to inhibit tissue-factor induced thrombin generation in protein S deficient plasma was assessed in the presence of protein S (9-150nM) with 10 μ M of either PC/PS ($\circ$); PC/PS/PE ($\blacksquare$); PC/PS/GlcCer ($\blacksquare$). The ETP was determined for each condition and 100% ETP was set as the ETP obtained in the absence of protein S for each phospholipid mixture. The mean ETP $\pm$ SEM at each protein S concentration was determined with each APC species in at least three independent experiments.

4.4 Protein S-dependent FVa proteolysis by APC-I18V is not stimulated by the presence of PE and GlcCer

To investigate the role of PE and GlcCer upon protein S dependent FVa proteolysis by APC, the rate of FVa inactivation in the presence of each phospholipid vesicle preparation was examined. In keeping with the APC response in plasma (Section 4.3), protein S-mediated FVa proteolysis by APC-WT was slow in the presence of PC/PS, retaining $57\pm3\%$ residual FVa cofactor activity at 50nM protein S (Figure 4.3A). However, FVa proteolysis by APC-WT was significantly increased by the presence of both PE (3.5-fold) and GlcCer (2-fold) (IC_{50} for PC/PS=50nM protein S, PC/PS/PE=13.4nM protein S, PC/PS/GlcCer=34.5nM protein S; Figure 4.3A).

APC-I18V did not display any increase in proteolysis in the presence of PE-/GlcCer (Figure 4.3B). However, the activity of APC-I18V in the presence of PC/PS was less than that of APC-WT (Figure 4.3). APC-I18V retained $72\pm15\%$ residual FVa cofactor activity at 50nM protein S. However, this was not a statistically significant difference. The IC_{50} for APC-I18V in the presence of PE and GlcCer could not be calculated. However, it was clear that PE and GlcCer incorporation into the phospholipid vesicles did not increase FVa inactivation by APC (Figure 4.3B).

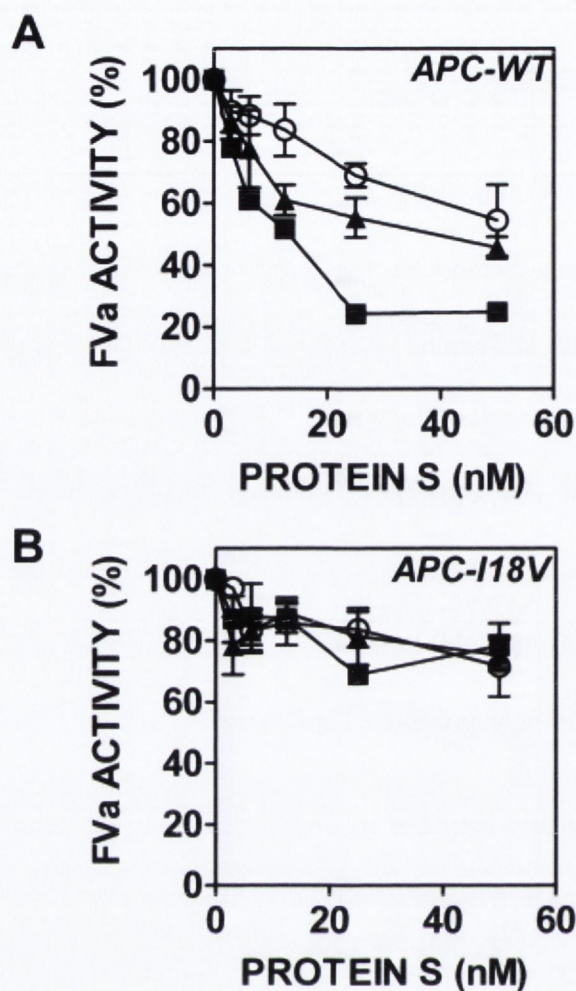


Figure 4-3: Protein S dependent FVa proteolysis by APC-I18V is not enhanced by the presence of PE and GlcCer.

FVa (7nM) was incubated with either 2nM **(A)** APC-WT or **(B)** APC-I18V in the presence of protein S (3.25-50nM) with 20 μ M of either PC/PS (○); PC/PS/PE (■); PC/PS/GlcCer (▲); lipid vesicles. After a 2 minute incubation at 37°C an aliquot was removed and residual FVa cofactor activity determined using a prothrombinase assay, as described in Chapter 2. All experiments were performed in triplicate and data represented as mean residual FVa cofactor activity $\pm$ SEM.

4.5 APC-I18V exhibits normal EPCR binding

The APC Gla domain binds EPCR to facilitate PAR1 dependent protective signalling on endothelial cells. To examine whether APC-I18V has aberrant EPCR binding in addition to insensitivity to PE and GlcCer, APC-WT and APC-I18V binding to recombinant soluble EPCR (sEPCR) was assessed using surface plasmon resonance on the Biacore (Section 2.3.7). After allowing sEPCR bind to the flow cell 2 of the CM5 sensor chip (flow cell 1 used as a control to account for non-specific binding), APC-I18V and APC-WT, in concentrations ranging from 16nM to 500nM, were passed over the chip. It was found that APC-I18V bound with similar affinity to sEPCR compared to APC-WT, with a K_D of approximately 120nM (Figure 4.4). This suggests that EPCR binding is not affected by the mutation of isoleucine18 in APC Gla domain.

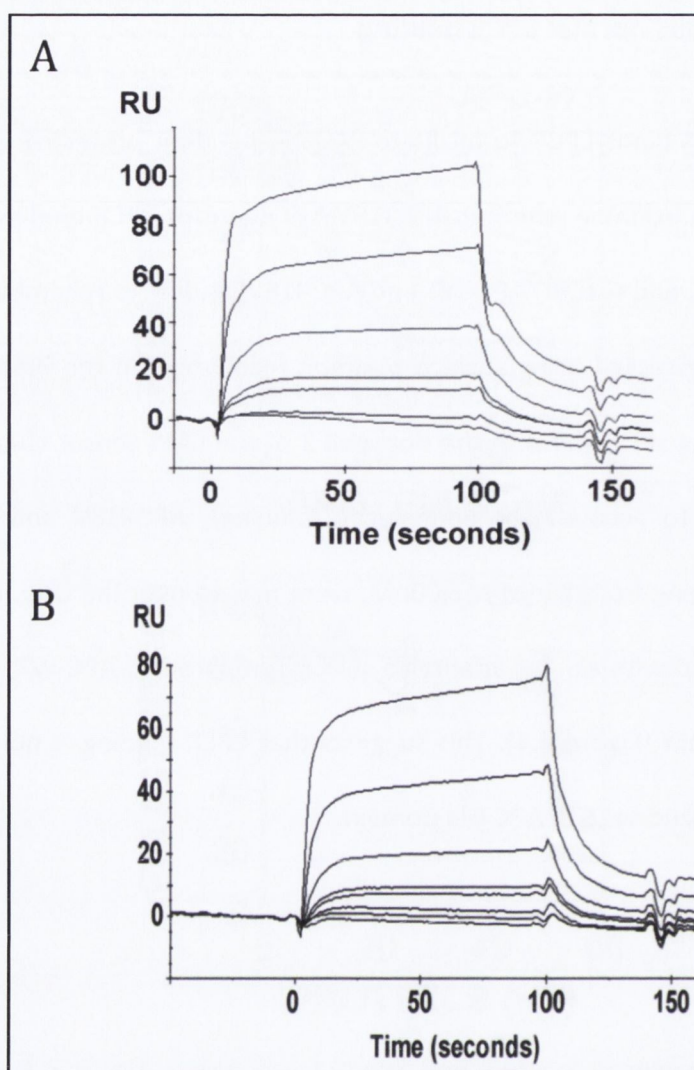


Figure 4-4: APC-I18V binds normally to sEPCR using surface plasmon resonance on Biacore. APC-I18V (**B**) was found to bind to sEPCR with similar affinity compared to APC-WT (**A**). RCR-2 was used to capture sEPCR on the CM5 sensor chip (Chapter 2.3.7). The concentrations of APC shown for both APC-WT and APC-I18V are 500nM, 250nM, 12.5nM, 6.25nM, 31nM and 16nM.

4.6 Effect of APC on thrombin induced endothelial barrier permeability

To investigate whether loss of specific lipid enhancement by APC affected APC signalling via EPCR-PAR1, an *in vitro* assay to detect thrombin induced barrier permeability assay was established using EA.hy926 cells as a model cell for endothelial cells (as described in Section 2.3.8). The mechanism by which thrombin induces increased permeability (see Figure 1.10) is believed to occur through cleavage and activation of PAR1 which signals through G proteins to activate RhoA causing barrier dysfunction.

Thrombin increased EA.hy926 cell barrier monolayer permeability (Figure 4.5), as determined by an increase in the OD, signalling an increased flow of Evans blue-BSA through the cell monolayer. APC-WT significantly protected EA.hy926 cells from thrombin-induced permeability (Figure 4.5). Cells treated with APC showed a much more gradual increase in the OD compared to thrombin treated cells (Figure 4.5).

