2002

THE ROLE OF CELLULAR STRESS DURING COLD ISCHAEMIC AND REPERFUSION INJURY

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PHD

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Award date:
2002

Awarding institution:
University of Plymouth

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by

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A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY (PhD)

Department of Molecular Medicine
Peninsula Medical School

October 2002

For Mum & Dad

ABSTRACT

The elemental physiology of the highly complex and regulated cellular response to stress remains poorly understood. Hypothermia and reperfusion are necessary and unavoidable stresses associated with the procurement, storage and transplantation of organs such as the kidney. Signal transduction pathways and transcription factors are evolutionarily conserved mediators of stress responses. This project has investigated the activation of the transcription factors Nuclear Factor kappa B (NFκB), Activator Protein 1 (AP1) and the Heat Shock Factor 1 (HSF1) as well as the mitogen activated protein kinases (MAPK), p38, JNK and ERK 1 /2, during hypothermic and reperfusion stress in cultured endothelial cells (HUVECS) as a model of kidney graft endothelial cells. HUVECS were subjected to 72 hours of hypothermia at 4^0C in a renal preservation solution. For reperfusion experiments cells were returned to 37^0C after 30 minutes or 12 hours of hypothermia. NFκB was activated within minutes of a hypothermic insult, correlating with the phosphorylation of the p38 and ERK 1 /2 MAPKs ($p<0.01$). Inhibition of p38 had no effect on NFκB translocation, but inhibition of ERK 1 / 2 prevented subsequent NFκB activation ($p<0.01$). In contrast AP1 was not significantly up-regulated until 12 hours of hypothermia and HSF1 was down regulated during hypothermia. The downstream effects of NFκB activation were investigated by measuring the production of the inflammatory cytokines IL-6, IL-8 and TNFα. All three cytokines were up-regulated during hypothermia and reperfusion and the inhibition of NFκB with a decoy oligonucleotide reduced the expression of these cytokines. HUVECS were not killed by hypothermia with greater than 95% cell viability for 48 hours. Similarly DNA fragmentation, an event that occurs during apoptosis was not seen during hypothermic or reperfusion stress in HUVECS. There was a consistent expression of the mitochondrial anti-apoptosis protein BCL-2 during

hypothermia. HUVECS did not release von willebrand factor (VWF), a marker of endothelial dysfunction during hypothermia, however, cells did produce lactate after prolonged storage indicating a degree of hypoxia developed during hypothermic stress. To extrapolate the *in vitro* work to a clinical setting 17 preservation solutions were collected from cadaveric donor kidneys. Levels of lactate, VWF, IL-6, IL-8, TNFα and the anti-inflammatory IL-4 were measured. A large range in values was found for all markers in the 17 preservation solutions, however, no correlation was found between increasing cold or warm ischaemic times, early graft function or rejection with the markers chosen. IL-6 was significantly increased in donor kidneys that had experienced greater than 30 minutes warm ischaemia ($p<0.05$). The response of a donor kidney may be regulated by polymorphisms which the graft cells contain. Microsatellite polymorphisms in the NFκB and TNFα genes and restriction enzyme sites in the IL-6 and NAD(P)H genes were investigated in 50 donor DNA samples. No correlation was found with rejection episodes with any of these polymorphisms. However, in the 17 preservation solutions, donors with the IL-6 allele which is linked to high protein production had higher IL-6 levels than donors with the low production allele. The role of IL-6 in transplantation remains unclear. However, this study has demonstrated that IL-6 may be a useful marker of stress. The ability to block cytokines by inhibiting transcription factors such as NFκB may have a therapeutic potential in ischaemic injury.

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ACKNOWLEDGEMENTS

I would like to thank Dr Andy Demaine and Dr Peter Rowe for their support throughout this project and for giving me the opportunity to study for a PhD.

I would like to thank the following staff at Derriford Hospital who have helped me collect data and samples.

Dr Ed Kaminski, Katherine Poole - For Donor DNA samples and data

The Transplant co-ordinators and Transplant surgeons
Mr Shaw, Mr Wilkin, Mr Akoh - For collecting the preservation solutions

Claire West - For all donor and recipient clinical data

I would like to thank all my friends 'the lab rats' past and present

Angela, Debbie, Nicole, Alison, Anna, Mo, Karen, Derek, Mark C.
Martin, Toby, 'Rohita', Nick, Ajith, Annwyne, Lisa, Mark P, Doha, Liz, Magda, 'Sparky'.

Thanks to Jane for the Birthday Cakes!

Dr Hongxin Zhou for advice and help with Statistics

Special thank you to Dr Bingmei Yang for always having time to help

A very special thank you! to Dr Andrea 'Hodgy' Hodgkinson for everything

AUTHOR'S DECLARATION

All of the studies presented in this thesis were performed by the author. At no time during registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

Signed: 

Date: 30/01/03

WORKSHOPS, CONFERENCES AND PUBLICATIONS

WORKSHOPS

The Biochemical Society, Signal transduction and homeostasis workshop. 11th –13th July 2001. Trinity college, Dublin.

British transplantation society and British society for histocompatibility and immunogenetics. 1999 summer school in transplantation. 5th –7th October 1999. Chancellors conference centre, University of Manchester.

CONFERENCES

Roberts JR, Rowe PA, Demaine AG: Analysis of IL-6 cytokine levels in renal preservation solutions after static storage.
The British Transplantation Society. 17th –19th April 2002. Robinson's college, Cambridge. Presented as a poster.

Roberts JR, Rowe PA, Demaine AG: NFκB is activated via the p38 MAP Kinase pathway in endothelial cells
The Renal Association Spring meeting 2001. 3rd-4th April 2001. East midlands conference centre, Nottingham. Presented as a poster.

Roberts JR, Rowe PA, Demaine AG: Transcription factor activation during hypothermic stress.
The Renal Association 50th Anniversary meeting. 27th-29th September 2000. St John's College Cambridge. presented as a poster.

Roberts JR, Rowe PA, Demaine AG: Interleukin-6 as a marker of reperfusion stress and injury.
The Renal Association Spring 2000 meeting. 3rd –4th April 2000. Royal College of Physicians, London. Presented as an oral presentation.

