VASOACTIVE PEPTIDES IN ACUTE RENAL ALLOGRAFT REJECTION AND RENAL DISEASES: THE ROLE OF ENDOTHELINS, ATRIAL NATRIURETIC PEPTIDE AND KININS

by

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AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted in any other form to any other 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 Experimental and Clinical Pharmacology, Nelson R Mandela School of Medicine, University of Natal, Durban, South Africa under the supervision of Professor K D Bhoola.

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PROFESSOR K. D. BHOOLA

DEDICATION

I dedicate this work to:

The sources of my inspiration, my mother, Mrs. Gonam Naicker and my late father,

Mr. Ramalingum Veerasamy Naicker

The various teachers who moulded my academic career

All my family for their encouragement and support

And God who makes all things possible

PUBLICATIONS ARISING FROM THIS DISSERTATION

1.1 REVIEW ARTICLES

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Bhoola KD, Ramsaroop R, Cassim B, Plendl, J, Dlamini, Z, Naicker S. Kallikrein and kinin expression in inflammation and cancer. Biological Chemistry, 2001; 382: 77-89

Naicker S. Endothelins in renal transplantation. Proceedings of the First International Nephrology Conference on the Internet, February 2000

1.2 PUBLICATIONS IN REFEREED JOURNALS

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ABBREVIATIONS

ACE, angiotensin converting enzyme

ADH, anti-diuretic hormone

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide

Anti-DIG AP, sheep anti-DIG IgG conjugated to alkaline phosphatase;

Anti-DIG FITC, sheep anti-DIG IgG conjugated to fluorescein isothiocyanate;

AII/ AT, angiotensin II

AVP, arginine vasopressin

B1, kinin B1 receptor; B2, kinin B2 receptor; BK, bradykinin;

BP, blood pressure

BSA, bovine serum albumin;

$[Ca^{2+}]$, calcium concentration

cAMP, cyclic adenine monophosphate

CNT, connecting tubule

CyA, cyclosporine A

DAB, diaminobenzidine;

des-Arg^{10}LBK, des-Arg^{10}kallidin; des-Arg^{9}BK, des-Arg^{9}bradykinin;

dH_2O, distilled water;

ECE, endothelin converting enzyme

ECFV, extracellular fluid volume

ED4, fourth extracellular domain of the B2 bradykinin receptor;

EDRF, endothelium derived relaxing factor

EGF, epidermal growth factor

ER, endoplasmic reticulum

ET, endothelin

FITC, fluorescien isothiocyanate;

GBM, glomerular basement membrane

GFR, glomerular filtration rate; SNGFR, single nephron glomerular filtration rate

H&E, hemotoxyllin and eosin staining;

HLA, human leucocyte antigen

HMWK, high molecular weight kininogen;

HOE 140, D-Arg-(Hyp^3,Thi^5,D-Tic^7,Oic^8)-BK (icatibant);

ICC, immunocytochemistry;

ID1, intracellular domain one of B2 bradykinin receptor; ID2, intracellular domain two of B2 bradykinin receptor;

IFN, interferon

Ig, immunoglobulin;

IGF, insulin growth factor

IL, interleukin

IMCD, inner medullary collecting duct

iNOS, inducible nitric oxide synthase

IP3, inositol 1,4,5 triphosphate; IP4, inositol 1,3,4,5 tetrakiphosphate

KI-CPM, kininase I-carboxypeptidase M; KI-CPN, kininase I- carboxypeptidase N;

KII-ACE, kininase II-angiotensin I-converting enzyme; KII-NEP, kininase II-neutral endopeptidase;

K_f, ultrafiltration coefficient

KKS, kallikrein-kinin system;

LBK, lys-bradykinin (kallidin); LMWK, low molecular weight kininogen;

LFA, leucocyte function-associated antigen

L-NAME, N^G-nitro -L-arginine methylester

L-NMMA, N^G-monomethyl-L-arginine

LPS, lipopolysaccharide

MAP, mitogen activated protein

MHC, major histocompatibility complex

Na, sodium; K, potassium; Cl, chloride; HCO_3, bicarbonate

NBT/BCIP, nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate;

NO, nitric oxide;

NS, physiological saline (0.9% NaCl);

PBS, phosphate buffered saline;

PFA, paraformaldehyde;

PDGF, platelet derived growth factor

pHi, intracellular pH

PKC, protein kinase C

PL, phospholipase

PG, prostaglandin

P_{UF} , net filtration pressure across glomerular capillary

Q_A, glomerular capillary flow rate

RBF, renal blood flow; RPF, renal plasma flow

ROC, receptor operated calcium channel; VOC, voltage gated calcium channel

RT, room temperature;

rTK, recombinant tissue kallikrein;

RT-PCR, reverse transcriptase-polymerase chain reaction;

RVR, renal vascular resistance

SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat

TCR, T cell receptor

TEM, transmission electron microscopy;

TGF, transforming growth factor;

TK, tissue kallikrein; PK, plasma kallikrein

TNF, tumour necrosis factor.

TXA_2, thromboxane A_2

ABSTRACT

Introduction

The kidneys play a pivotal role in maintaining fluid and electrolyte homeostasis and regulating blood pressure. Multiple vasoactive peptides interact to exert autocrine and paracrine influences on the renal circulation, tubular function and mitogenesis. Endothelin-1 is now known to be the most potent vasoconstrictor yet described, with natriuretic and mitogenic effects. Endothelin-1 exerts its effects via two receptors: the ET_A receptor mediates vasoconstriction; the ET_B receptor functions as a clearance receptor and has vasodilator effects by clearing endothelin-1 from the circulation as well as promoting natriuresis and diuresis. The natriuretic peptides exert potent natriuretic and diuretic effects. Circulating atrial natriuretic peptide is produced primarily in response to increased intravascular volume. Elevated levels of atrial natriuretic peptide are present in hypertension, nephrotic syndrome and acute and chronic renal failure. Kinins bind to their receptors at target organs and exert potent effects in vasodilatation, blood pressure reduction, vascular permeability, smooth muscle contraction, natriuresis, diuresis and renal blood flow. The kinin B2 receptor is the constitutive receptor and mediates most of the actions of kinins; the B1 receptor is induced by inflammation. The connecting tubule cells show a loss of the kinin generating enzyme, tissue kallikrein in hypertension and renal failure.

Aim:

The aim of this thesis was to study a group of vasoactive peptides of differing physiological actions, namely, endothelin-1, atrial natriuretic peptide and kinins in models

of human renal inflammation, namely acute renal allograft rejection and glomerulonephritis. The hypothesis is that these peptides are closely inter-related, both anatomically and functionally and counter-balance each other's molecular and cellular effects.

Methods:

Ethical permission for the study was obtained from the Ethics Committee of the Medical School, University of Natal.

1. Blood and urine samples and renal biopsies were collected from renal transplant recipients with acute rejection and patients with renal parenchymal disease who underwent routine diagnostic renal biopsies. Blood and urine samples were collected from renal donors and normal volunteers; normal kidney tissue was obtained from forensic autopsies carried out within 24 hours of death. Plasma and urinary endothelin-1 was measured by ELISA; plasma and urinary atrial natriuretic peptide by radio-immunoassay; basal and generated kinins were measured in urine by ELISA; urinary tissue kallikrein by an enzymic assay as well as by ELISA.

2. Immunocytochemistry was carried out on renal biopsy material for endothelin-1 and its receptors (ET_A and ET_B) using the peroxidase–antiperoxidase (PAP) method, immunofluorescent technique with confocal microscopy and immuno-electron microscopy. In addition endothelin-1 mRNA expression was detected by in situ reverse transcriptase-polymerase chain reaction in acute rejection biopsies and control kidney tissue. Atrial natriuretic peptide immunolabelling was carried out by the PAP method. Tissue kallikrein immunofluorescent labelling was examined by confocal microscopy. The PAP images were analysed by the Kontron KS 300 (Zeiss GmbH, Germany), running on Windows

95™. Confocal images were analysed by the Analysis 2.1 Pro system (Soft-Imaging GmbH, Germany).

3. Total body water was measured by bioelectrical impedance in 5 renal transplant patients, 4 patients with renal disease and 3 control subjects. Renal plasma flow was measured using sodium ^{131} iodohippurate in 6 renal transplant patients.

Results:

1. Endothelin-1 and ET_A and ET_B receptors

Plasma endothelin-1 levels were elevated in patients with chronic renal failure on dialysis, decreased after renal transplantation, rose again during acute rejection and subsequently decreased after treatment of rejection. Urinary endothelin-1 excretion was increased during acute rejection. Plasma endothelin-1 concentrations were elevated in both proliferative and non-proliferative glomerulonephritis, with the highest levels in proliferative glomerulonephritis, hypertensive glomerulonephritis patients and those on dialysis. Immunocytochemistry showed increased endothelin-1 labelling of distal tubules and the luminal brush border of proximal tubules during acute rejection. In addition endothelin-1 label was demonstrated in lymphocytes and plasma cells of the interstitial inflammatory infiltrate in acute rejection. Endothelin-1 immunolabelling was increased in proximal and distal tubules in proliferative glomerulonephritis. The ET_A receptor was upregulated in both acute rejection and glomerulonephritis in the proximal and distal tubules and collecting ducts. The ET_B receptor immunolabelling was decreased in glomeruli, proximal and distal tubules and collecting ducts in acute rejection and glomerulonephritis. Immuno-electron microscopy revealed endothelin-1 and endothelin receptor labelling in epithelial cells of proximal and distal tubules, endothelial cells of blood vessels and glomerular capillaries. Label was found in the intercellular system and within secretory vesicles and

vacuoles as well as mitochondria. Endothelin-1 label was demonstrated in lymphocytes and plasma cells during acute rejection. Endothelin-1 mRNA was upregulated in tubular epithelial cells and capillary endothelial cells, as well as the inflammatory infiltrate during acute rejection.

2. Atrial natriuretic peptide

Plasma and urinary atrial natriuretic peptide concentrations were elevated during acute rejection. Atrial natriuretic peptide immunolabelling of glomeruli and collecting ducts was decreased in acute rejection and glomerulonephritis, while labelling of distal tubules and blood vessels was similar to controls.

3. Tissue kallikrein and kinins

Urinary tissue kallikrein enzymic activity was reduced in dialysis patients prior to transplant, rose after transplant, decreased during acute rejection and rose again after treatment of rejection. Urinary tissue kallikrein enzymic activity and basal kinin excretion was reduced in stable transplant recipients and in kidney donors after nephrectomy; basal kinin urinary excretion rose during acute rejection. Kinin generation in the urine was decreased in renal transplant patients during acute rejection as well as the donors after nephrectomy. Urinary tissue kallikrein excretion was decreased in glomerulonephritis. Basal kinins in urine were similar in patients with glomerular disease and controls; however significantly decreased kinins were generated in glomerulonephritis patients. Reduced tissue kallikrein immunolabelling was observed in the distal tubule during acute rejection and with renal parenchymal disease.

4. Total body water was increased during acute rejection; effective renal plasma flow was reduced during acute rejection.

Discussion and Conclusions:

This thesis is the first study of endothelin-1 and its receptors in human renal inflammation and provides evidence of upregulation of ET_A receptors and downregulation of ET_B receptors in the kidney in renal inflammatory conditions (acute rejection and glomerulonephritis).

1) The downregulation of ET_B receptors may account for the fluid retention and hypertension that occur during acute rejection and glomerulonephritis; ET_B receptors in the tubule inhibit sodium and water reabsorption in the distal tubule; ET_B receptors are also clearance receptors and play a role in clearing circulating endothelin-1, thereby reducing its predominantly ET_A-mediated pressor actions. Elevated plasma endothelin-1 levels may be as a result of its impaired clearance, as well as increased production by mononuclear cells (macrophages and monocytes) in renal inflammation. Cytokines produced during rejection and glomerulonephritis may also be responsible for the elevation in plasma endothelin-1. None of the patients had histological or biochemical evidence of cyclosporine toxicity; cyclosporine therefore probably did not play a major role in increasing plasma endothelin-1 in this study.

2) The elevated plasma and urinary atrial natriuretic peptide levels are probably a response to increased intravascular volume and hypertension in these patients; in addition, atrial natriuretic peptide production may be stimulated by the increased endothelin-1 present. Reduced atrial natriuretic peptide immunolabelling in collecting ducts may be a

reflection of impaired natriuresis and diuresis, resulting in the fluid retention observed in these patients.

3) Decreased urinary tissue kallikrein activity may be a reflection of reduced distal nephron function and may mediate the hypertension that accompanies renal disease.

Multiple vasoactive mediators have an impact on renal function; future therapy should be inclusive of the functions regulated by endothelin-1, atrial natriuretic peptide and kinins. Current therapy suggestive of this approach are angiotensin converting enzyme inhibitors, which block angiotensin II and decrease endothelin-1 while enhancing nitric oxide, prostacyclin and kinins.

CHAPTER 1

INTRODUCTION

1.1 VASOACTIVE PEPTIDES IN THE KIDNEY

1.1.1 VASOACTIVE MODULATORS OF RENAL FUNCTION

Many vasoactive substances, both circulating and local, influence the tone of the afferent and efferent glomerular arterioles (Fig 1.1-1). These include a variety of vasoconstrictor molecules such as angiotensin, catecholamines, vasopressin and endothelin (ET)-1 as well as vasodilator substances, such as bradykinin, adrenomedullin, natriuretic peptides, adenosine and prostaglandins (Egido, 1996). Whereas some of these regulators of vascular tone are either released into or formed in the circulation, others are produced by endothelial cells, including those lining the glomerulus.

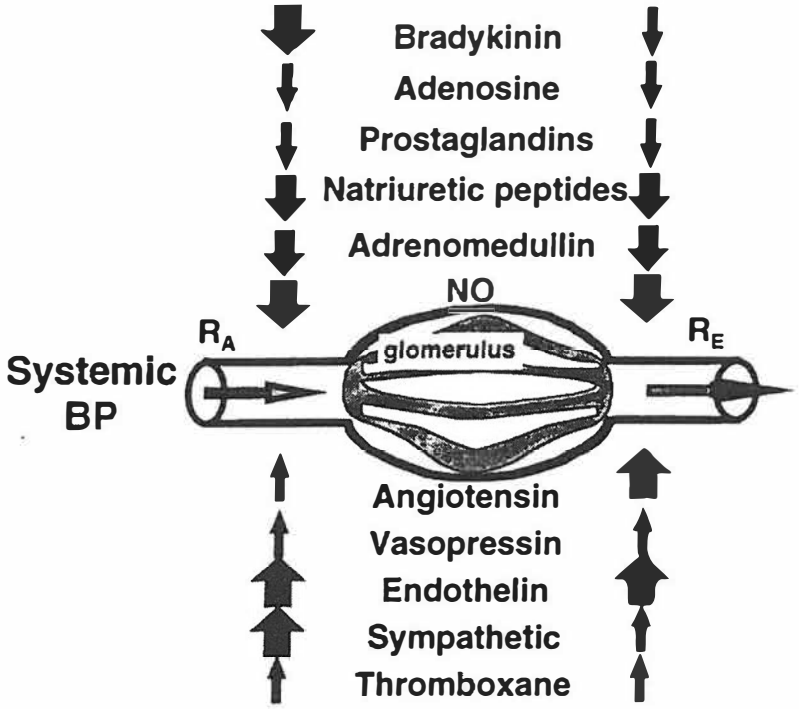


Fig 1.1-1: Vasoactive substances influencing tone and resistance in renal vasculature.
Ref.: Johnstone C I et al. (1998). Journal of Hypertension, 16 (4): S1-7 (S4)
Abbreviations: R_A =afferent artery; R_E =efferent artery; BP= blood pressure; NO= nitric oxide

The endothelium is a monolayer of cells that forms the inner lining of all blood vessels. Endothelial cells synthesize many active substances, including large molecules [such as fibronectin and heparan sulphate, interleukin (IL)-1, tissue plasminogen activator] and various growth-promoting factors as well as smaller molecules [such as prostacylin, endothelium-derived relaxing factor (EDRF)/nitric oxide (NO), platelet-activating factor and ET-1]. The outer surface of endothelial cells contains angiotensin-converting enzyme, which catalyses the formation of angiotensin II from its inactive precursor angiotensin I. It also inactivates bradykinin (reviewed by Vane et al., 1990). In this way, many of them interact with EDRF / NO which mediates signal transduction. Nitric oxide is a powerful regulator of intrarenal haemodynamics and its release is stimulated by pressure and sheer stress (Webb, 1997), as is ET-1 and angiotensin II.

Vasoconstrictors not only increase blood pressure but are also anti-natriuretic and neuro-excitatory, and stimulate growth and hypertrophy. In contrast, vasodilators lower blood pressure and are also natriuretic and neuro-inhibitory, and inhibit growth and hypertrophy. Some growth factors, such as transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF), may be vasoactive. Both angiotensin II and ET-1 are potent stimuli for cytokine production and extracellular matrix formation (Egido, 1996).

The relationship between ET-1 and cytokines is dual. Renal cells stimulated by TGFβ, PDGF and IL-1β, secrete endothelin-1. Endothelin-1 induces the expression and synthesis of various cytokines in mesangial cells. Interleukin-6 synthesis has been reported to be stimulated by ET-1, angiotensin II and arginine vasopressin in mouse mesangial cells (Fujibayashi et al, 1991). It is likely that vasoactive hormones, growth factors and

cytokines are activated and, conversely, inhibited by the same stimuli, including mechanical stress, stretch and pressure transmission and immunological injury.

Many vasoactive substances directly affect glomerular permeability and mesangial cell function. In the renal cortex, angiotensin II and ET-1 act as vasoconstrictors to decrease renal blood flow and glomerular filtration rate, whereas bradykinin causes vasodilation and increases glomerular capillary permeability. In the medulla, angiotensin II and ET cause vasoconstriction of the outer medullary descending vasa recta and thereby decrease vasa recta and papillary blood flow, whereas bradykinin exerts opposite effects (Navar et al., 1996). Receptor-mapping studies in the rat kidney have shown that the distribution of angiotensin II subtype 1 (AT_1), endothelin A and B (ET_A and ET_B) and kinin B_2 receptors closely overlaps at several anatomical sites, including the renal vasculature, glomeruli, and the inner stripe of the outer medulla. In the cortex, the distribution of AT_1 and ET_B receptors is similar in glomeruli and proximal tubules. In contrast, B_2 receptor density is low in the cortex. AT_1 receptors are predominantly in mesangial cells whereas ET_B receptors are present mainly in endothelial cells of glomerular capillaries. In the medulla, ET_B and B_2receptors are abundant in the inner medulla towards the tip of the papillae, whereas AT_1 receptors are not readily detected in this region. However, receptor binding sites for these peptides all occur in the inner stripe of the outer medulla, suggesting their role in the regulation of renal medullary haemodynamics, tubular transport processes and probably long-term blood pressure homeostasis. These peptides also promote cell proliferation and extracellular matrix synthesis in renal medullary interstitial cells (Zhuo et al., 1998).

1.1.2 ENDOTHELINS IN THE KIDNEY

Yanagisawa and colleagues (1988a) reported an endothelium-derived factor (a 21 amino acid peptide) as the most potent vasoconstrictor ever described to date. This substance was named endothelin (ET). Subsequent studies showed that ET was one of a family of 3 isopeptides, all of which were formed through a 2-step processing pathway from their respective precursor peptides that shared high sequence homology but were encoded by distinct genes. Endothelin isopeptides share a marked structural similarity to the sarafotoxins (SRTX), peptides isolated from the Israeli burrrowing asp, *Atractaspis engaddensis* [Kloog et al., 1988; (Fig 1.2-1)]. The ETs and SRTXs act through common receptors to evoke a multitude of biological effects. Isoforms of SRTX have been utilised as tools for the characterisation of ET receptors (Sokolovsky, 1994 a and b).

ET-1 is the major isopeptide produced by human endothelial cells and is present in the greatest concentration in blood. The concentrations of ET-1, though detectable in the human circulation, are very low (in the picomolar range). However, as ET-1 is released predominantly in an abluminal direction towards the underlying smooth muscle (Wagner et al., 1992), the tissue concentration is likely to be sufficiently high to activate local receptors. Recent studies, using inhibitors of ET synthesis or receptor antagonists, suggest that ET-1 is released tonically to maintain basal systemic vascular resistance in humans (Haynes and Webb, 1994). In this way, ET-1 might balance out the dilator effects of nitric oxide (NO), which is also thought to be released in a tonic manner [(Vallance et al., 1989; Haynes et al., 1993); Fig 1.2-2].

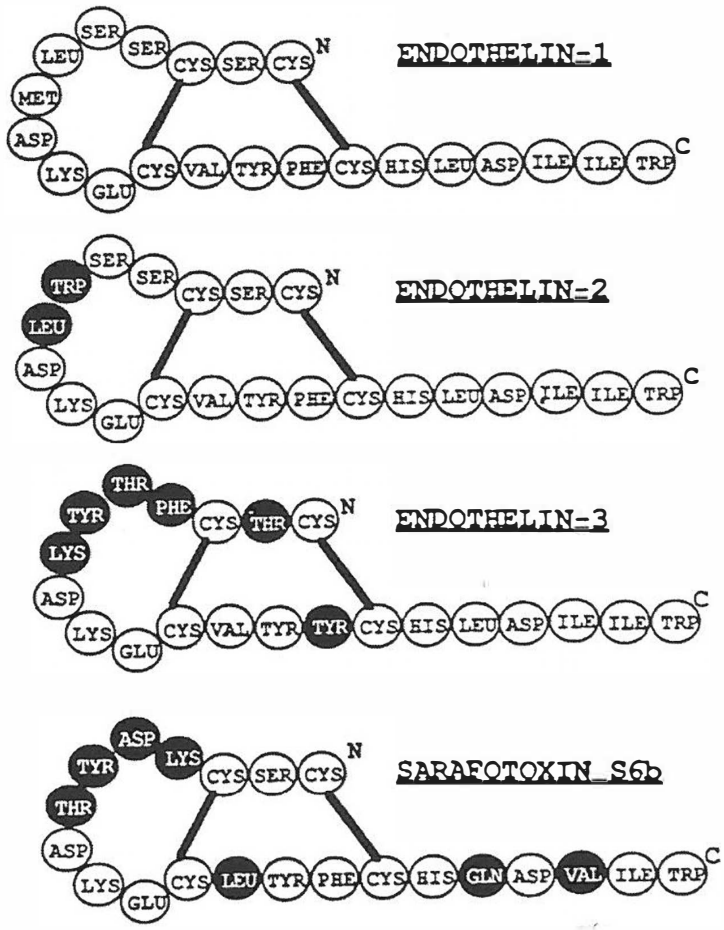


Fig 1.2-1: Amino acid sequence of endothelin isopeptides
Ref: Brenner B M et al. (1989), Journal of Clinical Investigation, 84: 1373-1378 (1375)

While the synthesis of NO can be increased within minutes in response to various stimuli, ET-1 synthesis is regulated at the transcriptional level with a resultant delay in release (Yanagisawa et al., 1988b; Boulanger and Lüscher, 1990). Nitric oxide has a short half-life, and its effects can be terminated quickly by cessation of release; in contrast endothelial ET-1 binds to its receptors on smooth muscles irreversibly, and its constrictor and pressor effects are of longer duration (Hirata et al., 1988; Yanagisawa et al., 1988 b).

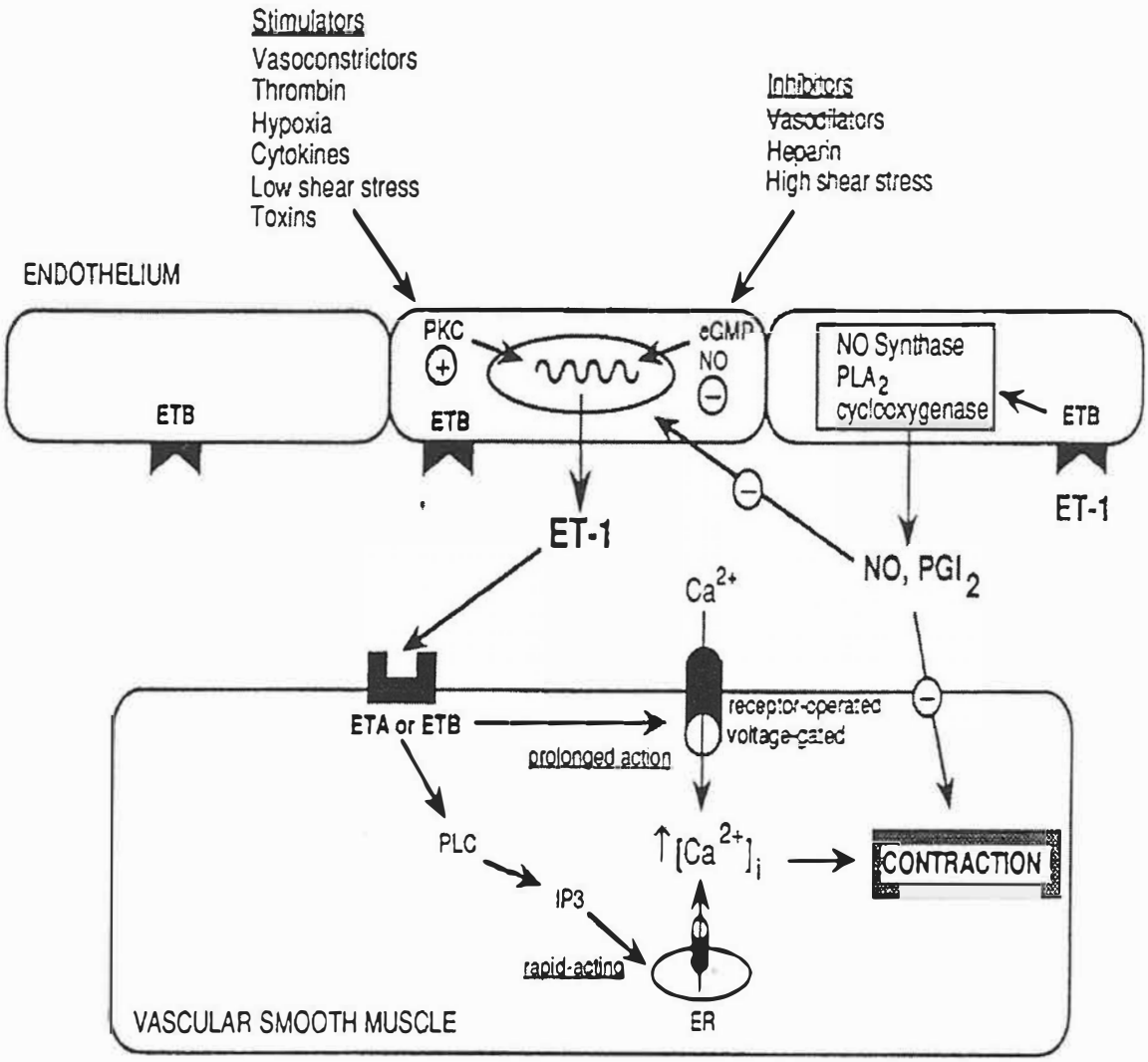


Fig 1.2-2: Endothelium-derived factors that regulate vascular smooth muscle

Ref: Kohan D E (1997), American Journal of Kidney Disease, 29: 2-26 (5)
Abbreviations: ER= endoplasmic reticulum; IP3= inositol triphosphate; PLC= phospho-lipaseC; $[Ca^{2+}]$= calcium concentration; PGI_2= prostacyclin; PKC= protein kinase C; PLA_2= phospho-lipase A_2

Intensive research into the ET system has greatly improved our understanding of the genes that encode for ET-1 and its isopeptides, of the enzymes involved in its synthesis and the receptors that by activating signaling pathways mediate its actions.

1.1.2.1 ENDOTHELIN GENERATION AND CLEARANCE

1.1.2.1.1 Endothelin genes and regulation

At least 3 genes encoding ET- like sequences in mammalian genomes (Inoue et al., 1989b), have been shown to encode the precursors of ET-2 and ET-3, in addition to prepro ET-1. In the human genome, the ET-1 gene is found on chromosome 6 (Bloch et al., 1989 a; Hoehe et al., 1993), the ET-2 gene on chromosome 1 (Bloch et al., 1991) and the ET-3 gene on chromosome 20 (Bloch et al, 1989 b). Endothelin gene expression has been demonstrated in the brain and spinal cord, lung, kidney, gut, eye, pituitary and amnion (reviewed by Simonson, 1993). The endothelium is the major site of ET gene expression. The genes encoding ET precursors have promotor regions through which external factors are able to modulate transcription (Hilkert et al., 1992; Benatti et al., 1994). Extracellular factors can influence ET-1 generation both positively and negatively through liberation of a series of intracellular mediators that modulate gene transcription. Several agents, including insulin, thrombin, low density lipoprotein, angiotensin II, vasopressin and ET-1 itself (Emori et al., 1991; Boulanger et al., 1992; Emori et al., 1992; Kohno et al., 1992; Benatti et al., 1994) enhance ET-1 generation via activation of protein kinase C [(PKC); Table 1.2-1]. Responsiveness to PKC is mediated by binding of the proto-oncogenes, *jun* and *fos* to the Activator Protein-1 (AP-1) transcription regulatory element of the ET-1 promotor (Curran and Franza, 1988; Lee et al., 1991). PKC activation is also thought to be a mechanism by which low levels of shear stress (1.8 dyne/cm2) enhance endothelial ET-1 release (Kuchan and Frangos, 1993; Wang et al., 1993). Higher levels of shear stress (>6 dyne/cm2) activate another mechanism that inhibits ET-1 mRNA transcription (Kuchan and Frangos, 1993; Malek et al., 1993). This effect is prevented by inhibitors of NO synthesis and by methylene blue, an inhibitor of guanylate cyclase, suggesting that endothelial cells release

NO in response to shear stress, and inhibit ET-1 synthesis through formation of cyclic GMP. Synthesis of ET-1 is inhibited by thrombin (Boulanger and Lüscher, 1990), heparin (Yokokawa et al., 1993), atrial and brain natriuretic peptides (Kohno et al., 1991) and by the prostanoids, prostaglandin E_2 and prostacyclin (Prins et al., 1994). One action of cyclic GMP is to reduce the availability of intracellular calcium, which may be relevant for the inhibition of ET-1 synthesis, as calcium chelation similarly reduces ET-1 release from endothelial cells (Emori et al., 1992).

1.1.2.1.2 Processing of endothelin precursors

Endothelin isopeptides arise from post-translation processing of large isopeptide-specific prohormones in a manner analogous to other peptides. The 212-amino acid pre-proET-1 undergoes proteolytic cleavage between Lys ^{52}-Arg ^{53} and Arg^{90}-Arg^{91} to release the 38 amino acid precursor, big ET-1; this step may be dependant on one of the proprotein convertases (Steiner et al., 1992; Seidah et al., 1993). Furin, a proprotein convertase of the constitutive secretory pathway, has been proposed as a likely candidate (Laporte et al., 1993). Big ET-1 is less active than ET-1 for displacement of binding to ET receptors and in stimulating vascular contraction *in vitro* (Hirata et al., 1990). The release of the biologically active 21 amino acid ET-1 requires selective cleavage of the Trp^{21}-Val^{22} bond in the carboxy terminal portion of big ET-1, catalysed by endothelin-converting enzyme activity [(ECE); Fig 1.2-3]. Several ECE-like enzyme activities representing different endopeptidase classes have been identified (reviewed by Opgenorth et al., 1992; Turner and Murphy, 1996). These include serine proteases (Yanagisawa et al., 1988 b; McMahon et al., 1989; Takaoka et al., 1990a; Kaw et al., 1992; Wypij et al., 1992), aspartate proteases such as pepsin (Takaoka et al., 1990 b) and Cathepsin D (Sawamura et al., 1990), and soluble thiol protease (Deng et al., 1992). The physiologically relevant ECE is a

membrane-bound, zinc-containing metalloprotease that is inhibited by phosphoramidon, a neutral (metallo) endopeptidase (NEP 24.11) inhibitor (Opgenorth et al., 1992). The activity of this ECE is not affected by thiorphan (another NEP inhibitor) or by inhibitors of the neutral metalloprotease angiotensin converting enzyme (ACE). Human ECE -1 has a neutral pH optimum and is inhibited by phosphoramidon (Schmidt et al., 1994; Yorimitsu et al., 1995). A second novel enzyme, ECE-2 has been cloned more recently (Emoto and Yanagisawa, 1995); this enzyme is also inhibited by phosphoramidon. ECE-1 is widely distributed but not found in neurons and glia in the brain. ECE-2, in contrast, seems to be most abundantly expressed in neural tissues (Emoto and Yanagisawa, 1995). Both ECE-1 and ECE-2 are predicted to be integral membrane proteins and therefore the primary site for cleavage of endogenous big ET-1 could be at the plasma membrane or at an intra-membranous site.

Exogenous big ET-1 can be converted to ET-1 *in vivo* (McMahon et al., 1991), *in vitro* (Auguet et al., 1992) and in *cos* cells transfected with the ECE-1 gene (Xu et al., 1994), consistent with localisation of ECE at an accessible plasma membrane site. Endogenous big ET-1 is most likely to be converted during its transit through the intracellular constitutive secretory pathways, especially within the Golgi apparatus. Formation of ET-1 in secretory vesicles that can recognise transport pathways could explain the directional release of ET-1 towards the abluminal surface of endothelial cells (Wagner et al., 1992).

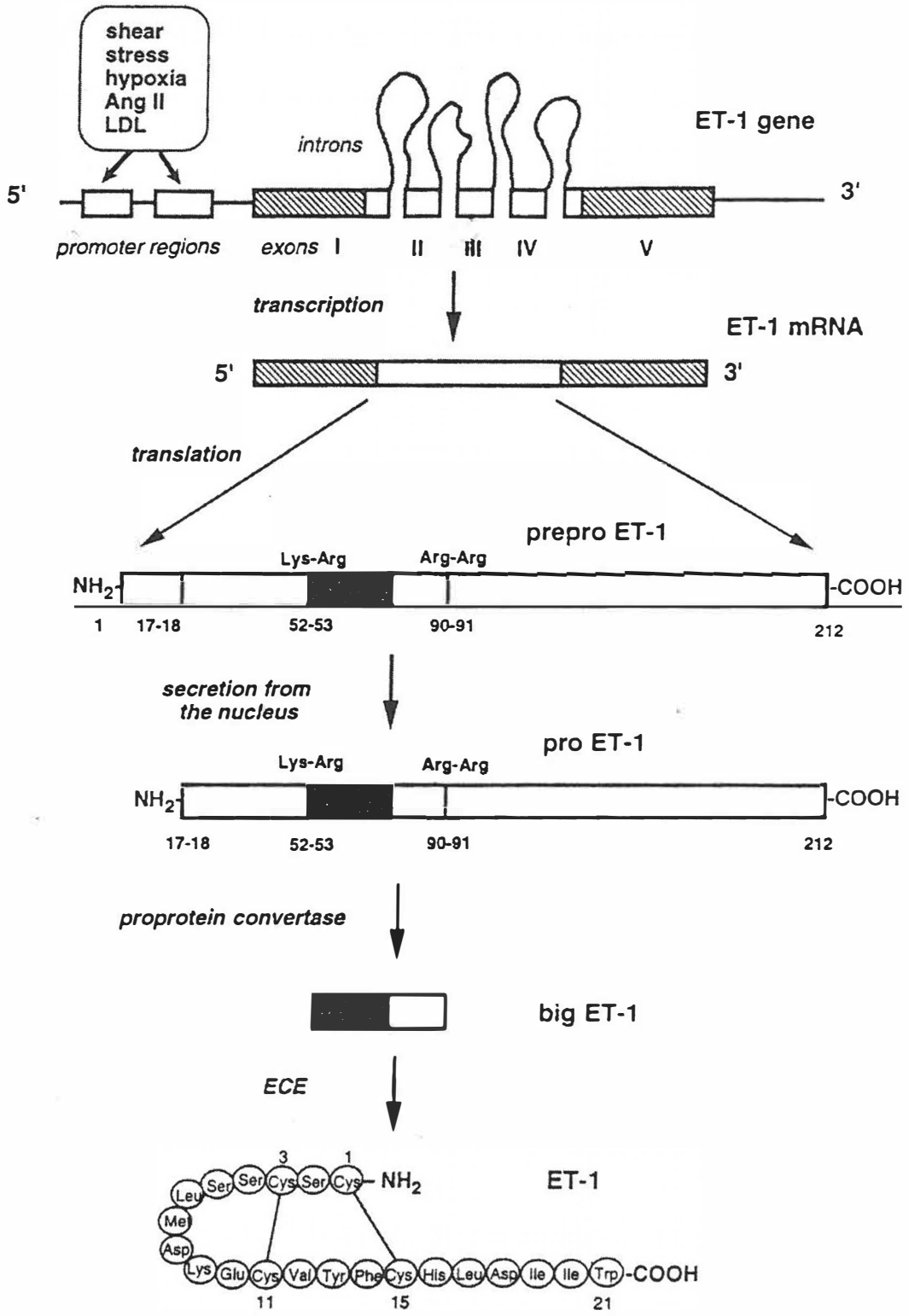


Fig 1.2-3: Processing pathway of ET-1
Ref: Gray, G A and Webb, D J (1996). Pharmacology and Therapeutics, 72: 109-148 (114)

1.1.2.1.3 Sites of endothelin synthesis and secretion

Endothelin-1 is secreted by a constitutive pathway but recent evidence suggests that in some cells, ET can be secreted by a regulated pathway via secretory granules. Endothelial cells in culture secrete about 80% of ET-1 into the basolateral compartment (Wagner et al., 1992). Stimulation with thrombin doubles ET-1 secretion. Numerous cells in culture, including endothelial cells (Yanagisawa et al., 1988a; Bloch et al., 1989a; Saito et al., 1989), vascular smooth muscle cells (Resink et al., 1990a; Yu and Davenport, 1995), mesangial cells (Sakamoto et al., 1990; Zoja et al., 1991), and glial cells (MacCumber et al., 1990), secrete a constant level of ET into the culture supernatant, suggesting a constitutive pathway for secretion, which is supported by immunocytochemical studies on endothelial cells (Nakamura et al., 1990). Immunoreactive ET-1 is detected primarily in the endothelial cell layer in human blood vessels (Howard et al., 1992; Tokunaga et al., 1992; Saetrum Opgaard et al., 1994; Properzi et al., 1995), consistent with the idea that ET-1 is released by endothelial cells to act on the underlying smooth muscle cells. *In situ* hybridisation for ECE-1 mRNA also shows the most intense labelling over vascular endothelial cells of most tissues (Xu et al., 1994). The secretory pathway involves the rough endoplasmic reticulum, Golgi cisternae, Golgi vesicles and small exocytic vesicles directly beneath the plasma membrane. A variety of stimuli increase ET secretion, as shown in Table 1-1. In most cases induction of ET-1 secretion above basal levels requires 2-5 h, and most likely results from ET gene induction. ET-1 secretion by endothelial cells is inhibited by atrial natriuretic factor (Saijonmaa et al., 1990; Kohno et al., 1991; Hu et al., 1992) and by co-culture with cells of the vascular media (Stewart et al., 1990), which might protect vascular target cells from excessive ET-1 production. Endothelial secretion is also inhibited by activation of protein kinase A (Sakamoto et al., 1992).

Table 1-1. Factors that influence ET-1 biosynthesis

Fluid-mechanical shear stress
Hypoxia
Thrombin
Transforming growth factor-β
Interleukin-1
Tumour necrosis factor
Bradykinin
Arginine vasopressin
Epidermal growth factor
Endotoxin
Oxidized low-density lipoprotein
Insulin
Insulin-like growth factor I
Thromboxane A_2
Epinephrine
High glucose
Ca^{2+} ionophores
Phorbol esters
Endothelin-1

Immunoreactive ET peptides have been demonstrated in secretory vesicles in the posterior pituitary of the rat (Yoshizawa et al., 1990). These are depleted upon water deprivation, suggesting that ET-1 is secreted in response to changes in extracellular fluid volume or osmolarity and might be involved in neurosecretion and osmolar regulation. Glomerular immunoreactive ET-1 is localised primarily in the endothelium and to a lesser extent, in the mesangium. Focal staining was also observed in the proximal tubule brush border (Wilkes et al., 1991). Experiments with primary cultures (derived from nephron segments) reveal that renal tubule cells secrete abundant amounts of ET-1 >ET-3; ET synthesis is much greater from medullary tubular segments with inner medullary collecting duct > medullary thick ascending limb > cortical collecting tubule >> proximal tubule (Kohan, 1991). It is thus possible to conclude that ET acts at multiple sites in the kidney, including the glomerulus (probably at afferent and efferent vascular smooth muscle and mesangial cells), medulla (probably loop of Henle and collecting ducts), vasa recta bundles and perhaps at the proximal tubules. ET might act at other renovascular sites, including the interlobular and arcuate arteries and possibly the vascular smooth muscle cells in the postglomerular medullary circulation. The proximal location of cells in the cortex and medulla expressing ET binding sites and preproET mRNA transcripts suggests that ET acts as an autocrine or paracrine peptide (Simonson, 1993).

1.1.2.1.4 Clearance and degradation of endothelin

The plasma half-life of ET-1 in humans is less than 1.5 min because of its efficient extraction by the splanchnic and renal vascular beds (Weitzberg et al., 1991; Gasic et al., 1992). Although ET-1 is also taken up by the lungs, pulmonary clearance is less important in humans than in other species (Ray et al., 1992; Hemsen et al., 1995). Extraction of ET-1 follows binding to cell surface receptors, which are then internalised, allowing degradation

to be carried out within the cell, perhaps in lysosomes (Löffler et al., 1991). ET_B receptors may have a role in clearance of ET-1, as suggested by increased circulating concentrations of ET-1 with mixed ET_A/ET_B receptor antagonists (Löffler et al., 1993), or a ET_B selective receptor antagonist (Fukuroda et al., 1994) but not by ET_A selective antagonists. A soluble protease found in human platelets, vascular smooth muscle and endothelial cells may be a possible candidate for an intracellular degrading enzyme (Jackman et al., 1992, 1993). A deamidase with similar characteristics was purified from rat kidney (Deng et al., 1994; Janas et al., 1994). The ETs can also be degraded by neutral endopeptidases [NEPs, (E.C. 24.11)], which are associated with arterial and venous endothelial cell plasma membranes (Llorens-Cortes et al., 1992). Activated polymorphonuclear leucocytes are able to rapidly inactivate ET-1 through release of a protease, believed to be cathepsin G, which degrades ET by cleavage of His^{16}- Leu^{17} (Fagny et al., 1992; Patrignani et al., 1992); this process may have a role in acute inflammation following adhesion of polymorphonuclear leucocytes to vascular endothelial beds.

1.1.2.2. ENDOTHELIN RECEPTORS

The ET_A type receptor is characterised by its very high (subnanomolar) affinity for ET-1 and ET-2, and its 70-100 fold lower affinity for ET-3, while the ET_Breceptor has high and equal affinity for all 3 isopeptides. The cDNAs encoding the human ET_A and ET_B receptors predict 427 and 442 aminoacids respectively (Fig 1.2-4), and the overall identity between the two mature proteins is reported to be between 55% and 64%, depending on the tissue studied. The ET_A and ET_B receptor genes, located on chromosomes 4 (Hosoda et al., 1992) and 13 (Arai et al., 1993) respectively, have similar structural organisation, suggesting that they originated from the same ancestral gene.

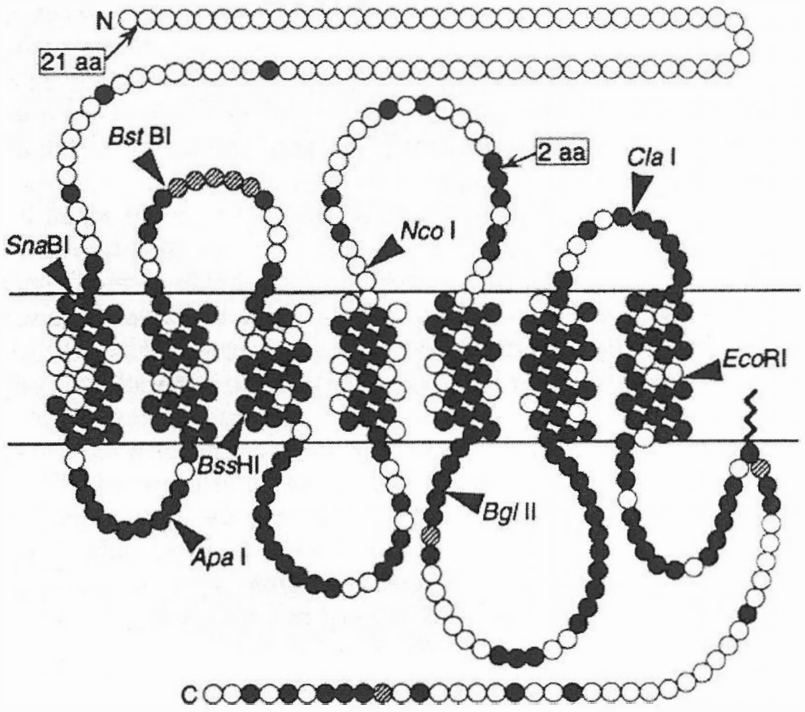


Fig 1.2-4: Transmembrane topography of endothelin receptors.

Ref: Sakamoto H et al. (1993), J Biol Chem, 268: 8547-8553 (8548)
The number of amino acids (circles) is based on the ETA sequence; striped circles depict the sequence introduced to ETB and the insertions by arrows. Closed circles denote amino acid residues that are identical in ETA and ETB receptors; open circles the non-identical residues. Position of the restriction sites used to construct chimeric receptors are shown with arrowheads with the names of the enzymes. Abbreviations: N= amino terminal; C= carboxy terminal; aa= amino acid.

Screening of amphibian cDNA libraries has revealed the existence of 2 alternate receptor clones: ETc receptor subtype showing relative selectivity for ET-3, cloned from *Xenopus* dermal melanophores (Karne et al., 1993); ET_{AX}, cloned from *Xenopus* heart, has a relatively high affinity for ET-1 (like the ET_A receptor) but an uncharacteristic low affinity for the ET_A selective ligand BQ-123 (Kumar et al., 1994).

As with the ET genes, the nontranscribed 5' flanking regions of the ET receptor genes contain a number of regions in the regulation of gene transcription (Hosoda et al., 1992;

Arai et al., 1993). Exogenous factors can act through these regions to increase receptor transcription, namely upregulation of ET receptor mRNA by insulin (Frank et al., 1993), or ET_B receptor mRNA by angiotensin II (Kanno et al., 1993). These mechanisms may be important in the regulation of responsiveness to the ETs in pathophysiological states. One of the major factors that reduces ET receptor numbers at the cell surface is prolonged exposure to ET-1, because of down-regulation or feedback inhibition of receptor expression (Hirata et al., 1988) or both in combination. All of the cloned ET receptor genes predict a heptahelical membrane spanning structure, common to members of the G-protein coupled receptor superfamily and similar to many neuropeptide receptors (Fig 1.2-4; Burbach and Meijer, 1992). The seven transmembrane domains and cytoplasmic loops of the receptors are highly conserved and the N-terminal and extracellular domains exhibit differences in both length and amino acid sequences (Ogawa et al., 1991; Elshourbagy et al., 1993; Sakamoto et al., 1993). The extracellular terminal regions of peptide G-protein coupled receptors are known to be important for ligand binding (Nagayama et al., 1991). The amino acid loops of the receptor proteins have been a specific source for the harvesting of anti-peptide antibodies.

In vascular tissue, ET_A receptor mRNA is expressed predominantly in smooth muscle, while ET_B receptor mRNA is most abundant in endothelial cells, suggesting that constriction of vascular smooth muscle is mediated predominantly by ET_A receptors, and that constriction is modified by release of relaxing factors from the endothelium via stimulation of ET_B receptors (reviewed by Gray and Webb, 1996). Any analysis of the renal actions of ET requires an understanding of the distribution and molecular characterization of ET receptors. Both glomeruli and the inner medulla express abundant

binding sites for ^{125} I-ET-1 autoradiography (reviewed by Simonson, 1993). Precise cellular localizations are unavailable. Equilibrium binding studies in cultured medullary interstitial cells confirm the existence of high affinity (Kd=57pM) ET receptors in the medulla. A high density of ET-1 binding sites is also present in longitudional bands over the inner stripe of the outer medulla. In humans, the distribution of binding sites for ET-1, ET-2 and ET-3 and sarafotoxin 6b is similar, supporting the notion that different ET isopeptides act on the kidney (Waeber et al., 1990). ET-1 binds nearly irreversibly to its receptor and is not displaced by peptides such as angiotensin II, atrial natriuretic peptide and arginine vasopression, and is unaffected by dihydropyridine, phenylalkylamine and benzothiazepine calcium channel blockers (Kohzuki et al., 1989; Grone et al., 1990). Equilibrium binding studies with the ET_B specific agonist sarafotoxin 6c and theET $_A$ receptor antagonist BQ123 demonstrate that the kidney cortex expresses equal numbers of ET_A and ET_B receptors (Nambi et al., 1992). Steady-state mRNA levels for both ET_A and ET_B receptors have been observed by Northern blot analysis in adult rat kidney. *In situ* hybridisation reveals differential localization of ET_A and ET_B receptors. ET_A mRNA is expressed in renal arteries and in afferent and efferent glomerular arterioles, implicating ET_A receptors in the intense renal vasoconstriction induced by ET-1. ET_A receptors have also been characterized from microdissected nephron segments of the glomerulus, vasa recta bundle and arcuate artery. ET_B receptors are abundantly expressed by glomerular endothelial cells and in vasa recta bundles; weak hybridization signals corresponding to ET_B mRNA were also observed over epithelial cells in thin segments of Henle's loop and over interstitial cells but not over epithelial cells of collecting ducts. In constrast, mRNA transcripts for ET_A and ET_B have been demonstrated by RT-PCR of RNA isolated from inner medullary collecting ducts *in vitro* (reviewed by Simonson, 1993). These studies demonstrate that ET binds to high-affinity, saturable, cognate receptors in the kidney. Moreover, the distribution

of ET_A and ET_B receptors suggests multiple actions on renal haemodynamics, glomerular function, medullary blood flow and perhaps, proximal tubular reabsorption or secretion. A large number of ET receptor antagonists (peptide and non-peptide, selective and non-selective) have become available and have confirmed the role of ET-1 in pathology, the first report using BQ-123 (Moreland, 1994).

1.1.2.3 MODE OF ACTION

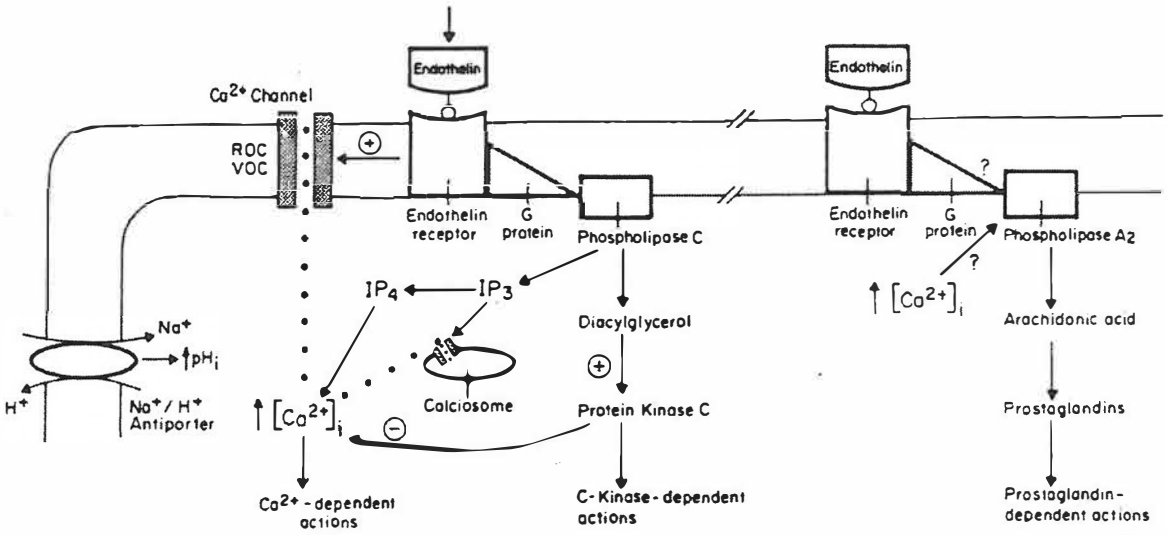
Low levels of plasma ET-1 are consistent with the predominantly basolateral secretion of ET-1 by endothelial cells. Thus it would appear that circulating ET-1 simply reflects spillover from local ET-1 release. The peptide is rapidly cleared from the plasma and degraded, consistent with a predominantly autocrine/paracrine action of ET. In some instances, circulating ET might be biologically active. Doubling of plasma ET levels by exogenous infusion causes a significant increase in peripheral and renal vascular resistance without affecting coronary vascular resistance or mean arterial pressure (Lerman et al., 1990). Moreover, elevated ET plasma concentrations have been reported in some pathophysiological states that might activate high-affinity ET receptors (Lerman et al., 1991).

1.1.2.3.1 Transmembrane signalling by endothelin

Endothelin peptides evoke complex, tightly regulated pathways of signal transduction that result in short-term (i.e. contraction, secretion) and long-term (i.e. mitogenesis) biological actions (Simonson and Dunn, 1990). Endothelin initially activates a membrane transduction process comprising a G protein-coupled cell surface receptor, coupling G-

protein(s) and phospholipase C or other G protein-activated effectors (Fig 1.2-5). Endothelin rapidly induces a dose-dependent increase in phosphatidylinositol turnover. Activation of phospholipase C by ET produces at least 2 second messengers: 1,4,5-triphosphate which diffuses to specific receptors on specialised compartments of the endoplasmic reticulum to release intracellular calcium; and 1,2-diacylglycerol, which remains in the plasma membrane and (with cofactors calcium and phosphatidylserine) activates phospholipase C. Calcium signalling is thought to mediate some short-term effects of ET such as vasoconstriction in the kidney and in other organ systems. Calcium signalling appears to be a nearly universal response to ET receptor activation (reviewed by Simonson, 1993). Calcium signalling induced by ET-1 requires conversion of proET-1 to ET-1. Endothelin peptides release calcium from intracellular stores. Endothelin-1 increases calcium influx from the extracellular space by activating multiple types of calcium channels in the plasma membrane. Evidence suggests that protein kinase C mediates both short and long-term events of ET. Evidence also suggests that ET-1 stimulates phospholipase A activity resulting in increased arachidonic acid-derived mediators such as prostaglandins and thromboxane. Both ET_A and ET_B receptors can stimulate phospholipase A_2 (Simonson, 1993). Endothelin activates the electroneutral Na^+- K^+ antiport: sarcolemmal Na^+- K^+ exchange has been demonstrated to sensitise cardiac myofilaments to intracellular calcium, thereby contributing to the inotropic activity of ET (Kramer et al., 1991). Endothelin has also been shown to increase the Na^+- K^+-Cl^- cotransport system in vascular smooth muscle cells (Rosati et al., 1990).

Fig. 1.2-5 Transmembrane signalling by ET-1



Ref: Simonson, M S (1993). Physiological Reviews, 73, 375-411 (383)
Abbreviations: ROC= receptor-operated calcium channel; VOC= voltage-gated calcium channel; pH_i= intracellular pH; IP_3 = inositol 1,4,5-triphosphate; IP_4= inositol 1,3,4,5-tetrakisphosphate; $[Ca^{2+}]$= intracellular calcium concentration

In addition to activating the serine/threonine-specific protein kinase C, ET also activates S6 kinase (Resink et al., 1990 b), which phosphorylates the sixth protein on the small ribosomal subunit, and is thought to contribute to mitogenic signalling. Endothelin-1 causes tyrosine and threonine phosphoryation and activation of mitogen-activated protein (MAP) kinase in mesangial cells, which is also postulated to contribute to mitogenic signalling (Wang et al., 1992).

1.1.2.3.2. Signal transduction in the nucleus

Cells proliferate when stimulated by high concentrations of ET. Endothelin has been reported to increase the expression of several genes including collagenase, prostaglandin endoperoxidase synthase and platelet-derived growth factor A and B chains. Further, ET-1 enhances the ability of epidermal growth factor to induce colony formation in semi-soft

agar. Endothelin-1 elevates the expression of transcription factors such as *c-fos, c-jun, c-myc* and VL-30. Induction of both *c-fos and c-jun* by ET is rapid and transient, consistent with a role for these *trans*-acting factors as a genetic switch. It is also likely that by induction of *trans*-acting factor genes, ET contributes to long-term changes in cell phenotype (reviewed by Simonson, 1993).

1.1.2.4 REGULATION OF RENAL HAEMODYNAMICS

Renal haemodynamics influence renal function by controlling, in part, glomerular filtration, tubular secretion and reabsorption, and urine concentration and dilution. Changes in renal haemodynamics act by regulating blood flow and hydraulic pressure in the glomeruli, peritubular and medullary micro-circulation (Dworkin et al., 1991). Renal vascular tone is regulated by α-and β-adrenergic sympathetic output, and by the levels of circulating vasoactive compounds such as angiotensin II, prostaglandins, arginine vasopressin, atrial natriuretic peptide and kinins. Renal vasomotor tone is exquisitely sensitive to ET-1 (Simonson, 1993). Systemic infusions of ET-1 cause a marked increase in renal vascular resistance (RVR), and a decline in renal plasma flow (RPF) and glomerular filtration rate (GFR). Changes in RPF occur rapidly and are dose-dependant: maximal decreases occur 20-30 min after infusion, after which RPF gradually increases and returns to baseline after 1-2 h. The increase in RVR following ET-1 infusion results from intense contraction of glomerular arterioles (afferent > efferent), and arcuate and interlobular arteries. ET-1 may induce renal vasoconstriction via platelet-activating factor and increase in cytosolic free calcium (Simonson and Dunn, 1992). Direct infusion of ET-1 into the renal artery in dogs causes a rapid transient increase in RPF followed by a marked long-lasting decrease (Stacy

et al., 1990). The decline in RPF is partially attenuated by dihydropyridine calcium channel blockers and by atrial natriuretic peptide (Katoh et al., 1990). On a molar basis, ET-1 is 30 times more potent than angiotensin II and 50 times more potent than noradrenaline (Cairns et al., 1989).

Hypotheses for a role for ET in local control of blood flow in the kidney and autoregulation are: (i) an increase in blood flow may be sensed at the endothelium by shear stress receptors, which then transduce a signal to increase the rate of ET-1 secretion by endothelial cells. Endothelin-1 would then diffuse to nearby vascular smooth muscle cells, bind to specific receptors and induce a phospholipase C-dependant signalling cascade to initiate contraction of the blood vessel via an excitation-contraction coupling, signal transduction process. The resultant increase in vasomotor tone would oppose the increase in blood flow, and function as a myogenic mechanism of autoregulation. Secretion of ET by endothelial cells would counteract the vasodilatory signals released by endothelial cells, such as endothelium derived relaxing factor (EDRF), kinin-forming enzymes and prostacyclin. Thus, ET-1 would function in concert with other endothelium-derived vasoactive factors to produce regional regulation of blood flow; (ii) a second hypothesis is that ET contributes to tubulo-glomerular feedback in response to increased NaCl delivery at the macula densa by inhibiting renin secretion (Simonson, 1993).

1.1.2.5 EFFECTS ON GFR AND GLOMERULAR FUNCTION

1.1.2.5.1 Endothelin and GFR

Single nephron GFR (SNGFR) is the product of net filtration pressure (P_{uf}) across the glomerular capillary and the ultrafiltration coefficient (K_f). ET-1 acts on both variables to regulate SNGFR. Mildly pressor doses of ET-1 cause a greater increase in efferent than afferent arteriolar contraction thereby increasing P_{uf} (King et al., 1989). While this would favour filtration, a decline in K_f and a modest decline in glomerular capillary flow rate (Q_A) cause SNGFR to remain relatively constant at these low doses of ET-1. Higher doses of ET-1 cause profound increases in afferent and efferent resistance, and a decline in Q_A and K_f, thereby reducing GFR. ET-1 evokes contraction of glomerular mesangial cells via excitation-contraction coupling, suggesting that a reduction in filtering surface area might mediate the fall in K_f by ET (reviewed by Simonson, 1993).

1.1.2.5.2. Endothelin and mesangial cells

Mesangial cells are specialized microvascular pericytes located in the central region of the glomerular tuft between capillary loops. Mesangial cells help regulate GFR by controlling K_f; they also process macromolecules (including immune complexes) trapped within the mesangium. In addition, mesangial cells synthesize and assemble the mesangial matrix, which is a major determinant of the visco-elastic properties of the mesangium. Endothelins have both contractile and pro-mitogenic actions on mesangial cells, which might contribute to the glomerular response to injury. Prostaglandins help regulate contraction and mitogenesis of mesangial cells in culture. ET-1 evokes arachidonic acid release and production of PGE_2 from mesangial cells. ET-1 is a potent growth factor for mesangial cells and stimulates quiescent mesangial cells to enter G_1 and proliferate. ET-1 also

increases mesangial DNA topoisomerase-I activity by a pertussis toxin-sensitive pathway. Mesangial cells in *vivo* are normally quiescent. Mesangial proliferation is a common finding in glomerular inflammation suggesting that local overproduction of ET-1 might serve as a pro-inflammatory signal in glomerular injury. Pro-inflammatory agents such as IL-1 and TGF_{β} stimulate ET secretion in endothelial and mesangial cells, supporting this hypothesis. ET-1 induces platelet-derived growth factor (PDGF) A and B chain expression in human mesangial cells; AP-1 transcription factors probably mediate induction of the collagenase gene by ET-1 in mesangial cells (reviewed by Simonson, 1993).

