ISOLATION, IDENTIFICATION, IMMUNOLOCALISATION AND ELUCIDATION OF THE ROLE OF PLASMA KALLIKREIN IN HUMAN TISSUES

by

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AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted in part or any other form to the University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Pharmacology, Faculty of Medicine, University of Natal, Durban, South Africa under the supervision of Prof D.M. Raidoo.

SIGNED:_____

M.E. CERF

I hereby certify that the above statement is correct.

SIGNED:_____

Prof D.M. Raidoo

DEDICATION

I would like to dedicate my work to God; my mother, Mrs R.M. Cerf; my late father, Mr B.M. Cerf, and to my family.

PUBLICATIONS ARISING FROM THIS DISSERTATION

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ABBREVIATIONS

α-PI, alpha1-proteinase inhibitor
APTT, activated partial thromboplastin time
BK, bradykinin
BSA, bovine serum albumin
DAB, diaminobenzidine
dH_20, distilled water
DK, darmkontrahierenderstoff
EDRF, endothelium-derived relaxing factor
ELISA, enzyme linked immunosorbent assay
EtOH, ethanol
HAE, hereditary angioedema
HK, high molecular weight kininogen
HNE, human neutrophil elastase
ICC, immunocytochemistry
IgG, immunoglobulin G
Igl, α-(2-indanyl)-glycine
IMI, intra-muscular injection
KI-CPM, kininase I-carboxypeptidase M
KI-CPN, kininase I-carboxypeptidase N
KII-ACE, kininase II-angiotensin converting enzyme
KII-NEP, kininase II-neutral endopeptidases
KKS, kallikrein-kinin system
LK, low molecular weight kininogen
Lys-BK, lysl-bradykin (kallidin)
MAC, membrane attack complex
MeOH, methanol
PAP, peroxidase anti-peroxidase
PBS, phosphate buffered saline
PCR, polymerase chain reactions
PK, plasma kallikrein
PKCS, plasma kallikrein-contact activation system
PKKS, plasma kallikrein-kinin system
PMN, polymorphonuclear
PPK, plasma prekallikrein
RT, room temperature
RT-PCR, reverse transcription polymerase chain reactions
SBTI, soybean trypsin inhibitor
SDS-PAGE, sodium dodecyl sulphate polyacylamide gel electrophoresis
TK, tissue kallikrein

ABSTRACT

Introduction: Plasma kallikrein (PK) is a cofactor in blood coagulation and modulates inflammation through the release of bradykinin (BK). Previously it was believed that plasma prekallikrein (PPK), the precursor of PK and a member of the serine protease superfamily, was synthesised exclusively by hepatocytes and secreted into circulation. However, recent studies show that various human tissues contain PPK mRNA. In this study we sought to determine in which human tissues PK is expressed.

Methods: Following approval by the Ethics Committee at the University of Natal, tissue samples from the spinal cord, 13 different regions of the brain, 7 different blood vessels and various other organs were collected at autopsy within 24h of death (n = 10). Sections were probed using polyclonal antibodies specific for PK. PK concentrations in extracts of these tissues were measured by competitive ELISA.

Results: A Western blot analysis demonstrated the monospecificity of the antibody for the PK protein. The presence of immunoreactive PK in cells of the pancreatic islets of Langerhans served as a positive control for each immunolabeling experiment. The hepatocytes, renal distal convoluted tubules and epithelial cells lining the bronchiole and pulmonary alveoli labeled positively for PK. In the gastrointestinal tract tissue, immunoreactive PK was visualised in the acinar cells of the salivary gland, in stromal and glandular duct cells of the

oesophagus, and in some chief and glandular cells in the stomach. Some of the above-mentioned tissues contained a few inflammatory cells which stained intensely for PK.

Immunoreactive PK was visualised in the endothelial cells and smooth muscle cells of the all the blood vessels examined, except the renal vein. Increased immunolabeling for PK in the endothelial cells, foam cells and macrophages was observed in arteries with atheromatous plaques.

In neural tissue immunoreactive PK was observed in neurons, ependymal cells, fibre tracts, and in secretory cells of the anterior pituitary gland. Immunolabeling for PK was visualised in some neurons of the spinal cord and in different brain regions viz. hypothalamus, cerebral cortex, thalamus, brain stem and hippocampus. In sections of the hypothalamus and spinal cord, we observed immunolabeling for PK in ependymal cells lining the third ventricle and central canal respectively. Positive labeling for PK was evident in fibre tracts of the pons, medulla and hippocampus. No immunoreactive PK was visualised in the choroid plexus or cerebellum.

High amounts of PK were measured by competitive ELISA in extracts of the pancreas (12.94 ± 2.04 μg/ml), the pons (1.67 ± 1.46 μg/ml) and aorta (0.44 ± 0.14 μg/ml). The basilar artery (0.09 ± 0.07 μg/ml) and spinal cord (0.09 ± 0.04 μg/ml) had the least PK concentrations.

Discussion and Conclusions: We have shown that the PPK mRNA demonstrated in various human tissues is most likely translated into protein by the immunolocalisation of PK within specific cells in the different tissues examined. The actions of PK within these tissues may be two fold, firstly by its kininogenase activity it may release BK from high molecular weight kininogen, or alternatively, PK may act as a proteolytic enzyme on other proteins. With respect to the latter, PK may be involved in the processing of protein precursors, for example precursors of the digestive enzymes found in saliva and in gastric secretion, insulin precursors in the pancreas, and hormonal precursors in the pituitary gland. The localisation of PK and B1 and B2 kinin receptors in the kidney, lung, stomach, blood vessels and brain suggests that the effects of PK in these tissues are mediated by BK-receptor interaction. These may include the regulation of glucose uptake in the pancreas, water and ion transport in the kidney, and local and systemic blood pressure in the cardiovascular system. The presence of immunoreactive PK in neurons suggests that BK-receptor mediated interaction may regulate neurophysiological processes such as synaptic transmission. Immunolabeling for PK in polymorphonuclear leukocytes observed in some of these tissue sections suggests the potential to mediate the inflammatory process.

CHAPTER ONE

1. The Kallikrein-Kinin System

1.1 Historical Overview

1.1.1 The discovery of kallikrein

In 1926, while undertaking research at the City Surgical Hospital in Munich, Frey and Kraut noted a considerable reduction in blood pressure when they injected human or animal urine into dogs. This effect was attributed to a specific substance designated *F-substance*, which was isolated from human urine and from the pancreas and other glandular tissues (Frey and Kraut, 1926). *F-substance* was later re-named *kallikrein* after a Greek synonym for the pancreas, since it existed in that organ in such a high concentration, that it was originally thought to be produced primarily in this gland (Kraut *et al.*, 1930). Urine from patients with postoperative reflex anuria was found to contain very high concentrations of *F-substance*. When Frey injected such urine into dog, it failed to produce any effect on the blood pressure suggesting the presence of a kallikrein inactivator. However it was soon discovered that the *F-substance* had been inactivated due to contamination of the urine from anuric patients' blood, and this principle was referred to as a serum inactivator of kallikrein (Kraut *et al.*, 1928).

1.1.2 The discovery of kallidin (Lys-BK)

In 1936, Werle was convinced that he was dealing with an enzyme during the course of his research with kallikrein, but the physiological importance of the enzyme was still unclear (Werle, 1936). He found that kallikrein preparations from human urine elicited contractions in isolated dog intestine. The kallikrein had been shown to possess two activities: induction of hypotension *in vivo*,

and contraction of isolated intestinal smooth muscle *in vitro*. In the course of his experiments, Werle discovered that kallikrein alone did not contract the intestine, but, rather, it had to be mixed with serum before spasmogenic activity was observed *in vitro* (Werle, 1936). Thus the possible *de novo* formation of an active substance in serum-treated kallikrein had to be considered. The pharmacological actions of this substance were the same as those of kallikrein. Furthermore, this low molecular weight thermostable substance could be concentrated. The substance, which was thought to be a peptide, was not a split product of kallikrein but, rather, was derived from a serum protein. It was named substance DK from the German *darmkontrahierenderstoff* (intestine-contracting substance) (Werle *et al.*, 1937). Shortly thereafter, in 1948, substance DK was renamed kallidin (KD) and its precursor was named kallidinogen. Owing to the fact that substance DK migrated in a broad pH range toward the cathode, it was established that basic amino acids were present in its composition (Werle and Däumer, 1940).

1.1.3 The discovery of bradykinin release by snake venom

The venom of *Bothrops jararaca*, an extremely poisonous snake occurring only in South America, was long known to cause vascular shock in the dog and other species, including man. A sample of the venom was brought to the Instituto Biológico in São Paulo, Brazil, where studies were performed to determine the role of the venom in releasing histamine, and to examine the mechanisms underlying venom-induced vascular shock. The assumption that the effects of *B. jararaca* venom were mediated by histamine was based on earlier work by Feldberg and Kellaway (1938) who had demonstrated the release of histamine by the venom of many Australasian snakes. When blood

incubated with *B. jararaca* venom was applied to guinea-pig ileum suspended in Tyrode's solution containing an anti-histamine and atropine, it surprisingly had a stimulating effect on the isolated smooth muscle. This effect was not due to the direct action of the venom, since the preparation had previously been made refractory to it. No desensitisation was observed after several additions of serum to the bath containing guinea-pig ileum. The contractions produced by the active peptide were slow in nature and exhibited a short latent interval when compared to those induced by histamine and acetylcholine. Rocha e Silva and collaborators (1949) coined a name from Greek for this substance, using the word *kinin* (indicating movement) with the prefix *brady* (indicating slow) to describe the slow effect of the substance on the guinea-pig ileum. Thus the new substance was termed *bradykinin* (BK).

BK was found to be thermostable, dialysable through cellophane, resistant to prolonged boiling in 0.1-1.0 N HCl solution, but rapidly destroyed if heated in alkaline solution (Rocha e Silva *et al.*, 1949). Significantly, in the seminal publications by Rocha e Silva and Beraldo (1949), these authors associated the actions of BK with the discipline of autopharmacology. This term was introduced to indicate the important phenomenon of endogenous chemical mediation of physiological and pathophysiological events in the body.

1.2 General Overview

Kallikreins are a group of serine proteases found in glandular cells, some body tissues, biological fluids and neutrophils (Bhoola *et al.*, 1992). Tissue kallikrein (TK) and plasma kallikrein (PK) are the two main types of kallikrein found in humans. PK occurs in zymogen form and differs significantly from TK

in its synthesis, localisation, biochemical, immunological and functional characteristics.

1.2.1 Tissue kallikrein

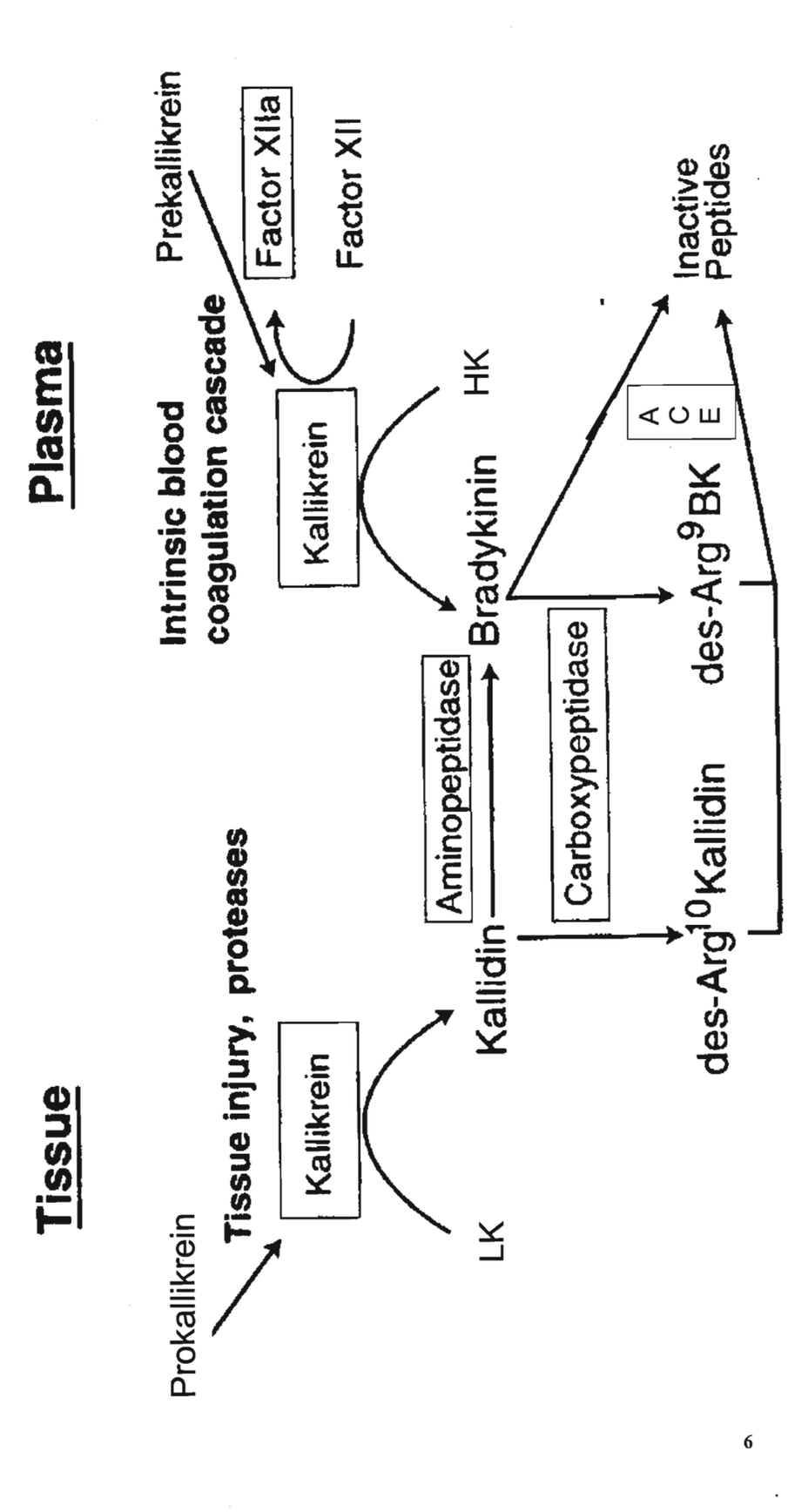
TK is the serine protease product of a family of 3 genes in humans, up to 20 genes in rat, and 24 genes in mouse. All TK genes, regardless of species, contain 5 exons and 4 introns (Margolius, 1996). The human genes, clustered on chromosome 19 at q13.2-13.4 is designated *h*KLK1 and found to be expressed in pancreatic and salivary gland duct cells as well as kidney and intestine (Margolius, 1996). The human neutrophil contains TK mRNA, indicating that expression can occur in these cells (Naidoo *et al.*, 1999b). The closely related *h*KLK3 gene is of interest because it is only expressed in prostate and codes for the prostate-specific antigen, a serine protease with high sequence homology to TK. The *h*KLK2 gene is also only expressed in prostate but the function of its product remains unknown.

True human TK is an acidic glycoprotein of 24-45 kDa (Margolius, 1996). Most of the variation in molecular weight is due to variable glycosylation; for example human urinary kallikrein has ~20% carbohydrate (Margolius, 1996). The purified renal enzyme is synthesised as a zymogen called prokallikrein. It has a 17 amino acid signal peptide preceding a 7-amino acid activation sequence, which must be cleaved to produce the active enzyme (Margolius, 1996). Several proteases, including a human renal thiol protease, are capable of activating prokallikrein (Figure 1.1). Once activated, TK cleaves low molecular weight kininogen (LK) at a Met-Lys NH_2-terminal bond and an Arg-

Figure 1.1 OVERVIEW OF THE TISSUE AND PLASMA KALLIKREIN-KININ SYSTEMS

TK and PK are the two protagonists of the kinin system. Tissue prokallikrein, upon activation by proteases and trauma, acts upon low molecular weight kininogen (LK) to form kallidin. Plasma prekallikrein is activated by the intrinsic contact system to PK where it acts upon high molecular weight kininogen (HK) to release bradykinin (BK). The action of carboxypeptidases upon kallidin and BK releases the peptides desArg^{10}-kallidin and desArg^{9}-BK respectively. Angiotensin converting enzyme (ACE) inactivates the carboxypeptidase cleavage products as well as BK. The lys residue of kallidin can be cleaved by aminopeptidase to form BK.

Figure 1.1 OVERVIEW OF THE TISSUE AND PLASMA KALLIKREIN-KININ SYSTEMS



Ser COOH-terminal bond to release Lys-BK (kallidin) (Figure 1.1). Conversion of Lys-BK to BK can occur via the action of aminopeptidases and since these peptides are not equipotent, such conversion is physiologically important. Several inhibitors of the enzymatic activity of TK are known, including aprotinin, the bovine basic pancreatic trypsin inhibitor (a 6.5 kDa, 58-amino acid polypeptide) and a new serine protease inhibitor (a serpin) called kallistatin (Margolius, 1996). Aprotinin is widely used experimentally as a TK inhibitor even though it is not specific since it is capable of efficiently inhibiting other serine proteases. Kallistatin, a 58 kDa acidic protein, slowly forms heat-stable complexes with active TK (and other serine proteases) that can be blocked by heparin (Zhou *et al.*, 1992). Although the protein is measurable in human plasma, its actual target *in vivo* and its role in regulating kallikrein activity has still to be elucidated.

1.2.2 Plasma kallikrein

1.2.2.1 Synthesis and circulation of PK

Plasma prekallikrein (PPK) is synthesised in the liver and secreted into blood as a single polypeptide with a molecular weight of 88 kDa (Mandle and Kaplan, 1977; Bouma *et al.*, 1980). It is present in plasma at a concentration of 35-45 μg/ml and circulates as a covalent complex with high molecular weight kininogen (HK) (Mandle *et al.*, 1976). Approximately 75% of the zymogen PPK circulates in plasma as a heterodimer complex with HK, while the remaining 25% circulates in a free form. Recent evidence suggests that the complex is attached to the external surface of the neutrophil membrane through binding sites on domains 3 and 5 of HK (Henderson *et al.*, 1992;

Figueroa *et al.*, 1992a). The neutrophil thereby provides a circulating platform for the HK-PPK complex.

1.2.2.2 Activation of PK

PK is activated by cleavage of an internal peptide bond in PPK by either activated clotting Factor XII (Factor XIIa) or Factor XII fragments. This cleavage results in the formation of one heavy chain (55 kDa), and a light chain (33 kDa) linked by disulphide bridges. The active site (catalytic domain) of PK is associated with the light chain of the molecule (Mandle and Kaplan, 1977). The heavy chain, with four large amino acid sequence repeats, contains the binding site for HK, and is required for the surface-dependent pro-coagulant activity of PK (van der Graaf *et al.*, 1982). Apart from protecting activated PK from its inhibitors, the PK-HK complex (in conjunction with Factor XII) is involved in the activation of the complement system. Furthermore, this complex plays an important role in the surface-dependent activation of Factor XII that results in coagulation of blood, formation of kinins, regulation of vascular tone and fibrinolysis. Once the cascade is triggered, clotting occurs to initiate thrombus formation together with the formation of PK and the release of kinins on endothelial and subendothelial surfaces.

1.2.2.3 Substrates and functions of PK

Substrates for PK include HK, LK, prorenin and Factor XII. HK is the preferred physiological substrate for PK, which releases BK from HK by hydrolysis of Lys-Arg and Arg-Ser bonds to yield the nonapeptide with arginine at both amino and carboxyl terminals (Figure 1.1). Despite LK being a poor substrate for PK, PK can form BK upon cleavage of LK in the presence of neutrophils.

This is possible because neutrophil elastase cleaves a fragment from LK from which PK readily releases BK. This reaction possibly occurs *in vivo* for kinin generation from LK. This activity has important implications because LK has been localised on the external membrane of the neutrophil (Figueroa *et al.*, 1992a).

PK has a significant effect on polymorphonuclear (PMN) leukocytes, and is therefore considered to play an important role in inflammation. PK shows chemotactic activity for PMN leukocytes (Kaplan *et al.*, 1972). Purified PK also results in marked aggregation of these cells with a similar potency to the chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (N-fMLP) (Schapira *et al.*, 1982). These effects seem to be attributable exclusively to active PK, since PPK or PK preincubated with soybean trypsin inhibitor (SBTI) appeared to be ineffective.

1.2.2.4 Molecular structure

A single gene codes for PPK, which is synthesised in the liver and secreted into blood as an inactive molecule (Bhoola *et al.*, 1992). PPK is a single chain glycoprotein with an isoelectric point of 8.9 and exists in two forms of 85 and 88 kDa in plasma (Mandel and Kaplan, 1977). The mature human enzyme is made up of 619 amino acids with 371 amino acids at the amino terminus linked to a catalytic chain of 248 residues (Bhoola *et al.*, 1992). Hydrolysis of a single Arg-Ile bond (in positions 371 and 372) results in the formation of a two-chain protease molecule held together by a disulphide bond (Seidah *et al.*, 1988). Both molecular forms are cleaved by Factor XIIa in a manner that results in the heavy chain being linked through the disulphide bridge to a light

chain of two different molecular weights, which is dependent on the initial molecular form involved (Bouma *et al.*, 1980).

The heavy chain comprises four domains arranged in sequential tandems of 90 and 91 residues (Bhoola *et al.*, 1992). Each of the four domains is bridged by six half-cysteine residues, except the last one which carries two additional half-cysteine residues to link the heavy and light chains together. The amino acid sequences of the repeat segments show considerable homology with Factor XI (Chung *et al.*, 1986). The active site of the enzyme resides in the light chain in a catalytic triad consisting of His^{415}, Asp^{464} and Ser^{559} (Mandel and Kaplan, 1977; van der Graaf *et al.*, 1982). An aspartate at position 559 provides specificity for substrate binding and the preferential cleavage of the C-terminus of basic amino acids.

The serine proteases, like PK, involved in blood coagulation and fibrinolysis, differ from the digestive proteases in that they have large non-catalytic segments present in the N-terminal regions of their molecules. These non-catalytic segments serve to mediate the binding of these proteases to their zymogens, to other proteins or surfaces and, through these interactions, determine the location and substrate specificity of these enzymes (Chung *et al.*, 1986). Also, these non-catalytic segments, which have been referred to as regulatory regions, are often organised into recognisable domains (Patthy, 1985). Five types of domains have been identified, including kringle domains, vitamin K-dependent calcium binding domains, growth factor domains, and type I and type II domains of fibronectin (Fung *et al.*, 1985; McMullen and

Fujikawa, 1985; Foster and Davie, 1984). These domains, including the kringle domain and type I and type II fibronectin domains, are also present in other proteins (Gray *et al.*, 1983). This indicates that these domains have evolved in genes other than those of the serine protease family, and have been transferred and fused to the serine protease domain during evolution by some translocational event.

The four tandem repeats that constitute the heavy chain of PK define a new type of domain that has yet to be observed in other proteases except for Factor XI (Fujikawa *et al.*, 1986). These tandem repeats are essential for the coagulant activity and neutrophil aggregation induced by PK (van der Graaf *et al.*, 1982; Schapira *et al.*, 1982). Cleavage of the heavy chain of PK by an unidentified protease(s) in acetone-activated plasma produces a modified form of PK, designated β-kallikrein (Chung *et al.*, 1986). This enzyme shows reduced activity in the cleavage of HK and fails to elicit neutrophil aggregation and elastase release (Colman *et al.*, 1985). The contribution of the heavy chain to coagulant activity can be attributed to the specific binding of the heavy chain to HK. This interaction is required prior to the proteolytic conversion of HK to an activated form that is composed of an N-terminal heavy chain and a C-terminal light chain (Chung *et al.*, 1986). This proteolytic modification of HK is associated with the expression of enhanced coagulant cofactor activity (Scott *et al.*, 1984). The role of the heavy chain in neutrophil aggregation is unclear. It is suggested that the heavy chain might bind to neutrophil membrane receptors and that such binding is required prior to the

induction of the kallikrein proteolytic activity that leads to neutrophil aggregation and elastase release (Colman *et al.*, 1985).

An analysis of the degree of homology among the four repeats in PPK indicates that repeats 1 and 3, and repeats 2 and 4, share the highest degree of homology (Chung *et al.*, 1986). This suggests that the time of divergence of repeat 3 from repeat 1, and repeat 4 from repeat 2, may be similar. This pattern of similarity is consistent with the proposal that the four tandem repeats in the heavy chain of PK resulted from two gene duplication events in which a single primordial repeat element was initially duplicated to give rise to two tandem repeats (Chung *et al.*, 1986). Subsequently, the entire locus containing the two contiguous repeats was duplicated to give rise to the present four tandem repeats (Chung *et al.*, 1986). PPK shows a high degree of identity (58%) with Factor XI, which also contains four homologous tandem repeats in the amino terminus of the molecule (Fujikawa *et al.*, 1986). The high degree of identity between these two proteins extends beyond the tandem repeats into the catalytic domain which suggests that the genes for these two proteins are closely related and have recently diverged from a common precursor. Homology alignments of the catalytic light chain of PK with the corresponding chains of other serine proteases indicate that the light chain of PK is closely related to those of Factor IX, Factor XII, protein C, and more distantly related to those of plasmin, urokinase, pancreatic kallikrein, and tissue plasminogen activator (Chung *et al.*, 1986). The fact that the heavy chain of PPK has no structural similarity with these serine proteases suggests that the primordial gene for PPK and Factor IX has probably diverged from a

common ancestor of Factor IX through a recombination event(s) that fused the catalytic chain to the heavy chain repeat elements.