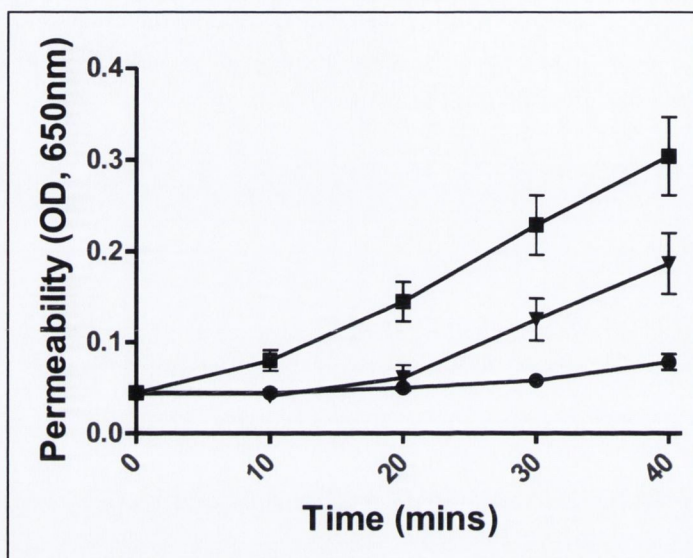


Figure 4-5: APC protects the endothelial barrier from thrombin.

EA.hy926 cells were preincubated with 20nM wild type APC for 3hrs (▲). Untreated cells (●) were used as a negative control. The cells were then treated with 5nM thrombin for 10min (cells treated with thrombin only; ■) and the endothelial barrier permeability was assessed over time using Evans Blue-bovine serum albumin (Section 2.3.8). Results represent the mean $\pm$ SEM for 3 independent experiments.

4.7 APC-I18V retains normal barrier stabilization activity

Using the permeability assay, the ability of APC-I18V to protect against thrombin induced permeability was investigated to determine if loss of PE and GlcCer enhancement of this variant's anticoagulant activity affected its cytoprotective signalling activities. Thrombin treated cells showed an increase in permeability compared with untreated cells (Figure 4.6). APC-I18V exhibited very similar endothelial cell barrier protective activity to that of APC-WT, as seen by the fact that both APC-WT and APC-I18V treated cells resulted in similar OD reading (Figure 4.6). This indicates that the loss of cell membrane lipid stimulation does not affect EPCR-dependent signalling via PAR1 by APC.

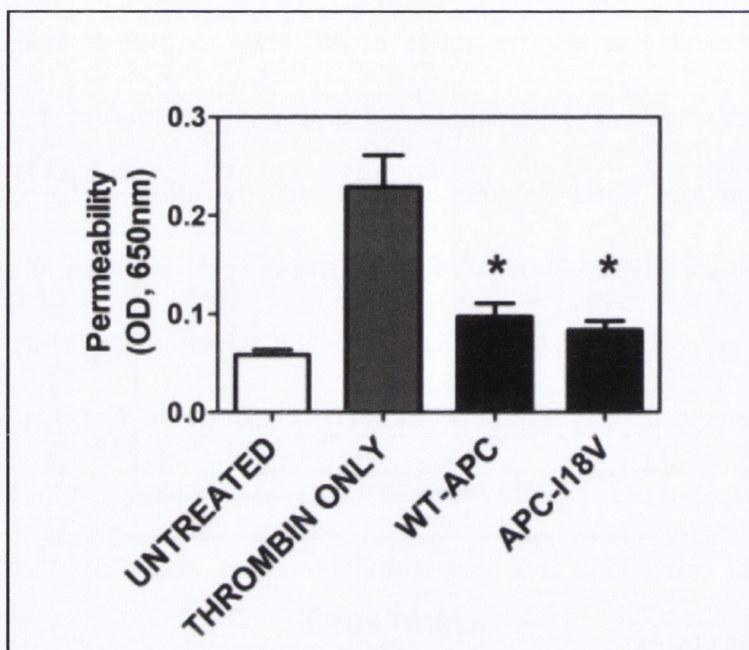


Figure 4-6:APC-I18V exhibits normal PAR1 dependent endothelial barrier protective activity.

To assess APC variant barrier stabilization on endothelial cells, EA.hy926 cells were pre-incubated with either 20nM APC-WT or APC-I18V for 3hrs (*black bars*). Untreated EA.hy926 cells (*white bar*) were used as a negative control. EA.hy926 cells were then treated with 5nM thrombin for 10min (thrombin only treated cells, *grey bar*) and endothelial barrier permeability was assessed after 30min using Evans Blue-BSA complex. Results represent the mean $\pm$ the SEM for 3 independent experiments. * $P<0.02$ compared to thrombin only treated EA.hy926 cells.

4.8 Inhibition of APC-WT and APC-I18V in plasma

The rate of inhibition of APC-WT and APC-I18V by serpins present in plasma was investigated to determine whether loss of neutral lipid enhancement effected APC-I18V inhibition. Normal pooled plasma (NPP) was incubated with the APC at 37°C for 60min and samples of the plasma was tested for APC activity using a chromogenic assay at various time points. Inhibition of both APC-WT and APC-I18V activity was closely comparable (Figure 4.7), with the half life for APC-WT of 17min and for APC-I18V of 18.3min. This was similar to the half-life of APC in human plasma of 22min³³. This shows that the substitution of I18V in APC Gla domain did not alter APC inhibition in plasma. It also suggests that the recombinant protein C variants have similar rates of inhibition compared to human protein C.

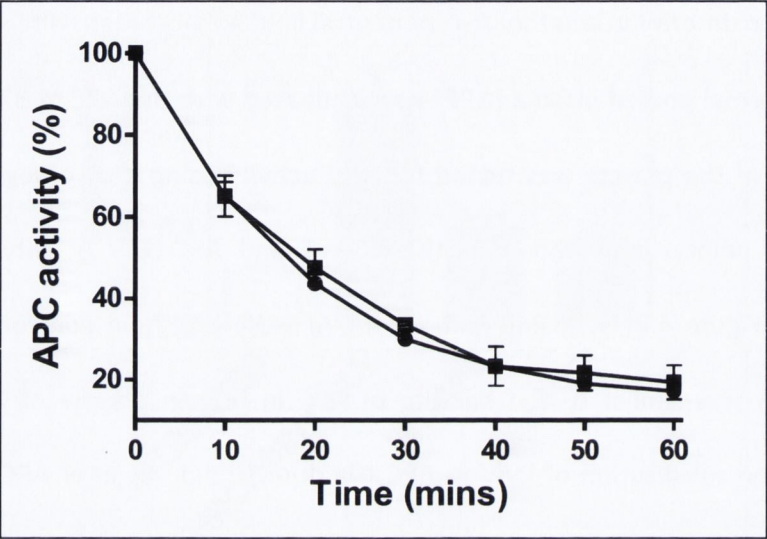


Figure 4-7: APC-WT and APC-I18V followed a similar pattern of inhibition in normal pooled plasma.

Inhibition of APC-WT (●) and APC-I18V (■) in NPP was determined by incubation of the plasma with 20nM APC at 37°C. At the designated time points, aliquots were removed, and the residual amidolytic activity was determined. The data represents the mean of three independent determinations $\pm$ SEM.

4.9 Discussion

4.9.1 Role of protein S and APC anticoagulant activity

Protein S is a vitamin K dependent protein, which is recognised as an important cofactor for the APC anticoagulant pathway. Protein S stimulates FVa and FVIIIa inactivation by APC. In the presence of protein S, FVa proteolysis by APC is strongly stimulated at Arg306 and mildly stimulated at Arg506. Deficiency of protein S results in a similar phenotype to protein C deficiency, demonstrating the direct link between these two proteins. Homozygous protein S deficiency results in life-threatening purpura fulminans⁵¹, while heterozygous protein S deficient patients are at increased risk of thrombotic disease^{52,53}.

Despite the clinical importance of protein S, the precise molecular mechanism behind protein S anticoagulant function is still unclear¹⁵⁹. There are many theories as to how protein S acts as a cofactor to APC anticoagulant activity. For example, protein S and APC have been suggested to form a complex on negatively charged phospholipids, which alters the active site of APC in relation to the membrane and FVa¹⁶⁰. This alteration of active site of APC then results in an increased rate of proteolysis of FVa¹⁶⁰. Another suggestion is that protein S and TFPI inhibit FXa¹⁶¹. FXa usually protects the cleavage of FVa by APC, and therefore remove of this protection leads APC free to inactivate FVa¹⁶¹. It is known that the Gla domain of APC is critical for the interaction of APC and protein S^{42,138}, specifically residue leucine-38⁴⁰. In relation to protein S it is suggested that both the Gla domain and EGF1 domain of protein S is important for

mediating its interaction with APC¹⁵⁹.

Apart from protein S role as a cofactor for APC, protein S also has APC-independent anticoagulant activity, which has been attributed to its enhancement of the interaction between TFPI and FXa⁵⁶. The mechanism of these functions of protein S is still unclear. Indeed, the well accepted fact that only free protein S can influence APC function has now been challenged. Protein S-C4b-binding protein complex has been found to enhance the APC inactivation of residue Arg306 of FVa, despite impairing the inactivation of Arg506 of FVa by APC¹⁶².