1.1.2.6 EFFECTS ON SODIUM

A major function of the kidney is to regulate extracellular fluid volume (ECFV) and thus mean arterial pressure, both of which may be regulated by ET. ECFV is primarily determined by total exchangeable sodium and therefore alterations in renal sodium excretion, which represents the difference between the filtered load of sodium and the amount of sodium reabsorbed at various tubular sites. ET-1 appears to have diverse effects on renal sodium handling and the precise role of ET-1 in renal sodium excretion is unclear. Systemic infusions of ET-1 decrease sodium excretion in some studies (Goetz et al., 1988; Hirata et al., 1989; Miller et al., 1989), but in others (King et al., 1989; Garcia et al., 1990), ET-1 was modestly natriuretic despite a decline in RBF. Intrarenal infusions of ET-1 cause no change in sodium excretion, whereas higher doses decrease sodium excretion (Katoh et al., 1990; Stacy et al., 1990), suggesting that ET has an intrarenal natriuretic effect but that the influence of other extra- and intrarenal mechanisms might oppose this action.

1.1.2.6.1 Endothelin and the filtered load of sodium

ET-1 reduces GFR and would therefore be expected to reduce the filtered load of sodium, resulting in decreased sodium excretion. The haemodynamic actions of ET-1 would reduce the peritubular capillary Starling pressures, thereby leading to enhanced removal of sodium from the peritubular interstitial space into the peritubular capillaries.

1.1.2.6.2 Endothelin and the Renin-Angiotensin-Aldosterone System

The renin-angiotensin system is the chief regulator of ECFV. Angiotensin II (AII) increases proximal tubular fluid reabsorption via alterations in renal haemodynamics and by directly stimulating proximal tubule sodium reaborption. AII also stimulates adrenal aldosterone secretion, which stimulates sodium reabsorption by the late distal tubules and collecting ducts. ET-1 stimulates the apical Na^{+}-K^{+} exchanger and the basolateral Na^{+}-$HC0^{-}_{3}$ cotransporter (Eiam-Ong et al., 1992). Both the haemodynamic and proximal tubular actions of ET-1 might be similar to those of AII. ET-1 infusion increases circulating levels of aldosterone (Goetz et al., 1988; Miller et al., 1989). Systemic infusion of ET-1 sharply increases plasma renin activity (Goetz et al., 1988; Miller et al., 1989) which may reflect activation of the intrarenal baroreceptor and macula densa-mediated pathways of renin secretion. In *vitro* experiments with superfused juxtaglomerular cells, cortical slices and isolated rat glomeruli demonstrate inhibition of renin release by ET-1 (Takagi et al., 1989). The inhibitory effect of ET-1 is attenuated in the absence of extracellular calcium.

ET-1 therefore has variable effects on renin release: when ET-1 is secreted locally, it likely inhibits renin release via a calcium-dependent mechanism, resulting in decreased aldosterone secretion, thereby reducing sodium reabsorption and increasing sodium

excretion; high levels of circulating ET-1 would increase renin secretion either directly at the adrenal glomerulosa, or indirectly as a result of profound renal vasoconstriction, resulting in increased aldosterone, enhanced sodium reabsorption and decreased sodium excretion. Thus the net effect of ET-1 on renal sodium handling would depend on whether ET-1 functions as a circulating or local peptide hormone.

1.1.2.6.3 Endothelin and atrial natriuretic peptide secretion.

Atrial natriuretic peptide (ANP), released from cardiac atrial myocytes in response to atrial stretch, regulates sodium excretion by directly stimulating natriuresis through guanylate cyclase linked ANP receptors, increases GFR by enhancing the filtered load of sodium and concomitantly inhibiting sodium reabsorption at the collecting duct. ANP also indirectly decreases sodium reabsorption by inhibiting renin secretion and AII-induced aldosterone release. ET-1 infusion elevates circulating levels of ANP (Goetz et al., 1988; Miller et al., 1989). ANP is a potent vasodilatory compound and the major function of ANP secretion could be to antagonise the contractile effects of ET-1 and AII on vascular smooth muscle and on glomerular mesangial cells (Neuser et al., 1990).

1.1.2.6.4 Endothelin and sodium-potassium-ATP in the collecting duct

Systemic infusions of ET-1 (at doses that do not markedly impair GFR) produced modest natriuresis. In addition to its effect on ANP stimulation, ET might also produce natriuresis by inhibiting Na^+-K^+-ATPase activity in the medullary collecting duct (Zeidel et al., 1989), thereby reducing the electrochemical gradient favouring sodium reabsorption. ET-1-induced inhibition of Na^+-K^+-ATPase is abolished by ibuprofen, suggesting that the inhibitory action is mediated by prostanoids. Table 1-2 summarizes the possible mechanisms by which ET modifies sodium excretion by the kidney.

Table 1-2. Effects of Endothelin-1 on renal sodium regulation

Natriuretic actions of endothelin
Inhibition of renin secretion: local effect on JG cells
Increase of atrial natriuretic peptide secretion: direct action at cardiac atrial myocytes
Inhibition of Na^+-K^+-ATPase in medullary collecting ducts: mediated by prostaglandin synthesis
Anti-natriuretic actions of endothelin
Reduction in filtered load of Na^+: reduction in glomerular filtration rate
Increase in plasma aldosterone: direct effect on adrenal glomerulosa or from decreased renal haemodynamics
Possible stimulation of Na^+ reabsorption by direct action of endothelin-1 on proximal tubule
Reduction of peritubular capillary Starling gradient in favour of Na^+ reabsorption

1.1.2.7 ENDOTHELIN AND RENAL WATER REABSORPTION

Renal water reabsorption is regulated indirectly by plasma osmolality, which evokes changes in pituitary arginine vasopressin (AVP) release. In the absence of AVP, collecting ducts are highly impermeable to water. AVP acts on renal collecting duct cells to stimulate insertion of preformed water channels into their apical membrane, causing the cells to become permeable to water. This results in movement of water from the tubular lumen to the interstitium. These effects of AVP are mediated by V_2 AVP receptors with consequent activation of adenylate cyclase (reviewed by Simonson, 1993). ET-1 increases urine flow rate despite a decrease in RBF and GFR (Goetz et al., 1988; Badr et al., 1989), suggesting

that ET-1 might also regulate water reabsorption. Infusion of ET-1 into conscious dogs decreases urine osmolality despite a constant level of circulating AVP (Goetz et al., 1989). Evidence suggests that ET-1 can antagonise AVP-dependent cyclic AMP accumulation. In microdissected nephron segments, ET-1 dose-dependently (0.1-10.0nM) inhibits AVP-evoked cAMP accumulation in the cortical collecting duct, outer medullary collecting duct and inner medullary collecting duct (Tomita et al., 1990). ET-1 has no effect on AVP-dependent cAMP accumulation in other nephron segments. This inhibitory effect is independent of calcium channel activity or prostaglandin synthesis but is dependent on protein kinase C activity.

Immunoreactive ET peptides have been demonstrated in secretory vesicles in the rat posterior pituitary, and the vesicles were depleted by water deprivation (Ptashne, 1988). Therefore ET-1 secretion, stimulated by water deprivation, could reduce water excretion directly by inhibiting baroreceptor reflexes or reducing GFR.

1.1.2.8 ENDOTHELIN IN RENAL PATHOPHYSIOLOGY

Endothelins affect three major aspects of renal physiology (i) vascular and mesangial tone, (ii) cell proliferation and matrix formation and (iii) sodium and water excretion. The evidence for a pathophysiologic role of ET-1 in renal dysfunction is reviewed.

1.1.2.8.1 Ischaemic acute renal failure

This is commonly precipitated by an initial episode of severe renal ischaemia followed by several days or weeks of the kidneys failing to function normally. Reduction of renal blood

flow is the hallmark of this maintenance phase of ischaemic acute renal failure. Increased tissue concentrations of ET-1 during the initial 24 hours of the maintenance phase (Shibouta et al., 1990), a 5-10 fold increase in ET-1 mRNA in glomeruli and inner medullary collecting ducts (Terada et al., 1992) and in renal tissue homogenate (Firth and Ratcliffe, 1992) during the first 48 hours, suggest a role for ET-1 in this phase of ischaemic acute renal failure. Elevated plasma ET-1 concentrations have been reported in patients with acute renal failure (Nakanishi et al., 1990). Beneficial effects of infusions of anti-ET antibody have been described on functional (Kon et al., 1989; Shibouta et al., 1990; Lopez-Farre et al., 1991), histologic and biochemical parameters (Shibouta et al., 1990) of the kidney. Anti-ET antibody treatment during the maintenance phase caused a fall in elevated afferent and efferent arteriolar resistances, improved glomerular plasma flow and increased single nephron filtration rate (Kon et al., 1989); however normal control values were not re-established by this treatment. Subsequent studies in rats and dogs have shown that administration of ET receptor antagonists ameliorated impaired GFR, RBF and sodium excretion following transient renal artery or abdominal aortic occlusion (Stingo et al., 1993; Brooks et al., 1994; Chan et al., 1994; Gellai et al., 1994, 1995; Krause et al., 1995; Kusumoto et al., 1994). Endothelin-1 production and binding were increased in ischaemic kidneys (Firth and Ratcliffe, 1992; Nambi et al., 1993). Increased ET-1 production may be due to hypoxia and oxygen radical production. Renal ischaemia causes tissue hypoxia which can increase renal ET-1 production. When renal perfusion is restored, there is an increase in reactive oxygen species due to metabolic impairment induced during ischaemia. These oxygen radicals (especially hydrogen peroxide) may augment ET-1 production (Hughes et al., 1996). Hence, once the initial ischaemic event is resolved, prolonged release of ET-1 may perpetuate a reduced RBF and GFR.

1.1.2.8.2 Nephrotoxic acute renal failure

Endothelin-1 may be involved in the pathogenesis of many toxic nephropathies, including that due to cyclosporine A (CyA), radiocontrast and endotoxaemia.

1.1.2.8.2.1 Cyclosporine

Endothelin-1 mediates acute CyA-induced renal vasoconstriction. Cyclosporine and related immunosuppressants such as Tacrolimus (FK506) directly stimulated ET-1 release from mesangial and /or endothelial cells (Langman and Yatscoff, 1994; Goodall et al., 1995; Kohno et al., 1995). Cyclosporine A increased renal ET-1 mRNA expression (Iwasaki et al., 1994). Anti-ET antibodies and ET receptor antagonists ameliorated acute CyA-induced renal vasoconstriction (Kon and Awazu, 1992; Lanese and Conger, 1993; Conger et al., 1994; Brooks and Contino, 1995; Kon et al., 1995). Salt-depleted rats given CyA daily for 5 weeks developed a reduced GFR and tubulo-interstitial fibrosis. The decrease in GFR was ameliorated by concurrent administration of an ET_A/ET_B receptor antagonist, while fibrotic changes were unaltered (Kon et al., 1995), suggesting that ET-1 is a mediator of the vasoconstrictor but not fibrotic effects of CyA.

1.1.2.8.2.2 Radiocontrast nephropathy

An increased risk of radiocontrast-induced acute renal failure is well known in the setting of the elderly patient suffering from diabetes mellitus, hypertension, arteriosclerosis and/or reduced renal function. Endothelin-1 stimulation has been observed with both ionic and non-ionic radiocontrast agents (Nakayama et al., 1991; Obialo et al., 1991). Decrease in renal blood flow by a third was demonstrated in rats following radiocontrast administration; however simultaneous infusion of the ET antagonist CP170687 partially prevented this (Cantley et al., 1992). Endothelin-1 receptor antagonists markedly inhibited

the decrease in renal perfusion induced by radiocontrast agents (Oldroyd et al., 1995). If prostaglandin formation is unimpaired, both the vasoconstrictive response to the radiocontrast and the degree of protection offered by ET receptor blockade are greatly reduced (Cantley et al., 1993).

1.1.2.8.2.3 Bacterial toxins

Gram-negative bacterial sepsis and endotoxaemia cause systemic vasodilatation and renal vasoconstriction. The endotoxin lipopolysaccharide directly stimulated endothelial cell ET-1 release and increased renal tissue levels of ET-1 (Nambi et al., 1994; Kaddoura et al., 1996). Anti-ET-1 antibodies or ECE-blockade ameliorated the renal vasoconstrictor effects of endothelin in this condition (Morise et al., 1994). Nonspecific ET receptor blockage may further reduce systemic blood pressure in septic shock, suggesting that ET-1 protects against lipopolysaccharide-induced hypotension (Kohan, 1997). Urinary ET-1 was elevated in children with post-diarrhoeal haemolytic-uraemic syndrome, while serum from these children increased endothelial cell ET-1 production (Kohan et al., 1994). Purified Shigella toxin stimulated ET-1 release by human glomerular endothelial cells (Kohan, 1997). Endothelin-1 also increased von Willebrand factor release, induced micro-thrombosis and caused a consumptive coagulopathy (Halim et al., 1994; Schulz et al., 1995). These studies suggest that ET-1 is involved in the pathogenesis of post-diarrhoeal haemolytic-uraemic syndrome.

1.1.2.8.3. Miscellaneous disorders

The following disorders are associated with increased renal ET-1 production: nephrotoxicity due to amphotericin B and cisplatin, glycerol-induced myoglobinuric renal failure, hepatorenal failure (Kohan, 1993; Karam et al., 1995), obstructive uropathy (Klahr

et al., 1991; Kelleher et al., 1992), acute vascular renal transplant rejection (Watschinger et al., 1991), renal vasculitides (Kanno et al., 1990), erythropoietin-induced hypertension (Sekino et al., 1991) and hypertensive disorders of pregnancy (Kamoi et al., 1990).

1.1.2.8.4 Proliferative glomerular disease

There is evidence to suggest that ET-1 is involved in the pathogenesis of proliferative glomerulonephritis. Renal ET-1 production was increased in experimental and human glomerulonephritis (Murer et al., 1994; Roccatello et al., 1994; Nakamura et al., 1995a; Yoshimura et al., 1995), while ET_B receptor expression was upregulated in glomerulonephritis in the rat (Yoshimura et al., 1995). ET-1 is a potent mitogen and partly mediated the proliferative effects of several cytokines (Bakris and Re, 1993; Kohno et al., 1994; Nitta et al., 1995). Inflammatory cytokines and proteinuria per se augmented renal ET-1 production (Zoja et al., 1995). ET-1 may activate and be chemotactic for monocytes, which can in turn secrete ET-1 (Martin-Nizzard et al., 1991; Achmad and Rao, 1992). ET receptor antagonists reduced mesangial cell proliferation in experimental mesangial proliferative glomerulonephritis (Fukuda et al., 1996) and decreased renal injury in murine lupus nephritis (Nakamura et al., 1995b). Renal ET-1 mRNA levels were elevated in polycystic kidney disease (Nakamura et al., 1993; Hocher et al., 1998).

1.1.2.8.5 Renal fibrosis

Endothelin-1 also contributes to excessive accumulation of extracellular matrix components and fibrosis by increasing renal cell fibronectin and collagen production, tissue inhibitor of metalloprotease levels and the release of cytokines that stimulate matrix accumulation (Ong et al., 1994; Ruiz-Ortega et al., 1994). ET-1 antagonism decreased matric accumulation in experimental models of glomerulonephritis (Fukuda et al., 1996).

Chronic treatment with an ET receptor antagonist attenuated increases in glomerular mRNA levels of collagen, laminin, tumour necrosis factor, TGF-β, PDGF and basic fibroblast growth factor in diabetic rats (Nakamura et al., 1995c). Once substantial renal scarring occurs, there is an inevitable progression to end stage kidney disease, a process involving gradual glomerular sclerosis and interstitial fibrosis. Endothelin receptor blockade reduced proteinuria and glomerulosclerosis and protected against hypertension and elevations in serum creatinine in the 5/ 6 nephrectomy rat model (Benigni et al., 1993 and 1996).

1.1.2.8.6 Renal endothelin-1 in essential hypertension

Alterations in ET-1 in the renal vasculature and renal tubules have differing effects on blood pressure. In the vasculature, increases in ET-1 predominantly cause vasoconstriction with a hypertensive effect. Increased ET-1 in the nephron probably enhances sodium and water excretion, favouring hypotension (reviewed by Markewitz and Kohan, 1995; Schiffrin, 1995). Renal vascular responsiveness to ET-1 in animal models of essential hypertension is controversial with both enhanced and unchanged renal vascular responsiveness reported. Whole kidney ET-1 expression has been reported to be increased, unchanged or decreased in hypertensive animals in different studies. Infusion of anti-ET antibodies into spontaneously hypertensive rats (SHR) resulted in a 50% increase in RBF while having no effect on Wistar Kyoto (WKY) rats (Ohno et al., 1992). ET_A receptor blockade increased RBF in SHR and DOCA-salt hypertensive rats but not in their normotensive controls (Fujita et al., 1996). Thus evidence suggests that enhanced intrarenal ET-1 activity augments renal vasoconstriction in animals with genetic hypertension. Urinary ET-1 excretion was markedly reduced in patients with essential hypertension (Hoffman et al., 1994). ET-1 production was much lower in inner medullary collecting duct

(IMCD) cells and in the outer and inner medulla from hypertensive SHR rats compared with WKY controls (Hughes et al., 1992). Bosentan, a mixed ET_A/ET_B receptor antagonist, administered to patients with mild-moderate essential hypertension was as effective as enalapril (inhibitor of angiotensin converting enzyme) in controlling blood pressure, suggesting that ET-1 contributes to hypertension in these patients (Krum et al., 1998).

1.1.2.8.7 Chronic renal failure and hypertension

Plasma ET-1 levels are frequently elevated in patients with chronic renal failure or on dialysis (Shichiri et al., 1990;Warrens et al., 1990; Stockenhuber et al., 1992). Further fractionation by gel permeation chromatography revealed borderline increased plasma ET-1 with marked elevations of big ET-1 and other large forms of ET, with an increase of about 500% above control (Saito et al., 1991). Although plasma ET-1 levels correlate with the degree of hypertension in chronic renal failure patients, this observation has not been uniformly noted. The kidney is a major site of ET-1 metabolism (Abassi et al., 1992); plasma ET-1 half-life was increased in bilaterally nephrectomised rats (Kohno et al, 1989). Dialysis may elevate ET-1 levels. The type of dialysis membrane may influence plasma ET-1 levels (Niwa et al., 1993; Ross et al., 1993); plasma ET-1 levels were reduced when high flux membranes are used. Thus patients with chronic renal failure or end stage renal disease have borderline elevated plasma ET-1 levels due partly to reduced ET-1 clearance while intrarenal generation of ET-1 may be increased.

1.1.2.8.8 Renal transplantation

Plasma ET-1 levels were reported to be elevated in predialysis patients with chronic renal failure and in patients on regular haemodialysis; plasma ET-1 levels were normal in stable renal transplant patients treated with CyA (Stockenhuber et al., 1992). An association

between increased plasma ET-1 levels and CyA was first suggested in a renal transplant patient receiving high doses of CyA (Fogo et al., 1990). After administration of CyA, plasma ET-1 levels increase transiently in transplant patients because ET-1 is rapidly metabolised and cleared from the systemic circulation (Grieff et al., 1993). The inflammatory process of acute cellular rejection is predominantly confined to the interstitium and marked intragraft upregulation of ET-1 may occur without significant changes in ET-1 plasma concentrations (Watschinger et al., 1995), unless there is endothelial damage as occurs with vascular rejection (Watschinger et al., 1994). Activated mononuclear cells that infiltrate the allograft secrete a variety of cytokines (Blancho et al., 1993; Halloran et al., 1993; Hancock 1984), that have been shown to influence the production of ET-1 by other cells in *vitro*. Tumour necrosis factor α increased ET-1 mRNA and ET-1 release in capillary endothelial cells, epithelial cells and rat mesangial cells (Kohan 1991); IL-1β induced ET-1 release from renal epithelial cells (Ohta et al., 1990). Platelet activation and intravascular coagulation (attributable to endothelial injury) appeared to trigger the release of TGFβ, thromboxane A_2, PDGF and thrombin from platelets accumulating in the graft. This resulted in ET-1 secretion and upregulation of ET-1 gene expression in endothelial, vascular smooth muscle and renal mesangial and epithelial cells (Kurihara et al., 1989; Watschinger and Sayegh, 1996). Renal allografts with chronic rejection and transplant-associated arteriosclerosis were reported to express 6-fold more ET-1 in the neointima of the vasculature, when compared to allografts with acute rejection or normal control kidneys (Simonson et al., 1998).

1.1.3 ATRIAL NATRIURETIC PEPTIDE IN THE KIDNEY

Following the observation by de Bold and colleagues (1981) that infusion of extracts of atrial tissue caused a copious diuresis, atrial natriuretic peptide, the first member of a family of peptides with potent natriuretic, diuretic and vasorelaxant activity was isolated and later cloned (Kangawa and Matsuo, 1984).

1.1.3.1 BIOCHEMISTRY

Three peptides comprise the natriuretic peptide family: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide [(CNP) Fig 1.3-1]. ANP is produced primarily from cardiac atria, with increased atrial wall tension (reflecting increased intravascular volume) as the major stimulus. Various substances for example endothelin, arginine vasopressin and catecholamines, directly stimulate the secretion of ANP. The precursor protein (pro ANP) is composed of 126 amino acids; cleavage releases a 98 amino acid amino-terminal fragment and a 28 amino acid carboxy-terminal portion that is the mature ANP (Fig 1.3-2). Both fragments circulate in the plasma and their concentrations are increased in patients with increased intravascular volume (for example, congestive heart failure). The ANP gene is also expressed in the kidney and a 32-amino acid peptide called urodilantin is generated from the precursor in the urine (Schulz-Knappe et al., 1988). Urodilantin may be important for the local regulation of sodium and water handling in the kidney.

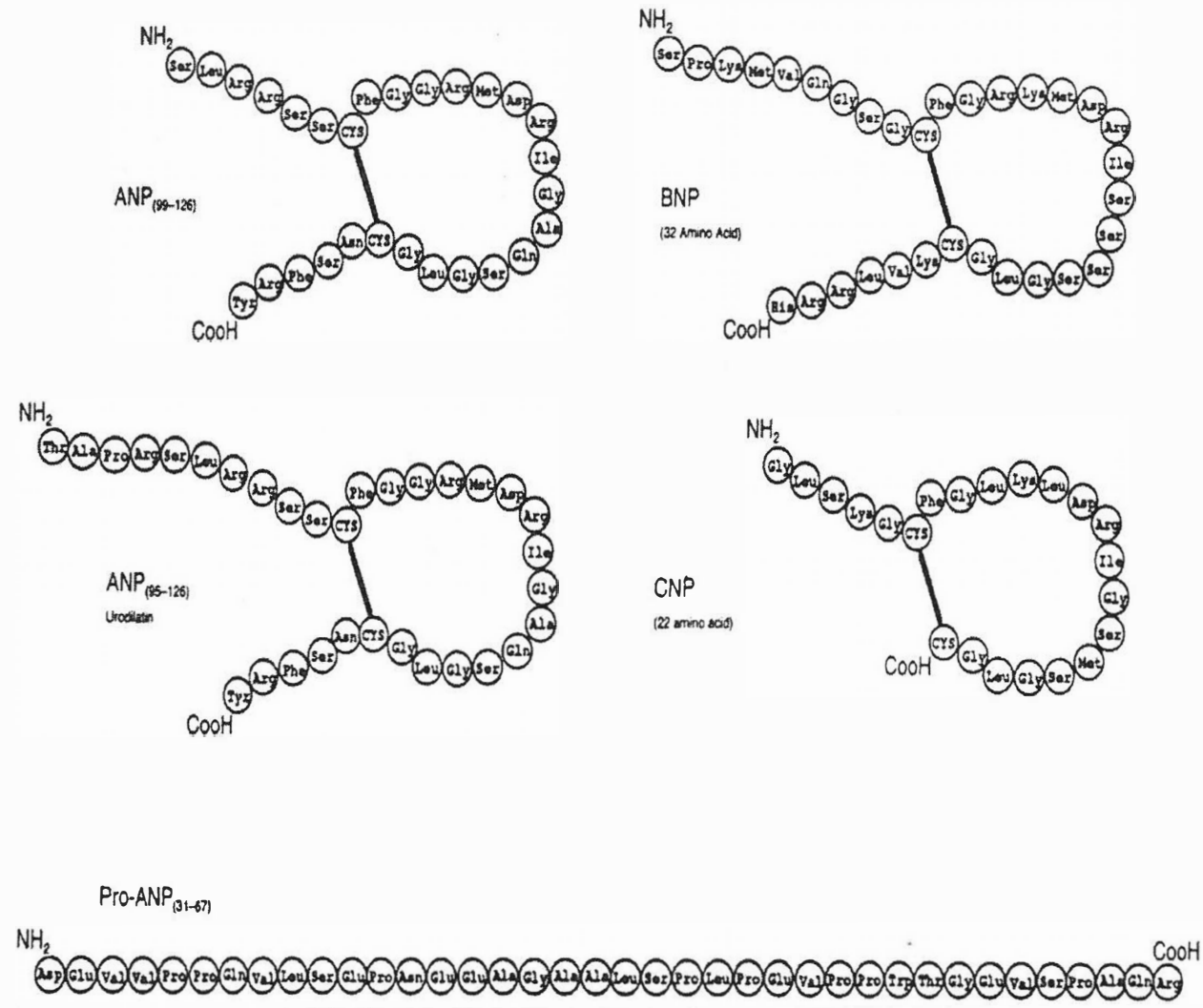


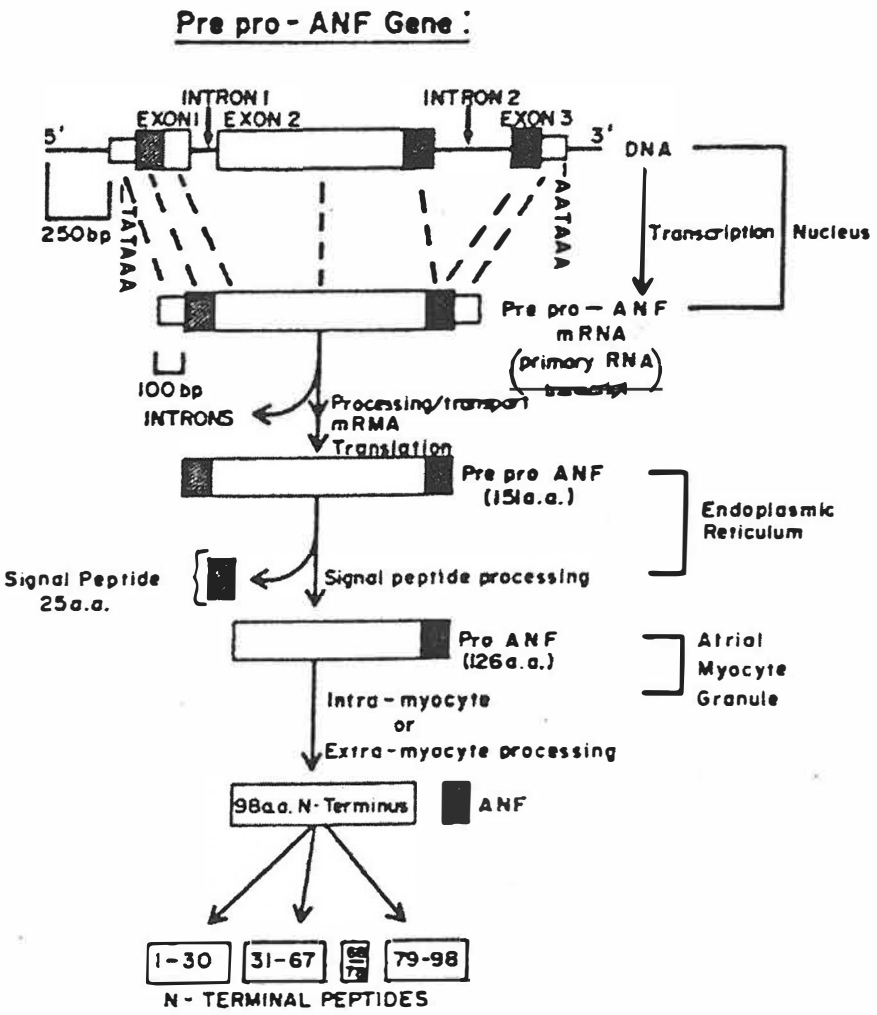
Fig 1.3-1: Amino acid sequence of the natriuretic peptides in humans

Ref: Gunning M E and Brenner B M (1992), Kidney Int, 42: S127-133 (S128)

In the kidney, ANP has been localised to the renal artery wall (Koseki et al., 1986), glomeruli (Koseki et al., 1986), descending vasa recta (Koseki et al., 1986; Naruse et al., 1988), inner medullary collecting tubule (Koseki et al., 1986; Bianchi et al., 1987) and distal convoluted tubular cells, intercalated cells of the connecting tubules and collecting ducts (Figueroa et al., 1990). BNP, a 32-amino acid molecule which is released from a 108 amino acid precursor, is present in brain and cardiac ventricles. It circulates in the plasma and concentrations are increased in patients with ventricular hypertrophy or congestive

heart failure (Levin et al., 1998). Two C-type natriuretic peptides (22 and 53 amino acids in length) have been identified. The 22 amino acid peptide predominates in the central nervous system, anterior pituitary, kidney and vascular endothelial cells. Its concentration in plasma is very low. Other related peptides, guanylin and uroguanylin, are 15- and 16-amino acid peptides produced primarily in the gastro-intestinal tract. They may regulate salt and water transport across the intestinal mucosa and co-ordinate intestinal reabsorption with renal excretion of sodium.

Fig 1.3-2: Processing pathway of ANP



Ref: Vesely D L (1994). Kidney: A Current Survey of World Literature, 3: 4-8 (5)

1.1.3.2 NATRIURETIC PEPTIDE RECEPTORS

Natriuretic peptides exert their effects through interaction with high-affinity receptors on the surface of target cells. Natriuretic peptide receptors A and B are linked to the cGMP-dependent signalling cascade, and mediate many of the cardiovascular and renal effects by activation of guanylylcyclase. The A receptor binds both ANP (preferentially) and BNP; the B receptor binds C-type natriuretic peptide. The A receptor is the most abundant type in large blood vessels (which also have some B receptors). B receptors predominate in the brain. Both receptors are present in adrenal glands and the kidney.

All three peptides bind with equal affinity to the natriuretic peptide receptor C, which is involved in clearance of the peptides. The natriuretic peptides bind to it, are internalized and enzymatically degraded, after which the C receptor returns to the cell surface (Maack et al., 1987). Circulating natriuretic peptides are also inactivated by cleavage by neutral endopeptidases present within renal proximal tubular cells and vascular cells. The plasma half-life of immunoreactive ANP is 2.5-4.5 min. Multiple organs (liver, kidney and lower limb) extract ANP from the blood. The kidneys clear approximately 20% of the total ANP (Vierhapper et al., 1990). The A receptor is expressed in the kidney, lung, heart and adipose tissue with its mRNA expressed in the renal glomerulus, adrenal zona glomerulosa, anterior pituitary, cerebellum and heart. The B receptor is present in the brain, lung, kidney, placenta and heart. Its mRNA is detected in the adrenal medulla, cerebellum and pituitary gland. The C receptor is abundantly present in the renal cortex, lung, placenta and heart; its mRNA is distributed in the renal glomerulus, adrenal gland, cerebral cortex, cerebellum and heart (reviewed by Nakao et al., 1996).

1.1.3.3 ACTIONS OF NATRIURETIC PEPTIDES

1.1.3.3.1 Renal Actions

The natriuretic and diuretic actions of natriuretic peptides are due to both renal haemodynamic and direct tubular actions. ANP causes dilatation of afferent arterioles and constriction of efferent arterioles, resulting in increased pressure within the glomerular capillaries (Marin-Grez et al., 1986). This causes a rise in GFR. ANP also increases cGMP in mesangial cells, relaxing these cells and thereby increasing the effective surface area for filtration (Fried et al., 1986; Stockand and Sansom, 1997). However, plasma concentrations of ANP that do not increase GFR cause natriuresis, indicating that ANP has direct tubular actions for example by locally produced peptides (such as urodilantin) or by systemic ANP. ANP can inhibit angiotensin II-stimulated sodium and water transport in proximal convoluted tubules (Harris et al., 1987). It inhibits tubular water transport in collecting ducts by antagonizing the action of vasopressin (Dillingham and Anderson, 1986), and stimulates cGMP production in the inner medullary collecting duct, blocking sodium reabsorption (Sonnenberg et al., 1986; Zeidel, 1995).

Infusions of ANP, that raise their plasma concentrations slightly above normal, result in diuresis and natriuresis without changes in blood pressure; they reduce plasma renin and aldosterone concentrations and inhibit angiotensin II-stimulated aldosterone secretion. HS-142-1, a competitive natriuretic peptide antagonist binding to receptor A or B, blocks natriuresis and diuresis, increases renal vascular resistance and increases plasma renin, aldosterone and catecholamine concentrations in normal, diabetic and cirrhotic rats with ascites (Sano et al., 1992; Angeli et al., 1994; Zhang et al., 1994).

In summary, ANP affects many aspects of renal function. It increases renal blood flow, and by selective action on glomerular arterioles, increases glomerular capillary hydraulic pressure and GFR. Glomerular ultrafiltration coefficient is also increased via inhibition by ANP of the actions of angiotensin II and ADH. In the proximal tubule, ANP opposes angiotensin II-mediated sodium reabsorption and in the distal tubule, aldosterone-mediated sodium reabsorption. In the collecting duct, ANP inhibits ADH-mediated water reabsorption and directly inhibits inner medullary collecting duct sodium transport. These effects, together with increased vasa recta blood flow and hydraulic pressure, favour increased excretion of sodium from the renal medullary interstitium into the urine. ANP directly inhibits renin secretion by the juxtaglomerular cells and aldosterone secretion by the adrenal zona glomerulosa.

1.1.3.3.2.Cardiovascular actions

Sustained low dose infusions in animals reduce peripheral vascular resistance (PVR) and lower blood pressure (Charles et al., 1993) but high doses increase PVR despite the decrease in blood pressure (Lappe et al., 1985). The decrease in blood pressure results partly from a reduction in cardiac preload caused by shift of fluid from the intravascular into the extravascular compartment. ANP increases venous capacitance and promotes natriuresis and also reduces sympathetic tone in the peripheral vasculature. CNP is a more potent dilator of veins than the other two peptides.

Natriuretic peptides have anti-mitogenic activity: ANP and CNP inhibit mitogenesis in cultured vascular cells and in balloon-injured carotid arteries in rats (Itoh et al., 1990;

Furuya et al., 1993), thereby modulating growth within the vascular wall in disorders such as atherosclerosis, hypertension and post-angioplasty restenosis.

1.1.3.3.3 Actions on the Central Nervous System

ANP and BNP do not cross the blood-brain barrier but they reach sites in the CNS outside this barrier for example the subfornical organ, hypothalamic median eminence and area postrema. All 3 natriuretic peptides (but especially CNP) are produced in the brain. Pressor substances for example endothelin, vasopressin and norepinephrine stimulate the release of ANP from cultured hypothalamic neurons. Natriuretic peptides in the brain inhibit the appetite for salt and water drinking, and inhibit the secretion of vasopressin and corticotrophin, thus co-ordinating central and peripheral actions in controlling fluid and electrolyte homeostasis.

1.1.3.4 ANP AND RENAL PATHOLOGY

1.1.3.4.1 Hypertension

Levels of ANP and BNP are elevated in hypertension, with a positive correlation between the severity of hypertension and degree of peptide elevation (Cheung and Brown, 1994). ANP immunoreactivity was reduced in hypertensive nephrosclerotic distal tubules, probably as a result of reduced renal tubular mass (Figueroa et al., 1990). Upregulation of the A receptor subtype occurred in hypertensive rats, together with increased levels of ANP and BNP (Yoshimoto et al., 1995). Restriction fragment length polymorphism exists in the second intron of the human ANP gene, a candidate gene for familial susceptibility to hypertension (Ramasawmy et al., 1994). Furthermore, restriction fragment length

polymorphism at the ANP gene locus has been reported in hypertension associated with aldosterone-producing adenoma (Tunny et al., 1994). Animal studies have defined the role of natriuretic peptides in preventing the development of hypertension: transgenic mice, that showed over-expression of the genes for ANP or BNP, had plasma natriuretic peptide levels that were at least 10 times that of normal litter mates, and systolic blood pressures that were 20-30 mmHg lower. Mice with homozygous inactivation of the ANP gene on a low salt diet had slightly elevated blood pressures; it rose markedly on a high salt diet. Heterozygotes (with a normal basal blood pressure) had a similar response. Mice with inactivation of the A receptor had an elevated basal blood pressure but did not respond to salt loading with increases in blood pressure (John et al., 1995). During aldosterone hypersecretion or exogenous mineralocorticoid administration, sodium is retained for a few days, after which there is an escape from sodium-retention; plasma ANP concentration rises, coincident with escape (Yokota et al., 1994). Administration of ANP reduced BP and promoted sodium excretion in patients with essential hypertension (Weder et al., 1987).

1.1.3.4.2 Nephrotic syndrome

Endogenous plasma ANP levels were increased in nephrotic syndrome (Woolf et al., 1989). A blunted natriuretic response, observed during head-out water immersion studies in nephrotic syndrome patients with avid sodium retention, suggests a relative hypo-responsiveness of the distal tubule to ANP (Peterson et al., 1988). Exogenous ANP caused an increase in proteinuria in patients with nephrotic syndrome (Zietse and Schalekamp, 1988), which may lead to further sodium retention.

1.1.3.4.3 Acute renal failure

Plasma levels of ANP and its second messenger cGMP were elevated during acute renal failure and tended to return towards normal values at recovery. ANP and cGMP correlated significantly with total blood volume and the fractional excretion of sodium (Kanfer et al., 1989). In a randomised controlled study of 53 patients, ANP administered parenterally to patients with acute renal failure due to acute tubular necrosis resulted in a significant improvement in GFR and reduced the need for dialysis (Rahman et al., 1994a). These results were not substantiated in a larger multicentre, randomized double-blind, placebo-controlled trial of anaritide (ANP) in 504 critically ill patients with acute tubular necrosis (Allgren et al., 1997). Prophylactic use of Ularitide (previously called Urodilantin), a natriuretic peptide recovered from human urine, resulted in significant reduction in requirement for haemodialysis or haemofiltration in patients with acute renal failure following cardiac surgery or organ transplantation (Meyer et al., 1996).

1.1.3.4.4 Chronic renal failure

Plasma concentrations of all three natriuretic peptides were elevated in chronic renal failure, probably because of reduced clearance (Prins et al., 1996). Infusion of doses of ANP slightly above the physiological range in patients with moderate chronic renal failure secondary to glomerulonephritis resulted in a natriuretic response similar to that of normal controls, together with a marked increment in the urinary excretion of urea, potassium and phosphate (De Nicola et al., 1997). The elevated levels of ANP in patients with chronic renal failure were significantly lowered by haemodialysis (Hasegawa et al., 1986; Niwa et al., 1993), in particular by haemodialysis with fluid removal or haemofiltration (Shiota et al., 1990). Plasma concentrations of ANP were not generally elevated in children on

peritoneal dialysis; in some children with elevated ANP levels, plasma ANP concentrations declined to normal values with fluid withdrawal (Bald et al., 1994).

1.1.3.4.5 Renal transplantation

ANP concentrations (elevated before transplantation) decreased after successful and increased after failed renal transplantation (Zuber et al., 1993). Studies in renal transplant recipients (Bricker et al., 1956), and in subjects with autonomic failure (Gill and Bartter, 1966) suggest that renal denervation impairs sodium conservation in the presence of dietary sodium restriction. Renal sympathetic nerve activity profoundly affects all aspects of renal function. Efferent sympathetic nerve stimulation increases tubular sodium and water reabsorption and produces a fall in GFR and renal blood flow mediated by preglomerular vasoconstriction (Kopp and Di Bona, 1982). Head-out water immersion studies in stable renal transplant recipients showed a diuretic and natriuretic response with elevation in plasma ANP and increased urinary tissue kallikrein excretion; plasma renin activity did not suppress with water immersion, probably as a result of a reduction in sympathetic nerve traffic to the juxtaglomerular apparatus (Al-Haidary et al., 1990). Low dose ANP infusion to stable renal transplant recipients resulted in immediate natriuresis and urinary cyclic GMP excretion as well as albuminuria, in contrast to the delayed response seen in normal subjects (Lipkin et al., 1992). Intravenous infusion of ANP during acute renal allograft rejection in a canine model resulted in increased urine flow rates and an increase in GFR, together with a fall in mean arterial pressure in the presence of an unchanged haematocrit (Lewis et al., 1993).

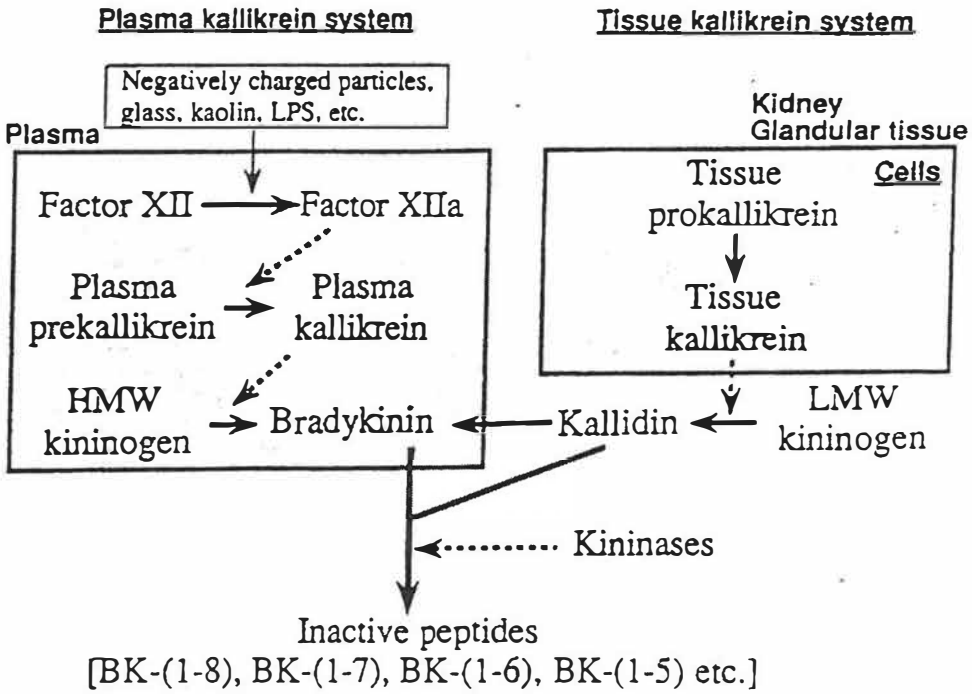
1.1.4 KININS IN THE KIDNEY

The early indication of tissue kallikrein in urine became evident when Abelous and Barder (1909) detected the presence of a hypotensive substance in normal human urine that they called urohypotensin. Further experiments on the kinin-forming enzymes commenced in 1926 when Frey and colleagues reported that injections of pancreatic extract, pancreatic secretion and urine into anaesthetized, normotensive dogs reduced arterial blood pressure (Frey, 1926; Frey et al., 1930; Frey and Werle, 1933). The active principle in urine was assumed to be identical with that in the pancreas, and therefore the hypotensive substance was named kallikrein from the Greek word "kallikreas" for pancreas. A decade later Rocha e Silva and colleagues (1949) found that incubating dog plasma with snake venom and trypsin produced an agent that lowered blood pressure, and *in vitro* caused a slowly developing contraction of guinea pig ileum. This peptide agent was named bradykinin from the Greek word "slow moving" because of the slow contraction of the guinea pig ileum. The vasoactive peptides formed by kallikrein (kallidin) and trypsin (bradykinin) from endogenous protein substrates (kininogens) were given the generic name of kinins.

In humans there are two kinin-forming enzymes, namely plasma and tissue (glandular) kallikrein and two substrates, high and low molecular weight, H-kininogen (HK) and L-kininogen [(LK); Fig 1.4-1]. Plasma prekallikrein (PPK), a cofactor of coagulation, is activated by factor Xlla to form plasma kallikrein (PK), which in turn, cleaves bradykinin (BK) from H-kininogen. Tissue kallikrein (TK), on the other hand, is either released mainly in its active form or requires to be proteolytically cleaved to an active state. Tissue kallikrein cleaves lysyl-BK (kallidin) from L-kininogen (Fig 1.4-2). The regulatory role of

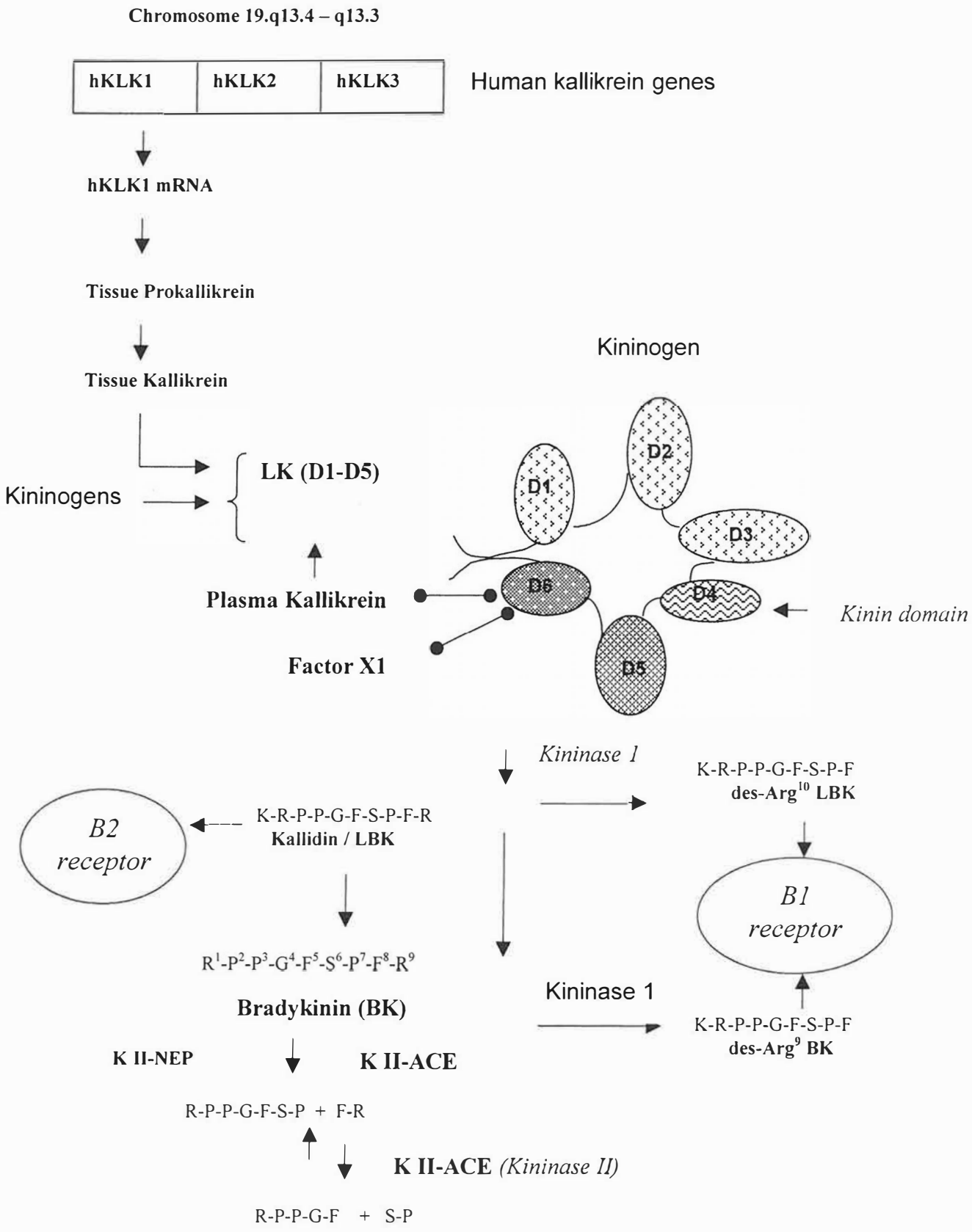
plasma and tissue kallikrein-kinin systems is different and functions independently of each other in *vivo*. The proportion in active forms varies in the different biological fluids.

Fig 1.4-1 The two kinin release systems involving plasma and tissue kallikrein



Kinins bind to their receptors at target organs and exert potent effects in vasodilatation, blood pressure reduction, vascular permeability, smooth muscle contraction, pain generation, natriuresis, diuresis and renal blood flow. The systemic half-life of kinins is very short (15-30 sec); however concentrations in biological fluids although low (10^{-11} mmol) in human plasma, have a longer half life. The binding capacity of tritiated BK along the nephron of the rabbit is maximal at cortical collecting ducts and outer medullary ducts (Kauker, 1980; Tomita and Pisano, 1984).

Fig 1.4-2: Overview of the kallikrein-kininogen-kinin- cascade



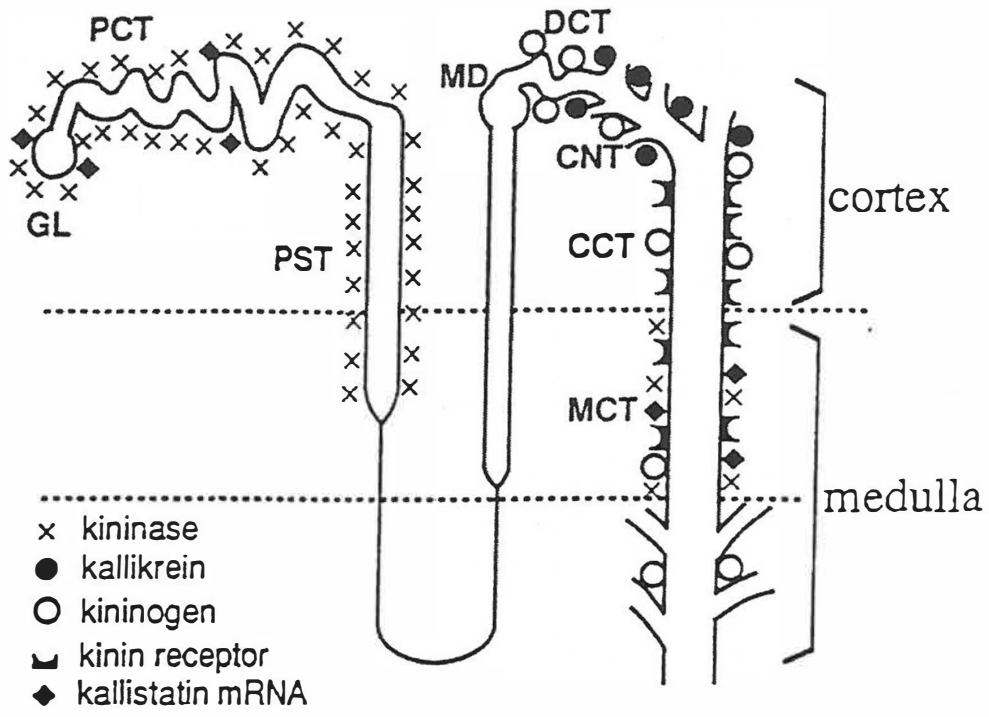
1.1.4.1 RENAL TISSUE KALLIKREIN

Tissue kallikrein is present in the plasma but the organ or tissue source is not known (Nustad et al., 1979; Geiger et al., 1980). The activity of TK is regulated by inhibitors present in plasma (Lawton et al., 1981) and urine. In spite of such inhibition, kinins are generated but in negligible measurable amounts (Scicli et al., 1982 and 1983).

1.1.4.1.1 Localisation

More than 85% of the active and inactive tissue kallikrein in the rat kidney is localised in the granular portions of the distal tubules and cortical collecting ducts [Tomita et al., 1981; Omata et al., 1982; Vio and Figueroa, 1985; (Fig 1.4-3)]. Kallikrein is present in the granular cells of the connecting tubule of the distal nephron, where it is concentrated mainly on the luminal side of the cells and at both sides of the nuclei, and to a lesser extent, is associated with plasma membranes and basolateral infoldings. The immunoreactivity is related to free polyribosomes, rough endoplasmic reticulum and Golgi complexes, suggesting that kallikrein is actively synthesised in these cells (Figueroa et al., 1984; Vio and Figueroa, 1985). Tissue kallikrein mRNA is expressed predominantly in the cells of the distal tubules but reported also in the vascular pole of the glomeruli (Xiong et al., 1989), and in the connecting tubules of the outer cortex (El-Dahr and Chao, 1992). Tissue kallikrein mRNA and protein are present in the walls of renal blood vessels (Cumming et al., 1994).

Fig 1.4-3: Localisation of components of the renal kallikrein kinin system



Ref.: Katori M and Majima M (1996). Japanese Journal of Pharmacology, 70: 95-128 (99). Abbreviations: GL=glomerulus; PCT=proximal convoluted tubule; DCT=distal convoluted tubule; PST=proximal straight tubule; MD=macula densa; CNT=connecting tubule; CCT=cortical collecting tubule; MCT=medullary collecting tubule

1.1.4.1.2 Stimuli for renal tissue kallikrein secretion

Renal perfusion pressure may be one of the major factors controlling urinary kallikrein excretion (Bevan et al., 1974; Lauar et al., 1982; Bhoola and Lauar, 1983; Misumi et al., 1983). A low sodium diet or salt deprivation accelerated renal kallikrein synthesis and excretion in humans (Geller et al., 1972; Bascands et al., 1987). The effects of a high sodium intake are controversial: acute sodium loading in rats induced an increase in urinary kallikrein excretion (Marin-Grez et al., 1984). Feeding rats a high salt diet for 10 days decreased the total immunoreactive kallikrein in the urine and kidney (Lieberthal et al., 1983). The increased kallikrein excretion due to prolonged sodium deprivation may be

mediated by aldosterone release through activation of the renin-angiotensin system and was reversed by spironolactone (Margolius et al., 1974a).

Experiments with isolated perfused rat kidney demonstrated the stimulatory effect of potassium on the secretion of tissue kallikrein (Lauar et al.,1982; Lauar and Bhoola, 1986). Patients with hyperaldosteronism excreted higher amounts of kallikrein in the urine (Margolius et al., 1972 and 1974b). Administration of spironolactone to patients with hyperaldosteronism decreased the high urinary kallikrein excretion (Margolius et al., 1974b). Removal of aldosterone-producing tumours reversed the increased excretion of urinary kallikrein (Miyashita, 1971). Urinary kallikrein excretion varied directly with potassium intake and parallelled aldosterone excretion in both normal and hypertensive subjects. The increase in urinary kallikrein in hypertensive subjects by potassium intake was less than that in normotensive subjects; the increase in white subjects was higher than in black subjects (Horwitz et al., 1978). Electron microscopy showed that a high potassium diet produced hypertrophy and hyperplasia of the kallikrein-containing cells of the connecting tubule, including hyperplasia of the Golgi complex and rough endoplasmic reticulum and secretory vesicles containing kallikrein (Vio and Figueroa, 1987). Intravenous infusion of vasopressin stimulated both the release of urinary kallikrein and the intrarenal formation of kinin in dogs and rats (Fejes-Toth et al., 1980). Other hormones affecting synthesis, activity or release of renal kallikrein are oestrogens, mineralocorticoids, glucocorticoids, testosterone, thyroxine, insulin, catecholamines and angiotensin (Bhoola et al., 1992; Margolius, 1995).

1.1.4.1.3 Gene expression of tissue kallikrein

Tissue kallikrein belongs to a multi-gene family, is clustered on chromosome 19 at q 13.2-13.4, is composed of 5 exons and 4 introns with its length being 4.5 kilobase pairs (Inoue et al., 1989a; Berg et al., 1992). The family is strictly conserved and comprises members with close sequence homology. In humans until recently, 3 genes were known: hKLK1 (glandular kallikrein), hKLK3 (prostatic specific antigen) and hKLK2, and present on the long arm of chromosome 19 (Riegman et al., 1992; Clements, 1994 and 1998; Mahabeer and Bhoola, 2000). Recent evidence suggests a larger family than hitherto realised, with the discovery of a new hKLK-L2 human gene (Yousef and Diamandis, 1999), hKLK4 (Stephenson et al., 1999) and hKLK-L4 (Yousef et al., 1999), and several other kallikrein-like genes (KLK-L) in humans (Diamandis, 2000). Table 1-3 shows the localisation of tissue kalikrein genes in different organs.

Table 1-3. Expression of Tissue Kallikrein Genes in Human Tissue

Kidney	Salivary	Pancreas	Breast	Endo metrium	Ovary	Prostate
KLK1	hKLK1	KLK1	KLK1	KLK1	KLK1	KLK1
				KLK1*		KLK3
KLK3*			KLK3*	KLK3	KLK2	KLK2
			KLK3*	KLK2	KLK3	KLK1*
				KLK4 *	KLK3*	KLK2
						KLK3*

*Induction in tumours

New kallikrein-like gene family

KLK-L1, -L2, L-3, L-14, L-15, L-16 (Yousef and Diamandis, 2000)

Organization of the human kallikrein gene (hKLK) family on chromosome 19q13.4-q13.3

hKLK1 – tissue prokallikrein (Mahabeer and Bhoola, 2000);
hKLK3 – prostate specific antigen (Rae et al., 1999)
hKLK2 – trypsin-like protease (Yousef et al., 2000)
hKLK4 – matrix serine protease (Stephenson et al., 1999)

Gene induction of tissue kallikrein and the subsequently formed kinins enhances the proliferation of tumour cells (Rae et al., 19999). KLK-L2 and KLK-L1 are upregulated by oestrogens and other hormones in the breast cancer cell line BT-474 (Yousef and Diamandis, 1999; Yousef et al., 1999); while KLK-L4 is downregulated in breast cancer (Yousef et al., 2000) and NES1 in breast and possibly other cancers (Diamandis et al., 2000).

1.1.4.1.4 Inhibitors of tissue kallikrein

Kallistatin, a tissue kallikrein inhibitor belonging to the serpin superfamily, inhibits human tissue kallikrein activity (both kininogenase and amidolytic activity) towards either kininogen or a tripeptide substrate (Chao et al., 1990, Zhou et al., 1992). Its major site of synthesis is the liver, with lower levels of expression levels in the pancreas and kidney. The mRNA of this protein is expressed in the inner medullary ducts of the kidney with small amounts in the outer medullary collecting duct, proximal convoluted tubules and the glomerulus (Yang et al., 1994). The human kallistatin gene displays the 5 exon-4 intron gene structure and is located on chromosome 14q31-32.1 (Chai et al., 1994). The circulating inhibitor of TK is α1-antitrypsin inhibitor, which also inhibits urinary tissue kallikrein (Geiger and Mann, 1976).

1.1.4.2 KININOGENS

The human kininogen gene, which codes the endogenous protein substrate for the kallikreins is localised to chromosome 3q26→qter (Fong et al., 1991). Kininogen has been detected in human urine (Hial et al., 1976; Pisano et al., 1978). Immunoreactive kininogen

was localized in the principal cells of the collecting duct and restricted to the luminal portion of the principal cells (Figueroa et al., 1988). The close relationship between the cells containing tissue kallikrein and its substrate, kininogen suggests that kinins could be generated in the lumen of the collecting tubules. The mRNA of L-kininogen is expressed in the renal cortex and medulla, suggesting the biosynthesis of L-kininogen in the distal tubule (Iwai et al., 1988). Infusion of partially purified rat L-kininogen into mutant kininogen-deficient Brown Norway-Katholiek rats increased kinin excretion in ureter urine, whereas infusion of kininogen caused only a slight increase in kinins in the urine (Hagiwara et al., 1994), suggesting that the kidney secretes L-kininogen, and that urinary kallikrein releases urinary kinin mainly from L-kininogen.

1.1.4.3 KININASES

The turnover of kinins depends on both the rate of formation and the rate of destruction. After kinins are formed, they are rapidly destroyed by the enzymic action of peptidases. Kininases, which inactivate plasma kinins, are distributed in 2 major portions of the nephron: in the proximal tubules and the medullary collecting ducts. Kininase II (angiotensin converting enzyme) is concentrated along the brush border membrane of proximal tubule cells and the S3 segments of the proximal tubules (Marchetti et al., 1987; Ikemoto et al., 1990). Almost all of the [^{3}H] BK injected into the proximal tubules is destroyed in the proximal tubules (Carone et al., 1976). Microdissection techniques indicate that kininase activity is present in both proximal tubules and medullary collecting ducts (Marchetti et al., 1987). Neutral endopeptidase (NEP) accounts for more than half of the renal kininases in humans (Ura et al., 1993), while accounting for 68% of the total

kininase activity in rat urine, with kininase II and kininase I accounting for 23% and 9% respectively (Ura et al., 1987). Neutral endopeptidase is present in the outer surface of the brush border of proximal tubules and to a lesser extent, in the vesicular organelles in the apical cytoplasm and basal infoldings of the proximal tubule cells (Schulz et al., 1988). Carboxypeptidases (M and N) cleave the C-terminal arginine of kinins, suggesting a ready source of substrate (arginine) for the endothelial synthesis of nitric oxide by NOS (Sakuma et al., 1988).