PUBLICATIONS

Roberts JR, Rowe PA, Shaw JF, Akoh J, Wilkin D, West MCT, Demaine AG: Cytokine levels in preservation solutions from donor human cadaveric kidney transplants. *Neph Dial Trans* (Submitted).

Roberts JR, Rowe PA, Demaine AG: Activation of nuclear factor kappa B and mitogen activated protein kinase cascades by hypothermic stress in endothelial cells. *Cryobiology* 44(2): 161-169, 2002

O'Reilly DA, Roberts JR, Demaine AG, Kingsnorth AN: Nuclear factor –κB and heat shock factor-1 are systemically activated in human acute pancreatitis. *Br J Surg* 88 (Suppl 1): 14, 2001

LIST OF ABBREVIATIONS

ADCC	Antibody Mediated Cell Cytotoxicity
AIP	Apoptosis Inducing Protein
AP1	Activator Protein 1
APAF	Apoptotic Protease Activating Factor
APC	Antigen Presenting Cell
ATP	Adenine Tri-Phosphate
B-cell	B-lymphocyte
C	Complement protein
Ca^{2+}	Calcium ion
CAPD	Continuous Ambulatory Peritoneal Dialysis
Cl^-	Chloride ion
CMV	Cytomegalovirus
DAG	1,2-diacylglycerol
DD	Death Domain
DGF	Delayed Graft Function
ELISA	Enzyme Linked Immunoabsorbant Assay
EMSA	Electrophoretic Mobility Shift Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ESRF	End-Stage Renal Failure
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor

GEF	Guanine nucleotide Exchange Factor
gp	Glycoprotein
H_20	Water
H_20_2	Hydrogen Peroxide
HLA	Human Leukocyte Antigen
HPK	Haematopoietic Progenitor Kinase
HSF	Heat Shock Factor
HSP	Heat Shock Protein
HTK	Histidine-Tryptophane-Ketoglutarate
HUVEC	Human Umbilical Vein Endothelial Cell
IAP	Inhibitor of Apoptosis
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP3	1,4,5-trisphosphate
ITAM	Immunoreceptor Tyrosine-based Activation Motif
iNos	Inducible Nitric Oxide Synthase
IκB	Inhibitor Kappa B
JNK	c-Jun N-terminal kinase
K^+	Potassium ion
KIRs	Killer Inhibitory Receptors
KDa	Kilo Dalton
L	Ligand
LFA	Lymphocyte Functional Antigen

MAbs	Monoclonal Antibody
MAC	Membrane Attack Complex
MAPK	Mitogen Activated Protein Kinase
Mg^{2+}	Magnesium ion
MHC	Major Histocompatibility Complex
MMP	Matrix Metaloproteinase
Na^{2+}	Sodium ion
NFAT	Nuclear Factor of Activated T-cells
NFκB	Nuclear Factor kappa B
NK	Natural Killer cell
NO	Nitric Oxide
ns	Non-significant
NSAID	Non-steroid Anti-inflammatory Drug
ODN	Oligodeoxynucleotide
PAI	Plasminogen Activator Inhibitor
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
RANTES	Regulated by Activation, Normal T-cell Expressed and Secreted
RIP	Receptor Interacting protein
RNA	Ribonucleic Acid
ROI	Reactive Oxygen Intermediate
ROS	Reactive Oxygen Species

RRT	Renal Replacement Therapy
RTK	Receptor Tyrosine Kinase
SAPK	Stress Activated Protein Kinase
SH	Src Homology domain
SOD	Superoxide Dismutase
TBP	TATA Binding Protein
Tc-cell	T-cytotoxic cell
T-cell	T-lymphocyte
TCR	T-cell Receptor
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF	Transforming Growth Factor
Th-cell	T-helper cell
TIC	Transcription Initiation Complex
TNFα	Tumour Necrosis Factor alpha
tPA	Tissue-type Plasminogen Activator
TRAF	TNF Receptor Associated Factor
UKTSSA	United Kingdom Transplant Special Support authority
UNOS	United Network for Organ Sharing
UV	Ultra Violet
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen
VNTR	Variable Number Tandem Repeat
VWF	Von Willebrand Factor

CHAPTER ONE: GENERAL INTRODUCTION

1.1. A Brief History of Renal Transplantation

The first description of a transplant dates back to the fifteenth century with the legend of Saint Cosmos and Saint Damien. Paintings from this time show the two Saints removing a diseased leg from a sleeping man and replacing it with a healthy leg that they have removed from a dead man. The 'laws of transplantation' were formulated by Little and Tyzzer at the beginning of the century have been paraphrased as 'Isografts succeed, allografts fail' (Halloran *et al* 1993). The two major periods of interest in the development of the transplantation of organs for the treatment of disease were in the early 1900's and later in the 1950's. At the beginning of the century surgical techniques were vastly improving and the establishment of the method of suturing by the Nobel Prize winner Alex Carrel in 1902 accelerated interest in vascular surgery. In the same year Emerich Ullman from Vienna demonstrated the first autotransplant of a kidney, when he removed the kidneys from a dog, and replaced one by implanting it in the neck area. In 1905, Carrel repeated Ullman's experiment but without success and Alfred Von Decastello demonstrated the first allograft dog-to-dog transplant. Small amounts of urine were passed but all transplanted animals died within hours of the operations. In 1906 Mattieu Jaboulay carried out two xenotransplants from pig and goat donors in to the arms or thighs of human patients with renal failure. The grafts were reported to have functioned for 1 hour. In 1909 Ernst Unger attempted a dog-to-dog transplant that was successful for 14 days. He performed many such transplants, including an unsuccessful monkey-to-human transplant. In 1923 Carl Williamson examined a failed kidney transplant, and was the first to use the term 'rejection' when describing the appearance of the kidney. This early work established the technical possibility that kidneys could be transplanted, but the uncertainty as to why the grafts were rejected led to a diminished interest in renal transplantation.