1.2.3 Kininogens

Kininogens are the substrates for kallikreins. Two main types of kininogens exist in humans: high molecular weight kininogen (HK) and low molecular weight kininogen (LK). A third, T-kininogen, has been found only in rats. The single human kininogen gene (K) is localised to chromosome 3q26-qter close to two related genes, the α-2-HS glycoprotein and the histidine-rich glycoprotein (Margolius, 1996). It codes for the production of both HK (626 amino acids; 88-120 kDa) and LK (409 amino acids, 50-68 kDa) via alternative splicing from 11 exons spread over a span of 27 000 base pairs (Kitamura *et al.*, 1985). The 9 exons upstream of the kinin sequence code for the same amino acids in both kininogens; the portion of exon 10 downstream of the kinin is unique to HK mRNA, whereas exon 11 is expressed only in LK mRNA (Margolius, 1996). The kininogen receptor is formed by a complex of three proteins: gC1qR, suPAR, and cytokeratin 1 (Schmaier, 1997).

All kininogens are single-chain glycoproteins composed of a N-terminal heavy chain, a smaller variable C-terminal light chain and the kinin moiety intervening (Figure 1.2) (Margolius, 1996). Multiple disulphide bridges are present in both HK and LK. Separate functions are known for each of the molecules, including inhibition of cysteine proteases such as cathepsins and calpains by the heavy chains, binding to endothelial surfaces and promotion of contact activation of clotting factors by the light chain of HK, and the

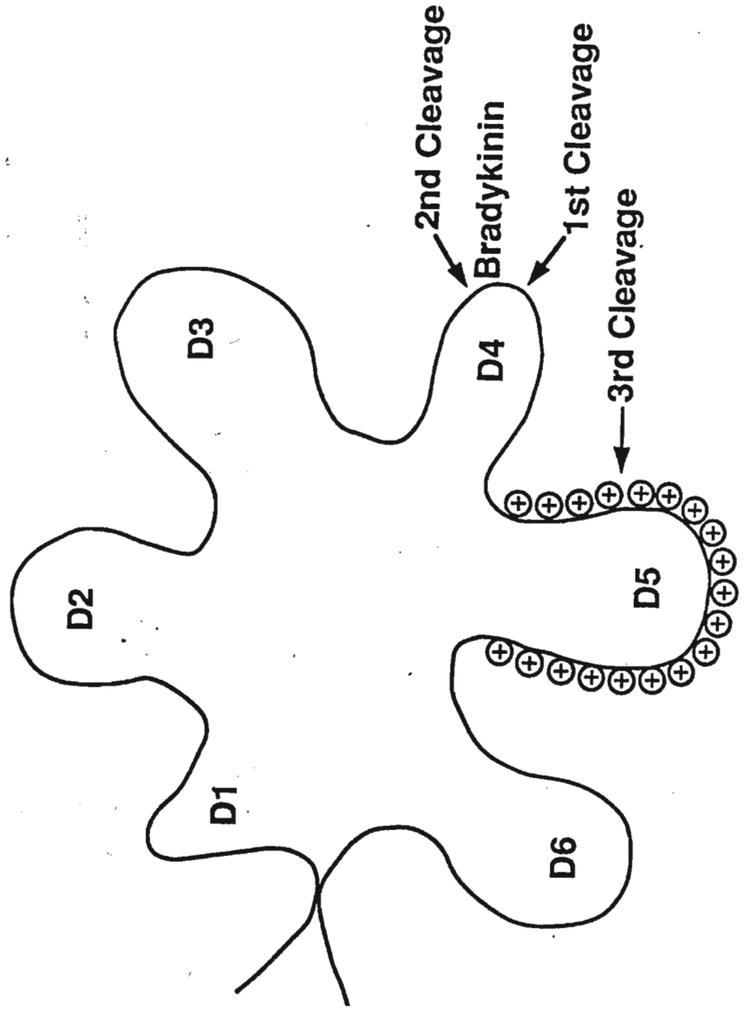


Figure 1.2 The domain structure of high molecular weight kininogen

Figure 1.2 THE DOMAIN STRUCTURE OF HIGH MOLECULAR WEIGHT KININOGEN

HK is divided into 3 regions: a heavy chain consisting of domains D1-3; the bradykinin region- domain D4; and a light chain consisting of domains D5 and D6. D1 binds calcium. D 2, a cysteine protease inhibitory region, particularly antagonistic to the actions of calpain. D3 which also comprises a cysteine protease inhibitory region, contains a major cell-binding site. D4 which contains BK, has an amino terminal end that has α-thrombin inhibitory activity and a carboxyl terminal that has cell-binding properties. D5 is the major cell-binding site and it also contains the artificial surface-binding site. Its positive charges facilitate interaction with anionic surfaces. D6 is the plasma prekallikrein and Factor XI binding sites. Note the cleavage sites on HK where plasma kallikrein acts to release bradykinin.

binding of various kinin receptors by kinins released from the molecules by the proteolytic kallikreins (Margolius, 1996).

The cardiovascular system is presented with kininogens that are synthesised primarily in the liver (Nakanishi, 1987), and then secreted and transported in plasma as well as on platelet and neutrophil cell membranes (Schmaier *et al.*, 1983; Gustafson *et al.*, 1989). Both HK and LK occupy high affinity binding sites on the unstimulated platelet, presumably forming a ready reservoir for kinin release after proteolysis. In addition, human vascular endothelial cells contain mRNA for HK (Schmaier *et al.*, 1988). BK upregulates the expression of kininogen binding sites on the surface of human vascular endothelial cells (Zini *et al.*, 1993). Because such binding protects at least HK from proteolytic cleavage from kallikreins, kinin-induced upregulation may serve to attenuate kinin generation at sites of vascular injury. It may also be possible that regulation of the kininogen gene expression affects systemic blood pressure regulation (Schmaier, 1992).

1.3 PK inhibitors

Guinea-pig plasma enhances vascular permeability when injected into guinea-pig skin, but because this effect is short lived it led to the search for a kallikrein inhibitor in guinea-pig plasma. The circulating inhibitors of kallikrein in plasma, first described by Werle, Kraut and colleagues were low molecular weight proteins (Heimburger *et al.*, 1971) isolated from bovine organs (Kraut *et al.*, 1928; Werle, 1934). Higher molecular weight inhibitors of PK isolated from plasma occlude or combine with the catalytic site of the kallikreins. These include α_2-macroglobulin, C1 inhibitor and antithrombin III for PK

(Ratnoff *et al.*, 1969; Vahtera and Hamberg, 1976), and α_1-antitrypsin for TK (Geiger *et al.*, 1981). α_2-macroglobulin and α_1-antitrypsin occur at the highest concentration in plasma (Heimburger *et al.*, 1971). C1 inhibitor combines with PK to form an inactive complex. α_2-macroglobulin also forms a complex with PK, suppressing approximately 75% of its functional activity. Antithrombin III is a minor inhibitor of PK.

PK can be rapidly inactivated by C1 inhibitor, which also has the capacity to inhibit Factor XII with high affinity (Cameron *et al.*, 1989). Further inhibition could occur with α_2-macroglobulin, antithrombin III, and mutant α_1-antitrypsins. After the PK-inhibitor complexes are formed, they are rapidly cleared from the circulation. It has been proposed that the combination of both PK (Schapira *et al.*, 1982) and Factor XIa (Scott *et al.*, 1982) with HK protects the former from inhibition by C1 inhibitor, α_2-macroglobulin and α_1-antitrypsin, thereby augmenting the plasma half life of the enzyme.

Kallikrein inhibitor proteins inhibit trypsin and elastase but not chymotrypsin. It has an inhibitory spectrum that is similar to that of contrapsin in mouse plasma (Imamura and Kambara, 1989). In pathological states such as hereditary angioedema (HAE) (Landerman *et al.*, 1962) and inflammatory joint disease, activation of PK and/or the complement system (C2) is believed to be responsible for the symptoms that patients experience. The clinical state in HAE (an autosomal disorder arising from a reduction in both the amount and efficiency of C1 inhibitor) is characterised by localised swellings, laryngeal oedema, and abdominal pain. Some studies support a complement C2-

dependent mechanism, but most report BK to be the primary inflammatory mediator formed in HAE, because during the swelling attacks concomitant with the formation of BK, there is a reduction in the circulating levels of prekallikrein and HK. Active PK has been detected in suction-induced blister fluids obtained from patients with HAE (Curd *et al.*, 1980). Of particular interest has been the finding that only during pregnancy did the plasma of a patient with HAE show sustained contact activation which resulted in the formation of kallikrein-α_2-macroglobulin and kallikrein-50 kDa HK complexes. These disappeared after delivery, with the subsequent return of the intact 120 kDa HK molecule (Chibber *et al.*, 1989). Replacement therapy with C1 inhibitor during attacks of swelling and oedema produced clinical improvement that was associated with an increase in circulating levels of HK (Kodama *et al.*, 1984). Another clinical condition in which cascade-formed kinins are considered to be involved, is the cutaneous late phase reaction. This reaction is immunoglobulin E (IgE)-dependent and is caused by the secretion of mediators from activated mast cells. In the early phase of the reaction when wheals and flares occur (appearing within 10 min and disappearing in 60 min), histamine, substance P, prostanoids, and 5-hydroxytryptamine (5-HT) may be involved, whereas during the late phase, there is strong evidence for a contribution by kinins (Kaplan *et al.*, 1989).

1.3.1 C1 esterase inhibitor

Serpin, a serine protease inhibitor of C1, is a single chain polypeptide with a molecular weight of 104 kDa, which is cleaved when it interacts with PK or plasmin (Heimburger *et al.*, 1971; Harpel *et al.*, 1975). However, the formation

of a reversible complex between PK and HK decreases the rate of inactivation of C1 inhibitor (Schapira *et al.*, 1981).

Factors XIIa, XIa, kallikrein and C1 protease are highly regulated by C1 inhibitor which is the only serpin known to inactivate C1s and C1r, and also contribute to over 90% of the plasma's capacity to inhibit Factor XIIa (De Agostini *et al.*, 1984). C1 inhibitor contributes most of the plasma capacity to inhibit kallikrein (Schapira *et al.*, 1981). The regulation of Factor XIa is achieved by Factor XIa forming a complex with the inhibitor (Scott *et al.*, 1982). The primary inhibitor of Factor XIa regulation is α_1-proteinase inhibitor (α_1 -PI), with α_2-antiplasmin (α_2-APO) and antithrombin III (AT-III) having less potent inhibitory roles. It is clear that sustained activation of the contact and complement systems, which can lead to the depletion of C1 inhibitor, will result in reduced inhibitory regulation of PK and Factor XIIa (Pixley and Colman, 1997). This decrease in the regulatory molecule allows the reciprocal activation to proceed between these two enzymes, which results in sustained release of BK and neutrophil activation (Pixley and Colman, 1997). Continued depletion of C1 inhibitor can also stimulate the complement system by leaving C1r and C1s unchecked, thereby allowing formation of a membrane attack complex (MAC) with ensuing cell lysis. It is interesting that C1 inhibitor has not been demonstrated to regulate proteases other that Factor XIIa, kallikrein and Factor XIa, generated in the intrinsic or extrinsic coagulation cascades. This suggests that low levels of C1 inhibitor may favour activation of the kallikrein-kinin system (KKS) in the absence of appreciable coagulation. In shock, where organ perfusion is reduced, a local environment exists in which C1

inhibitor is depleted and not further supplied from blood (Pixley and Colman, 1997). Thus, both the kinin and complement systems may be fully activated and uncontrolled, thereby further exacerbating inflammation.

1.3.2 α_2-Macroglobulin

α_2-macroglobulin contributes to 58% of the regulatory activity of kallikrein. The formation of kallikrein:α_2-macroglobulin complexes were monitored by developing an assay for contact activation in blood samples obtained from patients and primates with systemic inflammatory response syndrome (SIRS) (Pixley *et al.*, 1995). α_2-macroglobulin has a molecular weight of 725 kDa (Jones *et al.*, 1972; Hall and Roberts, 1978), is denatured below pH 4.0 (Barrett, 1981) and has an isoelectric point of 5-5.2 (Barrett *et al.*, 1979). Its defined inhibitory characteristics are: (i) it specifically traps proteases, (ii) it covalently links proteases and other molecules that follow into this trap, and (iii) it participates in a non-covalent, non-steric adherence reaction with a variety of molecules unrelated to their proteolytic activity for example thrombin (Starkey and Barrett, 1977). It has been speculated that each subunit of α_2-macroglobulin has a region which is highly susceptible to limited proteolysis, and when bound by proteases, causes a conformational change in the α_2-macroglobulin molecule, allowing it to trap the protease molecule within itself (Barrett, 1981). After the molecule is trapped, a barrier is formed at the opening which allows the entry of particles less than 10 kDa in size to pass through into the active site domain of α_2-macroglobulin. The largest trappable known enzymes are PK and plasmin. In this complex, the active centre remains accessible to small molecules (Haverback *et al.*, 1962) and as a

consequence the enzymes retain their ability to act on small synthetic substrates in the presence of the PK inhibitors. When PK or plasmin interacts with α_2-macroglobulin, the inhibitor undergoes cleavage (Harpel *et al.*, 1975) similar to a reaction occuring between neutrophil proteases and α_2-macroglobulin or antithrombin III (Movat *et al.*, 1976).

1.3.3 Antithrombin III

Antithrombin III, an inhibitor of thrombin (Steinbuch *et al.*, 1968), has a molecular weight of 63-65 kDa, an isoelectric point of 5.1 (Burrowes and Movat, 1977) and forms a complex with PK resulting in a molecule that ranges from 110-139 kDa in size (Venneröd *et al.*, 1976).

1.3.4 Heparin

Heparin is a catalytic cofactor that significantly accelerates the action of antithrombin III to inactivate Factor Xa and thrombin (Reddigari *et al.*, 1997). It contains a specific sulphated carbohydrate sequence that facilitates its binding to antithrombin III, resulting in a conformational change in the structure of the inhibitor to form a more efficient enzyme inactivator. However, heparin does not appreciably accelerate the inhibition of PK by antithrombin III.

1.3.5 Soybean trypsin inhibitor (SBTI)

SBTI is a double-headed inhibitor of proteases which has a configuration specific for the inhibition of trypsin-like enzymes. It has a molecular weight of 20 kDa and consists of 181 residues with two disulphide bridges (Ikenaka *et al.*, 1974).

1.4 Kinin receptors

Vasoactive peptides, like kinins, exert their actions by binding to specific high-affinity receptors located in the cell membrane of target cells. After occupancy, receptors undergo rapid phosphorylation, redistribution in the plane of the membrane, and endocytosis. The receptor-ligand interaction promotes signal transduction mechanisms in the cell membrane and cytosol which involve the activation of ion channels or enzymes to modulate the level of second messengers such as cyclic nucleotides, phosphoinositides and Ca^{2+}.

The two main receptor subtypes for kinins viz. B1 and B2 have been characterised and cloned. The B1 receptor shows some, but limited, homology to the B2 receptor. The cloned B2 (McEachern *et al.*, 1991) and B1 receptors (Hess *et al.*, 1994) belong to the superfamily of G protein receptors. They have seven characteristic membrane-spanning regions linked by three extracellular and four intracellular loop regions that are unique in their sequences (McEachern *et al.*, 1991). Most of the actions of BK and kallidin are, however, mediated by the activation of the B2 receptor. These effects include vascular smooth muscle relaxation, endothelial cell retraction, contraction of visceral and bronchial smooth muscle, and activation of peripheral afferent C-type nerve fibres (Riccioppo Neto *et al.*, 1974; Rehbock *et al.*, 1994). B2 receptor activation may stimulate the inositol triphosphate pathway via phospholipase C and/or the arachidonic pathway via phospolipase A_2 (Burch and Axelrod, 1987). The human B2 gene maps to chromosome 14 (Powell *et al.*, 1993). B2 receptor mRNA is present in human kidney (highest level), pancreas, colon, salivary gland, prostate, uterus,

skeletal muscle, adrenal glands, stomach, breast, ovary, brain, heart, arteries, veins and lung (Hess *et al.*, 1992; Ma *et al.*, 1994). The B1 receptor, however, is induced rather than being constitutively expressed. Expression can be induced in animals by exposure to low doses of interleukin-1 (IL-1) or endotoxin. The main endogenous agonist for the B1 receptor is des-Arg^9-BK, which is a metabolite that results from the hydrolysis of C-terminal arginine of the kinin moiety by kininase 1-carboxypeptidase N (KI-CPN) (Bhoola *et al.*, 1992; Marceau, 1995). During inflammation, or after exposure of tissue to lipopolysaccharides, the presence of B1 receptor becomes evident in smooth muscle cells and fibroblasts.

1.4.1 Kinin receptor antagonists

Replacement of the C-terminal phenylalanine of BK or kallidin by leucine produces an antagonist that is highly selective for B1 receptors. Replacement of the Pro^7 in BK (or Pro^8 in kallidin) by a D-aromatic amino acid, like D-phenylalanine, yields an effective antagonist that is selective for B2 receptors in many tissues and species. Although the presence of this D-amino acid residue impaired the degrading action of kininase II-angiotensin converting enzyme (KII-ACE), this first generation of B2 antagonists was relatively weak with half lives of only a few minutes *in vivo* in rats. The D-Arg residue at the N-terminus protected them from aminopeptidase degradation. They were, however, substrates for KI-CPN, which terminated their action on B2 receptors and converted them to B1 antagonists (Regoli *et al.*, 1986). Despite these limitations, antagonists such as DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DPhe-Thi-Arg (NPC-349) were critical for demonstration of the participation of kinins in both normal and pathological conditions.

The second generation of B2 antagonists came with the introduction of HOE-140 (DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg; icatibant) by Hoechst (Hock *et al.*, 1991; Wirth *et al.*, 1991). The proline-like Oic residue at position 8 made this antagonist resistant to the action of KI-CPN and conferred high potency and long duration of action *in vivo*. This antagonist has been used in human clinical trials with success in allergic rhinitis and allergic asthma (Akbary *et al.*, 1996; Austin *et al.*, 1994). A different antagonist, Bradycor (CP-0127)- a dimer of DArg-Arg-Pro-Hyp-Gly-Thi-Cys-DPhe-Leu-Arg in which the Cys residues were linked by bis-maleimidohexane, was also introduced (Cheronis *et al.*, 1992). Bradycor has been found to be effective in animal models of septic shock (Whalley *et al.*, 1992) and in closed head trauma in humans (Rodell, 1996). Several heterodimers having both B1 and B2 antagonist activity have also been introduced (Cheronis *et al.*, 1994).

A third generation of BK antagonists was characterised by the introduction of analogs containing the novel amino acid, α-(2-indanyl)-glycine (Igl) (Stewart and Gera, 1996). The presence of Igl residue at position 5 of these kinin receptor antagonists evidently conferred resistance to degradation by the neutral endopeptidase, enkephalinase, and yielded antagonists that appear to be resistant to all kininases and show remarkably long activity *in vivo*. Combined with a D-Igl residue at position 7, this new antagonist has about the highest potency known. Dimers of certain of these antagonists are selectively cytotoxic for cells of small cell lung cancer (SCLC) *in vitro* and *in vivo*. As examples, DArg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg (B9430), an extremely potent and long-acting antagonist of B1 and B2 receptors, is stable against

the endogenous kininase enzymes, and is active in various *in vivo* models, including intragastric administration (Hanson *et al.*, 1996).

1.5 The biological actions of kinins

BK and kallidin (Lys-BK) are biologically active nona- and deca-peptides respectively, released upon cleavage of kininogens by kallikreins. These kinins are the primary mediators produced during inflammation, a regular biological response to injury. They have the ability to cause vasodilation, venoconstriction and endothelial cell retraction that result in increased capillary permeability. By depolarising C-type nerve fibre terminals, they induce pain. Additionally, the biological actions of kinins include hypotension and contraction of smooth muscles of the bronchopulmonary tree, intestine and uterus. Kinins also have the capacity to release neurotransmitters, such as substance P; stimulate cytokine synthesis such as interleukin-1 and tumour necrosis factor; induce the formation of prostaglandins and leukotrienes by activating phospholipase A_2; and release endothelium-derived relaxing factor (EDRF; nitric oxide) from endothelial cells (Bhoola *et al.*, 1992). At a cellular level, kinins promote glucose and chloride transport, stimulate osteoclasts and increase DNA synthesis (Bhoola *et al.*, 1992).

1.5.1 Bradykinin

In the KKS, PK generates from HK the nonapeptide BK with the amino acid sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. BK induced receptor stimulation of endothelial cells promotes the synthesis of PGI_2 and release of tissue plasminogen activator. BK acts as a potent vasoactive peptide which has a number of effects, mostly involving the inflammatory response.

Although kinins act as potent dilators of arterioles resulting in hypotension (Mombouli and Vanhoutte, 1992), BK, however, can both contract and relax smooth muscle. This is dependent on the type of kinin receptor, the type and potency of kininases present on endothelial cells, and the presence of EDRF and PgE_2 (Regoli and Barabè, 1980). Increased cytosolic calcium causes the shortening of contractile proteins in endothelial cells, formation of fenestrations in the walls of microvascular tissue, and the extravasation of plasma constituents as well as specific cellular elements of blood, which results in an increase in BK-induced vascular permeability (Oleson, 1987; Wiemar *et al.*, 1994). Kinins also modulate the interaction between endothelial cells and cellular elements in blood (Kaplan and Silverberg, 1987).

Many bacteria release enzymes that increase kinin release by either direct cleavage of kininogen, or indirectly by activation of the prekallikreins to active kinin-generating enzymes. The increased vascular permeability caused by kinins produced in this manner appears to be an important factor promoting the spreading of bacterial infections through tissues (Maeda *et al.*, 1996).

1.5.2 Inactivation of kinins

The turnover of kinins depends both on the rate of formation, as outlined above, and the rate of destruction by the enzymic action of peptidases generally called kininases (Erdös, 1989; Erdös, 1990). These circulating and local enzymes, found in blood and tissues respectively, include the circulating kininase I-carboxypeptidase N (KI-CPN) and membrane-bound carboxypeptidase M (KI-CPM) which cleave the C-terminal arginine of BK to produce the B1 agonist, des-arg^9-BK. Other important kininase-II enzymes are

angiotensin converting enzyme (KII-ACE) and neutral endopeptidases (KII-NEP) which cleave and transform the kinin molecule into inactive fragments (Bhoola *et al.*, 1992). KII-ACE cleaves two internal peptide bonds, producing completely inactive metabolites. KII-NEP that occurs both in circulation and in tissues degrade the molecule into even smaller peptide fragments. Additional kinin-hydrolysing enzymes are prolidase (aminopeptidase P) and two endopeptidases (kininase A and B). Sequences for the regulatory and catalytic subunits for most of these enzymes have been ascertained by cDNA clones. A series of selective enzyme inhibitors (D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid, ethylenediamintetraacetic acid, o-phenanthroline for KI-CPN and KI-CPM, captopril for KII-ACE; phosphoramidon for KII-NEP; and amastin, bestatin, and puromycin for aminopeptidases) are available for regulating kininase activity (Bhoola *et al.*, 1992).

The relative importance of each of the peptidases in controlling kinin levels varies with species, type of biological fluid, and tissue site of peptide formation. In humans, circulating levels of kinins are primarily regulated by KI-CPN but on endothelial surfaces, particularly in the pulmonary vascular bed, by KII-ACE. In contrast, the most potent kinin-degrading enzyme in rat plasma is KII-ACE with little contribution from other peptidases (Ishida *et al.*, 1989).

1.5.2.1 KI-CPN

KI-CPN, originally discovered in the Cohn fraction IV of human plasma (Erdös and Sloane, 1962), is an arginine carboxypeptidase, optimally active at pH 7.4

with the capacity of being activated by $CoCl_2$. This carboxypeptidase is synthesised by the liver and secreted into the circulation. It accounts for approximately 90% of the BK-destroying activity in human plasma (Zacest *et al.*, 1974) and can also become associated with the vascular endothelium (Palmieri *et al.*, 1986). Although the primary importance of this enzyme in cleaving kinins within the circulatory system varies between species, it can also hydrolyse the C-terminal basic amino acid of several biologically active peptides like enkephalins, anaphylotoxins and fibrinopeptides.

1.5.2.2 KI-CPM

KI-CPM cleaves the C-terminal arginine of BK and Lys-BK, resulting in the formation of des-Arg^9-BK and des-Arg^9-Lys-BK. The optimal pH for degrading both kinins is in the neutral range. KI-CPM has been purified from human urine and human placental microvilli and its encoding gene has been cloned from a human placental cDNA library (Skidgel *et al.*, 1984; Skidgel *et al.*, 1989; Tan *et al.*, 1989). KI-CPM is a 62 kDa metallopeptidase inhibited by guanidino-ethylmercaptosuccinic acid and activated by cobalt (optimal at pH 5.5) which replaces zinc as a cofactor (Deddish *et al.*, 1989). Structurally, KI-CPM is a single-chain glycoprotein with 426 amino acids and six asparagine-linked glycosylation sites (Bhoola *et al.*, 1992).

1.5.2.3 KII-ACE

KII-ACE is a major kininase in most organs because of its wide distribution in mammals. Based upon its physical properties, enzymatic characteristics and immunological cross-reactivity, KII-ACE enzymes in different species and various tissues are quite similar and likely represent the two-domain somatic

KII-ACE (Skidgel and Erdös, 1993). KII-ACE purified from various human tissues is a single-chain glycoprotein. Its estimated molecular weight ranges from 140-170 kDa in SDS-PAGE (Skidgel and Erdös, 1993). KII-ACE is heavily glycosylated, the human renal enzyme containing approximately 25% carbohydrate (Weare *et al.*, 1982). The differences are, however, in the carbohydrate moieties. Owing to its high sialic acid content, KII-ACE is protected from uptake by liver lectins.

Being a zinc metalloenzyme, chelating agents and sulphydral compounds inhibit KII-ACE by complexing the active site Zn^{2+} (Erdös, 1979). KII-ACE has an optimum pH above neutral, and activity drops steeply at acidic pH primarily due to the dissociation of Zn^{2+} from histidine in the active centre (Ehlers and Riordan, 1990). Studies with rabbit lung KII-ACE showed that anion activation depends on both the structure of the substrate and the pH of the medium (Ehlers and Riordan, 1990). Accordingly, the substrates were divided into three classes, BK being class II substrate with a lower activation constant for Cl^- than angiotensin I, a class I substrate. Weare (1982) described two anion-binding sites in KII-ACE, with one being the primary binding site.