1.1.4.4 KININ RECEPTORS

Presently, kinin receptors are characterized as B1, B2 and perhaps B3 (Regoli and Barabe, 1980). B1 receptors are induced especially after an insult for example, with E Coli endotoxin or lipopolysaccharide, whereas B2 are constitutive and are present universally in mammalian tissues. The constitutive B2 receptor, that accounts for the majority of the physiological effects of kinins, is a member of the superfamily of G-protein-coupled (Ga and Gq) rhodopsin-like receptors characterized by seven transmembrane regions connected by three extracellular and three intracellular loops (Burch and Axelrod, 1987). Homology to other receptors of this family is most pronounced in the transmembrane regions, whereas the loop regions are more divergent in their sequence. The B2 receptor protein consists of 364 amino acids, is highly glycosylated, exists in multiple isoforms at 69 kDa with isoelectric points of pH 6.8-7.1, and contains the kinin binding site at the amino-terminal part of the third extracellular loop [(Figure 1.4-4); Abu Alla et al., 1993; 1996]. The human B2 receptor density is highest in the kidney but is also present in heart, lung, brain, uterus and testes (McEachern et al., 1991; Hess et al., 1992). In the rat kidney, the B2 receptor has

been found in the straight portions of the proximal tubules, distal straight tubules, connecting tubules and collecting ducts (Figueroa et al., 1995). The B2 receptors are present in the luminal membranes, basal infoldings of the tubular cells and in smooth muscle cells of cortical radial artery and afferent arterioles.

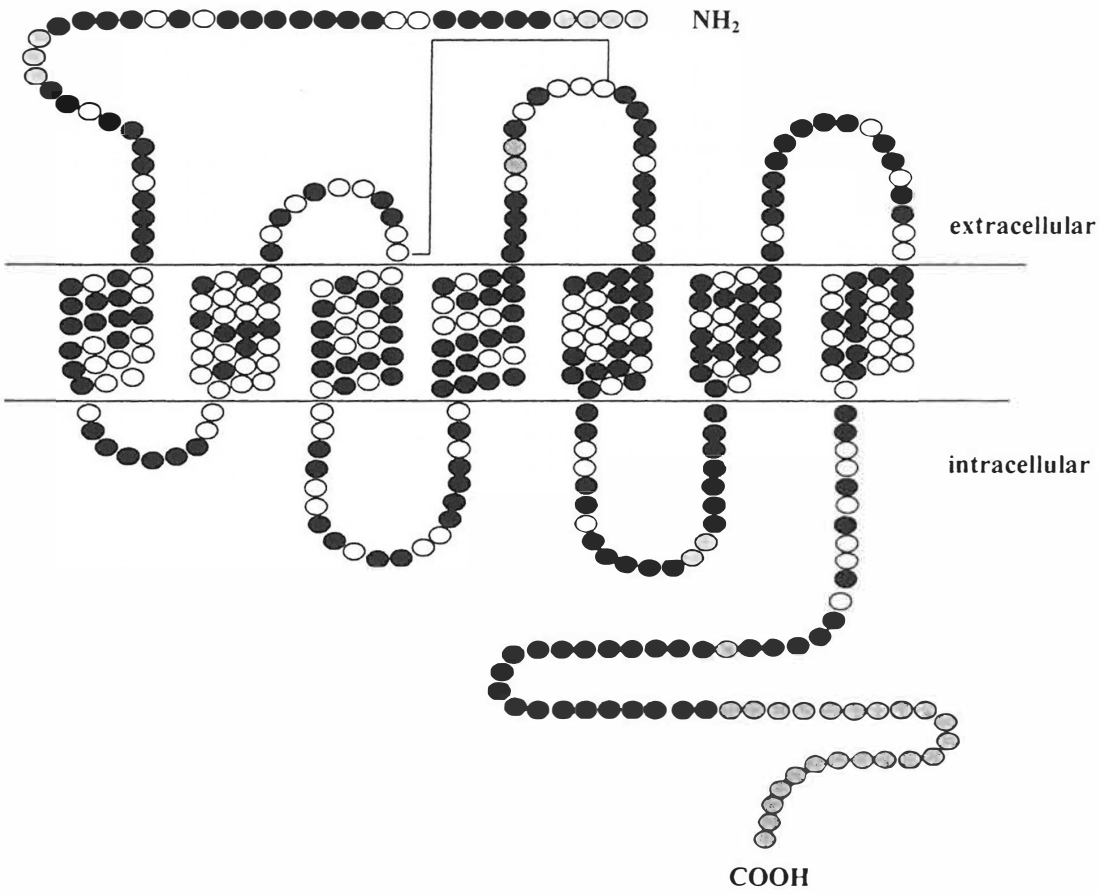


Figure 1.4-4: Comparison of the amino-acid sequences of human B_1 and B_2 receptors
Each circle represents a single amino acid, identical amino acids being denoted in white, differences in black and deletions in the B_1 receptor in light grey. Deletions in the B_2 receptor are shown in dark grey. The seven putative transmembrane-spanning domains are also shown. A putative disulfide bond between two cystine residues in the extracellular loops is shown by the line.

The B2 receptors are co-localized with tissue kallikrein and kininogen in connecting tubules and collecting duct cells respectively. The localization of the B2 receptor has been

mapped to chromosome 14q32, comprises more than 28kb and is organised in 3 exons and 2 introns (Powell et al., 1993; Ma et al., 1994). B2 binding sites, following chronic ACE inhibitor treatment and using the radio-labelled analogue (^{125}I-HPP-HOE 140) of the B2 receptor antagonist HOE 140, have been ultrastructurally mapped to the vasa recta bundles, capillary endothelial cells, epithelial cells of the thin limbs, distal tubule, collecting duct and renal medullary interstitial cells (RMIC) in the inner stripe of the outer medulla. In the inner medulla, B2 binding sites were localised to RMICs, loops of Henle, capillary endothelium and collecting duct epithelial cells (Dean et al., 1997). Kinins, acting via B2 receptors, increase intracellular calcium concentration, inhibit adenylate cyclase activity, stimulates the formation of inositol phosphates.

The kinin B1 receptor, which was initially defined as the one mediating the contractile effect of kinins on the isolated rabbit aorta (Regoli et al., 1977), apparently is rapidly upregulated in immunopathology under the influence of inflammatory mediators [cytokines (IL-1β), endotoxins (lipopolysaccharides)] and growth promoters (Marceau, 1995). Structurally, this receptor also has the characteristics of a classical G-protein-coupled receptor, and is composed of 353 amino acids, which have a 36% homology to the amino acid sequence of the B2 receptor [Figure 1.4-4; (Menke et al., 1994)]. The B1 receptor has been localised to chromosome 14q32 (Bachvarov et al., 1996, 1998; Chai et al., 1996). In smooth muscle, the B1 receptor appears to signal via phosphatidylinisitol hydrolysis (Butt, et al., 1995), while stimulation of B1 receptors on macrophages by the kinin degradation product des-Arg^9-BK, causes the release of IL-1 (interleukin) and TNF [tumour necrosis factor; (Tiffany and Burch, 1989)]. *In vivo* B1 receptor mediation includes the effect on

blood pressure (Tokamasu et al., 1995), persistent hyperalgesia (Perkins et al., 1993) and plasma extravasation (Cruwys et al., 1994).

Cellular actions following kinin binding to these G-protein coupled receptors are mediated by all of the known second messenger systems including the phospholipases C and A_2, which increases the synthesis of inositol triphosphate and arachidonic acid respectively (Mahan and Burch, 1990), as well as the participation of NO, especially on neurones and blood vessels. The metabolism of BK to des-Arg^{9}BK and L-arginine by the kininase 1 group of enzymes, either close to or within endothelial cells or in synaptic clefts, provides the primary substrate for the formation of NO [endothelium derived relaxing factor (EDRF)]. The released L-arginine acts as the substrate for NO synthetase, which rearranges the guanidino nitrogen to produce NO (Erdos, 1990; Bhoola et al., 1992).

The affinity of B1 receptors for kinin and kinin analogues differ markedly from that of B2 receptors. The effects of BK and lys-BK are mediated by the B2 receptors while those of the metabolites desArg^9-BK and lys-desArg^9-BK are mediated by the B1 receptor (Marceau, 1995). A third kinin receptor (B3) or a subtype may exist in bovine aortic endothelial cells, in microvasculature of guinea pig hindbrain and in cultured guinea pig smooth muscle cells (Burch et al., 1993; Pyne and Pyne, 1993). At these sites, variant responses to B1 and B2 agonists and antagonists suggest a possible interaction with another receptor subtype.

1.1.4.5 KININ ANTAGONISTS

As a result of the recent cloning of kinin receptor genes, considerable advances in kinin research have seen the emergence of numerous potent and highly specific kinin receptor antagonists that may have therapeutic value. Modifications saw the emergence of Lys-(Leu^8)des-Arg^{9}BK as a high-affinity B1 antagonist (Regoli and Barabe, 1980) and more recently, Ac-Lys-(MeAla^6, Leu^8)-des-Arg^{9}BK was found to be a metabolically multi-resistant B1 antagonist (Drapeau et al., 1993). Icatibant (HOE-140), a selective B2 antagonist more potent than NPC-567, was found to display greater resistance to degradation as it was metabolically stable (Hock et al., 1991). HOE 140 and NPC-17761, a bis-maleimidohexane-dimer analogue of HOE-140, became the "second-generation" kinin antagonists. The "third-generation" single-chain kinin antagonists (B-9224, B-9430, and B-9668), which have high potency at both B1 and B2 receptors (Burkard et al., 1996; Stewart et al., 1996) contain the novel amino acids α-(2-indanyl)-glycine at position 5 and D-α(2-indanyl)-glycine at position 7. B-9878 has been fashioned as a bi-kinin-like peptide.

Thus, several hundred analogues of the kinin nonapeptide have since been synthesized and have given mixed results due to their antagonistic behaviour towards other agonists. The "second-generation" kinin antagonists have been used in clinical trials with some success in allergic rhinitis (Austin et al., 1994) and atopic asthma (Akbary et al., 1996). The "third-generation" kinin antagonists have been shown to be selectively cytotoxic for cells of SCLC *in vitro* (Chan et al., 1996) and *in vivo* (Stewart et al., 1997). The recent discovery of the first nonpeptide competitive B2 receptor antagonist WIN 64338 (Sawutz et al., 1994), which is stable to proteolytic degradation, may have therapeutic roles in the near future. In

animals, kinin antagonists have also been shown to block kinin-induced hypotensive shock, as well as non-specific stimulus-induced pain, nasal allergies, and carageenan-and thermally-induced oedema (Rodell et al., 1995).

Inhibitors of ACE, a member of the K-11 group of enzymes that rapidly inactive kinins, and also convert the decapeptide angiotensin 1 to an octapeptide angiotensin 11, are probably the best examples of drugs acting on the kinin system that are used in clinical medicine. The mode of action of ACE inhibitors as antihypertensive agents have been shown to be due to both the inhibition of angiotensin 11 (a potent vasoconstrictor) production, as well as an increase in circulating levels of kinins (Shimamoto et al., 1990). The hypotensive efficacy of these inhibitors has also been shown to correlate with the reduced activity of ACE in brain, kidney, and vascular smooth muscle (Unger et al., 1987). Recent studies on isolated bovine coronary arteries indicate that the hypotensive effect of ACE inhibitors (moexiprilat and ramiprilat) are due to the relaxation of vascular smooth muscle, facilitated by the accumulation of endothelium-derived kinins in or at the vessel wall (Hecker et al., 1993).

1.1.4.7 KININS AND BLOOD PRESSURE

The observation of reduced urinary excretion of tissue kallikrein in untreated hypertensives was made as early as 1934 by Elliot and Nuzum, and confirmed more than 3 decades later in hypertensive humans and rats (Margolius et al., 1971 and 1974b; Carretero and Scicli, 1971). It has been reported that 20% of patients with essential hypertension have low kallikrein excretion (Zschiederich et al., 1980). White patients with uncomplicated

essential hypertension have been reported to have normal kallikrein excretion rates with normal plasma renin activity and aldosterone (Lawton and Fitz, 1977); only hypertensives over the age of 40 excreted a significantly lower kallikrein (Koolen et al., 1984a). Black people (adults and children) excreted markedly less kallikrein compared to whites, regardless of blood pressures, with black hypertensive subjects showing the lowest excretion of kallikrein (Zinner et al., 1976; Levy et al., 1977).

Gender differences in renal kallikrein excretion have been reported, with females excreting more kallikrein than males (Hughes et al., 1988); urinary kallikrein levels rise during the luteal phase and return to levels similar to males and postmenopausal females during the follicular phase (Albano et al., 1994). Urinary kallikrein activity increased in white females during the luteal phase but did not change in blacks (Kailasam et al., 1998).

Japanese patients with low renin hypertension showed significant reductions in urinary kallikrein and kinin excretion, together with increased levels of a kallikrein inhibitor and kininase in urine and with reduced levels of kininogen (Nakahashi et al., 1986). South African Indians with essential hypertension showed lower urinary kallikrein excretion compared to Black South Africans (Seedat et al., 1999). The urinary kallikrein excretion was significantly lower in salt-sensitive hypertensives than in salt-resistant hypertensives and showed an inverse correlation with plasma atrial natriuretic peptide levels (Ferri et al., 1994). Patients with malignant essential hypertension excreted less urinary kallikrein than those with non-malignant essential hypertension and normotensive control subjects (Hilme et al., 1992). A recent study involving 57 Utah subjects' pedigrees indicated that a dominant allele expressed as high urinary kallikrein excretion may be associated with a

decreased risk of essential hypertension (Berry et al., 1989): the "low homozygotes" would have a high risk of hypertension; the heterozygote genotype on a low potassium intake would have a high susceptibility to hypertension, whereby a low potassium diet in this group predisposes them to hypertension. This study showed a direct association between urinary kallikrein and urinary potassium and the risk of developing hypertension. A high potassium diet administered to SHRs resulted in a greater amount of urinary kallikrein excreted, paralleled by a reduction in blood pressure (Barden et al., 1988). Potassium appears to be a stimulus for tissue kallikrein (Lauar and Bhoola, 1986), increasing kallikrein excretion in the urine as well as producing hypertrophy and hyperplasia of CNT cells together with an increase in the number of immunoreactive secretory vesicles in these cells (Vio and Figueroa, 1987).

In the two kidney, one clip Goldblatt hypertensive rat, kallikrein levels were low in the urine from the stenotic kidney and normal or less reduced in the contralateral kidney (Girolami et al., 1983). B2 receptor density was increased to a greater extent in the contralateral kidney than in the stenotic kidney (Emond et al., 1991). Reduced urinary kallikrein excretion has been reported in genetically hypertensive rat models (Dahl, Milan, New Zealand, Fawn-hood, Sabra), in rats made hypertensive by deoxycorticosterone plus 1% salt, and in the Okamoto-Aoki spontaneously hypertensive rat (reviewed by Katori and Majima, 1996). Kininogen-deficient Brown Norway Katholiek rats became hypertensive on a moderate salt intake and non-pressor doses of angiotensin II.

Kallikrein gene therapy, in the form of human kallikrein DNA constructs under the control of the metallothionein metal response element, the cytomegalovirus promoter/enhancer or

the Rous sarcoma virus 3^1-LTR, caused a prolonged reduction in blood pressure for 8-10 weeks following a single injection given to the SHR adult male intravenously, intramuscularly, intraperitoneally, into the portal vein and intracerebroventricular route (Chao and Chao, 1997). This hypotensive effect was reversed by a subcutaneous injection of aprotonin, a potent tissue kallikrein inhibitor. Icatibant acetate (HOE 140), a B2 receptor antagonist, given together with captopril, attenuated the hypotensive effect of captopril by 53% in black and white subjects on a low sodium diet (Gainer et al., 1998). This study provides evidence that kinins contribute substantially to the hypotensive effects of ACE inhibition.

1.1.4.8 KININS AND RENAL DISEASE

1.1.4.8.1 Nephrotic Syndrome

Urinary kallikrein excretion was found to be markedly increased in patients with nephrotic syndrome, irrespective of the level of renal function (Cumming and Robson, 1985), while patients with glomerulonephritis (without nephrotic syndrome) had reduced urinary kallikrein excretion compared to healthy volunteers. Kallikrein excretion correlated with plasma renin activity but not with plasma volume (Cumming et al., 1989). Infusion of kallikrein into the renal artery of dogs caused proteinuria, which was abolished by aprotinin (Murakami et al., 1968). Kinins are potent stimulators of phospholipase A_2 and promote synthesis of arachidonic acid metabolites, including thromboxane A_2 (Regoli and Barabe, 1980). Increased glomerular synthesis of thromboxane has been suggested as a cause of proteinuria in nephrotic syndrome (Remuzzi et al., 1985).

1.1.4.8.2. Parenchymal renal disease and renal failure

Patients with renal parenchymal disease and hypertension with impaired renal function had a more marked decrease in urinary kallikrein excretion compared to hypertensive subjects with normal renal function, who also had a reduced urinary kallikrein excretion but less so (Mitas et al., 1978). Spontaneously hypertensive rats at 78 weeks showed a dramatic decrease in the number of tubules and cells immunostaining for tissue kallikrein, while there was no difference between SHR and WKY rats during 4-52 weeks. In humans with advanced hypertensive nephropathy similarly, there was a reduction in the percentage of tubules (CNT) and cells with immunoreactive tissue kallikrein (Figueroa et al., 1992). Prevalence of the $G^{699} \rightarrow C$ polymorphism of the B1 receptor was found to be present significantly less frequently in several aetiological subgroups of uraemic patients (Bachvarov et al., 1998). Thus, the polymorphism of the B1 receptor promotor may be a marker of prognostic significance for the preservation of renal function.

1.1.4.8.3 Dialysis

Patients with end stage renal failure on haemodialysis have fluctuations in their sodium and volume status. While urinary tissue kallikrein excretion was decreased in patients before haemodialysis, a significant increase in 24 hour urinary tissue kallikrein was observed in all patients after haemodialysis (Girolami et al., 1991). Immediate hypersensivity or anaphylactoid reactions have been reported within the first 10 minutes of commencing haemodialysis. Plasma kinin levels were noted to be significantly increased during hypersensitivty reactions during haemodialysis (Verresen et al., 1994). A higher incidence of hypersensitivity reactions was observed in patients treated with ACE inhibitors while undergoing haemodialysis with polyacrilonitrile membranes (Tielemans et al., 1990; Verresen et al., 1990). *In vitro* studies showed that plasma kallikrein activation and release

of bradykinin was related to electronegativity of the dialyzer membrane, as well as plasma dilution with residual saline rinsing solution at the commencement of dialysis and a fall in pH (Renaux et al., 1999).

1.1.4.8.4 Renal transplantation

Hypertension frequently accompanies renal transplantation. Proposed mechanisms for hypertension include acute rejection, chronic rejection, therapy with steroids and cyclosporine, renal insufficiency, presence of the recipient's own diseased kidneys, transplant renal stenosis, increased activity of the vasoconstrictor systems (eg. renin-angiotensin, endothelin) and decreased activity of vasodilator systems. Urinary kallikrein excretion was found to be decreased in hypertensive patients and in those with renal complications (more markedly decreased with acute tubular necrosis than acute rejection). Urinary kallikrein excretion was also lower in cadaver graft recipients who tend to be more hypertensive (O'Connor et al., 1982).

Urinary excretion of tissue kallikrein was reduced in renal transplant recipients and more markedly so following acute rejection (Moodley et al., 1996). TK immunoreactivity was reduced in acute rejection both on immunocytochemistry and electron microscopy; while TK was observed mainly at the luminal side of distal connecting tubules and collecting ducts, there was a shift in immunolabelling to the basolateral membranes (Ramsaroop et al., 1997). A marked rise in urinary kallikrein excretion occurred 1-3 days before the clinical diagnosis of acute rejection was made (Brouhard et al., 1982; Koolen et al., 1984b). Lower urinary kallikrein excretion was found in transplant recipients compared to controls, probably related to reduced renal function or reduced renal mass (Koolen et al., 1984b;

Marin-Grez et al., 1982). It has been suggested that tissue kallikrein excretion rate might be a useful indicator of functional distal tubular mass: kallikrein excretion was significantly decreased in renal transplant recipients and uninephrectomized donors (Spragg et al., 1985). Decreased kallikrein excretion followed cylosporine administration (Spragg et al., 1988; Martinez et al., 1990). Short term cyclosporine administration decreased kallikrein and kinin B2 receptor mRNA expression in rat kidney cortex (Bompart et al., 1996).

1.1.4.8.5 Obstructive uropathy

Intrarenal vasoconstrictor-vasodilator imbalance has been observed in obstructive uropathy, resulting in a marked increase in renal vascular resistance and profound reduction in renal blood flow and GFR. Excessive production of angiotensin II, thromboxanes, leukotrienes, vasopressin and endothelin-1 has been described. In rats with unilateral ureteric ligation, systolic pressure, plasma angiotensin II levels, plasma renin activity, angiotensin I and plasma angiotensin converting enzyme (ACE) - kininase II activity was elevated. Blockade of angiotensin II receptors with losartan normalised blood pressure. Renin mRNA content and angiotensin II were elevated in obstructed kidneys. ACE-kininase II activity was elevated in both the obstructed and contralateral kidneys. Total immunoreactive kallikrein content and tissue kallikrein mRNA levels were markedly reduced in obstructed kidneys (El-Dahr et al., 1993). Reduced intrarenal kinin generation may therefore aggravate vasoconstriction produced by high local levels of angiotensin II and ET-1 in the obstructed kidney.

1.1.4.8.6 Endotoxaemia

Lipopolysaccharide (LPS) induces arterial hypotension in endotoxaemia by releasing kinins via activation of plasma prekallikrein by limited proteolysis of H-kininogen (Kalter et al.,

1983; Muller-Esterl and Fritz, 1984). Administration of selective kinin antagonists to rat models with endotoxic shock has produced conflicting results: Weipert et al. (1988) reported attenuation of the hypotensive effect while Berg et al. (1989) found no effect on the hypotensive response to LPS. Bacterial LPS strongly induced the expression of B1 receptor mRNA in the efferent arteriole, the medullary and inner medullary thin limb and the distal tubule, with more moderate expression in the glomerulus, proximal tubules and medullary thick ascending limb and slight expression in the cortical duct (Marin-Castano et al., 1998); B1-receptor mRNA was not detected under physiological conditions. Induction of B1 receptor mRNA expression resulted in functional receptor expression, as increases in intracellular calcium were observed during B1-agonist stimulation. LPS treatment also increased expression of B2 receptor mRNA in all segments of the nephron except the glomerulus, inner medullary thin limb and outer medullary collecting duct; however no changes in intracellular calcium were found.

1.1.4.8.7 Nephrotoxic acute renal failure

Gentamycin is an antibiotic commonly used in life threatening gram-negative bacterial sepsis. Nephrotoxicity occurs in approximately a third of patients treated with gentamcyin for more than 7 days (Mathew, 1992). Gentamycin suppressed the urinary excretion of endogenous tissue kallikrein in rats. Systemic delivery of the human tissue kallikrein gene in the rat model of gentamycin-induced acute renal failure significantly increased renal blood flow, GFR and urine flow while attenuating renal tubular damage, cell necrosis and luminal protein casts (Murakami et al., 1998). The mechanism of improved renal function following kallikrein gene delivery appears to be mediated via kinin through a nitric oxide signal transduction pathway.

1.2. RENAL DISORDERS

1.2.1 ACUTE RENAL ALLOGRAFT REJECTION

Renal transplantation is the treatment of choice for patients with end stage renal disease. Success in transplantation is limited by the availability of donor organs and the occurrence of complications such as acute rejection, infections and chronic allograft nephropathy.

1.2.1.1 Immunology

Renal allograft rejection depends on the co-ordinated activation of alloreactive T cells and antigen-presenting cells (for example, monocyte-macrophages, dendritic cells and B cells). This process involves the activity of antibodies and inflammatory mediators, adhesion molecules, chemokines and cytokines (Fig 2-1). The extent and severity of the rejection response depends on genetic similarities between donor and recipient, circumstances surrounding organ harvest and early graft dysfunction, the type of tissue engrafted and effectiveness of immunosuppresion. The characteristic feature of acute rejection is the infiltration of the graft by host mononuclear cells (lymphocytes and macrophages). Immunohistologically these have been characterized as T and B lymphocytes, macrophages and natural killer cells (Medawar, 1944; Mason and Morris, 1986). Stimulated B lymphocytes differentiate into antibody-producing plasma cells which secrete nonspecific and specific anti-donor antibodies (Tilney et al., 1979). The immunological host response to foreign tissue comprises 2 limbs: an afferent or sensitizing limb and an efferent or effector limb (Gowans et al., 1962). T cell activation begins when T cells recognize intracellularly processed fragments of foreign proteins embedded in the groove of the

major histocompatibility complex (MHC) proteins, expressed on the surface of antigen presenting cells (Krensky et al., 1990; Weiss and Littman, 1994).

CD4 and CD8 proteins, expressed on peripheral blood T cells, bind to human leucocyte antigen (HLA) class II and class I molecules respectively (Miceli and Parnes, 1991). The complex of T cell-antigen receptor and CD3, CD4 and CD8 proteins physically associate with and activate several intracellular protein tyrosine kinases, resulting in mobilization of ionized calcium from bound intracellular stores by inositol triphosphate. The increased intracellular free calcium and sustained activation of protein kinase C function synergistically in promoting the expression of several nuclear regulatory proteins, and in the transcriptional activation and expression of genes central to T cell growth (Krensky et al., 1990; Weiss and Littman, 1994). Calcineurin, a calcium and calmodulin-dependent serine-threonine phosphatase, participates in signal transduction. Inhibition of the phosphatase activity of calcineurin is central to the immunosuppressant activity of cyclosporine and tacrolimus (Liu et al., 1991; Fruman et al., 1992).

Cytokines are soluble mediators of cellular communication and mediate interactions between leucocyte populations, and between leucocytes and cells in the donor organ. Cytokines of the interleukin (IL) family, derived from antigen-presenting cells (namely IL-1 and IL-6), also provide co-stimulatory signals that result in T cell activation. T cell-derived cytokines (for example IL-2 and IL-4) and contact between T and B cells through specific pairs of receptors and co-receptors provide signals essential for B-cell stimulation (Clark and Ledbetter, 1994). T cell proliferation is the result of IL-2 expression that is dependent on T cell activation. IL-2 triggers the activation of protein tyrosine kinases that

results in the expression of several DNA-binding proteins (including *c-jun, c-fos* and *c-myc*) and progression of the cell cycle.

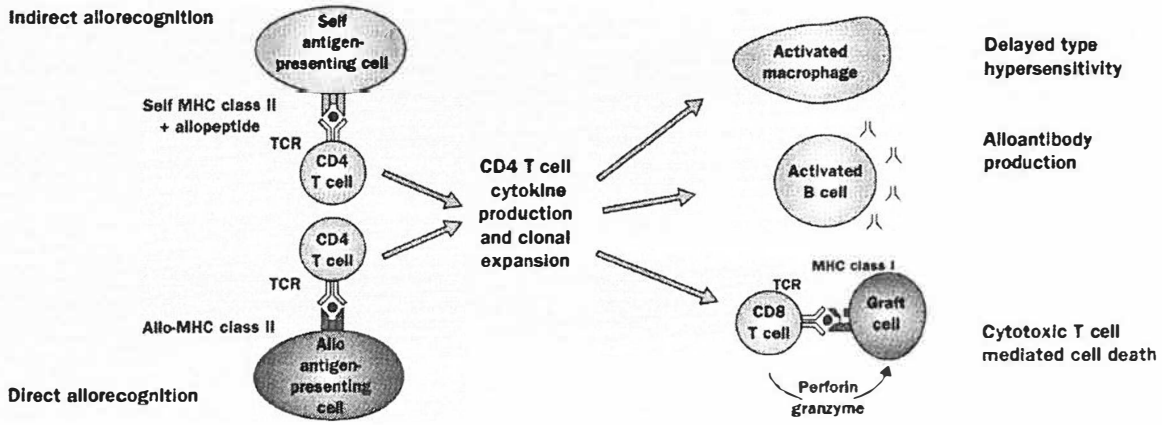


Fig 2-1: Cellular interactions that form the anti-allograft response

Ref: Denton M D et al. (1999), Lancet, 353: 1083-1091 (1084)
Abbreviations: TCR= T cell receptor; MHC= major histocompatibility complex

Activated macrophages elaborate IL-1, which causes $CD4^+$ T cells to produce a series of humoral mediators. Other macrophage-derived cytokines, important in graft destruction, include tumour necrosis factors (TNFβ and TNFα). The ability of activated $CD4^+$ T lymphocytes to elaborate lymphokines is critical in the early phases of acute rejection. CD^+4 cells can be divided into 2 subclasses: T helper 1(Th1) and T helper 2 (Th2). Th1 cells produce effector lymphokines, especially IL-2, which causes differentiation and proliferation of activated T lymphocytes and stimulates B cell maturation. Interferon (IFN)| derived from Th1 cells, induces and intensifies class I and II MHC antigen expression on the graft, and stimulates B cells to increase antibody production. It may also increase lymphocyte adhesiveness to an antigenic surface by upregulating leucocyte

function-associated antigen-1 (LFA-1) expression (Paineau et al., 1989; Patarroyo and Makgoba, 1989). Th 2 cells produce inhibitory products such as IL-4 and IL-10.

The net result of cytokine production is the emergence of antigen-specific, graft-infiltrating and destructive T cells. Cytokines also activate macrophages and other inflammatory cells, and the production of antidonor antibodies by stimulated T cells. Cytokines can amplify the ongoing immune response by up-regulating the expression of HLA antigens and co-stimulatory molecules (such as B7) on graft parenchymal cells and antigen-presenting cells. The co-stimulators direct T cell differentiation, for example into a $CD4^+$ Th1 cell which secretes lymphokines, facilitating cytotoxic T lymphocyte killing of cells; or differentiates into a $CD4^+$ Th 2 cell which stimulates antibody production by B cells (Dallman, 1995). Cell killing may occur via specific T-cell products, such as granzyme B (a serine esterase protein) and perforin (a pore-forming lytic protein), which have been reported to correlate closely with acute rejection of grafts (Clement et al., 1994). The type of organ grafted, HLA matching between donor and host and the degree of pre-sensitisation influence the acute rejection process. $CD4^+$ T helper cells are the primary, initiating and organizing component of host immuno-responsiveness against grafts. CD 8^+ cells are recruited secondarily to the site to complete the acute rejection process (Tilney et al., 1984; Mason and Morris, 1986; Mason, 1987).

With completion of the rejection episode and destruction of the graft, intrinsic control mechanisms return the host activated immune processes to baseline. There is progressive decrease in the expression of graft antigens, slowing of clonal expansion of lymphocyte subpopulations, with reversion to their resting state; elaboration of cell products and surface receptors gradually cease. Suppressor mechanisms may reverse the intense

inflammatory process. Th2 cells may produce cytokines (for example, IL-4 and IL-10) which may inhibit alloresponsiveness and counter the effector activity of Th1-derived IFN|, TNFα and IL-1β.

Long term graft survival rate has not improved despite major improvements in one year graft survival. The half-life of cadaveric renal allografts remains at approximately 7 years (Paul and Benediktsson, 1993). The major reason for graft loss is chronic rejection or chronic allograft nephropathy, which may be related to poor HLA matching, more frequent acute rejection episodes, cytomegalovirus infections, ischaemic and reperfusion injury to the graft, the initial amount of functioning renal mass and nephron number (Brenner and Milford, 1993). Periglomerular and perivascular macrophages secrete cytokines that are profibrogenic, including platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. This is associated with upregulation of the ET-1 gene expression in endothelial, vascular smooth muscle and renal mesangial and epithelial cells and results in ET-1 secretion (Kurihara et al., 1989; Watschinger and Sayegh, 1996). Increased intracellular adhesion molecule-1 (ICAM-1) expression is found on the glomeruli of rat renal allografts undergoing acute rejection (Azuma et al., 1994). The gradual functional deterioration caused by the development of glomerulosclerosis and arterial obliteration may also cause systemic hypertension which causes the remaining functional glomeruli to hyperfilter before eventually fibrosing, thus resulting in progressive renal damage (Neuringer and Brenner, 1992).

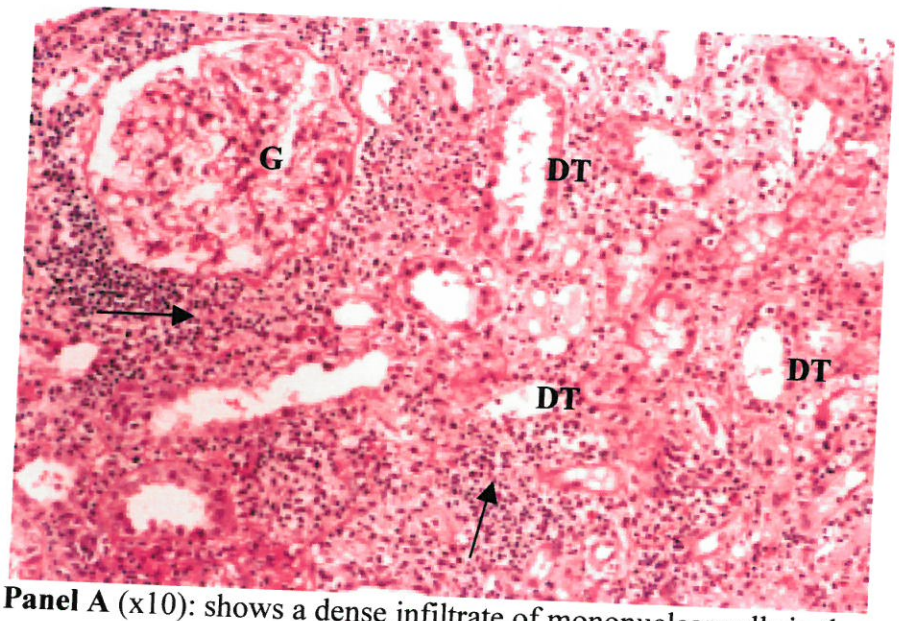
Summary: The primary cells involved in rejection are T lymphocytes and macrophages: T lymphocytes are important regulators and effectors in rejection; macrophages and dendritic cells are important in antigen presentation. Other cells involved are B cells, natural killer

cells, neutrophils, eosinophils and platelets. Soluble factors important in acute rejection include components of the complement system, coagulation factors, leukotrienes, kinins and inflammatory cytokines. Recipient T lymphocytes recognise foreign HLA class II molecules in the allograft, and are activated to proliferate, differentiate and secrete a variety of cytokines. Cytokines upregulate expression of HLA class II antigens, stimulate B lymphocytes to produce antibodies against the allograft and help cytotoxic T cells, macrophages and natural killers cells to develop cytotoxicity against the graft.

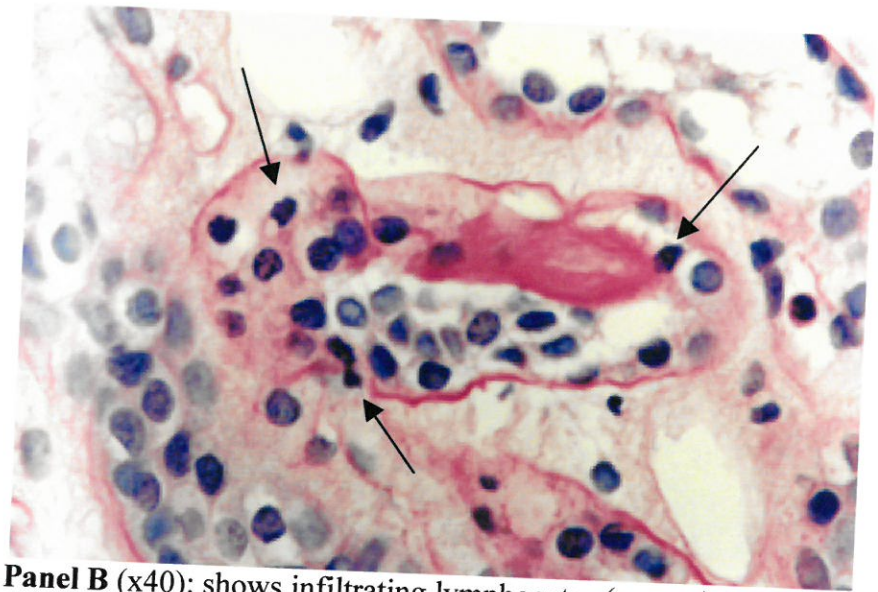
1.2.1.2 Histopathology

The early stage of acute cellular rejection in the renal allograft is characterized by a perivenular and periglomerular infiltrate of transformed lymphocytes and macrophages. This is followed by infiltration of the interstitium and tubular epithelium (Fig 2-2). The interstitial infiltrate is pleomorphic, consisting predominantly of variably sized lymphocytes as well as macrophages, eosinophils, plasma cells and neutrophils (Porter et al., 1964). The interstitial infiltrate is associated with oedema, and there may also be extravasation of erythrocytes. The tubular lumina become dilated, brush borders disappear and cell death occurs. With vascular involvement, arteries and arterioles undergo subendothelial infiltration by lymphocytes and macrophages. The endothelium is often lifted from its basement membrane and, in some cases, small deposits of fibrin and platelets are found in relation to the endothelial injury. The mononuclear cell inflammation uncommonly infiltrates transmurally, involving the blood vessel wall and resulting in an inflammatory necrotizing arteritis. The Banff classification standardized the criteria for the histologic diagnosis of renal allograft pathology internationally as depicted in Tables 1-4 and 1-5 (Solez et al., 1993; Racusen et al., 1999).

Fig 2-2 Histology of acute renal allograft rejection



Panel A (x10): shows a dense infiltrate of mononuclear cells in the interstitium of the transplant kidney (arrows)



Panel B (x40): shows infiltrating lymphocytes (arrows) invading the tubular epithelium (tubulitis) of the distal tubule

Abbreviations: G= glomerulus; DT= distal tubule

Table 1-4. Banff Classification: Criteria for acute rejection in renal allograft biopsies

Grade I, mild acute rejection

Cases with significant interstitial infiltration (>25% of parenchyma affected) and foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells)

Grade II, moderate acute rejection

Cases with (A) significant interstitial infiltration and foci of severe tubulitis >10 mononuclear cells/tubular cross section) and/or (B) mild or moderate intimal arteritis

Grade III, severe acute rejection

Cases with severe intimal arteritis and/or "transmural" arteritis with fibrinoid change and necrosis of medial smooth muscle cells. Recent focal infarction and interstitial haemorrhage without other obvious cause are also regarded as evidence for Grade III rejection

Table 1-5. Banff Classification of acute rejection. Numerical codes

g	0, 1, 2, 3	no, mild, moderate, severe glomerulitis (g3 = mononuclear cells in capillaries of all or nearly all glomeruli with endothelial enlargement and luminal occlusion)
i	0, 1, 2, 3	no, mild, moderate, severe interstitial mononuclear cell infiltration (In rejection oedema and lymphocyte activation usually accompany mononuclear cell infiltration; i3 = > 50% of parenchyma inflamed)
t	0, 1, 2, 3	no, mild, moderate, severe tubulitis (t3 = > 10 mononuclear cells per tubule or per 10 tubular cell in several tubules)
v	0, 1, 2, 3	no, mild, moderate, severe intimal arteritis (v3 = severe intimal arteritis and/or transmural arteritis and/or hemorrhage and recent infarction

1.2.2 GLOMERULONEPHRITIS

The severity of glomerular injury is determined by (1) the primary insult and secondary mediators involved (2) the site of injury and (3) the speed of onset, extent and intensity of disease.

1.2.2.1 Immune glomerular injury

Glomeruli are susceptible to a variety of inflammatory, metabolic, haemodynamic, toxic and infectious insults. Most human glomerular disease is triggered by immune attack, diabetes mellitus or hypertension. Similar clinicopathological presentations are provoked by different aetiologies. Infections and vasculitides can trigger acute proliferative glomerulonephritis; metabolic disorders such as diabetes mellitus and deposition diseases (for example, amyloid) can induce glomerulosclerosis with nephrotic syndrome. Glomerular injury at different sites results in characteristic disease patterns and clinicopathological presentations (Table 1-6).

Table 1-6. Correlation between site of glomerular injury and clinicopathologic presentation

Target of injury	Physiologic role	Response to injury	Renal disease
Endothelial cell	Maintains glomerular perfusion	Vasoconstriction	acute renal failure
	prevents leucocyte adhesion	leucocyte infiltration	focal/diffuse proliferative GN
	Prevents platelet aggregation	Intravascular microthrombi	Thrombotic microangiopathies
Mesangial cell	controls glomerular filtration surface area	Proliferation/↑matrix	Mesangioproliferative GN/glomerulosclerosis
Basement membrane	prevents filtration of plasma proteins	Proteinuria	membranous GN
Visceral epithelial cell	prevents filtration of plasma proteins	Proteinuria	minimal change disease/FSGS
Parietal epithelial cell	maintains Bowman's space	crescent formation	crescentic GN

Endothelial and subendothelial injury results in (1) recruitment of leucocytes (2) abnormal haemostasis leading to thrombotic microangiopathies and (3) vasoconstriction and mesangial cell contraction leading to acute renal failure. Mesangial injury, usually immunological in origin, results usually in asymptomatic abnormalities of the urinary sediment and mild renal insufficiency. Proteinuria is the dominant presentation with injury to the subepithelial aspect of the glomerular basement membrane and visceral epithelial cells.

Immune-mediated glomerulonephritis accounts for a large proportion of acquired renal disease. The majority are associated with deposition of antibodies within the glomerular tuft. indicating dysregulation of humoral immunity. Cellular immunity, by modulating antibody production also contributes to the pathogenesis of glomerulonephritis via antibody-dependent cytotoxicity. Formation of antigen-antibody complexes activates complement and leads to a significant inflammatory response. Localization of these complexes in the different structures of the glomerular capillary determines the presence and severity of the inflammatory response. The role of complement in immune-mediated renal injury has been clearly established in anti-glomerular basement membrane (GBM) antibody glomerulonephritis, membranous nephropathy and mesangial proliferative nephritis.

Secondary immune mechanisms involve the cascade of inflammatory mediators that may be recruited to propagate renal damage after the primary glomerular attack. These include cytokines, growth factors, reactive oxygen metabolites, platelet-activating factor and eicosanoids, proteases and vasoactive substances (ET and EDRF). The presence of an inflammatory infiltrate is associated with generation of multiple mediators that participate in

the decrease in glomerular filtration rate (GFR) associated with the different types of glomerulonephritis. Leukotrienes, especially leukotriene D4 (LTD4), generated by neutrophils, play a significant role in the glomerular haemodynamic changes associated with immune injury; LTD4 receptor blocker partially ameliorates the decrement in the ultrafiltration co-efficient in the anti-Thy-1 antibody model (Bresnahan et al., 1992). Leukotriene generation is antagonised by 15-lipoxygenase products (lipoxin A4 and lipoxin B4) produced by macrophages (Badr, 1992). The glomerular response to immune injury therefore depends on the interaction between leukotrienes derived from neutrophils and lipoxins generated by macrophages, both cell types stimulated by the presence of antigen-antibody complexes.

Thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2), generated by endothelial, mesangial and epithelial cells of the glomeruli, are involved in glomerular immune injury. TXA_2 is associated with renal vasoconstriction and reduction in the ultrafiltration co-efficient in the early phases of immune injury; normalization of GFR and renal plasma flow in the later stages depends on the generation of PGE_2. The highest concentrations of PGE_2 are present in the medullary collecting duct; its production is regulated by sodium chloride, molarity and bradykinin (Zusman and Keiser, 1977). Studies in humans and animals have shown that PGE_2 and PGI_2 are natriuretic (Johnston et al., 1967; Bolger et al., 1978) and can stimulate renin release. PGE_2 antagonizes arginine vasopressin (AVP)-induced water reabsorption (Grantham and Orloff, 1968).

Nitric oxide (NO) may contribute to immunologic renal disease by different and seemingly opposing mechanisms. Cytokines produced during renal injury stimulate nitric oxide synthase (NOS) to form NO in infiltrating macrophages as well as in glomerular endothelial and mesangial cells. In immune complex nephritis, isolated glomeruli and infiltrating macrophages produce large amounts of NO; cellular NO secretion and urinary NO excretion is augmented; inducible NOS (iNOS) mRNA expression is stimulated (Jansen et al., 1994). The L-arginine analogue, N^G-monomethyl-L-arginine(L-NMMA) is able to prevent the onset of glomerulonephritis in this model. Mesangial cells are stimulated by NO to increase capillary surface area and glomerular ultrafiltration coefficient, with consequent hyperfiltration and possible glomerular scarring (Zatz and DeNucci, 1991). Focal or segmental glomerulosclerosis occurs after reduction of renal mass due to the resulting hyperfiltration, and to increased intraglomerular hydraulic pressure. Inhibition of NO by N^G-nitro-L-arginine methylester (L-NAME) and L-NMMA leads to increased intraglomerular hydraulic pressure, accompanied by renal vasoconstriction, proteinuria and glomerular sclerosis (Reyes et al., 1993). Glomerular scarring can be prevented and renal vascular resistance ameliorated in renal mass-ablated animals that have been fed with L-arginine (Reyes et al., 1994), thus demonstrating the protective role of NO in glomerulosclerosis. NO also has anti-thrombotic and platelet-inhibitory effects in glomerular injury (Shultz and Raij, 1992). Thus, elevated NO levels in glomerulonephritis affect vascular tone, mesangial relaxation and tissue perfusion. The resulting glomerular hyperfiltration is thought to directly affect mesangial integrity and lead ultimately to glomerulosclerosis.

A hallmark of proliferative glomerulonephritis is an increase in glomerular cell number, which initially is due predominantly to infiltration of the glomerular tuft by leucocytes.

Subsequently, resident glomerular cells proliferate in response to growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and thrombospondin. Glomerular inflammation can resolve with complete recovery of renal function or with a variable degree of scarring and chronic renal insufficiency.

Transforming growth factorβ (TGFβ) stimulates production of extracellular matrix by most glomerular cells, inhibits synthesis of tissue proteases that normally degrade matrix proteins, and is a potent stimulus for scar formation following glomerular injury. Angiotensin II, PDGF and endothelins are other potential modulators of this process (Wilson, 1996). Moderate to severe glomerulonephritis is usually associated with tubulo-interstitial inflammation and scarring, which correlates closely with long term impairment of renal function.

1.2.2.2 Non-immunological glomerular injury

Nephropathy complicates approximately 30% of type 1 and 20% of type 2 diabetes mellitus. It is characterized clinically by proteinuria and progressive renal insufficiency and pathologically by glomerulosclerosis due to thickening of the glomerular basement membrane (GBM) and mesangial expansion with extracellular matrix. This may be triggered by glomerular hypertension, the direct effect of hyperglycaemia on mesangial cells, advanced glycosylation end products, growth factors such as growth hormone, insulin growth factor 1 (IGF_1) and angiotensin II, cytokines (such as TGFβ), hyperlipidaemia and cell sorbitol accumulation and myoinositol deficiency (secondary to activation of the aldose reductase polyol pathway). Haemodynamic factors also play a central role. High intraglomerular pressure is a major cause of glomerular injury and can result from systemic hypertension or a

local change in glomerular haemodynamics. Nephron loss, from any cause, is followed by compensatory hyperfiltration in the remaining functional glomeruli in an effort to maintain GFR. Over years, the hyperfiltering remnant nephrons develop focal and segmental glomerulosclerosis and eventually, global sclerosis (Neuringer and Brenner, 1992). TGFβ may be an important regulator of matrix accumulation in remnant nephrons. Other mediators are angiotensin II, PDGF and endothelins.

1.3. AIM OF STUDY

Vasoactive substances, with directly opposing effects, are present and functional in the kidney. Previous reports in the literature are predominantly those of animal experiments or human studies of isolated peptides. The aims of this thesis have been:

(1) To examine the role of functionally opposing vasoactive peptides (endothelin-1, atrial natriuretic peptide and kinins) in a human model of renal inflammation, namely acute renal rejection and glomerulonephritis. Comparison was made with various glomerular disorders in an effort to assess the effect of immunosuppressive drugs on these peptides.

(2) To study the localisation of ET_A and ET_B receptors in these renal disorders, as this has not been previously elucidated in human renal disease.

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 ETHICAL APPROVAL AND PATIENT/GUARDIAN CONSENT

Ethical permission for the study was obtained from the Ethics Committee of the Medical School, University of Natal. Permission for the collection of post-mortem tissue samples had been obtained from Professor J B Botha, Head - Department of Forensic Medicine, University of Natal. Normal kidney tissue was collected at autopsy with the co-operation of the attending Forensic Surgeon. Consent was obtained from patients, normal subjects and the family of the deceased (Appendix 2.1.1 and 2.1.2). Pathological kidney tissue was collected at the Renal Unit, Addington Hospital, Durban. All patients were informed by myself that blood and urine samples and a small piece of kidney tissue that will be removed at biopsy will be used for research purposes.

2.2 SAMPLE COLLECTION

2.2.1 RENAL DISORDERS

2.2.1.1 Acute renal allograft rejection

Samples of blood, spot urine and kidney biopsy material were collected from renal transplant patients undergoing graft dysfunction. Renal biopsies were undertaken for routine diagnostic purposes, and those patients with a diagnosis of acute rejection were included in the study. All relevant personal and clinical details together with all medications that had been administered are recorded in Appendix 2.2.1.1

2.2.1.2 Renal parenchymal disease

Blood, spot urine and renal biopsy material was collected from patients with haematuria, proteinuria, nephrotic syndrome or renal dysfunction who underwent routine diagnostic renal biopsies. The tissue was processed as in 2.3.1.1 below. All relevant clinical details are recorded in Appendix 2.2.1.2. This group of patients served as disease controls.

2.2.2 CONTROLS

2.2.2.1 Kidney donors

Blood and spot urine samples were collected from kidney donors prior to uni-nephrectomy; these served as controls and were compared with samples collected post-uninephrectomy.

2.2.2.2 Post mortem kidney tissue

Human kidney tissue was collected from individuals who had died in or soon after arrival at hospital, or were declared dead on arrival at hospitals in the Durban area, as a result of trauma not involving the abdomen or sudden unexplained death. The corpses were immediately refrigerated and maintained at 4°C in the hospital or state morgue. The age, sex, cause and time of injury and death was recorded, together with all medications that had been administered before death (Appendix 2.2.2). Kidney tissue was collected within 24h of death in 5% (v/v) formal saline and served as normal controls.

2.3 SAMPLE PROCESSING AND STORAGE

2.3.1 KIDNEY TISSUE

2.3.1.1 Tissue processing

Kidney tissue was processed as follows:

- 0.5cm^3 of tissue in 5% formal saline (v/v) fixative for 24 h at room temperature (RT) to be embedded in paraffin wax and used for light microscopy (Appendix 2.3.1.1a)
- 0.25cm^3 of tissue in 2% paraformaldehyde (PFA, w/v)/1% gluteraldehyde (v/v; pH 7.2) fixative for 2 h at 4°C to be embedded in resin and used for immuno-electron microscopy
- 0.25cm^3 of tissue in 4% PFA (w/v)/2% gluteraldehyde (v/v; pH 7.4) a modified Karnovsky fixative [Karnovsky, 1965], for 2 h at 4°C to be embedded in resin and used for transmission electron microscopy (TEM); Appendix 2.3.1.1b

2.3.1.2 Tissue fixation and wax embedding for light microscopy

Sections (5mm thick) of kidney were placed in tissue cassettes. These tissue samples were dehydrated and embedded in paraffin wax using absolute ethanol, xylene and wax under sterile conditions in an automatic tissue processor (Shandon), and these samples were used for both light microscopy and *in situ* RT-PCR. The automated schedule of steps outlining the fixation, dehydration, clearing, infiltration and embedding carried out by the Department of Histopathology, University of Natal are listed in Appendix 2.3.1.1a. Of these wax embedded tissue samples, three μm sections were adhered onto plain glass slides for histology, as well as poly-L-lysine (Sigma Chemicals, St. Louis) coated glass slides to be used for the localisation of endothelin and its receptors, TK and ANP by immunocytochemistry (ICC). Four micron

sections of these tissue samples were adhered onto glass slides coated with a 2% solution (v/v) of 3-aminopropyltriethoxysilane (Sigma Chemicals, St. Louis) in acetone for the demonstration of ET-1 expression by *in situ* RT-PCR.

2.3.1.3 Tissue fixation and resin embedding for electron microscopy

2.3.1.3.1 Tissue fixation for transmission electron microscopy (TEM)

Biopsy tissue for TEM was immediately immersed in Karnovsky's fixative for 90 min at 4°C. The specimen was then diced into 1mm^3 cubes and re-immersed in fresh Karnovsky's fixative for a further 30 min. Thereafter, the tissue was transferred to 0.2M sodium cacodylate (pH 7.2) maintained at 4°C until embedded and polymerised in low viscosity embedding media (Electron Microscopy Services, Pennsylvania, USA) as outlined in Appendix 2.2.1.1b [Glauert, 1975].

2.3.1.3.2 Tissue fixation for immuno-electron microscopy

Biopsy tissue for immuno-electron microscopy was immediately immersed in 2% PFA (w/v)/1 % gluteraldehyde fixative (v/v, pH 7.2) for 2 h at 4°C, then transferred to 0.2 M sodium cacodylate buffer (pH 7.2) maintained at 4°C before resin embedding.

2.3.1.3.3 Resin embedding for electron microscopy

The fixed tissue was dissected into 1mm^3 sections and placed in disposable baskets in a tissue processor (Reichert-Lynx). The machine was programmed to incubate the sections in a series of reagents as described in Appendix 2.3.1.1b. The tissue was then removed from the processor and polymerised in pure resin overnight at 40°C.

2.3.1.4 Sample storage

2.3.1.4.1 Wax embedded tissue

The wax embedded tissue samples were stored at room temperature (RT) for future microscopic and immunohistochemical analysis.

2.3.1.4.2 Resin embedded tissue

The resin embedded tissue samples were stored at room temperature (RT) for future microscopic and immuno- histochemical analysis as outlined in section 2.6.

2.3.2 URINE SAMPLES

2.3.2.1 Endothelin-1 (ET-1)

Spot urine samples (10 ml) were collected in 200 ul bacitracin [(bacteriostat); 2840 u/ml; Sigma, St Louis] and 200 ul Soyabean trypsin inhibtor [(SBTI, inhibitor of arginine proteases); 2720 u/ml, Sigma, St Louis] and centrifuged at 1400xg for 20 min at 4°C. One ml supernatant was aliquotted into microfuge tubes containing 200 ul mediator peptide inhibitor cocktail (see Appendix 2.3) and stored at -20°C until ET-1 measurements were made.

2.3.2.2 Tissue kallikrein

10 ml urine samples were decanted into corningware tubes containing 200 ul bacitracin and 200 ul SBTI (inhibitor of arginine proteases other than TK). Urine was centrifuged at 1400xg for 20 min at 4°C. Thereafter 1 ml of urine was aliquotted into eppendorf tubes containing 50 ul 0.2M Tris-HC1 (pH 8.2) and stored at –20°C until assayed.

2.3.2.3 Basal kinin generation

Spot urine samples were collected in corningware tubes (Corning, USA) containing 200 ul kininase inhibitor mediator peptide cocktail (Appendix 2.3), excluding STB1 and aprotinin (so that kinins could be generated from endogenous kininogen) and centrifuged at 1400xg for 20 min at 4°C. One ml aliquots were stored in eppendorf tubes at –20°C until the kinin generation assay.

2.3.2.4 ANP

Spot urine samples were collected in 200 ul bacitracin/SBT1 and centrifuged at 1400xg for 20 min at 4°C. One ml supernatant was aliquotted into eppendorf tubes containing 200 ul mediator peptide inhibitor cocktail and stored at –20°C until assayed.

2.3.3 BLOOD SAMPLES

2.3.3.1. Endothelin-1

Whole blood samples were collected from patients recumbent for 20 min in chilled sodium ethylenediamine tetraacetic acid (EDTA; 2mg/ml, w/v) tubes and centrifuged immediately at 1400xg for 20 min at 4°C. One ml plasma was transferred into polypropylene tubes (Corning, USA) containing 200ul peptide inhibitor cocktail and stored at –20°C until assayed.

2.3.3.2 ANP

Whole blood samples were collected as reported in 2.3.3.1 above and stored at –20°C until the assay was done.

2.4 ANTIBODY PROFILES

2.4.1 Endothelin-1 (ET-1) antibody

The ET-1 antibody was supplied by Biomedica (Vienna, Austria). The immunogen was human ET-1 coupled to bovine serum albumin (BSA) and keyhole limped haemocyanine (KLH). The antibody was raised in the rabbit, and produced as an immuno-affinity purified IgG, lyophilized in 0.05 M borate buffer (pH 8.5). The antibody specificity was 100% with ET-1, 142% with ET-2, 98% with ET-3, and less than 1% with Big ET 1-38 and Big ET-fragment 22-38, Sarafotoxin and ANP.

2.4.2 Endothelin receptor antibodies

2.4.2.1 Endothelin A (ET_A) receptor antibody

ET_A receptor antibodies (AS444) were kindly provided by Werner Müller-Esterl (Institute of Biochemistry, University Hospital Frankfurt, Theodore-Stern-Kai 7, D-60590, Frankfurt, Germany). AS444 raised in the rabbit against peptide sequences CDN 25 (ED1- from the NH2 terminal) and CTS 24 (ED4 –from the carboxy terminal) of the human ET_A receptor. The whole antiserum was lyophilized and reconstituted with distilled water.

2.4.2.2 Endothelin B (ET_B) receptor antibody

ET_B receptor antibodies (AS445) were also provided by Werner Müller-Esterl. AS445 was raised in the rabbit against peptides CGL-26 (ED1-from the amino terminal) and CLK (ED4-from the carboxy terminal) of the ET_B receptor. The whole antiserum was lyophilized and reconstituted with distilled water.

2.4.3 ANP antibody

ANP antibody, raised against human ANP 1-28, was supplied by the Peptide Institute (Osaka, Japan). The antiserum was lyophilized with 0.001 M PBS (pH 7.0). The immunogen was ANP (human, 1-28)-TG (bovine thyroglobulin). The specificity of ANP (human, 1-28) was reported to be 100 %, ANP (human, 7-28) 100 %, [Met $(0)^{12}$]-ANP (human, 1-28) 150%, ANP (rat, 1-28) 55 % and ß-ANP (human, 1-28 dimer) 100 %. The sensitivity (IC_{50}) was 0.28 pmol/ml. There was no cross-reactivity against Oxytocin, (Arg^8)-Vasopressin, Somatostatin, (Met^5)-Enkephalin and ß-Endorphin (human).

2.4.4. Tissue kallikrein antibody

2.4.4.1 Generation of anti-human rTK antibody

Anti tissue kallikrein antibody directed against recombinant tissue kallikrein (rTK) generated in *E. Coli* transfected with human tissue cDNA was raised in the goat. The recombinant TK (rTK) was supplied by Dr Michael Kemme (Institute for Technical University of Darmstadt, Darmstadt, Germany).

2.4.4.2 Antibody Production

A healthy goat was initially immunised by a single intramuscular injection of recombinant tissue kallikrein (rTK), conjugated in 125 µl Titermax® adjuvant. Thereafter, a booster programme using similar doses of conjugated antigen was initiated over a 4 month period. Serum from pre-booster (non-immune) and fortnightly venous bleeds was used to determine cross-reactivity, specificity and antibody titre. The titre was determined by a standard single site enzyme-linked immunosorbant assay (ELISA) using human urinary kallikrein (Protogen, Sweden) as the antigen. The titre increased from 1: 50 to an optimum of 1:800.

2.4.4.3 Antibody isolation and purification

The isolation and characterization of the IgG was performed according to the method described by Johnstone and Thorpe (1982). The serum proteins were double precipitated with 14 % $NaSO^4$ (w/v), and centrifuged at 3000xg. The precipitate was reconstituted up to 30 % of the original volume with distilled H_2O, and dialysed against 0.07 M sodium phosphate buffer (pH 6.3) for 24 h at 4°C. Isolation of the IgG was performed using a diethylaminoethyl sephadex (DEAE), a-25 ion exchange column (Sigma) with 0.02 M sodium phosphate buffer (pH 8.0). One ml fractions were collected. Those fractions showing the highest absorbance (0.311 - 0.827) at 280 nm were pooled. The protein concentration of this pooled fraction was 1.11 mg/ml. The IgG was then characterized on 7 % sodium dodecyl-sulphate polyacrylamide gel (SDS-PAGE) against IgG molecular weight markers (Sigma MW SDS 70) to determine purity. The purity, specificity and sensitivity of the antibodies were verified by a single site ELISA using human urinary kallikrein (HUK, which also determined the antibody titre), Western blot (immunoblotting), positive control tissue and preabsorption with rTK in immunocytochemical studies, as well as the use of control human urine (TK is present in human urine in readily measurable concentrations), in an ELISA which demonstrated the reproducibility of the results produced with these antibodies. The optimal rTK concentration detected was 7.5 ug and the optimal antibody dilution was 1: 2000; 30 µl aliquots were stored at -20°C.The methodology is described in Appendix 2.4.4.3 a-c.

2.5 MEASUREMENTS

2.5.1 ENDOTHELIN-1 ELISA

ET-1 was measured in plasma and urine by a sandwich-type enzyme immunoassay, based on the method of Suzuki et al. (1989), using a commercial kit supplied by Wako Chemical Industries, Osaka (Japan). A sandwich-type antigen-antibody complex < anti ET-1 monoclonal antibody: ET-1: enzyme-labelled anti ET-1 polyclonal antibody > was formed. Microplates coated with anti ET-1 monoclonal antibody were incubated with the samples and enzyme-labelled antibody (peroxidase-labelled anti ET-1 polyclonal antibody). After washing the plates with PBS and removing unbound material, the amount of enzyme (peroxidase) bound to the plate is directly proportional to the amount of ET-1 in the sample, and was determined by a colour reaction using 0-phenylenediamine and hydrogen peroxide as substrates measured at 492 nm. All measurements were made in duplicate. When 1 ml of plasma was spiked with a known concentration of ET-1, recovery rates of 97.3% and 98% were achieved. Repeated assays yielded an inter-assay co-efficient of variation of 6.1 %. Data report included with the kit indicated that cross-reactivities with ET-3 and Big ET-1 were below 0.4%, and a measurable range of 2-200 pg/ml (corresponding to 0.5-50 pg/ml when 1 ml of plasma is pre-treated). The detailed methodology is tabulated in Appendix 2.5.1a. The standard curve is shown in Appendix 2.5.1b.