4.9.2 Role of protein S in the PE and GlcCer enhancement of APC anticoagulant activity

This study investigated whether PE and GlcCer influenced protein S anticoagulant activity. It was found that PE and GlcCer had no effect on APC anticoagulant activity in the absence of protein S. This result highlights the importance of the interaction of PE and GlcCer with protein S for full APC anticoagulant activity. It suggests that the mechanism behind PE and GlcCer enhancement of APC anticoagulant activity is directly dependent on protein S.

In the presence of protein S, PE and GlcCer were found to enhance APC anticoagulant activity in the FVa inactivation assay. Interestingly, APC-I18V was found to be insensitive to this enhancement in both the thrombin generation assay and the FVa protein S dependent-inactivation assay. This supports the results from Chapter 3 showing that APC-I18V is completely insensitive to the effects of PE and GlcCer. These

results suggest that for full protein S cofactor function, and indeed full APC anticoagulant activity, APC must be sensitive to the enhancement effects of PE or GlcCer enhancement.

4.9.3 Mechanism of PE and GlcCer enhancement of APC anticoagulant activity

This study has found that isoleucine 18 in APC Gla domain is critical for mediating the PE and GlcCer enhancement of APC anticoagulant activity. Furthermore, using the APC-I18V variant it has been found that APC must be sensitive to PE and GlcCer for full protein S cofactor activity. However, the precise mechanism behind PE and GlcCer effect on APC anticoagulant activity is still unclear. Studies have shown that when incorporated into phospholipid vesicles, GlcCer enhances APC affinity for negatively charged phospholipids⁸². Interestingly, a study showed that GlcCer increases the affinity of APC for phospholipids only when PS is present⁸², possibly explaining the requirement for negatively charged phospholipids for APC anticoagulant activity. From FRET analysis, it was found that regardless of the presence or absence of GlcCer, phospholipids bind to the same end of APC⁸². From the FRET study the existence of GlcCer-binding sites on APC was suggested⁸². The results from this study support this idea and strongly suggest that isoleucine-18 in APC Gla domain is the binding site for neutral lipids.

Using APC-I18V, this study has found that APC must be sensitive to PE and GlcCer for full protein S cofactor activity. However, the reason for this requirement is unclear. It does suggest that neutral lipids and protein S are cooperating to enhance APC

anticoagulant activity.

4.9.4 APC and endothelial barrier permeability

Different mechanisms by which APC exerts its cytoprotective activity have been proposed, including binding to histones, integrin, ApoER2 and PAR1/EPCR. APC ability to mediate endothelial barrier stabilization has been proposed to be mediated through the EPCR/PAR1 signalling activity of APC¹²¹. It is suggested that APC first binds to EPCR which mediates PAR1 signalling. PAR1 signalling results in activation and upregulation of sphingosine kinase. This results in the activation of S1P, which in turn activates the S1P receptor-1¹²¹. This results in the activation of Rac-1 (a GTPase) which results in endothelial barrier protection due to rearrangements of the cytoskeleton which reduces the permeability of the endothelium¹²¹. For this APC/EPCR PAR1 signalling caveolin-1 must be present and it is suggested that the receptors are colocalised with specialised lipid rafts¹²¹.

However, the endothelial barrier stabilizing activity of APC might be due to a mechanism which is PAR1 independent. An inhibitory PAR1 antibody did not significantly reduce the barrier stabilization properties of APC¹⁶³. This suggests that PAR1 might be of little importance in mediating the endothelial barrier stabilization by APC. However, this mechanism has been found to be EPCR dependent, as RCR-252 (inhibitory EPCR antibody) reduced APC ability to protect the endothelium¹⁶³.

In this study it was found that APC-I18V has similar ability to protect the endothelial barrier from thrombin induced hyper permeability compared to APC-WT. This would

suggest that neutral lipid affinity of APC is unimportant for APC endothelial barrier stabilizing activity, and that PE and GlcCer do not play a role in this activity of APC. This observation is not surprising as for the barrier stabilizing effects of APC, the Gla domain of APC binds to EPCR. As reported here, loss of PE and GlcCer sensitivity does not effect EPCR binding as APC-I18V has normal affinity for EPCR.

In this study the Ea.hy 926 thrombin induced barrier permeability assay was used as it has been used extensively to investigate APC cytoprotective activity^{29,40,109}. In this study the immediate response of the pretreated cells to thrombin was reported. However, further work should look at how these Ea.hy 926 cells would recover after expose to thrombin. This should be done by washing the thrombin from the cells and then observing recover once place in normal culture media. Of interest to this study would be if there was any difference in the recover of the cells pretreated with APC-I18V compared to APc

4.9.5 Strategies to separate the anticoagulant and cytoprotective activities of APC

There have been many attempts to separate the anticoagulant and cytoprotective activities of APC. APC-L38D is a Gla domain variant which retains its cytoprotective signalling *in vitro*, as seen by normal anti-apoptosis and endothelial barrier permeability activity. However APC-L38D has dramatically reduced anticoagulant activity *in vitro*, due to a lack of protein S sensitivity⁴⁰. 5A-APC consists of two clusters of alanine mutations, Lys191-193 and Arg229/239 to Ala, and this disrupts the binding site of FVa exosite on APC resulting in decreased APC anticoagulant activity¹²². 5A-APC has

significantly reduced anticoagulant activity, however *in vivo* it was as effective as APC-WT at reducing mortality of sepsis and LPS challenge¹²².

A protein C variant which had an engineered disulfide bond in a critical Ca^{2+} binding loop of the serine protease domain (Cys⁶⁷-Cys⁸²) resulted in a APC variant that had almost eliminated anticoagulant activity⁴⁷. However, this variant, while needing higher concentrations than wild-type APC, still exhibited cytoprotective activity⁴⁷. The requirement for higher concentration of Cys⁶⁷-Cys⁸²-APC compared to wild-type APC is believed to be due to decreased EPCR affinity of this mutant⁴⁷.

Recently, an exciting new variant has emerged which has increased cytoprotective activity and decreased anticoagulant activity²⁹. This variant consists of the L38D mutant in the APC Gla domain and a mutant of one of the N-linked glycan in the serine protease domain, N329Q. APC-L38D/N329Q has minimal anticoagulant activity, however has dramatically increased cytoprotective signalling activity *in vitro*²⁹.

This study has identified a possible new strategy of separating out the cytoprotective signalling of APC from the anticoagulant activity. APC-I18V has normal endothelial barrier stabilizing activity with reduced anticoagulant activity due to decreased neutral lipid enhancement. Reduced, but not absent, anticoagulant properties may however make the APC-I18V variant useful in the treatment of diseases in which the beneficial effects of APC have been shown to require anticoagulant activity in addition to EPCR-PAR1 signalling. For example, a recombinant APC variant with no EPCR affinity was partially protective in a murine model of experimental autoimmune encephalomyelitis¹¹².

4.9.6 Summary

APC-I18V represents a novel mechanism of separating the anticoagulant and cytoprotective activity of APC. APC-I18V has reduced anticoagulant activity as it lacks PE and GlcCer lipid enhancement, however it retains normal EPCR binding affinity and has normal endothelial barrier stabilization properties. Also APC-I18V activity is inhibited by normal pooled plasma at a similar rate to wild type APC *in vitro*.

CHAPTER 5 Investigation of the effects of high density lipoprotein on the protein C pathway

5.1 HDL influence on the protein C pathway

Lipoproteins are complexes of proteins and lipids, which are known to affect coagulation. HDL concentration in blood is approximately 1.5mg/ml. HDL has been shown to enhance APC anticoagulant activity, while LDL had no effect on APC activity⁶⁹. However, these results showing HDL enhancement effect have recently been disputed. The original study also showed that unlike phospholipids which support procoagulant activity, HDL seems to only support anticoagulant activity⁶⁹. As was the case for PE and GlcCer, the mechanism of HDL enhancement of APC anticoagulant activity is unknown⁶⁹. Therefore this chapter will investigate the nature of HDL enhancement of APC anticoagulant activity.

HDL has important anti-inflammatory activity. However, it has not been investigated whether HDL has an effect on APC cytoprotective signalling activity. Therefore, the endothelial barrier permeability assay will be used to examine whether HDL has an influence on APC cytoprotective signalling activities.

5.2 HDL enhances APC anticoagulant activity

The effect of HDL on APC anticoagulant activity was investigated using the thrombin generation assay in protein C deficient plasma. To ensure that HDL alone had no anticoagulant activity in this assay, thrombin generation was assessed in the presence of 1.5mg/ml HDL in the absence of APC. HDL alone was found to have little effect on thrombin generation compared to the untreated sample with $P>0.4$. (Figure 5.1). As expected in this assay, APC alone significantly decreased the amount of thrombin generated ($P<0.02$). In the presence of HDL, APC anticoagulant activity was further enhanced with a statistically significant difference between APC treatment alone compared to HDL and APC ($P<0.04$).