The concentration of Cl^- ions *in vivo* appears to be high enough for KII-ACE to be fully active in most tissues, although at some sites, Cl^- concentrations may fluctuate sufficiently to regulate KII-ACE activity with some substrates. The Cl^- ion sensitivity of the membrane-bound recombinant KII-ACE showed that the Cl^- dependence of the N and C domains was the same as that determined for the soluble enzyme (Jaspard and Alhenc-Gelas, 1995).

1.5.2.4 KII-NEP

Neutral endopeptidase (Neprilysin, KII-NEP) is a zinc metallopeptidase with a single peptide site containing the canonical HEXXH sequence (Erdös and Skidgel, 1989). The enzyme is a trans-membrane, single-chain protein of 742 amino acids, but in contrast to KII-ACE, is bound via an uncleaved N-terminal signal peptide (Roy *et al.*, 1993). It cleaves peptide substrates with a molecular weight not usually exceeding 3 kDa at the N-termini of hydrophobic amino acids, and cleaves BK at the Pro^{7}-Phe^{8} bond (Almenoff *et al.*, 1981). KII-NEP is a second kininase II that releases the C-terminal Phe^{8}-Arg^{9} of BK (Gafford *et al.*, 1983). Active peptide substrates for KII-NEP include the B-chain of insulin (Kerr and Kenny, 1974), enkephalins (Schwartz *et al.*, 1985), endothelin (Vijayaraghavan *et al.*, 1990), atrial natriuretic peptide, substance P and a chemotactic peptide (Erdös and Skidgel, 1989). Sites where KII-NEP may be an important kininase include the epithelial cells of the respiratory tract (Dusser *et al.*, 1988), skeletal muscles (Dragovic *et al.*, 1996), neutrophils (Painter *et al.*, 1988), renal proximal tubules (Ura *et al.*, 1987) and possibly human coronary vessels (Graf *et al.*, 1995).

1.6 PK and haemostasis

1.6.1 Factor XII

Factor XII, an 80 kDa single chain β-globulin, occurs at a concentration of 30 μg/ml in blood (Reddigari *et al.*, 1997). As an initiator of the contact pathway for intrinsic coagulation, Factor XII undergoes conformational changes upon binding to negatively charged surfaces (Wachtfogel *et al.*, 1999). This conversion of zymogen to active enzyme is termed solid-phase activation.

Additionally, autoactivation of Factor XII yields a small quantity of Factor XII fragments, which can also convert PPK to PK. PK then further activates Factor XII in the presence of HK; the latter activation then proceeds exponentially (Wachtfogel *et al.*, 1999). In the absence of a negative surface, Factor XII can also be activated in the fluid phase by either Factor XI or plasmin. However, these activators are less potent than PK.

Two major forms of activated Factor XII exist: Factor XIIa (80 kDa), consisting of two disulphide-linked polypeptide chains (52 kDa and 28 kDa), and Factor XIIf (28 kDa), both of which are derived from the native molecule (Figure 1.3) (Wachtfogel *et al.*, 1999). Factor XIIa, which initiates the intrinsic coagulation cascade, arises from the cleavage of a single Arg-Val peptide bond in the zymogen. This cleavage initiates coagulant activity by exposure of the active site, which hydrolyses Arg substrates. Factor XII fragments result from further proteolysis of the Factor XIIa molecule, and mainly contain the catalytic light chain and a tiny fragment of the heavy chain. The surface binding property of Factor XIIa resides in its 52 kDa heavy chain, where the amino acid sequence contains putative collagen binding domains, growth factor regions, and a structure characterised by multiple disulphide loops (Wachtfogel *et al.*, 1999). Substrates of Factor XIIa include PPK, Factor XI, HK and Factor VII. Factor XII fragments are responsible for the non-immunological activation of the classic complement pathway. C1 inhibitor accounts for 90% of the inhibition of both Factor XIIa and Factor XII fragments, while antithrombin III is responsible for inhibiting the remainder of the activity (Wachtfogel *et al.*, 1999). In conditions where C1 inhibitor is absent or decreased, as in hereditary

Figure 1.3 STRUCTURE OF FACTOR XII , XIIa, AND XIIf

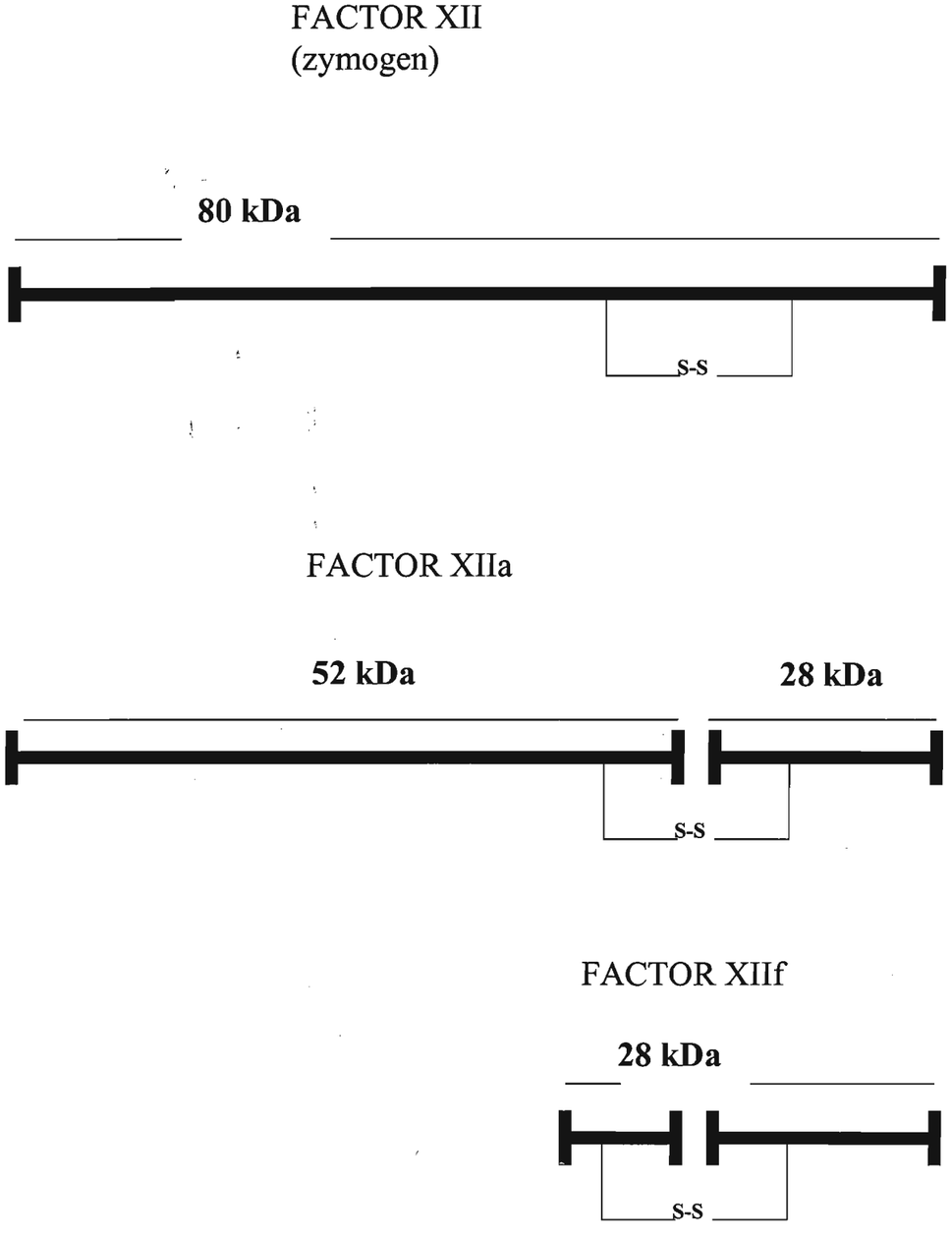


Figure 1.3 STRUCTURE OF FACTOR XII, XIIa, AND XIIf

This figure shows the structure of the zymogen Factor XII and its active components, Factor XIIa and XIIf. This zymogen has a molecular weight of 80 kDa, whereas the cleaved Factor XIIa (upon exposure of the catalytic active site) consists of two disulphide-linked polypeptide chains of 52 and 28 kDa. Factor XII fragments consist solely of the 28 kDa subunit.

angioedema, antithrombin III becomes the major, but less potent, inhibitor of Factor XIIa and Factor XII fragments.

1.6.2 Factor XI

Factor XI is a 160 kDa γ-globulin composed of two identical polypeptide chains of 80 kDa each (Reddigari *et al.*, 1997) linked by disulphide bridges (Wachtfogel *et al.*, 1999). It occurs in plasma at a concentration of only 5 μg/ml (Wachtfogel *et al.*, 1999). This protein exhibits a high degree of homology with PPK suggesting a common ancestral gene. Factor XI is activated to Factor XIa upon cleavage by Factor XIIa. This results in two heavy chains, each with a molecular weight of 50 kDa, and two light chains, with a molecular weight of 30 kDa each (Figure 1.4). The binding region of Factor XI is found on its heavy chains, while the active site is located on its light chains. Substrates of Factor XIa include HK, Factor IX and plasminogen (Wachtfogel *et al.*, 1999). Virtually all of Factor XI forms a complex with HK, thus protecting itself from its inhibitors. α_1-protease inhibitor is the major inhibitor of Factor XI. In purified systems, antithrombin III inhibits Factor XIa, but in plasma it is not a significant inhibitor even in the presence of heparin.

1.6.3 High molecular weight kininogen

High molecular weight kininogen (HK) is present in endothelial cells in culture (Schmaier *et al.*, 1988; van Iwaarden *et al.*, 1988), and in human vascular smooth muscle (Figueroa *et al.*, 1992a). This endogenous protein is synthesised by hepatocytes and secreted into the circulation (Kitamura *et al.*, 1985). It has six domains of which the first three are homologous to cysteine protease inhibitors (domains 2 and 3 retain their inhibitory activity), a fourth

Figure 1.4 STRUCTURE OF FACTOR XI AND XIa

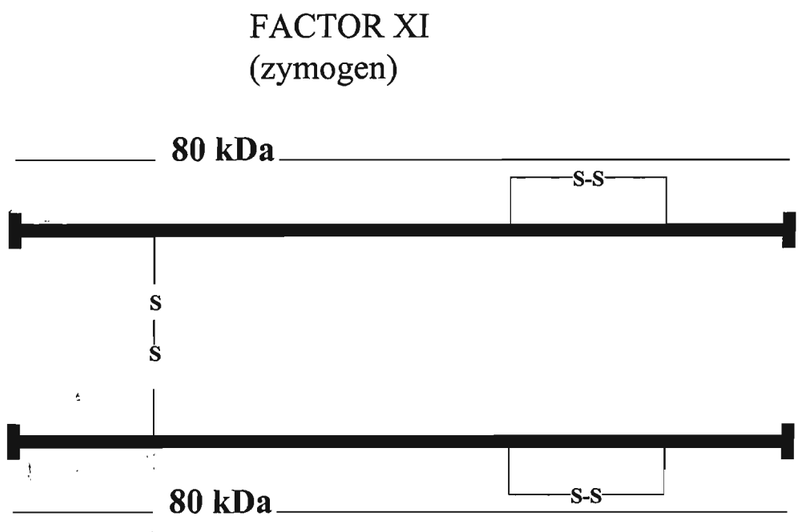


Figure 1.4 STRUCTURE OF FACTOR XI AND XIa

This figure shows the structure of the 160 kDa Factor XI, composed of two identical 80 kDa polypeptide chains linked by disulphide bridges. Cleavage of these polypeptide chains by Factor XIIa produces active Factor XI (Factor XIa) which comprises two 50 kDa heavy chains linked to two 30kDa light chains by disulphide bridges.

domain features the BK moiety, and domains 5 and 6 interact with surfaces and prekallikrein or Factor XI respectively (Kaplan *et al.*, 1997) (Figure 1.2). HK directly enhances the surface-mediated formation and function of Factor XII, PPK, and Factor XI so that activation proceeds optimally. Domain D5 contains positively charged histidine residues that facilitate interaction with anionic surfaces (Wachtfogel *et al.*, 1999). Domain D6 has specific amino acid sequences that bind either PPK or Factor XI (Tait and Fujikawa, 1987), thereby transporting these zymogens to the surface for cleavage by Factor XIIa (Wachtfogel *et al.*, 1999). HK is a profactor which does not effectively bind to activating surfaces in plasma until it has been cleaved by PK. The cofactor is destroyed when Factor XI cleaves the light chain of HK. PK splits HK in a three step sequential manner. The first cleavage produces a nicked kininogen composed of two disulphide-linked chains (56 and 64 kDa) (Schmaier *et al.*, 1997). The second cleavage yields BK (which mediates pain, vasodilation, increased vascular permeability) and an intermediate kinin-free protein (Schmaier *et al.*, 1997). The final cleavage results in a stable kinin-free protein composed of two disulphide-linked chains (45 and 64 kDa), and an additional peptide of 10 kDa (Wachtfogel *et al.*, 1999). Both HK and LK, which contain the same amino acid sequences in their heavy chain (D1-D3), are structurally and immunochemically identical to the cysteine protease inhibitors (Wachtfogel *et al.*, 1999).

Although recognised more than two decades ago as a defect associated with a prolonged activated partial thromboplastin time (APTT) (Colman *et al.*, 1975; Saito *et al.*, 1975; Wuepper *et al.*, 1975), the genetic deficiency of HK is

not associated with bleeding. Although the incidence of homozygous HK deficiency is rare, recognition of HK deficiency is greatly facilitated by the detection of a prolonged APTT. The prolonged APTT associated with HK deficiency may have contributed to the masking of the physiological importance of HK as an antithrombin, as well as its role in ordering the assembly and activation of a PK-dependent fibrinolytic pathway on biological membranes (Schmaier, 1997).

1.6.3.1 Participation of HK in fibrinolysis

In addition to its unique mechanism of antithrombin inhibition, the recognition of HK deficiency has purported the role of HK in fibrinolysis (Figure 1.5), although the specific physiological mechanism is unclear (Colman *et al.*, 1975; Saito *et al.*, 1975). It is known that contact activation can increase total plasma fibrinolysis (Niewiarowski and Prou-Wartelelle, 1959). Kallikrein, Factor XIIa and Factor XIa cleave plasminogen directly, albeit much less efficiently than tissue-type plasminogen activator or urokinase plasminogen activator (Mandle and Kaplan, 1979). However, PK has been characterised to be a kinetically favourable activator of single-chain urokinase *in vitro* (Ichinose *et al.*, 1986). Recent studies suggest that single-chain urokinase activation by kallikrein is most likely to occur on the platelet and endothelial cell surface (Gurewich *et al.*, 1993). In 1998, Motta and colleagues examined the relationship between prekallikrein assembly on endothelial cells and its participation in single-chain urokinase activation. When prekallikrein binds to HK on endothelial cells, the zymogen becomes activated to kallikrein, as indicated by the elaboration of amidolytic activity, changes in the structure of

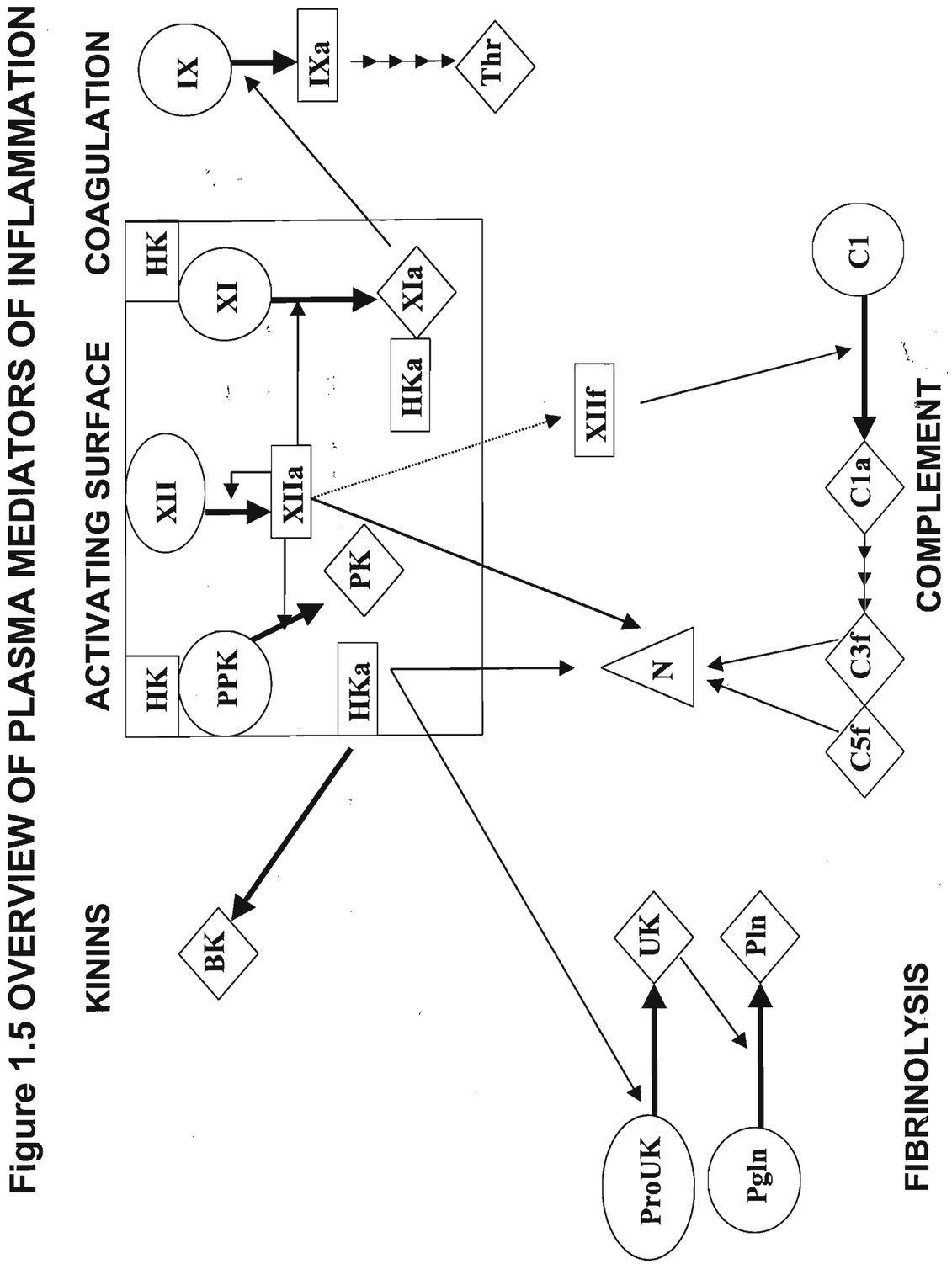


Figure 1.5 OVERVIEW OF PLASMA MEDIATORS OF INFLAMMATION

Figure 1.5 OVERVIEW OF PLASMA MEDIATORS OF INFLAMMATION

This figure shows the interactions of the plasma kallikrein-kinin system with the coagulation, complement and fibrinolytic pathways. An activated surface (anionically charged), HK, and PPK cleavage from activated Factor XII is required for its conversion to PK. PK in turn cleaves activated HK, thus yielding BK. PK also activates neutrophils along with activated Factor XII, and fragments of Complement components C3 and C5. PK activation is required for the conversion of prourokinase to urokinase. Urokinase then activates the conversion of plasminogen to plasmin, thus resulting in fibrinolysis. In the complement pathway, Factor XII fragments activates C1 which forms C3 and C5 fragments via a cascade mechanism. In the coagulation reaction, activated Factor XI converts Factor IX to its active form resulting in thrombin formation via a cascade mechanism.

FIGURE SYMBOLS

- ZYMOGEN
- ACTIVE ENZYME
- COFACTOR
- CONVERSION
- ACTIVATION
- CASCADE
- NEUTROPHIL

ABBREVIATIONS

BK	Bradykinin
C1	Complement component 1
C1a	Complement component 1- activated
C3f	Complement component 3- fragment
C5f	Complement component 5- fragment
HK	High molecular weight kininogen
HKa	High molecular weight kininogen-activated
PK	Plasma kallikrein
PPK	Plasma prekallikrein
Plgn	Plasminogen
Pln	Plasmin
ProUK	Prourokinase
Thr	Thrombin
UK	Urokinase
VII	Factor VII
VIIa	Factor VII- activated
IX	Factor IX
IXa	Factor IX- activated
XI	Factor XI
XIa	Factor XI- activated
XII	Factor XII
XIIa	Factor XII- activated

prekallikrein to kallikrein on gel electrophoresis and the cleavage of HK (Motta *et al.*, 1998). PPK activation occurs independently of the active forms of Factor XII, and its activating enzyme is a Ca^{2+}-requiring metalloprotease rather than a serine protease (Motta *et al.*, 1998). PPK activation on endothelial cells is kinetically similar to prekallikrein activation by Factor XII on an artificial surface. These data showed that contact protein assembly on endothelial cells results in prekallikrein activation in the absence of Factor XII and an artificial surface (Motta *et al.*, 1998). This assembly of contact proteins allows for a physiological pathway of contact activation. The degree of PPK activation is regulated by HK where increasing HK concentrations upregulate the enzyme that activates cell-bound PPK. Thus, HK regulates PPK activation, which then liberates more BK from cell-bound HK and removes HK from the surface to slow PPK activation (Motta *et al.*, 1998). Also, increased BK increases kininogen binding, which subsequently decreases soluble kallikrein from cleaving HK to liberate more BK (Zini *et al.*, 1993). The pathway for PPK activation and BK liberation is, therefore, tightly regulated.

The PPK activation pathway on endothelial cells results in a kinetically favourable conversion of single-chain urokinase into two-chain urokinase in an environment where there is a constitutive molar excess secretion of endothelial cell plasminogen activator, inhibitor-1 (Motta *et al.*, 1998). Formation of a two-chain urokinase results in a 4.3 fold increase in plasminogen activation. This system for plasminogen activation occurs in an environment where there is no tissue plasminogen activator or fibrin, and the mechanism for single-chain urokinase activation is another pathway for

cellular fibrinolysis that is either independent of or co-joined with single-chain urokinase activation associated with its binding to its receptor (Higazi *et al.*, 1995). This also provides a probable explanation of how two-chain urokinase can be formed in plasminogen knockout mice (Carmeliet and Collen, 1995).

HK and LK assemble on cell membranes allowing for BK to be liberated in a protected environment, wherein this potent, vasoactive peptide can activate its receptors to influence vascular function. Both kininogens participate in maintaining the constitutive anti-coagulant environment of the intravascular compartment by virtue of their antithrombin inhibitory activities. This contributes to the physiology of vasculature by regulating local blood flow, thrombosis and fibrinolysis.

1.6.4 The Contact Activation Cascade

The plasma kallikrein-contact activation system (PKCS) comprises a cascade of proteinases which, when activated, generates the nonapeptide, BK, from HK. There is also cross-activation of C1 protease (the initial protein of the classical complement pathway), hydrolysis of prourokinases to yield urokinases (which activates cell-associated fibrinolysis), and activation of Factor XI of the intrinsic blood coagulation pathway. The PKCS is triggered following activation of Factor XII by endotoxin (Kalter *et al.*, 1983) and microbial proteases (Molla *et al.*, 1989) to yield Factor XIIa, or indirectly by auto-activation through injury to endothelium.

Factor XIIa cleaves PPK to its active form PK, which can then further activate more Factor XII to Factor XIIa. HK associated with PPK forms a non-covalent complex and aids PPK in the feedback activation by carrying it to a common activating surface where Factor XII or Factor XIIa are present. When the PPK-HK complex is activated to a PK-HK complex, PK can cleave HK at two sites, causing the release of BK. BK, via activation of cell surface B2 receptors on the vascular endothelium, releases nitric oxide and prostacyclin, both potent vasodilators. Thus activation of the PKCS can contribute significantly to the hypotension that occurs in sepsis. Factor XIIa also contributes to coagulation by activating Factor XI to Factor XIa, thereby initiating the intrinsic cascade of coagulation.

PK can also stimulate neutrophil chemotaxis, aggregation, elastase release, and oxygen consumption *in vitro* (Goetzl and Austen, 1974; Schapira *et al.*, 1982; Wachtfogel *et al.*, 1983). Furthermore, neutrophil activation *in vivo*, assessed by increased complexes in plasma between human neutrophil elastase (HNE) and α_1-proteinase inhibitor (α_1-PI), occurs in sepsis and is associated with a poor prognosis (Egbring *et al.*, 1977; Duswald *et al.*, 1985; Nuijens *et al.*, 1992). Thus the KKS may contribute to HNE-mediated tissue damage seen in sepsis.

1.6.5 Regulation of Contact Activation

The auto-activation of Factor XII is very slow. However, the reciprocal reactions involving kallikrein contribute to the rapid activation of Factor XII. The *in vivo* source of this active enzyme is unknown, but may be formed by other plasma proteases, like plasmin or activation along cell surfaces. In fact,

very slow turnover of the cascade may always be occurring (Silverberg and Kaplan, 1982; Shibayama *et al.*, 1994) and controlled by plasma inhibitors (Weiss *et al.*, 1986). Introduction of a surface or other polyanionic substances could accelerate many thousand-fold the baseline turnover of Factor XII and PPK to accelerate the cascade. The addition of the cofactor, HK, promotes these reactions even further, but requires the surface to be present. The surface appears to create a local milieu in the contiguous fluid phase (Griffin and Cochrane, 1976; Griep *et al.*, 1985) where the local concentrations of reactants are greatly increased, which increases the rate of the reciprocal interaction. In addition, surface-bound Factor XII undergoes a conformational change that renders it more susceptible to cleavage (Griffin, 1978).