Before the revival of interest in transplantation the soviet surgeon Yu.Yu Voronoy carried out 6 transplants including the first recorded human allograft transplant in 1933. The patient died 2 days later. An important discovery was made between 1936 and 1937 by George Snell in the US and Peter Gorer in the UK, who used inbred strains of mice to transplant tumours and found that tumours grew in mice of the same strain, but were rejected by other strains. Snell identified the H2 'histocompatibility' gene in mice, which was associated with strong rejection of tumours. Later Gorer demonstrated that the H2 gene was in an identical locus to the human blood group antigen II. During the war years the large number of casualties dying from renal failure and requiring skin grafts, due to burns, intensified the need to understand tissue rejection. In the late 1940's and early 1950's surgeons Kuss, Sevelle and Dubost from Paris and David Hume from Boston attempted allografts without immunosuppression. No sustained function was achieved in any cases. During this time, Nobel Prize scientists Peter Medawar and MacFarlane Burnet produced pioneering work on the function of the immune system in transplantation using skin grafts from genetically disparate mice. It was established that grafts from disparate mice are rejected, whilst mice were tolerant to grafts from the same strain. They also showed that the immune system has a memory function. In 1944 the 'artificial kidney' was first used to treat acute renal failure by Willem Kolf. In the UK, artificial kidneys were first used at the Hammersmith Hospital, London in 1948. The increased interest in the kidney led to the formation of the Renal Association in 1952. In 1954, Joseph Murray performed the first isograft transplant between identical twins in Boston. The graft survived for 7 years and is widely regarded as the first truly 'successful' transplantation of a kidney in humans. The identification of the human leukocyte antigens (HLA) in 1958 by Dausset and Hamberger, led to the proposal that histocompatibility systems were common to all mammals.

Between 1959 and 1960 in Boston and Paris the first attempts at immunosuppression were carried out. These involved total body irradiation, but results were poor. In a 1959 paper by Dameshek and Schwartz, it was found that rabbits treated with the anti-cancer drug 6-mercaptopurine (6-MP) developed a poor immune response to foreign protein. Roy Calne saw the potential for 6-MP and used it as immunosuppression in dog allografts with improved survival. Calne and colleagues developed a less toxic derivative of 6-MP, known as BW57-322, (later Imuran/Azathioprine). During the next few years, 6-MP was used with total irradiation in a number of transplants with some success. In 1961 Imuran became available for human use. Improvements in dialysis methods in the mid 1960's allowed better preparation of patients and the introduction of immunosuppression encouraged the use of transplantation. Terasaki and colleagues, cross-matched donor cells with recipient serum and proposed leukocyte grouping as a basis for patient selection in 1965. In 1966 Bach and Voynow designed the mixed lymphocyte culture assay, which was based on stimulating the recipients lymphocytes with donor lymphocytes to identify positive cross-matches. The first international congress on transplantation was held in 1966. The 'London Transplant Club' was set up in 1971. The co-operation of John Hopewell from the Transplant Club and Roy Calne from the Immunological Society, led to the formation of a 'transplantation committee' of the Immunological Society, which held an inaugural meeting at the Royal Free Hospital, London, in 1972 chaired by Sir Peter Medawar. This committee later became the British Transplantation Society.

The 1970's brought the establishment of transplantation as the choice treatment for patients with end stage renal disease. This was the result of increased public awareness leading to improved cadaveric donation, the establishment of brain death, improvements in HLA typing methods, organ sharing schemes, and the important discovery by Opelz that regular blood transfusion during dialysis improved outcome. In the late 1970's a major advance in

immunosuppression was made with the discovery of a new immunosuppressive agent, cyclosporine. Cyclosporine was available for general use in 1982, and in conjunction with azathioprine and corticosteriods became the common regime for transplant immunosuppression.

1.2 The basic function of the kidney

The kidneys have both excretory and hormonal functions. The prime function of the kidney is the excretion of waste, water and electrolytes. The kidneys also synthesise hormones such as erythropoietin and renin and regulate insulin metabolism. The kidney is a vital organ subserving the diverse metabolic requirements of the body systems.

The basic functional unit of the kidney is the nephron. An adult kidney contains between 850,000 and 1,000,000 nephrons. A nephron consists of five main sections, the glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct leading to the bladder. The kidney achieves the regulation of homeostasis by three main processes. Firstly filtration of plasma at the glomerulus, followed by selective absorption and reabsorption by the tubules and production of ammonia and finally excretion of urine. Plasma is forced through the glomerulus by the hydrostatic pressure of the blood. The glomerular basement membrane does not allow macromolecules to pass through and hence the glomerular filtrate is a soluble concentrate containing solutes such as glucose and amino acids, and ions such as sodium, potassium, chloride, calcium, phosphorus and magnesium. The glomerular filtrate also contains waste products such as creatinine, urea and urate as well as acidification products such as bicarbonate and hydrogen ions. The net filtration rate of the glomeruli over 24 hours should be approximately 180 litres. The filtrate then enters the proximal tubule, which is the main area of reabsorption of glucose, amino acids and most electrolytes. The proximal tubule also absorbs 65-70% of the water from the glomerular

filtrate. The process of reabsoption in the proximal tubule is an active (energy requiring) event. The purpose of the proximal tubule is to prevent loss of fluid and solute. The filtrate then enters a thin loop structure with a descending and ascending limb, known as the loop of Henle. The loop of Henle is essentially a counter-current system that reabsorbs sodium and results in the eventual formation of hypotonic urine. The descending limb is freely permeable to water and sodium and the ascending limb is impermeable to water. As water diffuses out of the descending limb, the urine becomes more concentrated at the bottom of the loop. The ascending limb is the site of numerous sodium pumps that remove sodium from the urine into the medulla. The countercurrent system is achieved by this passive diffusion of water in the descending limb resulting in a hypertonic solution, an isotonic solution at the bottom of the loop, where the two limbs meets, and the removal of sodium ions in the ascending limb resulting in a more hypotonic solution at the top the loop where it joins to the distal tubule. The distal tubule plays a vital role in feedback from the tubules to the glomerulus and blood system (tubuloglomerular feedback). This feedback is vital in controlling ultrafiltration rates in the glomerulus. Cells in a region of the distal tubule known as the *macula densa*, can sense changes in glomerular filtration rate or sodium and chloride ion concentrations and produce vasoactive substances such as adenosine and prostaglandins that effect the haemodynamics of the glomerulus. Similarly the distal tubule controls the secretion of renin, which is a proteolytic enzyme that removes angiotensin from angiotensinogen and forms angiotensin II, the potent vasoconstrictor. The other function of the distal tubule is the secretion of hydrogen ions and the formation of ammonia. Ammonia is formed by the production of glutaminase by tubule cells, which converts glutamine to glutamate, a process that liberates ammonia. Ammonia binds to hydrogen ions to form the ammonium cation. The distal tubule is also sensitive to the anti-diuretic hormone (ADH), which is secreted by the pituitary gland in response to a message

from the hypothalamus, which contains osmoreceptors that react to hypo-osmotic blood. ADH acts by increasing the permeability of the distal tubule and collecting duct, allowing water to re-enter the blood.