2.5.2 ATRIAL NATRIURETIC PEPTIDE RIA

Blood samples collected in chilled sodium EDTA (2 mg/ml, w/v) tubes was centrifuged at 1400xg for 20 min at 4°C. One ml plasma was aliquotted into microfuge tubes containing 200 µl peptide inhibitor cocktail and stored at -20° until the assay. Urine samples were collected in

chilled tubes containing 200 ul thiomersal (Sigma, St Louis) and 200 ul SBTI (4mg/ml) and centrifuged at 1400xg for 20 min at 4°C. One ml supernatant was aliquotted into microfuge tubes containing 100 ul 0.2 M Tris buffer (pH 8.2). ANP was extracted from plasma and urine samples using Sep-Pak C18 cartridges. The cartridges were activated with 1ml 60 % acetonitrile in 1 % trifluoroacetic acid (TFA). The plasma sample was acidified with 0.1 % TFA and loaded onto Sep-Pak cartridges. After washing, ANP was eluted with 60 % acetonitrile in 1 % TFA. The eluant was evaporated to dryness under a nitrogen stream at 37°C in a water bath. The residues were reconstituted with a buffer solution and subjected to RIA using a commercial kit (Peninsula Laboratories, Belmont, Ca). Standard curve was generated on semi-log graph paper. To calculate the amount of peptide in the original sample, the concentration of the assayed sample was multiplied by the dilution factor used to prepare the sample. Measurements were expressed in pg/ml. Detailed methodology is outlined in Appendix 2.5.2a. The standard curve is shown in Appendix 2.5.2b.

2.5.3 ASSAYS FOR TISSUE KALLIKREIN (TK)

The presence of active TK in urine samples was determined by an amidolytic assay whereas total immunoreactive TK was measured by ELISA. Purified human urinary tissue kallikrein (HUK, Calbiochem, Lucerne) was used to validate both the amidolytic assay and ELISA, and these results were used to calculate both the intra- and inter-assay coeffficients of variation.

2.5.3.1 Controls for Amidase assay, TK ELISA and Kinin generation ELISA

A large volume of pooled human urine (from normal volunteers), a fairly good source of TK, was collected in thiomersal. Aliquots (1ml) were mixed with 50 ul of 40 mM Tris (pH 8.0) per ml of urine and stored at -20°C. Serial dilutions of this urine were included as controls for

both the amidolytic assay and TK ELISA. These values were used to calculate both the intra- and inter-assay coefficients of variation. For basal kinin values, aliquots were mixed with 100 ul of inhibitor cocktail (refer to Appendix 2.3) per ml of urine, and for the kinin generation assay, 100 ul kinin generating cocktail (Appendix 2.3) per ml of urine and stored at -20°C. These were used as controls for the kinin generation ELISA. One set of plain urine samples stored at -20°C, without any buffer, was used to measure total protein.

2.5.3.2 Enzymic assay (amidolytic microassay)

This is a colorimetric, end-point microassay using a microtitre plate for the measurement of the enzymic activity of TK in biological samples. The amount of TK is measured by assessing the activity of the enzyme on the selective, synthetic substrate, H-D-Val-Leu-Arg-pNA [(S2266, Kabivitrum, Sweden); Amundson, 1979] in the presence of soya bean trypsin inhibitor (SBTI) as modified by Lauar et al. (1982), and further developed as an end-point assay in a microtitre plate by Rahman *et al.* (1994b). The enzymic activity of TK is proportional to the release of para-nitroaniline (pNA) from H-D-Val-LeuArg-pNA, which has a peak absorbance at 405 nm. A microassay standard curve was constructed using human urinary kallikrein (HUK, Calbiochem, Lucerne) from which the concentration of TK in urine samples were measured in ng/ml (Appendix 2.5.3.2b). Control urine was included in each run to determine inter-assay coefficient of variation. During each assay, 2 sets of plates were processed simultaneously, one being the measurement of TK activity at zero time (blank), and the other the enzymic activity of TK after a 3 h incubation. All determinations were performed in triplicate as tabulated in Appendix 2.5.3.2a.

2.5.3.3 TK ELISA

Aliquots of urine were used to measure total TK (in triplicate) by a sandwich ELISA using goat anti-human rTK IgG and rabbit anti-human rTK IgG. The anti-species antibody, anti-rabbit IgG raised in sheep (Sigma, St Louis), was conjugated with the enzyme alkaline phosphatase, that hydrolyses the chromogenic substrate disodium p-nitrophenyl phosphate (pNPP). The stepwise procedure is described in Appendix 2.5.3.3a. The standard curve is depicted in Appendix 2.5.3.3b.

2.5.4 MEASUREMENT OF BASAL KININ LEVELS AND KININOGENASE ACTIVITY OF TK BY KININ GENERATION ELISA

The capacity to form kinins by TK from the kininogen in urine was determined by initially generating the release of its kinin moiety with endogenous TK followed by the quantitative analysis of the released kinin by ELISA. An important aspect of this assay is the use of two separate kininase/protease inhibitor cocktails (Appendix 2.3). Firstly, one set of aliquots of all samples and control urine were stored in inhibitor cocktail that contained proteins that inhibited the enzymic activities of TK, PK and other trypsin-like proteases (10 uM aprotonin, w/v, 10 uM SBTI, w/v), thereby preventing the release of kinin from endogenous kininogen. It also contained 10 uM captopril, an inhibitor of the KII family of kininases, and 10 uM phosphoramidon, another kininase inhibitor, to ensure that the basal kinins present in the samples were not destroyed. A second set of aliquots of all samples and control urine contained peptidases that prevented the destruction of kinins but permitted its formation. The second aliquot of each sample and control urine was stored in kinin generating cocktail, which was similar to the inhibitor cocktail except that it lacked aprotonin and SBTI. The absence of these protease inhibitors ensured that kinin could be released from endogenous kininogen and

protected by inhibitors of the peptidases. Activation of endogenous TK was achieved by incubating equal volumes of urine and generating cocktail for 60 min at 37°C in the presence of kininase inhibitors to limit the degradation of the generated kinin (Appendix 2.5.4). The released kinin was then extracted with acid-alcohol (absolute alcohol/3 mM HCl, v/v) and measured by a competitive ELISA following overnight incubation with a standard amount of monoclonal mouse anti-BK IgG (SBK1) at 4°C, to bind the released kinin. Known amounts of standard BK (1.25 - 150 ng BK/ml, Sigma, St. Louis) were also allowed to react overnight with standard amounts of SBK1. The wells of a Nunc Immulon Maxisorp^{TM} ELISA plate (Nunc, UK) were coated with a standard amount of BK conjugated to cytochrome C using the linker N-succimidyl 3- (2-pyridyldithio) propionate (SPDP). The kinin-SBK1 reaction mixtures were then added to each well and incubated for 3 h at 37°C so that any remaining free antibody could react with the BK-SPDP-cytochrome C conjugate bound to the ELISA plate. The secondary antibody was an anti-mouse IgG labelled with the enzyme alkaline phosphatase, which converted the colourless disodium p-nitrophenyl phosphate (pNPP) to a yellow chromogen that has a maximal absorbance at 405 nm. The absorbance values obtained for the standard BK was used to plot a standard curve of absorbance versus log concentration from which the basal and generated kinin contents of the tissue extracts were determined (Appendix 2.5.4b). The basal and generated kinin contents of control urine were also measured during each run to determine the inter- and intra-assay coefficients of variation. The detailed stepwise method for the competitive ELISA is tabulated in Appendix 2.5.4a.

2.6 IMMUNOLOCALISATION OF ET-1 AND ET_A AND ET_B RECEPTORS, ANP AND THE KININ FORMING ENZYME TISSUE KALLIKREIN

2.6.1 LIGHT MICROSCOPY: PEROXIDASE-ANTIPEROXIDASE (PAP) IMMUNOLABELLING METHOD

Three um thick sections of wax-embedded tissue were adhered onto adhesive coated (poly-L-lysine, Sigma Chemicals) slides. The technique used was adapted from that described by Robinson et al. (1990), MacLennan et al. (1990) and Marriott and Carlton (1990). The kidney sections were placed on a heating mantle at 60°C until the wax melted. The PAP method required dewaxing the tissue sections using xylene and rehydration with increasingly dilute alcohol solutions (100%, 90%, 70% ethanol) and distilled water as the final rehydrant. When the tissue was partially rehydrated, it was immersed in absolute methanol (99.9%; Saarchem, S Africa) for 20 min to quench endogenous peroxidases. The tissue was then heated in 0.1 M sodium citrate buffer (pH 6.0) at 80°C for 8 min for antigen retrieved in a Sharp R-4A52 microwave oven (Sharp Electronics, Japan). The tissue was further blocked with 5% H202/ 95% MeOH for 5 min to quench endogenous peroxidases. Immunolabelling for ET_A and ET_B receptors, ANP and TK was carried out similarly at the following dilutions: ET-1 1:50; ET_A 1: 650; ET_B 1: 500; ANP 1: 100. Incubation with the specific primary antibody in 0.1 M phosphate buffer (pH 7.4)/1% BSA (v/v)] was carried out for 2 min. Next the tissue was incubated with biotin link (DAKO K0690) for 2 min, and then treated with Strepavidin (DAKO K0690) for 2 min. The sections were washed between incubations by submerging the slides for 5 min in 0.01 M PBS (pH 7.4) containing 27 mM potassium chloride, 0.137 M sodium chloride (w/w) (Sigma) for 5 min and the tissue sections were not allowed to dry out. All dilutions were made up in 0.01 M PBS/1% BSA (pH 7.4, v/v). Labelled slides were stored

in the dark to preserve the immunoprecipitant label. The labelling antibody, containing the PAP immunoenzyme complex, was visualised by incubating the sections for 2-5 min with liquid 3,3' diaminobenzidine (DAB) precipitant (DAKO, K3465). The sections were counterstained with Mayers' haematoxylin (Sigma, St Louis) for 3-5 min. Sections were then dehydrated and mounted with a permanent medium (Entellen, Merck Gmbh) and immunovisualised by conventional light microscopy under a Nikon photomicroscope (Nikon Optiphot, Japan). The detailed methodology is described in Appendix 2.6.2.

2.6.2 CONFOCAL MICROSCOPY: FLUORESCENT IMMUNOLABELLING METHOD

This process required melting the wax on a heating mantle, dewaxing in xylene and rehydrating the 3 micron sections with increasingly dilute alcohol solutions (100 %, 90 %, 70 %) and distilled H_2O as the final rehydrant. Endogenous peroxidases were quenched by immersing the sections in 100% MeOH for 20 min. Antigen retrieval was carried out by heating the tissue in 0.1 M sodium citrate buffer (pH 6.0) at 80°C for 3 min in a microwave oven (Sharp R-4A52), and nonspecific sites blocked with human IgG (Sigma) for 2 min. Immunolabelling for ET-1, ETA and ETB receptors, ANP, and TK was carried out similarly at the following dilutions: ET-1 1: 50; ET_A 1: 650; ET_B 1: 500; TK 1: 1000. The tissue sections were incubated with the specific primary antibody diluted in 0.1 M PBS (pH 7.4) for 18h at 4°C. The bound primary antibody complex was conjugated to a fluorescein labelled [fluorescein isothiocyanate (FITC), 525 nm, Sigma] anti-rabbit IgG for 30 min at RT. All washing steps were carried out in 0.01M PBS (Sigma; pH 7.4). Fluorescent labelling was analysed using the Leica TD4 confocal microscopy system (Leica, Germany). All dilutions were made up in 0.01M PBS/0.1% BSA (v/v, pH 7.4) and tissue sections were not allowed to

dry out between incubations. Labelled slides were stored in the dark at 4°C to limit bleaching of the fluorescent tag. The detailed method is outlined in Appendix 2.6.3.

2.6.3 ELECTRON MICROSCOPY: IMMUNOLOCALISATION OF ENDOTHELIN-1 AND ITS RECEPTORS IN THE HUMAN KIDNEY

Fresh tissue was immersed in 1% glutaraldehyde (v/v) and 4% paraformaldehyde (w/v) fixative in a 0.2 M cacodylate buffer (pH 7.2) for 4 h at 4°C, then transferred to sodium cacodylate buffer (0.2 M, pH 7.2) until processed. The fixed tissue was divided into 1 mm cubes, and processed in an automatic Reichert-Lynx tissue processor. The specimens were then rotated through a pre-programmed sequence of dehydration in graded alcohol solutions (70%, 90% and absolute alcohol) for 15 min each, immersed in an intermediate solvent (propylene oxide) for 30 min and then in spurr-epoxy resin for 120 min (2 changes of 60 min each). The sections were then embedded in pure resin overnight at 60°C and 1 μm sections cut with a Reichert Ultra cut ultramicrotome (Jung, Germany). Sections were collected onto glass slides, heat fixed, stained with 1% alkaline toluidine blue (Sigma) and examined with a Nikon microscope. Fields of interest were selected, 50 nm sections cut and collected onto uncoated Nickel grids prior to immunostaining. The sections were incubated with rabbit anti ET1, ETA and ETB antibodies (dilutions of 1:100, 1: 1000 and 1: 1000 respectively) and goat anti-rabbit IgG (1:100 dilution) conjugated with a 10 nm Aurogold probe. Sections were counterstained with 2% uranyl acetate and 1% Reynold's lead citrate (Pelco International, California, USA) and examined under a Jeol 1010 transmission electron microscope. The detailed methodology is outlined in Appendix 2.6.4.

2.6.4 CONTROLS FOR ICC

2.6.4.1 Negative controls for ICC

Loss of immunolabelling following preabsorption of the primary antibody with an excess of rTK demonstrated the specificity of the antibody. The goat anti-human rTK antibody was diluted 1:500 with 0.01M phosphate buffer (pH 7.2) and an equal volume added to 500 ul of a 2 mg/ml stock solution of rTK to yield a final concentration of 1 mg antigen (rTK) per ml. This was mixed and incubated overnight at 4°C to allow formation of antigen antibody complexes. Following centrifugation (2200 x g, 4°C, Biofuge 13R), the preabsorbed antibody was used to replace the primary antibody. An additional control was the replacement of the primary antibody by 100 ul PBS. The immunolocalisation procedure is described in section 2.6.1.

2.6.4.2 Positive tissue controls for Immunocytochemistry

Since TK is abundant in the duct cells of the human salivary glands, samples of fresh human salivary gland were collected at post-mortem, fixed in 5% formal saline and embedded in paraffin wax. During each labelling run, this appropriate positive control tissue demonstrated the presence of TK in the apical region of duct cells in human salivary glands (Schachter, 1980; Orstavic, 1980). Placental tissue and cardiac atria were used as positive control tissue for ET-1 and ANP respectively.

2.7 EXPRESSION OF ENDOTHELIN-1 mRNA IN THE HUMAN KIDNEY

2.7.1 *In situ* reverse transcriptase polymerase chain reaction (RT-PCR)

Four μm thick wax impregnated tissue sections, placed on pre-treated glass slides, were dewaxed in xylene and rehydrated in ethanol into water. The tissue was then treated with

20μg/ml Proteinase K [in 100 mM Tris HCl (pH 8.0), 50m M EDTA v/v] for 25 min at 37°C to permeabilise the cell membranes. This was followed by a DNAse treatment overnight to destroy all DNA present. cDNA synthesis using the Moloney murine leukaemia virus (M-MuLV) reverse transcriptase and the First Strand Synthesis Kit (Pharmacia, Sweden) was then carried out at 37°C for 1 h. The PCR reaction was followed in the presence of the appropriate primers and a freshly prepared colour substrate (1:50 dilution of nitroblue tetrazolium/ 5-bromo-4-chloro-3-indoyl-phosphate (Roche) and incubated at RT in the dark until a reddish-purple colour precipitate was visible. The detailed methodology is outlined in Appendix 2.7.

2.8 MEASUREMENT OF RENAL PLASMA FLOW

Effective renal plasma flow (ERPF) was measured in 6 stable renal transplant patients (on day 3 post transplant) and one control subject in the Medical Physics Department at Addington Hospital, Durban, using sodium ^{131} iodohippurate. Simultaneous measurement of GFR was made using 99^{M} Technetium-diethelene triamine penta acetate (Tc-DTPA). ERPF was repeated in 3 of the patients, 2 during an episode of acute rejection.

2.9 MEASUREMENT OF TOTAL BODY WATER

Assessment of excess fluid distribution by total body water (TBW) was made by bioelectrical impedance analysis (BIA) using the Biostat body composition analyser (BodyTrak, Cape Town) in 5 transplant patients, 4 patients with renal disease and 3 controls. Electrodes are placed on the right hand and right foot with the subject relaxed in the supine position. A high

frequency electrical current discriminates between the different impedances (or resistances) of muscle and fat. A comprehensive body composition analysis is given which calculates data on ideal body weight, fat weight, lean weight, TBW, BMR and estimated average energy requirement and corresponding normal ranges. BIA correlations against densitometry and dilution techniques such as D20 and H20 have enabled accurate determinations of TBW to be simply and rapidly made.

2.10 PHOTOMICROGRAPHY

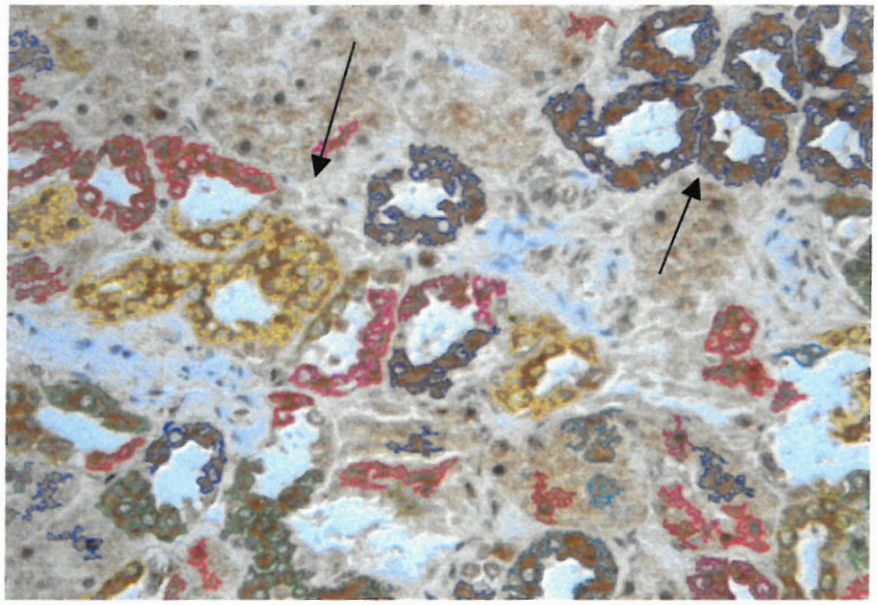
Initially for light microscopy, tissue sections processed by the PAP method and stained with DAB were examined, and areas of interest photographed, with a Nikon binocular Optiphot photomicroscope (Nikon, Japan) with objective magnifications ranging from 10x to 100x using Kodak 160 ASA (Eastman-Kodak, USA) colour film. For tissue sections labelled with a fluorescent tag, immunofluorescence was observed with a Leitz DM IRB confocal microscope (Leica, Germany) attached to Diamond Pro 17 (Mitsubishi) and Diamond Pro 21T (Mitsubishi) monitors. Confocal images were recorded at a pixel density of 225 x 225 pixels in 8 bit tagged image file (tif) format. Subsequently, PAP and fluorescent-labelled tissue sections were viewed under a Leica DMLB microscope and the images captured with a Leica DC 100 digital camera (Leica, Germany). These 24 bit images were also recorded in tagged image file (tif) format.

2.11 IMAGE ANALYSIS

2.11.1 Analysis of PAP images

PAP immuno-stained tissue slides were viewed by a Nikon Optiphot microscope (Nikon, Japan) which was interphased to a 3 CCD digital camera system (Sony Corp., Japan). The quantitative image analyser used to determine the labeling intensity of the PAP immuno-localisation was the Kontron Elektronic KS 300 (Zeiss GmbH, Germany), running on Windows 95™, (Microsoft Corp., USA). The digital images captured were processed and converted to grey image ranging in grey density from 0 to 255. The areas of PAP labelling were segmented. This involved the immuno-positive areas being separated from their environments on the basis of their grey values (set up threshold factor), thereby creating a binary image for quantification. The grey values in the image represent the intensity of the label over the entire area of possible x-y co-ordinates. A coloured contour was superimposed on the binary image to complement previous threshold areas. This image was then masked onto the original image of the chosen areas. The median maximal density of immuno-labelling was then calculated as pixels per unit area. Figure 2.11.1 is a photomicrographic representation of this procedure.

Fig 2.11.1 PAP IMAGE ANALYSIS



Coloured contours are superimposed over areas of immunolabelling (arrows)

2.11.2 Analysis of immunofluorescent images

Determination of the amount of ET-1, ET_A and ET_B receptors and TK were computed by image analysis. Confocal scanning laser microscopy is a powerful imaging technique in which the staining intensity of fluorescently labelled tissue section could be viewed. An important capability of the confocal microscope is that it can optically cut sections through tissue. This feature was used to determine the middle plane of the cell or tissue section, and an image representative of the whole cell was then generated.

Confocal images were typically recorded at a pixel density of 225x225 pixels. The grey scale ranged from 0 to 256, and was divided into 8 equal phases (POLI Look-Up-Table), with each phase having a lower and upper threshold value on the grey scale. All the pixels in each phase were attributed with a colour for easy visible distinguishing between the phases. The breakdown of the phases is as follows:

PHASE	Lower and upper threshold	Fluorescence intensity
1 purple 2 blue	0 – 56	No fluorescence
3 cyan	56 – 88	Low fluorescence
4 green	89 – 154	Medium fluorescence
5 yellow		
6 orange	155 – 256	High fluorescence
7 red		
8 white		

The analysis performed in this study was based on a breakdown of the number of pixels per phase of the grey scale in the cytoplasm of various structures in the normal and diseased kidney. The amount of antigen was estimated by analysis of the computer generated confocal

images using the Analysis 2.1 Pro system (Soft-Imaging Software GmbH, 1996, Germany). In order to analyse the cytoplasm only of each structure of interest (proximal-, distal-, collecting tubules and glomerulus), a mask had to be generated for each type of structure. The regions of interest (ROI) – first the whole tubule, then the tubule duct and nuclei - are encircled. These images are then turned into black and white images and the one deducted from the other to generate the cytoplasm-only mask. A histogram was generated showing the pixel-breakdown in the cytoplasm of the different structures. This information was used to calculate the number of pixels falling within each phase, as well as the area analysed. This data, exported to Microsoft Excel was computed to calculate the mean intensity of immunolabelling per phase in (n) number of cells applying the unit pixel/μm^2.

2.12 STATISTICAL ANALYIS

Statistical analysis was carried out by the Instat 2 computer programme and reviewed by a statistician. Results are presented as the mean and standard error of mean (SEM). Significance was calculated by a two-tailed, unpaired Student's t-test, the Mann-Whitney test and the Kruskal-Wallis test. Levels of significance were determined using a 95 % confidence interval; a p-value < 0.05 was taken to be statistically significant.

APPENDIX (A) OF BUFFERS, REAGENTS AND METHODS

Appendix A 2.1.1 Consent form for Controls

A 2.1.1.1 Normal subjects

We plan to study hormones produced by patients with kidney disease and to compare this with normal subjects as yourself. You will be required to bring a 24-hour collection of urine and have blood taken from a vein in your arm.
You are free to agree or refuse to participate in this study.

Sicabanga ukwenza ucwaningo ngo'ju olukhiqizwa zindlala (hormones) emzimbeni Kololu cwaningo sizo qhathanisa ama hormones abantu abaguliswa yi zinso – na bantu abangaguli ndawo. Kuzo dingeka umchamo oqongelelwe ilanga lonke nobusuku bonke– uma useletha – kuzo thathwa igazi engalweni nje ngoku jwayelekile.
Ukhululekile ukwala uma ungathandi uku zibandakanya na lolu cwaningo.

_____ _____
Name/ Igama Date

A 2.1.1.2 Informed Consent for the Collection of Autopsy tissue

To the guardian

I wish to determine the role of peptides in kidney disease and compare the changes in disease with normal human tissue. To accomplish the above objective I need small samples of normal human tissue, which is most easily obtained from the post- mortem procedure.
Please consent to the above so that I may be able to accomplish this investigation.

Ngifisa ukubheka iqhaza elibanjwa ngama-enzyme ezifeni zezinso. Bese ngiziqhathanisa nalezo zabantu abangaka ngenwa yilezozifo. Ukuze lolocwaningo luphumelele ngingatho koza ukuthola isicutshana senso engenasifo. Lesosicutshana singatholakala kalula uma udokotela enza uqhaqho lokuthola imbangela yokufa komuntu, obengaphethwe yisifo sezinso. Ngicela usayine; usinike imvume yokukwenza lokhu, ngoba ngayo ngiyethemba lolucwaningo luyoba yimpumelelo.

_____ _____
Igama Lomzali/ Umbheki kamufi
Name of guardian Date

Appendix A 2.1.2 Consent form for patients

As you know, your kidney is not functioning normally. It is essential that you have a procedure called "kidney biopsy" so that the cause of the kidney failure can be determined, in order that you may be treated correctly. A scan of the kidney will be done in the Ultrasound Department, and a fine needle will be passed into the kidney tissue (which will be made pain-free by an injection of local anaesthetic) and a small amount of kidney tissue will be removed and will be sent to the Laboratory for diagnosis of your condition. A portion will be used for research to study localisation of peptides in kidney disease. After the biopsy, you may find blood in the urine, this usually settles in about 2 days. If it does not settle (which is rare), various treatments (including surgery) may be undertaken to stop the bleeding.
Blood will be taken from a vein in the arm and urine will be collected on the day of the biopsy to study hormones produced by your kidney.
You are free to agree or refuse permission for any of these procedures.

Njengoba wazi ukuthi izinso zakho azisebenzi ngendlela efanele; kubalulekile ukuba kuthathwe isicutshana ensweni yakho; sihlolwe, ukuze kutholakale ukuthi kungani ingasebenzi ukuhlanza igazi nokwenza umchamo ngendlela efaneleyo. Lokhu kuyosiza kakhulu ekwelashweni kwakho. Uyoyiswa emafutheni (Ultrasound Department) lapho okuyofike kuthathwe khena isithombe sezinso. Kuyobe sekufakwa inalithi encane kwenye yesinzo zakho, kuthathwe isicutshana esincane senso. Yisona-ke esizoya kohlolwa ukuze kutholakale imbangela yokungasebenzi kwezinso zakho. Uyoqale unikwe umjovo, oyokwenza ukuba kube ndikindiki; ukuze kungabibuhlungu.

Kuyobe sekuthathwa igazi engalweni yakho, kanye nomchamo ukuze kuyohlolwa kubhekwe izinjengezi (hormones) ezibalulekile empilweni yomuntu; ezikhiqizwa yizinso.
Emva kokuthathwa kwesicutshana ensweni, kunokwenzeka umchamo wakho ube negazi. Ngokujwayelekile lokhu kuyaphela ezinsukwini mhlawumbe ezimbili. Uma kungapheli zikhona izindlela zokunqamula lokho kopha. Kungenzeka-ke nokho ugcine usuhlingwa ukuze kunqanyulwe ukopha, kodwa yinto engajwayelekile leyo neze neze.
Imiphumela yalolucwaningo iyosiza impilo yakho; kodwa-ke ukhululekile ukwala uma ungathandi ukuzibandakanya nalolucwaningo.

_____ _____

Name (Igama) Date

Appendix A 2.3: Reagents for Sample Collection (2.2), Processing and Storage (2.3)

1. Sterile normal saline (NS) (0.9% NaCl, w/v, pH 7, Sabax)
2. SBT1 (2720 units/ml in 0.2 M Tris, Sigma)
3. Bacitracin (2840 units/ml in 0.2 M Tris, Sigma); 0.0147g in 10 ml
4. PBS (0.2 M; pH 7.4). $NaH2PO_4$ 3.12g; NaH_2HPO_4 11.32g; NaCl 8.5g
5. 5% formal saline (41% formaldehyde / 0.9% NaCl, 1:8 v/v) – dilute formaldehyde (35%, Saarchem, SA) 1:7 in 0.9% NaCl
6. 2% (w/v) paraformaldehyde (PFA) and 1% (v/v) gluteraldehyde - dissolve 2 g PFA (Saarchem, SA) in 100 ml sodium cacodylate buffer (0.2 M, pH 7.2) at 60^0C, and add 4 ml of a 25% Gluteraldehyde solution (Saarchem, SA). Store at 4^oC
7. Karnovsky's fixative (4% PFA, w/v and 2% gluteraldehyde v/v, pH 7.4) - dissolve 4 g PFA in 100 ml sodium cacodylate buffer (0.2 M, pH 7.2) at 60^0C, and add 8 ml of a 25% Gluteraldehyde solution (Saarchem, SA). Store at 4^oC
8. Sodium cacodylate buffer (0.2 M, pH 7.2) – dissolve 4.28g Cacodylic acid Na salt (Acros Organics, USA) in 100 ml dH_2O. To 25 ml of this stock add 2.1 ml 0.2 M HCl {1.7 ml conc HCl (Saarchem, SA) made up to 100 ml with dH_2O} and make up to 100 ml to adjust to pH 7.2.
9. TK cocktail 40 mM Tris, pH 8 - dissolve 4.8 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml dH_2O, adjust pH to 8.0 with HCl and adjust volume to 1 L.
10. Kinin generating cocktail: (60 mM EDTA, 6 mM phenanthraline, 10 uM captopril and 10 uM phosphoramidon) – all w/v and dry reagents purchased from Sigma Chemicals, St. Louis
11. Basal kinin and kinin inhibitor cocktail : (60 mM EDTA, 6 mM phenanthraline, 10 uM captopril, 10 uM phosphoramidon, 10 uM SBTI and 10 uM aprotonin) – all w/v and dry reagents purchased from Sigma Chemicals, St. Louis.

Appendix A 2.3.1.2 Fixation, Dehydration and Embedding schedule for light microscopy

Reagents

1. 5% formal saline (41% formaldehyde / 0.9% NaCl, 1:8 v/v) – dilute formaldehyde (35%, Saarchem, SA) 1:7 in 0.9% NaCl
2. Absolute ethanol (99% ethanol, Saarchem, SA)
3. Xylene (AR, Saarchem, SA)
4. Paraffin wax (Paraplast plus, Sherwood Medical, St. Louis, USA)

Procedure

STEP	SOLUTION	TEMP	TIME
1.	Fixation - 5% formal saline	24°C	1 h
2.	Fixation - 5% formal saline	24°C	1 h
3.	Dehydration – absolute ethanol	24°C	1 h
4.	Dehydration – absolute ethanol	24°C	1 h
5.	Dehydration – absolute ethanol	24°C	1 h
6.	Dehydration – absolute ethanol	35°C	1 h
7.	Dehydration – absolute ethanol	35°C	1 h
8.	Dehydration – absolute ethanol	35°C	1 h
9.	Dehydration – absolute ethanol	35°C	1 h
10.	Clearing – xylene	35°C	1 h
11.	Clearing – xylene	35°C	1 h
12.	Vacuum infiltration 1 – paraffin wax	60°C	1 h
13.	Vacuum infiltration 2 – paraffin wax	60°C	1 h
14.	Embedding – paraffin wax	20°C	20 min

Appendix A 2.3.1.3 Fixation, Dehydration and Embedding schedule for electron microscopy

Reagents

1. 2% (w/v) paraformaldehyde (PFA) and 1% (v/v) gluteraldehyde - dissolve 2 g PFA in 100 ml sodium cacodylate buffer (0.2 M, pH 7.2) at 60^0C, and add 4 ml of a 25% Gluteraldehyde solution (Saarchem, SA). Store at 4^oC
2. Karnovsky's fixative (4% PFA, w/v and 2% gluteraldehyde v/v, pH 7.4) - dissolve 4 g PFA in 100 ml sodium cacodylate buffer (0.2 M, pH 7.2) at 60^0C, and add 8 ml of a 25% Gluteraldehyde solution (Saarchem, SA). Store at 4^oC
3. Sodium cacodylate buffer (0.2 M, pH 7.2) – dissolve 4.28 g Cacodylic acid Na salt (Acros Organics, USA) in 100 ml dH_2O. To 25 ml of this stock add 2.1 ml 0.2 M HCl {1.7 ml conc HCl (Saarchem, SA) made up to 100 ml with dH_2O} and make up to 100 ml to adjust to pH 7.2.
4. Absolute ethanol (99% ethanol, Saarchem, SA)
5. Propylene oxide (Sigma Chemicals, St. Louis)
6. Araldite epoxy resin (Spurr, Electron Microscopy Sciences, Pennsylvania, USA)

Procedure

STEP	SOLUTION	TEMP	TIME
1.	Fixation for TEM - Karnovsky's fixative	RT	90 min
2.	Fixation for Immuno-electron microscopy - 2% PFA/1% gluteraldehyde	4^oC	2 h
3.	Second fixation for TEM – Karnovsky's fixative	RT	30 min
4.	Store fixed tissue from 2 and 3 above in Sodium cacodylate buffer (0.2 M, pH 7.2) before embedding	4^oC	20 min
5.	Sodium cacodylate buffer (0.2 M, pH 7.2)	RT	20 min
6.	Dehydration - 70% ethanol	RT	30 min
7.	Dehydration - 90% ethanol	RT	30 min
8.	Dehydration - absolute ethanol	RT	15 min
9.	Dehydration - absolute ethanol	RT	15 min
10.	Dehydration - absolute ethanol	RT	15 min
11.	Dehydration - absolute ethanol	RT	15 min
12.	Clearing - Propylene oxide	RT	30 min
13.	Infiltration - 50/50 propylene oxide and resin	RT	30 min
14.	Infiltration - Pure resin	RT	60 min
15.	Infiltration - Pure resin	RT	60 min

Appendix A 2.4.4.3a Isolation of IgG from serum

Reagents

1. Diethylaminoethyl sephadex (DEAE A-25, Sigma Chemicals, St. Louis) – in 50 ml wet settled volume of 0.07 M phosphate buffer.
2. 14% Sodium sulphate - dissolve 0.14 g anhydrous Na_2SO_4 (Saarchem, SA) in 1 ml serum
3. 0.07 M Sodium phosphate pH 6.3 - dissolve 54.6 g NaH_2PO_4 (Saarchem, SA) in 5 l dH_2O

Method

STEP	SOLUTION	TEMP.	TIME
1.	Warm serum in water bath	25°C	15 min
2.	Add anhydrous sodium sulphate to make a 18% (w/v) solution, stir to dissolve and incubate	25°C	30 min
3.	Centrifuge at 3000xg	25°C	30 min
4.	Discard supernatant, note volume of protein precipitate and redissolve in warm H_2O up to half the original volume	25°C	
5.	Add anhydrous sodium sulphate to make a 14% (w/v) solution, stir and incubate	25°C	30 min
6.	Centrifuge at 3000xg	25°C	30 min
7.	Discard supernatant, redissolve precipitate in warm water up to one third of starting volume and dialyse against 0.07M phosphate buffer	4°C	ON
8.	Equilibrate ion exchanger (DEAE) with the 0.07 M phosphate buffer	4°C	ON
9.	Pack hydrated ion exchanger into a polypropylene column and wash with 3 column volumes 0.07 M phosphate buffer at a flow rate 1 ml/min	RT	
10.	Load - dialysate	RT	
11.	Elute – with 0.07 M phosphate buffer, collect 1 ml fractions	RT	
12.	Measure absorbance at 280 nm	RT	
13.	Pool fractions with highest absorbance		

Appendix A 2.4.4.3b Titre determination for anti-rTK antibody in rabbit and goat serum

Reagents

1. Human Urinary Kallikrein (HUK, Calbiochem, USA)
2. Coating buffer (Na_2CO_3/$NaHCO_3$, pH 9.6) - dissolve 1.59 g Na_2CO_3 (Saarchem, SA) and 2.93 g $NaHCO_3$ (Saarchem, SA) in 1 l dH_2O
3. 0.01 M PBS/ 0.5% (v/v) Tween - dilute 100 ul Tween 20 (Sigma Chemicals, St. Louis) in 200 ml 0.01M PBS {1 PBS tablet (Sigma Chemicals, St. Louis) dissolved in 200 ml dH_2O}
4. 5% (w/v) Milk protein blocker - dissolve 5g Country Pasteur fat free milk powder (Nutritional Foods, SA) in 100 ml PBS. Make fresh.
5. Rabbit anti-goat (Sigma Chemicals, St. Louis) or sheep anti-rabbit (Boehringer Mannheim, Germany) IgG conjugated to alkaline phosphatase
6. 0.1M PBS/0.1% BSA (w/v) – dissolve 1.56 g NaH_2PO_4 (Saarchem, SA), 5.66 g Na_2HPO_4 (Saarchem, SA) and 4.25 g NaCl (Saarchem, SA) in 800 ml dH_2O, adjust to pH 7 with HCl, add 1 g BSA (Fraction V, Boehringer mannheim) and make up to 1 l. Store at 4°C.
7. Phosphatase substrate (pNPP, Sigma Chemicals, St. Louis)

Procedure

STEP	SOLUTION	TEMP.	TIME
1.	Dilute HUK (stored at -20°C in aliquots of 1200 ng/400 ul NS) in 4ml coating buffer (Na_2CO_3/$NaHCO_3$, pH 9.6) to obtain 5 ug HUK/ml coating buffer	4°C	
2.	Coat ELISA plate (Corning) by adding 100ul of diluted HUK to each well	4°C	ON
3.	Wash plate with 0.1 M PBS/Tween	RT	3X3min
4.	Block plate twice with 5%Elite/5%BSA	RT	2x30min
5.	Dilute serum 1/200; 1/400; 1/800; 1/1600 and 1/3200 with 0.1 M PBS/0.1% BSA	4°C	
6.	Load 100ul of diluted serum (triplicate). Blanks are wells filled with 100 ul 0.1 M PBS/0.1% BSA	37°C	60 min
7.	Wash plate with 0.1 M PBS/Tween	RT	3X3min
8.	Load 100ul of alkaline phosphatase conjugated anti-goat or anti-rabbit IgG diluted 1/250 in 0.1M PBS/0.1% BSA	37°C	60 min
9.	Wash plate with 0.1 M PBS/Tween	RT	3X3min
10.	Load plate with 100 ul 1mg/ml phosphatase substrate and read at 405nm, until absorbance readings peak at 1.0-1.5 units	RT	

Appendix A 2.4.4.3c Western Blot Analysis for Rabbit and Goat anti- human rTK IgG

Reagents

1. Recombinant tissue kallikrein (rTK, 750 ug/ml, supplied by Dr. Michael Kemme, Institute for Biochemistry, Technical University of Darmstadt, Germany).
2. PBS pH 7.2 – dissolve 1 PBS tablet (Sigma Chemicals, St. Louis) in 200 ml dH_2O
3. 22 µm Nitrocellulose paper (Amersham, England)
4. 3 µm Whatman paper (Whatman, England)
5. Blocking solution (Boehringer Mannheim, Catalogue No. 258576)
6. Rabbit and goat anti-human rTK IgG isolated in 2.4.3
7. TBST {10 mm Tris pH 9.5, 150 mM NaCl, 0.05% (v/v) Tween 20}
8. 3% (w/v) BSA in TBST – dissolve 3 g BSA (Fraction V, Boehringer Mannheim)
9. Secondary antibodies:

9.1 Alkaline phosphatase conjugated rabbit anti-goat IgG (Sigma chemicals, St. Louis) diluted 1:10000 with TBST

9.2 Sheep anti-rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) diluted 1:3000 with TBST

10. Detection buffer (0.1M Tris-HCl, 0.05M $MgCl_2$, 0.1M NaCl, pH 9.5)
11. Chromogen 0.375 mg/ml NBT (nitro blue tetrazolium chloride)/0.188 mg/ml BCIP (5-bromo-4-chloro-3-indoyl-phosphate)
12. 0.05M EDTA (ethylenediamine-tetra-acetic acid) (Sigma, St. Louis) pH 8
13. 40% Acrylamide Stock solution (w/v) – dissolve 40 g acrylamide (Sigma Chemicals, St. Louis) and 1.07 g N,N'-Bisacrylamide (BDH, UK) in 100 ml dH_2O by stirring in the dark with a bit of Amberlite mixed bed ion exchange resin (Sigma chemicals, St. Louis). Filter through 0.45 um millipore filter and store at 4°C
14. 1.125 M Tris, pH 8.8 (w/v) – dissolve 136.12 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml DEPC treated H_2O, adjust to pH 8.8 with HCl and make up to 1 L.
15. 10% Sodium dodecyl sulphate (SDS, w/v) – dissolve 10 g SDS (Amresco, Ohio, USA) in 100 ml DEPC treated H_2O
16. TEMED (BDH, UK)
17. 10% Ammonium peroxodisulphate (APS, w/v) – dissolve 10 g APS (BDH, UK) in 100 ml dH_2O.
18. 0.5 M Tris, pH 6.8 – dissolve 61.5 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml DEPC treated H_2O, adjust to pH 6.8 with HCl and make up to 1 L.
19. Loading buffer (10% SDS, 1% bromophenol blue, 5% ß-mercaptoethanol, 1% glycerol, 0.5M tris-HCl pH 6.8)
20. Prestained low range standards - Ovalbumin 43 kDa, Carbonic anhydrase 29 kDa, Lactoglobulin 18.4 kDa, Lysozyme 14.3 kDa, Bovine Trypsin Inhibitor 6.3 kDa, Insulin 2.9 kDa (Life Technologies former Gibco/BRL, Germany)

21. 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 and phosphorylase B 97.4 kDa (Biorad, USA)
22. Running Buffer (0.4M glycine, 0.02M SDS, 0.12M Tris-HCl, pH 8.3)
23. Transfer buffer (10% methanol, 0.025M Tris-HCl, 0.192M glycine, pH 8.3)
24. 0.4% Tween 20/PBS (200 µl Tween 20 in 50 ml PBS)
25. India ink solution {50 µl India ink (Pelikan drawing ink A, Pelikan, Germany) in 50 ml 0.3% Tween 20/PBS}
26. Composition of Running and Stacking gels

	15% Running gel	**5% Stacking Gel**
40% Acrylamide Stock	18 ml	2.5 ml
1.125 M Tris, pH 8.8	16 ml	5 ml
dH_2O	14 ml	12.5 ml
10% SDS	480 ul	200 ul
TEMED	20 ul	25 ul
10% APS	200 ul	150 ul

Procedure

CHECKERBOARD TEST FOR ANTIGEN AND ANTIBODY DILUTION			
STEP	**PROCEDURE**	**TEMP**	**TIME**
1.	Serially dilute rTK stock (250 to 0.9 ng/ml) with PBS		
2.	Cut Nitrocellulose paper into 8 mm x 8 cm strips		
3.	Spot 1µl of each dilution of rTK 1 cm apart on each of the nitrocellulose strips and dry on Whatman paper	RT	1h
4.	Block non-specific binding sites with 1x blocking solution with shaking	RT	2x10 min
5.	Prepare serial dilutions of rabbit & goat anti-human rTK IgG with 3% BSA in TBST and incubate with shaking	4°C	ON
6.	Wash in TBST	RT	3X10 min
7.	Incubate with secondary antibody	RT	3 h
8.	Wash in detection buffer	RT	3X10 min
9.	Incubate with chromogen solution in the dark	RT	few min
10.	Stop reaction with 0.05M EDTA	RT	5-10 min
11.	Wash with dH_2O	RT	30 min
12.	Press dry between Whatman paper, mount, seal in cellophane & determine optimal rTK conc.& Ab dilution		

SDS-PAGE			
1.	Clean Mini Protean® II Electrophoretic cell (Biorad, USA) with 70% alcohol and dH_2O and assemble		
2.	Prepare 15% running gel and pour into gel tank	RT	1 h
3.	Mix 20 ul of 1 mg/ml concentrations of MWM, antigens and control proteins with 14 ul loading buffer	95 ^{0}C Ice	5 min Until use
4.	Prepare 5% stacking gel and pour into gel tanks	RT	1 h
5.	Load wells with 10 ul of each protein from 3 in duplicate		
6.	Assemble apparatus and electrophoresis at 160 mV		1 h
ELECTROPHORETIC TRANSFER			
1.	Cut Whatman paper & nitrocellulose membrane		
2.	Equlibrate gel, Whatman paper, nitrocellulose membrane and Biorad fibre pads in transfer buffer	RT	2-3 min
3.	Assemble Mini Transblot® Electrophoretic transfer cell (Biorad, USA) with gel (reversed left to right) closer to negative electrode & nitrocellulose near +ve eloctrode		
4.	Perform protein transfer at 90 Ma		2 h
5.	Remove and cut nitrocellulose membrane		
IMMUNOBLOTTING			
1.	Block non-specific binding sites with 1x blocking solution	RT	30 min
2.	Wash in PBS	RT	3X10min
3.	Incubate with 1^o Ab diluted in in 1x blocking solution	4^{0}C	overnight
4.	Wash in TBST	RT	3X10 min
5.	Incubate with 2^o Ab diluted in TBST	RT	3 h
6.	Wash in detection buffer	RT	3X10 min
7.	Incubate with chromogen solution in the dark	RT	few min
8.	Stop reaction with 0.05M EDTA	RT	5-10 min
9.	Wash with dH_2O	RT	30 min
10.	Press dry between Whatman paper, mount, seal in cellophane & determine optimal rTK conc.& Ab dilution		
PROTEIN STAINING			
1.	Wash with 0.4% Tween 20/PBS	RT	2x 5 min
2.	Place in ink solution	RT	15 min
3.	Destain with multiple washes of PBS	RT	Few min
4.	Dry on Whatman paper, mount and seal in cellophane		

A-2.5.1 ENDOTHELIN-1 ELISA (PLASMA AND URINE)

(A) SAMPLE COLLECTION AND PRE TREATMENT

1) Collect whole blood from recumbent patients in chilled sodium EDTA (2 mg/ml) tubes and centrifuge immediately at 1400xg for 20 min at 4°C.

2) Transfer plasma into polypropylene tubes with 200 ul kinin cocktail and store at > -20°C.

3) Collect urine in 200 ul/ Bacitracin/SBTI solution; centrifuge at 3000 rpm for 20 min at 4°C.

4) 1 ml of supernatant is placed into polypropylene tubes with 200ul of kinin cocktail and stored at -20°C.

(B) SAMPLE PREPARATION

1) Holding the Sep-Pak C-18 column (Waters, Milford, USA) vertically, wash it by injecting 5ml of the following solutions, in the order shown : eluent, methanol, distilled water, 4% acetic acid. The eluant comprised 10ml dH2O, 4ml acetic acid and 86ml ethanol.

2) Fix a 10ml syringe (without the plunger) to the activated Sep-Pak column and add a small amount of 4% acetic acid with a micropipette to remove air bubbles in the connection between the column and the syringe.

3) Add 3ml 4% acetic acid into a polypropylene tube containing 1ml plasma and mix well.

4) Transfer this mixture of plasma and 4% acetic acid into the syringe and let the specimen pass through the Sep-Pak column by gravitation.

5) Wash the wall of the polypropylene tube with 1ml 4% acetic acid and transfer this into the syringe and let it pass through the column.

6) Wash the column with 10 ml of distilled water.

7) After all the water has passed through the column, remove the column from the 10 ml syringe and fix it to a 5ml syringe and elute the absorbed fraction with 4ml of eluent into a polypropylene tube.

8) The eluate is then evaporated to dryness under a stream of nitrogen at 37°C.

9) Add 1ml ethanol to the dry residues thus obtained, mix well with a vortex mixer to obtain a suspension of the residues.

10) Evaporate the suspension to dryness under a stream of nitrogen at 37°C.

11) The dry residues are reconstituted with 250ul of buffer solution (50mM Good's buffer, pH 8.0) mixed with a vortex mixer and sonicated for 10 min.

12) The suspension is transferred to a microfuge tube and centrifuged (12000 rpm r 5 min.

13) The supernatant obtained is used for Endothelin-1 assay.

(C) ENDOTHELIN-1 MEASUREMENT

The solution from the microplate wells (Wako Chemical Industries, Osaka, Japan) is removed before use and the plate washed three times with washing solution (PBS).

1) Prepare Endothelin-1 standards by adding 1ml of buffer solution to one bottle of standard Endothelin-1 (1 ng/ml), dissolve and transfer the solution to a polypropylene tube.

3) Serial dilutions with Good's buffer were made using five polypropylene tubes to obtain standards of 200, 40, 8,2, and 0 pg/ml.

4) Add 50ul of buffer solution to all wells and add 100ul of standard solutions prepared above and samples in duplicate.

5) Mix gently, seal the plates with parafilm and incubate for 24 hours at 4°C.

6) Wash the plate 4 times with PBS using an automated plate washer (BioTek Instruments Inc. Model ELP-40).

7) Add 100ul of the peroxidase labelled anti-ET_1 polyclonal antibody (1.5 μg/ml; raised in the rabbit) solution to all wells except those containing the reagent blanks.

8) Add 100ul buffer solution to wells with reagent blanks.

9) Mix gently, seal the plate with parafilm and incubate at 4°C for 24 h.

10) Remove the reaction mixture and wash the plate 4 times with PBS.

11) Add 100 ul colouring reagent (O-phenylenediamine 14.4mmol/l) to all wells, mix gently and allow it to stand for 60 min at room temperature in the dark.

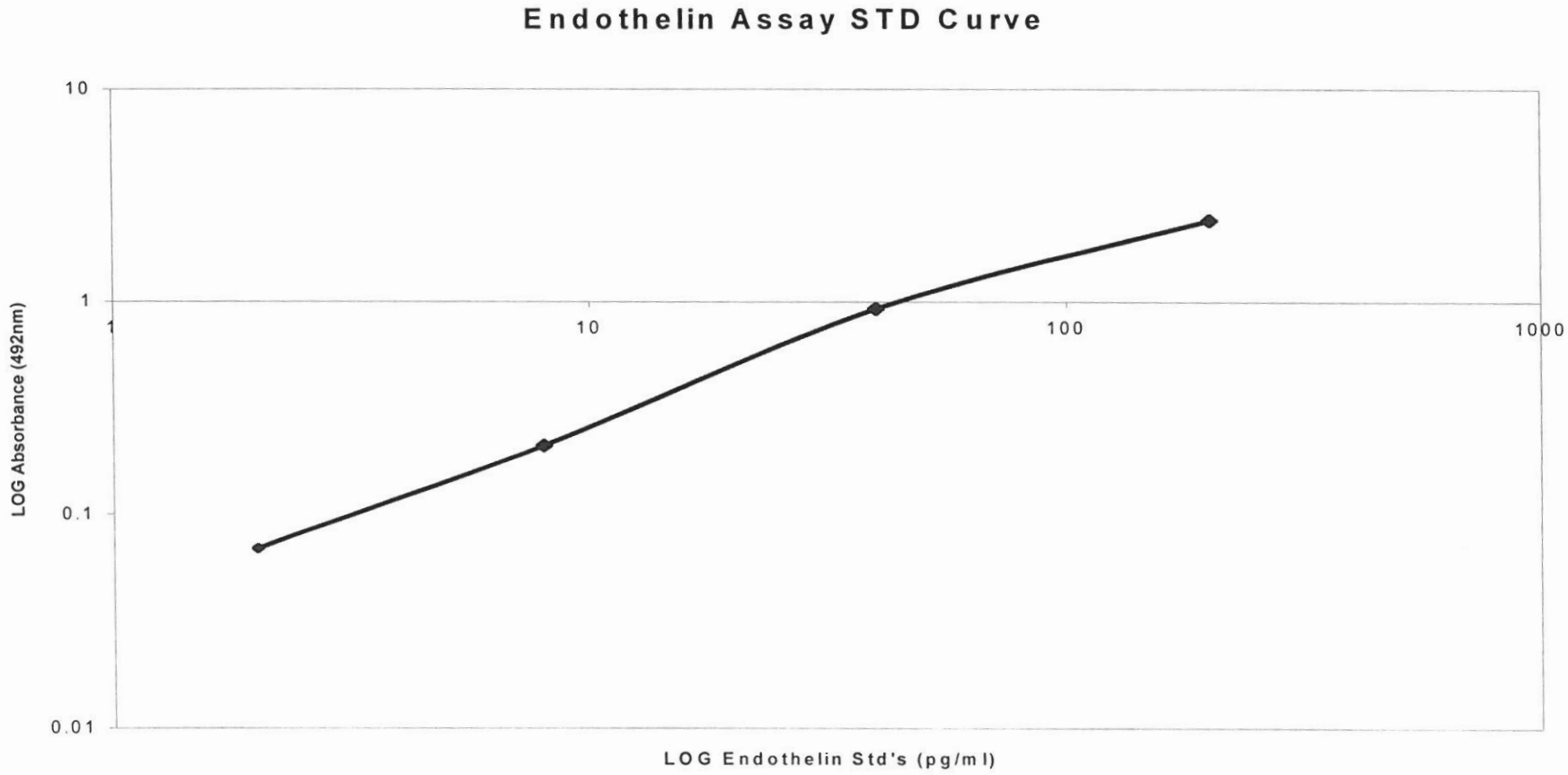
12) Stop the reaction by adding 100 ul reaction terminator (sulphuric acid 49mg/ml) to all wells.

13) Measure the absorbances of the samples and standards against the reagent blank by a microplate reader (Bio-tek Model ELX800) at 492 nm.

14) A graph is plotted on logarithmic graph paper using the absorbance of each standard (ordinate) against its Endothelin-1 concentration (abscissa).

15) Endothelin-1 concentration of the samples can be read directly from this curve by plotting the measured absorbance and dividing by 4, as the actual specimens used for endothelin-1 are concentrated 4 times.

Figure 2.5.1


Endothelin Assay STD Curve
10
1
0.1
0.01
1
10
100
1000
LOG Absorbance (492nm)
LOG Endothelin Std's (pg/ml)

A-2.5.2 ANP RIA PROCEDURE

MATERIALS

- RIA Buffer Concentrate (4x), 50ml
- Standard Peptide; 12.8 g lyophilized powder
- Rabbit antiserum specific for the peptide; lyophilized powder for 13 ml
- ^{125}1-Peptide; lyophilized powder (1.5 μCi)
- Goat Anti-Rabbit IgG serum (GARGG); lyophilized powder for 13 ml
- Normal Rabbit Serum (NRS); lyophilised powder for 13 ml
- Instructions/flow sheet of RIA protocol and guidelines for the calculation results
- Data sheet

DAY 1 :

1.With distilled water, dilute the RIA Buffer Concentrate to a final volume of 200ml.Vortex at room temperature. This buffer will be used to reconstitute all of the other components in this kit and should be used for dilution of samples, if needed.

2. Reconstitute the standard peptide with 1 ml of RIA buffer.

3. Reconstitute the rabbit anti-peptide serum with 13 ml of RIA buffer.

4. Reconstitute the samples with the RIA buffer. Prepare a dilution series of the standard ranging from 0.1 pg- 64 pg/tube.

5. Set up the initial RIA reactions in 12x75 mm polystyrene tubes.
   a) Label tubes : TC-1, TC-2, NSB-1, NSB-2, TB-1, TB-2 and #7-22 for the standards (where TC is total counts, NSB is non-specific binding and TB is total binding).
   b) Label tubes #23-125 for the unknown samples.
   c) Pipette 200 μl of RIA Buffer into the TC and NSB tubes.
   d) Pipette 100 μl of RIA Buffer into the TB tubes.
   e) Pipette 100 μl of the standards I through B into duplicate tubes #7-22.
   f) Pipette 100 μl of primary antibody (rabbit anti-peptide serum) into TB-1, TB-2 tubes, #7-22 tubes, and tubes #23 and higher. **DO NOT ADD ANTIBODY TO THE TC AND NSB TUBES.**
   g) Vortex the contents of each tube.
   h) Cover and incubate overnight (16-24 h) at 4°C.

6. Cover and store all rehydrated solutions at 4°C, except for the standard peptide solution which should be kept frozen at -20°C, or lower, for maximum stability.

DAY 2 :

7. Reconstitute the ^{125}I-Peptide with 1 ml of RIA buffer. Mix thoroughly (this is your **stock solution**). After rehydration, cover and store the tracer solution at -20°C, or lower, for maximjum stability.
8. Combine X µl of ^{125} I-Peptide with X ml of RIA buffer and mix well. Measure with a gamma counter to achieve between 10000-15000 cpm.
9. Add 100µl of the tracer to each solution test tube in the assay.
10. Vortex each test tube.
11. Cover and incubate overnight (16-24 h) at 4°C.

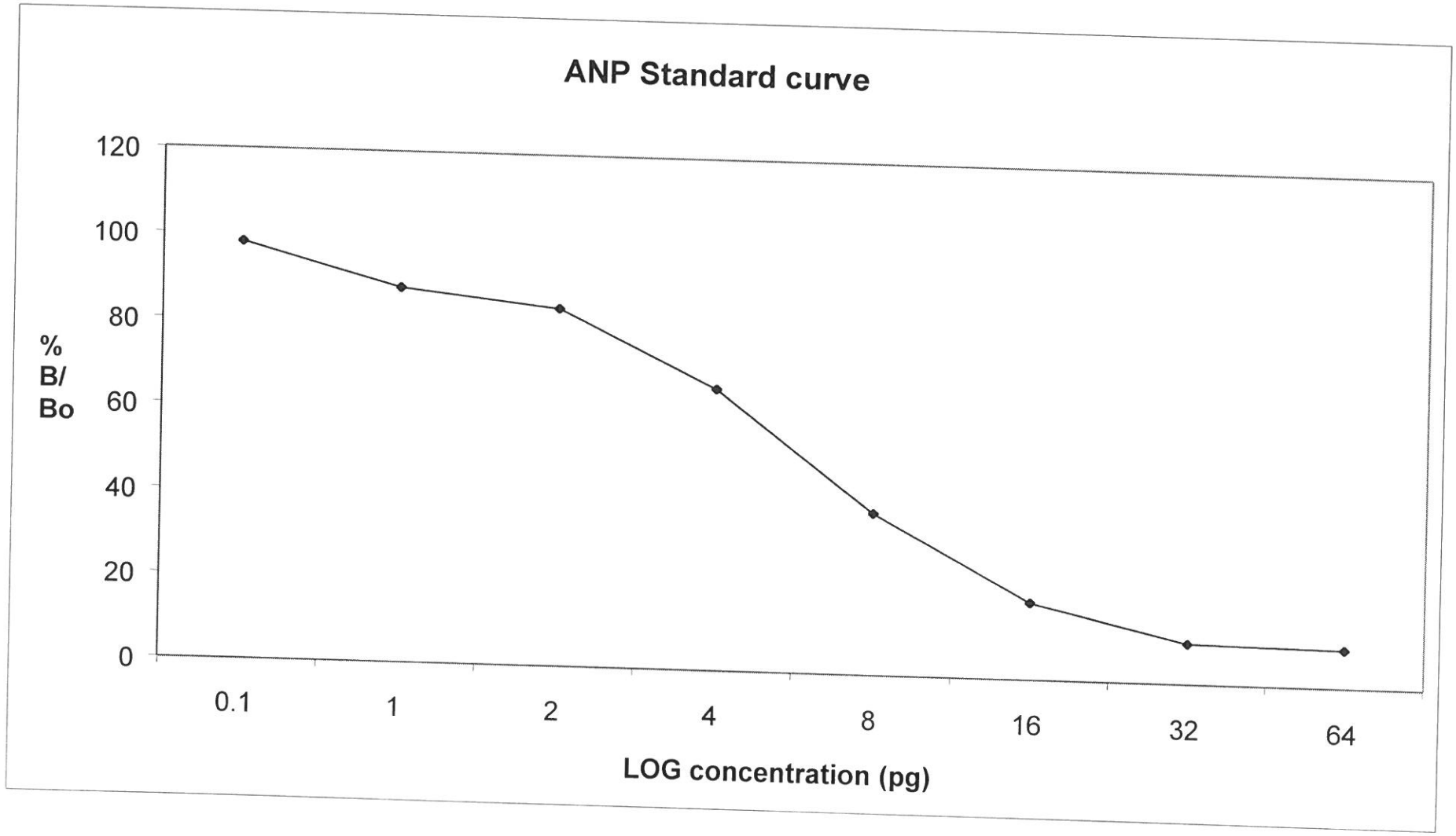
DAY 3 :

12. Reconstitute the Goat Anti-rabbit IgG serum (GARGG) with 13ml of RIA buffer.
13. Reconstitute the Normal Rabbit Serum (NRS) with 13 ml of RIA buffer.
14. Add 100 µl of GARGG to every test tube.
15. Add 100 µl of NRS to every test tube.
16. Vortex each tube and incubate at room temperature for 90 minutes.
17. Add 500 µl of RIA Buffer to each tube and vortex.
18. Centrifuged the samples at 3,000 rpm (approx. 1700xg) for 20 minutes at 4°C.
19. Set aside the TC tubes. **DO NOT ASPIRATE THE TC TUBES.**
20. For the remaining tubes, carefully aspirate the supernatant (do not decant since the pellet may be lost or excess liquid may be left in the test tube).

SUMMARY OF ASSAY PROTOCOL

1. Add sample or standard and antiserum.
2. Vortex and incubate overnight (16-24 h) at 4°C.
3. Add ^{125}I-Peptide.
4. Vortex and incubate overnight (16-24 h) at 4°C.
5. Add GARGG and NRS.
6. Vortex at room temperature for 90 min.
7. Add RIA buffer.
8. Vortex and centrifuge for 20 minutes at 1700xg.
9. Aspirate off the supernatant (**except TC tubes**).
10. Count assay tubes.
11. On semilog graph paper, determine the concentration of samples against known standards.