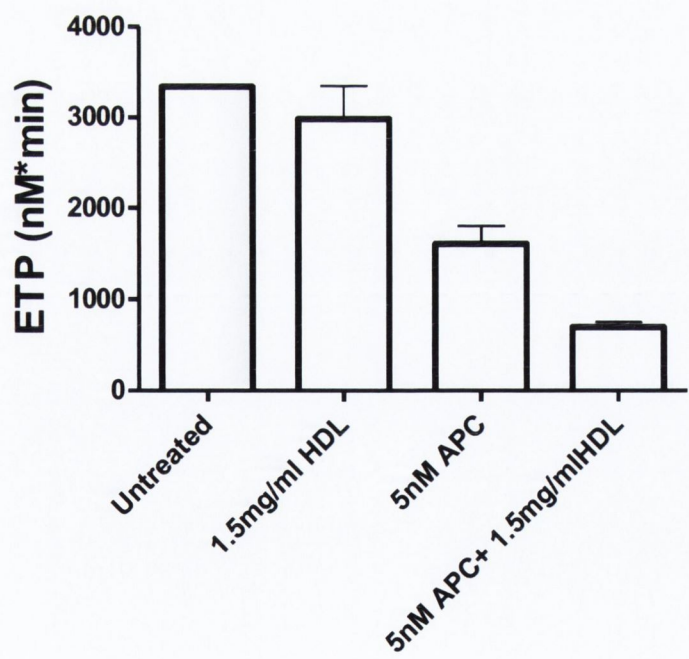


Figure 5-1: HDL enhances APC anticoagulant effect.

Thrombin generation was determined in 80 μ l protein C-deficient plasma in the presence of 5nM APC, 1.5mg/ml HDL and 20 μ l of a PPP reagent (Total volume per well 120 μ l). 1.5mg/ml HDL only was included as a control. The results represent the mean ETP $\pm$ SEM for 3 independent experiments.

5.3 Effect of protein S on HDL enhancement of APC anticoagulant activity

Next it was investigated whether the HDL effect on APC anticoagulant activity was dependent on protein S. Using the thrombin generation assay previously established to investigate protein S sensitivity (Section 2.3.4), the effect of HDL has on APC anticoagulant activity was determined.

Interestingly, HDL enhanced APC anticoagulant activity even in the absence of protein S, as seen in the reduced ETP in the presence of APC and HDL compared to APC alone at zero protein S concentration (Figure 5.2A).

However, when protein S was titrated back into the protein S deficient plasma, the presence of HDL did not affect APC sensitivity to protein S, as the slope of the lines for both presence and absence of HDL are very similar (Figure 5.2B).

Therefore, this would suggest that a) HDL effect on protein C anticoagulant activity is independent of protein S and b) that HDL has no effect on protein C sensitivity to protein S.

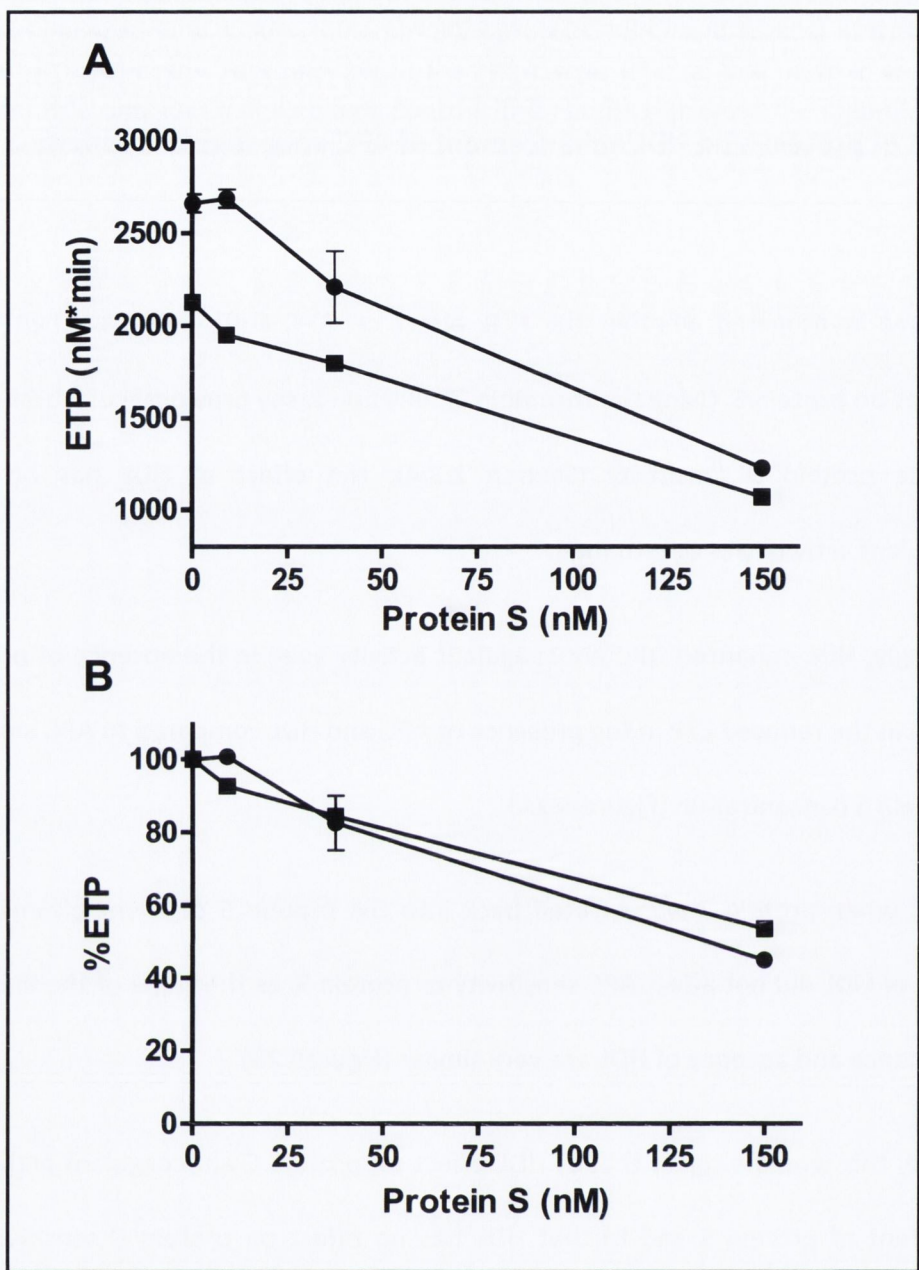


Figure 5-2: HDL does not affect APC sensitivity to protein S.

The ability of 10nM hAPC (circles) or 10nM hAPC + 1.5mg/ml of HDL (squares) to inhibit tissue-factor induced thrombin generation in protein S deficient plasma was assessed in the presence of protein S (9-150nM). The mean ETP $\pm$ SEM at each protein S concentration was determined in at least three independent experiments.

5.4 Effect of HDL on APC barrier stabilization activity

After confirming that HDL does increase APC anticoagulant activity, it was investigated if HDL has any effect on APC cytoprotective signalling. The endothelial barrier permeability assay, which was already established (Section 2.3.8) was utilized. HDL was co-incubated with APC on EA.hy926 cells for 3h before thrombin was added to the cells for 10min to induce permeability.

HDL, in the absence of APC, had little effect on thrombin induced hyper-permeability (Figure 5.3). However, HDL enhanced APC ability to attenuate thrombin induced hyper-permeability (Figure 5.3). HDL was found to significantly enhance APC signalling ($P=0.001$) for HDL and APC treated cells compared to APC only treatment.

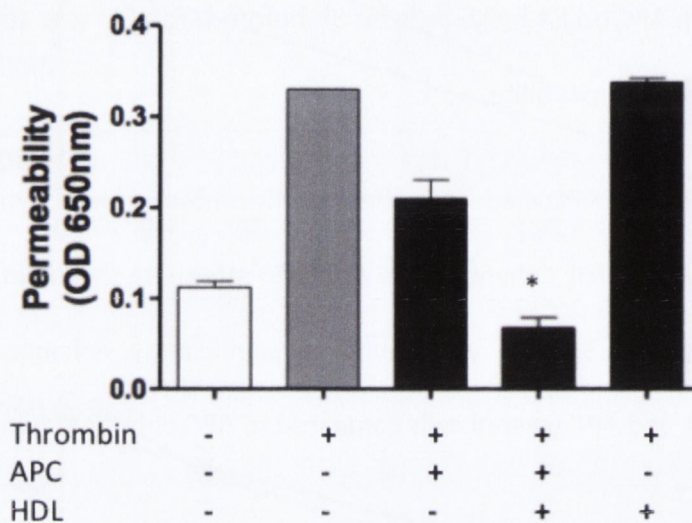


Figure 5-3: HDL enhances the barrier stabilising properties of APC.