The maintenance of fluid balances and the secretion of waste products and hydrogen ions are vital in maintaining homeostasis. A reduction in glomerular filtration rate would lead to a reduced excretory capacity. Any reduction in urine output by the kidney is a serious problem for the maintenance of internal pH, fluid balance and availability of solutes in the body.

1.3 Recipients of kidney transplants.

The progressive loss of kidney function leads to renal insufficiency, which may develop to advanced end stage renal failure (ESRF). The major causes of ESRF are autoimmune disease such as glomerulonephritis, systemic disease such as diabetes and inherited conditions such as polycystic kidney disease (See Table 1.1). Chronic renal failure is the result of long standing nephron loss and damage leading to glomerular sclerosis and uraemia. Acute renal failure is the sudden and often reversible loss of kidney function leading to the rapid accumulation of nitrogenous waste such as urea or creatinine and an inability to regulate electrolytes. Many substances can cause acute renal failure due to their nephrotoxic properties including heavy metals (lead, mercury), certain antibiotics (gentamicin) and alkylating agents (streptozotozin). Renal failure can also occur as a consequence of heart disease, sepsis, anaphylactic shock or liver disease. The loss of kidney function leads to the need for renal replacement therapy (RRT). One means of RRT is dialysis. Dialysis is the process of filtering the blood, hence removing fluid, toxins and waste materials. Dialysis via the blood is known as haemodialysis, and patients require 4-6 hours, 3 times a week. Continuous ambulatory peritoneal dialysis (CAPD) is

Autoimmune Diseases	Systemic Diseases
Glomerulonephritis	Lupus (SLE)
Pyelonephritis	Vasculitits
Diabetes	Amyloidosis

Other	Inherited Disease
Infection (Cystitis)	Polycystic Kidneys
Obstruction (kidney stones)	
Disease of renal blood vessels	
Hypertension	

Table 1.1. Common reasons for the development of chronic ESRF leading to the need for RRT (Data from the UK Renal Registry 2001).

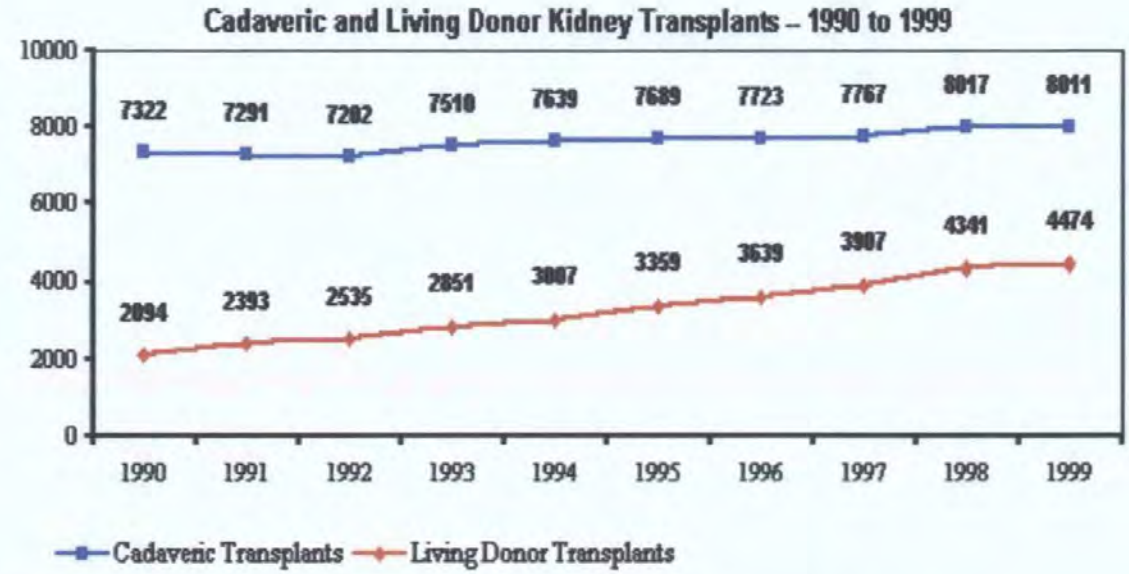
performed via a permanently fitted tube in the abdomen. Approximately 2 litres of dialysis fluid is passed through the abdomen, 4 times a day. CAPD is less efficient but allows the patient to perform dialysis at home. Dialysis can not replace the hormonal functions of the kidney, although a major improvement in the quality of life for dialysis patients has been the introduction of synthetic erythropoietin (Evans *et al* 1990).

A second means of RRT is the transplantation of a kidney from a donor, thus restoring organ function more effectively than dialysis. Kidney transplantation is the most common allograft organ transplantation. World wide in 1997 there were an estimated 24,200 renal transplants (Van Den Berghe 1998). Transplantation is the choice treatment for patients with end stage renal failure as it greatly improves the quality of life for the patient and reduces the substantial cost of dialysis to the health service. According to the united network of organ sharing (UNOS) in the US, dialysis costs $70,000 a year. Transplantation in the first year costs the same, but subsequent care costs $12,000 a year (UNOS 2000). Transplantation offers the chance of returning to a near normal lifestyle. It has been shown that 91.6% of successful transplant recipients are able to return to full time or part time work (Jacobs *et al* 1977). In the US, 75% of patients receiving a successful cadaveric transplant return to work, whereas only 25-59% of dialysis patients are employed (Evans *et al* 1985, Manninen *et al* 1991). In a subjective assessment of the quality of life for transplant patients compared to dialysis patients, as measured by eating and sleeping behaviour, symptoms, general satisfaction and happiness, it was found that 79% of patients with a transplant felt that they function at a near normal level compared with 47%-59% of dialysis patients (Evans *et al* 1990). According to data in the UK, one-year patient survival for patients on RRT in 2000 was 83.7% for dialysis compared with 97.3% for transplant patients (UK Renal Registry report 2001).