Figure 2.5.2


ANP Standard curve
120
100
80
60
40
20
0
% B/ Bo
0.1
1
2
4
8
16
32
64
LOG concentration (pg)

Appendix A 2.5.3.2a T K Functional Microassay (Amidase)

Colorimetric assay for the measurement of functionally active and total TK in biological samples

Reagents:

Amidase buffer:	0.2 M Tris-HCl, pH 8.2
Assay buffer:	300 ug/ml SBTI and 375 ug/ml EDTA in 0.2 M Tris-HCl pH8.2
S2266 Solution:	1.5 M in deionised water, (Chromogenix, Sweden)
SBTI/Bacitracin:	1.47 g/ml of SBTI (Sigma); 2720units/mg, 40 mg/ml Bacitracin (Sigma) in 0.2 M Tris-HCl, pH 8.2

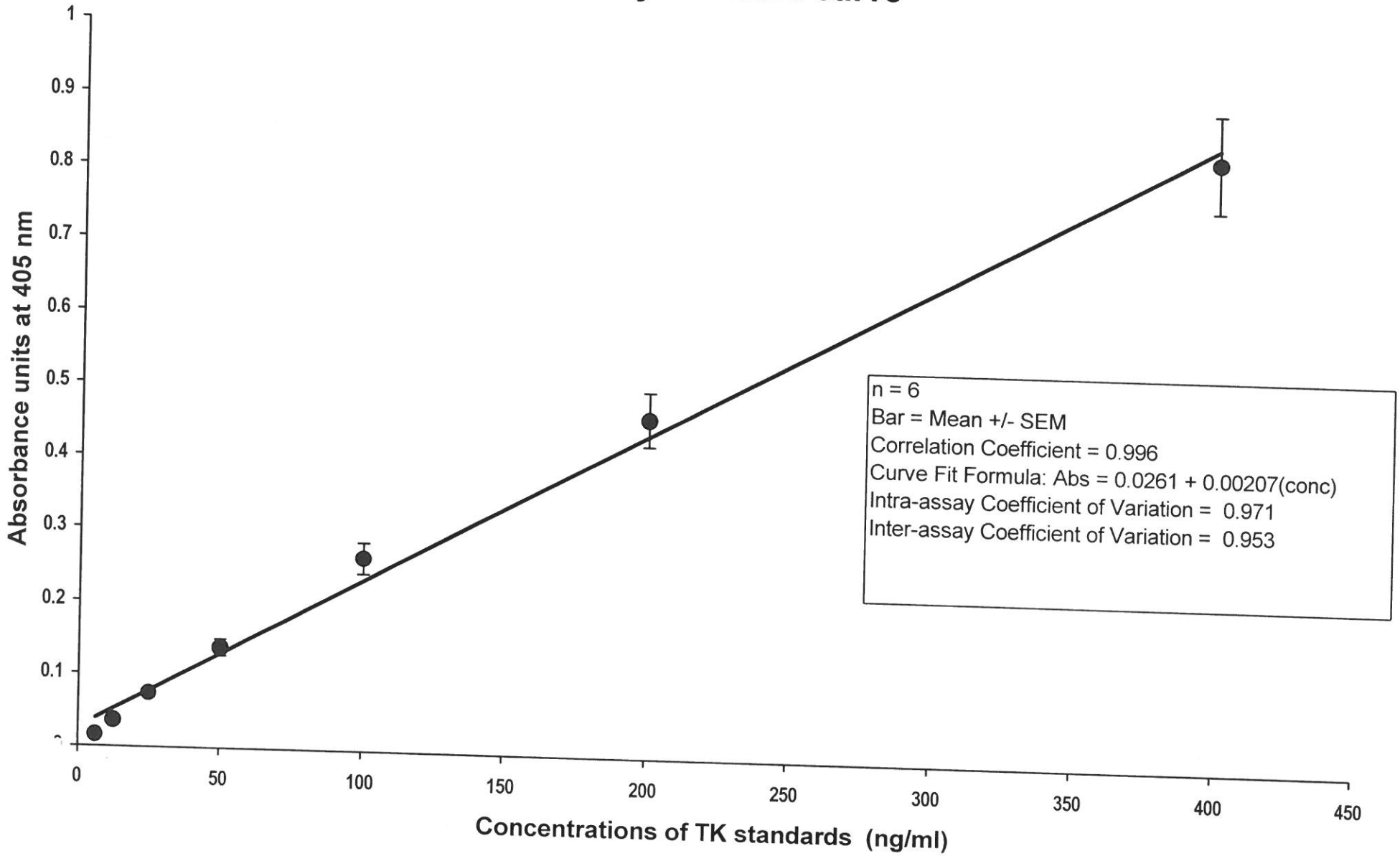
Sample collection:

1. Urine samples (approx 15ml) collected immediately in 200 ul SBTI/Bacitracin /Tris-HCl buffer at 4^0c, and thereafter stored in 200 ul 0.2 M Tris-HCl pH 8.2 per 1000 ul sample, in 1 ml aliquots, at -20^oc.
2. One set of plain urine samples collected and stored, without any buffer, for total protein determination, at -20^oc

Method:

1. On two sets of plates, 50 ul HUK (Protogen) of each standard human urinary kallikrein (400 ng/ml - 12.5 ng/ml, in 0.2 M Tris-HCl pH8.2) is to be added to 50 ul Assay buffer containing SBTI and EDTA. One plate is incubated at 37^oc for 30 min. (on tissue culture plates, Corning Cell Wells™, 25860, Corning, New York). To the other plate, 50 ul dH_2O is added to each of the wells and the plate is read at 405 nm on Biorad Microplate Reader (model 3550). This would determine the zero activity blank.
2. To the incubated plate, add 50 ul of the substrate S2266 and incubate again for 3 h at 37^oc.
3. Read at 405 nm on microplate reader.
4. 50 ul Standard/samples done in triplicate
5. Blanks will only contain assay buffer.
6. Total protein measurement done according to Bradford determination.
   (300 ul Biorad bradford reagent, diluted 1/5 in dH_2O, added to 30 ul of sample/Std. and read at 595 nm. Blanks are dH_2O.)
7. Activity expressed as ngTK/ ug protein

Figure 2.5.3.2a TK Amidase assay standard curve



Appendix A 2.5.3.2b Measurement of Total protein [Bradford, 1976]

Reagents

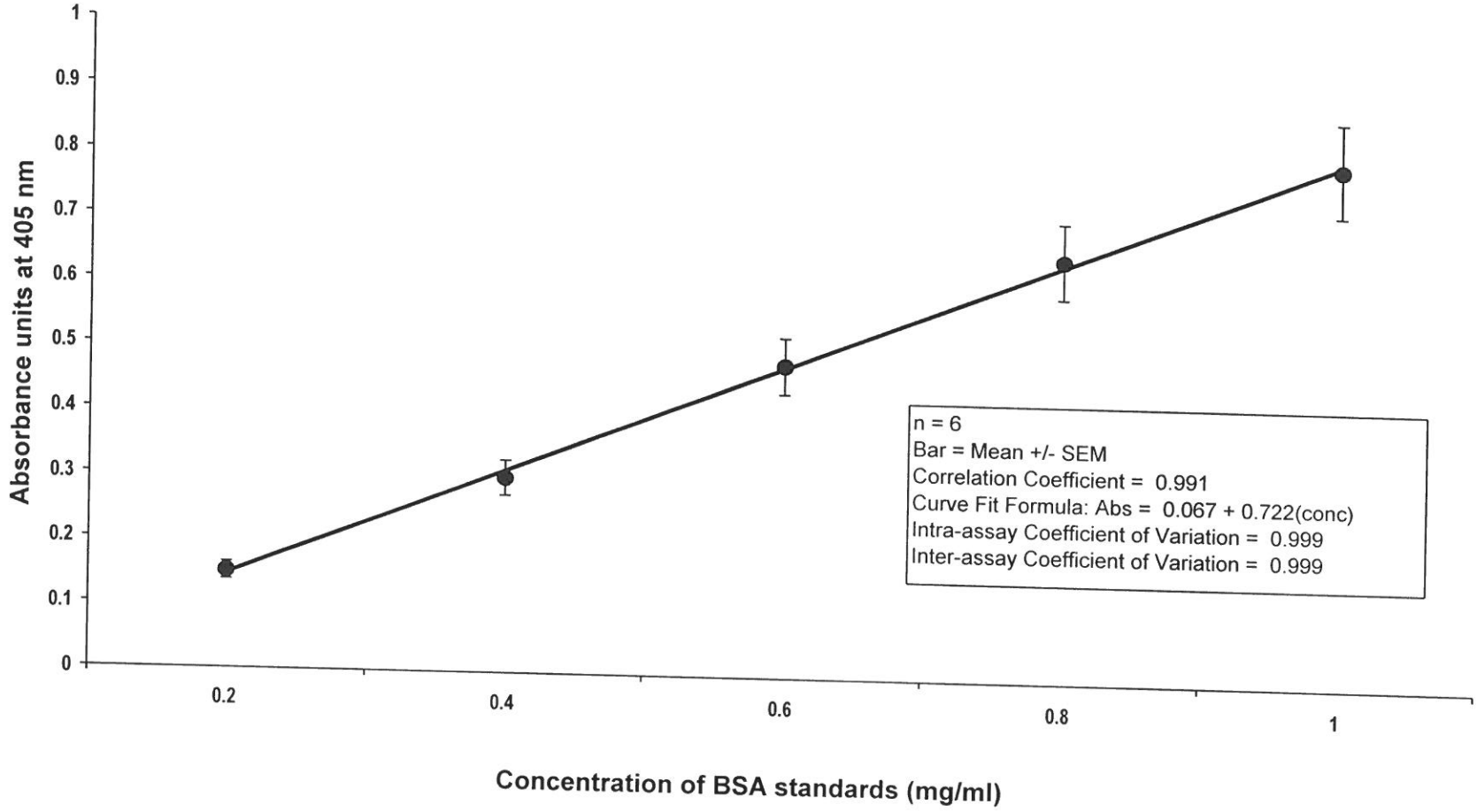
1. Bovine Serum Albumin (BSA, Fraction V, Boehringer Mannheim)
2. Biorad Protein Assay Reagent (Biorad, UK)

Procedure

STEP	SOLUTION
1.	Protein standard – 100 ml of 1 mg/ml BSA was prepared and stored in 1 ml aliquots at –20C. A single 1 ml aliquot of this standard was serially diluted with dH_2O from 1000 to 16 ug/ml. 30 ul of each dilution was added to a microassay plate in triplicate.
2.	Blank - 30 ul dH_2O was added to 3 wells of the plate
3.	Samples - were added in triplicate to the plate
4.	Biorad Protein Assay Reagent - was diluted 1/5 in dH_2O and 300 ul was added to each well of the plate
5.	Read absorbance at 595 nm immediately
6.	Calculation – subtract the absorbance of the blanks from the absorbance of the standards and samples. Plot absorbance vs concentration of BSA standards. Read protein concentration of samples from this graph.
7.	TK enzymic activity is expressed as ng TK / ug protein

All stages are performed at RT

Figure 2.5.3.2b Standard curve of Bradford protein



Appendix A 2.5.3.3 TK ELISA

Reagents and Immunochemicals:

1. Coating buffer ($Na_2CO_3/NaHCO_3$, pH 9.6)
2. Substrate buffer (5 mM $MgCl_2$/10% Diethanolamine, pH 9.8)
3. Milk protein blocker (5% Elite)
4. PBS/Tween (0.01M phosphate buffered saline/0.5% Tween 20)
5. Human Urinary Kallikrein
6. Goat anti-human TK IgG (30 ng/ml)
7. Rabbit anti-human TK IgG (25 ng/ml)
8. Anti-rabbit alkaline phosphatase (Sigma)
9. diphenylparanitroalaninephosphate substrate tablets (pNPP, Sigma)

Procedure:

1. Coat the ELISA plate (Corning) with 100 ul, 30 ng/ml Goat anti- human TK in coating buffer and incubate overnight at 4^0c. *(Make one vial up to 10ml)*
2. Wash the plate 3x3min with PBS/Tween. Block with 200 ul of milk blocker for 30 min at room temperature (RT). Wash again. Repeat blocking step. Wash.
3. Load the plate with 100 ul of sample or standard and incubate for 1 hr at 37^0c (water bath). Wash.
4. Load the plate with 100 ul, 25 ng/ml of rabbit anti- human TK and incubate for 1 hr at 37^0c (water bath). *(Make one vial up to 12ml).* Wash.
5. Incubate with 100 ul of anti-rabbit alkaline phosphatase (1:250 dilution in 0.01M PBS). Wash
6. Add 100 ul of 1 mg/ml pNPP substrate to the wells (1 tablet in 5 ml of substrate buffer) and allow colour to develop for 1 hr.

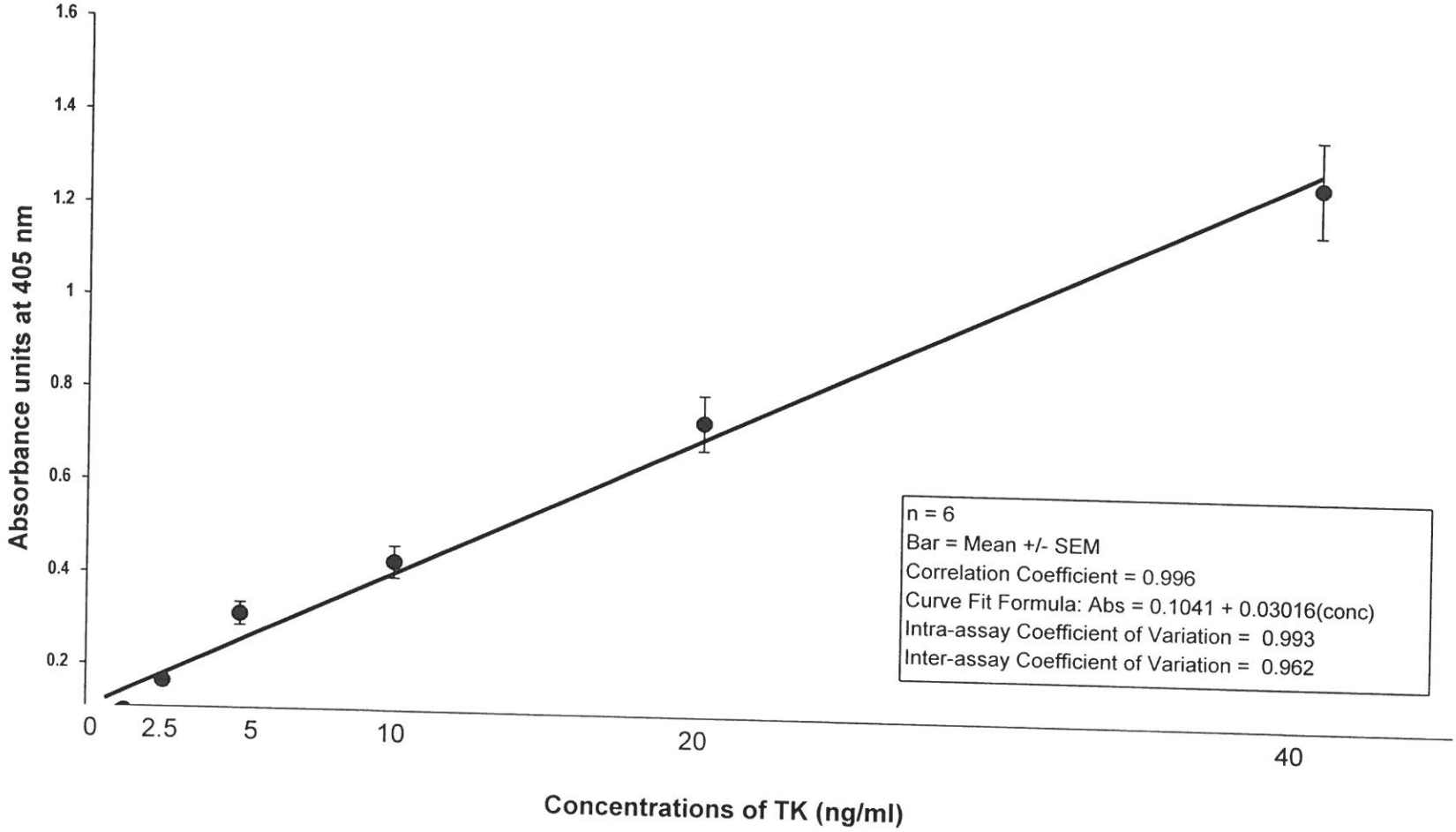
Controls: Urine pool controls double-diluted from 1/8 to 1/512 in 0.01 M PBS.

Also, known samples are rerun to test for interassay variation

Blanks: Use **0.01 M PBS** instead of samples or standards.

Standard Curve: Different concentrations of Human Urinary Kallikrein were run and a linear-linear relationship was obtained -200 ng/ml double diluted down 6X .

Figure 2.5.3.3 TK ELISA standard curve



Appendix A 2.5.4 KININ ELISA

Conjugation

Reagents:

1. SDP- 13 uM in 0.1M Phosphate buffer pH 8.6, 20% Ethanol

   5 mg SPDP (Sigma P3415) + 500 ul phosphate buffer + 200 ul ethanol

   first dissolve SPDP in EtOH, and then add buffer

2. Cytochrome C- 10 uM in 0.1M phosphate buffer pH 8.6

   1 mg cytochrome C (Sigma C6913) + 833.33 ul phosphate buffer.

3. Bradykinin 1mM

   1 mg BK (Sigma) + 943 2 ul 0.01M PBS pH 7.4

Method:

1. Mix 1 volume SPDP with 1 volume Cytochrome C, and incubate at 22^0c for 60 min.
2. Filter solution through ultra-free (Millipore) 10 000 nominal weight cut off filters to remove any unconjugated SPDP (may require 2-3 h).
3. Resuspend top part in two volumes of 0.3 M phosphate buffer pH 6 (i.e. to give original volume)
4. Add one volume of BK and incubate at 22^0c for 60 min. Each batch of conjugate has to be tested for optimal concentration before use in ELISA (usually this is between 3-4 ug/ml protein or approx. 1:160 dilution).
5. Store at -200c in aliquots after testing for optimum concentration.

ELISA procedure:

1. Coat Nunc Immulon Maxisorp™ microtitre plate overnight with 100 ul/well of cytochrome-SPDP-BK conjugate (optimal concentration determined for each batch: ± 3-4 ug/ml) in sodium carbonate/coating buffer, pH 9.6 at 4^0c.
2. Also overnight at 4^0c, incubate sample or standard with equal volume of SBK1 α-BK antibody (1:1000 in PBS)
3. The next day, wash plate with 0.01M PBS/ 0.5% Tween, 3X3min; and then block plate with 200 ul of 5% Milk blocker.

4. Incubate for 30 min at room temperature (RT). Wash with PBS/Tween 3X3min and repeat blocking step. Wash again.
5. Incubate 100 ul/well of preincubated sample or standard for 3 hrs/ 37^0c. Wash.
6. Incubate 100 ul/well with alkaline phosphatase labelled α-mouse IgG (1:250 in PBS) for 2 hrs at 37^0c. Wash.
8. Finally add 100 ul of the substrate pNPP (1mg/ml in coating buffer) to the plate and incubate at RT for approx. 1 hr, or until sufficient maximum absorbance values obtained at 405 nm (±1.8 AU) on Biorad microplate reader

Blanks:

Non-specific binding to the plate was determined by incubating only SBK1 α-BK (1/1000 in PBS (preincubated with equal volume of PBS buffer).

The wells used for the blanks were coated with coating buffer only, and no conjugate.

Endogenous (basal) kinins and Kinin Generation for ELISA measurement.

Urine

Reagents:

1. Kininase inhibitor cocktail: kinin cocktail excluding SBTI and Aprotinin
2. Acid-Alcohol: absolute Ethanol/ 0.1% conc. HCl

Methods:

A: Endogenous (basal) kinins

1. Thaw urine and centrifuge at 5000xg/10 min/4^0c (stored **in KI cocktail wth SBTI and Aprotinin**)
2. Remove 500 ul and add to 500 ul acid-alcohol and place at -20^0c for 90 min.
3. Centrifuge (5000xg/10 min/4^0c) and keep supernatant.
4. Wash pellet with 500 ul of acid-alcohol (1:2 in dH_2O) and centrifuge (5000xg/10 min/4^0c).
5. Pool supernatants and place in 24-well cell culture plate and evaporate to dryness at 55^0c overnight.
6. Resuspend dry residue in 500 ul (original volume) of assay buffer and centrifuge(5000xg/10 min/4^0c). Use clear supernatant for ELISA measurements.

B: Kinin Generation

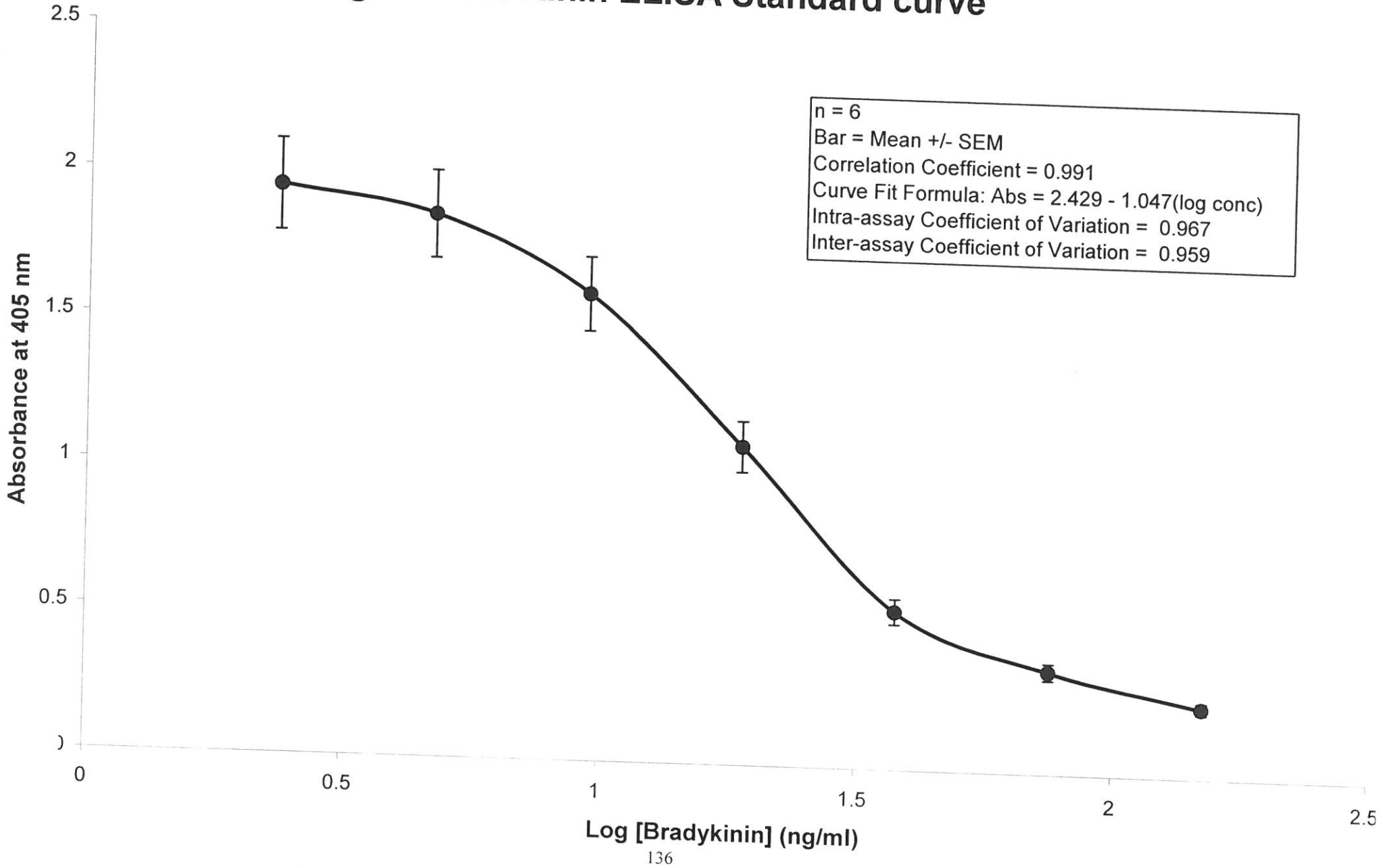
1. Thaw urine and centrifuge at 5000xg/10 min/4^{0}c (stored in **KI cocktail without SBTI and Aprotinin**)
2. Mix 500 ul of urine with 500 ul kinin cocktail and incubate for 60 min at 37^{0}c.
3. Remove 500 ul and add to 500 ul acid-alcohol and place at -20^{0}C for 90 min.
4. Centrifuge(5000xg/10 min/4^{0}c) and keep supernatant.
5. Wash pellet with 500 ul of acid-alcohol (1:2 in dH_2O) and centrifuge (5000xg/10 min/4^{0}C).
6. Pool supernatants and place in 24-well cell culture plate and evaporate to dryness at 55^{0}c overnight.
7. Resuspend dry residue in 500 ul (original volume) of assay buffer and centrifuge (5000xg/10 min/4^{0}c). Use clear supernatant for ELISA measurements.

C: Controls

1. BK STD. CURVE: Bradykinin diluted to give concentrations of 150 ng/ml double-diluted down to 2.34 ng/ml in PBS buffer.
2. CONTROLS: Urine pool, spiked at 2 concentrations of BK (100 & 50 ng/ml) Acid-alcohol extraction. Gives the variation for the extraction procedure and ELISA at two concentrations of BK.

   Urine pool samples are spiked with BK and the double-diluted to determine whether concentrations of BK linearly diluted out with dilutions of urine

Figure 2.5.4 Kinin ELISA Standard curve



Appendix A 2.6.2 IMMUNOCYTOCHEMISTRY: PAP METHOD

1. For wax-embedded tissue, place on heating mantle until wax melts.

2.Dewax in:	Xylene	.....2 x10 min/ RT
Dehydrate in:	100 % EtOH	.....2 x 5 min/ RT
	100 % MeOH	.....1 x 20min/RT
	90 % EtOH	.....2 x 4 min/ RT
	70 % EtOH	.....1 x 3 min/ RT
	dH_2O	.....1 x 5 min/ RT

3. Boil (80^0c) in 0.1M Na-citrate pH 6.0 (microwave) .....± 3min HIGH
.....5min LOW
Allow to cool to room temp .....± 20min
place in dH_2O .... ± 5min

4. Use Dako PAP marker and wash in 0.01M PBS/1 %BSA.

5. Incubate in 10% H_2O_2/ 95 % MeOH .....2 x 15min/RT
Wash in 0.01M PBS.

6. Incubate with 1^0 antibody (diluted in Maleic acid/ milk blocker)
Incubate overnight at 4^0c under humid conditions.
Wash in 0.01M PBS

7. Incubate with Biotin link (DAKO K0690) ..... 20 min/RT
Wash in 0.01M PBS .

8. Incubate with Strepavidin 2^0 antibody (DAKO K0690) ..... 20 min/RT
Wash in 0.01M PBS.

9. Incubate with liquid DAB (Dako) 1-5 min/RT

10. Counterstain in Mayers' Hemotoxylin .3-5min/RT
Wash in tap H_2O .5min

11. Dehydrate tissue through EtOH into Xylene
Mount in Entellen (Merck)

* Method controls are 1^0 antibody replaced with buffer or non-immune serum.
* All dilutions made up in 0.01M PBS/ 1 %BSA.
* Do not allow tissue sections to dry out.

Appendix A 2.6.3 IMMUNOFLUORESCENCE

1. For wax-embedded tissue, place on heating mantle until wax melts

2. Dewax in	Xylene	....2 x 10 min/ RT.
Dehydrate in	100 % EtOH	....2 x 5 min/ RT.
	100 % MeOH	.....1 x 20 min/RT.
	90 % EtOH	.....2 x 4 min/ RT.
	70 % EtOH	....1 x 3 min/ RT.
	dH_2O	....1 x 5 min/ RT.

3. Boil (80^0c) in 0.1M Na-citrate pH 6.0 (Ag retrieval) ±3. min HIGH
....5 min MED LOW

Allow to cool to room temp ....±20 min
place in dH_2O

4. Use Dako marker and wash in 0.01M PBS/0.1 %BSA
Incubate with 1% Human IgG ....15 min/RT.
Wash in 0.01M PBS.

5. Incubate with 1^0 antibody (diluted in 0.01M PBS/1 %BSA)
Incubate overnight at 4^0c under humid conditions.
Wash in 0.01M PBS.

6. Incubate with anti-species Fluorescent conjugate .....30 min/RT.
Wash in 0.01M PBS.

7. Mount with 10 % PBS/90 % glycerol.

*Method controls are 1^0 antibody replaced with buffer
* All dilutions made up in 0.1 M PBS/0.1 %BSA.
* Do not allow tissue sections to dry out.
* Store labelled slides in the dark.
* Only antigen retrieval done in microwave.

Appendix A 2.6.4 IMMUNO-ELECTRON MICROSCOPY

Tissue fixation and processing for Immunoelectron microscopy.

Biopsy tissue for immuno-electron microscopy was immediately immersed in 4 % PFA/ 1 % gluteraldehyde fixative for 2 h at 4°C, then transferred to 0.2 M sodium cacodylate buffer, pH 7.2, maintained at 4°C. The fixed tissue was dissected into $1mm^3$ sections and processed in an automatic Reichert-Lynx em tissue processor. During processing the specimens were retained in baskets at a fixed processing station and agitated. The specimens rotated through a preprogrammed sequence as outlined in Table 1.

TABLE 1. Dehydration and Embedding schedule for immunoelectron microscopy

STEP	SOLUTIONS	TEMP	TIME
1	Sodium cacodylate buffer (0.2M, pH 7.2)	4°C	10 min
2	Sodium cacodylate buffer (0.2M, pH 7.2)	4°C	10 min
3	Dehydration - 70% ethanol	4°C	30 min
4	Dehydration - 90% ethanol	20°C	30 min
5	Dehydration - absolute ethanol	20°C	15 min
6	Dehydration - absolute ethanol	20°C	15 min
7	Dehydration - absolute ethanol	20°C	15 min
8	Dehydration - absolute ethanol	20°C	15 min
9	Intermediate solvent – Propylene oxide	20°C	30 min
10	Infiltration - 50/50 propylene oxide and epoxy resin	20°C	30 min
11	Infiltration - Spurr epoxy resin (Spurr ARJ, 1969)	60°C	60 min
12	Infiltration - Spurr epoxy resin	60°C	60 min
13	Polymerization - Spurr epoxy resin	60°C	48 h

Ultramicrotomy

Semi-thin section (1μm) were cut with a Reichert Ultra cut ultramicrotome using glass knives. Sections were collected onto glass slides, heat-fixed, stained with 1 % alkaline toluidine blue and examined with a Nikon Optiphot photomicroscope. Fields of interest were selected and located on the block face, and the block trimmed to produce a "mesa" with a trapezoidal shape. Ultrathin section (50-60nm) were cut and collected onto uncoated Nickel grids prior to immunostaining

Immunostaining (see Table 2)

a. Etch Nickel grid by floating on a droplet of 5 % hydrogen peroxide for 5 min.

b. Place grid in a drop of distilled water for 1 min then jet wash into 10 ml distilled water and dry on fibre free paper.

c. Submerge grid in blocking medium (non-immune serum from species in which primary antibody was raised) for 30 min and blot dry on filter paper.

d. Incubate grid in a 30 μl droplet of primary antibody for 3 h. Optimised antibody dilution in 50 mM Tris. (pH 7.2).

e. Submerge grid in droplet of 50 mM Tris (pH 7.2) for 1 min then jet wash grid with 20ml 50 mM Tris. (pH7.2).

f. Submerge grid in droplet of 50 mM Tris containing 0.2 % BSA (pH 7.2) for 5 min, then jet wash grid with 20ml 50 mM Tris containing 0.2 % BSA (pH 7.2).

g. Submerge grid droplet of 50 mM Tris containing 1% BSA (pH 8.2) for 5 min.

h. Incubate grid secondary antibody (IgG against primary antibody-raised in a different species conjugated to 10nm immunogold particles) diluted 1 : 200 in 50 mM Tris (pH 8.2) for 1 h.

i. Droplet wash (1 min) and jet wash grid with 20ml of the following series of reagents.

a) 50 mM Tris containing 0.2 % BSA (pH 7.2).

b) 50 mM Tris (pH 7.2).

c) Distilled water.

d) Counterstain with saturated ethanolic uranyl acetate for 5 min.

e) Jet wash in 20 ml distilled water and blot dry.

f) Incubate grid in Reynolds lead citrate for 3 min.

g) Jet wash well with distilled water and blot onto filter paper.

TABLE 2 : Immunostaining

STEP	PROCESS	REAGENT	TIME
1	Etching	11202 (5 %)	3 min
2	Wash	10 ml grid distilled	
3	Blocking	Blocking medium (nonimmune serum from rabbit	30min
4	Incubation	Primary antibody (see notes)	180min
5	Jet wash	50mM Tris (pH 7.2) –20 ml per grid	
6	Jet wash	50mM Tris + 0.2% BSA (pH 7.2) 20ml/grid	
7	Jet wash	50 mM Tris + 1% BSA (pH 8.2) 20ml/grid	5 min
8	Incubation	Secondary antibody (anti-rabbit) conjugated to 10nm immunogold particles 1 :100) IN 50mM Tris pH 8.2	60 min
9	Jet wash	50 mM Tris + 0.2% BSA pH 7.2 20 ml per grid	
10	Jet wash	50mM Tris pH 7.2 20 ml per grid	
11	Jet wash	Distilled water 20 ml per grid	
12	Stain	Saturated ethanolic uranyl acetate	5 min
13	Jet wash	Distilled water 20 ml per grid	
14	Counterstai n	Reynold's lead citrate (Reynolds ES, 1963)*	3 min
15	Jet wash	Distilled water 20 ml per grid	

Grid can be stored at room temperature in a grid box until viewed.

Control samples :

Method Control : Step (d) : instead of primary antibody, 50 mM Tris pH 7.2 was used.

Primary antibodies :

ET_1 : rabbit anti ET_1 antibody (Biogenesis, Poole, UK)

ET_A receptor : rabbit anti ET_A antibody (donated by Werner Müller-Esterl)

ET_B receptor : rabbit anti ET_B antibody (donated by Werner Müller-Esterl)

Dilutions :

ET_1 1 : 100

ET_A 1 : 1000

ET_B 1 : 1000

A 2.7 *IN SITU* REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (*in situ* RT-PCR)

SOLUTIONS

2 % 3-Aminopropyltriethoxysilan in acetone

Add 2 ml 3-Aminopropyltriethoxysilan to 98 ml of acetone.

0.1 % DEPC dH_2O

- Add 100 µl of DEPC to 100 ml dH_2O i.e. 1 ml of DEPC to 1 L dH_2O

1M Phosphate Buffered Saline (PBS), pH 7.4 (1L)

29.25g Na_2HPO_4 x H_2O ; 4.90g KH_2PO_4 ;160.00g NaC

- Dissolve the above in 1000 ml distilled H_20.
- **Autoclave**

0.05 M Phosphate Buffered Saline (PBS), pH 7.4 (1L)

- 50 ml 1M PBS, pH 7.4 (1 l) + 950 ml dH_2O

4 % Paraformaldehyde in PBS

- Dissolve 40 g of Paraformaldehyde in 800 ml of 0.05M PBS.
- Place on a heating plate with stirrer and heat the solution to about 80 °C.
- The paraformaldehyde has dissolved when the solution is clear.
- Top up the volume to 1000 ml with 0.05 M PBS and allow to cool.
- Filter the solution through 2 layers of Whatman filter paper No. 1 and store at 4°C.

1 M Tris HCl , pH 7.5

- Dissolve 121 g Tris Base in 800 ml DEPC dH_2O
- Adjust pH to 7.5 with concentrated HCl.
- Cool, determine final pH and bring volume to 1L.

50 mM Tris HCl , pH 7.5

- Dilute 10 ml 1 M Tris HCl , pH 7.5 with 190 ml DEPC dH_2O

(Final Volume 200ml)

0.5 % Acetic Anhydride

- Mix 25 ml 1M Tris HCl, pH 7.5; 225 ml DEPC dH_2O and1250 µl Acetic Anhydride. Prepare just before use

Proteinase K Solution (1 mg/ml)

- Dissolve 1 mg Proteinase K in 50 mM Tris-HCl, pH7.5, 5 mM EDTA (Stock Solution) (**) Required dilution needs to be determined for each tissue.

20 X SSC

- Dissolve 175.3 g NaCl and 88.2 g Sodium Citrate in 800 ml H_2O.
- Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH.
- Adjust volume to 1L.
- Autoclave.

2 X SSC

- Dilute 100 ml 20 X SSC with 900ml of DEPC dH_2O . (Final Volume 1L.)

SILANISATION OF SLIDES

Require:

Slides

3-Aminopropyltriethoxysilan- obtained from Fluka Chemika- 09324 (100 ml)

Chloroform

Absolute Ethanol

Acetone

Distilled H_2O

Method

- Put slides into slide holder and immerse into chloroform for 30 min at room temperature.
- Remove slides, shake off excess chloroform and immerse the slides into absolute ethanol for 30 min at room temperature.
- Air dry the slides for one h.
- Immerse slides in 2 % 3-Aminopropyltriethoxysilan in Acetone for 5 min.
- Quickly dip the slides into acetone - 2 times, and shake off excess acetone.
- Wash the slides by dipping into distilled H_2O and shaking off excess water.
- Dry in 42 ^{o}C oven overnight and store in slidebox.

CUTTING TISSUE

Require

Microtome

Wax embedded tissue (Place in the freezer for ½ an hour)

Silanated slides

RNase Away 70 % Ethanol

Water bath with DEPC dH_2O

Method

1. Clean all equipment with RNAse away
2. Wipe the blade with xylene to remove any wax .
3. Fill a water bath with 0.1 % DEPC dH_2O and maintain at 60 ^{o}C.
4. Cut 4 µm thick sections place on silan pre-treated slides and dry at 55 ^{o}C for 2h store in a clean dust free slide box.

NOTES : Ensure that wax block is cold before cutting.

Do not touch the blade or covering plate with your fingers (Becomes warm)..

FIXATION OF TISSUE

Require

Freshly prepared 4 % Paraformaldehyde in PBS (pH 7.5)

0.05 M PBS

Ethyl Alcohol series (70 %, 80 %, 90 % and 100 %)

Xylol (Xylene)

0.1 M HCl

0.5 % Acetic Anhydride

Method

Dewax in xylene 3 X 10 min.

Rehydrate in an alcohol series as follows:

100 % - 2 X 5 min; 90 % - 1 X 5 min; 70 % - 1 X 5 min; 50 % - 1 X 5 min;

DEPC dH_2O – 1X 5 min.

Place in 4 % Paraformaldehyde - 1 X 60 min.

Wash in 0.05 M PBS - 1 X 10 min.

Wash in 0.1 M HCl - 1 X 10 min (Denature proteins)

Wash in 0.05 M PBS - 2 X 10 min.

Wash in 0.5 % Acetic Anhydride - 1 X 10 min.

Wash in 0.05 M PBS – 1 X 10 min.

Dehydrate in an ethyl alcohol series: 50 % - 1 X 5 min; 70 % - 1 X 5 min; 90 % - 2 X 5 min and 100 % -2 X 5 min. Store at 4 ^{o}C in a dust free environment.

PRE-TREATMENT OF SLIDES

Require

Frames

Proteinase K Solution (1 mg/ml) in 50 mM Tris-HCl, pH7.5, 5 mM EDTA (Stock Soln). (The Proteinase K concentration may need to be optimised)

0.05 M PBS

DNase (Boehringer Mannheim)

50 mM Tris-HCl, pH7.5, 5 mM $MgCl_2$

RNase Inhibitor (Boehringer Mannheim)

Method

1. Place a frame around the tissue so as to form a well.
2. Treat the tissue with pre-digested Proteinase K (10μg/ml) (Sigma) in a moist chamber at 37^{0}C for 30 min (Proteinase K treatment must be empirically determined).
3. Wash with PBS – 3 X 10 min.
4. Incubate the slides at in a moist chamber at 37 ^{o}C overnight in the presence of a DNase solution that consists of

	μl
RNase-free DNase (Concentration)	3
RNase inhibitor (Concentration)	2
1 M Tris HCl pH 7.5	2
25 mM $MgCl_2$	8.5
DEPC dH_2O	46.5

5. Wash with 50 mM Tris-HCl, pH7.5 – 3 X 10 min.

REVERSE TRANSCRIPTION

Require

DTT

dNTPs

Buffer

DEPC Treated H_2O

RNase inhibitor

25 mM $MgCl_2$

Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase

The above is obtained in the First Strand cDNA Synthesis Kit (Pharmacia Biotech)

Specific Primer

Method

1. Make up RT mix using

Primer	2 µl (10 mM)
DTT	2 µl
DEPC dH_2O	36 µl
Bulk (Kit)	10 µl
Total	50 µl

2. Remove excess buffer from the slides.
3. Place 50 µl of the RT mix over the tissue and covered with parafilm
4. Incubate at 37 °C for 1 h
5. At this point the slides can be stored at -20 °C until the PCR step.
6. Incubate in the presence of 5 % BSA for 30 min at 37 °C.
7. Wash in PBS for 10 min
8. Incubate in the presence of 10 % Milk Blocker for 15 min
9. Wash in PBS for 10 min

POLYMERASE CHAIN REACTION

Require

10 X Taq buffer
25 mM $MgCl_2$
dATP, dCTP, dGTP and dTTP (100 mM stock each)
Primer 1
Primer 2
dH_2O
nitroblue tetrazolium/ 5-bromo-4chloro-3ndoyl-phosphate (1:50 dil)- colour substrate
Taq DNA Polymerase
Cover slips (Plastic)
Mineral Oil

Method

1. Make up the PCR mix as follows:

	µl
10 X Taq Buffer	5
25 mM $MgCl_2$	7
(100 mM) dATP, dCTP, dGTP and dTTP	4 µl altogether (1 µl each)
Primer 1	2
Primer 2	2
dH_20	27
DNA Taq	1

2. Carefully remove the RT solution off the tissue sections.

3. Place 50 µl of the PCR mix onto the tissue.

4. Cover with plastic cover slips and surround cover slip with Mineral Oil.

5. Place on the PCR block and set programme with the required parameters.

6. After the PCR reaction is complete, remove the plastic cover slips.

7. Place the slides into xylene for about 10 - 20 sec to remove mineral oil (if used).

8. Wash slides twice in 0.1 X SSC at 45 ^{o}C for 20 min with continuous shaking.

9. Cover slides with freshly prepared colour substrate solution in 1x detection buffer (1mM Tris, 1mM NaCl, 0.5 mM $MgCl_2$, pH 9.5) and incubate in the dark at RT until a reddish-purple colour precipitate is visible

10. Examine the slides under a Leica DMLB microscope.

CHAPTER 3

RESULTS

3.1 ASSESSMENT OF EXCESS FLUID DISTRIBUTION BY BIOELECTRICAL-IMPEDANCE ANALYSIS

Fluid retention frequently accompanies renal disease and renal failure. Measurement of total body water (TBW) by bioelectrical impedance analysis (BIA) is useful in assessing the state of hydration. Bioelectrical-impedance analysis of 5 transplant recipients, 4 patients with renal parenchymal disease and 3 control subjects was made, using the Biostat body composition analyser (BodyTrak, Cape Town, South Africa).

Bioelectrical-impedance measurements

Patient	Weight (kg)	BM1	TBW (L)	TBW (%)	Weight Change (kg)
TP_1-	63.3	19.7	41.5	65.8	Baseline
TP_1-Day 3	68.8	21.5	46.5	67.3	5.5
TP_1-Day6- AR	66.2	20.6	47.4	71.8	2.9
TP_1-Week 6	63.2	19.7	42.9	68.1	-0.1
TP_2	65	22.5	35.8	55.1	Baseline
TP_2-Day 3	70.2	24.2	41.8	59.7	5.2
TP_2-Day 9- AR	68	23.5	39.3	57.7	3
TP_2-Day 35	67.5	23.2	36.8	54.9	2.5
TP_3	57	22.5	26.6	46.7	Baseline
TP_3-Day 3	62	24.8	31.1	50.1	5
TP_3-Day 11	56.4	22.4	27.0	48.2	-0.6
TP_4- AR	89.5	28.7	39.7	44.6	
TP_5- AR	71.0	23.5	43.2	60.9	
RD_1- MCNS	34	16.0	21.7	63.8	
RD_2- MCNS	79	28	36.2	45.8	
RD_3- MCNS	65.4	28.1	30.5	46.9	
RD_4- MN	64	23.2	36.6	57.2	
Control 1	60	26.3	27.5	45.9	
Control 2	58	22.4	28.7	49.4	
Control 3	43.3	17.0	22.7	52.8	

Abbreviations: BMI= body mass index; kg= kilograms; L= litres; TP= transplant recipient; RD= primary renal disease; TBW= total body water; AR= acute rejection; MCNS= nephrotic syndrome due to minimal change disease; MN= membranous nephropathy

As expected, body weight correlated with BMI ($p<0.0001$, linear regression; $p=0.0076$, Spearman's rank correlation). There was a significant correlation between body weight and total body water ($p=0.0007$, linear regression; $P=0.0002$, Spearman's rank correlation) and

between BMI and total body water (p=0.0024, linear regression; p=0.0023, Spearman's rank correlation), in keeping with the fluid retention present during acute rejection and renal disease.

3.2 MEASUREMENT OF RENAL PLASMA FLOW

Effective renal plasma flow (ERPF) was measured in 6 stable renal transplant recipients on day 3 post-transplant and one control subject using Sodium ^{131}Iodohippurate. The glomerular filtration rate (GFR) was measured simultaneously, using ^{99m}Technium-diethelenetriamine pentaacetate (Tc-DTPA). ERPF measurement was repeated in 3 of the 6 patients, 2 of whom (patients 1 and 5) were undergoing an episode of acute rejection. ERPF was reduced during acute rejection.

Patient	Post TP Day	GFR ml/min	ECF ml	ERPF ml/min
1	3	44	16495	381
1 - AR	8	37	16495	190
2	3	40	11711	184
3	3	85	11794	340
4	3	93	15066	372
5	3	61	16177	277
5 -AR	6	51	10050	224
6	3	35	22472	331
6 - stable	6	41	21795	444
Control	-	114	18498	573

There was no correlation between GFR and ERPF (p=0.0535) and between GFR and ECF (p=0.6829, linear regression) probably because of the small sample size; it would be anticipated that a fall in GFR is the consequence of a decrease in renal blood flow and ERPF, resulting in an increase in ECF.

3.3 ENDOTHELIN STUDY

3.3.1 PATIENT DEMOGRAPHICS

The total number of subjects in this sector of the study was one hundred and one. Of these, twenty six were kidney transplant recipients undergoing acute rejection, twenty nine were patients with glomerular and other renal disorders and twenty four were patients with end stage renal failure on dialysis. The twenty two controls comprised seventeen kidney donors prior to nephrectomy and five healthy volunteers. For the purposes of statistical analysis for measurement of ET-1 in plasma and urine, n= patient numbers.

3.3.1.1 Acute Rejection Group

Twenty six renal transplant patients with acute rejection were studied in this group. There were 24 males and 2 females; of these, 17 had received kidneys from living related donors, 2 had received kidneys from living unrelated donors and 7 from cadaver donors. Their mean age was 38.27 years (range 16-53 years). There was no difference in age when compared to the 22 control subjects (p=0.495, unpaired t test). In the acute rejection group, rejection was mild in 7 patients, moderate in 12 and severe in 7 (Appendix 3.3.1.1). The severity of rejection was categorised according to the Banff 1995 classification.

3.3.1.2 Renal Disorders

Twenty nine patients with various renal disorders were studied in this group. Of these, 16 were males and 13 females, with a mean age of 33.17 years (range 13-59 years). Six patients had mesangiocapillary glomerulonephritis (MCGN), 4 had class IV lupus nephritis (SLE), 5 membranous nephropathy (MGN), 5 minimal change disease with nephrotic syndrome (MCNS), 4 immunoglobulin A (IgA) nephritis, 2 hypertensive nephrosclerosis (HPT neph), 2 chronic interstitial nephritis (CIN) and 1 patient with mesangioproliferative glomerulonephritis [(MPGN); Appendix 3.3.1.2].

3.3.1.3 Control Group

Twenty two subjects formed the control group; 17 were kidney donors who were studied prior to uni-nephrectomy; 5 were healthy volunteers. There were 13 males and 9 females with a mean age of 34.32 years (range 21-48 years). Their details are presented in Appendix 3.3.1.3

3.3.2 PLASMA ENDOTHELIN-1 VALUES

Literature suggests that plasma ET-1 concentrations are elevated in the circulation in renal failure (Shichiri et al., 1990; Stockenhuber et al., 1992) or show a borderline increase (Saito et al., 1991). It has been suggested that plasma ET-1 levels are elevated during acute rejection only if there has been concomitant endothelial cell damage (Watschinger et al., 1994).

3.3.2.1 Controls

The mean value for plasma ET-1 in 22 control subjects measured 0.76 pg/ml, median of 0.69 pg/ml and range of 0.4-1.55 pg/ml (Table 3.3.2)

3.3.2.2 Acute Rejection Group

i) The mean value for plasma ET-1 was 1.71 pg/ml with a median value of 1.50 pg/ml and range of 0.63-5.25 pg/ml in the acute rejection group (n=26). There was no correlation between serum creatinine and plasma ET-1 in the patients with acute rejection; the correlation coefficient (r) equalled 0.29 and a p value of 0.17 (linear regression).

ii) Circulating ET-1 levels were significantly increased in the rejection group when compared to control subjects, with a p value of 0.0002 (unpaired-t test), as indicated in Table 3.3.2.

Table 3.3.2 Plasma Endothelin-1 levels in acute rejection and controls

	Control	Rejection
n	**22**	**26**
Mean ± SEM	**0.76 ± 0.065**	**1.71 ± 0.21**
SD	**0.31**	**1.05**
Median	**0.69**	**1.5**
Range	**0.40 – 1.55**	**0.63 – 5.25**
p (unpaired t-test)	**0.0002**	

n= Patient numbers; measurements: pg/ml

3.3.2.2.1 Serial plasma endothelin-1 levels after renal transplantation

Plasma ET-1 levels were measured serially in renal transplant patients. The mean ± SEM and median values for plasma ET-1 were 2.01 ± 0.23pg/ml before transplantation (n=17), 1.42 ± 0.14 pg/ml following transplantation on day 3-4 (n=18), 1.74 ± 0.18 pg/ml during acute rejection (n=16) and 1.37 ± 0.12 pg/ml after treatment and recovery from rejection (n=11) respectively; the median values were 1.84 pg/ml; 1.3 pg/ml, 1.56 pg/ml and 1.2 pg/ml

respectively and tabulated in 3.3.2.2.1. Plasma ET-1 levels showed cyclical changes during acute rejection and the subsequent treatment phase. Following transplantation, plasma ET-1 levels declined significantly (p=0.036, Mann Whitney test), but levels rose during acute rejection (p=0.47, Mann-Whitney test). Once the rejection episode was treated, plasma ET-1 levels decreased again (p=0.12, Mann-Whitney test). This post-treatment value was lower than that measured prior to transplantation (p=0.0412, unpaired t-test).

Table 3.3.2.2.1 Serial Plasma Endothelin 1 levels in renal transplantation

	(i) Pre-TP	(ii) Post-TP	(iii) Acute rejection	(iv)After Rejection Rx
n	17	18	16	11
Mean ± SEM	2.01 ± 0.23	1.42 ± 0.14	1.74 ± 0.18	1.37 ± 0.12
SD	0.93	0.61	0.72	0.41
Median	1.84	1.3	1.56	1.20
Range	1.1 – 4.29	0.71 – 2.64	0.87 – 3.76	0.85 – 2.16
p(Mann-Whitney test)	(i) vs (ii) : 0.036		(iii) vs (iv) : 0.12	
	(i) vs (iv) : 0.0412*; (ii) vs (iii) : 0.18 ; (i) vs (iii) : 0.47			

n= patient numbers *Unpaired t-test ; measurements: pg/ml

3.3.2.3 Renal diseases

The mean plasma value for ET-1 measured 1.98 pg/ml in this group (n=29), with a median value of 1.6 pg/ml and a range of 0.45-5.56 pg/ml; the mean value was significant when compared to control subjects with a p value of <0.01 (Tukey Kramer multiple comparisons). There was a significant correlation between plasma ET-1 and serum creatinine levels in this

group, with r=0.55 and a two-tailed p value of 0.0019 (linear regression). For the purposes of further analysis, the renal diseases were grouped into proliferative and non-proliferative disorders.

3.3.2.3.1 Proliferative glomerulonephritis

Thirteen patients with proliferative glomerulonephritis (prolif. GN) had a mean plasma value for ET-1 of 2.81pg/ml, median of 3.0 pg/ml and range of 0.6-5.56 pg/ml.

3.3.2.3.2 Non-proliferative glomerulonephritis

The 16 patients with non-proliferative disorders had a mean plasma value for ET-1 of 1.3 pg/ml, median of 1.07 pg/ml and range of 0.45-3.0 pg/ml.

Table 3.3.2.3 Plasma Endothelin-1 in Renal Disorders

	(i)Control	(ii)Whole Group	(iii)Prolif GN	(iv) Non Prolif GN
n	**22**	**29**	**13**	**16**
Mean ± SEM	**0.76 ± 0.065**	**1.98 ± 0.26**	**2.81 ± 0.42**	**1.30 ± 0.21**
SD	**0.31**	**1.39**	**1.50**	**0.85**
Median	**0.69**	**1.60**	**3.0**	**1.07**
Range	**0.40-1.55**	**0.45-5.56**	**0.60-5.56**	**0.45-3.0**
p*	**(i) vs (ii) : <0.01**		**(iii) vs (iv) < 0.01**	
	(i) vs (iii) : <0.001; (i) vs (iv) 0.0096 (unpaired t-test)			

n= Patient numbers; *Tukey Kramer multiple comparisons ; measurements: pg/ml

The difference between these two histological groups was statistically significant, with the highest levels of plasma ET-1 in proliferative GN ($p<0.01$). The difference between patients

with proliferative GN and control subjects was highly significant with a p value of <0.001. Plasma ET-1 was increased in patients with non-proliferative diseases compared to control subjects (p=0.0096, unpaired t-test).

3.3.2.3.3 Hypertensive glomerulonephritic subjects

Fifteen patients were hypertensive (HPT) in this group, with a mean plasma value for ET-1 of 2.52 pg/ml, median of 2.53 pg/ml and range of 0.6-5.56 pg/ml.

3.3.2.3.4 Normotensive glomerulonephritic subjects

The 14 normotensive (NBP) patients had a mean plasma value for ET-1 of 1.39 pg/ml, median of 0.87 pg/ml and range of 0.45-4.0 pg/ml.

Table 3.3.2.3 Plasma Endothelin-1 in Renal Disorders

	(i) Control	(ii) HPT	(iii) NBP
n	**22**	**15**	**14**
Mean ± SEM	**0.76 ± 0.065**	**2.52 ± 0.37**	**1.39± 0.30**
SD	**0.31**	**1.44**	**1.11**
Median	**0.69**	**2.53**	**0.87**
Range	**0.40-1.55**	**0.60-5.56**	**0.45-4.0**
p (unpaired t-test)		**(ii) vs (iii) : 0.0261**	
	(i) vs (ii) :0.0001*; (i) vs (iii) : 0.0155		

n= patient numbers; * Mann Whitney test ; measurements: pg/ml

Plasma ET-I was significantly increased in hypertensive patients when compared with normotensive patients, with a p value of 0.0261 (unpaired t test); the p value of 0.0001 was

highly significant when comparing the hypertensive patients with control subjects. Plasma ET-1 was increased in patients with normotensive renal disorders compared with controls (p=0.0155, unpaired t test).

3.3.2.3.5 Dialysis patients

Patients on dialysis (n =26) had a mean value for plasma ET-1 of 2.4 pg/ml; median 2.07 pg/ml; range 0.29-5.56 pg/ml.

3.3.2.3.6 Patients not requiring dialysis

In comparison, 26 patients who were not dialysis-requiring had a mean value for plasma ET-1 of 1.7 pg/ml, median of 1.6 pg/ml and range of 0.45-4.12 pg/ml.

Table 3.3.2.3 Plasma Endothelin-1 in Renal Disorders

	(i) Control	(ii) Dialysis	(iii)Non-dialysis
n	**22**	**26**	**26**
Mean ± SEM	**0.76 ± 0.065**	**2.40 ± 0.25**	**1.70 ± 0.22**
SD	**0.31**	**1.27**	**1.13**
Median	**0.69**	**2.07**	**1.60**
Range	**0.40-1.55**	**0.29-5.56**	**0.45-4.12**
p (unpaired t-test)	**(i)vs(ii)<0.0001; (ii) vs (iii) : 0.0428 (i) vs (iii) : 0.0004**		

n= patient numbers ; measurements: pg/ml

Plasma ET-1 was significantly increased in both the dialysis and non-dialysis requiring groups. The difference between the dialysis-requiring and non-dialysis groups was significant

(p=0.0428); and also when comparing the dialysis-requiring group with control subjects (p<0.0001) and non-dialysis patients with control subjects (p=0.0004).

3.3.3 URINE ENDOTHELIN-1 VALUES

It has been suggested that ET-1 is generated in situ in the kidney in renal disorders and levels in the urine may reflect renal synthesis of the peptide.

3.3.3.1 Urine endothelin-1 levels in acute rejection

The mean value for urinary ET-1 was 1.67 pg/ml in 17 patients with acute rejection with a median value of 1.23 pg/ml and range of 0.7-4.7 pg/ml; corresponding control values (n=11) were mean = 0.37 pg/ml, median = 0.2 pg/ml and range of 0.0-1.58 pg/ml (Table 3.3.3). The difference was significant, with a p value of 0.005 (Mann Whitney test). There was no correlation between serum creatinine and urine ET-1 levels, with r = 0.11 and p=0.66.

Table 3.3.3 Urine Endothelin-1 levels

	(i) Control	(ii) Rejection	(iii) GN
n	**11**	**17**	**11**
Mean ±SEM	**0.37 ± 0.13**	**1.67 ± 0.35**	**0.41 ± 0.13**
Median	**0.20**	**1.23**	**0.38**
Range	**0 – 1.58**	**0.70 – 4.70**	**0 – 1.09**
p	**(i) vs (ii) : 0.005***		**(i) vs (iii) : 0.80***

n= patient numbers; *Mann Whitney; measurements: pg/ml

3.3.3.2 Urine endothelin-1 levels in renal disease

Urinary ET-1 excretion in glomerulonephritis (n=11) measured a mean ± SEM of 0.41 ± 0.13 pg/ml, median of 0.38 pg/ml and range of 0.0 - 1.09 pg/ml. This was similar to controls (p=0.80; Mann-Whitney test) where the mean ± SEM was 0.37 ± 0.13 pg/ml, median of 0.20 pg/ml and range of 0.0 - 1.58 pg/ml

3.3.4 LOCALISATION OF ENDOTHELIN-1 AND THE ENDOTHELIN RECEPTORS ET_A AND ET_B IN THE KIDNEY

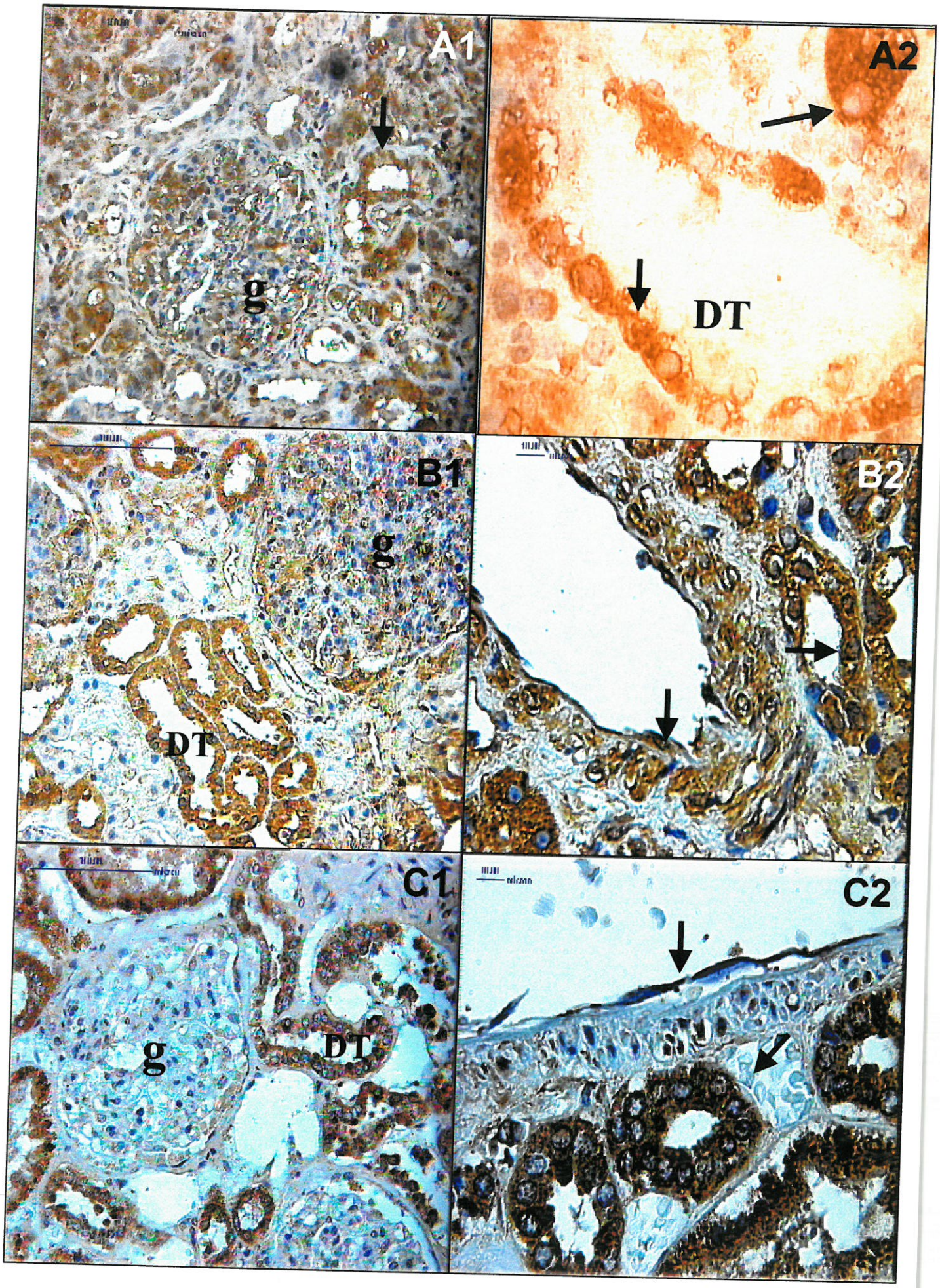
Histological sections of one per patient, when viewed at a magnification of x40 showed 1-2 glomeruli per section, whereas the tubules were numerous. Field means of immunolabel were derived in pixels per micron^2 for the different regions of interest, namely glomeruli, tubules and renal blood vessels and compared statistically. Because of the complex nature of the study and access to tissue samples, it was not possible to always receive adequate numbers of tissue samples in each category. Therefore non-parametric tests were used, since it was not known whether the study population was normally distributed.