In plasma, the involvement of HK in the contact activation system was discovered in a study on abnormal persons who had a prolonged partial thromboplastin time (PTT) and no BK could be generated upon incubation of their plasma with kaolin, but they were not deficient in Factor XII or PPK (Colman *et al.*, 1975; Wuepper *et al.*, 1975; Donaldson *et al.*, 1976). This phenomenon was explained by the identification of HK as a non-enzymatic cofactor in contact activation. It appeared to accelerate activation of both Factor XII and PPK as well as Factor XI (Meier *et al.*, 1977; Revak *et al.*, 1977; Wiggins *et al.*, 1977). The discovery that PPK and Factor XII circulate bound to HK, provided the mechanistic key to an explanation (Thompson *et al.*, 1977). One function of HK is to present the substrates of Factor XIIa in a conformation that facilitates their activation. Thus, PPK that is bound to the surface in the absence of HK is not subsequently activated by Factor XIIa

(Silverberg *et al.*, 1980). A synthetic peptide encompassing the HK binding site for PPK can interfere with contact activation by competitively interfering with the binding of PPK to the HK light chain (Tait and Fujikawa, 1987). Similarly, a monoclonal antibody to this binding site inhibits coagulation and kinin formation in plasma (Reddigari and Kaplan, 1989). Factor XI activation is almost totally dependent upon the formation of a surface-binding complex with HK.

1.6.6 The Complement System (Figure 1.6)

The classic complement pathway is activated by immune complexes, whereas the alternative pathway is activated on the surfaces of damaged host tissues and invading micro-organisms. The precursor protein, C1 (800 kDa), is composed of a 460 kDa subunit (C1q) associated with two molecules each of the 83 kDa polypeptides, C1s and C1r $C1q(r,s)_2$ (Pixley and Colman, 1997). Upon activation, C1q dissociates and activates the protease form of C1s. Activated C1s initiates a cascade resulting in formation of the membrane attack complex (MAC), as well as release of C3a, C4a and C5a (Pixley and Colman, 1997). These anaphylatoxins, acting on cell-surface receptors of mast cells and basophils, release histamine and serotonin, thereby contributing to microvascular leakage. C3a and C5a are also chemotactic for monocytes and neutrophils (Pixley and Colman, 1997). Inactivation of C4b2a occurs by dissociation of C2a from the complex and cleavage of bound C4b. Factor I cuts C4b2a which occurs by dissociation of C2a from the complex and cleavage of bound C4b (Pixley and Colman, 1997). Factor XI cuts C4b at

Figure 1.6 CLASSICAL AND ALTERNATIVE PATHWAYS OF COMPLEMENT ACTIVATION

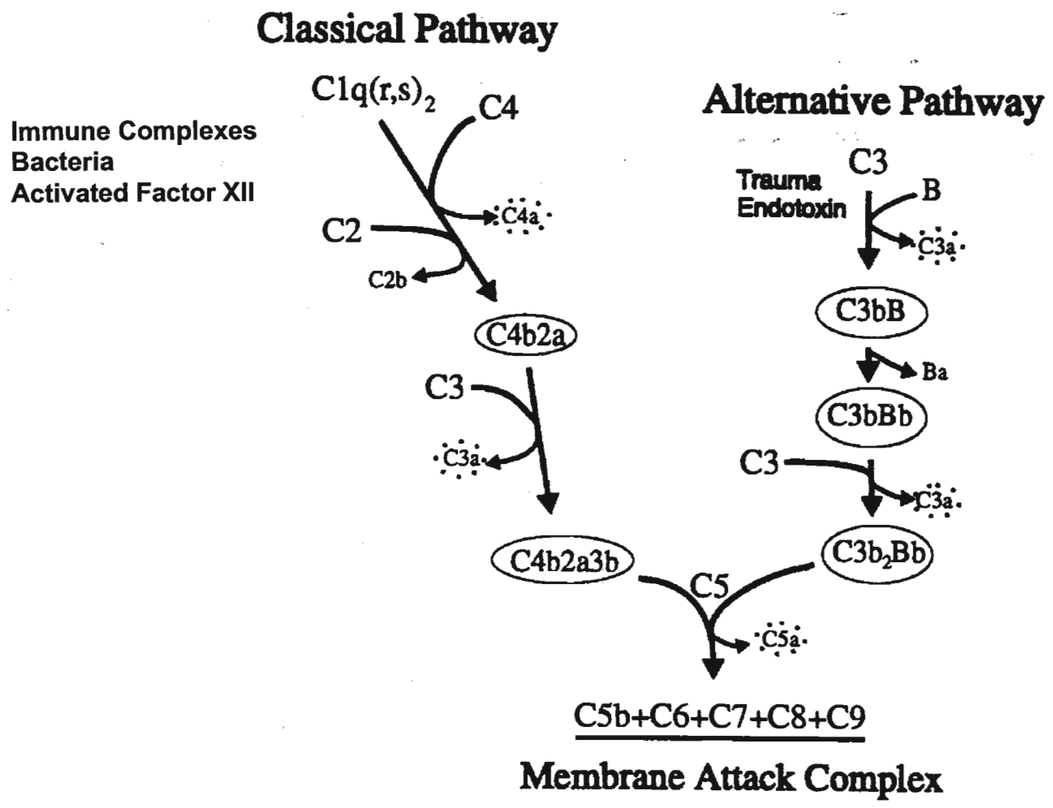


Figure 1.6 Classical and alternative pathways of complement activation

This figure shows the formation of a membrane attack complex (MAC) via the classical and alternative complement activation pathways that are activated by immune complexes and damaged host tissue surfaces/micro-organisms respectively. Subunits of C1, the precursor protein, initiate a cascade resulting in the formation of MAC. Detailed descriptions of these systems are described in the text (see **1.6.6**).

two sites releasing a large fragment C4c, and leaving the C4d attached to the cell membrane. Inactivation of C3b occurs in a similar manner.

Direct activation of complement by bacteria and their products occurs, since endotoxin and intact bacteria can activate complement via the alternate pathway (Morrison and Kline, 1977). However, *in vitro* studies have demonstrated a mechanism for activation of the classical pathway where another cleavage product of Factor XIIa, Factor XIIf (30 kDa), can activate C1 through cleavage of C1r (Ghebrehiwet *et al.*, 1981; Ghebrehiwet *et al.*, 1983). Alternatively, kallikrein can cleave the C1 components, resulting in destruction of C1. Kallikrein has been demonstrated to replace Factor D in the alternative pathway, generating C3 convertase by cleaving Factor B (Di Scipio, 1982). Kallikrein can generate C5a from C5 (Wiggins *et al.*, 1981), suggesting that the KKS may also activate complement.

1.7 PK in tissues

Immunocytochemical studies using monoclonal and polyclonal antibodies showed that PPK occurs bound to domain 6 of the kininogen molecule located on the external surface of the human neutrophil (Figueroa *et al.*, 1992b). Probing of the kinin moiety by a specific antibody demonstrated that kininogen molecules bound to the neutrophil cell membrane were intact and contained the kinin sequence, domain 4. Thus it appears that neutrophils may provide a circulating platform for the surface activation of PPK and clotting factors (Henderson *et al.*, 1992). PK also appears to be involved in the conversion of prorenin to renin (Sealey *et al.*, 1978; Derkx *et al.*, 1979).

PPK synthesis in the liver was originally identified after reduced levels of PPK were measured in the plasma of patients with liver cirrhoses (Colman and Wong, 1979). Direct evidence of hepatic PPK synthesis came from immunocytochemical studies using confocal microscopy which demonstrated PPK in hepatocytes (Henderson *et al.*, 1992). Previously, the inability to find PK mRNA transcripts in any tissue other than the liver suggested that PK does not normally function as a processing enzyme cleaving selected pairs of basic amino acids in prohormones *in vivo* (Seidah *et al.*, 1988). However, in more recent studies PPK mRNA was demonstrated in RNA preparations from whole tissues of kidney, adrenal gland and placenta by reverse transcription polymerase chain reactions (RT-PCR) (Ciechanowicz *et al.*, 1993; Hermann *et al.*, 1996). PPK mRNA is also expressed in the human brain, heart, lung, trachea, endothelial cells, leukocytes, as well as a variety of fibroblast and epithelial cell lines (Hermann *et al.*, 1999). Expression of PPK mRNA in fibroblasts, endothelial cells and leukocytes suggests that PPK mRNA detected from whole tissue may originate from these ubiquitously occurring cells. However, PPK mRNA expression in various epithelial cell lines demonstrate that tissue-specific cells also transcribe the PPK gene. The presence of PPK mRNA in non-hepatic cells indicates their capacity to synthesise the protein. It appears therefore, that PPK may participate in local actions within tissues as well as contributing to the PPK pool in blood plasma. PK immunolocalisation has been demonstrated in human arteries suggesting that kinins may play a role in maintaining and regulating vascular tone and local blood flow (Cerf *et al.*, 1999).

Both PK and Factor XIIa can activate granulocytes (Wachtfogel *et al.*, 1983; Wachtfogel *et al.*, 1986) and the complement system (Ghebrehiwet *et al.*, 1981; Ghebrehiwet *et al.*, 1983; Di Scipio, 1982). Factor XIIa activates Factor XI and thus triggers the intrinsic blood coagulation cascade. PK is an effective activator of the precursor for urokinase-type plasminogen activator and therefore provides a connection to the fibrinolysis system (Ichinose *et al.*, 1986; Hauert *et al.*, 1989).

1.8 Hypothesis and aims of this study

Only a few studies, and that only recently, have reported on the cellular expression of plasma kallikrein (PK) in mammalian tissues. Therefore, the aim of this study is to identify, isolate and immunolocalise PK and to examine its possible role in human tissues viz. the liver, pancreas, kidney, lung, salivary gland, oesophagus, stomach, blood vessels (aorta, renal, coronary, basilar and carotid arteries, inferior vena cava, renal vein) as well as the spinal cord and various brain tissue (hypothalamus, frontal lobe, occipital lobe, pre-central and post-central gyri, thalamus, pituitary gland, medulla, pons, hippocampus, midbrain, choroid plexus and cerebellum). With the availability of specific antibodies to PK, the capability to demonstrate PK in different human tissues became possible. Also, evidence was needed to demonstrate whether extra-hepatic synthesis of PK occurs in human tissue.

CHAPTER TWO

2.1 Sample collection

2.1.1 Ethical approval and Patient/Guardian Consent

Ethical permission for this study was obtained from the Ethics Committee of the Nelson R. Mandela School of Medicine, University of Natal. Permission for the collection of post-mortem tissue samples was obtained from Professor M.A. Dada, Head - Department of Forensic Medicine, University of Natal. Autopsy samples for liver, pancreas, kidney, lung, submandibular salivary gland, oesophagus, stomach; blood vessels (aorta, renal, coronary, basilar and common carotid arteries, inferior vena cava, renal vein), upper cervical portion of the spinal cord and brain tissue [frontal lobe, occipital lobe, pre-central and post-central gyri, hypothalamus, thalamus, midbrain, pons, medulla, hippocampus, pituitary gland (both anterior and posterior), choroid plexus and cerebellum] were collected at post-mortem, performed within 24h of death, with the co-operation of the attending forensic surgeon. Tissue samples of the liver, kidney, lung and salivary gland were collected from all 10 patients. Pancreas and oesophagus samples were collected from 9 patients, and only 4 sections of the stomach were collected due to autolysis at post-mortem. Aorta samples were collected from 10 patients, whereas 9 sets of each of the other blood vessels were collected. For the different brain regions, sections were collected from 7 patients. All the tissue collected was placed in physiological saline (0.9 % NaCl, pH 7; Sabax, SA) kept at 4^0C. The samples were then separated into 3 pieces: one placed in a cryotube and stored at -70^0C, another in physiological saline for homogenising, and the third fixed in 5% formal saline (35% formaldehyde/physiological saline; Saarchem, SA) overnight for paraffin-wax embedding. Consent was obtained from the families of the deceased (See Annexure: Table 2.1.1).

2.2 Sample Processing and Extract Preparation

2.2.1 Preparation of tissue extracts

The tissue stored in physiological saline was homogenised on ice in 1.5 ml physiological saline with a hand held homogeniser (10 ml Down's) for 10 strokes. To remove cellular debris and high molecular weight components, the homogenates were centrifuged at 9923 xg (Heraeus Biofuge 13R, Heraeus Sepatech, Germany) at 4^0C for 10 min. The pellets containing cells and debris were discarded, and the supernatant was stored for measurement of PK concentration by ELISA.

2.2.2 Tissue processing: fixation and wax embedding for light microscopy

Formal saline fixed tissue samples were orientated and set in plastic tissue cassettes. The tissue samples were then processed by routine tissue fixation and embedding techniques. Briefly, the tissue samples were dehydrated using absolute ethanol (Saarchem, SA) and xylene (Saarchem), and then embedded in paraffin-wax under sterile conditions, in an automatic tissue processor (Shandon, UK). The automated fixation, dehydration, clearing, infiltration and embedding were carried out by the Department of Histopathology, University of Natal. (For detailed methodology see Raidoo, 1999).

2.2.3 Haematoxylin and Eosin (H&E) staining of wax-embedded tissue

Ultra-thin (3 μm) sections of the wax-embedded tissue were cut on a rotary microtome (Jung RM2035, Leica, Germany) and floated onto glass slides (Lasec, SA). The slides were allowed to air-dry and then stained with Mayer's haematoxylin (Sigma, St. Louis) and eosin (Sigma) (H&E) to confirm the absence of disease in the tissue samples and to ensure that tissue processing was optimal. The following staining method was performed at room temperature (RT). The

tissue was dewaxed in xylene (Saarchem, SA) twice for 5 min, then rehydrated through a series of increasingly dilute ethanol (Saarchem) in distilled water (dH_2O) (100%, 90%, 70% v/v) for 1 min each, and finally into dH_2O for 5 min. The slides were then immersed in Mayer's haematoxylin for 5 min at RT, washed under running tap water for 5 min to intensify the stain, immersed in eosin for 6 min, and then rinsed in 95% ethanol for 30 seconds. The stained tissue slides were then dehydrated in absolute ethanol twice for 1 min each, and finally into xylene. These were then mounted with glass coverslips using a permanent mountant, Entellen (Merck, Germany) (For detailed methodology see Raidoo, 1999).

2.3 Sample storage

2.3.1 Extracts for PK ELISA

A volume of 40 µl 40 mM Tris-HCl, pH 8 (PK cocktail) (Saarchem, SA) was added to 1ml aliquots of each tissue extract to prevent PK degradation, and then stored at -20^{0}C for the subsequent determination of total PK by ELISA.

2.3.2 Wax-embedded tissue

The wax-embedded tissue samples were stored at RT for future microscopic and immunohistochemical analysis.

2.4 Anti-PK antibodies

Polyclonal anti-PK antibodies, raised in rabbit, were directed against PK (hPK 1060L, Enzyme Research Laboratories, USA). The lyophilised PK protein was reconstituted in sterile physiological saline at a concentration of 1.654 mg/ml, frozen in 50 µl aliquots and maintained at -20^0 C.

2.4.1 Generation of Rabbit anti-human PK antibody

Two healthy free-range adult rabbits were used to raise polyclonal antibodies to PK. The animals were obtained from, and housed at the Biomedical Resource Centre, University of Durban-Westville, Durban (ethical permission was obtained from the University of Durban-Westville Ethics Committee) where all procedures were performed under ketamine (100 mg/ml) anaesthesia at a dose of 2-3 mg/kg body weight by intra-muscular injection (IMI). A volume of 2 ml blood was removed from an ear vein of each rabbit prior to inoculation to test the non-immune serum for cross-reactivity. For the initial inoculation, 150 µg antigen was mixed with 150 µl Freunds complete adjuvent (Sigma, St. Louis). The rabbits were initially immunised with 75 µl conjugated antigen by IMI into each hind limb. For the first booster injection, administered three weeks later, 100 µg of antigen was mixed with 100 µl Freunds incomplete adjuvent (Sigma), and 50 µl was injected into each hind limb. For the remainder of the booster programme 50 µg of PK, dissolved in 50 µl 0.01M phosphate buffered saline (PBS), pH 7.4 (Sigma), was conjugated to 100 µl Freunds incomplete adjuvent, and 100 µl conjugated antigen injected into a hind limb of each animal. Subsequent similar booster injections were rotated between limbs and administered at four-week intervals over a 4-month period. Two days before each booster injection, and weekly after the final booster, 2 ml of blood, removed from an ear vein, was used to determine cross-reactivity, specificity and sero-conversion. The antibody titre was determined by a standard single site ELISA using PK. When the antibody titre increased from 1:100 and finally plateaued at an optimum of 1:7000 after the final booster injection, the animal was exsanguinated by cardiac puncture. The whole blood removed at each bleed was allowed to clot at RT, centrifuged at 88 xg (Heraeus Megafuge 1.0R, Heraeus Sepatech, Germany) for 20 min at RT, and the serum collected was

stored at -20^{0}C.

2.4.2 Isolation and purification of Immunoglobulin G (IgG) from rabbit serum

A Protein G Sepharose^R **4** Fast Flow Column (Pharmacia Biotech, Sweden) was used to separate IgG from serum. The column was stored in 20% ethanol (v/v). Firstly, the ethanol was decanted and discarded. Then the sepharose column (5.5 ml in volume) was equilibrated with 3 bed volumes (15-20 ml) of 0.1M Tris-HCl, pH 8 (Sigma, St. Louis). 1 ml of rabbit anti-human PK anti-serum (from section 2.4.1) was added to the column. The column was connected to a fractionator pump (Gilson Pump, Abimed, Germany) at a flow rate of 1 ml/min, and the flow-through was collected. A volume of 3 ml 0.1M Tris-HCl, pH 8 was added when the column was almost dry and also collected. The column was stopped and 60 ml 0.1M Tris-HCl, pH 8 was added without the use of the pump, and collected. This was repeated using 60 ml 0.01M Tris-HCl, pH 8. The addition of 0.1M and 0.01M Tris-HCl, pH 8 was to facilitate the complete washing of all serum components out of the gel, except for the IgG. To elute the IgG, 40 ml 0.1M glycine, pH 3 (Sigma) was added to the column, which was connected to the pump (1 ml/min) and the fractionator (2112 Redirac Fraction Collector, LKB, Sweden), and eluted into 44 ependorfs in 1 ml fractions. The pH of the eluted IgG fractions were checked with litmus paper to determine at which fractions a pH of 8 was achieved, which is optimal for the elution of IgG fractions from the column. The absorbances of the eluted IgG fractions were read at OD_{280} using a Uvikon spectrophotometer 920 (Kontron, Italy) with 0.1M glycine, pH 3 as a blank. OD_{280} = 1.33 was taken as 1 mg/ml IgG concentration. IgG fractions with the highest absorbances were pooled. Sodium azide (0.02% w/v; Sigma) was added to the IgG pools, which were then stored at -20^{0}C. The column was then washed with 3 bed volumes of 0.1M

glycine, pH 3 followed by washing with 0.01M Tris-HCl, pH 8 and finally stored in 20% ethanol at 4^0 C (See Annexure: Table 2.4.2).

2.4.3 Quality control

The purity, immunospecificity and sensitivity of the antibodies were verified by Western immunoblotting, a single site ELISA using PK, positive control tissue and pre-adsorption with PK in immunocytochemical studies.

2.4.3.1 Titre determination

The stepwise determination of the anti-PK antibody titre in rabbit serum by a standard single site ELISA using PK was as follows-

An aliquot of PK (hPK 1060L, Enzyme Research Laboratories, USA) (1.654 mg/ml in sterile physiological saline stored at -20^0 C; Sabax, SA) was thawed at 4^0C and diluted to 5 µg/ml in coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH 9.6; Sigma, St. Louis). An ELISA microtitre plate (Corning, USA) was coated with 100 µl/well overnight at 4^0C. The plate was washed 3X3 min each with 0.01M PBS, pH 7.4 (Sigma)/0.05% Tween 20 (Saarchem, SA) (PBS/Tween) at RT. All wells were then blocked twice with 200 µl milk blocker [10% fat-free bovine milk (Elite, SA)/5% bovine serum albumin (BSA); Boehringer-Mannheim, Germany)] for 30 min each at RT, with a PBS/Tween wash step in-between for 3X3 min. Serial dilutions of the anti-serum (1:200; 1:400; 1:800; 1:1600 and 1:3200) were made up in 0.01M PBS/1% BSA (v/v) (PBS/BSA). Each dilution (100 µl) was added in triplicate to the plate and incubated for 1h at 37^0C in a shaking water bath (Tecator, UK). Blank wells were filled with 100 µl PBS/BSA. The plate was then washed 3X3 min each with PBS/Tween at RT. Next, each well was loaded with 100 µl of alkaline phosphatase conjugated anti-rabbit IgG (Sigma), diluted 1:250 in 0.01M PBS/1% BSA (v/v), and the plate incubated for 1h at 37^0C in a shaking

water bath. The plate was then washed 3X3 min each with PBS/Tween at RT. Finally, to facilitate chromogenic colour development, the plate was loaded 100 µl/well with 1 mg/ml phosphatase substrate, disodium paranitrophenyl phosphate substrate (pNPP, Sigma, 1 tablet in 5 ml of $MgCl_2$/diethanolamine, pH 9.6, substrate buffer, Sigma) and absorbances read at 405 nm on a Biorad Microplate Reader 3550 (Biorad, UK) utilising Biorad Microplate Manager software, until readings peaked at 1.0-1.5 absorbance units (For detailed methodology see Raidoo, 1999).

2.4.3.2 Western blot analysis for rabbit anti-human PK IgG

2.4.3.2.1 Checkerboard Test

A serial dilution (200 ng/ml to 12.5 ng/ml) of PK antigen (hPK 1060L, Enzyme Research Laboratories, USA) in PBS, pH 7.4 (Sigma) was done. Nitrocellulose paper (20 µm pore size, Amersham, England) was cut into 8 mm wide strips between cellophane wrapping and handled with forceps. Diluted PK antigen (1 µl) was then spotted 1 cm apart on the nitrocellulose paper. The protein was allowed to air-dry for 1h between 3 µm Whatman paper (Whatman, England) and covered with a plastic container to protect it from dust. Non-specific binding sites were then blocked 2x10 min in 3 ml 10% blocking reagent (10% casein protein in maleic acid buffer; Boehringer-Mannheim) in a Biorad transfer plate and placed on a shaker (Adams Nutator 1106, USA). Strips were then incubated in 3 ml of rabbit anti-human PK antibody diluted 1:4000; 1:8000; 1:16000 at 4^0C overnight on a shaker. The nitrocellulose strips were then washed in 0.01M PBS, pH 7.4 (3X10 min/RT), blocked with 10% blocking reagent for 30 min/RT, and incubated with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:5000 with 10% blocking reagent for 1h at RT. The strips were then washed in detection buffer (0.1M Tris-

HCl, 0.05M $MgCl_2$, 0.1M NaCl, pH 9.5; Boehringer-Mannheim) (3X10 min/RT). A 0.375 mg/ml NBT (nitro blue tetrazolium chloride)/0.188 mg/ml BCIP (5-bromo-4-chloro-3-indoyl-phosphate) chromogen solution (Boehringer-Mannheim) was used to develop the membrane for a few minutes in the dark at RT. When sufficient colour development showed visible immunoprobed protein bands, the reaction was terminated with 0.05M EDTA (ethylenediamine-tetraacetic acid, pH 8; Sigma) for 5-10 min and then quenched with dH_2O for 30 min. The membrane strips were then scanned on a Scan Jet II C flat bed scanner (Hewlett-Packard, USA).

2.4.3.2.2 Western Blotting

Rabbit anti-human PK IgG (from section 2.4.2) was characterised by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot transfer for specificity and purity as follows:

Electrophoresis: PK protein (hPK 1060L, Enzyme Research Laboratories, USA) was run on a 15% SDS polyacrylamide gel. The 15% resolving gel and 5% stacking gel were prepared according to the method of Sambrook (1989) (See Annexure: Buffers and Reagents). 10 µg of PK protein was added to 7 µl of sample loading buffer (10% SDS w/v, 1% bromophenol blue w/v, 5% ß-mercaptoethanol w/v, 1% glycerol w/v, 0.5M Tris-HCl, pH 6.8), heated on a heating mantle (Thermolyne Dri-Bath, Barnstead/Thermolyne, USA) at 42^0C for 1h, and then immediately plunged into ice. Low range molecular weight markers (lysozyme 14.4 kDa, trypsin inhibitor 21.5 kDa, carbonic anhydrase 31 kDa, ovalbumin 45 kDa, serum albumin 66.2 kDa, phosphorylase B 97.4 kDa; Biorad, UK) were also added to sample loading buffer (1:19) and heated. 10 µl of denatured protein and denatured molecular weight markers was loaded onto the

SDS gel and electrophoresed at 160 mV for 1h using a discontinuous buffer system (0.4M glycine, 0.02M SDS, 0.12M Tris-HCl, pH 8.3) on a Biorad Mini-Protean® Electrophoresis Cell (Biorad, USA) using a Biorad Powerpac 3000 power source. Controls for the electrophoretic run were 1 mg/ml human albumin (Sigma, St. Louis) and rTK (generated in *E. coli* transfected with human TK cDNA, donated by Dr. Michael Kemme, Institute for Biochemistry, Technical University of Darmstadt, Darmstadt, Germany; similar concentration and denaturing conditions as for PK). On completion of the run (determined when the dye front had reached within 1 cm of the base of the gel), the gel was removed from its apparatus.

Western blotting: The gel was equilibrated in transfer buffer (10% methanol, 0.025M Tris-HCl, 0.192M glycine, pH 8.3) for 15 min, as was filter blotting paper (Whatman, cut to the same size as the gel) and 0.4 μm Biorad nitrocellulose membrane. The 'gel sandwich' was assembled according to the Biorad Mini Trans-Blot® Electrophoretic Transfer Cell instruction manual (Biorad), and protein transfer was performed for 2h at 90 mA.