1.4 Waiting lists and organ donation.

In 1990 there were 3668 people on the kidney transplant waiting list in the UK. In 1998 this had risen to 5693, with only 1637 cadaveric organs retrieved from donors and in 2001 there were 2,339 kidney transplants with 6284 people on the waiting list (UK Transplant 2002). There has been a steady increase in the number of living donors, with 183 in 1996, 245 in 1998 and 358 in 2001 in the UK (UK Transplant 2002). However, in Europe, living related donors make up only 9% of all donors, whereas in the US, 30% are from live donors (UNOS) (Fig 1.1.1). The majority of cadaveric donors are in the 18-59-age range. Improved safety in the motoring industry has resulted in a reduced amount of donors from road traffic accidents, with 29% in 1989 and 16% in 1998 (UK Transplant). The largest proportion of donors are from cardiac arrest deaths (61%), with natural causes and trauma constituting the final 23% (Fig 1.1.2). With organs in such short supply, attention is now centring on increasing living related donors, as well as making best use of cadaveric organs when available. In the US, an average of 3.68 organs are removed from each cadaveric donor (Van Den Berghe 1998). The system in the US for organ donation is different from the UK and mainland Europe. The US has a system of required consent, whereby the next of kin must give permission for an organ to be removed with no expectation that a donation will result. In the UK, the current system for organ donation relies on 'opting in' or required consent. This means that organ donation is a voluntary act and individuals can opt to be a donor by registering their intentions or by carrying a donor card, which states their intentions. The next of kin is still approached to give consent for an organ to be removed, but are approached without expectation of donating organs. In certain European countries such as Spain and Belgium a system of 'opting out' or presumed consent has been legislated. This means all individuals are presumed to be donor unless they have registered

A.



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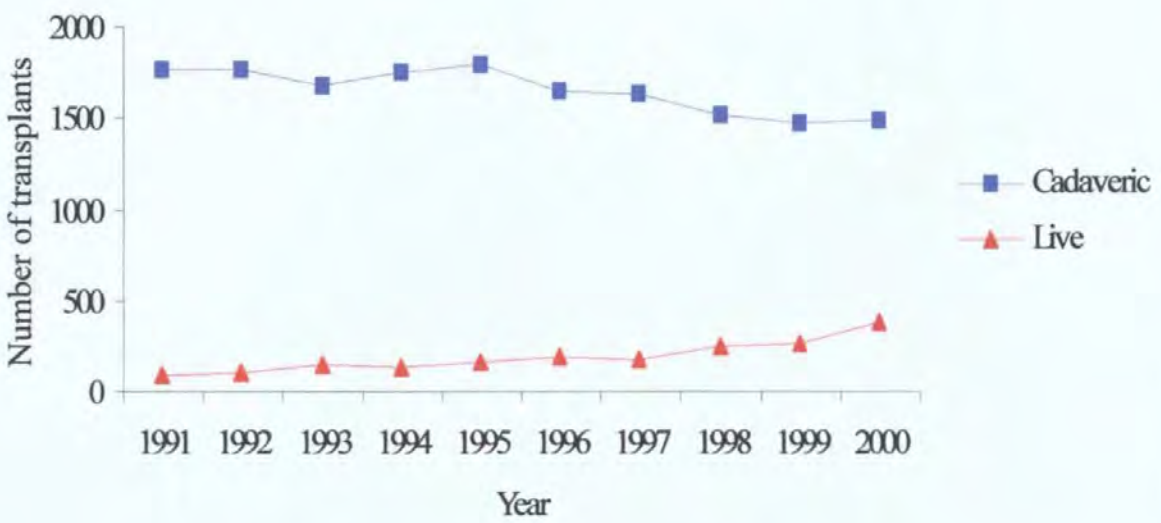
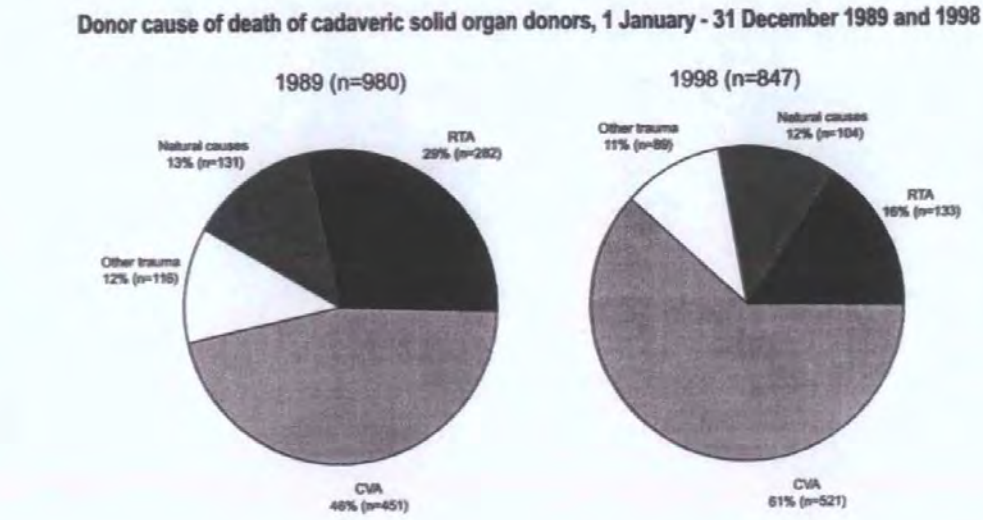


Figure 1.1.1. The increase in living donors in the US over the last decade to numbers approaching cadaveric donors (A). Estimates suggests more than 30% of donors in the US are living compared to only 9% currently in Europe (data from the UNOS registry). In the UK, there has been a gradual increase in living donors (B) (data from UK Transplant 2002).