To assess HDL effect on APC barrier stabilization, EA.hy926 cells were pre-incubated with either 10nM APC alone or 10nM APC with 1.5mg/ml HDL for 3hrs (*black bars*). Untreated EA.hy926 cells (*white bar*) were used as a negative control. EA.hy926 cells were then treated with 5nM thrombin for 10min (thrombin only treated cells, *grey bar*) and endothelial barrier permeability was assessed after 30min using Evans Blue-BSA complex. Results represent the mean $\pm$ SEM for 3 independent experiments.

5.5 Effect of APC concentration on HDL enhancement of barrier permeability

Next it was investigated whether HDL could enhance signalling of APC at very low concentrations. Using a concentration of 0.75mg/ml HDL (approximately half normal plasma concentration), a range of APC concentrations (0.75-10nM) was investigated for its ability to protect the endothelial barrier.

Cells which were not incubated with either HDL or APC and were untreated with thrombin, represented by the white square in Figure 5.4, were used as a control. In the absence of APC, there was no difference in thrombin induced permeability in cells treated with HDL (black squares Figure 5.4) or untreated (triangles) cells. However, in the cells treated with HDL and increasing APC concentrations there was a dramatic increase in endothelial barrier protection (Figure 5.4: black squares) compared to cells treated with APC alone (Figure 5.4: triangles). The maximum protection of the endothelial cells was observed at 2.5nM APC and 0.75mg/ml HDL (Figure 5.4), these cells had a similar result to the control cells.

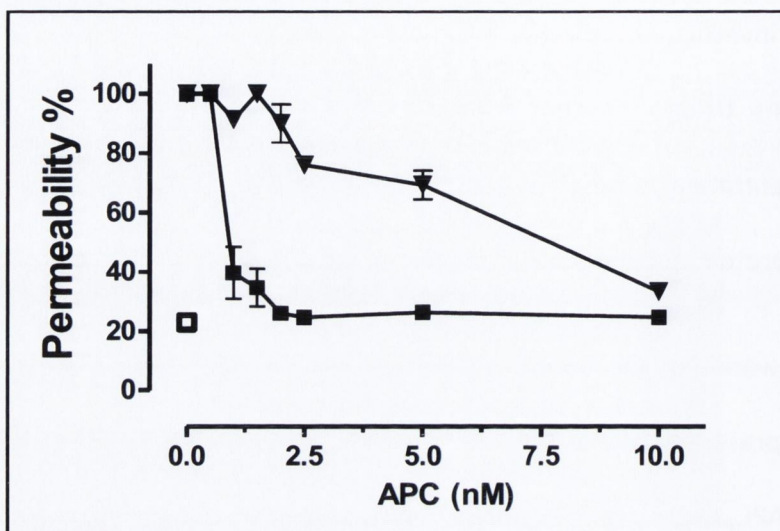


Figure 5-4: Range of APC concentrations with or without 0.75mg/ml of HDL.

To determine the role of APC concentration on HDL signalling effect, the permeability assay was used. EA.hy926 cells were pre-incubated with the indicated APC concentration with (black squares) or without (triangles) 0.75mg/ml of HDL for 3hrs. Untreated EA.hy926 cells were used as a negative control (white square). EA.hy926 cells were then treated with 5nM thrombin for 10min and endothelial barrier permeability was assessed after 20min using Evans Blue-BSA complex. Results represent the mean $\pm$ the SEM for 3 independent experiments.

5.6 Effect of HDL concentration on APC endothelial barrier permeability

After demonstrating HDL's effect on APC cytoprotective signalling, the effect of different HDL concentrations on APC signalling was investigated (Figure 5.5). A range of HDL concentration was tested from 0.25mg/ml to 1.5mg/ml for its ability to enhance 5nM hAPC barrier stabilizing properties.

Cells which were not incubated with either HDL or APC and were untreated with thrombin, represented by the white square in Figure 5.4, were used as a control. The enhancement effect of HDL was found to be maximal at 0.75mg/ml HDL in the presence of 5nM hAPC (Figure 5.5). However, even at the lowest HDL concentration tested (0.25mg/ml) there was still an increase in APC protective signalling (Figure 5.5).

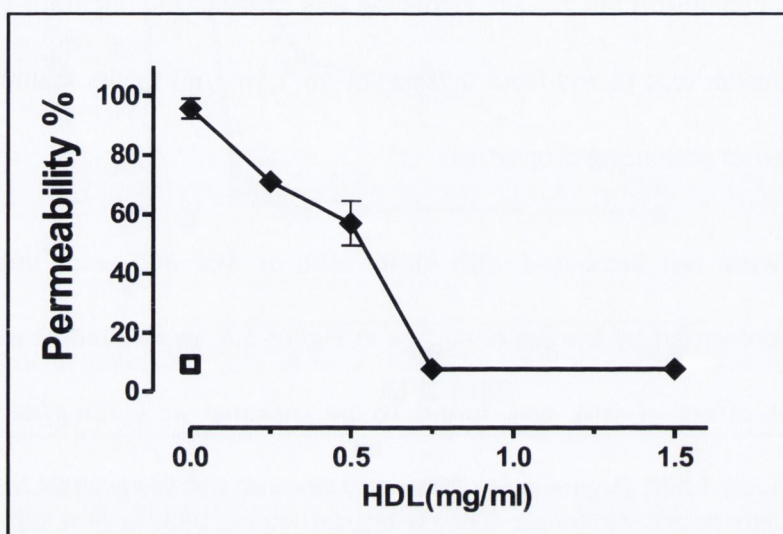


Figure 5-5: Range of HDL concentrations with 5nM hAPC.

To determine the role of HDL concentration on its signalling effect, the permeability assay was used. EA.hy926 cells were pre-incubated with 10nM hAPC with or without the indicated HDL concentration for 3hrs. Untreated EA.hy926 cells were used as a negative control. EA.hy926 cells were then treated with 5nM thrombin for 10min and endothelial barrier permeability was assessed after 20min using Evans Blue-BSA complex. Results represent the mean $\pm$ the SEM for 3 independent experiments.

5.7 HDL enhancement of APC signalling is independent of EPCR

To establish whether HDL enhancement of APC cytoprotective signalling activities was dependent on EPCR, the permeability assay was performed in the presence of a blocking antibody, RCR252, which prevents APC signalling through EPCR.

In this experiment 20nM APC was used because at this concentration APC is seen to be protective at preventing thrombin induced permeability. Therefore, it could be shown that the EPCR antibody, RCR252 does prevent APC protective signalling in our assay (Figure 5.6). Interestingly, in the presence of the anti-EPCR antibody, HDL was still observed to have an effect on APC protective signalling (Figure 5.6). This suggests that the mechanism behind HDL effect on APC protective signalling is EPCR independent.

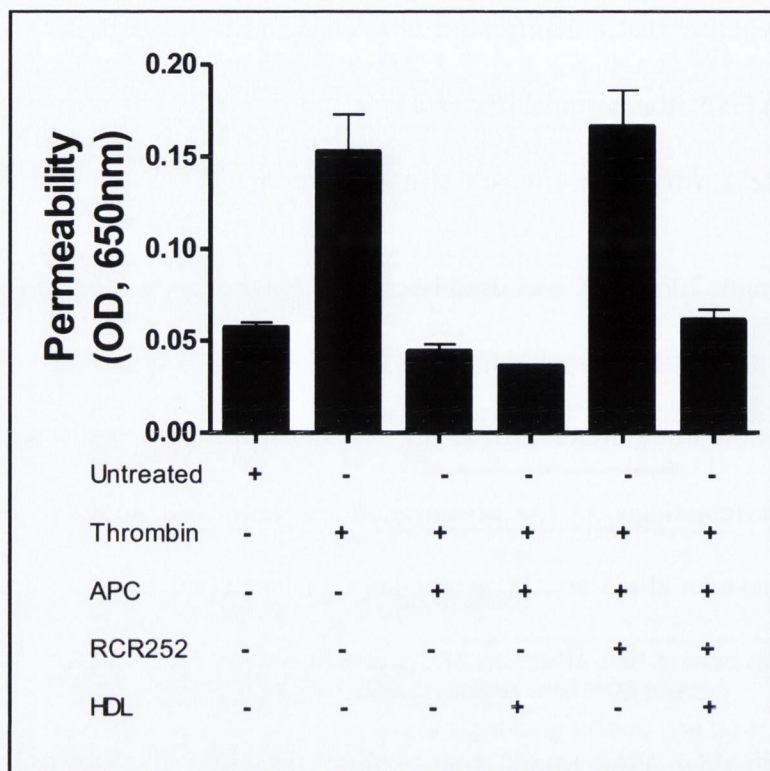


Figure 5-6: HDL barrier stabilizing effect is independent of EPCR.

To determine the role of EPCR in HDL signalling effect, the permeability assay was used. EA.hy926 cells were pre-incubated with 20nM hAPC with or without 0.75mg/ml of HDL for 3hrs. The anti-EPCR antibody, RCR252 was added to the required samples for this 3hr incubation. Untreated EA.hy926 cells (control) were used as a negative control. EA.hy926 cells were then treated with 5nM thrombin for 10min (thrombin) and endothelial barrier permeability was assessed after 20min using Evans Blue-BSA complex. It was found that HDL effect on APC anti-inflammatory action was independent of EPCR. Results represent the mean $\pm$ the SEM for 3 independent experiments.