Immunoblotting: Upon completion of the protein transfer, the membrane was cut in two. One part of the membrane was stained with India ink [50 μl ink (Pelikan Drawing ink A, Pelikan, Germany) in 50 ml of 0.3% Tween 20 (Saarchem, SA)/0.01M PBS, pH 7.4 (Sigma)], the other was immunoblotted. For India ink staining: The blot was washed in 0.4% Tween 20/0.01 M PBS, pH 7.4, with two changes of 25 ml at 5 min each (200 μl Tween 20 in 50 ml PBS). The blot was placed in ink solution for 15 min (up to 18h) at RT- longer incubations increased sensitivity. The blot was then destained by multiple washes with PBS, dried on

Whatman paper, and then sealed in plastic for storage.

After transfer was completed, the membrane was blocked in 10% blocking reagent [10% casein protein in maleic acid buffer; Boehringer-Mannheim, Germany] for 30 min at RT on a shaker. The membrane was then washed in PBS (3X10 min/RT) on a shaker (Adams Nutator 1106, USA). Next, the membrane was incubated with rabbit anti-human PK IgG (1:4000 in 10% blocking reagent) overnight at 4^0C on a shaker. The membrane was then washed in PBS (3X10 min/RT), blocked with 10% blocking reagent for 30 min/RT, and incubated with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:5000 with 10% blocking reagent for 1h at RT. The membrane was then washed in detection buffer (0.1M Tris-HCl, 0.05M $MgCl_2$, 0.1M NaCl, pH 9.5; Boehringer-Mannheim) (3X10 min/RT). A 0.375 mg/ml NBT (nitro blue tetrazolium chloride)/0.188 mg/ml BCIP (5-bromo-4-chloro-3-indoyl-phosphate) chromogen solution (Boehringer-Mannheim) was used to develop the membrane for a few minutes in the dark at RT. When sufficient colour development showed visible immunoprobed protein bands, the reaction was terminated with 0.05M EDTA (ethylenediamine-tetra-acetic acid, pH 8; Sigma) for 5-10 min and then quenched with dH_2O for 30 min. The membranes were then scanned on a Scan Jet II C flat bed scanner (Hewlett-Packard, USA).

2.5 Immunocytochemistry (ICC)

2.5.1 Positive tissue and method controls ICC

Initially, normal human liver was used as positive control tissue to demonstrate localisation, upon previous evidence of PK abundance in such tissue (Bhoola *et al.*, 1992). Samples of fresh control liver were collected at post-mortem, fixed in

5% formal saline and embedded in paraffin-wax (refer to section 2.2.2). During each immunolabeling experiment, this tissue demonstrating the presence of PK in the hepatocytes, served as a method control. Later, the pancreas was the preferred positive control tissue, since more specific immunolabeling for PK was demonstrated in cells of the islets of Langerhans.

2.5.2 Negative method controls for ICC

The absence of positive specific immunolabeling following preabsorption of the primary antibody with an excess of PK demonstrated the specificity of the antibody utilised. The rabbit anti-human PK antibody was diluted 1:500 with 0.01M PBS, pH 7.4 and added to a 2 mg/ml stock solution of PK to yield a final concentration of 1 mg/ml antigen (PK). This antibody-antigen conjugate was incubated overnight at 4^0C to allow maximum formation of antigen-antibody complexes. Following centrifugation (427 xg at 4^0C; Heraeus Biofuge 1.3 R, Heraeus Sepatech, Germany) the supernatant replaced the primary antibody in the ICC experiments. Normal pancreas was used as a method control (both positive and negative) for each initial labeling experiment to detect PK immunolocalisation in other tissues. Those tissue samples, from this initial labeling experiment, that showed a positive signal were then repeated with a respective negative control where incubation with the primary antibody was omitted and replaced with either PBS, non-immune serum or primary antibody that had been preabsorbed with an excess of antigen. Negative controls that showed false positive results were subjected to biotin blocking experiments to eliminate endogenous biotin labeling.

2.5.3 Immunolocalisation of PK by immunoprecipitation

Sections of the wax-embedded tissue (3 μm) were adhered onto adhesive coated

(poly-L-lysine; Sigma, St. Louis) slides and used for the detection of immunoreactive PK by standard histochemistry techniques, using polyclonal rabbit anti-human PK IgG as the primary antibody, and conjugated antibodies containing the peroxidase-antiperoxidase (PAP) immunoenzyme complex and diaminobenzidine (DAB) as the chromogen visualised by light microscopy. Slides containing wax-embedded tissue sections were placed on a hot plate (Nickel Electro, Clifton, England) at 60^0C. The tissue was then dewaxed in xylene (Saarchem, SA) for 2x10 min/RT. The tissue was rehydrated: 2x5 min/RT in 100% EtOH (Saarchem); followed by 1x20 min/RT in 100% methanol (MeOH) (Saarchem); 2x4 min/RT in 90% EtOH; 2x3 min/RT in 70% EtOH; and 1x5 min/RT in dH_2O. The tissue was then boiled (80^0C) in 0.01M sodium citrate ($Na_3C_6H_5O_7.2H_2O$, pH 6.0; Saarchem) for antigen retrieval (Shi *et al.*, 1991) at 3 min high and then at 5 min low in a microwave oven (Sharp R-4A52, Japan). The tissue was allowed to cool at RT for 20 min, then placed in dH_2O for 5 min. A Dako PAP marker (Dako, Denmark) was used to encircle the tissue which was then washed in PBS (Sigma)/1% BSA (Boehringer-Mannheim, Germany). The tissue was incubated in 20% hydrogen peroxide [(H_2O_2 (v/v); Saarchem/80% MeOH (v/v)] for 4X10 min/RT under humidified conditions to quench endogenous peroxidase activity, and then washed in PBS. It was further blocked for 20 min in 10% blocking reagent (10% casein protein in maleic acid buffer, Boehringer-Mannheim). Endogenous biotin present in the liver, kidney and salivary gland samples was blocked with extravidin alkaline phosphatase conjugate (Sigma) diluted 1:20 in blocking reagent for 15 minutes under humidified conditions. The tissue was incubated with rabbit anti-human PK IgG (from section 2.4.2) [primary antibody; diluted in 10% blocking solution- 1:500 for tissues (liver, pancreas, kidney, lung, salivary gland, oesophagus and stomach), 1:800 for blood

vessels, and 1:250 for brain sections were optimal] overnight at 4^0C under humidified conditions. The tissue was washed in PBS. The tissue was incubated with Biotin link (Dako, Denmark) for 20 rnin/RT under humidified conditions, then washed in PBS. The tissue was incubated with streptavidin (undiluted, secondary antibody; Dako) for 20 min/RT, then washed in PBS. The tissue was incubated with liquid DAB (Dako) for 1-5 min/RT in darkness, washed in PBS, and counterstained in Mayer's haematoxylin (Sigma) for 5min/RT. The tissue was washed in tap water for 5 min, then dehydrated through 70% EtOH into xylene, and mounted in Entellan (Merck, Germany). The tissue was examined using a light microscope (Leica, Germany) (For detailed methodology see Raidoo, 1999).

2.6 PK ELISA

96 well microtitre ELISA plates (Corning, USA) were loaded 100 µl/well with 0.6 µg/µl PK (hPK 1060L, Enzyme Research Laboratories, USA) in coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH 9.6; Sigma, St. Louis) and incubated at 4^0C overnight. PK standards were prepared in ependorf tubes as follows: 600 µl of 2 µg/ml PK was serially diluted in 0.01M PBS, pH 7.4/1% BSA (Boehringer-Mannheim, Germany) (PBS/BSA) to 0.008 µg/ml. A volume of 640 µl 1:320 dilution of human plasma in PBS/BSA was serially diluted in a similar manner to 1:20 480. Then, an equal volume of 1:400 (previously determined as the optimal dilution by serial dilutions) rabbit anti-human PK IgG was added to each dilution of PK standards and human plasma, resulting in a final concentration of 1:800 rabbit anti-human PK IgG. For the tissue samples, 200 µl of each tissue extract (refer to section 2.2.1) was pre-incubated with 200 µl of 1:400 rabbit anti-human PK IgG. Oesophagus tissue extracts were diluted 1:7 in PBS/BSA for detection within the

curve limits. All standards and samples were incubated for 18h at 4^0C. The following day the wells were then washed with 200 µl 0.01M PBS (Sigma)/0.5% Tween 20 (Saarchem, SA) (PBS/Tween) 3X3 min each at RT. The wells were blocked for 30 min at RT with 200 µl 5% fat-free milk (Elite, SA) in PBS/Tween, which constituted the milk blocker. Next, 100 µl of each standard and tissue sample (in triplicate) was added to the wells. Blank wells (in triplicate) were filled with 100 µl of milk blocker. The plate was then incubated at 37^0C for 1h in a shaking water bath (Tecator, UK). The wells were then washed with 200 µl PBS/Tween 3X3 min each at RT. Next, the microtitre plates were incubated 100 µl/well with 1:1000 anti-rabbit IgG biotin conjugate (Sigma) for 1h at 37^0C in a water bath. The wells were again washed with 200 µl PBS/Tween 3X3 min each at RT, then incubated 100 µl/well with 1:250 extravidin alkaline phosphatase (Sigma) for 1h at 37^0C in a water bath. Finally, following a PBS/Tween wash, the wells were all loaded with 100 µl of a 1 mg/ml chromogenic disodium paranitrophenyl phosphate substrate (pNPP, Sigma, 1 tablet in 5 ml of $MgCl_2$/diethanolamine, pH 9.6- substrate buffer) and the colour was allowed to develop until the highest absorbances peaked at 1 to 1.5 absorbance units. Readings were taken at RT with a Biorad Microplate Reader 3550 (Biorad, UK) using Biorad Microplate Manager software. The mean blank absorbance was subtracted from the standards, controls and samples, and a curve of absorbance versus log PK concentration was plotted. The log concentrations of the samples were extrapolated from this graph and the concentrations were then calculated (See Annexure: Table 2.6).

Annexure

Appendix of buffers, reagents and methods

Table 2.1.1. Informed consent for the collection of autopsy tissue

To the surgical patient
I am Mr. M.E. Cerf. I wish to localise and examine the role of plasma kallikrein in human tissues. To accomplish the above objective I will need a small piece of tissue that the surgeon will remove during surgical procedure. Please consent to the above so that I may be able to accomplish this investigation. You may refuse to participate without suffering any prejudice in consequence.

_____ _____
(Name of patient) (Date)

Isiguli esizohlinzwa
Ngingu Mnumzane M.E. Cerf, usisayensi. Ngifisa ukucubungula ngeplasm kallikrein emzimberi womuntu. Ukuze ngiphumelele kulokhu ngizodinga isicutshana esincane esizosikwa ngudokodela ozokuhlinza. Ngicela kuwe imvume yalokhu ngoba ngemvume yakho ngiyethemba ukuthi lolucwaningo luzoba yimpumelelo. Uma uthanda ungenqaba ukukwenza lokhu futhi akakho ozokuhlukumeza uma wenqabile.

_____ _____
(Igama lesiguli) (Date)

To the guardian
I am Mr. M.E. Cerf. I wish to localise and examine the role of plasma kallikrein in human tissues. I will need small samples of normal tissue that will most easily be obtained from post-mortem procedure. Please consent to the above so that I may be able to accomplish this investigation.

_____ _____
(Name of guardian) (Date)

Ummeki wesiguli
Ngingu Mnumzane M.E. Cerf, usisayensi. Ngifisa ukucubungula ngeplasma kallikrein emzimbeni womuntu. Ukuze ngiphumelele kulokho ngizodinga izicutshana ezincane esidunjini somufi ngenkathi kulolwa imbangela yokushona kukamufi.Ngicela kuwe imvume yalokhu ngoba ngemvume yakho ngiyethemba ukuthi lolucwaningo luzoba yimpumelelo.

_____ _____
(Igama lommeli wesiguli) (Date)

Table 2.4.2 Isolation and purification of IgG from rabbit antiserum

STEP	SOLUTION	TEMP
1	Equilibrate the Protein G Sepharose® 4 Fast Flow Column (Pharmacia Biotech, Sweden) with 20 ml 0.1M Tris-HCl, pH 8 (Sigma, St. Louis).	RT
2	Add 1 ml rabbit anti-human PK anti-serum to the column.	RT
3	Connect the column to a fractionator pump (Gilson Pump, Abimed, Germany) at a flow rate of 1 ml/min, and collect the flow-through.	RT
4	Add 3 ml of 0.1M Tris-HCl, pH 8 as column runs dry, and collect it.	RT
5	Stop the column and add 60 ml 0.1M Tris-HCl, pH 8 (without the use of the pump) and collect it.	RT
6	Repeat using 60 ml 0.01M Tris-HCl, pH 8.	RT
	Addition of 0.1M and 0.01M Tris-HCl, pH 8 washes out all the serum components from the gel, except for the IgG.	
7	Add 40 ml 0.1M glycine, pH 3 (Sigma) to the column, which is reconnected to the pump (1 ml/min) and to the fractionator (2112 Redirac Fraction Collector, LKB, Sweden). Now elute into 44 ependorfs in 1 ml fractions.	RT
8	Determine (with litmus) at which fractions pH 8 is achieved- optimal for elution of fractions from column.	RT
9	Read absorbances of the eluted IgG fractions at OD_{280} on a Uvikon spectrophotometer 920 (Kontron, Italy).	RT
10	Use 0.1M glycine, pH 3 as a blank. Take OD_{280} = 1.33 as 1 mg/ml IgG concentration.	RT
11	Pool IgG fractions with the highest absorbances.	RT
12	Add sodium azide (0.02% v/w; Sigma) to the pooled IgG fractions and store at -20°C.	RT
13	Wash the column with 15 ml of 0.1M glycine, pH 3, then with 0.01M Tris-HCl, pH 8 and store in 20% EtOH at 4°C.	RT

Table 2.6 PK ELISA

STEP	SOLUTION	TEMP	TIME
1.	Coat the ELISA plate (Corning, USA) with 100 µl of 0.6 µg/ml PK in coating buffer (dissolve 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 litre dH_2O; Sigma, St. Louis).	4^0C	18h
2.	Pre-incubate the standards [600 µl of 2 µg/ml PK (hPK 1060L, Enzyme Research Laboratories, USA) serially diluted in 0.01M PBS/1% BSA (PBS/BSA) to 0.08 µg/ml; 640 µl of 1:320 human plasma serially diluted in PBS/BSA to 1:20 480] and tissue samples (200 µl of each) with an equal volume of 1:400 rabbit anti-human PK IgG.	4^0C	18h
3.	Wash the plate with 0.01M PBS/0.5%Tween 20 (PBS/Tween).	RT	3X3 min
4.	Add 200 µl of 10% fat-free bovine milk (Elite, SA) in PBS/Tween (milk blocker) to each well.	RT	30 min
5.	Wash the plate with PBS/Tween.	RT	3x3 min
6.	Incubate the plate with 100 µl/well milk blocker (blanks), standards and for each of the samples (all in triplicate).	37^0C	1h
7.	Wash the plate with PBS/Tween.	RT	3x3 min
8.	Incubate the plate with 100 µl/well with 1:1000 anti-rabbit IgG biotin conjugate (Sigma).	37^0C	1h
9.	Wash the plate with PBS/Tween.	RT	3x3 min
10.	Incubate the plate with 100 µl/well with 1:250 extravidin alkaline phosphatase (Sigma).	37^0C	1h
11.	Chromogen- 100 µl of 1 mg/ml pNPP substrate was added to each well [1 tablet in 5 ml of substrate buffer (5 mM $MgCl_2$/10% diethanolamine, pH 9.8; Sigma)] and allow colour to develop.	37^0C	0-60 min
12.	Calculation- subtract the absorbance of the blanks from the absorbance of the standards, controls and samples. Plot absorbance versus concentration of PK standards. Read PK concentration of samples and controls from this graph.		

Buffers and Reagents

Reagents for sample collection, processing, dissection, embedding, storage and histological staining

1. Sterile normal saline (physiological saline) (0.9% NaCl, pH 7; Sabax, SA).
2. 5% formal saline (35% formaldehyde/0.9% NaCl, 1:7 v/v; Saarchem, SA).
3. Absolute ethanol (99% ethanol, analytical grade; Saarchem).
4. Xylene (analytical grade; Saarchem).
5. Paraffin-wax (Paraplast plus).
6. Mayer's haematoxylin (Sigma).
7. Eosin (Sigma).
8. Poly-L-lysine (Sigma)- dilute 1:10 in dH_2O in plastic-ware and store at 4^oC for 3 months.
9. PK cocktail 40 mM Tris-HCl, pH 8- dissolve 4.8 g Trizma base (Sigma) in 800 ml dH_2O, adjust pH to 8 with HCl and volume to 1 litre.
10. Giemsa Stain (Sigma).
11. Acetic acid (analytical grade; Saarchem).

Reagents for isolation of IgG from serum

1. Protein G Sepharose^R 4 Fast Flow Column (Pharmacia Biotech, Sweden).
2. Rabbit anti-human PK antiserum from section 2.4.1.
2. 20% ethanol (v/v) (Saarchem, SA).
3. 0.1M Tris-HCl, pH 8- dissolve 12.11g Trizma base (Sigma, St. Louis) in 800 ml dH_2O, adjust pH to 8 with HCl and volume to 1 litre.
4. 0.01M Tris-HCl, pH 8- dissolve 1.211g Trizma base (Sigma) in 800 ml dH_2O, adjust pH to 8 with HCl and volume to 1 litre.
5. 0.1M glycine, pH 3- dissolve 7.507g glycine (Sigma) in 800 ml dH_2O, adjust pH to 8 with HCl and volume to 1 litre.

Reagents for Western blot analysis for rabbit anti-human PK IgG

1. Rabbit anti-human PK IgG from section 2.4.2.
2. Human plasma kallikrein (hPK 1060L, Enzyme Research Laboratories, USA)- 1.654 mg/ml in sterile physiological saline, stored at -20^{0}C.
3. 0.01M PBS, pH 7.4 (w/v)- dissolve 1 PBS tablet (Sigma, St. Louis) in 200 ml dH_2O.
4. 22 µm Nitrocellulose paper (Amersham, England).
5. 3 µm Whatman paper (Whatman, England).
6. Blocking reagent- 10% casein protein in maleic acid buffer (Boehringer-Mannheim, Germany).
7. Anti-rabbit IgG alkaline phosphatase conjugate (Sigma).
8. Detection buffer- 0.1M Tris-HCl, 0.05M $MgCl_2$, 0.1M NaCl, pH 9.5 (Boehringer-Mannheim).
9. Chromogen- 0.375 mg/ml NBT (nitro blue tetrazolium chloride)/0.188 mg/ml BCIP (5-bromo-4-chloro-3-indoyl-phosphate) chromogen solution (Boehringer-Mannheim).
10. 0.05M EDTA (ethylenediamine-tetra-acetic acid, pH 8; Sigma).
11. 30% acrylamide stock solution (w/v)- dissolve 30g acrylamide (Sigma) and 1.07 g N,N'-bisacrylamide (BDH, UK) in 100 ml dH_20.
12. 1.5 M Tris-HCl, pH 8.8 (w/v)- dissolve 181,65g Trizma base (Sigma) in 800 ml dH_20, adjust pH to 8.8 with HCl and make up to 1 litre.
13. 10% SDS (sodium dodecyl sulphate, w/v; Amresco, USA)- dissolve 10g SDS in 100 ml dH_20.
14. TEMED (BDH).

15. 10% ammonium persulphate (APS w/v)- dissolve 10g APS (BDH) in 100 ml dH_20.
16. 0.5 M Tris-HCl, pH 6.8 (w/v)- dissolve 60.55g Trizma base (Sigma) in 800 ml dH_20, adjust pH to 6.8 with HCl and make up to 1 litre.
17. Loading buffer (10% SDS, 1% bromophenol blue, 5% β-mercaptoethanol, 1% glycerol, 0.5 M Tris-HCl, pH 6.8; Sigma).
18. Prestained low range molecular weight markers- lysozyme 14.4 kDa, trypsin inhibitor 21.5 kDa, carbonic anhydrase 31 kDa, ovalbumin 45 kDa, serum albumin 66.2 kDa, phosphorylase B 97.4 kDa (Biorad, UK).
19. Running buffer (0.4M glycine, 0.02M SDS, 0.12M Tris-HCl, pH 8.3).
20. Transfer buffer (10% methanol, 0.025M Tris-HCl, 0.192M glycine, pH 8.3).
21. 0.4% Tween 20/PBS (200 µl Tween 20 in 50 ml PBS).
22. India ink solution [50 µl India ink (Pelikan Drawing ink A, Pelikan, Germany) in 50 ml 0.3% Tween 20/PBS].

Composition of Running and Stacking gels

Reagents	10% Resolving gel	5% Stacking gel
30% Acrylamide stock	5 ml	830 µl
1.5 M Tris-HCl, pH 8.8	3.8 ml	630 µl (0.5 M Tris-HCl, pH 6.8)
dH_20	5.9 ml	3.4 ml
10% SDS	150 µl	50 µl
TEMED	6 µl	5 µl
10% APS	150 µl	50 µl

Reagents for immunocytochemical localisation of PK by immunoprecipitation

1. Poly-L-lysine (Sigma, St. Louis)- dilute 1:10 in dH_2O in plastic-ware and store at 4°C for 3 months.
2. Xylene (Saarchem, SA).
3. EtOH (Saarchem).
4. 0.01M Sodium-citrate, pH 6.0 (w/v)- dissolve 2.94g Tri-sodium citrate in 800 ml dH_2O, adjust to pH 6 with HCl and make up to 1 litre.
5. 20% H_2O_2/80% MeOH (v/v)- mix 200 µl H_2O_2 (Saarchem) with 800 µl MeOH (Saarchem) just before use.
6. 0.01M PBS, pH 7.4 (w/v)- dissolve 1 PBS tablet (Sigma) in 200 ml dH_2O.
7. 1% non-immune rabbit serum.
8. Rabbit anti-human PK IgG from section 2.4.2.
9. Anti-goat IgG Biotin link (LSAB K0690, Dako, Denmark).
10. Streptavidin-peroxidase (LSAB K0690, Dako).
11. Chromogen- liquid DAB (K3465, Dako).
12. Counterstain- Mayers haemotoxylin (Sigma).
13. Mountant- Entellen (Merck, Germany).

Reagents and immunochemicals for PK ELISA

1. Coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH 9.6; Sigma, St. Louis)- dissolve 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 litre dH_2O.
2. Substrate buffer (5 mM $MgCl_2$/10% diethanolamine, pH 9.8) - dissolve 0.10163 g $MgCl_2$ in 80 ml dH_2O, add 10 ml diethanolamine (Sigma), adjust pH to 9.8 with HCl and make up to 100 ml with dH_2O.
3. 0.01M PBS, pH 7.4 - dissolve 1 PBS tablet (Sigma) in 200 ml dH_2O.

4. 5% protein blocker- dissolve 5g fat-free bovine milk powder (Elite, SA) in 100 ml PBS. Make fresh.
5. 0.01M PBS/0.5%Tween- dilute 100 µl Tween 20 (Sigma) in 200 ml 0.01M PBS.
6. Rabbit anti-human PK IgG from section 2.4.2.
7. Anti-rabbit IgG alkaline phosphatase (Sigma).
8. Disodium paranitrophenyl phosphate substrate (pNPP, 5 mg tablets, Sigma)- dissolve 1 tablet in 5 ml substrate buffer. Make fresh and use within 90 min.

CHAPTER THREE

3.1 Patient Demographics

Following ethical approval for the study from the University of Natal Ethics committee, permission for the collection of post-mortem tissue samples from the attending Forensic Surgeon and consent from the families of the deceased, the tissue samples were collected at autopsy. The details of the 10 patients in whom autopsies were performed within 24 h from death are summarised in Table 3.1. Briefly, there were 2 females and 8 males, whose ages ranged from 10 to 65 years (mean age = 36 years). The causes of death were chest injuries (n = 2), gun shot injuries (n = 2), stab wounds (n = 2), myocardial infarction (n = 1), hanging (n = 1), strangling (n = 1) and drowning (n = 1) with no injury to the head. From each of the 10 individuals, 28 different tissues were collected.

3.2 Validation of the anti-PK antibodies raised

The specificity of the rabbit anti-human PK IgG was verified by Western blot analysis and the localisation of PK in hepatocytes of human liver samples obtained at autopsy. The optimal concentration of antigen and dilution of antibody for Western blot analysis, determined by a checkerboard test, was 50 ng/ml PK (hPK 1060L) and 1:4000 dilution for rabbit anti-human PK IgG (Figure 3.1 A).

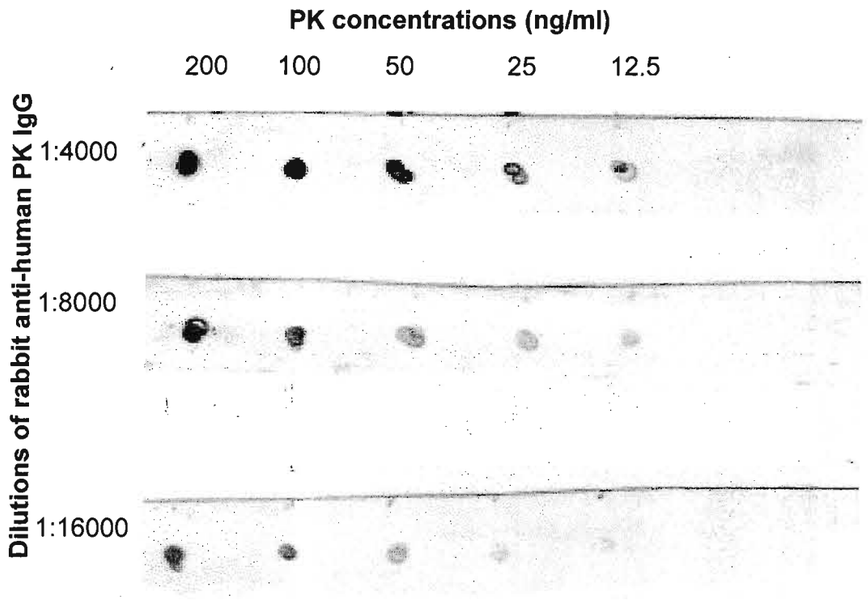
The Western blot analysis (Figure 3.1 B) showed that the IgG was specific for PK, since there was no cross-reactivity between the rabbit anti-human PK IgG and tissue kallikrein (lane 9), human albumin (lane 7), as well as the proteins in the low molecular weight marker {carbonic anhydrase, ovalbumin, serum albumin and phosphorylase B (lane 6)}.