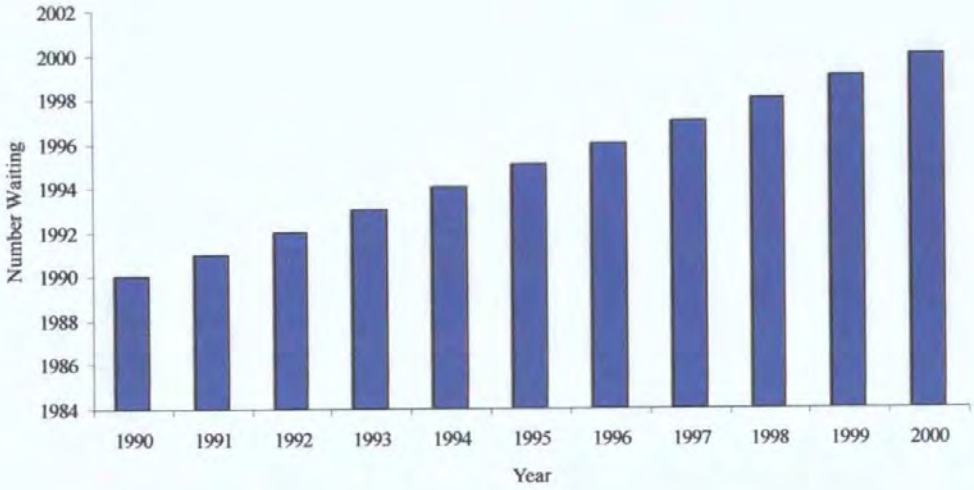


Figure 1.1.2. The reduction in donors from road traffic accidents has been reflected by the increase in waiting lists for renal transplants in the UK over a ten-year period (Data from UK Transplant).

their wish not to donate. The next of kin are therefore approached with the expectation of receiving consent to remove organs. Presumed consent has increased the donation rates in countries such as Germany and Italy that have adopted this system within the past 5 years (Rev by Andrews 2002). Another means of increasing the donor pool would be to use a greater number of marginal donors. Marginal donors are from donors with factors which may cause the kidney to function poorly including; extremes of age (<14 years or >65 years), diabetic or hypertensive/hypotensive donors, donors with primary brain tumours and donors with impaired renal function. Non-heart beating donors (NHBD) represent a further pool of donors. Normally donors will remain ventilated until the organs are removed. NHBD have organs retrieved without ventilation and suffer from increased graft damage as a result. It has been suggested that the use of more marginal donor and NHBDs can increase the organ donation rate by 20-40% (Cho *et al* 1998, Koostra 1997, Andrews 2002). However, the higher number of transplants may be matched by poorer outcomes.

1.5 Kidney allocation

Kidneys are allocated in the UK on the basis of a 3-tier system of priority (Rev by Fuggle *et al* 2001). In each tier, priority is first given to paediatric patients and then adults and local patients before national patients (Fig 1.2). Tier 1 represents 000 mismatched patients and tier 2 represents favourably matched patients with 100, 010 or 110 mismatches. Tiers 1 and 2 give priority to DR-homozygotes before DR-heterozygotes if the donor is a DR-homozygote as well. Tier 1 also gives priority to highly sensitised patients. Tier 3 represents non-favourably matched patients that are normally allocated a kidney from the nearest local transplant unit, depending on the units own policy for allocation or sharing.