5.8 HDL does not affect protein C activation

Next, it was investigated if HDL influenced protein C activation by the thrombin/TM complex on the surface of endothelial cells. To study protein C activation, EA.hy926 cells were used as an endothelial cell model. Protein C was incubated on EA.hy926 cells in the presence of thrombin for 30min and the amount of activation was determined by using a chromogenic substrate for APC. Protein C activation was found to occur at very similar rates in the presence or absence of HDL (Figure 5.7). The K_m , which is the substance concentration at which half maximal velocity occurs, was calculated by both the Michaelis Menten method (Figure 5.7A) and by the Lineweaver Burke plot method (Figure 5.7B). By the Michaelis Menten method the K_m for protein C alone was $117.4 \pm 50 \text{ nM}$ and for protein C with HDL the K_m was $79.24 \pm 30 \text{ nM}$. By the Lineweaver Burke plot the K_m for protein C alone was 115 nM and for protein C with HDL the K_m was 95 nM .

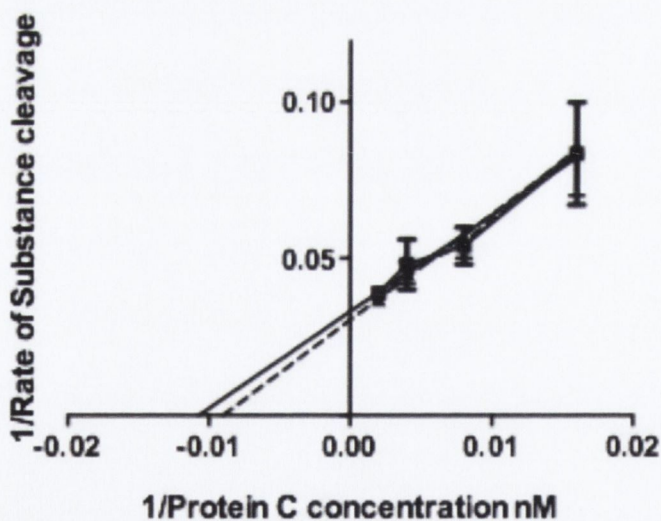
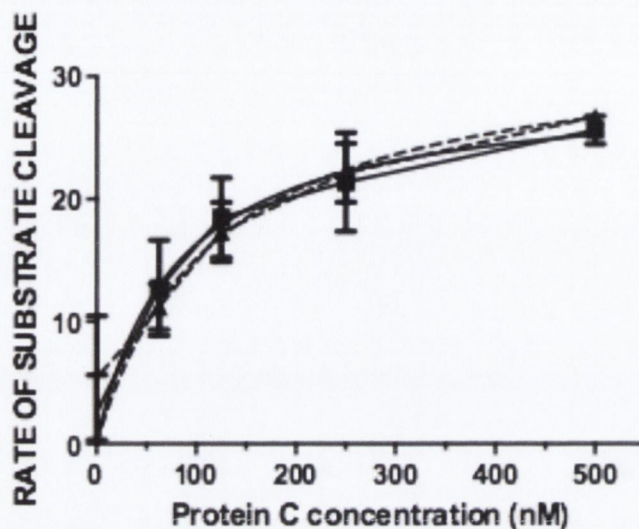


Figure 5-7: Activation of protein C with or without HDL on the surface of EA.hy926 cells. Protein C (62.5-500nM) with 1.5mg/ml of HDL (squares) or on its own (triangles with dotted lines) was added to EA.hy926cells in HBSS containing 3nM CaCl_2 and 0.6mM MgCl_2 . Activation was initiated by the addition of thrombin. After 30min, thrombin was inhibited with hirudin. Results are presented as the mean $\pm$ SEM for 3 individual experiments. (A) represents the Michaelis Menten data while (B) represents the Lineweaver Burke plot of the same data. Rate of substance is the V_{max} .

5.9 HDL does not affect FXa barrier stabilizing ability

As well as APC, FXa is known to protect against thrombin induced permeability on endothelial cells¹⁶⁴. Therefore, it was investigated whether HDL has any effect on FXa signalling, using the barrier permeability assay. First, it was determined that FXa was protective against thrombin induced permeability in this assay (Figure 5.8).

It was found that HDL had little effect on FXa protective signalling (Figure 5.8). Therefore, this suggests the mechanism behind FXa and APC signalling are distinct and, whatever effect HDL is having on the endothelial cells/APC is unique to that pathway.

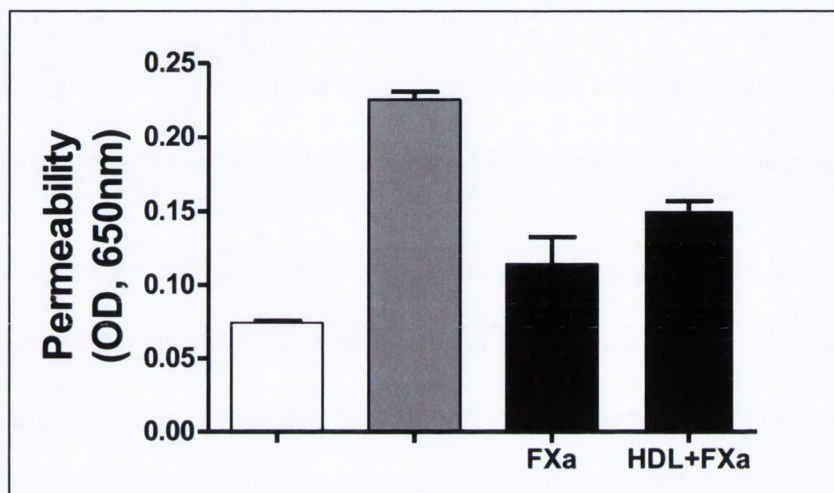


Figure 5-8: HDL does not affect FXa cytoprotective signalling.

To assess HDL effect on FXa barrier stabilization, EA.hy926 cells were pre-incubated with either 20nM FXa or 20nM with 1.5mg/ml HDL for 3hrs (*black bars*). Untreated EA.hy926 cells (*white bar*) were used as a negative control. EA.hy926 cells were then treated with 5nM thrombin for 10min (thrombin only treated cells, *grey bar*) and endothelial barrier permeability was assessed after 30min using Evans Blue-BSA complex. Results represent the mean $\pm$ the SEM for 3 independent experiments.

5.10 Discussion

5.10.1 Role of HDL on APC anticoagulant activity

This study aimed to clarify the conflicting reports in relation to HDL enhancement of APC anticoagulant activity. In this study, it was found that HDL does enhance APC anticoagulant activity. It has been suggested that the reason Oslakovic et al⁹⁷ did not observe HDL enhancement of APC anticoagulant activity was due to their use of frozen HDL^{165,166}. In this study, the HDL used was from human plasma and was not frozen; and was certified to be an essentially purified sample, free of contaminating lipoproteins.

Interestingly, the results presented in this study suggest that the enhancing ability of HDL is independent of protein S. This is a novel finding. This result suggests that HDL is mediating a protein S-independent effect on APC which results in increased anticoagulant activity. It has been suggested that HDL particles may have organized phospholipid domains which support APC anticoagulant activity⁶⁹. The precise nature of HDL enhancement is still unclear. However, it is known that the phospholipid content of HDL is essential for mediating HDL anticoagulant effect⁶⁹. As the phospholipid composition of HDL and LDL is not significantly different⁶⁹, the reason why only HDL enhances APC anticoagulant activity is unclear. However, it may be due to interaction between APC and apolipoproteins which are unique to HDL⁶⁹.

It is possible that *in vivo* both protein S and HDL may act together as cofactors to APC, each having a unique effect on APC. However the observation that deficiency of protein S results in a similar phenotype to protein C deficiency (Section 4.9.1) suggests that protein S has an unique influence on APC anticoagulant activity, via a mechanism that HDL cannot replicate. However, HDL may also play a vital role. It has been found that the sensitivity of plasma to the APC-protein S anticoagulant activity is significantly correlated with plasma levels of large HDL particles¹⁶⁵, suggests that *in vivo* there may well be a requirement for both protein S and HDL in mediating APC anticoagulant activity. It is possible that *in vivo* HDL is providing an attractive phospholipid surface for APC to bind to which enables APC to interact with protein S, allowing for full APC anticoagulant activity.

5.10.2 Possible role of HDL in influencing APC cytoprotective activity

HDL was investigated for its possible role as a cofactor to APC cytoprotective signalling activity. This was investigated to try and explain why APC can be cytoprotective *in vivo* at very low concentration while *in vitro* APC cytoprotective signalling is not seen at these concentrations. Therefore, it was hypothesized that HDL might be acting as a cofactor for APC signalling.

The results here show that normal plasma levels of HDL increase APC ability to protect the endothelial barrier for thrombin induced hyper-permeability. Even at very low levels of APC (0.75nM), HDL still resulted in an ability to protect the endothelial barrier. These results indicate a role for HDL in mediating of APC cytoprotective signalling.