Table 3.1 Demographics of patients from whom tissue samples were obtained at autopsy

Age	Race	Sex	Cause of Death	Time between autopsy and death
38	Black	Male	Blunt injuries to chest and neck	15 h
44	Black	Male	Penetrating chest injury	12 h
45	Asian	Male	Myocardial Infarction	17 h
18	Black	Male	Hanging	23 h
28	Black	Male	Multiple gun-shot wounds to chest and abdomen	22 h
46	Black	Male	Multiple stab wounds to chest and abdomen	6 h
65	Asian	Female	Strangled	16 h
33	Black	Male	Multiple stab wounds to chest and abdomen	23 h
10	Black	Male	Drowning	18 h
32	Black	Female	Multiple gun-shot wounds to chest and abdomen	7 h

Figure 3.1 Characterisation of rabbit anti-human PK IgG

A) Checkerboard Test



B) India ink stain and Western blot

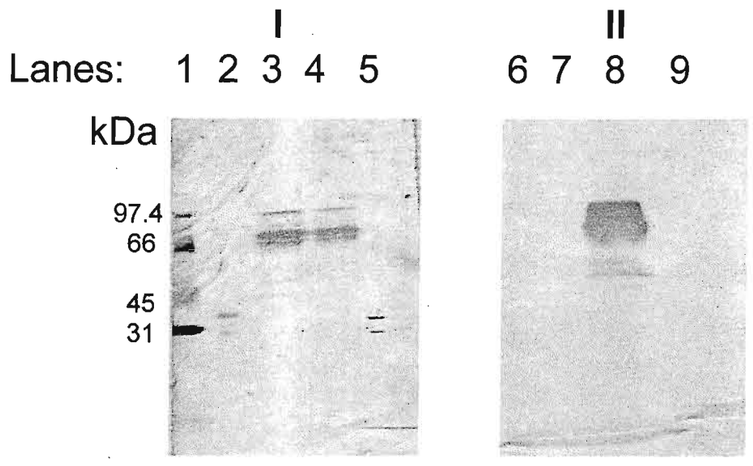


Figure 3.1 Characterisation of rabbit anti-human PK IgG

A) Checkerboard test for rabbit anti-human PK IgG

A double dilution series of PK (hPK 1060L) antigen was spotted onto strips of nitrocellulose membrane then incubated with various dilutions of rabbit anti-PK IgG. The bound antigen-antibody complex was detected with an alkaline phosphatase conjugated secondary antibody and the chromogen NBT/BCIP. The optimal concentration of the antigen and dilution of the antibody for Western blot analysis, determined by this checkerboard test, was 50 ng/ml PK and 1:4000 dilution for rabbit anti-human PK IgG.

B) India Ink stain and Western blot analysis

I India Ink stain (lanes 1-5): Proteins were resolved on a 15% SDS polyacrylamide gel, electro-transferred onto a nitrocellulose membrane, and then stained with India Ink. The first lane contained pre-stained low molecular weight markers [carbonic anhydrase 31 kDa, ovalbumin 45 kDa, serum albumin 66.2 kDa, phosphorylase B 97.4 KDa (Biorad, UK)]. Lane 2 contained 15 μg human albumin. Lanes 3 and 4 contained 10 μg PK and lane 5 contained 10 μg rTK.

II Western Blot analysis (lanes 6-9): Proteins from SDS gel were electro-transferred onto a nitrocellulose membrane and immunoblotted with rabbit anti-human PK IgG diluted 1:4000. The bound antigen-antibody complex was detected with an alkaline phosphatase conjugated secondary antibody (1:5000) and the chromogen NBT/BCIP. We demonstrate no cross-reactivity between rabbit anti-human PK IgG and the proteins in the low molecular weight markers {carbonic anhydrase, ovalbumin, serum albumin and phosphorylase B (lane 6)}, human albumin (lane 7) as well as tissue kallikrein (lane 9).

3.3 Immunohistochemical localisation of PK in human tissues

3.3.1 Immunohistochemical localisation of PK in positive control tissue

With the exception of a few inflammatory cells in the liver sections, histopathological examination revealed that all of the hepatic (Figure 3.2 A) and pancreatic (Figure 3.2 B) tissues were free from disease. The immunolabeling of PK observed in the liver was diffuse as most of the hepatocytes demonstrated some degree of immunolabeling (Figure 3.2 C), whereas in the pancreas immunoreactive PK was specific in cells of the islets of Langerhans and some acini (Figure 3.2 D). Therefore, the pancreas was used as a positive control for each immunolabeling experiment. Intense immunolabeling for PK was evident in the few inflammatory cells observed in samples of hepatic tissue.

3.3.2 Immunohistochemical localisation of PK in other tissue

Kidney: H&E staining showed normal histology although five sections of the tissue samples showed the presence of scattered inflammatory cells Immunoreactive PK was evident in cells of the distal convoluted tubules (Figure 3.3 A). No distinct immunolabeling for PK was visualised in cells of the proximal convoluted tubules, loops of Henle, collecting ducts or in the glomeruli.

Lung: H&E staining revealed that this tissue was essentially normal with the exception of scattered inflammatory cells as well as anthracotic deposits in nine of the ten samples that were collected. The epithelial cells of the bronchiole and alveoli showed immunolabeling for PK (Figure 3.3 C).

Salivary gland: Some acini within the lobules showed immunolabeling for PK.

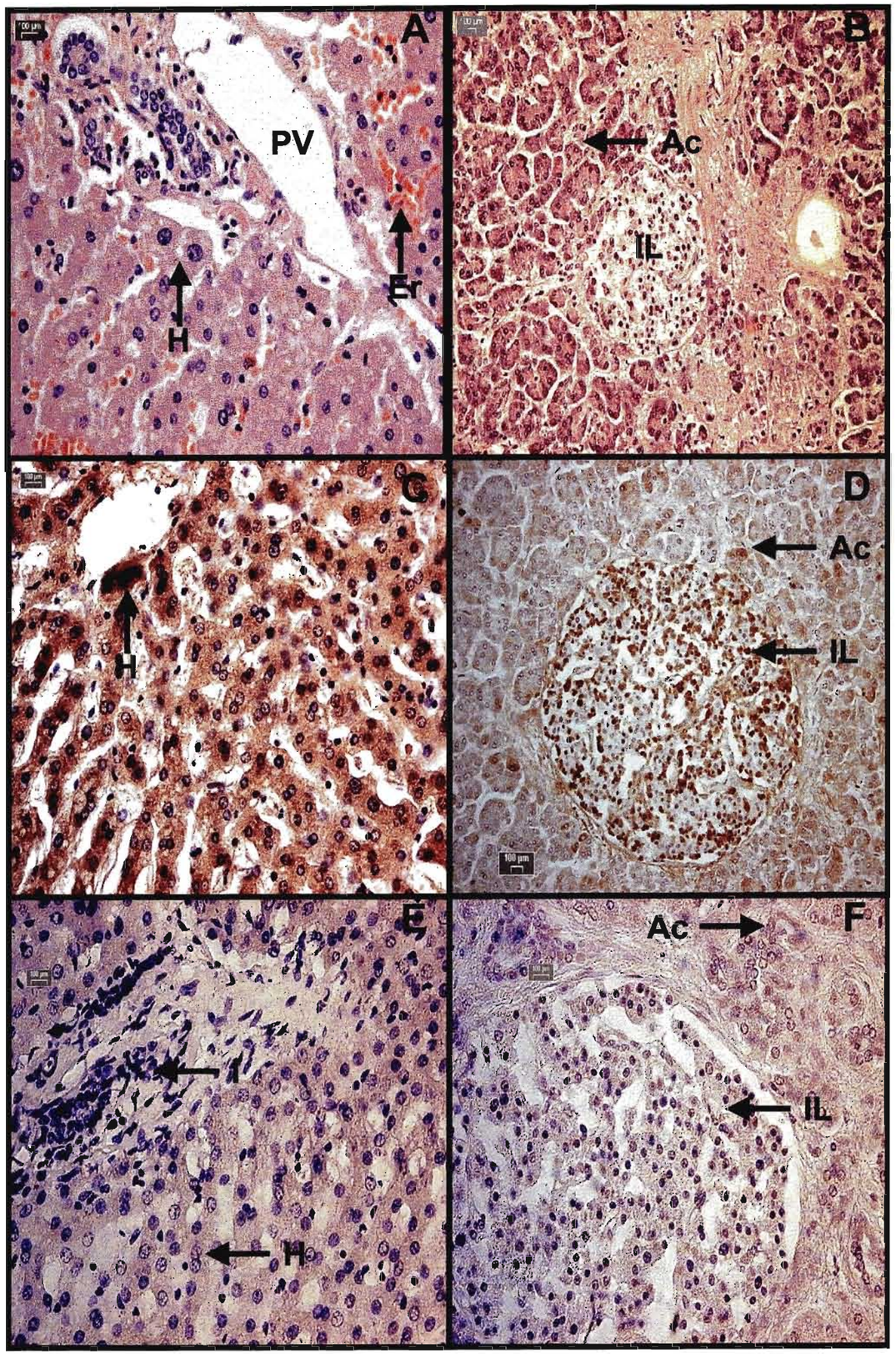


Figure 3.2 Immunolocalisation of PK in control tissue: liver and pancreas

Liver: Hepatocytes **H**, erythrocytes **Er** and the portal vein **PV** of the H&E stained tissue were visualised (A). Diffuse immunolabeling for PK was demonstrated in the hepatocytes **H** (C). No immunoreactive PK was present in the hepatocytes **H** or in scattered inflammatory cells **I** in the negative control (E).

Pancreas: The H&E stained tissue (B) demonstrated the acini **Ac** and cells of the islets of Langerhans **IL**, and also confirmed the absence of disease and preservation of normal histological structure. Immunoreactive PK was localised in cells of the islets of Langerhans **IL** and in some acini **Ac** (D). No immunolabeling for PK in the cells of the islets of Langerhans **IL** or acini **Ac** was evident in the negative control (F) where the primary antibody was preabsorbed with an excess of antigen.

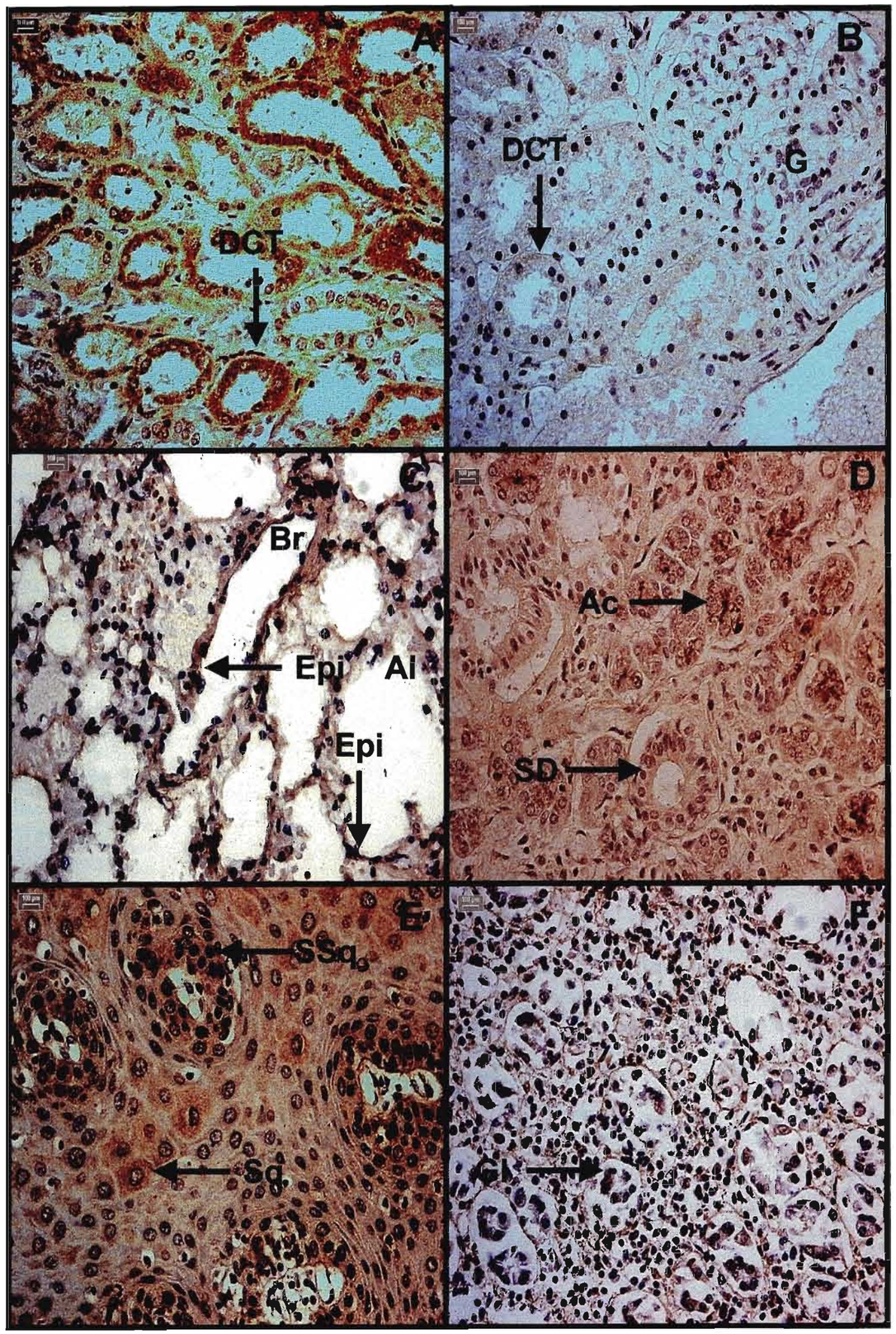


Figure 3.3 Immunolocalisation of PK in human tissue

Kidney: Immunoreactive PK was demonstrated in cells of the distal convoluted tubules **DCT** (A). In the negative control (B), the glomerulus **G** and distal convoluted tubule **DCT** showed no immunolabeling for PK.

Lung: Immunoreactive PK was present in epithelial cells **Epi** of the bronchioles **Br** and alveoli **Al** (C).

Salivary gland: Some acini **Ac** within the lobules were immunolabeled for PK. No immunoreactive PK was visualised in the striated ducts **SD** (D).

Oesophagus: Squamous epithelial cells **Sq** and stratified squamous epithelial cells **SSq** of the submucosa labeled positively for PK (E).

Stomach: Immunoreactive PK was evident in deep glandular cells **GI** within the fundus (F).

No PK immunoreactivity was demonstrated in either the striated ducts or in the septa (Figure 3.3 D).

Oesophagus: Intense PK immunolabeling was observed in the glandular duct and stromal cells of the submucosa (Figure 3.3 E). Diffuse immunolabeling for PK was also seen in some smooth muscle cells of the muscularis mucosa.

Stomach: The H&E stained sections revealed the presence of inflammatory cells in three of the stomach sections. Immunoreactive PK was present in chief and glandular cells within the fundus (Figure 3.3 F), some cells in the muscularis mucosa, and in the vascularised lamina propria.

PK was intensely immunolabeled in the few inflammatory cells observed in some of the above-mentioned tissues. In all of the above-mentioned tissues as well as in the blood vessels and brain sections, discussed subsequently, the distribution of immunoreactive cells was similar. Since this was a qualitative study, no quantitative assessment such as image analysis was done.

3.3.3 Immunohistochemical localisation of PK in blood vessels

Histopathological examination of all the blood vessels displayed normal morphology, with the exception of atheromatous plaques observed in one sample of the aorta (n = 10); five of the nine coronary arteries, four carotid, three basilar and two renal arteries. The various layers of the blood vessels, viz. the tunica intima, the tunica media and tunica adventitia showed varying degrees of immunostaining, with the tunica intima showing the most intense immunolabeling for PK and tunica adventitia the least. The endothelial cells in the tunica intima and smooth muscle cells in the tunica media of all the

arteries examined, demonstrated immunolabeling for PK. Intense labeling was visualised in the endothelial cells of the aorta (Figure 3.4 A), renal (Figure 3.4 C) and coronary (Figure 3.4 D) arteries. The foam cells, macrophages and inflammatory cells present in the atheromatous plaques also showed intense labeling for PK (Figures 3.4 C and D). Of the two veins examined, viz. the inferior vena cava (Figure 3.4 E) and the renal vein (Figure 3.4 F), only the former showed immunolabeling for PK as visualised in the endothelial and smooth muscle cells. In the basilar (Figure 3.5 A) and the carotid (Figure 3.5 B) arteries immunoreactive PK was also observed in the endothelial cells. In all of the arteries examined, immunolabeling for PK observed in endothelial cells was of greater intensity relative to labeling in smooth muscle cells.

3.3.4 Immunohistochemical localisation of PK in brain

H&E staining confirmed all the brain sections to be free of disease, injury and the preservation of normal morphology (Figure 3.5 C). Immunolabeling for PK was seen in small neurons of the supra-optic nucleus of the hypothalamus (Figure 3.5 D), and in neurons within the grey matter (Figure 3.5 E). In the hypothalamic sections, ependymal cells lining the third ventricle also labeled positively. In non-pyramidal neurons scattered in all layers of the grey matter of the four cerebral cortical regions examined, viz. the frontal lobe, occipital lobe, pre-central and post-central gyri (Figures 3.6 A-D respectively), immunolabeling for PK was observed. Immunoreactive neurons were also seen in specific nuclei within the thalamus (Figure 3.6 E) and in thalamic nerve fibres. The identity of the thalamic nuclei needs to be elucidated in further studies. Immunoreactive PK was visualised in secretory cells of the anterior pituitary gland (Figure 3.6 F). No immunolabeling for PK was

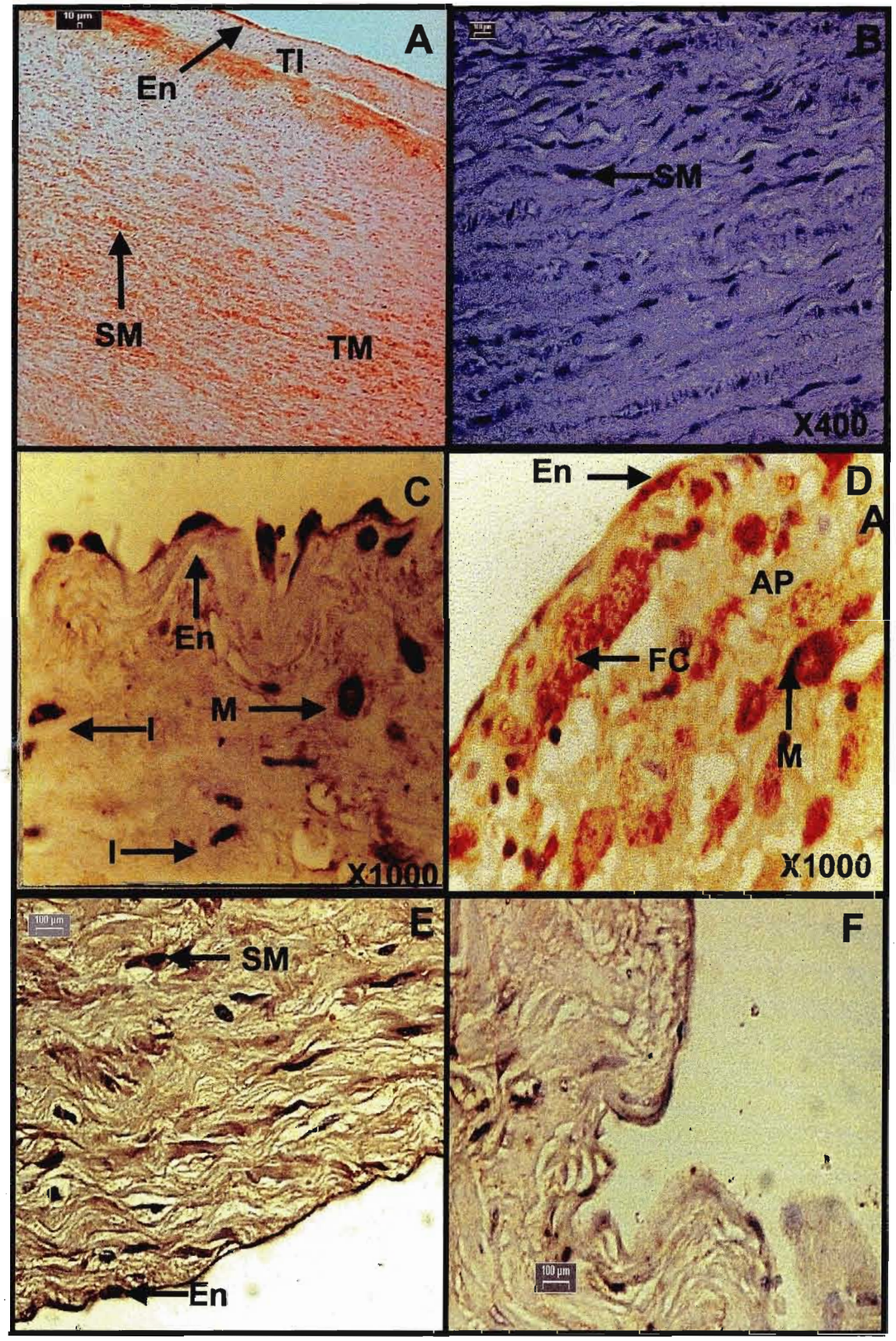


Figure 3.4 Immunolocalisation of PK in human blood vessels

Aorta: Positive labeling for PK was evident in the endothelial cells **En** of the tunica intima **TI** and smooth muscle cells **SM** of the tunica media **TM** (A). No immunoreactive PK was visualised in the smooth muscle cells **SM** of the negative control (B) following preabsorption of the primary antibody with an excess of antigen.

Renal artery (**X1000**): The endothelial cells **En**, macrophages **M** and inflammatory cells **I** showed immunolabeled PK (C).

Coronary artery (**X1000**): Intense PK immunoreactivity was visualised in the endothelial cells **En** as well as in the foam cells **FC** and macrophages **M** in the atheromatous plaque **AP** (D).

Inferior vena cava: Immunoreactive PK was visualised in both endothelial **En** and smooth muscle cells **SM** (E).

Renal vein (**X400**): No immunoreactive PK was detected in either the endothelial or smooth muscle cells (F).

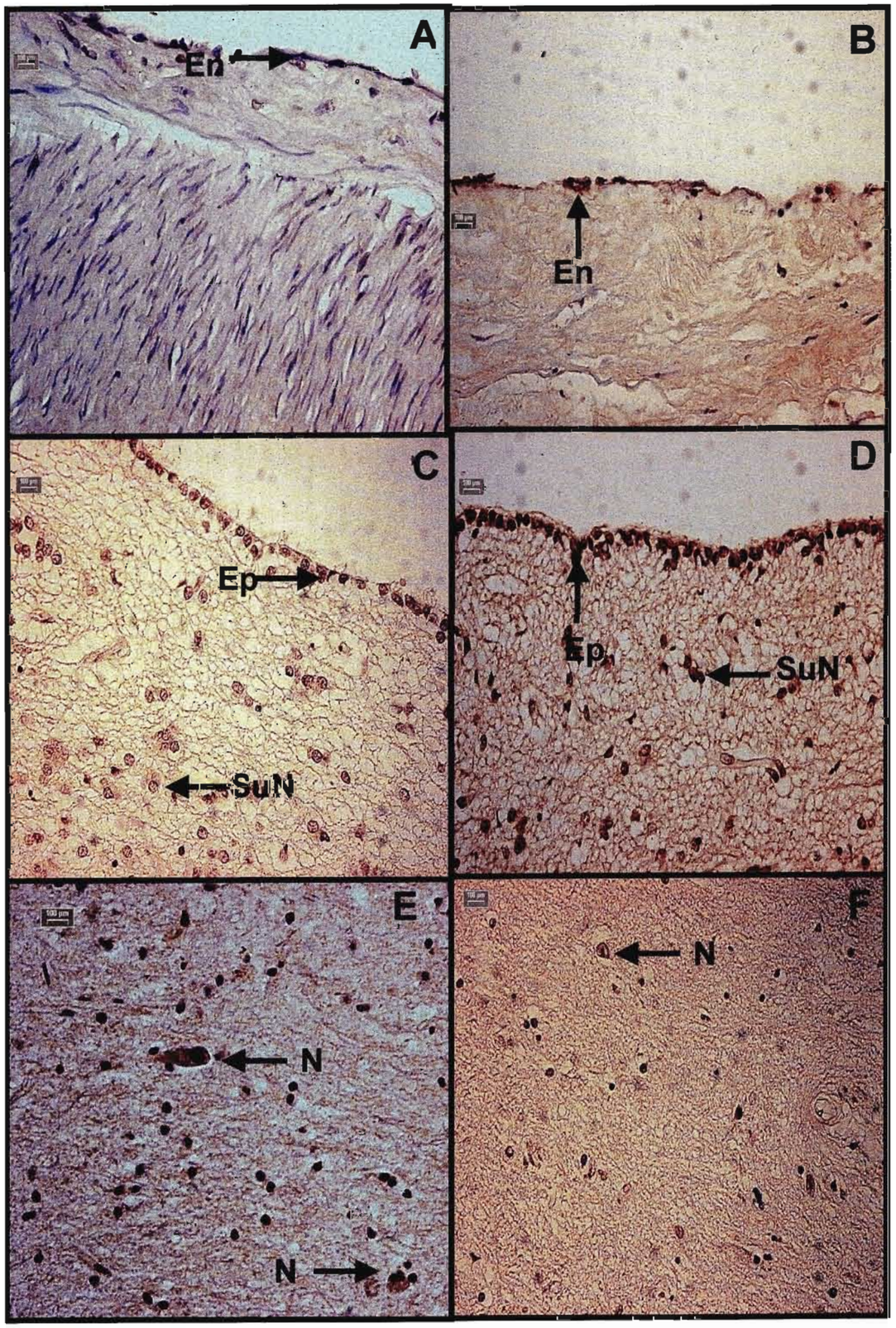


Figure 3.5 Immunolocalisation of PK in cerebral blood vessels and the hypothalamus

Basilar (A) and **carotid** (B) arteries: Immunoreactive PK was visualised in endothelial cells **En** of both cerebral arteries.