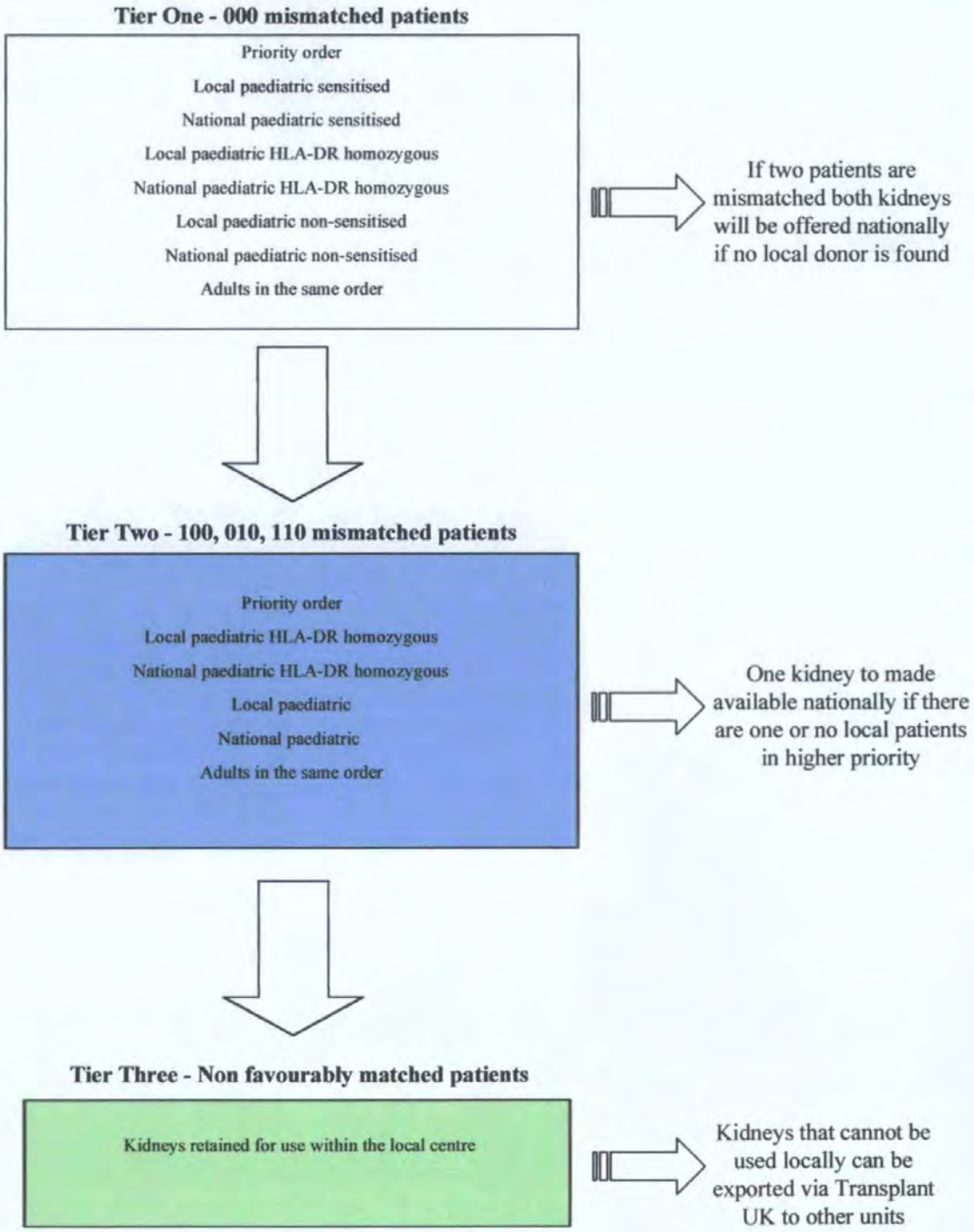


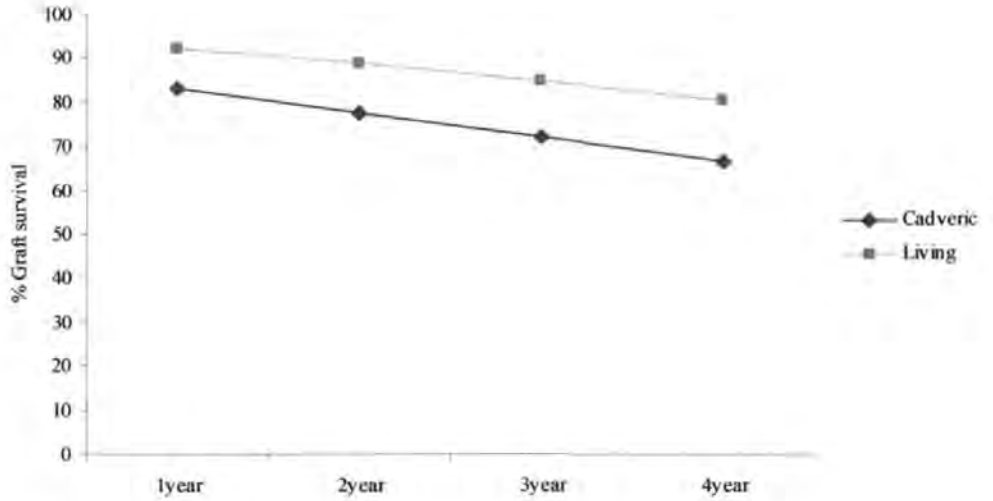
Figure 1.2. The kidney allocation system in current use in the UK (Adapted from Fuggle *et al* 2001).

Within each Tier, patients are matched for blood group by the ABO system. This means that a patient with a more common blood group is more likely to receive a transplant than someone with a less common group. Currently in the UK the most common blood group is O, which accounts for 45% of the population. 43% of the population are group A and groups B and AB account for 9% and 3% respectively (data from the National Blood Service). This is reflected by the waiting list which had 50% group O, 30% group A, 17% group B and 3% group AB in 1998 (UK Transplant). 84% of the population are rhesus positive and 16% rhesus negative. However, allocation does not take account of rhesus antigens.

1.6 Transplant Success

One-year graft survival rates for cadaveric grafts are currently above 90%, with ten-year survival now at 60% (UK Transplant 2002). One year patient survival is currently 95%, with five-year rates now at 87% and ten year survival rates now above 65% (Parrott 1995, UK Transplant 2002). Monozygotic twins have the best graft and patient survival rates with a 55% graft survival rate at 25 years (Tilney 1986). The longest surviving and functioning cadaveric transplant is 36 years which was received by a 19 year-old in 1966, and the longest surviving live donor transplant is 39 years, which was received by a 39 year-old in 1963 (UNOS 2002). Living related donors have better survival rates for both the graft and patients then cadaveric donors (see fig 1.3). One-year graft survival is 97.3% in live donors compared with 93.7% in cadaveric donors and four-year graft survival is 93.8% in live donors compared with 84.9% in cadaveric donors (UNOS 2002). The reason for this is due to the better HLA match between donor and recipient as well as living related donor sharing common 'minor' antigens with the recipient that are not matched by routine tissue typing. Living donor transplants also benefit from minimal cold ischaemic

A.



B.

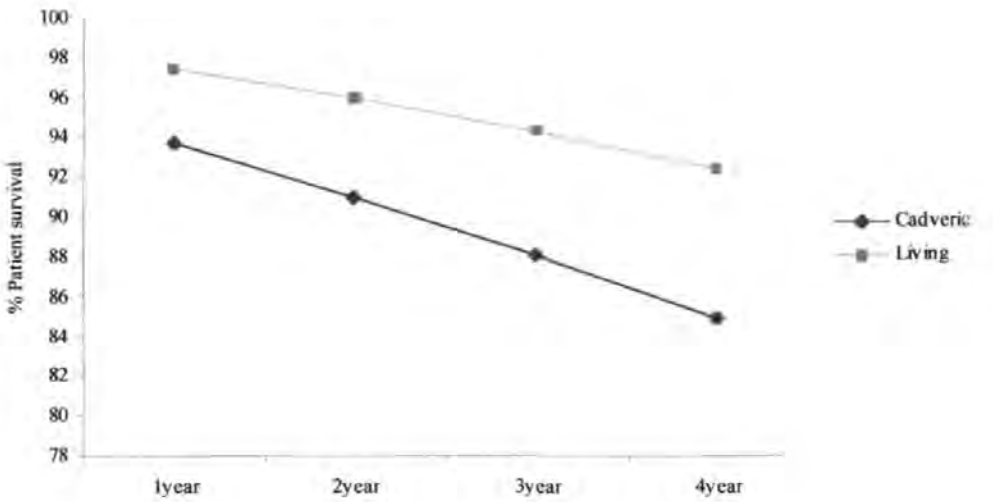


Figure 1.3. Success rates in living donors are higher for both graft (A) and patient (B) survival compared with cadaveric renal transplants. Data from UK Transplant.