HDL was found to enhance APC mediated barrier protection in an EPCR-independent manner. This contrasts with APC alone which is known to only be barrier stabilizing when EPCR is present. One of the proposed theories for the EPCR dependency of the APC signalling, is the requirement for PAR1 to be located within specific lipid rafts¹¹⁹. It is proposed that APC bound to EPCR is colocalised with PAR1 in caveolin-1 enriched lipid rafts¹¹⁹. When the EPCR and PAR1 receptors are colocalised in this fashion it sets up protective signalling through PAR1 mediated by GTPase, Rac1¹¹⁹. Therefore, from the results of this study, it is possible that HDL might be providing an alternate surface for APC to bind and signal through. HDL might then mediate a similar colocalising of APC and PAR1 in these caveolin-1 enriched rafts.

The results reported here showing that HDL enhances protective APC signalling is the first known description of a possible *in vivo* cofactor for APC cytoprotective effect. HDL could be mediating its effects on APC signalling through S1P. HDL is the main carrier for S1P in the plasma. As discussed in Section 4.9.4, S1P is upregulated by APC leading to activation of S1P receptor-1 (S1P1). However, as HDL has little effects in the endothelial barrier assay in the absence of APC in the assay used in this study, it is not simply a case of HDL supplying S1P that resulted in the protective effects. Instead, it is possible the APC, HDL and S1P are all cooperating to maximize endothelial barrier stabilization.

HDL effect on barrier stabilising was found to be APC-dependent, as HDL was found to have no effect on FXa cytoprotective signalling. Despite this FXa alone was found to be protective against thrombin induced permeability, as previously reported¹⁶⁴. FXa is reported to signal through both PAR1 and PAR2 to mediate its cytoprotective signalling activities¹⁶⁴. The fact that HDL has no effect on FXa signalling is an indicator that there

are separate signalling cascades through which APC and FXa signal. The results suggest that whatever effect HDL is mediating on APC signalling is unique to the APC signalling cascade.

5.10.3 Protein C activation

Protein C activation occurs via the thrombin/TM complex upon protein C binding to EPCR (Section 1.4). However, in the microvasculature where there is negligible EPCR present the mechanism by which protein C is activated is unclear. Platelet factor-4 (PF-4) has been found to enhance APC generation in cynomolgus monkeys⁴⁴. Therefore, it was investigated whether HDL could also affect protein C activation. In this study, it was found that HDL did not influence protein C activation on the surface of EA.hy926 cells in the presence of thrombin. However, as EA.hy926 cells express EPCR this result does not exclude the possibility that HDL might influence protein C activation in the absence of EPCR. This is a possibility as the HDL enhancement effect on APC cytoprotective signalling was found to be EPCR-independent.

5.10.4 Summary

In this study, it was found that HDL enhances APC anticoagulant activity and that this effect was independent of protein S. HDL was found to enhance APC endothelial barrier stabilizing ability and this was in an EPCR-independent manner. This study indicates that HDL is an important *in vivo* modulator of APC function.

CHAPTER 6 General discussion of this study

Prior to this study it was known that certain lipids play a role in enhancing the anticoagulant activity of APC. Furthermore, there is evidence that the interaction between neutral lipids and APC is clinically important⁸¹. However, the precise role these lipids play in mediating APC function was unclear. Therefore, the aim of this study was to investigate the role of both membrane lipids and lipoproteins on the protein C pathway.

Firstly, the role of PE and GlcCer in mediating APC anticoagulant activity was investigated. In keeping with previous studies, this study found that APC anticoagulant activity was enhanced approximately 3-fold in the presence of either PE or GlcCer. The precise nature of the PE and GlcCer interaction with APC anticoagulant activity was investigated.

Interestingly, this study identified a critical residue, isoleucine-18, in APC Gla domain that mediated neutral lipid enhancement of APC anticoagulant activity. While other residues in APC Gla domain may be involved in mediating this reaction between neutral lipids and APC, the identification of this critical residue in the Gla domain should significantly enhance our understanding of the mechanism of APC-neutral lipid enhancement. Using an atomic model of anionic lipid membrane-bound FVII Gla domain it was suggested that there is deep insertion of the ω -loop into the membrane bilayer¹⁵⁶. In this model, the corresponding FVII amino acid residue to APC isoleucine-18 (Lys-18) is located close to the membrane surface. If the APC ω -loop inserts into the membrane bilayer in a similar manner to FVIIa, the expected localization of isoleucine

18 at the membrane surface provides a possible rationale for how isoleucine 18 could mediate putative lipid head group interactions and consequently, lipid cofactor enhancement of APC anticoagulant activity. As reported in this study isoleucine 18 resides on the outer surface of the APC Gla domain in a location where it might well be mediating this interaction.

Furthermore, this study showed that PE or GlcCer are only effective in the presence of protein S. This suggests that the PE and GlcCer enhancement of APC anticoagulant activity is a protein S dependent mechanism and for full APC anticoagulant activity both protein S and PE or GlcCer must be present. Therefore it may be that *in vivo* there are microdomains/lipid rafts on the cell surface which are enriched with PE or GlcCer which facilitate optimal binding of protein S and APC. Support for this is seen by the observation that GlcCer is not disrupted uniformly throughout the cell but instead exists in lipid rafts⁷⁹. Further work is required to fully establish the nature of the neutral lipid enhancement of APC.

Recombinant APC was licensed for the treatment of late-stage sepsis and may also be therapeutic in a number of other disease states, including inflammatory bowel disease¹¹¹, multiple sclerosis¹¹² and ischemic stroke¹¹³. Noteworthy, the beneficial effect of recombinant APC in murine models of sepsis is believed to be not associated with its anticoagulant activity, but instead arises from EPCR-dependent PAR1 signalling¹²². APC signalling via EPCR-PAR1 exerts broad anti-inflammatory and anti-apoptotic signalling on endothelial cells¹⁰⁷. Several approaches to limit anticoagulant activity by disruption of APC substrate and cofactor molecular interactions, whilst preserving anti-inflammatory function, have been described^{40,47,105,106}.

This study has shown that APC-I18V exhibits impaired anticoagulant activity, but retained normal EPCR-dependent PAR1 signalling function. The precise amount of anticoagulant activity that APC-I18V has *in vivo* is unpredictable, given the incomplete understanding of the relative contributions of membrane lipid cofactors to APC anticoagulant function within the vasculature. However, reduced but not absent anticoagulant properties may make the APC-I18V variant useful in the treatment of diseases in which the beneficial effects of APC have been shown to require anticoagulant activity in addition to EPCR-PAR1 signalling. For example, a recombinant APC variant with no EPCR affinity was partially protective in a murine model of experimental autoimmune encephalomyelitis¹¹².

The effect of the lipoprotein HDL on APC function was also examined in this study. HDL was found to enhance APC anticoagulant activity, supporting the idea that HDL is a cofactor for APC⁶⁹. The nature of the lipoprotein HDL enhancement of APC anticoagulant activity has been reported to be distinct from the phospholipid enhancement⁶⁹. The results of this study suggest that the HDL enhancement of APC anticoagulant activity is independent of protein S. Therefore it is possible that *in vivo* protein S and HDL are cooperating to maximise APC anticoagulant activity.

This study has demonstrated that HDL can enhance APC barrier stabilizing activity. This is the first study that has demonstrated a cofactor to APC cytoprotective signalling that may be occurring *in vivo*. The results from this study suggest that HDL might be mediating its anti-inflammatory activity through its enhancement of the APC cytoprotective signalling pathway.

This study had hypothesized that HDL might be the missing cofactor for APC

cytoprotective signalling and may explain why APC can be cytoprotective *in vivo* at very low concentration while *in vitro* APC cytoprotective signalling is not seen at these concentrations. The results from this study support this idea. Even at very low levels of APC (0.75nM), HDL enabled APC to protect the endothelial barrier.

Interesting, and in contrast to APC alone, HDL was found to enhance APC mediated barrier protection in an EPCR-independent manner. It is proposed that the EPCR dependency of APC signalling is due to the requirement for PAR1 to be located within specific lipid rafts and that EPCR facilitates this arrangement¹¹⁹. This suggests that HDL might be mediating a similar colocalization of APC and PAR1 into caveolin-1 enriched rafts, as seen when APC signals through EPCR. Therefore in the presence of HDL, the requirement for the EPCR dependent colocalizing of receptors may be alleviated. Interesting the effect of HDL on endothelial barrier stabilisation was found to be dependent on APC, HDL did not affect FXa cytoprotective signalling. This provides evidence that the effect HDL is mediating on APC signalling is unique to the APC signalling cascade.

6.1 Clinical translation of APC therapeutics

The use of APC products in treating disease has been studied intensively since the 2001 PROWESS clinical trial reported that recombinant APC enhanced survival from severe sepsis. PROWESS was a randomised double-blind placebo-controlled trial on adults with severe sepsis and it reported a significant reduction in 28-day mortality¹⁰². The relative reduced in death in the treated group was 19.4%. The trial reported an increase risk of severe bleeding in the treated group (3.5% compared to 2%). After this trial Drotrecogin alfa (activated), a recombinant form of human activated protein C, was

licensed for the treatment of severe sepsis.