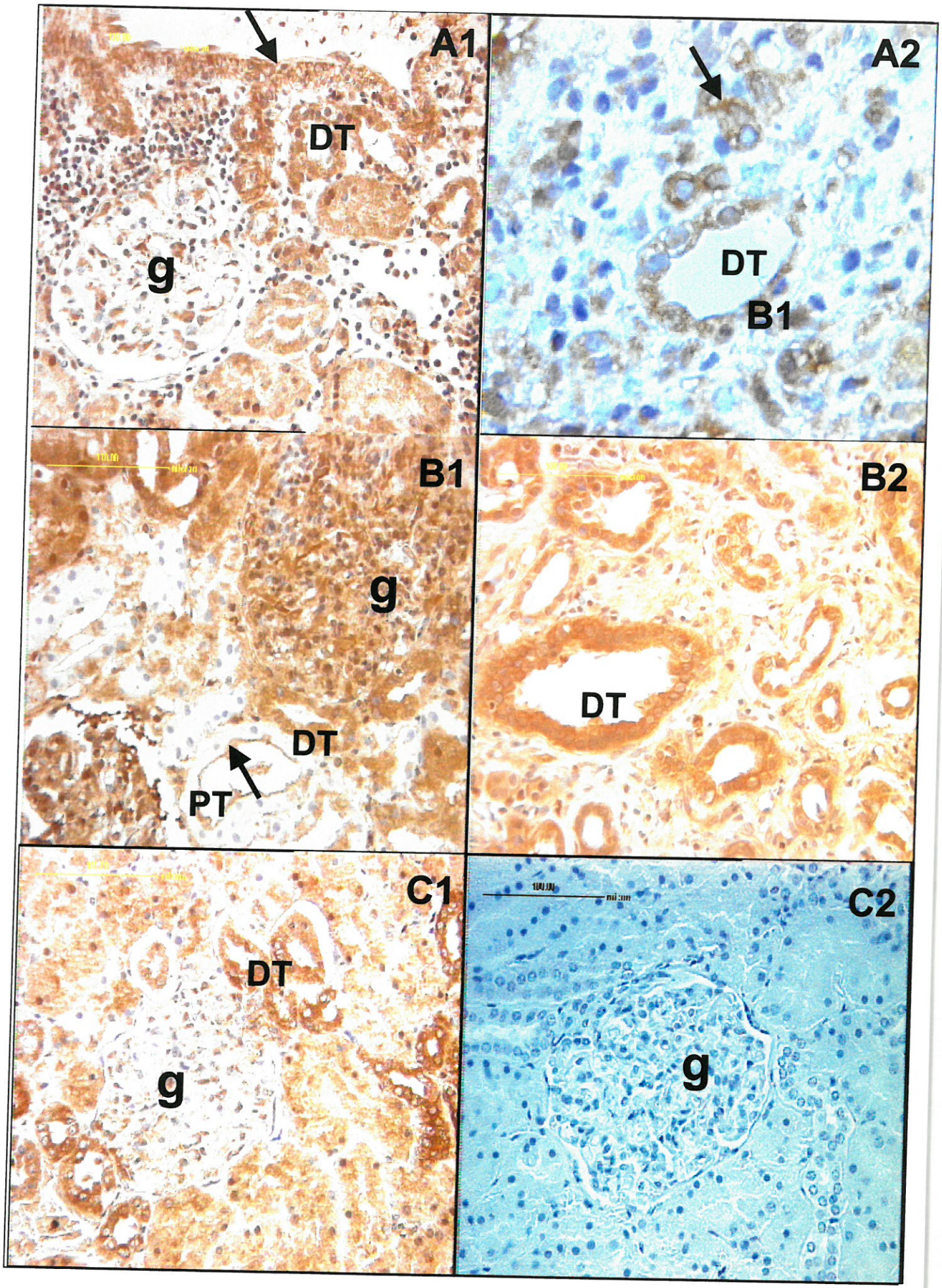
3.3.4.1 Immonoperoxidase: Peroxidase-antiperoxidase (PAP) labelling

The PAP-immunolabelled images were quantitatively analysed by the Kontron Elektron KS 300 (Zeiss GmbH, Germany) analyser, running on Windows 95. The digital images were converted to grey images ranging in grey scale density from 0 to 255. The median maximal density of immunolabelling was calculated as field means in pixels per micron^2 and statistically analysed.

3.3.4.1.1 Control kidney tissue

ET-1 immunolabelling was observed in glomerular capillary loops, distal tubules and collecting ducts as well as the endothelium and smooth muscle cells of media of arterioles of control kidney tissue. [Fig 3.1 (C)]. Endothelin$_A$ (ET_A) receptor immunolabelling was visualised in distal tubules and collecting ducts. Minimal labelling was observed in glomeruli and blood vessels [Fig 3.2 (C)]. Endothelin$_B$ (ET_B) receptor was immunovisualised in distal tubules and collecting ducts and minimally focally in the mesangium in control kidney tissue. In addition, immunolabelling of endothelium and smooth muscle cells of the media of blood vessels was observed [Fig 3.3 (C)].


A1
g
A2
DT
B1
g
DT
B2
C1
g
DT
C2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DT
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C2
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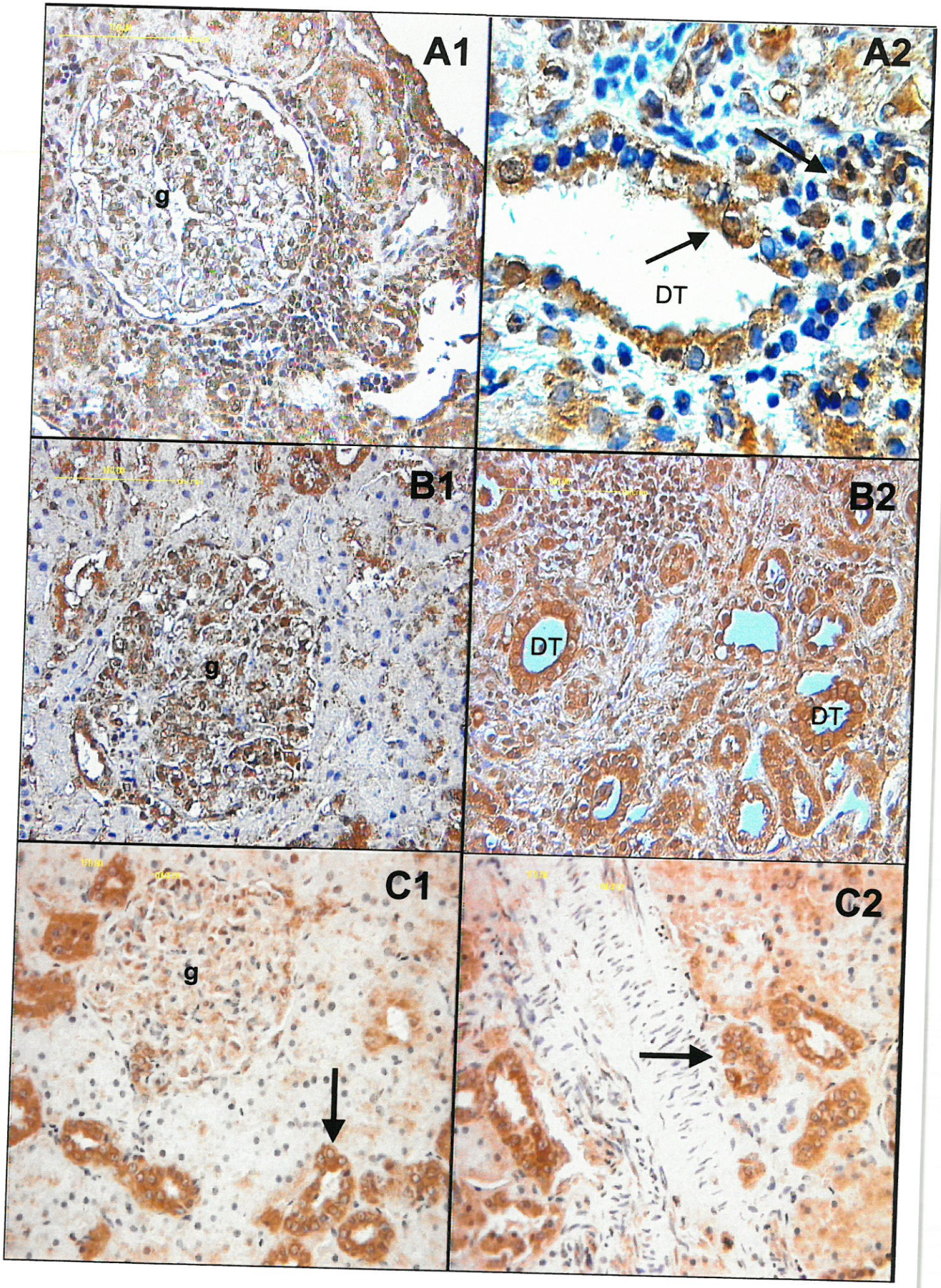
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3.3.4.1.2 Acute rejection

Endothelin-1

There was decreased immunolabelling for ET-1 in glomeruli in acute rejection compared to control glomeruli [p=0.0095, Mann-Whitney test; Fig 3.1 (A)]. Although the values for rejection tubules were lower when compared to controls, the difference was not significant (p=0.20); the difference was not significant when comparing tubules in mild rejection (n=3) and severe rejection (n=2) with controls and with each other.

Table 3.3.4.1 ET-1 in acute rejection (PAP immunolabelling)

	GLOMERULI		TUBULES	
	Control	Rejection	Control	Rejection
n	4	6	4	4
Mean ± SEM	138.33 ± 4.38	100.55 ± 5.37	94.01 ± 6.86	81.48 ±6.68
SD	8.76	13.16	13.75	13.35
Median	139.4	101.85	87.74	77.49
Range	127.77-146.74	81.06-116.69	85.97-114.6	70.3-100.66
p(Mann-Whitney test)	0.0095		0.20	

n = number of histological sections; one section per patient;
measurements: pixels/micron^2

Endothelin Receptors

ET_A Receptor

While there was increased ET_A receptor labelling in acute rejection, the site and intensity of immunolabelling of the ET_A receptor in glomeruli (p=0.14, Mann-Whitney test) and tubules (p=0.075) was not significantly increased in acute rejection when compared to

control kidney tissue (Table 3.3.4.1). There appeared to be no difference when comparing glomeruli from mild rejection (n=2) and severe rejection (n=5) or tubules from mild rejection (n=4) and severe rejection (n=6) with each other and with controls. The images are depicted in Figure 3.2.

Table 3.3.4.1 ET_A Receptor in acute rejection (PAP labelling)

	GLOMERULI		TUBULES	
	Control	**Rejection**	**Control**	**Rejection**
n	6	7	5	10
Mean ± SEM	113.64 ±5.01	127.14 ±7.71	127.52±31.92	147.16±4.59
SD	12.26	20.41	71.36	14.52
Median	116.74	136.55	97.94	147.76
Range	90.85-124.61	96.84-144.03	91.1-255.0	116.29-165.71
p(Mann-Whitney test)	0.14		0.075	

n = number of histological sections (one per patient); measurements: pixels/micron^2

ET_B Receptor

There was significantly increased ET_B receptor immunolabelling of glomeruli in acute rejection (p=0.0022, Mann-Whitney test) and decreased immunolabelling of tubules (p=0.0076, Mann-Whitney test) when compared to control kidney tissue. Glomerular labelling in both mild (n=3) and severe (n=3) rejection was significantly increased when compared to controls ($p<0.05$, Tukey-Kramer multiple comparisons). There was no difference in the glomerular labelling when comparing mild and severe rejection. There was a significant decrease in tubular labelling when comparing mild rejection (n=3;

p=0.047) and severe rejection (n=3; p=0.038) with controls (Mann-Whitney test). The images are shown in Figure 3.3.

Table 3.3.4.1 ET_B Receptor in glomeruli in acute rejection (PAP labelling)

	GLOMERULI			
	(i) Control	(ii) Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n	6	6	3	3
Mean ± SEM	77.16±14.79	138.73±3.22	136.32±5.0	141.14±4.59
SD	36.22	7.89	8.66	7.94
Median	78.96	136.7	132.32	138.7
Range	30.81-115.84	130.39-150.02	130.39-146.26	134.71-150.02
p*	(i)vs(ii) 0.0022; (i)vs(iii):0.023		(i)vs(iv):0.0238; (iii)vs(iv):0.4	

*Mann-Whitney test; n = number of histological sections (one per patient); measurements: pixels/micron^2

Table 3.3.4.1 ET_B Receptor in tubules in acute rejection (PAP labelling)

	TUBULES			
	(i) Control	(ii) Rejection	(iii)Mild Rejection	(iv)Severe Rejection
N	6	9	3	4
Mean±SEM	149.56±4.98	129.12±3.54	129.26±3.57	126.68±6.21
SD	12.20	10.62	6.17	12.42
Median	153.92	128.51	128.51	129.14
Range	129.85-162.03	111.18-145.97	123.49-135.77	111.18-145.97
p(Mann-Whitney)	(i)vs(ii):0.0076; (i)vs(iii):0.0476		(i)vs(iv): 0.038	

n = number of histological sections (one per patient); measurements: pixels/micron^2

3.3.4.1.3 Glomerulonephritis

ET-1

ET-1 immunolabelling was decreased in glomeruli (p=0.049, Mann-Whitney test), and similarly in tubules (p=0.90) in glomerulonephritis when compared to control kidney tissue. Glomerular labelling was significantly decreased in proliferative glomerulonephritis (n=6) when compared to controls (p<0.001), and non-proliferative glomerulonephritis (n=2; p<0.01; Tukey-Kramer multiple comparisons). There was no difference in tubular labelling in proliferative (n=13) and non-proliferative (n=3) glomerulonephritis. The images are depicted in Figure 3.1 (B).

Table 3.3.4.1.3 ET-1 in glomeruli in renal disease (PAP labelling)

	GLOMERULI			
	(i)Control	**(ii) GN**	**(iii)Non-prolif GN**	**(iv)Prolif GN**
n	**4**	**8**	**2**	**6**
Patients	**4**	**8**	**2**	**6**
Mean±SEM	**138.33±4.38**	**104.27±8.23**	**136.67±14.2**	**93.48±4.44**
SD	**8.76**	**23.27**	**20.08**	**10.87**
Median	**139.4**	**91.08**	**136.67**	**90.42**
Range	**127.77-146.74**	**85.11-150.86**	**122.47-150.86**	**85.11-115.19**
p(Tukey-Kramer)	**(i)vs(ii):0.049**; (i)vs(iii)>0.05**		**(i)vs(iv)<0.01; (iii)vs(iv)<0.01**	

**(Mann-Whitney test); n = number of histological sections ; measurements: pixels/micron^2

Table 3.3.4.1.3 ET-1 in tubules in renal disease (PAP labelling)

	TUBULES	
	Control	GN
n	4	18
Mean ± SEM	94.01± 6.86	91.3± 6.6
SD	13.75	28.02
Median	87.74	100.78
Range	85.97-114.6	25.52-135.81
p (Mann-Whitney test)	0.90	

n = number of histological sections (one per patient); measurements: pixels/micron^2

ET_A Receptor

The images are depicted in Figure 3.2 (B).

Table 3.3.4.1.3 ET_A receptor in glomeruli in renal disease (PAP labelling)

	GLOMERULI	
	Control	GN
n	6	7
Mean ± SEM	128.34±8.44	129.43±5.93
SD	20.68	15.69
Median	121.29	129.98
Range	113.42-167.3	106.3-150.65
p (Mann-Whitney test)	0.92	

n = number of histological sections (one per patient); measurements: pixels/micron^2

ET_A receptor immunolabelling was significantly increased in tubules (p=0.026, Mann-Whitney test) and similarly in glomeruli (p=0.92) of renal biopsies with glomerulonephritis when compared to control kidney tissue (Table 3.3.4.1.3)

Table 3.3.4.1.3 ET_A receptor in tubules in renal disease (PAP labelling)

	(i) Control	(ii Non-prolif GN	(iv) Prolif GN
n	**7**	**11**	**3**
Mean ± SEM	**109.1±9.8**	**133.22±5.52**	**130.2±6.49**
SD	**25.94**	**18.31**	**11.24**
Median	**100.85**	**130.87**	**135.94**
Range	**90.96-167.21**	**105.33-162.82**	**117.25-137.41**
p (Mann Whitney test)	**(i) vs(ii) :0.026**	**(i)vs(iii) >0.05**	**(ii)vs(iii) >0.05**

n = number of histological sections (one per patient); measurements: pixels/micron^2

ET_B Receptor

ET_B receptor immunolabelling was increased in glomeruli of glomerulonephritis kidney tissue but the increase was not statistically significant; the labelling was decreased in tubules in glomerulonephritis (non-proliferative p=0.0008, proliferative GN p= 0.0001; Mann-Whitney test; Table 3.3.4.1.3). There was no difference when comparing proliferative and non-proliferative glomerulonephritis. The images are depicted in Figure 3.3 (B).

Table 3.3.4.1.3 ET_B receptor in renal disease (PAP labelling)

	GLOMERULI			DISTAL TUBULES		
	(i)Control	(ii)Non Prolif GN	(iii) Prolif GN	(a)Control	(b)Non Prolif GN	(c) Prolif GN
n(sections)	7	8	5	7	11	13
Mean ± SEM	89.05 ± 17.25	127.62 ± 4.31	124.5 ± 3.49	151.79 ± 3.78	125.33 ± 3.92	129.39 ± 1.98
SD	45.64	12.18	7.8	10.0	13.0	7.13
Median	93.97	129.79	125.86	153.94	124.24	129.74
Range	30.81 – 160.39	108.31 – 142.82	113.07 – 131.83	134.16 – 163.64	106.21 – 154.79	116.82 – 144.15
p (Mann-Whitney)	(i) vs (ii) : 0.0541			(a) vs (b) : 0.0008		
	(i) vs (iii) : 0.1061; (ii) vs (iii) :0.62			(a) vs (c) : 0.0001; (b) vs (c) :>0.05		

n= number of patients also; measurements: pixels/micron^2

3.3.4.2 Confocal microscopy: Immunofluorescent labelling

The staining intensity of flourescent-labelled tissue sections was viewed by confocal scanning laser microscopy and quantitatively analysed using the Analysis 2.1 Pro system (Software GmbH, 1996, Germany). The mean intensity of immunolabelling was expressed in pixels x 100 per micron^2 and statistically analysed.

3.3.4.2.1 Acute rejection

ET-1

ET-1 labelling of proximal tubules in acute rejection was significantly increased in the rejection group as a whole when compared to controls (p=0.0016), and in mild rejection

compared to controls ($p=0.0001$) and severe rejection ($p<0.05$). Increased labelling was noted in distal tubules in mild rejection in comparison to severe rejection ($p=0.0058$) and controls ($p=0.0499$). While glomerular labelling was increased in rejection in comparison to controls, there was no statistical difference. The images are depicted in Figures 3.4 (A and B) and 3.5.

Table 3.3.4.2.1 ET–1 in acute rejection (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	**(ii)Rejection**	**(iii)Mild Rejection**	**(iv)Severe Rejection**
n (structures)	**5**	**77**	**60**	**17**
Sections	**4**	**19**	**15**	**4**
Mean ± SEM	**11.6 ± 0.8**	**58.8 ± 7.6**	**69.3 ± 7.3**	**19.4 ± 6**
Median	**12.1**	**60.7**	**63.5**	**15.1**
SD	**1.6**	**33.1**	**28.6**	**12.0**
Range	**9.4-12.9**	**10.5-128.6**	**22.4-128.6**	**10.5-37**
p	**(i) vs (ii) : 0.0016(Mann Whitney); (i) vs (iv):>0.05**** **(i) vs (iii) : 0.0001(Kruskal Wallis); (iii) vs (iv) <0.05****			

** Dunn's multiple comparisons; measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET–1 in acute rejection (immunofluorescent labelling)

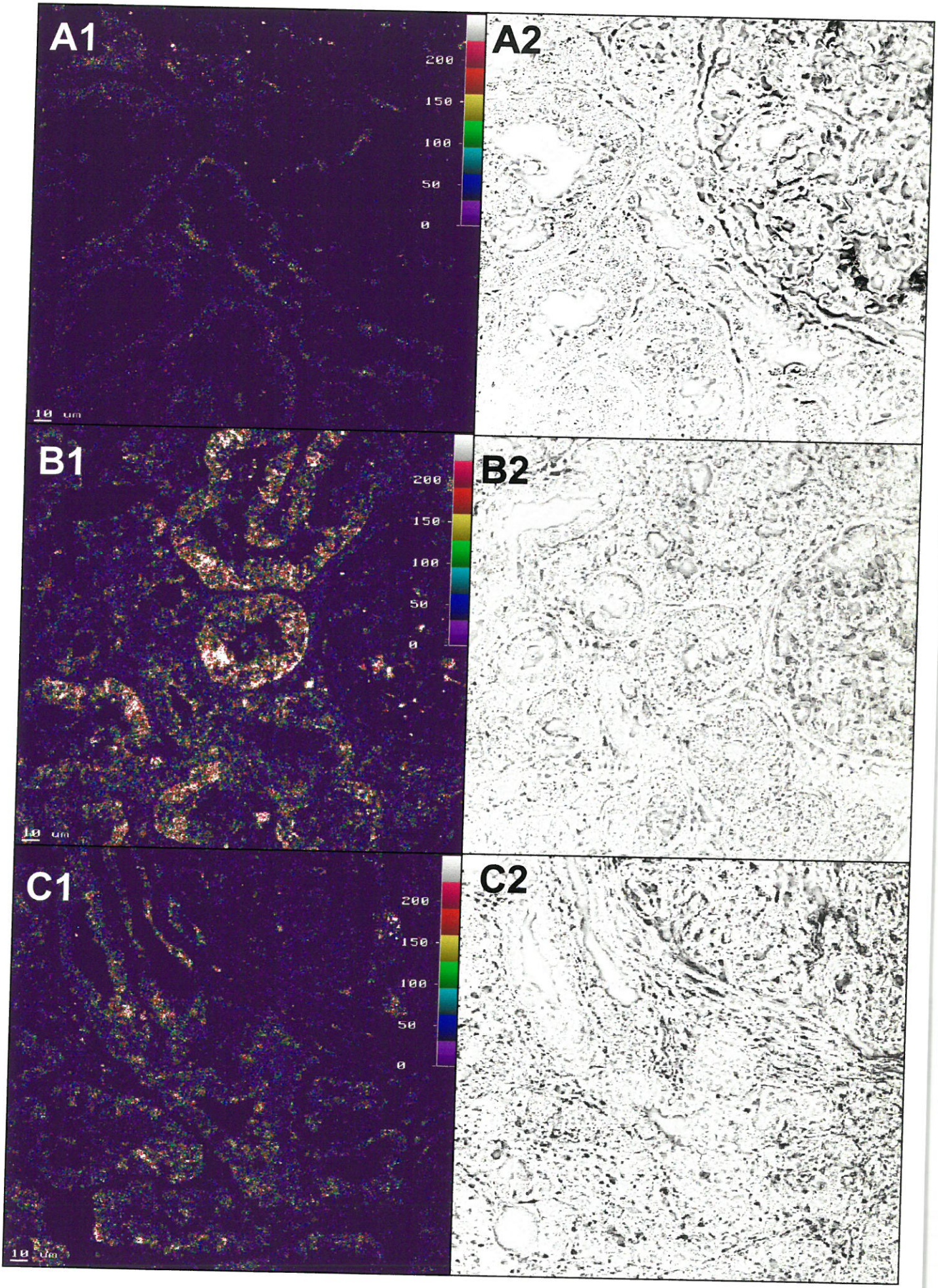
	DISTAL TUBULES			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	4	56	52	4
Patients	4	19	16	3
Mean ± SEM	23.09±1.09	55.8 ± 7.8	63.8 ± 7.7	13 ± 1.8
Median	22.7	53.6	69	13.7
SD	1.09	34	30.8	3.1
Range	21.14-25.8	9.7-107	12.2-107	9.7-15.7
p(Mann Whitney)	(i) vs (ii) : 0.47; (i) vs (iv):0.0571 (i) vs (iii) :0.0499; (iii) vs (iv): 0.0058(Kruskal-Wallis)			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET–1 in acute rejection (immunofluorescent labelling)

	GLOMERULI			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	3	13	9	4
Patients	3	13	9	4
Mean ± SEM	4.2 ± 0.16	13.1 ± 3.5	15.4 ± 3.9	12.1 ± 5.6
Median	4.29	10.2	10.7	9.7
SD	0.28	11.7	11.7	11.2
Range	3.9-4.4	2.7-37.7	2.7-37.7	2.7-26.5
p (Mann Whitney)	(i) vs (ii) : 0.64; (i) vs (iv):>0.05** (i) vs (iii) :0.33; (iii) vs (iv): 0.0727			

**Dunn's multiple comparisons; measurements: pixelsx100/ micron^2
n = number of structures in biopsies from the same number of patients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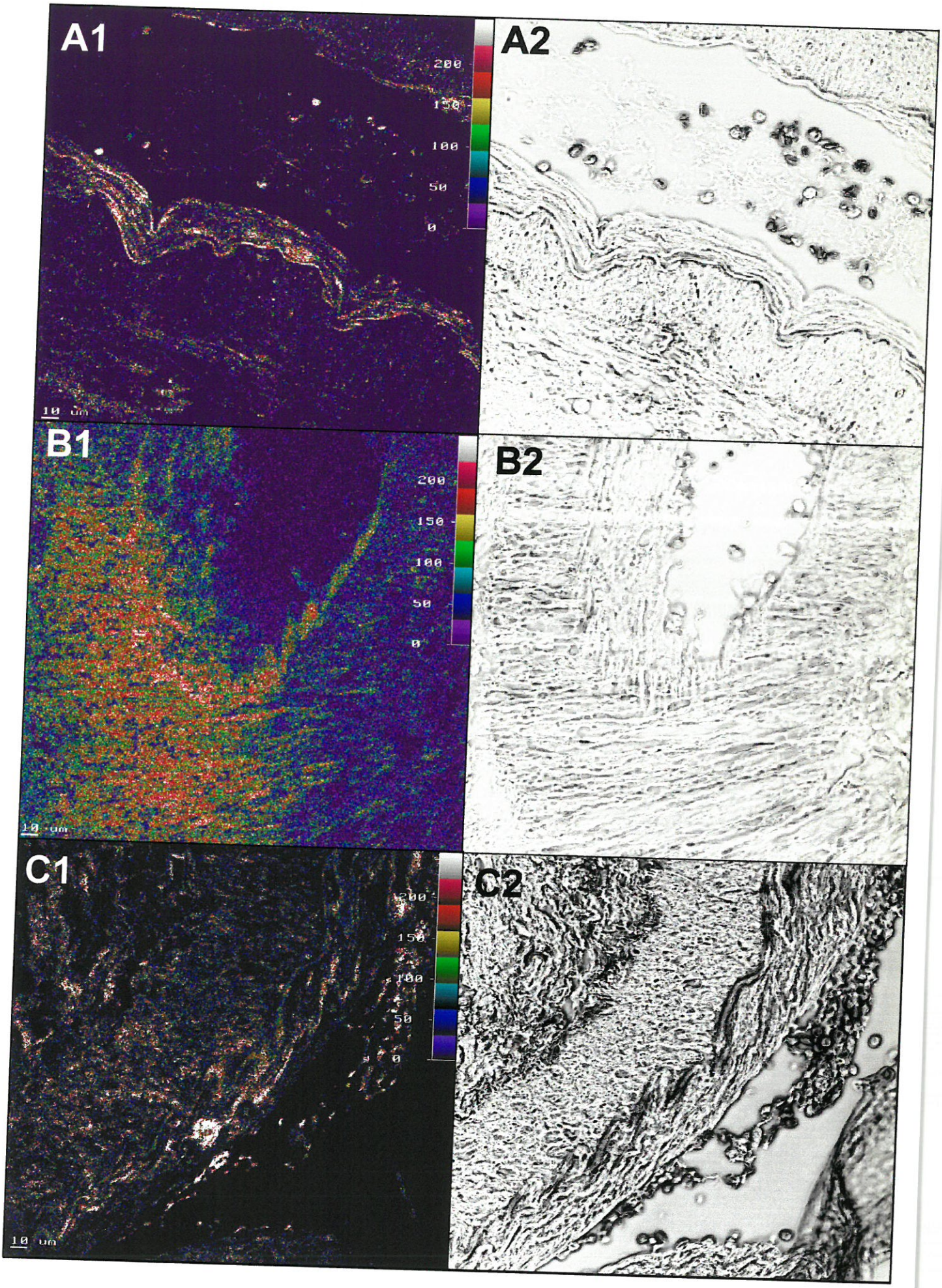
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Endothelin receptors

ET_A receptor

While ET_A receptor immunolabelling of proximal tubules was increased in both mild and severe rejection, it was not significantly so when compared to the control kidney. Similarly, while ET_A receptor labelling of distal tubules was increased in rejection, it was not significant when compared to control kidney ($p=0.09$, Mann Whitney test), probably because of the small sample numbers. ET_A receptor labelling of glomeruli was similar in rejection and control kidney. ET_A receptor labelling of collecting ducts was significantly increased in rejection ($p=0.0238$, Mann Whitney test) compared to control kidney. The images are depicted in Figures 3.6 (A and B).

Table 3.3.4.2.1 ET_A receptor in acute rejection (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	**(ii)Rejection**	**(iii)Mild Rejection**	**(iv)Severe Rejection**
n (structures)	**17**	**17**	**9**	**8**
Patients	**3**	**7**	**3**	**4**
Mean ± SEM	**27.5 ± 8.9**	**92.1 ± 31.6**	**69.1 ± 33.7**	**109.2 ± 51.9**
Median	**35.2**	**84.7**	**84.1**	**109.2**
SD	**15.4**	**83.6**	**58.3**	**103.8**
Range	**9.7-37.5**	**4.8-199.2**	**4.8-118.5**	**19.3-199.2**
p (Kruskal Wallis)	**(i) vs (ii) : 0.52**; (i) vs (iv):0.69 (i) vs (iii) :>0.05; (iii) vs (iv)>0.05**			

**Mann Whitney; measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET_A receptor in acute rejection (immunofluorescent labelling)

	DISTAL TUBULES			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	8	17	12	5
Patients	3	6	3	3
Mean ± SEM	7.3 ± 6.4	43.6 ± 10.7	32.8 ± 9.8	54.4 ± 19
Median	1.7	41.1	32.2	73.4
SD	11.1	26.3	17.1	32.9
Range	0.1-20.1	16.1-73.5	16.1-50.1	16.4-73.5
p (Kruskal Wallis)	(i) vs (ii) : 0.09 (Mann Whitney); (i) vs (iv):0.17 (i) vs (iii) :>0.05; (iii) vs (iv) >0.05			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET_A receptor in acute rejection (immunofluorescent labelling)

	COLLECTING DUCTS			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	15	25	19	6
Sections	3	6	3	3
Mean ± SEM	21.8 ± 3.6	102.7 ± 15.5	82.1 ± 26.4	123 ± 9.2
Median	19.6	114	45.7	114
SD	6.2	38	101.9	16
Range	17.1-28.9	29.9-141.8	29.9-114.6	113.5-141.8
p(Mann Whitney)	(i) vs (ii) : 0.0238; (i) vs (iv):<0.01** (i) vs (iii) :>0.05; (iii) vs (iv) >0.05			

**Tukey Kramer multiple comparisons; measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET_A receptor in acute rejection (immunofluorescent labelling)

GLOMERULI		
	(i) Control	(ii) Rejection
n	3	4
Patients	3	4
Mean± SEM	19.3 ± 9.4	7.3 ± 1.2
Median	18.3	7
SD	16.3	2.4
Range	3.5–36.1	5–10.5
p(Mann Whitney)	(i)vs(ii) : 0.63	

measurements: pixelsx100/ micron^2

ET_B receptor

ET_B receptor labelling of proximal tubules was significantly decreased in rejection ($p<0.0364$, Mann Whitney test) when compared to control kidney. While the label was decreased in distal tubules in severe rejection, the difference was not significant, probably because of small numbers.

Table 3.3.4.2.1 ET_B receptor in acute rejection (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	15	32	17	15
Patients	3	9	5	4
Mean ± SEM	112.8 ± 5.2	29.6 ± 15	41 ± 27	15.3 ± 4
Median	98.9	15.3	14.2	18.1
SD	89.2	45.1	60.5	8.1
Range	31.3-208.1	3.9-148.9	9.3-148.9	3.9-21.2
p(Tukey Kramer)	(i) vs (ii) : 0.0364**; (i) vs (iv): <0.01 (i) vs (iii) :<0.01; (iii) vs(iv).:>0.05			

** Mann Whitney; measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET_B receptor in acute rejection (immunofluorescent labelling)

	DISTAL TUBULES			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	7	23	18	5
Patients	3	7	4	3
Mean ± SEM	43.6 ± 1.1	27.8 ± 22.9	44.6±40.2	5.4 ± 0.9
Median	51.2	4.9	5.6	4.9
SD	11.1	60.6	80.4	1.6
Range	21.7-58	2-165.1	19.9-165.1	4-7.1
p (Mann Whitney)	(i) vs (ii) : 0.12; (i) vs (iv):0.1 (i) vs (iii) :0.4;(iii) vs (iv) 0.99			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.1 ET_B receptor in acute rejection (immunofluorescent labelling)

	GLOMERULI			
	(i)Control	(ii)Rejection	(iii)Mild Rejection	(iv)Severe Rejection
n (structures)	4	7	3	4
Patients	4	7	3	4
Mean ± SEM	47.4 ± 5.2	2.8 ± 0.2	2.7 ± 0.21	2.9 ± 0.2
Median	49.2	2.7	2.7	2.8
SD	10.4	0.4	0.3	0.5
Range	34.3-57	2.3-3.5	2.3-3	2.5-3.5
p**	(i) vs (ii) : <0.05; (i) vs (iv):<0.05 (i) vs (iii) :<0.05; (iii) vs (iv):>0.05			

** Dunn's multiple comparisons; measurements: pixelsx100/ micron^2

Glomerular labelling, was significantly decreased in rejection when compared to control kidney. The images are depicted in Figures 3.7 (A and B).

3.3.4.2.2 Glomerulonephritis

ET-1

ET-1 immunolabelling of proximal tubules was very significantly increased in non-proliferative (p=0.0059) and proliferative GN (p=0.0159, Mann Whitney test) when compared to control kidney; however there was no difference between non-proliferative and proliferative GN. ET-1 labelling of distal tubules was increased in GN when compared to control kidney (p=0.0286). There was a significant increase in tubular labelling when comparing proliferative and non-proliferative GN (p=0.0346). Glomerular labelling was

significantly increased in GN when compared to control kidney (p=0.044). The images are depicted in Figures 3.4 (C) and 3.5 (C).

Table 3.3.4.2.2 ET-1 receptor in renal disease (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	5	66	50	16
Patients	4	18	13	5
Mean ± SEM	11.7 ± 0.8	94.8 ± 24.3	88.8 ± 32	98.1 ± 14.9
Median	12.1	78.7	45	112
SD	1.6	100.3	115.4	33.4
Range	9.5-12.9	11-449.5	11-449.5	45.5-132.5
p(Mann Whitney)	(i) vs (ii) : 0.007; (i) vs (iv):0.0159 (i) vs (iii) : 0.0059; (iii) vs (iv): 0.12			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET-1 receptor in renal disease (immunofluorescent labelling)

	DISTAL TUBULES		
	(i)Control	(ii)Non-prolif GN	(iii)Prolif GN
n (structures)	4	45	13
Patients	4	13	4
Mean ± SEM	23.1 ± 1.1	50.5± 7.8	103.1 ± 13.7
Median	22.7	49	108.4
SD	2.2	28.1	27.4
Range	21.1-25.8	15-93.2	66.3-129.5
p (Mann Whitney)	(i) vs (ii) :0.16; (i) vs (iii):0.0286 (ii) vs (iii): 0.0346		

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET–1 in renal disease (immunofluorescent labelling)

	GLOMERULI			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	3	12	9	3
Patients	3	12	9	3
Mean ± SEM	4.2 ± 0.16	47.7 ± 15.9	45.9 ± 20.8	53 ± 19.4
Median	4.3	31.4	31.4	63.8
SD	0.28	55.2	62.4	33.7
Range	3.9-4.4	5.5-208.1	5.5-208.1	15.3-80
p(Mann Whitney)	(i) vs (ii) : 0.044; (i) vs (iv):0.20 (i) vs (iii) :0.0727; (iii) vs (iv): 0.48			

measurements: pixelsx100/ micron^2

Endothelin receptors

ET_A Receptor

While ET_A receptor labelling of proximal tubules was increased in GN, it was not significantly increased when compared to control kidney. ET_A receptor labelling of distal tubules was significantly increased in proliferative GN when compared to non-proliferative GN ($p=0.004$, Kruskal Wallis test) and control kidney ($p<0.05$). ET_A receptor labelling of glomeruli was similar in GN and control kidney. The labelling of collecting ducts was similar in both non-proliferative and proliferative GN compared to controls. The images are depicted as Figures 3.6 (C).

Table 3.3.4.2.2 ET_A receptor in renal disease (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	17	46	9	37
Patients	3	17	4	13
Mean ± SEM	27.5 ± 8.9	76.6 ± 15.3	47.8 ± 10	85.4 ± 19.2
Median	35.2	61.9	45.9	65.8
SD	15.4	62.9	20	69.4
Range	9.7-37.5	0.8-204.3	30-69.4	0.7-204.3
p (Kruskal Wallis)	(i) vs (ii) : 0.26**; (i) vs (iv):0.34 (i) vs (iii) :>0.05; (iii) vs (iv)>0.05			

**Mann Whitney; measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET_A receptor in renal disease (immunofluorescent labelling)

	DISTAL TUBULES			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	8	46	9	37
Patients	3	16	4	12
Mean ± SEM	7.3 ± 6.4	29.3 ± 8.9	2.8 ± 1.4	38.1 ± 10.8
Median	1.7	11.3	2.1	26.1
SD	11.1	35.6	2.9	37.2
Range	0.1-20.1	0.3-127.1	0.2-6.7	6.1-127.1
p (Kruskal Wallis)	(i) vs (ii) : 0.37 (Mann Whitney); (i) vs (iv)<0.05 (i) vs (iii) :>0.05; (iii) vs (iv) <0.05			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET_A receptor in renal disease (immunofluorescent labelling)

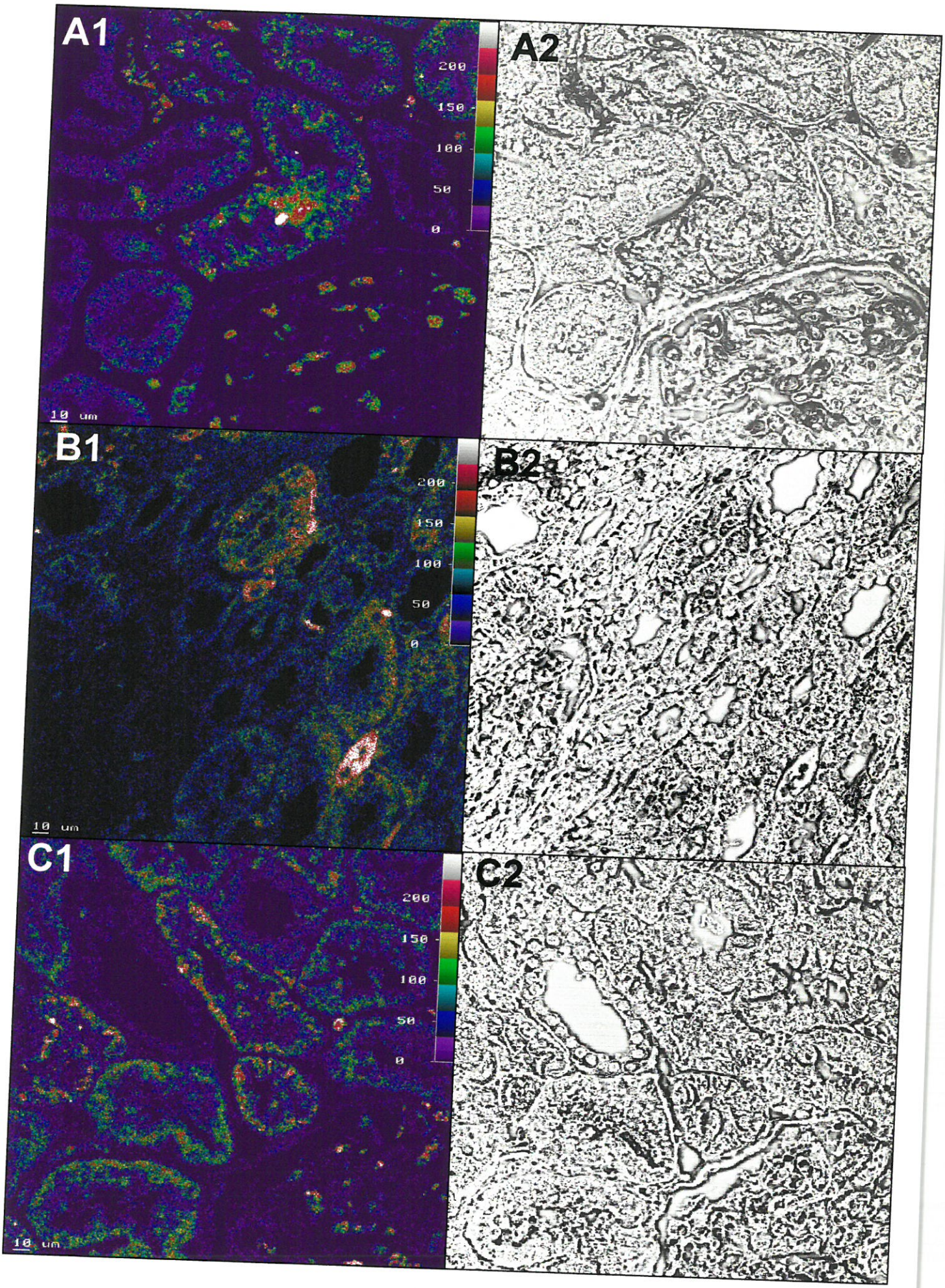
	COLLECTING DUCTS			
	(i)Control	(ii)GN	(iii) Non-prolif GN	(iv)Prolif GN
n (structures)	15	40	6	34
Patients	3	8	3	5
Mean ± SEM	21.8 ± 3.6	34 ± 7.3	21 ± 7	41.9 ± 9.6
Median	19.6	28.8	24.5	35.8
SD	6.2	20.5	12.1	21.4
Range	17.1-28.9	7.5-70.5	7.4-30.9	19.2-70.5
p (Kruskal Wallis)	(i) vs (ii) : 0.38 (Mann Whitney); (i) vs (iv):>0.05 (i) vs (iii) :>0.05; (iii) vs (iv) :0.29			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET_A receptor in renal disease (immunofluorescent labelling)

	GLOMERULI			
	(i)Control	(ii)GN	(iii) Non-prolif GN	(iv)Prolif GN
n (structures)	3	9	3	6
Patients	3	9	3	6
Mean±SEM	19.3 ± 9.4	8.4 ± 2.7	12.9 ± 5.8	6.1 ± 2.8
Median	18.3	4.9	15.2	3.1
SD	16.3	8.1	10	6.8
Range	3.6-36.1	0.1-15.6	2-21.6	0.1-15.6
p (Kruskal Wallis)	(i) vs (ii) : 0.21 (Mann Whitney); (i) vs (iv):>0.05 (i) vs (iii) :>0.05; (iii) vs (iv) :>0.05			

measurements: pixelsx100/ micron^2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ET_B receptor

While ET_B receptor labelling was decreased in GN in proximal and distal tubules, collecting ducts and glomeruli, the difference was not significant probably because of the small sample size. The images are depicted in Figure 3.7 (C).

Table 3.3.4.2.2 ET_B receptor in renal disease (immunofluorescent labelling)

	PROXIMAL TUBULES			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	15	43	11	32
Patients	3	11	4	7
Mean ± SEM	112.8± 51.8	35.8 ± 10.1	44.5 ± 23.7	30.8 ± 9.8
Median	98.9	25.9	27.1	18.8
SD	89.2	33.5	47.4	25.9
Range	31.2-208.1	9.1-114.5	9.4-114.5	9.1-78.8
p(Mann Whitney)	(i) vs (ii) : 0.0604; (i) vs (iv):0.067 (i) vs (iii) :0.229; (iii) vs (iv):0.788			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET_B receptor in renal disease (immunofluorescent labelling)

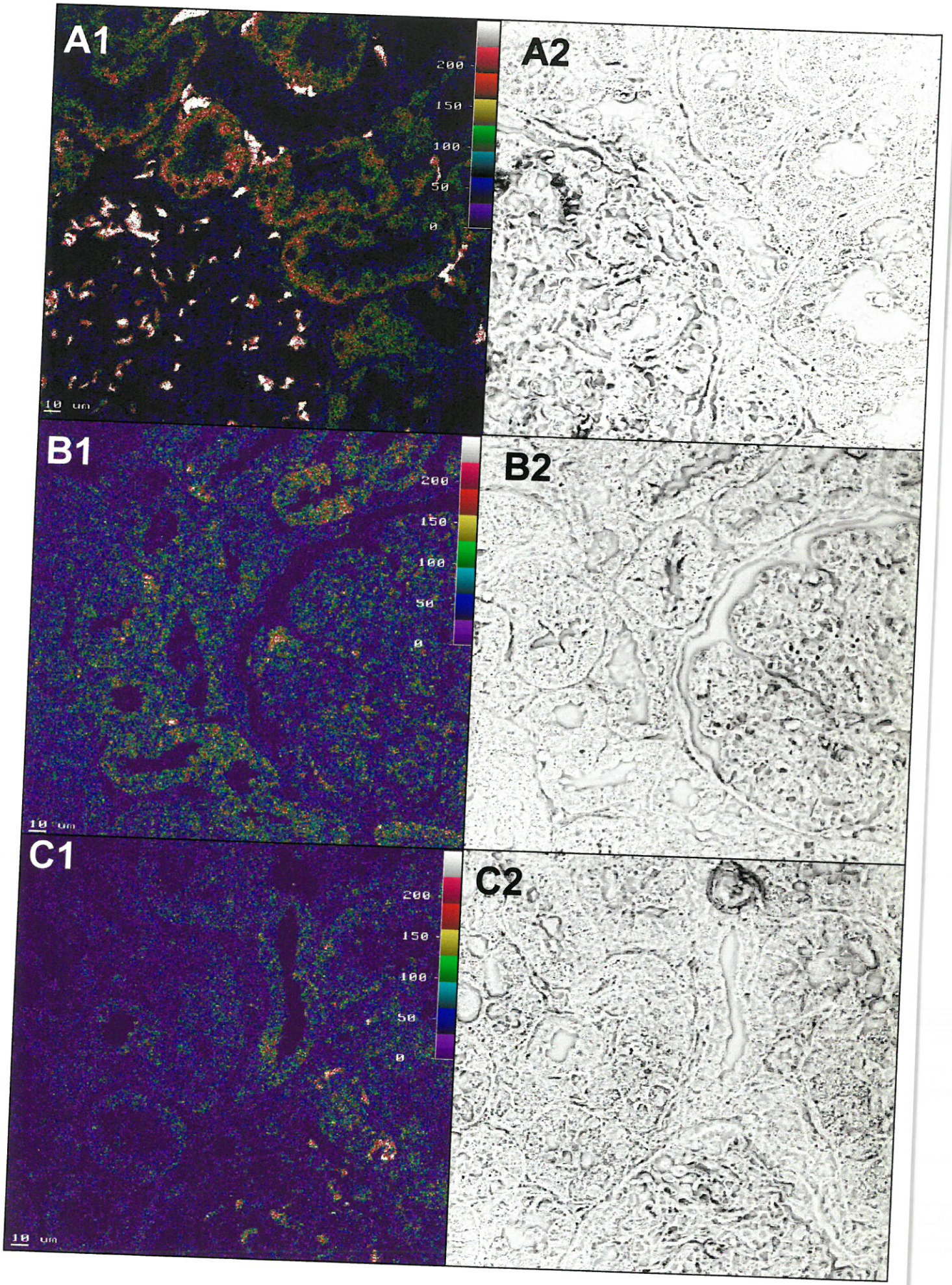
	DISTAL TUBULES			
	(i)Control	(ii)GN	(iii)Non-prolif GN	(iv)Prolif GN
n (structures)	7	25	11	14
Patients	3	11	4	7
Mean ± SEM	43.6 ± 11.1	24.9 ± 8.6	22.6 ± 4.8	26.2 ± 13.6
Median	51.2	14	20.2	76.6
SD	19.3	28.5	9.5	36.1
Range	21.7-58	0.6-96.5	14-36	0.6-96.5
p (Mann Whitney)	(i) vs (ii) : 0.17 ; (i) vs (iv):0.267 (i) vs (iii) :0.23; (iii) vs (iv) :0.315			

measurements: pixelsx100/ micron^2

Table 3.3.4.2.2 ET_B receptor in renal disease (immunofluorescent labelling)

	GLOMERULI		COLLECTING DUCTS	
	(i)Control	(ii)GN	(i)Control	(ii)GN
n (structures)	4	5	10	30
Patients	4	5	4	3
Mean ± SEM	47.4 ± 5.2	22.3 ± 8.4	51.7 ± 8.3	39.6 ± 3
Median	49.2	18.2	49.6	41.8
SD	10.4	18.8	16.7	6.9
Range	34.3-57	5.7-51.9	34.6-72.8	31.8-45.1
p(Mann Whitney)	0.38		0.4	

measurements: pixelsx100/ micron^2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C2

ELECTRON MICROSCOPY: IMMUNOGOLD LABELLING

3.3.3.2 Acute rejection

ET-1 immuno-labelling was visualised in proximal and distal tubules (in the cytoplasm and, and to a lesser extent, in the nucleus), collecting ducts, endothelial cells of arteries and glomerular capillaries in control kidney. Gold particles occurred in clusters or singly, mainly along infoldings of the basolateral membranes, intercellular system, cytoplasmic vacuoles, mitochondrial cristae and endoplasmic reticulum. The distribution of gold particles was increased along the basolateral membranes of distal tubules during acute rejection, predominantly for ET-1 antibody. ET_A and ET_B receptor immuno-labelling was in a similar distribution to ET-1. Figures 3.8, 3.9 and 3.10 show the ultrastructural localisation of ET-1 and its receptors. Table 3-1 depicts the score of the immunogold particles for the sections examined.

Table 3-1. IMMUNOGOLD LABELLING COUNTS IN ACUTE REJECTION

ET-1	PTL	PTB	DTL	DTB
Control (total count)	41 (10)	75 (13)	47 (11)	39 (12)
Rejection (total count)	152 (19)	193 (26)	184 (25)	190 (22)
Control (mean/section)	4.1	5.77	4.27	3.25
Rejection(mean/section)	8	7.42	7.36	8.64
ETA				
Control (total)	90 (9)	81 (10)	57 (5)	85 (7)
Rejection (total)	290 (21)	254 (23)	257 (21)	257 (23)
Control (mean/section)	10	8.1	11.4	12.14
Rejection(mean/section)	13.8	11.04	12.24	11.17
ETB				
Control (total)	23 (3)	55 (5)	31 (4)	29 (3)
Rejection (total)	174 (21)	189 (26)	230 (20)	256 (21)
Control (mean/section)	8.3	11	7.75	9.67
Rejection(mean/section)	8.29	7.27	11.7	12.19

PTL = Proximal tubule, luminal portion; DTL = Distal tubule, luminal portion
PTB = Proximal tubule, basal DTB = Distal tubule, basal; Figures in superscript = number of sections

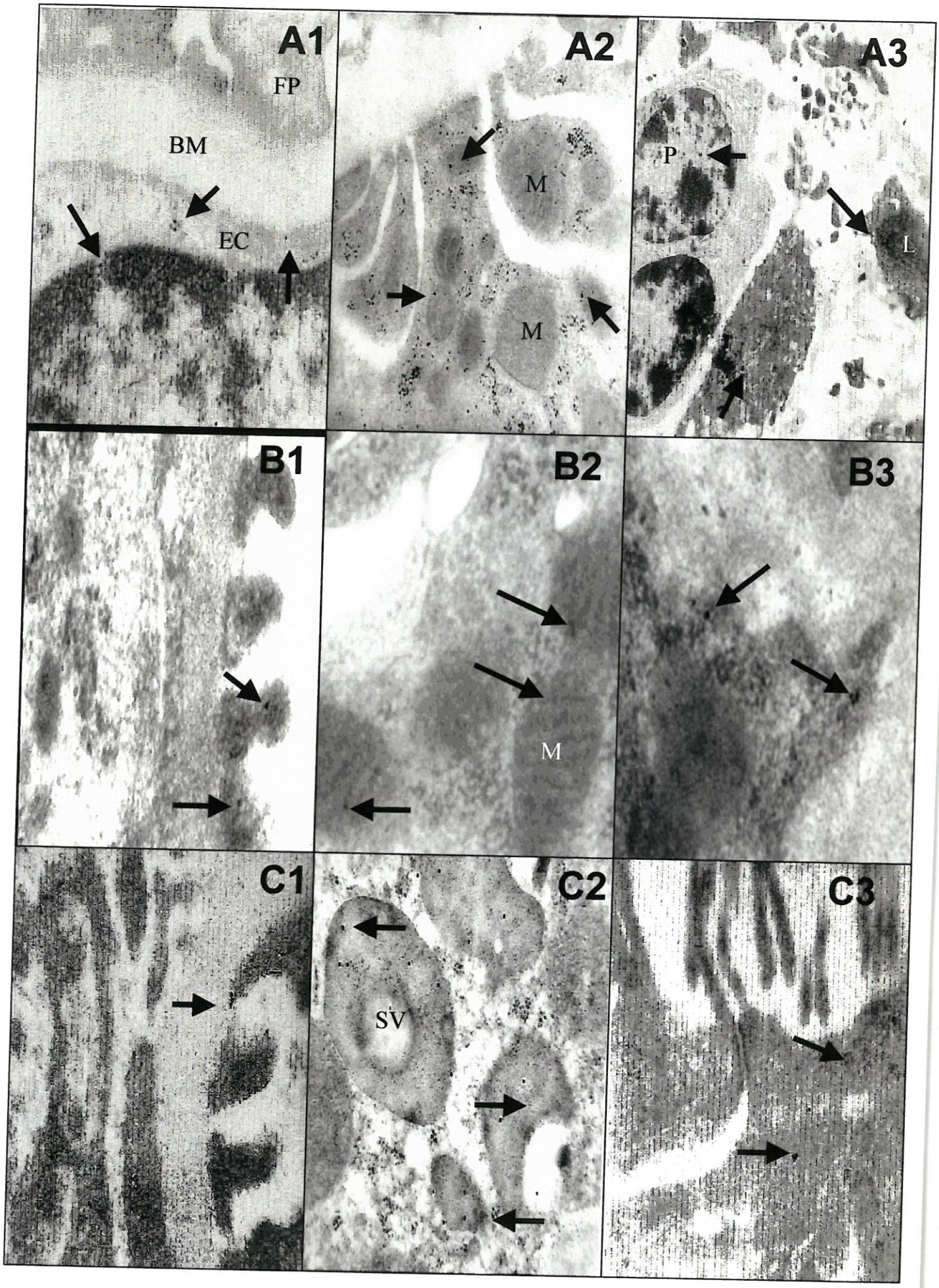
3.3.4.1 Glomerulonephritis

ET-1 immuno-labelling was visualised in proximal and distal tubules (in the cytoplasm and, and to a lesser extent, in the nucleus), collecting ducts, endothelial cells of arteries and glomerular capillaries in control kidney. ET_A and ET_B receptor immuno-labelling was visualised in a similar distribution to ET-1.Gold particles occurred in clusters or singly, mainly along infoldings of the basolateral membranes, intercellular system, cytoplasmic vacuoles, mitochondrial cristae and endoplasmic reticulum, in a similar distribution as in acute rejection. Figures 3.8, 3.9 and 3.10 show the ultrastructural localisation of ET-1 and its receptors. Table 3-2 depicts the score of the immunogold particles for the sections examined.