Hypothalamus: The H&E stained section (C) demonstrated ependymal cells **Ep** lining the third cerebral ventricle, neurons of the supraoptic nuclei **SuN**, and verified the absence of disease and preservation of histology. PK immunoreactivity was evident in neurons of the supraoptic nuclei **SuN** and in ependymal cells **Ep** (D) as well as in neurons **N** within the grey matter (E). Immunolabeling for PK was absent in neurons **N** of the negative control (F) where the primary antibody was preabsorbed with an excess of antigen.

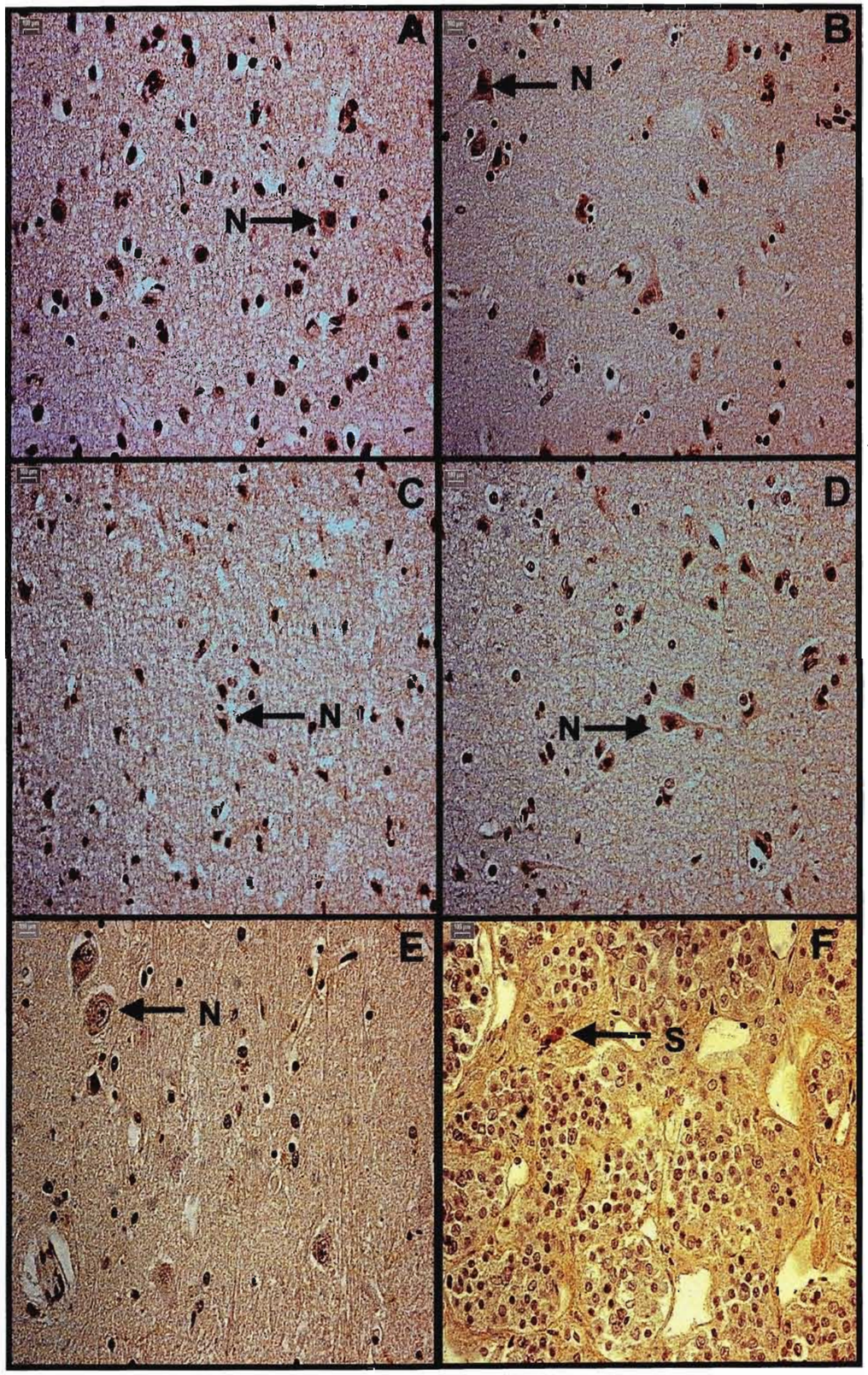


Figure 3.6 Immunolocalisation of PK in regions of the brain

Frontal lobe (A), **occipital lobe** (B), **pre-central** (C) and **post-central gyri** (D): Immunoreactive PK was visualised in cell bodies of the neurons **N** in the grey matter in all of these regions.

Thalamus: In the cytoplasm of the cell bodies in some neurons **N** within the thalamus, immunolabeling for PK was evident (E).

Pituitary gland: Immunoreactive PK was demonstrated in a few secretory cells **S** in the anterior lobe (F).

visualised in the posterior pituitary gland. In the spinal cord, labeling for PK was observed in some neurons throughout the grey matter as well as in ependymal cells lining the central canal (Figure 3.7 A). Immunoreactive PK was observed in certain neurons of the medulla (Figure 3.7 B), pons (Figure 3.7 C) and midbrain (Figure 3.7 D). Fibre tracts in the medulla, pons and hippocampus (Figure 3.7 E) also demonstrated the presence of immunoreactive PK. Generally, immunolabeling of PK was more intense in the grey matter than in the white matter, the only exceptions being the medulla, pons and hippocampus where the myelinated nerve fibre tracts of the white matter showed more intense immunolabeling. No immunoreactive PK was visualised in the capillaries, epithelial cells or stromal tissue of the choroid plexus (Figure 3.7 F) or in neurons in the outer molecular layer, inner granular layer or Purkinje cells of the cerebellum.

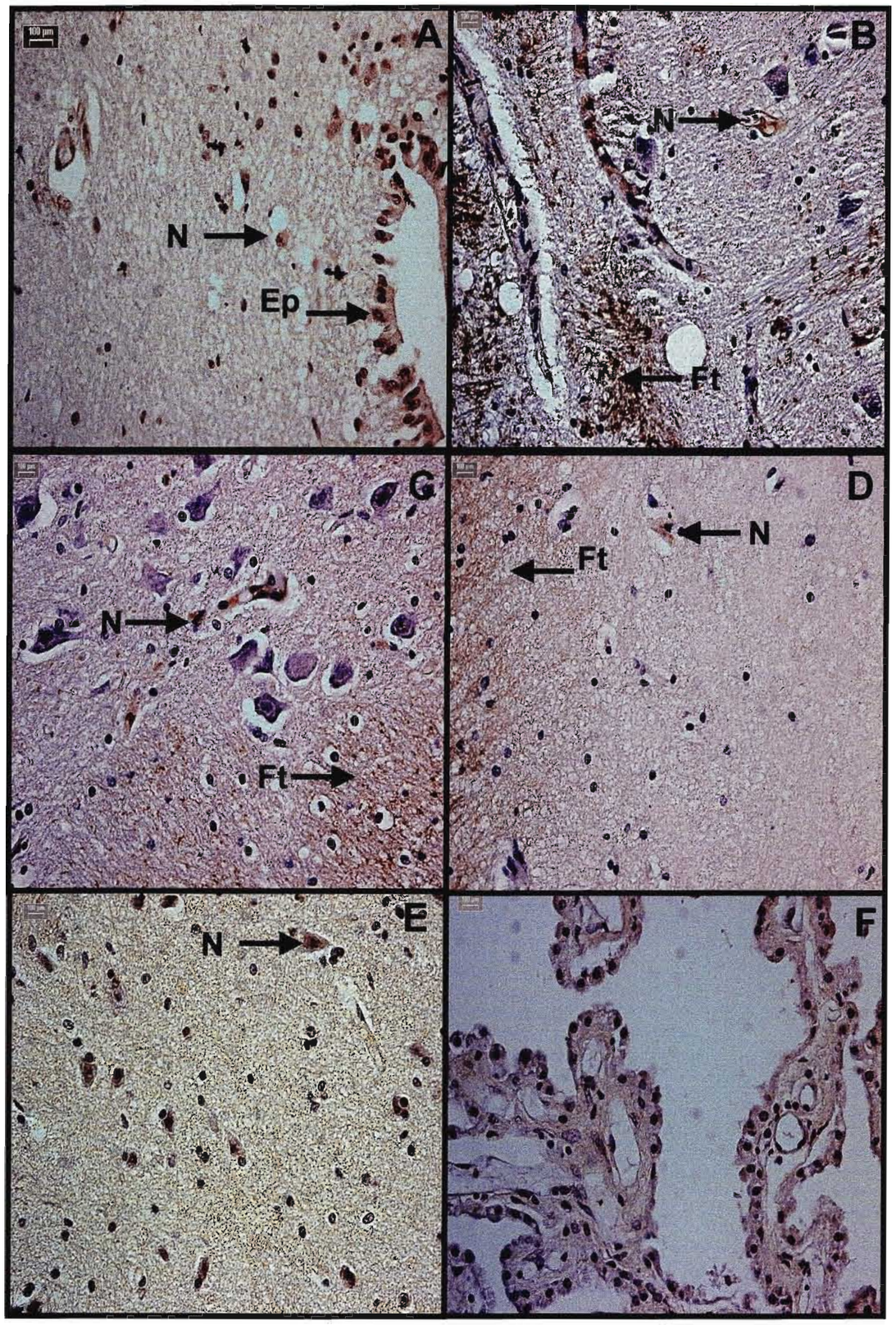


Figure 3.7 Immunolocalisation of PK in the spinal cord and various brain regions

Spinal cord: Immunoreactive PK was evident in the cell bodies of the neurons **N** in the grey matter as well as in the ependymal cells **Ep** lining the central canal (A).

Medulla (B), **pons** (C) and **hippocampus** (D): The presence of immunolabeled PK in the cell bodies of some neurons **N** as well as in the myelinated fibre tracts **Ft** of the white matter was demonstrated.

Midbrain: Here immunoreactive PK was visualised in cell bodies of the neurons **N** scattered throughout the midbrain (E). The nuclear localisation and identity of the neurons remain to be verified.

Choroid plexus: No immunoreactive PK was visualised in the capillaries, epithelial cells or stromal tissue (F).

3.4 Measurement of PK by enzyme linked immunosorbent assay (ELISA)

A standard curve measuring varying amounts of PK/PPK in a competitive ELISA was plotted (Figure 3.8). The detection limits of the curve were 0.008 - 2 μg/ml for the PK standards. For each ELISA experiment a sample of pooled human plasma was used as controls. The plasma was extracted from whole blood of a normal volunteer and stored in aliquots at -20^{0}C. A serial dilution of these plasma standards (1:320 to 1:20 480 in PBS/BSA) was added in triplicate to the wells as a positive method control for each ELISA experiment, and to determine the inter-assay (0.998) and intra-assay (0.967) coefficients (Figure 3.9).

The highest amounts of immunoreactive PK were measured in pancreas (12.94 ± 2.04 μg/ml - diluted 1:7 in PBS/BSA in order to be detected within the limits of the standard curve) and oesophagus (1.84 ± 1.45 μg/ml) (Figure 3.10).

In the various blood vessels examined, the aorta (0.44 ± 0.14 μg/ml), coronary (0.21 ± 0.07 μg/ml) and carotid (0.21 ± 0.08 μg/ml) arteries had the highest PK concentrations (Figure 3.10). In the brain sections, the pons (1.67 ± 1.46 μg/ml), thalamus (0.67 ± 0.34 μg/ml), pre-central gyrus (0.38 ± 0.24 μg/ml), frontal lobe (0.36 ± 0.16 μg/ml) and pituitary gland (0.36 ± 0.13 μg/ml) had the highest PK concentrations relative to the other brain regions (Figure 3.10). Tissues with the least amount of immunoreactive PK (less than 0.10 μg/ml) were the basilar artery and the spinal cord (Figure 3.10).

Figure 3.8 STANDARD CURVE FOR PK

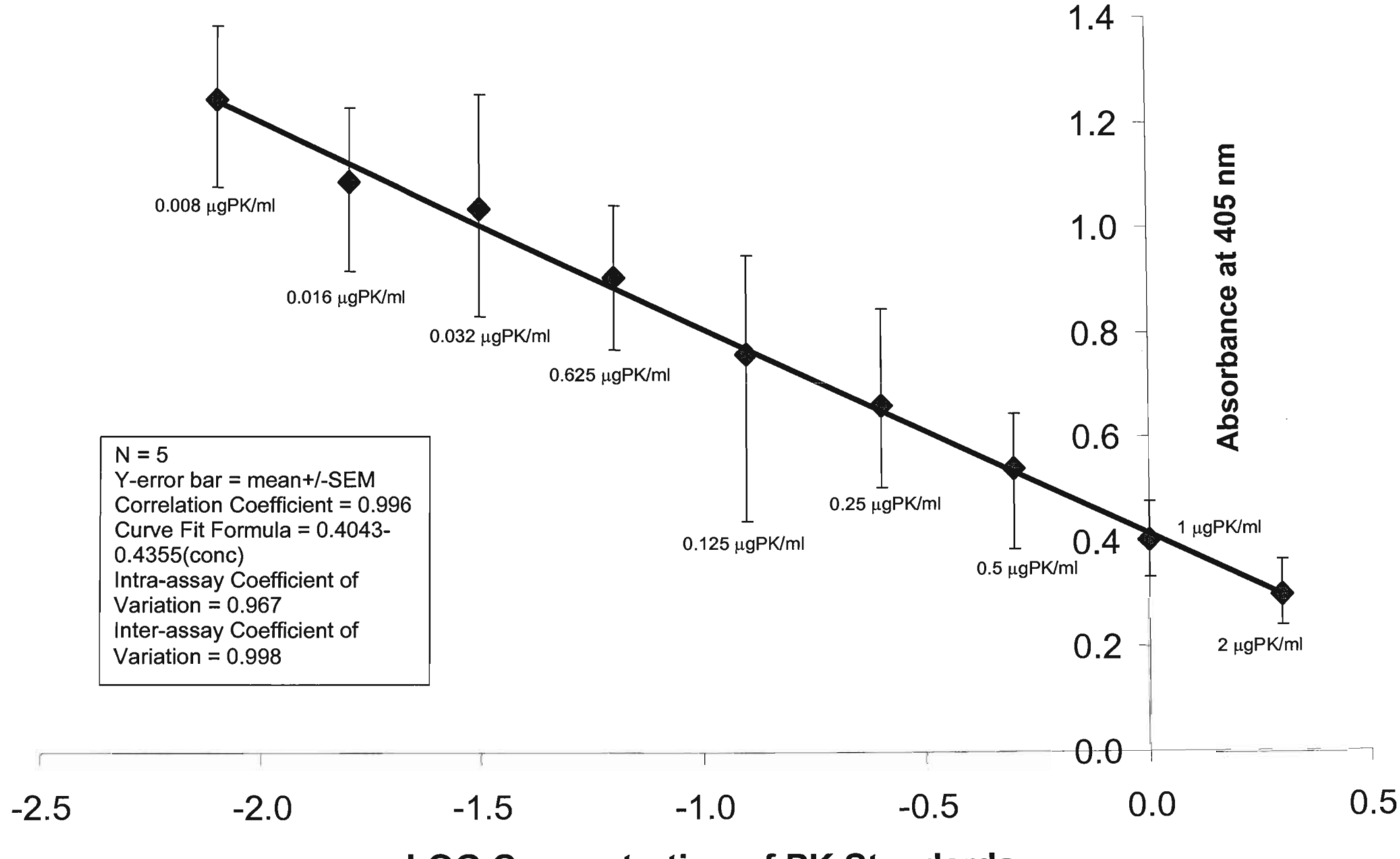


Figure 3.9 Plasma Standard Curve

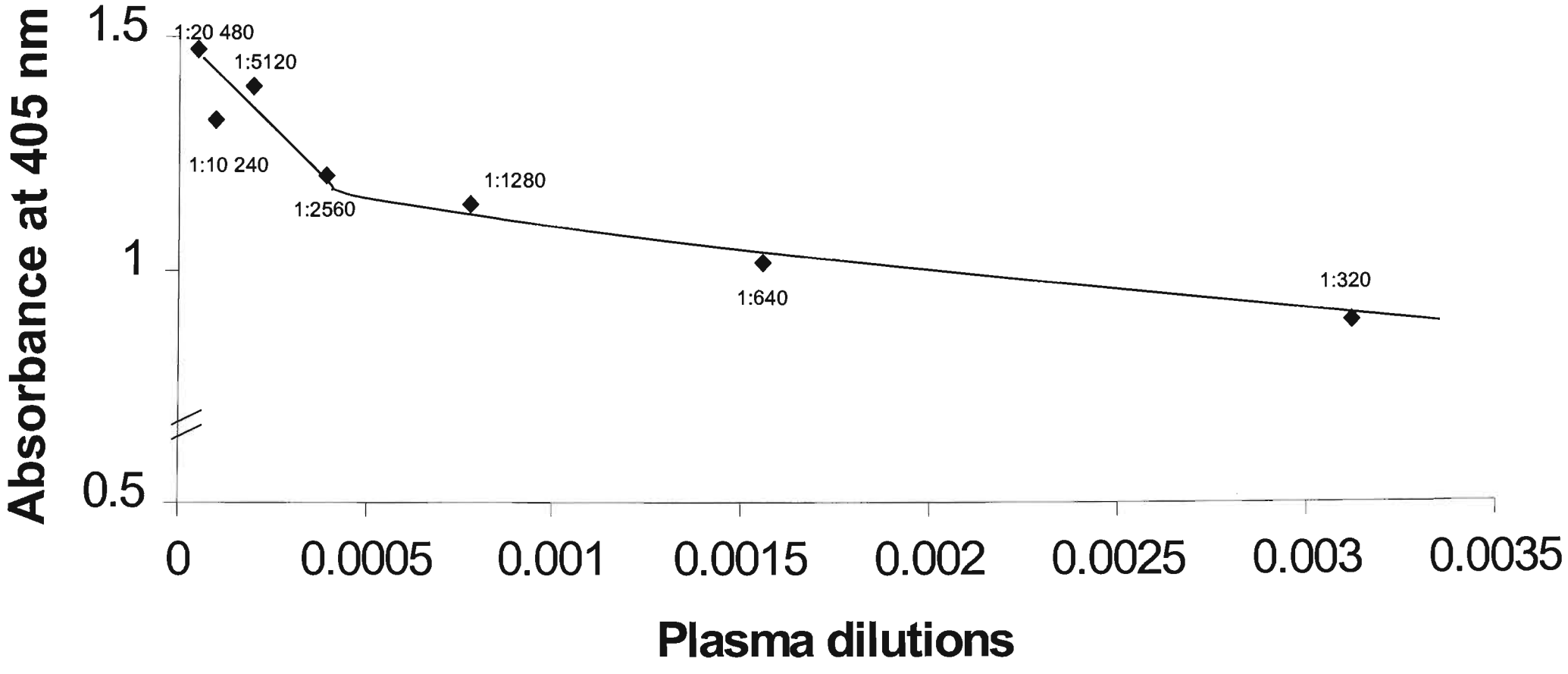


Figure 3.10 Measurement of PK by ELISA in human tissue

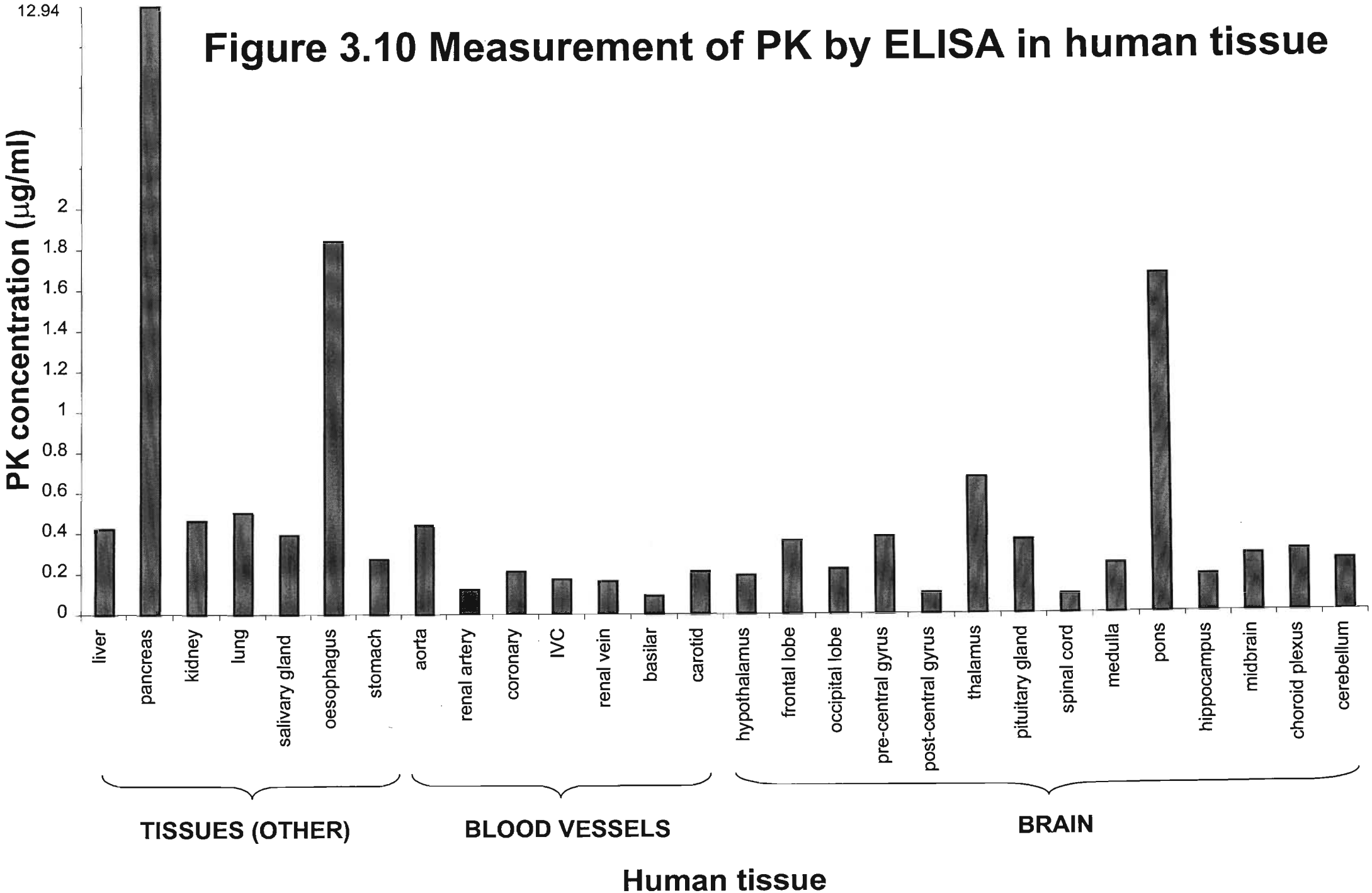


Table 3.2 PK immunolocalisation in human tissue

Tissue type	PK Immunolocalisation			Mean ELISA PK concentration (μg/ml) ± SEM
Liver	Hepatocytes			0.42 ± 0.08
Pancreas	Islets of Langerhans	Acini		12.94 ± 2.04
Kidney	Cells in distal convoluted tubules			0.46 ± 0.09
Lung	Epithelial cells in bronchioles and alveoli			0.50 ± 0.19
Salivary gland	Acini			0.39 ± 0.15
Oesophagus	Squamous and stratified squamous epithelial cells			1.84 ± 1.45
Stomach	Glandular cells			0.27 ± 0.10
BLOOD VESSEL	**Endothelial cells**	**Smooth muscle cells**		
Aorta	+	+		0.44 ± 0.14
Renal artery	+	+		0.12 ± 0.06
Coronary artery	+	+		0.21 ± 0.07
Inferior vena cava	+	+		0.17 ± 0.05
Renal vein	-	-		0.16 ± 0.04
Basilar artery	+	+		0.09 ± 0.07
Carotid artery	+	+		0.21 ± 0.08
BRAIN AND SPINAL CORD	**Neurons**	**Ependymal cells**	**Fibre tracts**	
Hypothalamus	+	+	-	0.19 ± 0.07
Frontal lobe	+	-	-	0.36 ± 0.16
Occipital lobe	+	-	-	0.22 ± 0.07
Pre-central gyrus	+	-	-	0.38 ± 0.24
Post-central gyrus	+	-	-	0.10 ± 0.03
Thalamus	+	-	-	0.67 ± 0.34
Pituitary gland*	-	-	-	0.36 ± 0.13
Spinal cord	+	+	-	0.09 ± 0.04
Medulla	+	-	+	0.24 ± 0.1
Pons	+	-	+	1.67 ± 1.46
Hippocampus	+	-	+	0.18 ± 0.1
Midbrain	+	-	-	0.28 ± 0.18
Choroid plexus	-	-	-	0.30 ± 0.15
Cerebellum	-	-	-	0.25 ± 0.13

+ = immunolabeling for PK - = no immunolabeling for PK

*immunolabeling for PK was visualised in the secretory cells

Table 3.2 Detection of PK in human tissues

This table demonstrates the specific localisation (by immunocytochemistry) of plasma kallikrein as well as its concentration (given in μg/ml as determined by ELISA) in various human tissues, vasculature and brain regions. In the control tissues, pancreas and liver, PK was present in the islets of Langerhans cells and some acini in the pancreas as well as in hepatocytes. In kidney, the distal tubules showed immunoreactive PK, whereas the epithelial cells in the bronchioles and alveoli of the lung also labeled positively for PK. In gastrointestinal tract tissues, acinar cells in the lobules of the salivary gland, squamous and stratified squamous epithelial cells of the oesophagus, and some glandular cells of the stomach demonstrated the presence of immunoreactive PK.