Recently, the PROWESS-SHOCK clinical trial reported that recombinant APC treatment did not affect survival from sepsis shock¹⁶⁷. PROWESS-SHOCK was a randomised, double blind, placebo control trial with multiple trial centres. The PROWESS-SHOCK trial had very strict criteria for inclusion of patient with severe sepsis and septic shock. They reported no significant difference between the treated or untreated groups in either the 28day or 90day survival. The reason for the discrepancy between the two PROWESS trials is unclear especially considering all the *in vitro* and *in vivo* APC studies which have some that APC has anti-inflammatory and cytoprotective functions. The PROWESS-SHOCK trial as a whole reported relatively low mortality rate in the setting of sepsis¹⁶⁸. This might be of key importance in relation to APC as it was believed that APC worked best in the patients who are serious unwell with sepsis.

The results of the PROWESS-SHOCK clinical trial led to the FDA withdrawing the license for recombinant APC therapy. While this is a disappointing development for those working with APC, it underlines the importance of furthering our understanding of the beneficial effects of APC *in vitro* and in murine models. Through a deeper understanding of these mechanisms of APC it is hoped that novel APC therapeutics will emerge which will be effective in the treatment of sepsis and other diseases.

This study has reported a novel mechanism for separating the anticoagulant and cytoprotective function of APC, through the use of APC-I18V. APC-I18V is expected to have much reduced anticoagulant activity *in vivo* but maintain normal cytoprotective signalling properties. Therefore, APC-I18V should be of use in disease setting that required both cytoprotective and anticoagulant activity but where APC-WT would have

a high bleeding risk. APC-I18V might be useful in the treatment of multiple sclerosis and acute kidney injury as in murine models of experimental autoimmune encephalomyelitis¹¹⁴ and acute kidney injury¹¹⁵ it was found that both cytoprotective and anticoagulant activities of APC were required for any beneficial effects of treatment.

Furthermore, this is the first study to report of a possible *in vivo* cofactor to APC cytoprotection, i.e. HDL. In light of this discovery the results from clinical trials with APC should be reviewed with consideration of the lipid profile of the patients. If HDL is enhancing APC cytoprotective signalling *in vivo* to a similar degree as seen in this study *in vitro*, then one might expect a poor clinical response to APC treatment in patients with low HDL levels.

6.2 Recommendations for further work

This study has furthered our understanding of the role of lipids in the protein C pathway. However, due to the clinical importance of this pathway and the potential therapeutic benefits of APC, further study is required. Further study of the precise nature of neutral lipid enhancement of APC will be helped by the use of APC-I18V, the first variant described that is insensitive to the effects of PE and GlcCer. The effect of PE and GlcCer on APC-WT and APC-I18V binding to phospholipids should be elevated. Biacore surface plasmon resonance could be utilised to investigate this interaction. Wild type protein C or protein C-I18V could be immobilised on a sensor chip prior to PC/PS or PC/PS/PE or PC/PS/GlcCer vesicles been passed over chip. Hopefully, this

would allow any differences between the binding of protein C-I18V compared to protein C-WT to be quantified. Alternatively, an ELISA technique could be developed to study this interaction.

The precise amount of anticoagulant activity of APC-I18V *in vivo* is unknown, however using animal models it may be possible to establish this. Using mouse models, APC-I18V or APC-WT could be infused and the effectiveness of the anticoagulant activity of the APC could be evaluated through tail bleeding time or thrombin generation time from blood samples¹⁶⁹. This should provide us with some detailed information about the contribution of neutral lipid enhancement of APC anticoagulant activity *in vivo*. Hopefully, through the use of APC-I18V, we should be able to establish the precise contribution of neutral lipid enhancement to APC anticoagulant activity *in vivo*.

As neutral lipid enhancement of APC anticoagulant activity is a protein S dependent effect, the precise mechanism of the protein S-APC anticoagulant activity will probably need to be resolved before a full understanding of neutral lipid enhancement of APC can be achieved. Recent work is mapping the interaction between APC and protein S and has shown that in addition to EGF2 domain of protein S, both the Gla domain and EGF1 domain of protein S are involved in the protein S cofactor interaction with APC¹⁵⁹. These authors also suggest that residues in APC Gla domain, especially leuince-36 and residues in EGF-1 are co-operating to facilitate full protein S cofactor activity.

It seems apparent now that HDL is an important cofactor to APC functions. Further work on the precise mechanism of HDL enhancement of APC anticoagulant activity is required. This work should focus on the different subfractions of HDL to identify whether specific subfractions are responsible for HDL enhancement of APC anticoagulant activity. Recent work has shown that it may be the large particles of HDL

that are most effective in enhancing APC anticoagulant activity¹⁶⁵. There is emerging evidence that there may be subfractions of HDL that are mediating different HDL functions. Indeed, a subfraction of HDL containing apolipoprotein C-III may be proatherogenic and be directly associated with risk of coronary heart disease¹⁰¹. As HDL is so vital *in vivo*, the precise function of each subfraction of HDL should be determined. Further work should isolate these subfractions of HDL from normal pooled plasma and investigate their properties in the thrombin generation assay used in this study. It will be challenging to determine precisely what the nature of the circulating HDL particles are *in vivo*.

Further work is required to understand the nature of HDL and APC cytoprotective signalling. The nature of the signalling pathway of APC in combination with HDL should be determined. This study showed that HDL ability to enhanced APC mediated barrier stabilization is via an EPCR-independent mechanism. In addition to the barrier stabilizing properties of APC and HDL, the other cytoprotective signalling pathways of APC should be investigated to examine if HDL enhances these activities too. A recent study has shown that HDL enhances APC anti-apoptotic signalling. Therefore, further work should investigate whether HDL enhances APC anti-apoptotic properties. This can be done using the staurosporine induced apoptosis assay with Ea.hy 926 cells²⁹ and determining whether co-incubation of APC and HDL is more protective then APC alone. As HDL is known to be an important anti-inflammatory agent, the effect of HDL on APC anti-inflammatory pathway should also be examined. For example, it is known that APC interacts with integrins so it should be seen if HDL influences this reaction. APC is known to be anti-inflammatory activity and alters the inflammatory markers on endothelial cells. Therefore, it should be investigated if the addition of HDL to APC-

treated cells leads to enhancement of anti-inflammatory phenotype of the endothelial. For example, flow cytometry could be used to measure the expression of the inflammatory markers in Ea.hy 926 cells treated with either APC alone or APC and HDL. Also, as HDL has the ability to enhance both APC anticoagulant and cytoprotective signalling activity, it should be determined if a common mechanism exist. For example, it should be studied whether the interaction between APC and HDL results in a more active serine protease domain. Recently an APC glycan variant, APC-N329Q, has been shown to possess increased anticoagulant activity in addition to enhanced cytoprotective activities²⁹. While the reason for the increased anticoagulant activity is unknown, it was suggested that the increased cytoprotection was due to APC-N329Q having an altered interaction site for PAR1²⁹.

The cytoprotective signalling activity of APC is believed to require lipid rafts that are present on the cell membrane of endothelial cells¹⁰⁹. Previous research has established the required for caveolin-1 to be present for APC to exert its activity¹⁰⁹. Therefore, it should be investigated whether HDL enhancement of APC cytoprotection is mediated through this lipid rafts. Firstly, it should be determined whether lipid rafts are required for HDL to mediate its enhancement of APC. This could be done by disrupting the lipid rafts of the Ea.hy 926 cells prior to performing the thrombin induced permeability assay. Once it is known whether lipid rafts are required or not, the downstream signalling effects of HDL and APC treatment of the Ea.hy 926 cells should be investigated by analysis of the various G proteins and protein kinases involved.

Once the interaction between APC and HDL is further characterised *in vitro*, animal models should be used to examine the interaction *in vivo*. For example, one could investigate the cytoprotective effect of APC treatment in mice that are deficient in HDL

to try and determine whether HDL is required for APC cytoprotective *in vivo*. The anti-inflammatory properties of HDL subfractions *in vivo* should also be determined. Therefore, further human clinical therapies might be able to target increasing the 'protective' HDL subfraction in patients together with APC treatment to maximise the cytoprotective properties. These therapies could lead to huge improvements in patient care.

6.3 Summary

This study has further characterised the effect of membrane lipids and lipoproteins on APC function. This study has shown the importance of both neutral lipids and HDL in enabling full APC function. APC-I18V is a novel APC variant that separates APC anticoagulant and cytoprotective activity through its insensitivity to PE and GlcCer. This study suggests that HDL may be an important cofactor to both APC anticoagulant and cytoprotective activity. It is hoped that the results of this study will help to develop new approaches to the treatment of sepsis and other diseases. These second generation APC therapeutics will hopefully have the maximal beneficial effects of APC with minimal side-effects.

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