Table 3-2. IMMUNOGOLD COUNTS IN GLOMERULONEPHRITIS

ET-1	**PTL**	**PTB**	**DTL**	**DTB**
Control (total count)	18 (6)	22 (6)	32 (5)	31 (6)
GN (total count)	76 (28)	72 (25)	113 (26)	89 (21)
Control (mean/section)	3	3.7	6.4	5.2
GN (mean/section)	2.7	2.9	4.3	4.3
ETA				
Control (total)	28 (9)	25 (10)	31 (8)	33 (8)
GN (total)	73 (23)	59 (17)	64 (13)	70 (16)
Control (mean/section)	3.1	2.5	3.9	4.1
GN (mean/section)	3.2	3.5	4.97	4.4
ETB				
Control (total)	13 (6)	15 (8)	15 (6)	37 (9)
GN (total)	39 (25)	48 (22)	77 (26)	88 (25)
Control (mean/section)	2.2	1.9	2.5	4.1
GN (mean/section)	1.6	2.2	3.0	3.5

GN= glomerulonephritis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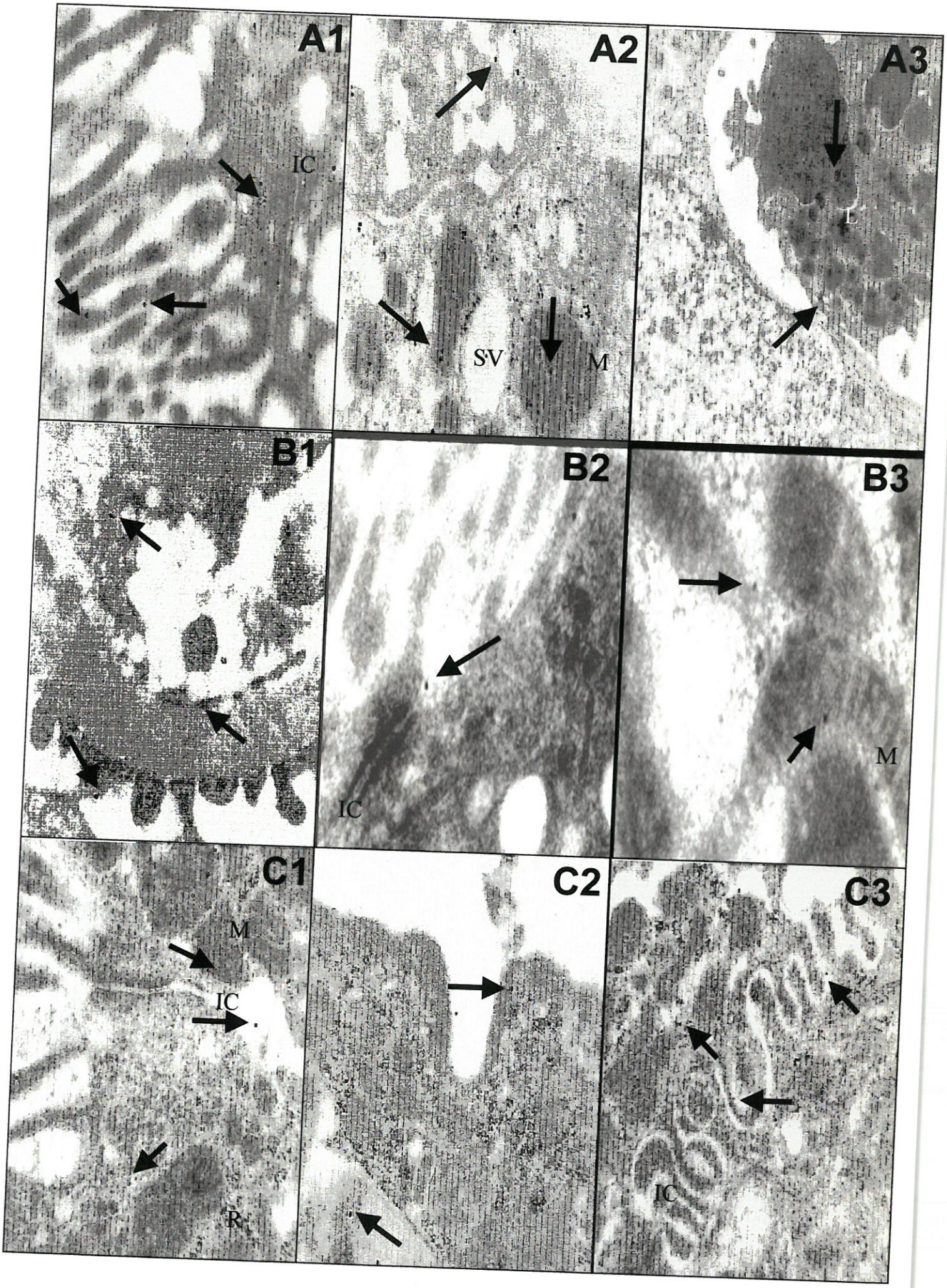
C2
SV
C3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SV
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C1
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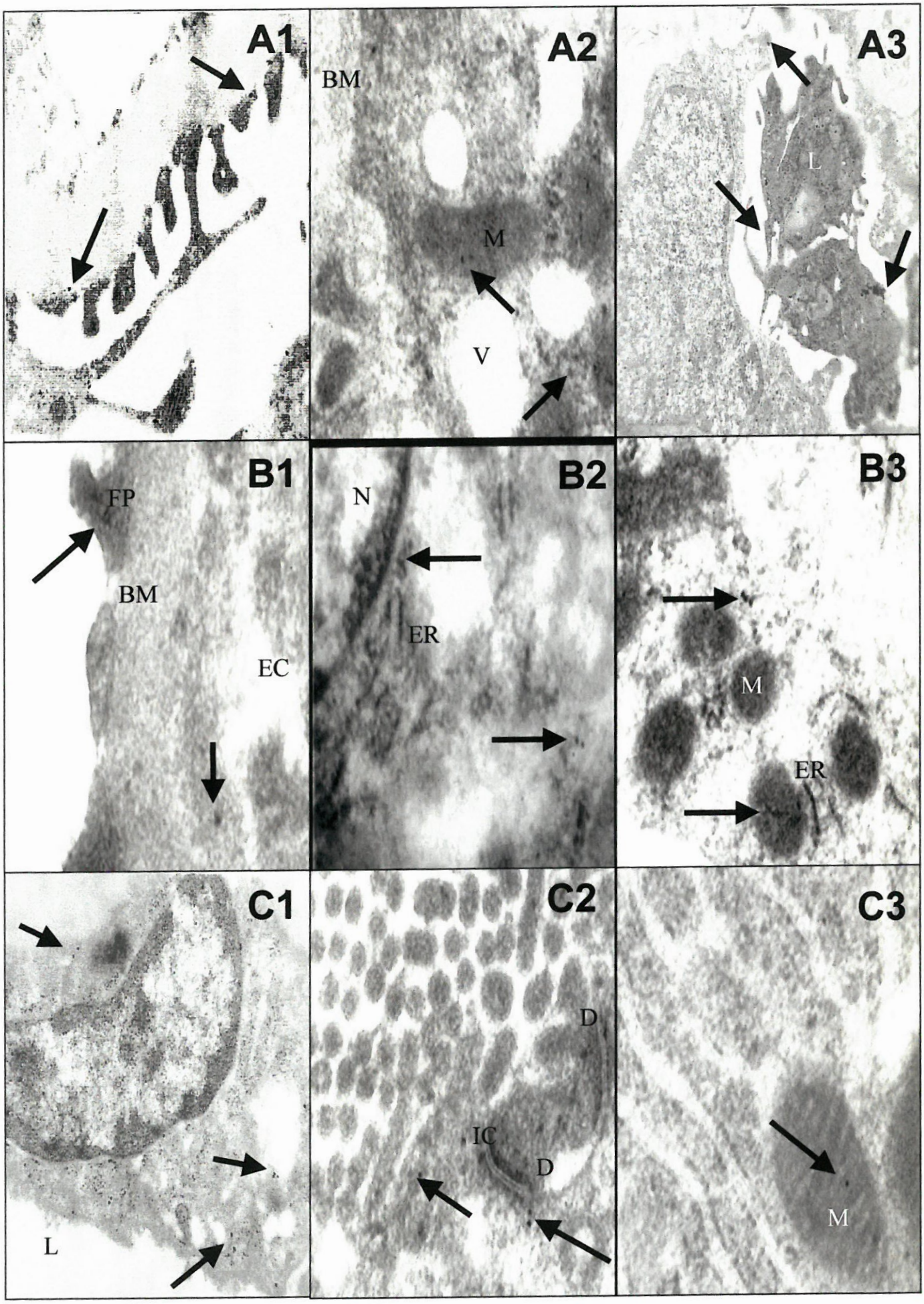
C3
IC


A1
A2
BM
M
V
A3
L
B1
FP
BM
EC
B2
N
ER
B3
M
ER
C1
L
C2
D
IC
D
C3
M

3.3.5 LOCALISATION OF ENDOTHELIN-1 mRNA IN THE HUMAN KIDNEY BY RT-PCR

Table 3-3: Sequence of Endothelin-1 primers and expected length of PCR product

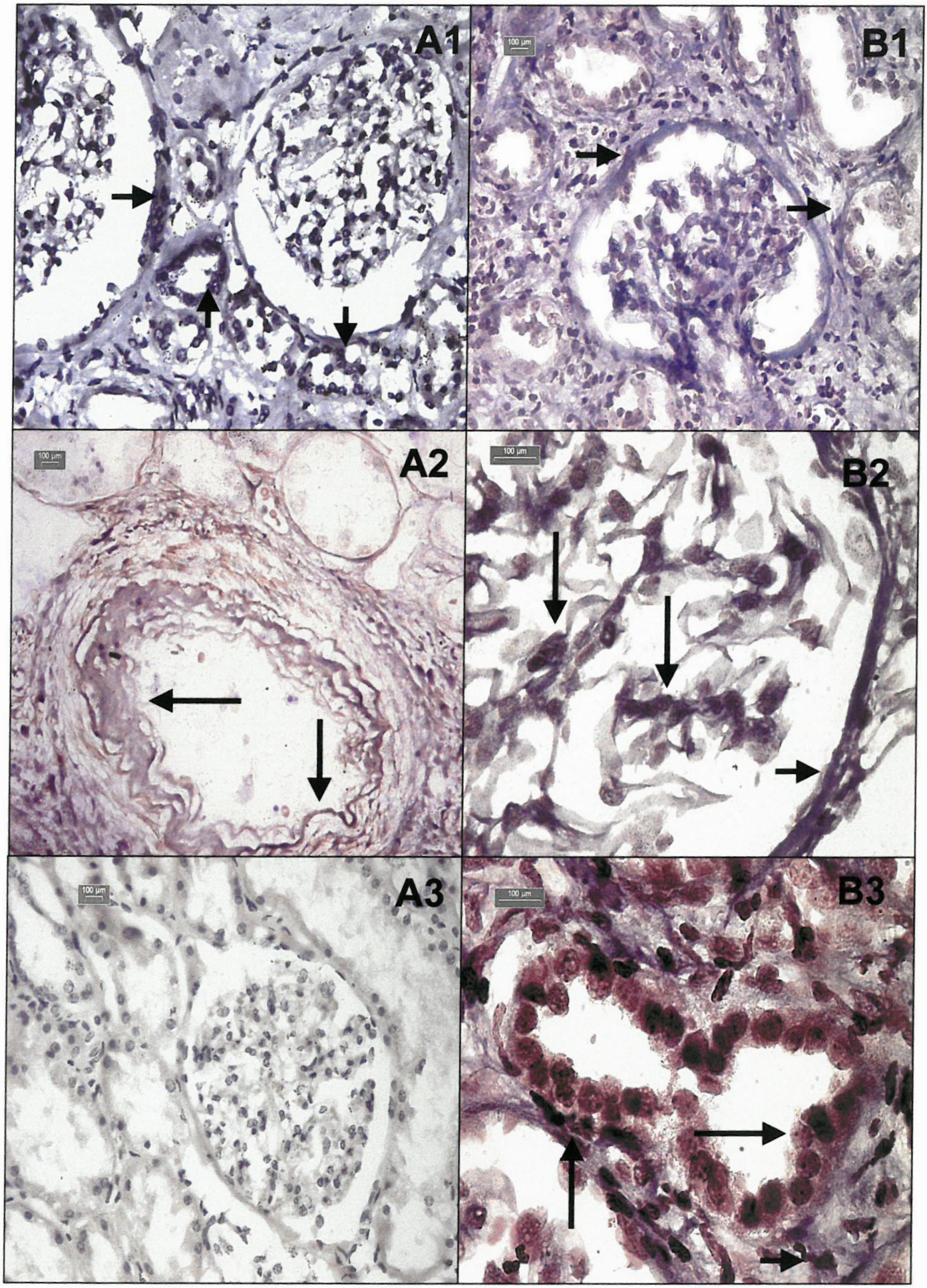
Primer	Size (bases)	a. Primer Sequence		PCR	PCR Product (bp)
ET-1-268F	21	5'-ATGGATTATTTGCTCATGATTTT-3'		✔	
ET-1-683R	21	5'-CAGTCTTTCTCCATAATGTCTTCAGC-3'		✔	415
ET-1-849R	17	5'-CTTGGGATCATGAAAAGATGATTT-3'	✔		

F : Forward Primer; R : Reverse Primer

Specific reverse primers for reverse transcriptase (RT) reactions, and forward & reverse primer sets for non-nested polymerase chain reactions (PCR) used in the *in situ* RT-PCR detection of mRNAs for ET-1. For the RT-PCR, a predicted length of cDNA product is expected.

Microscopy

Renal biopsies from ten patients with acute rejection were studied. Label was detected in epithelial cells of the distal tubule and collecting duct in control kidney. Increased label was observed in kidney biopsies with acute rejection; in addition, label was observed in endothelial cells of arterioles and glomerular capillaries as well as the epithelial cells of Bowman's capsule, as illustrated by the images in Figures 3.11.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100 µm
A3
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B3
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ATRIAL NATRIURETIC PEPTIDE

Electrolyte and volume status is altered in patients with renal disease. Changes in atrial natriuretic peptide (ANP) would be anticipated in renal disorders.

3.4.1 Plasma ANP

Plasma ANP was measured by radio-immunoassay (RIA) in 16 patients with acute rejection and 14 control subjects. In acute rejection, the mean plasma value for ANP was 47.36 pg/ml (SEM ± 5.26 pg/ml), median of 48.8 pg/ml and range of 13.0-80.0 pg/ml. In control subjects, the mean value was 23.93 pg/ml, SEM ± 2.78 pg/ml, median of 20.65 pg/ml, range of 11.7-42.5 pg/ml (Table 3.4.1). Plasma ANP was significantly increased during acute rejection when compared to control subjects (p=0.0011, Mann-Whitney test). There was no correlation between plasma ANP and serum creatinine levels (p=0.33, linear regression; p=0.69, Spearman rank correlation). Similarly, there was no correlation between plasma ANP and the degree of peripheral oedema.

Table 3.4.1 ANP RIA results

	PLASMA ANP (pg/ml)	
	Control	**Acute Rejection**
n (patient numbers)	**14**	**16**
Mean ± SEM (pg/ml)	**23.93 ± 2.78**	**47.36 ± 5.26**
SD	**10.41**	**21.06**
Median	**20.65**	**48.8**
Range	**11.7 – 42.5**	**13.0 – 80.0**
p (Mann-Whitney test)	**0.0011**	

3.4.2 Urinary ANP results

Urine ANP was measured in 13 patients with acute rejection and 4 control subjects. The mean ± SEM urinary ANP value was 29.68 ± 5.01 pg/ml, median of 25.0 pg/ml and range of 4.5-73.6 pg/ml in acute rejection; control values were 2.65 ± 0.14 pg/ml, 2.65 pg/ml and 2.3-3.0 pg/ml respectively.

Table 3.4.2 Urine ANP RIA results

	URINARY ANP (pg/ml)	
	Control	**Acute Rejection**
n (patient numbers)	**4**	**13**
Mean ± SEM (pg/ml)	**2.65 ± 0.14**	**29.68 ± 5.01**
SD	**0.29**	**18.07**
Median	**2.65**	**25.0**
Range	**2.3 – 3.0**	**4.5 – 73.6**
p (Mann-Whitney test)	**0.0008**	

Urinary ANP was significantly increased during acute rejection when compared to controls (p=0.0008, Mann-Whitney test).

3.4.3 RENAL CELLULAR LOCALISATION OF ANP: LIGHT MICROSCOPY: IMMUNOPEROXIDASE (PAP) METHOD

ANP immunolabelling by the PAP method was visualised predominantly in distal tubules and collecting ducts in acute rejection and glomerulonephritis as well as in control kidney

tissue. In addition, labelling was observed in epithelial cells of Bowman's capsule, and endothelium and smooth muscle cells of the media of arteries and arterioles.

3.4.3.1 Analysis of ANP labelling in acute rejection

There was significantly decreased immunolabelling of glomeruli in biopsies of acute rejection (p= 0.002, Kruskal-Wallis test) compared to control kidney tissue; the labelling was more significantly decreased in mild rejection. The images are depicted in Figures 3.12.

Table 3.4.3.1 ANP image analysis in acute rejection

	GLOMERULI			
	(i) Control	**(ii) Rejection**	**(iii)Mild Rejection**	**(iv)Severe Rejection**
n	**6**	**6**	**4**	**3**
Patients	**6**	**6**	**4**	**3**
Mean ± SEM	**124.12 ± 1.93**	**104.96 ± 4.94**	**98.47 ± 4.02**	**114.9 ± 3.85**
SD	**4.72**	**12.11**	**8.04**	**6.66**
Median	**125.91**	**104.64**	**96.53**	**114.59**
Range	**118.23 – 128.24**	**92.01 –121.71**	**92.01 – 108.8**	**108.4 – 121.71**
p(Mann Whitney)	**(i)vs(ii):0.002**; (i)vs(iii):0.0095**		**(i)vs(iv):0.095; (iii)vs(iv) 0.11**	

n= number of sections ; **Kruskal-Wallis test) ; measurements:pixels per micron^2

Immunolabelling of tubules in rejection was similar to control kidney tissue. ANP immunolabelling of arteries was similar to control kidney in acute rejection. There was significantly decreased immunolabelling of collecting ducts in acute rejection (p=0.0087, Mann-Whitney test) compared to controls.

Table 3.4.3.1 ANP image analysis in acute rejection

	ARTERIES	
	Control	Rejection
n	6	6
Mean ± SEM	100.47 ± 5.49	104.36 ± 3.23
SD	13.44	7.91
Median	105.08	103.16
Range	77.41 – 113.51	96.57 – 117.59
p(Mann Whitney)	0.4	

n= number of sections (one per patient); measurements: pixels per micron^2

Table 3.4.3.1 ANP image analysis in acute rejection

	DISTAL TUBULES	
	Control	Rejection
n	6	7
Patients	6	7
Mean ± SEM	116.49 ± 5.52	107.99 ± 3.40
SD	13.52	8.99
Median	110.79	110.65
Range	106.99–142.03	95.04 –120.5
p	>0.05 (Mann Whitney)	

n= number of sections; measurements: pixels per micron^2

Table 3.4.3.1 ANP image analysis in acute rejection

	COLLECTING DUCTS			
	(i) Control	(ii)Rejection	(iii(Mild Rejection	(iv)Severe Rejection
n	6	6	4	2
Patients	6	6	4	2
Mean± SEM	136.84± 2.36	113.48± 5.75	102.5 ± 4.46	82.38 ± 22.44
SD	5.78	14.08	8.91	31.73
Median	138.88	110.18	102.8	82.38
Range	126.48–42.19	95.76–134.23	93.87-110.47	59.94-104.81
p(Mann-Whitney)	(i)vs(ii):0.0087 (iii)vs(iv):0.53		(i)vs (iii) 0.0095 (i) vs (iv) <0.01**	

n= number of sections; ** Tukey-Kramer; measurements: pixels per micron^2

3.4.3.2 Analysis of ANP labelling in glomerulonephritis

There was significantly decreased immunolabelling of glomeruli in glomerulonephritis (p= 0.002, Kruskal-Wallis test) compared to control kidney tissue.

Table 3.4.3.2 ANP image analysis of glomeruli in renal disease

	GLOMERULI			
	(i) Control	(ii) GN	iii)Non-prolif GN	(iv)Prolif GN
N	6	6	2	3
Mean ± SEM	124.12 ± 1.93	108.03 ± 1.64	104.73 ± 0.52	109.98± 2.78
Median	125.91	107.24	104.73	108.04
Range	118.23–128.24	104.21–15.47	104.21-105.25	106.44-115-47
p(Mann Whitney)	(i)vs(ii):0.002*; (i)vs(iii):0.07		(iii)vs(iv):0.2; (i)vs (iv):0.02	

n= number of sections and number of patients; *Kruskal-Wallis; measurements: pixels per micron^2

Table 3.4.3.2 ANP image analysis of arteries in renal disease

	ARTERIES	
	Control	GN
n	6	6
Patients	6	6
Mean ± SEM	100.47 ± 5.49	106.9 ± 5.96
Median	105.08	104.1
Range	77.41– 117.59	93.87 – 133.98
p	>0.05 (Mann Whitney)	

n= number of sections; measurements: pixels per micron^2

Table 3.4.3.2 ANP image analysis in renal disease

	DISTAL TUBULES			
	(i) Control	(ii) GN	(iii)Non-prolif GN	(iv)Prolif GN
N	6	6	2	3
Patients	6	6	2	3
Mean± SEM	116.49 ± 5.52	110.42 ± 5.27	126.35 ± 4.78	101.92 ± 1.97
Median	110.79	104.82	126.35	101.34
Range	106.99– 142.03	98.84 – 131.13	121.58 - 131.13	98.84 - 105.59
p*	(i)vs(ii): >0.05; (i)vs(iii) 0.29		(i)vs(iv) 0.024; (iii)vs(iv) : 0.2	

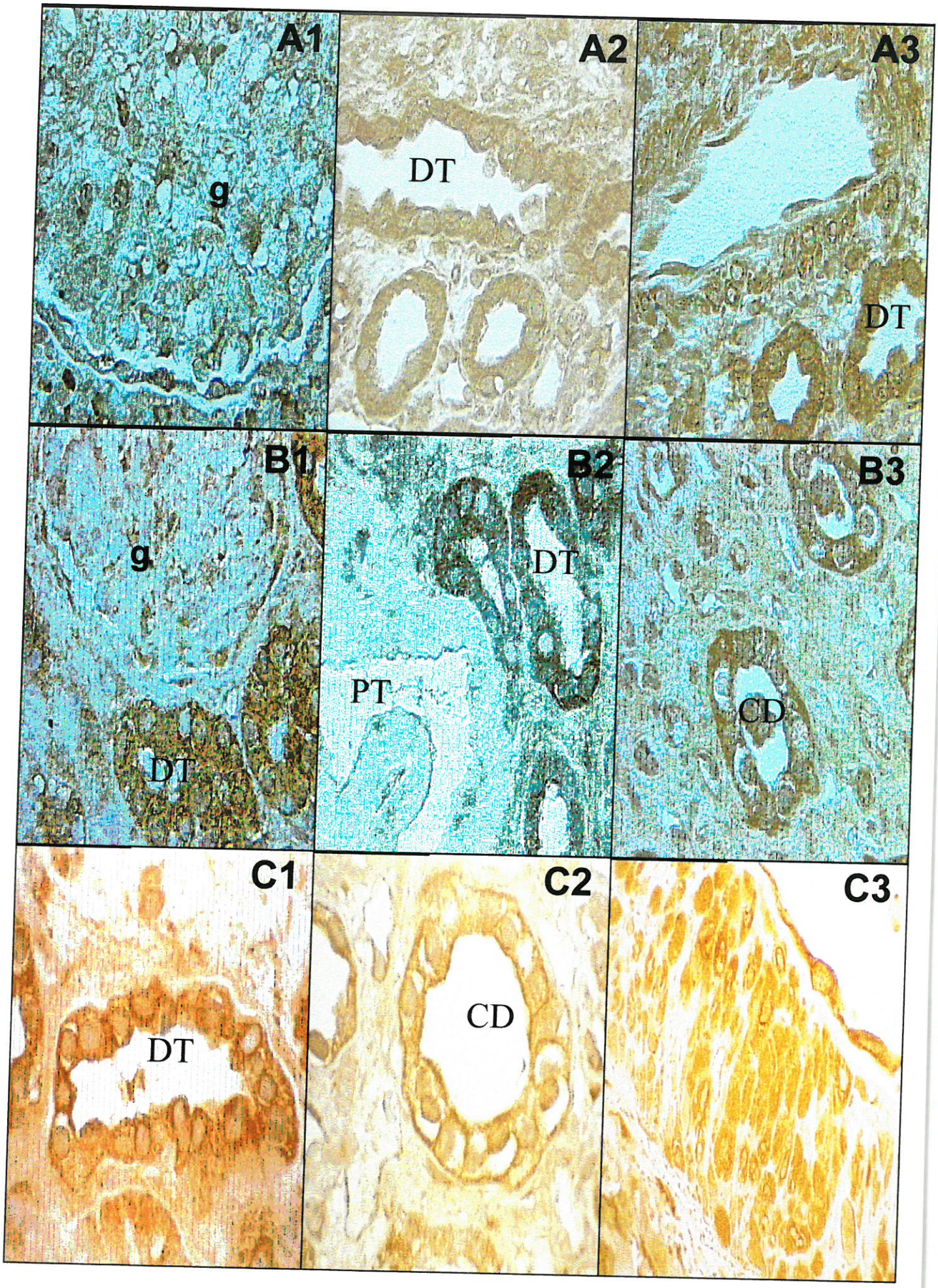
* Mann Whitney test; n= number of sections; measurements: pixels per micron^2

Table 3.4.3.2 ANP image analysis in renal disease

	COLLECTING DUCTS			
	(i) Control	(ii) GN	(iii)Non-prolif GN	(iv)Prolif GN
n	6	8	5	3
Patients	6	8	5	3
Mean± SEM	136.84 ± 2.36	98.6 ± 8.07	123.88 ± 6.14	91.84 ± 2.66
Median	138.88	94.5	125.71	94.25
Range	126.48– 142.19	59.94 – 138.12	103.87-138.12	86.52-94.74
p*	(i) vs (ii):0.0027;(i)vs (iii) 0.095		(i) vs (iv) 0.0238; (iii) vs (iv) 0.0357	

*Mann-Whitney test; n= number of sections; measurements: pixels per micron^2

Immunolabelling of tubules was reduced in proliferative GN (p=0.024, Mann-Whitney test), compared to control kidney tissue. Labelling of collecting ducts was reduced in glomerulonephritis compared to control kidney tissue (p= 0.0027); labelling was reduced in proliferative glomerulonephritis compared to control kidney tissue (p=0.0238) and non-proliferative glomerulonephritis (p=0.0357). The images are depicted in Figures 3.12.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DT
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URINARY TISSUE KALLIKREIN

The kallikrein-kinin system is known to be involved in inflammation and renal disease. This study measures tissue kallikrein as well as basal (endogenous) and generated kinin in urine in patients with acute renal allograft rejection and various renal disorders, the first time that such a study has been conducted. Kallikrein and kinins are produced in the kidney in renal disease; thus their assay in urine would reflect their status in renal disorders.

3.5.1 PATIENT DEMOGRAPHICS

Acute Rejection Group

Twenty renal transplant patients with acute rejection were studied: there were 18 males and 2 females; of these, 13 had received living related donor kidneys, 3 had received kidneys from living unrelated donors and 4 from cadaver donors. Their mean age was 35.8 years (range 16-52 years). Mild rejection was present in 5 patients, moderate rejection in 10 patients and severe rejection in 5 patients (Appendix 3.3.1.1).

Renal Disorders

Twenty nine patients with various renal disorders were studied: 20 were males and 9 females, with a mean age of 34 years (range 13-60 years). Six patients had mesangiocapillary glomerulonephritis (MCGN), 3 had class IV lupus nephritis (SLE), 1 membranous nephropathy (MGN), 3 minimal change disease with nephrotic syndrome (MCNS), 3 immunoglobulin A (IgA) nephritis, 3 hypertensive nephrosclerosis (HPT neph), 10 with miscellaneous causes (reflux nephropathy, obstructive uropathy, end stage diabetic nephropathy); 14 patients were on dialysis at the time of the study (Appendix 3.3.1.2}.

Control Group

Twenty subjects formed a control group; these were kidney donors who were studied prior to uni-nephrectomy. There were 8 males and 12 females with a mean age of 34.8 years (range 22-48 years). Their details are presented in Appendix 3.3.1.3. Further controls were a group of 24 healthy volunteers (8 males and 16 females), mean age 45.9 years, (range 27-64 years); 16 kidney donors (12 females and 4 males), mean age 34.5 years (range 25-52 years) following nephrectomy who served as single-kidney controls; and 20 stable kidney transplant recipients, 12 males and 8 females, mean age 35.7 years (range 17-58 years) who served as immunosuppressive controls.

3.5.2 MEASUREMENT OF TISSUE KALLIKREIN

3.5.2.1 ENZYMIC ACTIVITY

Tissue kallikrein (TK) was measured in the urine of 20 kidney donors pre-operatively and 16 donors at 7 months to 6 years post-nephrectomy by an amidase assay; these served as 2 and single kidney controls respectively; 20 stable transplant recipients served as an immunosuppressive control group. Twenty transplant recipients with acute rejection and 29 patients with glomerulonephritis were studied. Annexure 3.5.1 outlines the details.

3.5.2.1.1 Control subjects

Kidney donors

Kidney donors (prior to nephrectomy) had a mean ± SEM urinary TK excretion of 50.19 ± 20.39 ng/ug protein (range 0.02-314 ng/ug protein), with a median value of 8.09 ng/ug protein. The results are summarized in Table 3.5.1

Kidney donors (post nephrectomy)

Following uni-nephrectomy, mean ± SEM urinary TK excretion measured 2.13 ± 0.42 ng/ug protein (range 0 – 5.59 ng/ug protein), with a median of 1.47 ng/ug protein. The difference between these donor groups before and after nephrectomy was significant, with a two-tailed p value of 0.017 (Mann-Whitney test).

Stable kidney transplant recipients

Urinary TK excretion in stable transplant recipients measured a mean ± SEM of 2.07 ± 0.4 ng/ug protein (range 0 – 6.94 ng/ug protein) and median of 2.03 ng/ug protein. Urinary TK excretion was similar in stable transplant recipients and donors post-uninephrectomy (p=0.799; Mann-Whitney test) but was very significantly decreased when compared to donors pre-nephrectomy (p=0.0069; Mann-Whitney test).

Table 3.5.1 Urinary tissue kallikrein enzymic activity

	(i) Donors Pre-op	(ii) Donors Post-op	(iii) Stable Transplants
n	**20**	**16**	**20**
Mean ± SEM	**50.19 ± 20.39**	**2.13 ± 0.42**	**2.07 ± 0.4**
SD	**91.16**	**1.69**	**1.77**
Range	**0.02 – 314.0**	**0.0 – 5.59**	**0.0 – 6.94**
Median	**8.09**	**1.47**	**2.03**
p (Mann-Whitney test)	**(i) vs (ii): 0.017; (i)vs(iii) : 0.0069; (ii)vs(iii): 0.799**		

n= patient numbers; measurements: ng/ug protein

3.5.1.1.2 Acute rejection

Mean ± SEM urinary TK excretion measured 1.35 ± 0.37 ng/ug protein during acute rejection, with a median of 0.63 ng/ug protein and range 0 – 5.67 ng/ug protein. TK was very significantly decreased during rejection when compared to donors pre-nephrectomy (p=0.001). While urinary TK excretion was decreased during acute rejection, it was not significant when comparing the rejection group with donors post-uninephrectomy (p=0.0672) and stable renal transplant recipients (p=0.133; Mann-Whitney test).

Table 3.5.2.1 Urinary tissue kallikrein enzymic activity

	(i)Donors Pre-op	(ii) Donors Post-op	(iii) Stable Transplants	(iv) Acute Rejection
n	**20**	**16**	**20**	**20**
Mean ± SEM	**50.19 ± 20.39**	**2.13 ± 0.42**	**2.07 ± 0.4**	**1.35 ± 0.37**
SD	**91.16**	**1.69**	**1.77**	**1.65**
Range	**0.02 – 314.0**	**0.0 – 5.59**	**0.0 – 6.94**	**0.0 – 5.67**
Median	**8.09**	**1.47**	**2.03**	**0.63**
p (Mann-Whitney test)	**(i)vs(iv):0.001**		**(iii) vs (iv) : 0.133**	
	(ii) vs (iv) : 0.0672; (ii) vs (iii) : 0.799			

n= patient numbers; measurements: ng/ug protein

3.5.2.1.3 Serial urinary TK enzymic measurements following transplantation

While TK excretion increased after renal transplantation, decreased during acute rejection and increased again after treatment of rejection, there was no significant difference in serial urinary TK excretion pre- and post- transplant.

Table 3.5.2.1.3 Serial urinary TK enzymic activity following renal transplantation

	(i) Pre-TP	(ii) Post TP (D3-4)	(iii) Acute Rejection	(iv) After R_x of Rejection
n (patients)	12	12	17	16
Mean ± SEM	1.99 ± 0.69	3.41 ± 0.85	1.22 ± 0.40	3.05 ± 1.57
SD	2.37	2.93	1.63	6.26
Range	0.01 – 8.36	0.008 – 7.55	0.38 – 2.06	0.0 – 24.31
Median	1.30	2.88	0.45	0.87
p(Dunn's multiple comparisons)	(i) vs (ii) : > 0.05		(iii) vs (iv) : > 0.05	
	(i) vs (iii) : > 0.05; (i) vs (iv) : > 0.05			

measurements: ng/ug protein

3.5.2.4 Renal disease

Urinary TK excretion in this group measured a mean ± SEM of 7.37 ± 2.42 ng/ug protein, with median of 3.0 ng/ug protein and range 0.01–48.02 ng/ug protein. TK excretion was markedly decreased in renal disease compared to controls [(donors prior to nephrectomy), p=0.0158; unpaired t test]. Urinary TK excretion was significantly decreased during acute rejection when compared to renal disease (p=0.0068; Mann-Whitney test).

Table 3.5.2 Urinary tissue kallikrein enzymic activity

	(i)Controls (Donors Pre-op)	(ii) Acute Rejection	(iii) Renal Disease
n (patients)	20	20	29
Mean ± SEM	50.19 ± 20.39	1.35 ± 0.37	7.37 ± 2.42
SD	91.16	1.65	13.02
Range	0.02 – 314.0	0.0 – 5.67	0.01 – 48.02
Median	8.09	0.63	3.0
p(Mann-Whitney test)	(i) vs (iii) : 0.0158; (ii) vs (iii) : 0.0068		

measurements: ng/ug protein

3.5.2.5 Correlation between enzymic urinary TK activity and serum creatinine

There was no correlation between enzymic TK activity and serum creatinine by linear regression during acute rejection (p=0.874) and with renal disease (p=0.8566)

3.5.3 URINARY TISSUE KALLIKREIN ELISA ASSAY

Four groups served as controls: kidney transplant donors pre-and post-nephrectomy, volunteer subjects and stable renal transplant recipients. Immunoreactive tissue kallikrein was measured by ELISA in these control groups, serially following renal transplantation, during acute rejection and in patients with renal disease. Results are depicted in Table 3.5.3

3.5.3.1 Control subjects

Donors pre-nephrectomy

The mean ± SEM value for TK in 19 donors pre-nephrectomy measured 46.88 ± 9.4 ng/ml, median of 42.0 ng/ml, range of 0 – 165.4 ng/ml.

Donors after uni-nephrectomy

Sixteen donors studied post-nephrectomy had TK measurements of mean ± SEM of 18.48 ± 8.3 ng/ml, median of 8.1 ng/ml and range of 0 – 128.24 ng/ml. Tissue kallikrein excretion was significantly decreased in donors post- nephrectomy compared to pre - nephrectomy (p=0.0328; unpaired t test).

Combined group (normal volunteers and donors pre-nephrectomy)

Normal volunteer subjects (n=24) and kidney donors pre-nephrectomy (n=19) were combined for statistical analysis and the combined mean ± SEM was 61.14 ± 6.14 ng/ml, median of 66.25 ng/ml and range of 0-165.39 ng/ml.

Stable renal transplant recipients

In 21 stable transplant recipients, TK values were mean ± SEM of 47.54 ± 10.64 ng/ml, median of 33.2 ng/ml and range of 0 – 129.39 ng/ ml. The difference between the stable transplant recipients and combined controls was not significant (p=0.24; unpaired t test); however, TK was significantly increased in stable transplant recipients compared to donors post-nephrectomy (p=0.0482; unpaired t test).

Table 3.5.3.1 Urinary Tissue Kallikrein ELISA measurements

	(i) Donors Pre-op	(ii) Donors Post-op	(iii)Donors preop+volunteers	(iv) Stable transplants
n	**19**	**16**	**43**	**21**
Mean ± SEM	**46.88 ± 9.37**	**18.48 ± 8.30**	**61.14 ± 6.14**	**47.54 ± 10.64**
SD	**40.83**	**33.21**	**40.25**	**48.74**
Range	**0.0 – 165.39**	**0.0 – 128.24**	**0.0 – 165.39**	**0.0 – 129.39**
Median	**42.0**	**8.1**	**66.25**	**33.2**
p (unpaired t-test)	**(i) vs (ii) : 0.0328**		**(iii) vs (iv) : 0.24**	
	(ii) vs (iv) : 0.0482			

n= patient numbers; measurements: ng/ml

3.5.3.2 Acute rejection

Urinary TK excretion during 20 episodes of acute rejection was a mean ± SEM of 48.50 ± 11.22 ng/ml, median of 42.5 ng/ml and range of 0 – 157.14 ng/ml. Urinary TK ELISA was significantly increased during acute rejection compared to donors post-nephrectomy (p=0.0474; unpaired t test); however there was no difference when compared to the stable transplant group (p=0.95).

Table 3.5.3.2 Urinary Tissue Kallikrein ELISA measurements

	(i) Donors Pre-op	(ii) Donors Post-op	(iii)Donors +volunteers	(iv) Stable transplants	(v) Acute rejection
N	**19**	**16**	**43**	**21**	**20**
Mean± SEM	**46.88± 9.37**	**18.48 ± 8.30**	**61.14± 6.14**	**47.54± 10.64**	**48.5 ± 11.22**
SD	**40.83**	**33.21**	**40.25**	**48.74**	**50.18**
Range	**0.0– 165.39**	**0.0 – 128.24**	**0.0– 165.39**	**0.0 – 129.39**	**0.0 – 157.14**
Median	**42.0**	**8.1**	**66.25**	**33.2**	**42.5**
p(unpaired t-test)	**(ii) vs (v) : 0.0474; (iii) vs (iv) : 0.24; (iv) vs (v) : 0.95**				

n= patient numbers; measurements: ng/ml

3.5.3.3 Serial urinary tissue kallikrein measurements following renal transplantation

The mean, median and ranges for urinary TK are shown in Table 3.5.3.3. There was no statistical difference in TK excretion during the different phases at any period post-transplantation in this group.

Table 3.5.3.3 Serial Tissue Kallikrein ELISA following renal transplantation

	(i) Pre-TP	(ii) Post-TP (D3-4)	(iii) During acute Rej	(iv) After Rx of Rejection
n(patient numbers	11	13	17	17
Mean ± SEM	30.2 ± 16.42	36.20± 13.92	40.57 ± 11.79	40.91 ± 7.45
SD	54.45	50.19	48.60	30.72
Range	0.0 – 165.36	0.0 – 132.36	0.0 – 157.14	0.0 – 81.0
Median	0.0	8.0	35.0	46.0
p (unpaired t-test)	(i) vs (ii) : 0.78		(iii) vs (iv): 0.98	
	(i) vs (iv) : 0.51; (ii) vs (iii) : 0.81; (i) vs (iii) : 0.60			

measurements: ng/ml

3.5.3.4 Renal disease

Urinary TK excretion in 13 patients with renal disease measured a mean ± SEM of 31.47 ± 14.56 ng/ml, median of 0 and range of 0 – 165.39 ng/ml (Table 3.5.3.4). TK was significantly decreased in this group compared to the combined 2-kidney control group (p=0.0347; unpaired t test).

Table 3.5.3.4 Urinary tissue kallikrein ELISA measurements

	(i)Donors and Volunteers	(ii) Acute Rejection	(iii) Renal disease
n=numbers	43	20	13
Mean ± SEM	61.14 ± 6.14	48.5 ± 11.22	31.47 ± 14.55
SD	40.25	50.18	52.45
Range	0.0 – 165.39	0.0 – 157.14	0.0 – 165.39
Median	66.25	42.5	0.0
p (unpaired t-test)	(i) vs (iii) : 0.0347; (ii) vs (iii) : 0.3566		

measurements: ng/ml

3.5.3.5 Correlation between urinary tissue kallikrein and serum creatinine

There was no correlation between TK ELISA and serum creatinine by linear regression during acute rejection (p=0.7021) and with renal disease (p=0.2556).

3.5.4 KININS

Basal urinary kinin excretion was measured in 32 normal subjects (kidney donors and normal volunteers), 15 kidney donors post-nephrectomy, 22 stable transplant recipients, 20 kidney transplants with acute rejection and 19 patients with glomerulonephritis, followed by measurement of generated kinin in their urine. Table 3.5.4 summarizes their measurements. Basal kinin excretion in the urine was decreased in kidney donors post-nephrectomy when compared to the normal subjects ($p < 0.001$).

Table 3.5.4 Basal urinary kinin measurements

	(i)Normal controls	(ii)Donors post-op	(iii)Stable TP	(iv)Ac rejection	(v) GN
n=numbers	32	15	22	20	19
Mean ± SEM	3.88 ± 0.62	0.02 ± 0.41	0.44 ± 0.11	15.45 ± 6.86	27.39± 23.22
SD	3.50	1.60	0.52	30.67	101.19
Median	3.18	1.40	0.27	5.45	2.80
Range	0.26 – 18.41	0.40 – 6.40	0.19 – 2.60	0.20 – 137.5	0.20 – 444.8
p (Dunn's multiple comparisons)	(i) vs (ii) : < 0.001;		(iii) vs (iv) : <0.001		
	(i) vs (iii) :< 0.001; (ii) vs (iii) :< 0.05; (i) vs (v) :> 0.05				

measurements: ng/ml

Basal kinin excretion was significantly decreased in stable transplant recipients compared to normal subjects ($p<0.001$), and increased when compared to donors post nephrectomy ($p<0.05$; Dunn's multiple comparisons). Basal urinary kinin excretion was significantly increased during acute rejection when compared to the stable transplant recipients ($p=<0.001$). While basal kinin excretion was increased in glomerulonephritis when compared to normal controls, this did not reach statistical significance ($p>0.05$). There was no correlation between serum creatinine and basal kinin by linear regression during acute rejection ($p=0.63$) and renal disease ($p=0.59$).

Urinary kinin generation was decreased in kidney donors post nephrectomy compared to normal control subjects ($p=<0.001$). Significantly decreased kinin was generated in the urine during acute rejection ($p=0.001$) and glomerulonephritis ($p<0.01$; Dunn's multiple comparisons test), compared to normal controls. There was no correlation between serum creatinine and generated kinin during acute rejection ($p=0.56$) and renal disease ($p=0.43$).

Table 3.5.4 1 Generated urinary kinin measurements

	(i)Normal controls	(ii)Donors post-op	(iii)Stable TPs	(iv)Acute Rejection	(v) GN
n=numbers	**33**	**15**	**22**	**20**	**19**
Mean ± SEM	**88.57 ± 10.64**	**18.67 ± 6.45**	**46.6 ± 4.05**	**25.35 ± 5.91**	**40.19± 9.63**
SD	**61.11**	**24.99**	**18.98**	**26.43**	**41.97**
Median	**71.72**	**0.0**	**41.65**	**24.20**	**29.4**
Range	**20.25 – 270.8**	**0.0 – 70.5**	**19.0 – 92.3**	**12.98 – 37.72**	**0.0-138**
p*	**(i) vs (ii) : < 0.001; (i) vs (iv) : 0.001; (i) vs (v) : < 0.01**				

*Dunn's multiple comparisons; measurements: ng/ml

3.5.5 CONFOCAL MICROSCOPY: IMMUNOFLUORESCENT TK LABELLING

Confocal images of anti-TK antibody immunolabelling in kidney biopsies of acute rejection, glomerulonephritis and control kidney tissue were subjected to image analysis.

3.5.5.1 TK

TK was immuno-visualised maximally in the connecting tubules and collecting ducts of control kidney tissue (Figure 3.13).

3.5.5.1.1 Acute rejection

There was decreased TK immunolabelling in the distal tubules during rejection, significantly so in severe rejection when compared to control kidney (p=0.0357; Mann-Whitney test). The data is shown in Table 3.5.5.1.1

Table 3.5.5.1.1 TK immunofluorescence: Distal tubule

	(i) Control	(ii)Mild Rejection	(iii)Severe Rejection
n(structures)	15	14	5
Patients	3	4	5
Mean ± SEM	162 ± 30.3	57.8 ± 32.1	44.6 ± 15.3
Median	154	35	30
Range	114-218	9-152	12-87
SD	52.4	64.1	34.1
p*	(i) vs (ii): 0.11; (i) vs (iii): 0.0357		

*Mann- Whitney; measurements: pixelsx100/micron^2

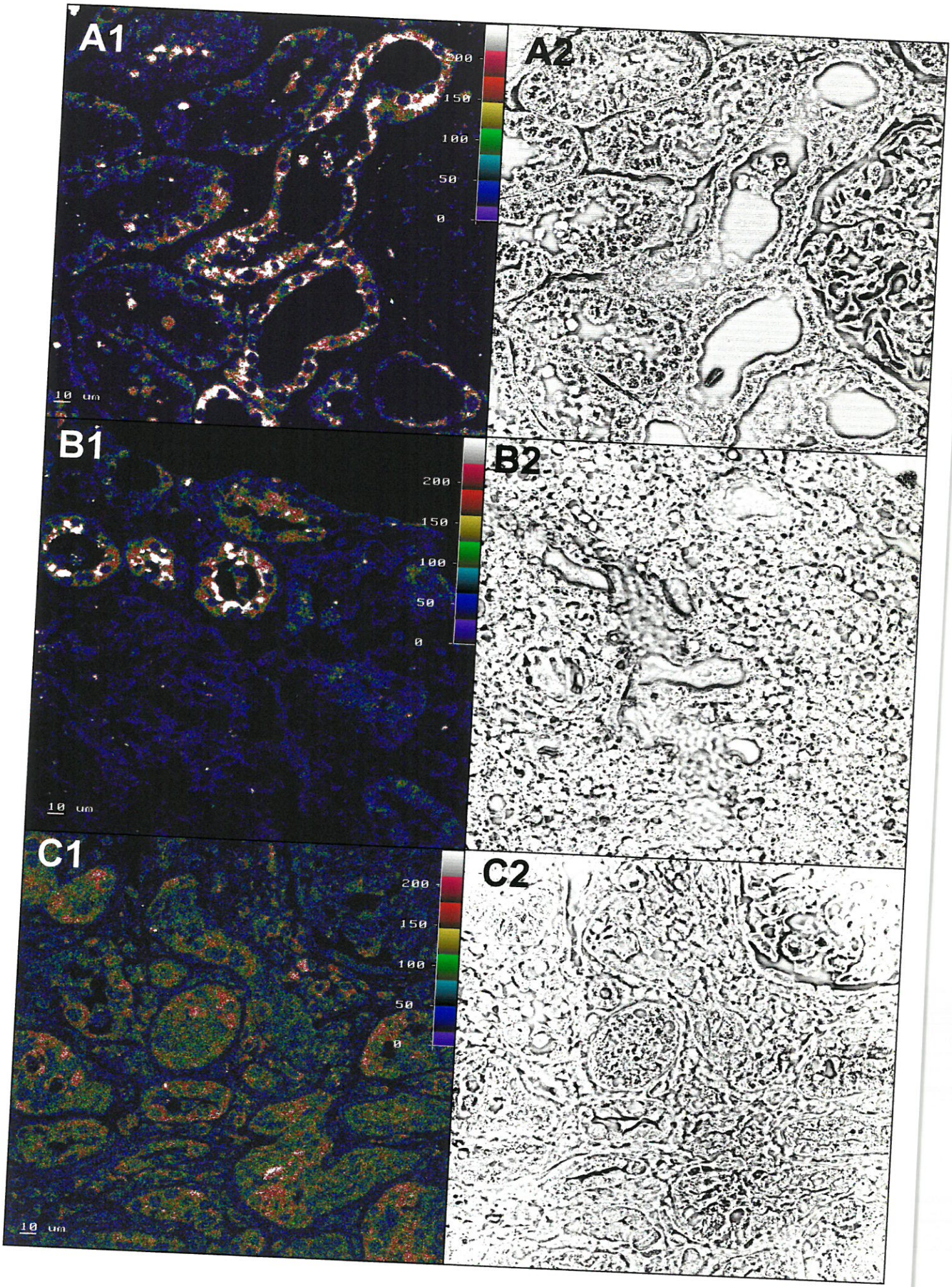
3.5.5.1.2 Glomerulonephritis

There was decreased immunolabelling of distal tubules in both non-proliferative and proliferative disorders; labelling was significantly reduced in non-proliferative GN ($p=0.0238$; Mann-Whitney test) when compared to control kidney.

Table 3.5.5.1.2 TK immunofluorescence: Distal tubule

	(i) Control	(ii)Non Proliferative GN	(iii)Proliferative GN
n(structures)	15	7	3
Patients	3	6	3
Mean± SEM	162.0 ± 30.3	23 ± 13.5	40.6 ±29.2
Median	154	10.1	16.5
Range	114-218	2-89.5	6.5-98.7
SD	52.5	33.1	50.6
p*	(i) vs (ii) :0.0238; (i) vs (iii) :0.10		

*Mann-Whitney test; measurements: pixelsx100/micron^2


A1
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APPENDIX B

Appendix B 3.1 Demographics of patients from whom kidney tissue samples were obtained at autopsy

AGE	RACE	SEX	CAUSE OF DEATH	TIME BETWEEN AUTOPSY AND DEATH
21 y	African	Male	Stab chest	16 h
25 y	African	Male	Stab chest	15 h
25 y	African	Male	Stab chest	16 h
30 y	African	Male	Gun shot chest	16 h
40 y	Indian	Male	Acute myocardial infarction	23 h
24 y	Caucasian	Male	Acute carbon monoxide poisoning	22h
46 y	Indian	Male	Hanging	6 h
31 y	Indian	Male	Hanging	6 h

Appendix B 3.2.1: Patient Profile: ET-1, TK and kinins in Acute Rejection

Pt	Age	Sex	Race	Type of TP	Days from TP	Serum Creat	BP	Treatment	Ac Rej	Renal Histology	ET-1 (pg/ml) Plasma	ET-1 (pg/ml) Urine	TK Amidase*	TK ELISA	KININ (ng/ml) Basal	KININ (ng/ml) Gen
S1	51	M	I	LRD	6	326	H	CAPD/CCB/Triple Rx	Severe	$G_1T_3I_3V_1$	1.56	1.8	0.1	70	137.5	0
S3	42	M	I	LRD	800	173	H	HD/CCB/ACEI/Triple Rx	Mod	$G_0T_2I_2V_0$	1.86	1.23	0	64	5.7	19.6
S5	35	M	I	LRD	7	278	H	CAPD/CCB/BB/VD/Triple Rx	Mild	$G_0T_1I_1V_0$	0.87	1.32	5.67	60	12.5	0.6
S10	51	M	I	LRD	44	437	H	CCB/BB/Triple Rx	Severe	$G_0T_3I_3V_0$	1.02	4.7	0.06	140.6	11.4	0.4
S11	43	M	I	LRD	518	183	H	HD/ACEI/Triple Rx	Mild	$G_0T_1I_1V_0$	0.93	0.51	0.2	3	2.9	36.6
S18	20	M	W	LRD	10	169	H	HD/VD/Triple Rx	Mod	$G_1T_2I_2V_0$	1.5		1.51	0	0.2	0
S28	16	M	I	LRD	9	666	H	CAPD/CCB/BB/Triple Rx	Mod	$G_0T_2T_2V_0$	5.25	1.74	0.2	73		
S36	22	M	B	LRD	12	146	H	HD/CCB/Triple Rx	Mild	$G_0T_2I_2V_0$	2.3	3.63				
S37	43	M	I	C	15	175	H	HD/Triple Rx	Severe	$G_0T_3I_3V_0$	2.39	0.07	0.29	3.7	2.9	51.6
S38	23	F	I	LUD	240	219	N	CAPD/Triple Rx	Severe	$G_0T_3I_3V_0$	0.63	4.65	3.19	15	1.3	81.6
S39	33	M	I	LRD	8	254	H	HD/Triple Rx/OKT3/CCB	Severe	$G_0T_2I_3V_0$	1.71		0.45	157.4	2.3	67.5
S40	42	M	I	C	3	923	H	CAPD/Triple Rx/CCB	Mod	$G_0T_2I_3V_0$	1.98					
S41	52	M	I	LRD	9	829	H	CAPD/CCB/VD/Triple Rx	Mod	$G_0T_2I_3V_0$	2.42	0.45	0.92	49		
S42	35	M	I	LRD	7	247	H	CAPD/VD/Triple Rx	Mod	$G_0T_2I_2V_0$	1.98	0.6	4	0	5.2	46
S43	45	M	I	C	150	193	H	Triple Rx/CCB	Mod	$G_0T_2I_2V_0$	1.5	1.3	0.31	16	0.2	40.7
S44	30	M	W	LUD	75	304	H	CAPD/CCB/Triple Rx /Zocor	Mod	$G_0T_2I_3V_0$	0.68		2	0		
S45	37	M	B	LRD	8	145	H	HD/CCB/VD/Triple Rx	Mild	$G_0T_1I_1V_0$	1.55	0.7	0.8	35	45.2	0.6
S48	23	M	I	LRD	6	194	H	CAPD/ACEI/CCB/VD Triple Rx	Mod	$G_0T_2I_3V_0$	0.83	2.93	1	55	11.8	0
S49	38	M	M	LRD	7	700	H	HD/CCB/Triple Rx	Severe	$G_0T_3T_3V_0$	1.05	0.98				
S54	25	M	B	LRD	10	208	H	Triple Rx/CCB	Mod	$G_1T_2I_2V_0$	1.25	0.95	0	0	6.8	46.7
S35	40	M	I	C	8	905	H	CAPD/Triple Rx/CCB/BB/VD	Severe	$G_0T_3T_3V_0$	1.38				6.5	0.3
E1	37	M	I	LRD	1927	251	H	CCB/Triple Rx/Ketoconazole	Mild	$G_0T_1I_1V_0$	4.24					
E3	53	M	W	C	4198	140	N	CS/Aza	Mild	$G_0T_1I_1V_0$	1.13					
E4	52	M	B	C	780	190	H	CS/Claforan	Mod	$G_0T_3T_2V_0$	2.2					
N3	33	F	I	C	1335	249	H	Triple Rx/CCB	Mod	$G_0T_3I_2V_0$	0.85		0.1	147.23	24.86	0.21
S47	21	M	I	LRD	8	442	H	Triple Rx/CCB/BB/VD	Mild	$G_0T_1I_1V_0$	1.23				20.8	
S17	40	M	B	C	1095	828	H	Triple Rx/CCB/ACEI	Mild	$G_0T_1I_1V_0$			2.36	48	4.2	0
S14	39	M	B	LUD	1670	290	H	Triple Rx/CCB/VD	Mild	$G_0T_1I_1V_0$			3.87	0	2.8	28.8

LRD= living-related donor; LUD= living unrelated donor; C=cadaver donor; M= male; F= female; I= indian; B= black; W= white; H BP= high blood pressure; HD= haemodialysis; CAPD= continuous ambulatory peritoneal dialysis; Triple Rx= triple therapy [cyclosporine A, azathioprine (Aza), corticosteroids (CS)]; CCB= calcium channel blocker; VD= vasodilator; BB= beta blocker; *TK Amidase units: ng/µg protein; **ELISA units: ng/ml; serum creatinine:µmol/l

Appendix 3.2.2 : Demographics of Control Group- Endothelin-1

Subject	Age	Sex	Race	Plasma ET-1 (pg/ml)	Urine ET-1 (pg/ml)
1	40	F	I	1.38	0.3
2	35	M	I	0.7	0.2
3	40	M	I	0.9	0
4	31	F	I	0.83	0.13
5	44	F	B	0.75	0.17
6	30	F	I	0.68	
7	30	M	I	0.73	
8	43	F	I	0.8	
9	22	M	B	1.55	0.7
10	48	F	I	0.75	0.38
11	28	M	I	0.58	0.13
12	25	F	B	0.55	0.35
13	27	F	I	0.72	
14	45	M	I	0.68	
15	30	F	I	0.63	1.58
16	31	M	W	0.6	
17	32	M	W	0.6	
18	33	M	B	0.4	
19	21	M	I	0.68	
20	46	M	I	0.4	
21	27	M	I	0.43	
22	47	M	I		0.16

I - Indian

B - Black

W - White

M - Male

F - Female

Appendix B.3.2.3 Patient profile and plasma ET-1 in renal disease

Pt	Race	Age	Sex	BP	Creatinine µmol/l	Proteinuria g/24 h	Renal Histology	Plasma ET-1 (pg/ml)	Urine ET (pg/ml)
1	I	18	F	H	Dialysis	3	SLE IV	4,5	
2	B	30	M	H	132	12,4	MCGN	1,6	
3	B	16	M	H	92	0,67	MPGN	0,6	0.04
4	B	35	M	N	180	2,2	MCGN	4,0	
5	I	43	F	H	Dialysis	4,5	SLE IV	3,0	0.5
6	I	34	M	H	108	0,3	IgA-MPGN	1,6	0
7	I	25	M	H	113	3,6	MCGN	3,0	
8	I	45	F	N	84	3,9	IgA-MPGN	2,1	
9	I	24	M	H	250	3,0	MCGN	3,3	
10	W	48	M	H	396	1,0	MCGN	0,65	
11	B	39	M	H	393	21	MCGN	2,53	0.4
12	I	26	F	H	113	3,1	SLE IV	4,12	0.95
13	I	47	F	H	Dialysis	5,2	SLE IV	5,56	
14	I	13	M	N	56	3,2	MCNS	1,41	0
15	B	21	F	N	65	13,9	MGN	0,45	0
16	B	33	F	N	53	19,7	MCNS	0,63	0.38
17	B	59	M	H	325	3,9	MGN	1,94	1.09
18	C	22	M	N	140	21	IgA and NS	2,0	0.22
19	B	24	M	H	290	1,3	CIN	2,8	
20	I	35	F	N	120	0,5	IgA Neph	0,6	
21	B	30	F	N	77	10	MCNS	0,6	
22	B	32	F	N	80	8	MCNS	0,48	
23	B	32	F	N	72	8,2	MGN	0,55	
24	B	26	F	N	70	6,9	MGN	0,48	
25	I	26	M	N	91	19,8	MCNS	1,1	
26	B	34	F	N	86	2,4	MGN	3,0	
27	B	55	M	N	350	1,0	CIN	2,1	
28	I	38	M	H	156	5,8	HPT Neph	1,6	
29	B	52	M	H	766		HPT Neph	1,04	0.98

Blood pressure : H = hypertensive; N= normotensive
SLE IV = Class IV lupus nephritis
GN = glomerulonephritis

MCNS = nephrotic syndrome due to minimal change disease
MPGN = mesangioproliferative GN
MGN = membranous GN
MCGN = mesangiocapillary GN
HPT neph = hypertensive nephrosclerosis
CIN = chronic interstitial nephritis
NS = nephrotic syndrome
M = male B = black I = Indian
F = female W = white C = coloured

Appendix B 3.2.4:Plasma ET-1 in patients with end stage renal failure on dialysis

Subject	Age	Sex	BP	Diagnosis	Plasma ET-1 (pg/ml)	Dialysis
1	51	M	H	Membranous GN	1,68	CAPD
2	35	M	H	MCGN	1,23	CAPD
3	43	M	H	Diabetic nephropathy	4,63	HD
4	12	M	H	MCGN	4,29	CAPD
5	52	M	H	Unknown	1,04	HD
6	53	M	N	Unknown	2,14	HD
7	33	M	H	Unknown	3,39	HD
8	16	M	H	Unknown GN	2,34	CAPD
9	22	M	H	Unknown	1,89	HD
10	23	F	H	Reflux nephropathy	1,9	CAPD
11	33	M	H	Unknown	3,2	HD
12	52	M	H	Unknown	1,11	CAPD
13	35	M	H	Unknown	2,4	CAPD
14	37	M	H	Unknown	2,63	HD
15	23	M	H	Unknown	1,43	CAPD
16	47	F	H	SLE	5,56	HD
17	42	F	H	SLE	2,13	CAPD
18	35	M	H	IgA	1,35	CAPD
19	28	M	H	Unknown	3,38	HD
20	26	M	H	Unknown	1,84	CAPD
21	36	M	H	Unknown GN	2,0	HD
22	35	M	N	Renal cortical necrosis	1,4	HD
23	60	F	N	Chronic interstitial Nephritis	0,29	HD
24	36	F	H	Unknown	1,6	CAPD

M = male; F = female
H = hypertension; N = normotension
GN= glomerulonephritis
MCGN =mesangiocapillary GN
SLE = systemic lupus erythematosis
IgA= immunoglobulin A nephritis
CAPD= continuous ambulatory peritoneal dialysis; HD = haemodialysis

B3.2.5 Serial ET-1 measurements before and following transplantation

Pt	Pre-TP	Post-TP	Acute Rejection	Post R_x Rejection
1	1.68	2.64	1.56	1.12
2			1.86	1.12
3	1.23	0.82	0.87	1.12
4			0.93	1.11
5	4.29	2.0		
6	1.1	0.75	1.5	
7	1.1	0.95		
8	2.34	0.96	3.76	1.25
9	1.84	1.55		
10	1.9	1.1	2.3	1.04
11		0.8	2.33	2.16
12	1.1	1.78	1.78	
13	2.4	1.45	1.98	1.5
14	2.63	1.28	1.55	
15	1.43	1.59	1.23	0.85
16	3.13	2.35		
17	1.35	1.03		
18	3.38		1.25	
19	2.12	1.32		
20	1.11	2.4	2.42	1.94
21			1.5	1.73
22		0.71	1.05	

Table 3.3.1 ANP levels during rejection

REJECTION								CONTROLS				
Patient	Age	Sex	Race	Plasma ANP	Urine ANP	Serum Creat	Oedema	Age	Sex	Race	Plasma ANP	Urine ANP
S 1	51	M	I	57.5	42.5	326	4+	43	F	I	40	2.6
S 5	35	M	I	21	17	278	2+	22	M	B	15.8	2.3
S10	51	M	I	62.5	35	437	4+	46	M	I	11.7	3.0
S28	16	M	I	50	-	666	3+	48	F	I	-	2.7
S35	40	M	I	-	32.5	850	4+	39	F	W	19.5	
S39	33	M	I	79	73.6	347	2+	33	F	W	30	
S42	35	M	I	80	-	250	3+	45	F	W	42.5	
S44	30	M	W	42.5	19	304	+	47	F	W	20	
S47	21	M	I	52.6	-	442	4+	27	F	C	22.5	
S41	52	M	I	42	-	626	4+	39	F	I	16	
S48	23	M	I	47.6	-	194	0	30	F	I	39	
S45	37	M	B	57.9	17.1	145	0	46	F	W	14	
S43	45	M	I	27.6	22.1	193	0	42	F	I	21.3	
S38	23	F	I	23	19.5	219	0	25	F	W	28.7	
S37	43	M	W	26.6	25.5	175	+	53	F	W	14	
S18	20	M	I	75	52.5	169	2+					
S11	43	M	I	13	2.5	183	0					
S3	42	M	I	-	4.5	173	0+					

B 3.4.1.Donor Controls

Donor	Age	Sex	Race	TK Amidase ng/ug protein	TK ELISA ng/ml	BASAL KININ (ng/ml)	GEN KININ (ng/ml)
S1D	38	M	I	12.77	70		
S5D	40	F	I	3.29	73		
S8D	27	F	B	14.3	56		
S12D	30	F	I	28.63	15.23		
S23D	45	M	I	50.68	65		
S27D	35	M	I	24.22	26	1.2	98.2
S28D	40.	M	I	1.37	69	2.05	100.7
S32D	26	F	I	225.9	101	2.0	174.6
S36D	44	F	B	0.02	18		
S39D	35	F	I	1.37	0.0	1.9	89.2
S41D	30	M	I	64.2	70		
S42D	43	F	I	314	7	2.7	113.7
S45D	22	M	B	2.43	18		
S48D	48	F	I	2.79	43	3	80.9
S51D	25	F	I	222		7.4	
S52D	28	M	I	29.5	42	5.0	113.7
S53D	27	F	C	3.41	12.12		
S54D	25	F	B	0.02	40	7.6	40.7
S15D	39	F	I	2.52	0.0		
S18D	48	M	C	0.46	165.39		

B3.4.2 Volunteers Controls

Patient	Age	Sex	TK ELISA ng/ml	TK AMIDASE ng/ugprot	KININ Basal	Gen KININ
249	46	F	75.97	2.18	6.05	52.78
250	36	F	7.9	0.05	6.04	21.83
251	42	M	25.88	0.06	8.52	41.68
385	47	F	0	0.08	5.25	20.25
386	38	F	50.24	0.5	4.46	20.94
387	27	M	75.83	0.18	6.02	20.9
388	40	M	48.01	0.18	5.86	20.45
390	52	F	0	1.4	18.41	56.09
448	59	F	85.13	0.15	4.27	71.72
449	35	F	114.47	1.92	3.99	120.01
450	50	F	86.48	0.37	3.35	68.17
451	47	F	68.76	0.25	1.18	68.09
453	40	M	74.92	0.31	2.18	126.65
454	43	M	118.02	0.42	1.37	74.12
455	39	F	115.6	0.24	4.3	55.42
456	52	M	111.91	1.23	3.96	71.13
457	64	M	106.38	0.47	0.26	45.75
460	42	F	88.33	0.49	0.75	67.74
461	50	F	66.25	0.94	1.76	270.8
462	42	F	56.98	1.11	1.54	251.5
463	40	F	55.27	0.38	0.97	164.4
464	48	M	85.67	0.77	1.11	155.4
505	58	F	121.02	1.57	0.89	110.1
506	64	F	104.35	0.12	1.14	99.5

B 3.4.3 Donors - Post Nephrectomy

	Age	Sex	Race	Period Post Sx	Serum Creat	Creat Cl (ml/min)	24 hr urine protein (g	BP	Wt (kg)	TK Amidase	TK ELISA	KININ Basal	KININ Gen
N4	32	F	I	5y	87			129/60	57	1.15	0.47	1.2	0
N5	30	F	B	14m	95			123/67	71.8	3.78	4.3	0.5	0
N6	25	M	B	7m	105	56	0.07	125/64	54.2	0.52	19.16	2.7	52.5
N7	42	F	I	21m	79			122/72	53.8	0.37	0	2.6	36.7
N8	35	F	I	3y 3m	69	56	0	138/73	46.4	1.59	0	0.5	0
N9	27	F	I	2y 2m	72	83	0	108/50	47.8	3.41	12.12	0.4	0
N10	28	M	I	3y 2m	92	75	0	123/72	57.6	2.51	64.52	2.6	0
N12	27	M	I	3y	112			122/67	72.6	0	5.84	0.9	0
N14	32	F	I	6y				149/96	102.2	2.57	0	3.3	33.1
N15	52	F	I	3y 3m	110	38	0	119/67	60.2	1.02	8.23	1.4	0
N16	39	F	I	2y 10m	80			106/68	48.6	5.4	20.43	0.75	0
N17	33	M	I	2y	144	60	0	109/64	91.6	5.59	10.44	1.1	0
N18	43	F	I	5y 3m	87	141	0	121/63	84.2	0.95	128.24	6.4	43.1
N19	27	F	I	4y 8m	55	129	0.03	117/64	62.8	1.35	7.97	2.8	70.7
N20	37	F	I	4y	84	84	0.06	130/52	71	1.32	13.24	3.1	44.5
N21	43	F	I	1y	80	70	0	105/79	71.8	2.55	0.67		

Serum creatinine: μmol/l; TK amidase: ng/μg protein; TK ELISA: ng/ml; Kinins: ng/ml

B 3.4.4 Demographics of GN group

Pt	Age	Sex	Race	BP	Renal Disease	Proteinuria	Serum creat	creat cl	Treatment	TK Amidase	TK ELISA	Basal Kinin	Gen Kinin
2	26	F	I	N	SLE IV	3.16g	113	36	CS/Zantac	1.0		444.8	0.75
4	60	F	C	N	ATN +Int neph	?	895 HD		Pen/Claforan/HD	0.51		14.8	55.2
7	13	M	I	N	MCNS	3.3g	56	69	CS/Diuretic	44.75			
9	21	F	B	N	MCNS	14g	65	140	Diuretic	48.02			
13	33	F	B	N	MCNS	12.5g	53	132	Diuretic	7.46		0.3	0
20	52	M	B	H	ESKD	?	766		CCB/VD/Diuretic	0.76	22	8.7	29.4
24	59	M	B	H	Membr + HPT	3.9g	325		CCB/VD/BB/Diuretic	18.05		3.2	50.05
26	33	M	B	H	HPT nephro		1034		CCB/VD	2.38			
30	38	M	I	N	IgA nephritis	2.2g	149	80	Nil	3.89		0.7	92.4
31	38	M	I	H	HPT neph	5.8g	156	40	ACEI/CCB	4.49		2.8	55.5
32	26	M	I	H	ESKD		CAPD		CCB/VD	0.05	91.2		
33	39	M	B	H	MCGN	21g	393		CCB/BB/VD	3.89		0.8	117.4
34	22	M	C	N	IgA NS	21.2g	142	67	Diuretics	3.04		10.9	35.1
36	22	M	B	H	ESKD		H/D		CCB	0.01	0		
N1	28	M	B	N	MCGN-NS	15.7	135	77	Diuretics	4.9		0.4	0
N2	35	F	W	N	IgA	0	72	99	Nil	1.44	0	1.7	69.3
N13	21	F	I	H	MCGN	1.02	328	9	CCB/BB/Diuretic	2.1	0	0.3	0
S12	12	M	I	H	MCGN		H/D		CCB/Diuretic	1.21	62		
S23	53	M	I	H	HPT/ESKD		H/D		Diuretic	3.86	0	0.5	0
S45	37	M	B	H	HPT/ESKD		H/D		CCB/VD	0.68			
S50	50	F	I	H	SLE		H/D		CCB/CS	0.1		.7	18
S39	35	M	I	H	CGN/ESKD		H/D		CCB/VD/BB	0.46	165.4		
S6	43	M	I	H	DM/ESKD		H/D		CCB/VD	5.99	79	3.4	22.3
S8	33	F	B	N	VUR/ESKD		H/D		HD/CCB				
S5	35	M	I	H	MCGN		CAPD		CCB/VD/BB	8.36	13.9	2.9	67.1
S52	35	M	I	H	ESKD		CAPD		CCB/VD	3		15	13.1
S53	38	M	M/C	H	MCGN		H/D		CCB	-			
S54	28	M	B	H	ESKD		Dialysis			1.39	0		
S51	32	F	I	H	SLE/ESKD		H/D		CCB/CS	3	0		
S18	20	M	W	H	Obstr Uro		H/D			1.54	0	0.2	0
S28	16	M	I	H	CGN		CAPD			37.28	0	2.1	138

M= male; F= female; I= Indian; B= black; W= white; M/C= mixed race

CCB= calcium channel blocker; VD= vasodilator; BB= beta blocker;
HD= haemodialysis; CAPD= continuous ambulatory peritoneal dialysis
SLE IV = Class IV lupus nephritis; ESKD= end stage kidney disease; SLE= systemic lupus erythematosis; CGN= chronic glomerulonephritis; DM= diabetes mellitus; HPT= hypertensive
MCNS = nephrotic syndrome due to minimal change disease; MPGN = mesangioproliferative GN
Membr = membranous GN; MCGN = = mesangiocapillary GN; NS = nephrotic syndrome
HPT neph = hypertensive nephrosclerosis; CIN = chronic interstitial nephritis; VUR= vesico-ureteric reflux; obstr uro= obstructive uropathy

B 3.4.5 Stable Transplant Patients

Patient	Sex	Age	TK ELISA	TK AMIDASE	KININ BASAL	KININ GEN
475	M	20	0	0.76	0.22	19
476	M	30	60.94	2.4	0.34	56.5
477	M	23	72.09	2.52	0.26	47.9
478	F	42	0	2.42	0.37	30.1
481	F	24	0	1.14	0.25	36.2
482	M	25	33.2			
483	F	41	129.39	6.94	0.23	23.7
484	M	43	0	0.83	0.2	40.3
485	F	58	5.79	2.57	0.23	51
487	M	21	9.67	0	0.48	41.7
488	M	46	125.84	4.2	0.27	31.7
489	M	32	0	0.12	0.53	80.4
490	F	43	116.02	2.41	0.27	44.2
491	M	35	66.23	4.55	0.57	92.3
492	F	27	71.35	1.97	0.33	82.8
493	M	47	91.74	0	0.19	40
494	F	36	115.02	3.71	0.19	36.8
495	M	39	0	2.09	0.36	35
497	F	46	0	1.39	0.26	47.4
498	M	44	86.29	0.11	0.28	65.3
499	M	17	14.70	1.24	0.21	36.5

CHAPTER 4

DISCUSSION

4.1 FLUID VOLUME STATUS AND EFFECTIVE RENAL PLASMA FLOW

Total body water (TBW) was measured in 5 transplant recipients immediately pre-transplant following dialysis, when they were assessed as having reached "dry" weight, namely showing no evidence of fluid retention. TBW was measured following transplantation and during rejection in 3 patients, when fluid retention was apparent; and again when kidney function had stabilised, demonstrating reduction in body weight and fluid retention and hence a reduction in TBW. Effective renal plasma flow was measured in 6 transplant patients, and was shown to be reduced in patients during rejection.