The endothelial cells and smooth muscle cells of all the blood vessels examined were immunolabeled with PK, except for the renal vein where no immunoreactive PK was detected in either cell type.

In the spinal cord and brain, the cell bodies of some neurons in all of the various brain regions examined were immunolabeled for PK, apart from the choroid plexus and cerebellum. The presence of immunoreactive PK was also evident in ependymal cells lining the third ventricle and central canal of the hypothalamus and spinal cord respectively. Fibre tracts of the pons, hippocampus and medulla as well as secretory cells of the pituitary gland also demonstrated immunoreactive PK.

CHAPTER FOUR

4.1 Western blot analysis for rabbit anti-human PK antibody

Lane 8 for PK on the immunoblotted membrane (Figure 3.1 B) showed three bands, and not a sharply defined distinct single band of 88 kDa. This is probably due to the cleavage of the PPK molecule during the denaturing step at 42^0C for 1h. Hence, we observe a band of PPK at 88 kDa, and two others representing the heavy and light chains of PK at 55 kDa and 33 kDa respectively. The purpose of characterising the antibody is to demonstrate its specific interaction with the antigen and no non-specific cross-reactivity with other control proteins. Although three bands are observed in lane 8, this does not compromise the specificity of the antibody since the membrane clearly shows that the only interaction occurring with the rabbit anti-human PK IgG is with the PK protein exclusively. Therefore we can conclude that our anti-human PK IgG raised in rabbit is specific for the PK protein.

4.2 PK in control tissue (liver and pancreas)

PK has been immunolocalised in human hepatocytes (Henderson *et al.*, 1992) and is long believed to be synthesised in these cells (Mandle *et al.*, 1976). The liver is the main organ involved in PK clearance from circulation *in vivo* (Borges and Kouyoumdjian, 1992). Ciechanowicz *et al.* (1993) have shown the presence of PPK mRNA in human and rat liver using RT-PCR. The findings of these studies would support the diffuse immunolabeling for PK visualised in hepatocytes and the PK concentration of 0.42 ± 0.08 μg/ml measured by competitive ELISA. It appears that PK, after synthesis in hepatocytes, is released into the circulatory system upon demand. Although the liver is regarded as the major source of circulating PK similar to other

plasma proteins such as albumin, we speculate that PK may also be involved in regulating some aspects of hepatic function such as the processing of nutrients and detoxifying pathogens.

The pancreas is a complex gland characterised by the richly vascularised mucous and serous acini (that contain secretory zymogenic granules), connective tissue and ducts constituting the exocrine portion; and the islets of Langerhans which are endocrine in nature (Junqueira *et al.*, 1995). The latter are clusters of epithelial cells (Craigmyle, 1986) which produce and secrete the blood-glucose regulatory hormones insulin (β-cells), glucagon (α-cells) and somatostatin (δ-cells) directly into the blood via fenestrated capillaries (Seeley *et al.*, 1996). Although whole pancreas extracts demonstrated high kallikrein activity decades ago (Kraut *et al.*, 1930), and only recently human pancreas extracts were shown to have a high copy number of PPK mRNA by real time RT-PCR (personal communication: Mr Peter Neth), the exact cellular localisation had not yet been described. We show, for the first time, distinctive, specific immunolabeling for PK in cells of the islets of Langerhans and in some acini. The ELISA results showed that pancreatic extracts had the highest PK concentration (12.94 ± 2.04 μg/ml) amongst all the tissues examined. Frey et al. (1932) first reported the hypoglycaemic effect of kallikrein where a decrease in circulating glucose was noted in diabetic patients. Studies have shown that infused BK doubled muscle glucose uptake in patients with postoperative stress syndrome (reviewed in Bhoola *et al.*, 1992). Since we have not established in which cell type of the islets of Langerhans PK is expressed, an extension of this study using specific stains

for these cells is required. However, we postulate that PK in these cells may have a regulatory role in the processing and for secretion of insulin and thereby control glucose uptake and utilisation in muscle cells, which may be of clinical significance in conditions such as diabetes mellitus. B2 receptor mRNA has been demonstrated in whole pancreas extracts by competitive PCR using human cDNA (Hess *et al.*, 1992). Hence, PK may release BK, which could act at B2 receptor level to regulate pancreatic function. Therefore an endogenous PKKS may be implicated, possibly in conjunction with other physiological systems, to maintain pancreatic homeostasis. We propose that PK may be involved in the processing of insulin precursors, and that by the release of BK, may subsequently result in B2-mediated insulin release. Lembeck and Griesbacher (1996) have postulated that kinins may have a modulatory role in the production of pancreatic juice and in microcirculatory regulation of the pancreas.

4.3 PK in kidney

The nephron, the functional and structural unit of the kidney (comprising of glomeruli, proximal tubules, loops of Henle, distal tubules and collecting ducts), is involved in water and electrolyte regulation. Studies employing real-time RT-PCR showed that human whole kidney extracts had high copy numbers of PPK mRNA (personal communication: Mr Peter Neth). We have demonstrated the cellular localisation of PK in the distal convoluted tubules by ICC. Also, a PK concentration of 0.46 ± 0.09 μg/ml was determined by ELISA in whole kidney extracts. Other components of the KKS that have been demonstrated in the kidney include TK and kinin receptors. The localisation of

TK in distal tubules and the connecting duct cells is well established (Schacter, 1980; Naicker *et al.*, 1999). Subsequent studies show that B2 receptor mRNA had the highest expression in the kidney compared to various other tissues (Hess *et al.*, 1992). In normal human kidney, B2 receptor was immunolocalised in the nephron using specific antibodies directed against the B2 receptor peptide and confocal microscopy (Naicker *et al.*, 1999). In addition, B1 receptors were immunolabeled in glomeruli, distal tubules and collecting ducts of patients with renal diseases such as acute tubular necrosis, nephritis and hypertensive nephrosclerosis, suggesting upregulation of these receptors in renal parenchymal disease (Naicker *et al.*, 1999). Wang *et al.* (1996) localised B1 receptor mRNA in epithelial cells of the parietal layer of Bowman's capsule and in loops of Henle of normal kidney, as well as in tumour cells of renal cell carcinoma patients by *in situ* hybridisation using a digoxigenin-labeled probe. We postulate that PK, visualised in the distal tubules elicits physiological effects via the release of BK, which would be mediated by B2 receptors found in the nephron. These effects may include a role in influencing urine concentration and volume as Margolius (1996) postulated, as well as in ion and water homeostasis. Intrarenal kinins modulate renal excretory function predominantly by effects on renal vasculature (Margolius *et al.*, 1996). Components of the KKS demonstrated in the kidney suggests that it acts in a paracrine fashion to regulate glomerular function, renal haemodynamics as well as ion and water excretion (Margolius *et al.*, 1996; Sharma *et al.*, 1996).

4.4 PK in lung

Alveoli form the mass of lung parenchyma (Di Fiore, 1974), open into alveolar ducts, and are the main sites of gaseous exchange between inspired air and blood (Junqueira *et al.*, 1995). Recently Hermann *et al.* (1999) reported the expression of PPK mRNA in whole extracts of human lung using RT-PCR. We immunolocalised PK in epithelial cells of the bronchioles and alveoli and demonstrated PK in whole tissue extracts of the lung (0.5 ± 0.19 μg/ml) by ELISA. Since the PPK gene is also transcribed in epithelial cell lines originating from different organs (Hermann *et al.*, 1999), the expression of PPK mRNA may take place in tissue-specific epithelial cells throughout an organism. This would provide a feasible explanation for the presence of immunoreactive PK in the epithelial cells of the lung. In bronchoalveolar lavage fluid of patients with asthma, kininogenase activity similar to TK was detected by cleavage of a radiolabeled HK (^{125}I-HK) (Christiansen *et al.*, 1987). High levels of kinins have also been detected in bronchoalveolar lavage fluid of patients with acute pneumonia and chronic bronchitis by specific radioimmunoassay, as well as an increase in PPK/PK concentration measured by sandwich ELISA (Zhang *et al.*, 1997). We propose that the PPK/PK measured in these lavages was due to the presence of PK in the epithelial cells of the bronchioles and alveoli as demonstrated in our study. TK has been immunolocalised in serous cell granules of the seromucous glands of human extrapulmonary and intrapulmonary bronchi (Poblete *et al.*, 1993). The presence of PK, TK and B2 mRNA (Hess *et al.*, 1992) in the human lung suggests that the KKS may be actively involved lung pathophysiology. During asthmatic attacks, concentrations of free circulating kinins have been shown

to increase from 0.1 ng/ml to 8 ng/ml which is significant since kinins are highly active bronchoconstrictors (Werle, 1972) and potent inducers of edema in human asthma (Christiansen *et al.*, 1987). PK visualised in epithelial cells could release BK that may act on smooth muscle cells causing bronchoconstriction thereby eliciting an asthmatic response. Werle (1972) suggested that kinins may contribute to the development and aggravation of emphysema. Immunofluorescence for B1 receptors in transbronchial tissue of patients with sarcoidosis and interstitial lung disease has been visualised in areas of fibrosis, in thickened interstitium, as well as in basement membranes of capillaries and alveoli (Nadar *et al.*, 1996). The presence of B1 receptors in fibrosis is perpetuated by kinins, suggesting kinin involvement in the pathogenesis of interstitial lung disease (Nadar *et al.*, 1996). Immunolabeled PK, as demonstrated in the epithelial cells of the alveoli, could generate BK, thereby potentiating lung inflammation via B1 receptor mediation.

Antagonists, like HOE-140 (icatibant) have shown success in down-regulating the pathophysiological effects of kinins in clinical trials. In patients with allergic rhinitis induced by house-dust mite, this B2 receptor antagonist, administered by intranasal aerosol, significantly reduced nasal blockage induced by antigen challenge (Austin *et al.*, 1994). Other studies have shown that nebulized icatibant results in a dose-dependent improvement in objective pulmonary function tests in adult patients with chronic asthma, suggesting an anti-inflammatory effect (Akbary *et al.*, 1996).

4.5 PK in gastro-intestinal tract tissue

4.5.1 Salivary gland

Salivary glands have mucous and serous acini, which secrete saliva (containing mucous, enzymes, antibodies and inorganic ions) via secretory ducts into the mouth (Di Fiore, 1974). Although immunolabeling for TK has been demonstrated in the apical region of duct cells (Schacter, 1980; Naidoo *et al.*, 1999b), PK has not yet been localised in the salivary gland. In this study we have shown PK immunolocalisation in salivary gland acini. We have not established whether these acini are serous or mucous in nature. A PK concentration of 0.39 ± 0.15 μg/ml was measured by competitive ELISA in salivary gland extracts. Both TK (Bhoola *et al.*, 1992) and kinins (Margolius, 1996) are known to occur in saliva. We speculate that PK could release BK in saliva, or alternatively, it may be involved in the processing of the proteolytic enzymes secreted in saliva.

4.5.2 Oesophagus

The mucosa, submucosa, muscularis externa and adventitia constitute the wall of the oesophagus; with the submucosa (broad connective tissue layer) characterised by adipose cells, blood vessels and glands that secrete mucous (Junqueira *et al.*, 1995). Application of a BK patch to the epicardia of the left ventricle produced transient relaxation of the lower oesophageal sphincter in dogs (Caldwell *et al.*, 1994). Other studies have shown that BK stimulation of motor nerves associated with the muscularis mucosa results in concentration-dependent contraction of the oesophageal muscularis mucosa in rabbits (Percy *et al.*, 1997). Hence it appears that BK has a regulatory role in

oesophageal function. Since smooth muscle cells transport food down the oesophageal tract by peristalsis, the presence of immunoreactive PK in squamous cells suggests that BK may be released which then acts on the smooth muscle cells resulting in peristaltic contractions. A PK concentration of 1.84 ± 1.45 μg/ml was measured by ELISA in oesophageal extracts. More recently TK (in giant tumour and mast cells of squamous cell carcinoma) as well as both B1 and B2 receptors (in giant cells) were immunolocalised in human oesophageal carcinoma, suggesting an influence of the KKS in tumorigenesis of the oesophagus (Dlamini *et al.*, 1999).

4.5.3 Stomach

The stomach (consisting of the cardia, fundus, body and the pylorus), is characterised by the mucosa, submucosa, muscularis externa, serosa (Junqueira *et al.*, 1995), and gastric glands in the lamina propria which contain the chief and parietal cells (Di Fiore, 1974). Recently TK was immunolocalised in parietal cells of the pylorus, with elevated levels measured by ELISA in gastric lavage fluid of patients with gastritis (Naidoo *et al.*, 1999a). Immunohistochemical studies have demonstrated the presence of B2 receptors in parietal cells of normal stomach, and B1 receptors in neck glands of regenerating stem cells in patients with gastritis (Bhoola *et al.*, 1997). The presence of PK in glandular cells, which secrete digestive enzymes and gastric acid, suggests that the PKKS may be important in processing digestive enzymes. Furthermore, a PK concentration of 0.27 ± 0.10 μg/ml was determined by ELISA in stomach extracts. This relatively low PK concentration (compared to the control tissues, kidney, lung and other gastro-

intestinal tract tissues) may be due to PK degradation by autolysis in the stomach. We speculate that the PKKS may be involved in maintaining gastric homeostasis, and has an aggravating role in gastritis via the generation of BK as postulated by Bhoola *et al.* (1997) and Naidoo *et al.* (1999).

4.6 PK in inflammation and disease

Neutrophils, isolated using cell fractionation techniques and then incubated with immune complexes, granules and lysosomes, released an enzyme capable of liberating a kinin-like substance from kininogen (Bhoola *et al.*, 1992). HK has been identified in lysates of washed neutrophils by competitive ELISA (Gustafson *et al.*, 1989). Immunohistochemical studies demonstrating TK in the human kidney, showed that neutrophils infiltrating a region of infection within renal parenchyma had an intense granular pattern of immunostaining (Bhoola *et al.*, 1992). Blood smears from patients with chronic myeloid leukemia have also shown intense immunostaining for TK in neutrophil precursor cells, notably in myelocytes and metamyelocytes (Figueroa and Bhoola, 1989).

In this study, intensely labeled PK was visualised in inflammatory cells of the liver, lung and stomach. Inflammatory cells were expected in the liver since it is involved in detoxification. Anthracotic deposits evident in some lung sections would be responsible for the occurrence of scattered inflammatory cells. Since gastric contents are acidic (due to the secretion of HCl) and involved in chemical digestion, inflammatory cells would be present as part of the homeostatic interface. It appears that the PKKS, through the release of BK, contributes to the inflammatory response. Vasodilation (which increases

blood flow to the area of pathology), nociception (which would be experienced during inflammation), increased vascular permeability (which would allow the easy transport of ions and water across the cell membrane ultimately resulting in edema), and increased cell proliferation (for example macrophages which occur during inflammation), are all kinin-produced effects which contribute to the inflammatory response. Evidence that activation of circulating PK may contribute to the aetiology of asthma, blood transfusion reactions, pancreatitis, pulmonary edema and shock induced by haemorrhage, burns and endotoxins was provided by Webster (1968).

4.7 PK in blood vessels

Arteries and veins, which are involved in blood transport, nutrient distribution and some aspects of temperature regulation, have a common basic structure comprising of the tunica intima, a single layer of flattened epithelial cells (endothelium), an intermediate smooth muscular layer constituting the tunica media, and the outer supporting tissue layer known as the tunica adventitia (Young and Heath, 2000).

TK, as well as B1 and B2 receptors have been immunolocalised in endothelial and smooth muscle cells of various human blood vessels using specific antibodies for TK, B1 and B2 receptors respectively (Raidoo *et al.*, 1997). In this study we have shown immunolabeling for PK in the endothelial and smooth muscle cells of the aorta; in endothelial cells of the renal, coronary, basilar and carotid arteries; and in both the endothelial and smooth muscle cells of the inferior vena cava. In addition, the highest PK concentrations measured by ELISA were 0.44 ± 0.14 μg/ml in the aorta, 0.21 ± 0.07 μg/ml in

the coronary artery and 0.21 ± 0.08 μg/ml in the carotid artery; with lower levels detected in the renal (0.12 ± 0.06 μg/ml) and basilar (0.09 ± 0.07 μg/ml) arteries. Immunolabeling for PK in medium to small muscular arteries, viz. the coronary, carotid and basilar, suggests a functional role for kinins in the maintenance and regulation of vascular tone, the loss of which may contribute to the pathogenesis of hypertension. Kinins have been shown to promote arterial dilation, constriction of veins, and capillary permeability thereby enhancing the extravasation of plasma proteins and fluid. In severe hypotension, these actions increase the severity of shock. Sharma *et al.* (1996) postulated that the KKS is involved in cardiovascular protection and in regulating blood pressure, therefore reduced formation of BK due to genetic effects/environmental alterations, may enhance hypertension.

Local blood flow in tissues is regulated by changes in the diameter of arterioles produced by relaxation and constriction of vascular smooth muscle. BK released by the action of PK, in the endothelial and smooth muscle cells of the various blood vessels examined, may play a role in controlling local blood flow via its vasodilatory action. We speculate that PK localised in the endothelial cells may release BK, which subsequently acts on smooth muscle cells resulting in smooth muscle relaxation and increased blood flow.

4.8 PK in atheromatous disease

With advancing age, the accumulation of cholesterol and associated atherosclerosis cause the arterial system to lose its elasticity thereby increasing peripheral resistance and thus arterial blood pressure (Young and Heath, 2000). An increase in immunoreactive TK has been demonstrated in

endothelial cells and macrophages within atheromatous plaques of various arteries (Raidoo *et al.*, 1997). Furthermore, intense labeling for B1 and B2 receptors in endothelial cells, macrophages, inflammatory cells and proliferating fibroblasts suggests that these receptors are upregulated within atheromatous plaques (Raidoo *et al.*, 1997). The intense immunolabeling for PK that we observed in macrophages and foam cells of arteries afflicted with atheromatous disease viz. the aorta, coronary, carotid, renal and basilar arteries, suggests that its expression may be increased in atherosclerosis resulting in the stimulation of BK release to overcome the vasoconstrictor effect of the plaque as Raidoo *et al.* (1997) hypothesised. This may possibly be mediated by both the B1 and B2 receptors localised within the atheromatous plaques.

4.9 PK in the brain

The brain is a large complex organ composed of neurons (the functional units of the nervous system made up of cell bodies, dendrites and axons) as well as supporting neuroglial cells (ependyma, oligodendrocytes, microglia and astrocytes). Groups of cell bodies and dendrites in the cerebral cortex, brain stem, cerebellum, central region of the spinal cord and basal ganglia form grey matter, whereas bundles of myelinated axons constitute the white matter forming fibre tracts for the conduction of nerve signals (Seeley *et al.*, 1996). Each region of the brain has various specialised functions, for example the hypothalamus has control centres including one for the regulation of body temperature; the pituitary gland synthesises and secretes numerous hormones; and other regions viz. the spinal cord, thalamus and hypothalamus

work together in the modulation of pain as well as subserving other functions (Seeley *et al.*, 1996).

High copy numbers of PPK mRNA in total human brain extracts, demonstrated by real time RT-PCR (personal communication: Mr Peter Neth) identified a potential study to define where exactly in the brain PK is regionalised. In this study, we immunolocalised PK in some neurons of the spinal cord and in different brain regions viz. hypothalamus, cerebral cortex, thalamus, brain stem and hippocampus. PK concentrations measured in brain extracts by ELISA were 1.67 ± 1.46 μg/ml in the pons, 0.67 ± 0.34 μg/ml in the thalamus, 0.38 ± 0.24 μg/ml in the pre-central gyrus and 0.36 ± 0.16 μg/ml in the frontal lobe. The absence of immunolabeled PK in the choroid plexus and cerebellum is not fully understood. It is not always possible to obtain conclusive correlation using different techniques. The ELISA method is more sensitive than the ICC method since it greatly enhances detection by the use of specific conjugates such as horse radish peroxidase. In addition, the severe fixation and labeling procedures of these tissues for ICC could result in degradation of proteins, ultimately resulting in a lower detection signal.

The other main serine protease of the KKS, TK has previously been demonstrated in various regions of the rat brain by specific radioimmunoassay techniques using sheep anti-rat urinary kallikrein antiserum (Chao *et al.*, 1987). Subsequent studies immunolocalised TK in neurons of the hypothalamus, thalamus, cerebral grey matter, reticular areas of the brain stem, cells of the anterior pituitary and in epithelial cells of the choroid plexus

in human brain (Raidoo *et al.*, 1996). TK mRNA has been detected in the rat brain using a TK cDNA probe (Chao *et al.*, 1987) as well as in neurons of the human hypothalamus and thalamus, in ependymal cells lining the cerebral ventricles, and in interstitial tissue cells of the choroid plexus by *in situ* hybridisation (Raidoo, 1999). The presence of immunoreactive PK in neurons of the various brain regions and spinal cord, as well as in ependymal cells, suggests that PK, via the generation of BK, may be implicated in neurophysiological processes. Further, we speculate that immunolabeling in the myelinated fibre tracts of the pons, medulla and hippocampus may be due to PK reacting with axonal proteins, the actual function of which remains to be elucidated.

B2 receptors are known to occur in animal neural tissue (Steranka *et al.*, 1988), primary brain cultures (Robert and Gulick, 1989), neuronal cell lines (Snider and Richelson, 1984) and in neurons of the human cerebral cortex, hypothalamus, thalamus and brain stem (Raidoo and Bhoola, 1997). In addition, B2 receptor mRNA was found to be expressed in total human brain extracts (Hess *et al.*, 1992). Immunolabeling for B1 receptors have been observed in neurons of the human hypothalamus, thalamus and spinal cord, where it appears to modulate the cellular actions of kinins in neural tissue (Raidoo and Bhoola, 1997). The presence of PK and both B1 and B2 receptors in the hypothalamus, thalamus and spinal cord suggests that the PKKS may play a receptor-mediated role in pain modulation. However, this needs to be elucidated by further studies to demonstrate PK expression in specific areas involved in pain perception such as the thalamic somato-

sensory relay and intralaminar nuclei, and the dorsal horn of the spinal cord. The localisation of both PK and B2 receptors in the various regions of the cerebral cortex (viz. frontal lobe, occipital lobe, pre-central and post-central gyri) and brain stem (viz. medulla, pons and midbrain) indicates that the cellular actions of the PKKS may be mediated by B2 receptors in these areas.

TK processes neuronal growth factors that maintain the integrity of neurotransmitters and synaptic function (Bhoola *et al.*, 1992). Jones *et al.* (1992) demonstrated the presence of immunoreactive TK in prolactin-secreting cells of the normal anterior pituitary gland as well as in prolactin-secreting adenomas. Furthermore, it appears that TK may play an important role in pituitary physiology and could also be involved in the intracellular processing of prolactin prior to secretion (Jones *et al.*, 1992). Since the pituitary gland orchestrates the endocrine system via hormone synthesis and secretion, the presence of immunoreactive PK in these secretory cells suggests involvement in hormonal regulation probably by precursor activation. A further function may be the regulation of cell proliferation since epidermal and other growth factors are known to occur in the pituitary gland. PK concentration, determined by ELISA, was 0.36 ± 0.13 in pituitary gland extracts.

Recently kininogen mRNA was shown to be expressed in cultures of rat meningeal cells from the leptomeninges/choroid plexus which propose that they are a major source of kininogens in rat brain (Takano *et al.*, 1999). PK measured by ELISA in the choroid plexus (0.30 ± 0.15 μg/ml) may cleave HK

to release BK, which would subsequently be transported via CSF throughout the brain. We speculate that the PKKS may be endogenous in specific brain area/s, on stand-by, until the onset of cerebral inflammation or injury, upon which time it will be circulated upon demand to the affected brain areas.

4.10 Regulation of the PKKS in cerebral disease

Maier-Hauff *et al.* (1984) postulated that in pathological conditions such as cerebral contusion, focal ischaemia and tumours activation of the PKKS may occur resulting in damage to the blood brain barrier. A recent study in male patients showed that during the acute stage of schizophrenia, there is an associated increased activity of the PKKS, due to enhanced functional activity of the α1-proteinase inhibitor (Shcherbakova *et al.*, 1999). Kinins also appear to be implicated in Alzheimer's disease. Amyloid beta-(1-40) was found to stimulate cyclic GMP production via the release of kinins in endothelial cells *in vitro*; demonstrated by a 3-4 fold increase of kinins in the supernatants of these cells; in addition cyclic GMP production was inhibited by both icatibant as well as a novel selective PK inhibitor, Pefabloc PK (Wirth *et al.*, 1999).

Numerous possibilities exist for antagonists to inhibit the PKKS. Specific sites of inhibition could include Factor XII (by C1 esterase inhibitor or antithrombin III) since it activates PK; PK (by C1 esterase inhibitor, α_2-macroglobulin, antithrombin III or Pefabloc PK); and HK, since it is the preferred substrate for PK resulting in BK generation. The cellular actions of BK could be inhibited by B1 receptor antagonists such as B-9224 and by the potent B2 receptor antagonist, icatibant. Kininases that regulate the rate of formation and

destruction of kinins (Erdös, 1989; Erdös, 1990) could also be manipulated to allow for the control of kinin levels by selective enzyme inhibitors such as captopril for KII-ACE, phosphoramidon for KII-NEP, and puromycin, amastatin or bestatin for aminopeptidases (Bhoola *et al.*, 1992).

CHAPTER FIVE

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