4.2 ENDOTHELINS IN RENAL FUNCTION AND RENAL DISEASE

Endothelin-1 (ET-1) affects renal physiology by influencing sodium and water excretion and vascular and mesangial tone. It also acts as a growth factor by promoting cell proliferation and matrix formation. ET-1 may activate and be chemotactic for monocytes, which can in turn secrete ET-1 (Martin-Nizzard et al., 1991; Achmad and Rao, 1992), and may thus influence renal inflammation.

4.2.1 Endothelins in acute renal allograft rejection

ET-1 in the circulation and urine

Plasma ET-1 levels were reported to be elevated in pre-dialysis patients with chronic renal failure and in patients on regular haemodialysis whereas they were found to be normal in stable renal transplant patients treated with cyclosporine A [(CyA); Stockenhuber et al., 1992]. An association between increased plasma ET-1 levels and CyA was first suggested

in a renal transplant patient receiving high (toxic) doses of CyA (Fogo et al., 1990). After administration of CyA, plasma ET-1 levels increase only transiently in transplant patients, because ET-1 is rapidly metabolised and cleared from the systemic circulation (Grieff et al., 1993). Cyclosporine and related immunosuppressants such as Tacrolimus (FK506) directly stimulate ET-1 release from mesangial and/or endothelial cells (Langman and Yatscoff, 1994; Goodall et al., 1995; Kohno et al., 1995). Cyclosporine A increases renal ET-1 mRNA expression (Iwasaki et al., 1994). Anti-ET antibodies and ET receptor antagonists ameliorated acute CyA-induced renal vasoconstriction (Kon and Awazu, 1992; Lanese and Conger, 1993; Conger et al., 1994; Brooks and Contino, 1995; Kon et al., 1995). Chronic oral administration of CyA increases urinary ET-1 in the rat. Both acute and chronic CyA toxicity are associated with reduced renal function and elevated plasma ET-1. As CyA levels reach therapeutic levels and renal function improves, urinary ET-1 levels return to baseline (Perico et al., 1992). Mixed ET antagonist SB209670 prevented acute vasocontriction and reduction of renal function induced by high dose CyA (Brooks and Contino, 1995). Little is known about the effect of steroids on ET-1 production and release. In vitro, pre-treatment of rat vascular smooth muscle cells with dexamethasone resulted in an attenuated response to ET-1 due to down-regulation of ET_A receptors. Other steroids (prednisolone and hydrocortisone) gave similar responses but were less effective (Nambi et al., 1992).

In the present study, plasma endothelin-1 levels were measured serially, in patients prior to renal transplantation, post-transplantation when stable, during acute rejection and subsequently after treatment of rejection. The highest levels of ET-1 were observed prior to transplant, in patients with chronic renal failure on dialysis. Following transplantation, ET-

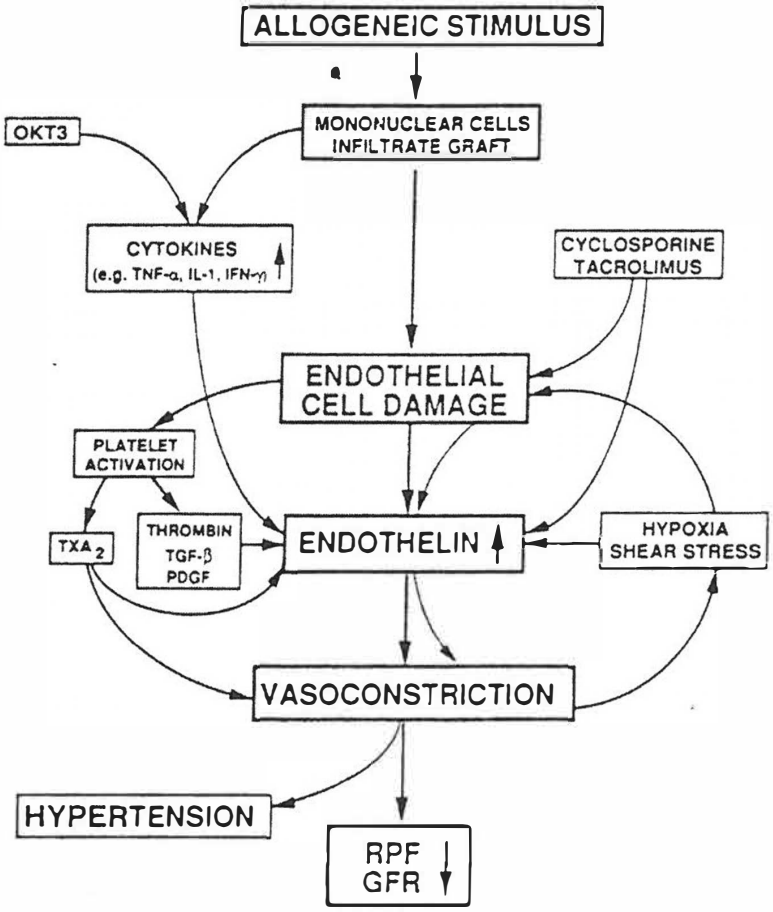
1 levels decreased, increased during episodes of acute rejection and decreased again after treatment of rejection. This corresponded with elevated urinary ET-1 levels during acute rejection, suggesting that ET-1 is produced by the kidney, probably by inflammatory cells within the renal cellular structures and interstitium, reflecting endogenous renal ET-1 synthesis (Abassi et al., 1992).

Cellular localisation of ET-1

Immunofluorescent studies showed increased ET-1 labelling of proximal and distal tubules during rejection Electron microscopy revealed ET-1 immunolabelling in epithelial cells of proximal and distal tubules and endothelial cells of blood vessels and glomerular capillaries, specifically in cytoplasm of cells, endoplasmic reticulum and mitochondria. Label was found in increased amounts adjacent to the intercellular system and within vacuoles and secretory vesicles. ET-1 is stored within these vesicles and released at the cell surface after an appropriate stimulus. The characteristic feature of acute rejection is infiltration of the graft by host mononuclear cells; these lymphocytes and macrophages infiltrate the interstitium and tubular epithelium. The interstitial infiltrate is associated with oedema. In this study, ET-1 label was also demonstrated in the mononuclear cells infiltrating the tubules during acute rejection by electron microscopy as well as by the immunoperoxidase method. ET-1 mRNA was upregulated in the tubular epithelial cells and capillary endothelial cells, as well as the inflammatory infiltrate, during acute rejection as demonstrated by in situ RT-PCR. The inflammatory process of acute cellular rejection is predominantly confined to the interstitium, and marked intragraft upregulation of ET-1 may occur without significant changes in ET-1 plasma concentrations (Watschinger et al., 1995), unless there is endothelial damage as occurs with vascular rejection (Watschinger et

al., 1994). Possible causes and effects of increased ET-1 production during acute renal allograft rejection are suggested in Fig 4.1.

Fig 4.1: Possible causes and effects of increased ET-1 production during acute renal allograft rejection.



Ref: Watschinger B and Sayegh M H (1996), Am J Kid Dis, 27: 151-161(156).
Abbreviations: RPF= renal plasma flow; GFR= glomerular filtration rate; TXA_2= thromboxane; OKT3= Orthoclone; PDGF= platelet-derived growth factor

Activated mononuclear cells infiltrate the allograft and stimulate the secretion of cytokines which influence the production of ET-1 by other cells *in vitro*: tumour necrosis factor α increases ET-1 mRNA and causes secretion of ET-1 by capillary endothelial cells,

epithelial cells and rat mesangial cells (Kohan, 1991); similarly IL-1β induces ET-1 release from renal epithelial cells (Ohta et al., 1990). Platelet activation triggers the release of transforming growth factor (TGFβ), thromboxane A2, PDGF and thrombin from platelets that accumulate in the graft as a consequence of the rejection process. This results in ET-1 secretion and upregulation of ET-1 gene expression in endothelial, vascular smooth muscle, renal mesangial and epithelial cells (Kurihara et al., 1989; Watschinger and Sayegh, 1996). Increased expression of the intracellular adhesion molecule-1 (ICAM-1) expression occurs in the glomeruli of rat renal allografts undergoing acute rejection (Azuma et al., 1994).

Cellular localisation of endothelin receptors

Immunoperoxidase localisation showed decreased ET_B receptor labelling in tubules during acute rejection; as well as an increase in ET_A receptor immunofluorescent labelling in collecting ducts during rejection. The small numbers in each sub-category made statistical analysis difficult. Electron microscopy revealed ET receptor immunolabelling in epithelial cells of proximal and distal tubules and endothelial cells of blood vessels and glomerular capillaries. The clinical features of hypertension and oedema that occur during acute rejection may be mediated by the ET_A receptor (promoting vasoconstriction) and by down-regulation of the ET_B receptor, resulting in an anti-natriuretic effect respectively.

Chronic rejection

Chronic rejection is recognized as a sequelae of acute rejection, and may thus follow on as a result of the secretion of growth factors. Periglomerular and perivascular macrophages

secrete cytokines that are profibrogenic, including platelet-derived growth factor (PDGF) and TGF-β. The gradual functional deterioration caused by the development of glomerulosclerosis and arterial obliteration may also cause systemic hypertension which results in the remaining functional glomeruli to hyperfilter before eventually fibrosing, a process that progressively leads to renal damage (Neuringer and Brenner, 1992). Renal allografts with chronic rejection and transplant-associated arteriosclerosis have been reported to express 6-fold more ET-1 in the neointima of the vasculature, when compared to allografts with acute rejection or normal control kidneys (Simonson et al., 1998).

4.2.2 Endothelins in renal parenchymal disease

ET-1 in the circulation and urine

This study demonstrated that increased plasma ET-1 levels were present in renal disease, compared to normal controls; the elevated plasma ET-1 levels were found in both proliferative and non-proliferative glomerulonephritis (GN), with significantly higher levels in proliferative glomerulonephritis. Both hypertensive and normotensive patients with glomerulonephritis had increased plasma ET-1 levels, but with significantly higher levels in the hypertensive patients. Plasma ET-1 values were elevated in both dialysis- and non dialysis-requiring patients when compared to normal controls; plasma ET-1 was significantly higher in dialysis patients compared to patients not requiring dialysis. Urinary ET-1 levels were similar in patients with glomerulonephritis and controls.

ET-1 may be important in increasing vascular resistance in hypertension. ET-1 potentiates the vasoconstrictor response to sympathetic stimulation (Haynes and Webb, 1998). The endothelin system in human hypertension resembles animal models of salt-sensitive

hypertension; ET-1 is elevated in black hypertensives compared to white hypertensives and black normotensives (Ergul et al., 1996). Endothelin levels were significantly elevated in normotensive black adolescents with a family history of hypertension; ET-1 was markedly increased in response to acute stressors (Treiber et al., 2000).

Cellular localisation of ET-1

Immunofluorescent immunocytochemistry showed increased ET-1 labelling of proximal and distal tubules in proliferative GN biopsies. Proximal tubular labelling was observed mainly in the brush border, as reported by Wilkes et al. (1991). Electron microscopy demonstrated the presence of ET-1 in the proximal and distal tubules and glomeruli as well as blood vessels; the localisation was predominantly in the cytoplasm, as well as mitochondria and within secretory vesicles. ET-1 label was present in endothelial cells of glomerular capillaries and arterioles as well as vascular smooth muscle cells. ET-1 acts as an autocrine/ paracrine substance, as circulating plasma levels are lower than those required to elicit most of the biological actions of ET-1. The concentration of ET-1 at the vascular smooth muscle may be several orders of magnitude higher than that in plasma (Yoshimura et al., 1995; Benigni, 2000). Angiotensin II stimulates the expression of ET-1 gene in endothelial and renal cells. Part of the mitogenic effect of Angiotensin II is mediated by ET-1, as suggested by studies with monoclonal antibodies against ET-1 (Bakris and Re, 1993). Data in animals with progressive nephropathies suggest that angiotensin converting enzyme inhibitors (ACE-I), besides reducing glomerular protein traffic, also inhibit the exaggerated synthesis of ET-1 (Zoja et al., 1998). ET-1 synthesis in proximal tubular cells can be influenced by proteinuria (Zoja et al., 1995).

Cellular localisation of endothelin receptors

ET_A receptor labelling was increased in distal tubules in proliferative GN. ET_B receptors, visualised by immunoperoxidase, showed decreased labelling in tubules in GN biopsies. The small numbers in each sub-category made statistical analysis difficult; further studies with larger sample numbers would help in confirming these findings. Electron microscopy demonstrated the presence of ET receptors in the proximal and distal tubules and glomeruli as well as blood vessels. ET receptor label was present in endothelial cells of glomerular capillaries and arterioles as well as vascular smooth muscle cells. The role and interaction of the two endothelin receptors *in vivo* remains unclear. Activation of ET_A or ET_B receptors on vascular smooth muscle cells produces a pressor response through vasoconstriction, while activation of ET_B receptor on the vascular endothelium produces a depressor response by evoking the release of vasodilators. ET_B receptors on the vascular endothelium may play a role in clearing circulating ET-1, thereby reducing its predominant ET_A mediated pressor actions. Activation of ET_B on the renal tubular epithelium can act as a depressor mechanism by promoting natriuresis and diuresis.

ET-1 in renal inflammation

Mesangial proliferation is a common finding in glomerular inflammation, suggesting that local overproduction of ET-1 might serve as a pro-inflammatory signal in glomerular injury. Pro-inflammatory agents, such as IL-1 and TGF_β, stimulate ET-1 secretion in endothelial and mesangial cells, supporting this hypothesis. ET-1 induces platelet-derived growth factor (PDGF) A and B chain expression in human mesangial cells; AP-1 transcription factors probably mediate induction of the collagenase gene by ET-1 in mesangial cells (reviewed by Simonson, 1993). There is evidence to suggest that ET-1 is involved in the pathogenesis of proliferative glomerulonephritis. Renal ET-1 production

was increased in experimental and human glomerulonephritis (Murer et al., 1994; Roccatello et al., 1994; Nakamura et al., 1995a; Yoshimura et al., 1995). ET-1 is a potent mitogen and partly mediated the proliferative effects of several cytokines (Bakris and Re, 1993; Kohno et al., 1994; Nitta et al., 1995). Inflammatory cytokines and proteinuria per se augmented renal ET-1 production (Zoja et al., 1995). ET_B receptor expression was upregulated in glomerulonephritis in the rat (Yoshimura et al., 1995). ET receptor antagonists reduced mesangial cell proliferation in experimental mesangial proliferative glomerulonephritis (Fukuda et al., 1996) and decreased renal injury in murine lupus nephritis (Nakamura et al., 1995b).

Endothelin-1 also contributes to excessive accumulation of extracellular matrix components and fibrosis by increasing renal cell fibronectin and collagen production, tissue inhibitor of metalloprotease levels and the release of cytokines that stimulate matrix accumulation (Ong et al., 1994; Ruiz-Ortega et al., 1994). ET-1 antagonism decreased matrix accumulation in experimental models of glomerulonephritis (Fukuda et al., 1996). Chronic treatment with an ET_A receptor antagonist (FR 139317, Fujisawa, Osaka, Japan) attenuated increases in glomerular mRNA levels of collagen, laminin, tumour necrosis factor, TGF-β, PDGF and basic fibroblast growth factor in diabetic rats (Nakamura et al., 1995c). Once substantial renal scarring occurs, there is an inevitable progression to end stage kidney disease, a process involving gradual glomerular sclerosis and interstitial fibrosis. Endothelin receptor blockade reduced proteinuria and glomerulosclerosis, and protected against hypertension and elevations in serum creatinine levels in the 5/6 nephrectomy rat model (Benigni et al., 1993 and 1996). Endothelin receptor antagonists (ET_A and combined ET_A/ET_B antagonists) reduced proteinuria and the amount of periodic

acid Schiff (PAS) positive material as well as decreasing the over-expression of fibronectin and typeIV collagen in diabetic nephropathy in rats with streptozotocin-induced diabetes (Hocher et al., 2001).

ET-1 and endothelin receptors in renal function

Fluid retention and hypertension accompany renal disease. Clinically, glomerular disease presents with oedema and an elevated blood pressure. Alterations in ET-1 in the renal vasculature and renal tubules have differing effects. In the vasculature, increases in ET-1 predominantly cause vasoconstriction with a hypertensive effect. Increased ET-1 in the nephron probably enhances sodium and water excretion, favouring hypotension (reviewed by Markewitz and Kohan, 1995; Schiffrin, 1995). The ET_B receptor was down-regulated in proximal and distal tubules and collecting ducts, and is probably implicated in impaired natriuresis. This observation may account for the fluid retention and hypertension occurring with acute rejection and glomerulonephritis: ET_B receptors have a potentially important hypotensive effect via inhibition of sodium and water reabsorption in the distal nephron (Webb et al., 1998). The amiloride-sensitive renal epithelial sodium channel is an essential component for the regulation of sodium balance, blood volume and blood pressure. In vitro studies suggest that activation of ET_B receptors inhibits the activity of the renal epithelial sodium channel in the renal collecting duct epithelium (Gallego and Ling, 1996). Activation of ET_B receptors leads to down-regulation of the epithelial sodium channel in the renal tubule via increased production of nitric oxide (Plato and Garvin, 1999). Mean arterial pressure was significantly higher in ET_B receptor deficient mice; excess dietary salt upregulated renal ET-1 in these rodents (Ohuchi et al., 1999). Thus ET_B receptor activation has a hypotensive effect via promotion of renal sodium and water excretion.

Endothelin receptor antagonists have been shown to be reno-protective, namely, to decrease blood pressure, improve renal function and reduce proteinuria in models of renal disease, for example, combined $ET_{A/B}$ receptor antagonist in immune-complex nephritis (Gomez-Garre et al., 1996); ET_A receptor antagonist FR 139317 (Fujisawa, Osaka, Japan) in murine lupus nephritis (Nakamura et al., 1995b); ET_A receptor antagonist LU 135252 (Knoll AG, Ludwighafen, Germany) and ACE inhibitor had an additive effect compared to either agent alone in accelerated passive Heymann nephritis (Benigni et al., 1998a); ET_A receptor antagonist (Nakamura et al., 1995c) and mixed $ET_{A/B}$ receptor antagonist (Benigni et al., 1998b) in Streptozotocin-induced diabetes. Bosentan, a mixed ET_A/ET_B receptor antagonist, administered to patients with mild-moderate essential hypertension was as effective as enalapril (inhibitor of angiotensin converting enzyme) in controlling blood pressure, suggesting that ET-1 contributes to hypertension in these patients (Krum et al., 1998).

4.3 MODULATION OF RENAL FUNCTION AND ROLE IN RENAL DISEASE OF ATRIAL NATRIURETIC PEPTIDE

Circulating atrial natriuretic peptide (ANP) is produced primarily as a response to increased intravascular volume; elevated levels of ANP are found in hypertensive patients, nephrotic syndrome and acute and chronic renal failure.

4.3.1 ANP and acute rejection

Plasma concentrations of all three natriuretic peptides were elevated in chronic renal failure, probably because of reduced clearance (Prins et al., 1996). Infusion of doses of ANP slightly above the physiological range in patients with moderate chronic renal failure secondary to glomerulonephritis resulted in a natriuretic response similar to that of normal controls, together with a marked increment in the urinary excretion of urea, potassium and phosphate (De Nicola et al., 1997). Plasma and urinary ANP concentrations were significantly elevated during acute rejection, compared to normal control subjects in the current study. ANP concentrations decrease after a successful kidney transplant and levels increase again when the transplant fails (Zuber et al., 1993). In these patients, increased plasma ANP levels in acute rejection may occur as a response to increased intravascular volume and hypertension: ANP production may be stimulated by excess ET-1 being present. ANP inhibited uptake of IgG complexes by macrophages and increased cGMP levels in macrophages. This alteration in macrophage-dependant phagocytosis may be important in the modulation of immune complex mediated tissue injury (Mattana and Singhal, 1993). Immunocytochemistry revealed decreased labelling of glomeruli and collecting ducts during acute rejection whereas immunolabelling of tubules and arteries was similar to control kidney.

Studies in renal transplant recipients (Bricker et al., 1956) and in subjects with autonomic failure (Gill and Bartter, 1966) suggest that renal denervation impairs sodium conservation in the presence of dietary sodium restriction. Efferent sympathetic nerve stimulation increases tubular sodium and water reabsorption and produces a fall in GFR and renal blood flow mediated by preglomerular vasconstriction (Kopp and Di Bona, 1982). Head-out water immersion studies in stable renal transplant recipients showed a diuretic and

natriuretic response with elevations in plasma ANP and urinary tissue kallikrein excretion; plasma renin activity did not suppress with head-out water immersion, probably as a result of a reduction in sympathetic nerve traffic to the juxtaglomerular apparatus (Al-Haidary et al., 1990). Low dose ANP infusion to stable renal transplant recipients resulted in immediate natriuresis and urinary cyclic GMP excretion as well as albuminuria, in contrast to the delayed response seen in normal subjects (Lipkin et al., 1992). Intravenous infusion of ANP during acute renal allograft rejection in a canine model resulted in increased urine flow rates and an increase in GFR, together with a fall in mean arterial pressure in the presence of an unchanged haematocrit (Lewis et al., 1993).

4.3.2 ANP and renal parenchymal disease

Plasma ANP is elevated in animals and humans with acute and chronic renal failure, due to enhanced release from the atria following volume expansion and reduced clearance by the kidneys. Predialysis ANP levels were higher in haemodialysis than CAPD or healthy subjects and fell to levels similar to CAPD following dialysis. Haemodialysis patients with moderate and severe hypertension had higher levels of plasma ANP, proANP and cGMP compared to normotensive or mild hypertensives. ANP was increased in moderate and severe hypertension and correlated with blood pressure and left ventricular hypertrophy. In nephrotic syndrome, plasma ANP was normal or elevated, reflecting the plasma volume status of these patients; however the urinary sodium excretion in response to ANP infusion is blunted, irrespective of plasma ANP levels (reviewed by Awazu and Ichikawa, 1993). ANP stimulates kallikrein excretion transiently and is dependent on distal sodium delivery (Klein et al., 1989). Low supraphysiological doses of ANP to patients with moderate chronic renal failure due to glomerulonephritis were natriuretic, together with an increased

excretion of urea, potassium and phosphate (resulting in a 15-20% decrease in plasma levels) without any drop in blood pressure.

There was decreased immunolabelling of glomeruli and collecting ducts in glomerulonephritis; labelling of tubules and arteries was similar to that of control kidney tissue. ANP immunoreactivity was reduced in hypertensive nephrosclerotic distal tubules, probably as a result of reduced renal tubular mass (Figueroa et al., 1990). Upregulation of the A receptor subtype occurred in hypertensive rats, together with increased levels of ANP and BNP (Yoshimoto et al., 1995). Renal ANP gene expression is upregulated in the 5/6 nephrectomised Munich-Wistar rats, suggesting that local synthesis of ANP may participate in increasing renal sodium excretion to maintain sodium and water homeostasis in the face of reduced nephron numbers (Totsune et al., 1998).

This study confirms the widespread immunolocalization of ANP in the normal human kidney. In the present study, the decreased immunolabelling of the collecting ducts during acute rejection and glomerulonephritis may be a reflection of impaired natriuresis and diuresis, resulting in fluid retention occurring in these patients.

4.4 THE KALLIKREIN KININ CASCADE IN RENAL FUNCTION AND DISEASE

Kallikreins are proteolytic enzymes which interact with their substrate the kininogens to form the vasoactive peptides, kinins. Kinins bind to their receptors at target organs and exert potent effects in vasodilatation, reduction of blood pressure, vascular permeability, natriuresis, diuresis and renal blood flow.

4.4.1 Tissue kallikrein in renal transplantation

Urinary tissue kallikrein enzymic activity was significantly decreased in stable renal transplant recipients and in kidney donors after unilateral nephrectomy, compared to normal controls. This supports the findings of Spragg et al., (1985) that tissue kallikrein excretion rate may be a useful indicator of distal tubular mass. Serial measurements of urinary tissue kallikrein enzymic activity were made prior to kidney transplant, on day 3 or 4 post transplant when the patient was stable, during an episode of acute rejection and with recovery after treatment of rejection. While tissue kallikrein levels rose from their pre-transplant levels on day 3 or 4 after renal transplant, decreased again during rejection and rose again after treatment of rejection, the difference did not reach statistical significance.. Lower urinary kallikrein excretion was found in transplant recipients compared to controls, probably related to reduced renal function or reduced renal mass (Koolen et al., 1984b; Marin-Grez et al., 1982).

There was no correlation between serum creatinine and tissue kallikrein during acute rejection. Serial ELISA measurements did not show any difference in total immunoreactive tissue kallikrein at any stage of transplantation. Tissue kallikrein showed a significant decrease in donors post-nephrectomy compared to pre-nephrectomy; an increase in urinary tissue kallikrein was observed during rejection, compared to levels in donors post nephrectomy. Urinary excretion of tissue kallikrein was reduced in renal transplant recipients and more markedly so following acute rejection (Moodley et al., 1996). Increase in urinary kallikrein excretion 1-3 days before clinical rejection suggests that activation of the kallikrein kinin cascade is associated with acute rejection (Brouhard et al., 1982; Koolen et al., 1984b). In addition to kallikrein, elevations in factor XIIa, plasminogen and antithrombin III have been observed 2-3 days before clinical signs of rejection (Schrader et

al., 1988). Decreased kallikrein excretion follows cylosporine administration (Spragg et al., 1988; Martinez et al., 1990). Short term cyclosporine administration decreaseskallikrein and kinin B2 receptor mRNA expression in rat kidney cortex (Bompart et al., 1996).

Hypertension frequently accompanies renal transplantation. Proposed mechanisms for hypertension include acute rejection, chronic rejection, therapy with steroids and cyclosporine, renal insufficiency, presence of the recipient's own diseased kidneys, transplant renal stenosis, increased activity of the vasoconstrictor systems (for example renin-angiotensin, endothelin) and decreased activity of vasodilator systems. Urinary kallikrein excretion was found to be decreased in hypertensive patients and in those with renal complications (more markedly decreased with acute tubular necrosis than acute rejection). Urinary kallikrein excretion was also lower in cadaver graft recipients who tend to be more hypertensive (O'Connor et al., 1982).

Renal tissue kallikrein is predominantly localized in the granular portions of the distal tubule and cortical collecting ducts (Tomita et al., 1981; Omata et al., 1982; Figueroa et al., 1984), where it is concentrated mainly on the luminal side of the cell and at both sides of the nuclei. Decreased labelling was observed in the distal tubules during acute rejection. TK immunoreactivity was reduced in acute rejection both on immunocytochemistry and electron microscopy; while TK was observed mainly at the luminal side of distal connecting tubules and collecting ducts, there was a shift in immunolabelling to the basolateral membranes (Ramsaroop et al., 1997).

4.4.2 Kinins in renal transplantation

Basal urinary kinin excretion was decreased in donors following nephrectomy and in stable transplant recipients compared to normal control subjects but rose significantly during acute rejection. Kinin generation in the urine was significantly decreased in donors post-nephrectomy and during acute rejection, compared to controls. The turnover of kinins depends on both the rate of formation and the rate of destruction. After kinins are formed, they are rapidly destroyed by the enzymic action of peptidases. Kininases, which inactivate plasma kinins, are distributed in 2 major portions of the nephron: in the proximal tubules and the medullary collecting ducts and may contribute to the decreased levels in the urine.

4.4.3 Kinin receptors in acute rejection

The kinin B2 receptor localises in the entire nephron in the normal control kidney and is down-regulated in acute rejection (Naidoo et al., 1996). The B1 receptor is not present in the normal control kidney and but is strongly induced during acute rejection (Bhoola et al., 2001).

4.4.4 Role of the kallikrein-kinin system in renal transplantation

Several factors may affect the kallikrein-kinin system in renal transplantation. The uninephrectomised donor and the renal allograft recipient have a single functioning kidney: therefore, the lower urinary tissue kallikrein excretion demonstrated in these subjects may be a reflection of decreased distal tubular mass. Cyclosporine A forms part of the immunosuppressive regimen in the majority of kidney transplant recipients. Although it is effective in increasing graft survival, it is also well known that its therapeutic benefits are counterbalanced by major side effects, including vascular toxicity, hypertension and renal insufficiency (Sturrock and Struthers, 1994); the hypothesis being that CyA may induce an

imbalance between renal vasoconstrictor (endothelins, renin-angiotensin, thromboxane) and vasodilator (nitric oxide, prostaglandins, kallikrein-kinins) systems, leading to an increase in renal vasoconstriction. A decreased kallikrein excretion has been reported in renal transplant patients receiving Cyclosporine (Martinez et al., 1990) as well as a reduction in kinin B2 receptor mRNA expression in the cortex of the kidney (Bompart et al., 1996).

The renal transplant recipient is frequently hypertensive; the blood pressure is further elevated during episodes of acute rejection together with deterioration in renal function; both of these factors have been associated with a reduced kallikrein excretion, which may be partially responsible for the sodium and water retention known to occur in these patients (and thereby further increase the blood pressure). The acute rejection process, by damaging the distal tubule, would decrease tissue kallikrein excretion.

4.4.5 Tissue kallikrein in renal parenchymal disease

Urinary kallikrein excretion is markedly increased in patients with nephrotic syndrome, irrespective of the level of renal function (Cumming and Robson, 1985), whereas patients with glomerulonephritis (without nephrotic syndrome) show reduced urinary kallikrein excretion compared to healthy volunteers. Kallikrein excretion correlates with plasma renin activity but not with plasma volume (Cumming et al., 1989). Urinary tissue kallikrein enzymic activity was significantly decreased in patients with renal disease compared to controls and was even further decreased during acute renal allograft rejection compared to renal disease. ELISA measurements confirmed the reduced excretion of urinary tissue kallikrein in renal disease. There was reduced immunolabelling of TK in distal tubules. In patients with renal parenchymal disease and hypertension with impaired renal function, a

more marked decrease in urinary kallikrein excretion was recorded, compared to hypertensive subjects with normal renal function, in whom a reduced urinary kallikrein excretion was also noted but less so (Mitas et al., 1978). Similarly in subjects with advanced hypertensive nephropathy, there was a reduction in the percentage of tubules and cells with immunoreactive tissue kallikrein (Figueroa et al., 1992).

4.4.6 Kinins in renal parenchymal disease

Although basal kinin levels in urine were similar for renal disease and controls, significantly decreased kinins were generated in the urine of patients with glomerulonephritis, reflecting a reduction in the kinin-producing enzyme TK during renal inflammation. Kinins are potent stimulators of phospholipase A_2, and promote synthesis of arachidonic acid metabolites, including thromboxane A_2 (Regoli and Barabe, 1980). Increased glomerular synthesis of thromboxane has been suggested as a cause of proteinuria in nephrotic syndrome (Remuzzi et al., 1985).

4.4.7 Kinin receptors in renal disease

The constitutive B2 receptor, present along the entire nephron, was down-regulated in renal disease. A novel finding has been the increased expression of the inducible kinin B1 receptor in these patients (Naicker et al., 1999). Prevalence of the $G^{699} \rightarrow C$ polymorphism of the kinin B1 receptor was reported to be significantly less frequently in several aetiological subgroups of uraemic patients (Bachvarov et al., 1998). Thus, the polymorphism of the kinin B1 receptor promotor may be a marker of prognostic significance for the preservation of renal function.

4.4.8 Role of kallikrein kinin system in renal parenchymal disease

The decreased urinary kallikrein activity may be a reflection of reduced distal nephron function in these patients and may mediate the hypertension that accompanies renal disease (Margolius et al., 1971). Clinical studies have shown that blood pressures of hypertensive patients can be temporarily lowered by oral administration of porcine pancreatic kallikrein (Overlack et al., 1981). Long-term infusion of tissue kallikrein via mini pumps has been shown to have beneficial effects by attenuating glomerulosclerotic lesions and tubular injury in hypertensive Dahl salt-sensitive rats (Uehara et al., 1990). A single injection of the human tissue kallikrein gene into the spontaneously hypertensive rat has been shown to reduce blood pressure for up to 10 weeks (Chao and Chao, 1997). Sub-depressor doses of purified rat urinary kalikrein infused for 4 weeks in Dahl salt-sensitive rats fed a high salt diet resulted in decreased urinary protein excretion, increased GFR and attenuated glomerulosclerosis, arterial and tubular injury, together with increased urinary excretion of NO and cGMP. These effects were diminished by co-administration of HOE-140, suggesting that reno-protection is mediated by the kinin B2 receptor (Hirawa et al., 1999).

Urinary kallikrein activity was lower in hypertensive compared to normotensive individuals and more markedly diminished in patients with renal parenchymal disease and hypertension. Inhibitors of ACE, a member of the kininase (K-II) group of enzymes that rapidly inactivate kinins and also convert the decapeptide angiotensin 1 to an octapeptide angiotensin II, are probably the best examples of drugs acting on the kinin system that are used in clinical medicine. The mode of action of ACE inhibitors as antihypertensive agents have been shown to be due to both the inhibition of angiotensin 11 (a potent vasoconstrictor) production, as well as an increase in circulating levels of kinins (Shimamoto et al., 1990). The hypotensive efficacy of these inhibitors has also been shown

to correlate with the reduced activity of ACE in the brain, kidney, and vascular smooth muscle (Unger et al., 1987). Angiotensin converting enzyme (ACE) inhibitors prevent the conversion of Angiotensin I to Angiotensin II and increase urinary kinin excretion, while the effect on plasma kinins is controversial with different studies showing an increase, a decrease or no change (Carretero and Scicli, 1981). The hypotensive effect of the ACE inhibitor, perindopril, in spontaneously hypertensive rats, on low and high sodium diets, was attenuated by the kinin B2-receptor antagonist HOE 140 (Bouaziz et al., 1994). The beneficial effects of ACE inhibitors may be related also to the formation of nitric oxide and prostacyclin by kinins (Linz et al., 1993). Icatibant acetate (HOE 140), a B2 receptor antagonist, given together with captopril, attenuated the hypotensive effect of captopril by 53% in black and white subjects on a low sodium diet (Gainer et al., 1998). This study provides evidence that kinins contribute substantially to the hypotensive effects of ACE inhibition.

4.5 INTER-RELATIONSHIP OF VASOACTIVE PEPTIDES IN RENAL FUNCTION AND DISEASE

Genetic and environmental factors interact to determine an individual's blood pressure. The kidney plays an important role in maintaining body fluid and electrolyte balance, and blood pressure homeostasis through actions of various humoral and paracrine/autocrine factors on renal medullary haemodynamics, tubular reabsorption and urine concentration. Nephron development is coordinated by the interaction of cell adhesion molecules, components of the extracellular matrix and peptide growth factors. Renal ontogeny and function are modulated through complex molecular interactions of endothelins, kinins, atrial natriuretic peptides and angiotensin.

Recent studies have shown that the endothelins are essential for normal foetal development, and that endothelin ET-1 plays an important physiological role in the regulation of basal vascular tone and blood pressure in healthy humans (Parris and Webb, 1997). The spotted lethal rat carries a naturally occurring deletion of the ET_B receptor gene that prevents expression of the ET_B receptor and results in aganglionic megacolon. These animals also represent a model of salt sensitive hypertension; the animal is normotensive until challenged by a high salt diet, which significantly increases arterial pressure. The hypertension is completely ameliorated by the epithelial sodium channel inhibitor, amiloride (Pollock, 2000). In a rat model of renal disease progression, upregulation of ET-1 during development of renal injury occurs progressively, initially in tubules and subsequently in glomeruli. ET-1 plays a role in the progression of chronic renal disease in different experimental models, including renal mass reduction, lupus nephritis and streptozotocin-induced diabetes (Bruzzi et al., 1997).

Endogenous ANP may help to maintain basal renal function in the normal foetal kidney (Silberbach et al., 1995). In addition to its vasodilator and natriuretic effects, ANP has antiproliferative effects. Infusion of CNP inhibits mesangial proliferation and matrix accumulation in a model of proliferative glomerulonephritis independent of haemodynamic changes (Wolf et al., 2000).

The developing kidney expresses an endogenous, functionally active kallikrein-kinin system (KKS). Gene expression of the KKS is activated post-natally and appears to be regulated primarily at the transcriptional level. Ontogenetic studies have revealed that the kinin B2 receptor is over-expressed in the developing rat kidney. As kinins are potent vasodilator and growth-promoting factors, it has been suggested that endogenous kinins

mediate developmental renal growth and differentiation and modulate the maturational changes in renal haemodynamics (el-Dahr, 1997).

All components of the renin-angiotensin system are highly expressed in the developing kidney. Antagonism of angiotensin (AT) suggests that the renin-angiotensin system may be an important contributor to renal ontogeny in the developing human. Maternal dietary protein restriction caused suppression of the foetal renin-angiotensin system, resulting in impaired structural and functional development (Woods, 2000). Pregnant women treated with ACE inhibitors for hypertension have an increased rate of foetal wastage, oligohydramnios, and intrauterine growth retardation; infants of ACE inhibitor-treated mothers have an increased incidence of severe systemic hypotension and anuria, and the kidneys have various structural abnormalities. Targeted mice, homozygous for a null deletion of the angiotensinogen gene, show a decreased maturity of glomeruli, together with renal vascular hypertrophy and increased glomerular matrix, associated with marked upregulation of TGFβ. Mice devoid of ACE showed a similar phenotype. Administration of an AT_1 receptor blocker in neonatal rats induced irreversible renal abnormalities, including tubulointerstitial inflammation, papillary atrophy and pelvic dilatation and a 42 % reduction in the number of nephrons. An increased incidence of congenital anomalies of the kidney and urinary tract was detected in mice deficient in the angiotensin II type 2 (AT_2) receptor. Impairment of AT_2 receptor function may cause pelvi-ureteric junction obstruction and primary obstructive megaureter, as this receptor is postulated to be involved in the development of the lumen of the ureter through apoptic resorption of cells. The AT_2 receptor was also shown to tonically decrease ACE activity in mice (reviewed by Hohenfellner et al., 1999).

ET-1, ANP, kinins and angiotensin II have been implicated in the regulation of renal medullary function; studies have shown that these vasoactive peptides either act alone or interact with each other to influence medullary/papillary blood flow and urinary water and sodium excretion (Cowley et al., 1995; Navar et al., 1996; Chou et al., 1990). In the cortex, angiotensin II and ET-1 act as vasoconstrictors to decrease renal blood flow and GFR, whereas kinins cause vasodilatation and increase glomerular capillary permeability. In the medulla, angiotensin II and ET-1 cause constriction of the outer medullary vasa recta and thereby decrease vasa recta and papillary blood flow, whereas kinins exert opposite effects (Navar et al., 1996). The renin-angiotensin-aldosterone system plays an essential role in salt and blood pressure homeostasis and is governed mainly by renin. Local factors (prostaglandins, NO, ET-1) produced in the immediate vicinity of the juxtaglomerular apparatus (JGA) affect renin secretion and renin gene expression. Prostaglandin E_2 and prostacyclin increase renin secretion and gene expression by activating cAMP produced in JG cells. The effect of NO on JG cells is not very clear but appears to stimulate renin via cAMP and cGMP-induced inhibition of cAMP-phosphodiesterase III. ET-1 inhibits renin by cAMP acting via calcium-phosphokinase C-related mechanisms (reviewed by Wagner et al., 1998).

The distribution of AT_1, ET_A, ET_B and kinin B2 receptors closely overlaps at several anatomical sites, including renal vasculature, glomeruli and the inner stripe of the outer medulla. In the cortex, the distribution of AT_1 and ET_B receptors is similar in the glomeruli and proximal tubules; kinin B2 receptor density is low in the cortex. In the inner medulla, ET_B and B2 receptors are abundant, whereas AT_1 receptors are not readily detected in this region. Effects of angiotensin II, ET-1 and kinins on cell proliferation and extracellular matrix synthesis in renal medullary interstitial cells imply an important interactive role in

chronic progressive renal disease (Zhuo et al., 1998). Angiotensin II upregulates the expression of growth factors and cytokines [TGFβ, TNFα, osteopontin, vascular cell adhesion molecule-1, nuclear factor-κB (NF-κB), PDGF, bFGF, IGF], most of which promote cell growth and fibrosis. Angiotensin II also stimulates oxidative stress, which may potentiate its vasoconstrictor effect partly by increased catabolism of NO. Angiotensinogen gene is stimulated by NF-κB activation. Angiotensin converting enzyme inhibitor inhibits NF-κB activation in renal disease (Klahr and Morrissey, 2000). Multiple vasoactive peptides interact to exert endocrine and/ or paracrine influence on renal medullary microcirculation, tubular function and mitogenesis; NO, ANP and kinins may counteract the vasoconstrictor and mitogenic effects of ET-1 and Angiotensin II.

CHAPTER 5

SUMMARY AND CONCLUSIONS

1. The kidneys play a pivotal role in maintaining body fluid and electrolyte balance and blood pressure homeostasis through actions of various humoral and paracrine/autocrine factors on renal medullary haemodynamics, tubular reabsorption and urine concentration. Multiple vasoactive peptides interact to exert autocrine and/ or paracrine influence on the renal microcirculation, tubular function and mitogenesis. ET-1, ANP, kinins and angiotensin II have been implicated in the regulation of renal medullary function; studies have shown that these vasoactive peptides either act alone or interact with each other to influence medullary/papillary blood flow and urinary water and sodium excretion. Local factors (prostaglandins, NO, ET-1) produced in the immediate vicinity of the juxtaglomerular apparatus affect renin secretion and renin gene expression. NO, ANP and kinins may counteract the vasoconstrictor and mitogenic effects of ET-1 and Angiotensin II.

2. Total body water (TBW) was measured in 5 transplant recipients immediately pre-transplant following dialysis, when they were assessed as having reached "dry" weight, namely showing no evidence of fluid retention. TBW was measured following transplantation and during rejection in 4 patients, when fluid retention was apparent, and again when kidney function had stabilised, demonstrating reduction in body weight and TBW. Effective renal plasma flow was measured in 6 transplant patients and was shown to be reduced in patients during rejection, a reflection of the increased vasoconstriction present in this condition.

3. The primary aim of my study was to determine circulatory and cellular actions of ET-1, ANP and kinins in human models of renal inflammation: in acute renal allograft rejection and renal parenchymal disease (mainly glomerular disease).

4(i) Plasma ET-1 levels were measured serially in patients prior to renal transplantation, post- transplantation when stable, during acute rejection and subsequently, after treatment of rejection. The highest levels of ET-1 were observed prior to transplant and in patients with chronic renal failure on dialysis. Following transplantation, ET-1 levels decreased, increased during episodes of acute rejection and decreased again after treatment of rejection. This corresponded with elevated urinary ET-1 levels during acute rejection, suggesting that ET-1 is produced by the kidney, specifically by inflammatory cells within the renal substance, reflecting endogenous renal ET-1 production. Increased plasma ET-1 levels were present in renal disease, compared to normal controls; the elevated plasma ET-1 levels were found in both proliferative and non-proliferative glomerulonephritis (GN), with significantly higher levels in proliferative glomerulonephritis. Both hypertensive and normotensive patients with glomerulonephritis had increased plasma ET-1 levels, with significantly higher ET-1 levels in hypertensive patients. Plasma ET-1 was elevated in both dialysis- and non dialysis-requiring patients compared to normal controls; plasma ET-1 was significantly higher in dialysis patients compared to patients not requiring dialysis. Urinary ET-1 levels were similar in patients with glomerulonephritis and controls.

4(ii) Immunocytochemistry, using immunoperoxidase and fluorescent methods, showed increased ET-1 labelling of proximal and distal tubules during rejection, mediated by the ET_A receptor as suggested by increase in ET_A receptor labelling in collecting ducts during

rejection. Endothelin-B receptor labelling was decreased in distal tubules and glomeruli during rejection. Immunocytochemistry showed increased ET-1 labelling of proximal and distal tubules in proliferative GN biopsies. Proximal tubular labelling was observed mainly in the brush border, as reported by Wilkes et al. (1991). ET_A receptor labelling was increased in distal tubules in proliferative GN. ET_B receptor labelling was decreased in tubules in GN biopsies. Further studies with larger sample numbers are required to confirm these findings.

4(iii) Immuno-electron microscopy revealed ET-1 and ET receptor labelling in epithelial cells of proximal and distal tubules, endothelial cells of blood vessels and glomerular capillaries, specifically in cytoplasm of cells, endoplasmic reticulum and mitochondria. Label was found in increased amounts adjacent to the intercellular system and within vacuoles and secretory vesicles. ET-1 is stored within these vesicles and released at the cell surface after an appropriate stimulus. The characteristic feature of acute rejection is infiltration of the graft by host mononuclear cells; these lymphocytes and macrophages infiltrate the interstitium and tubular epithelium. In this study, ET-1 label was also demonstrated in the mononuclear cells infiltrating the tubules during acute rejection by electron microscopy as well as peroxidase-antiperoxidase method. ET-1 mRNA was upregulated in the tubular epithelial cells and capillary endothelial cells, as well as the inflammatory infiltrate, during acute rejection as demonstrated by in situ RT-PCR.

4(iv) The down-regulation of the ET_B has not been previously reported in renal inflammation and is probably implicated in impaired natriuresis. This observation may account for the fluid retention and hypertension occurring with acute rejection and

glomerulonephritis: ET_B receptors have a potentially important hypotensive effect via inhibition of sodium and water reabsorption in the distal nephron. In vitro studies suggest that activation of ET_B receptors inhibits the activity of the renal epithelial sodium channel in the renal collecting duct epithelium. Activation of ET_B receptors leads to down-regulation of the epithelial sodium channel in the renal tubule via increased production of nitric oxide. Thus ET_B receptor activation has a hypotensive effect via promotion of renal sodium and water excretion. Activation of ET_A or ET_B receptors on vascular smooth muscle cells produces a pressor response through vasoconstriction, while activation of ET_B receptor on the vascular endothelium produces a depressor response by evoking the release of vasodilators. ET_B receptors on the vascular endothelium may play a role in clearing circulating ET-1, thereby reducing its predominant ET_A-mediated pressor actions.

4(v) Endothelin receptor antagonists have been shown to be reno-protective, that is to decrease blood pressure, improve renal function and reduce proteinuria in models of renal disease for example, combined $ET_{A/B}$ receptor antagonist in immune-complex nephritis (Gomez-Garre et al., 1996); ET_A receptor antagonist in murine lupus nephritis (Nakamura et al., 1995b); ET_A receptor antagonist and ACE inhibitor had an additive effect compared to either agent alone in accelerated passive Heymann nephritis (Benigni et al., 1998a); ET_A receptor antagonist (Nakamura et al., 1995c) and mixed $ET_{A/B}$ receptor antagonist (Benigni et al., 1998b; Hocher et al., 2001) in Streptozotocin-induced diabetes. Bosentan, a mixed ET_A/ET_B receptor antagonist, administered to patients with mild-moderate essential hypertension was as effective as enalapril (inhibitor of angiotensin converting enzyme) in controlling blood pressure, suggesting that ET-1 contributes to hypertension in these patients (Krum et al., 1998). Angiotensin II stimulates the expression of ET-1 gene in

endothelial and renal cells. Part of the mitogenic effect of Angiotensin II is mediated by ET-1. Data in animals with progressive nephropathies suggest that ACE-I, besides reducing glomerular protein traffic, also inhibit the exaggerated synthesis of ET-1 (Zoja et al., 1998). Chronic treatment with an ET receptor antagonist attenuated increases in glomerular mRNA levels of collagen, laminin, tumour necrosis factor, TGF-β, PDGF and basic fibroblast growth factor in diabetic rats (Nakamura et al., 1995c). Once substantial renal scarring occurs, there is an inevitable progression to end stage kidney disease, a process involving gradual glomerular sclerosis and interstitial fibrosis. Endothelin receptor blockade reduced proteinuria and glomerulosclerosis and protected against hypertension and elevations in serum creatinine in the 5/6 nephrectomy rat model (Benigni et al., 1993 and 1996).

5. Plasma and urinary ANP concentrations were significantly elevated during acute rejection, compared to normal control subjects in this study. Increased plasma ANP levels in acute rejection may be a response to increased intravascular volume and hypertension in these patients. ANP production may be stimulated by excess endothelin present. ANP stimulates kallikrein excretion transiently and is dependent on distal sodium delivery (Klein et al., 1989). Immunocytochemistry revealed decreased labelling of glomeruli and collecting ducts during acute rejection and glomerulonephritis; reduced immunolabelling of tubules was present in proliferative GN. This study extends knowledge of the widespread immunolocalization of ANP in the normal kidney. The decreased immunolabelling of the collecting ducts during acute rejection and glomerulonephritis may be a reflection of impaired natriuresis and diuresis, resulting in fluid retention occurring in these patients.

6(i) Urinary tissue kallikrein enzymic activity was significantly decreased in stable renal transplant recipients and in kidney donors after unilateral nephrectomy, compared to normal controls. This supports the findings of Spragg et al., (1985) that tissue kallikrein excretion rate may be a useful indicator of distal tubular mass. Serial measurement of urinary tissue kallikrein enzymic activity were made prior to kidney transplant, on day 3 or 4 post transplant when the patient was stable, during an episode of acute rejection and after treatment of rejection. Tissue kallikrein levels rose from their pre-transplant levels on day 3 or 4 after renal transplant, decreased again during rejection and rose again after treatment of rejection. Basal urinary kinin excretion was decreased in donors following nephrectomy and in stable transplant recipients compared to normal control subjects but rose significantly during acute rejection. Kinin generation in the urine was significantly decreased in donors post-nephrectomy and during acute rejection, compared to controls. The turnover of kinins depends on both the rate of formation and the rate of destruction. This finding may be a reflection of the reduction in tissue kallikrein as a consequence of decreased renal mass and renal inflammation. Confocal microscopy demonstrated decreased TK immunolabelling in the distal tubules during rejection.

6(ii) Urinary tissue kallkrein enzymic activity was significantly decreased in patients with renal disease compared to controls and was even further decreased during acute renal allograft rejection compared to renal disease. ELISA measurements confirmed the reduced urinary tissue kallikrein in renal disease. There was reduced TK immunolabelling in distal tubules in renal disease. While basal kinin levels in urine were similar in renal disease and controls, significantly decreased kinins were generated in the urine of patients with glomerulonephritis, reflecting the reduction in the kinin-producing enzyme TK during renal

inflammation. The decreased urinary kallikrein activity may be a reflection of reduced distal nephron function in these patients and may mediate the hypertension that accompanies renal disease.

6(iii) A previous study showed that the constitutive B2 receptor, present along the entire nephron, was down-regulated in renal disease. The B1 receptor was induced in the kidney in these patients (Naicker et al., 1999; Bhoola et al., 2001). Other studies provide evidence that reno-protection is mediated by the B2 receptor (Hirawa et al., 1999) and that kinins contribute substantially to the hypotensive effects of ACE inhibition (Gainer et al, 1998). Promising studies have shown that blood pressures of hypertensive patients can be temporarily lowered by oral administration of porcine pancreatic kallikrein (Overlack et al., 1981); infusion of tissue kallikrein via mini pumps has been shown to have beneficial effects by attenuating glomerulosclerotic lesions and tubular injury in hypertensive Dahl salt-sensitive rats (Uehara et al., 1990); a single injection of the human tissue kallikrein gene into the spontaneously hypertensive rat has been shown to reduce blood pressure for up to 10 weeks (Chao and Chao, 1997); sub-depressor doses of purified rat urinary kalikrein infused for 4 weeks in Dahl salt-sensitive rats fed a high salt diet resulted in decreased urinary protein excretion, increased GFR and attenuated glomerulosclerosis, arterial and tubular injury.

6(iv) Inhibitors of ACE, a member of the K-11 group of enzymes that rapidly inactivate kinins, and also convert the decapeptide angiotensin 1 to an octapeptide angiotensin 11, are probably the best examples of drugs acting on the kinin system that are used in clinical medicine. The mode of action of ACE inhibitors as antihypertensive agents have been

shown to be due to both the inhibition of angiotensin 11 (a potent vasoconstrictor) production, as well as an increase in circulating levels of kinins (Shimamoto et al., 1990). The hypotensive efficacy of these inhibitors has also been shown to correlate with the reduced activity of ACE in brain, kidney, and vascular smooth muscle (Unger et al., 1987). Angiotensin converting enzyme (ACE) inhibitors prevent the conversion of Angiotensin I to Angiotensin II and increase urinary kinin excretion, while the effect on plasma kinins is controversial with different studies showing an increase, a decrease or no change (Carretero and Scicli, 1981). The hypotensive effect of the ACE inhibitor, perindopril, in spontaneously hypertensive rats on low and high sodium diets was attenuated by the kinin B2-receptor antagonist HOE 140 (Bouaziz et al., 1994). The beneficial effects of ACE inhibitors may be related to the formation of nitric oxide and prostacyclin, enchanced by the kinins released (Linz et al., 1993). Icatibant acetate (HOE 140), a B2 receptor antagonist, given together with captopril, attenuated the hypotensive effect of captopril

7. Thus, as multiple mediators impact on renal disease, single agent therapy similarly has its limitations; exceptions are agents such as ACE inhibitors which block angiotensin II and affect ET-1, NO, prostacyclin and kinins.

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