times. It has been found that a well-matched cadaver organ with a minimal cold ischaemic time will function as well as living related donor (Cicciarelli & Terasaki 1991). With modern immunosuppression there is also evidence to suggest non-related living donors (e.g. spousal) can function as well as cadaveric donor organs (Terasaki *et al* 1985, Opelz *et al* 1993). Independent analysis of risk factors for outcome of primary cadaveric transplants in the UK, found a significant relationship between matching, donor and recipient age, cold ischaemia time and a centre effect in 6363 primary cadaveric transplants carried out in the UK between 1986 and 1993 (Morris *et al* 1999).

1.7 The Immune Response

The immune response involves an innate or 'nonspecific' response, mediated by phagocytes, natural killer cells and plasma enzyme systems which functions to prevent the entry of potential pathogens into the body. Innate defences include the protective outer surface of the skin and secretions such as mucus (mucocilliary escalator), tears (lachrymal glands) and urine (urinary flush). The bodies internal defences include the acidic environment found in potential entry sites for pathogens including the stomach, commensals and skin. Non-specific cells of the innate system act as scavenger cells as they can detect a wide range of antigens, internalise and destroy with lysosomal enzymes any potential pathogen.

1.7.1 Leukocytes

Phagocytes are scavenger leukocytes, which are commonly one of three cell types, macrophages, monocytes or polymorphonuclear neutrophils (PMNs). PMNs are further split into three classes, neutrophils, eosinophils and basophils. Phagocyte cells are differentiated into two main types depending on whether or not they present antigen to T-

cells. Neutrophils do not act as antigen presenting cells (APCs) but they have the ability to migrate into tissue. When a pathogen is engulfed by a neutrophil it internalises and enzymatically digests it. After encountering a pathogen neutrophils die. Generally, phagocytic cells have a short life span of 2-3 days in the circulation. When in the circulating blood phagocytes are known as monocytes, however, when migrating into a tissue, monocytes differentiate into mature antigen presenting macrophages. Monocytes have a longer life span than other phagocytes such as PMN cells.

1.7.2 Antigen presenting cells (APCs)

APCs present antigen to T-cells and B-cells via class I or class II MHC expression. Macrophages, dendritic cells and langerhan cells are the most common APCs, but cells such as endothelial and epithelial are able to act as APCs in certain circumstances such as during an inflammatory or active immune response. APCs present antigen by one of two mechanisms depending on whether they are presenting via the class I or class II MHC molecule. During antigen presentation via the class II pathway an antigen is first internalised by endocytosis. The pathogen is then degraded by proteolysis within the endoplasmic reticulum. Processed antigen peptides are then displayed within the class II receptor with proteins such as CLIP and DM facilitating the loading of peptides into the MHC groove in a structure known as the MIIC compartment. The MHC receptor is then exocytosed to the membrane, where they are displayed to the T-cells in the surrounding environment of the APC. Class I antigen presentation occurs within the cell where internal antigens and 'self' peptides are placed in the class I binding groove by TAP-1 and TAP-2 transporters. The class I receptor is then expressed on the cell surface with the peptide on display to circulating $CD8^+$ T-cells.

Cell	Features
T-cell	Lymphocytes from the thymus. 15% of circulating leukocytes. Many functions including inducing cytotoxic killer activity and antigen presentation
B-cell	Develop in the fetal liver and bone marrow.1-3% of circulating leukocytes Divide into mature plasma cells secreting immunoglobulin.
Natural Killer cell	3% of circulating leukocytes. Involved in Antibody dependent cell cytotoxicity (ADCC) via numerous receptors including the Fc receptor.
Macrophage	Vital for antigen presentation. Phagocytes which account for 5% of leukocytes.
Neutrophil	65% of circulating leukocytes, contain granules. Involved in phagocytosis and ADCC.
Eosinophil	2-5% of circulating leukocytes, also contain granules and are involved in phagocytosis.
Basophil	Less than 0.2% of circulating leukocytes. Important in allergy.
Mast cell	Inflammatory mediators. Found throughout the body's tissues.

Table 1.2. A list of the major cells of the immune system and their basic features. Adapted from Van de Berghe 1999, Male 1998

1.8 Complement

The complement system is a group of serum proteins that function in the innate immune response, but also co-operate with components of the adaptive response. The complement system was first described in 1900 by Jules Bordet.

The major function of the complement proteins is lysis of cells that are coated with antibody by puncturing the cell membrane. Complement also co-ordinates with the specific immune response by combining with antibody and natural killer (NK) cells to attack cells displaying viral or bacterial peptides in the antibody dependent cell cytotoxic (ADCC) reaction. Complement proteins also mediate phagocyte recruitment into inflamed tissue, by coating target cells (opsonisation). Phagocytes have a receptor for complement proteins known as CR1. When complement binds to a target cell it displays C3b and/or C4b on the cell surface, which bind CR1 and attract phagocytes to these target cells. There are collectively 17 proteins in the complement system that function in a cascade fashion, and account for a large amount of the globulin fraction of serum. Complement proteins exist in an inactive state in the plasma and serum. There is an order of activation by immunoglobulins, with complement more reactive to IgM then IgG. When complement proteins bind to an antigen-antibody complex to lyse the cell the reaction is known as complement fixation. This has been the basis of the complement fixation test, where an antigen-containing serum is incubated with test serum. If the serum contains antibodies against the test antigen, the antigen it will bind to the antibody. Complement will then 'fix' to the antigen-antibody complex when added. Red blood cells and anti-erythrocyte antibodies are then added and if complement has been fixed the cells will lyse (negative result) which can be visualised.

Complement can be activated via two pathways, the classical and alternative pathways. Both result in the cleavage of C3 to C3a and C3b, which is mediated by a convertase

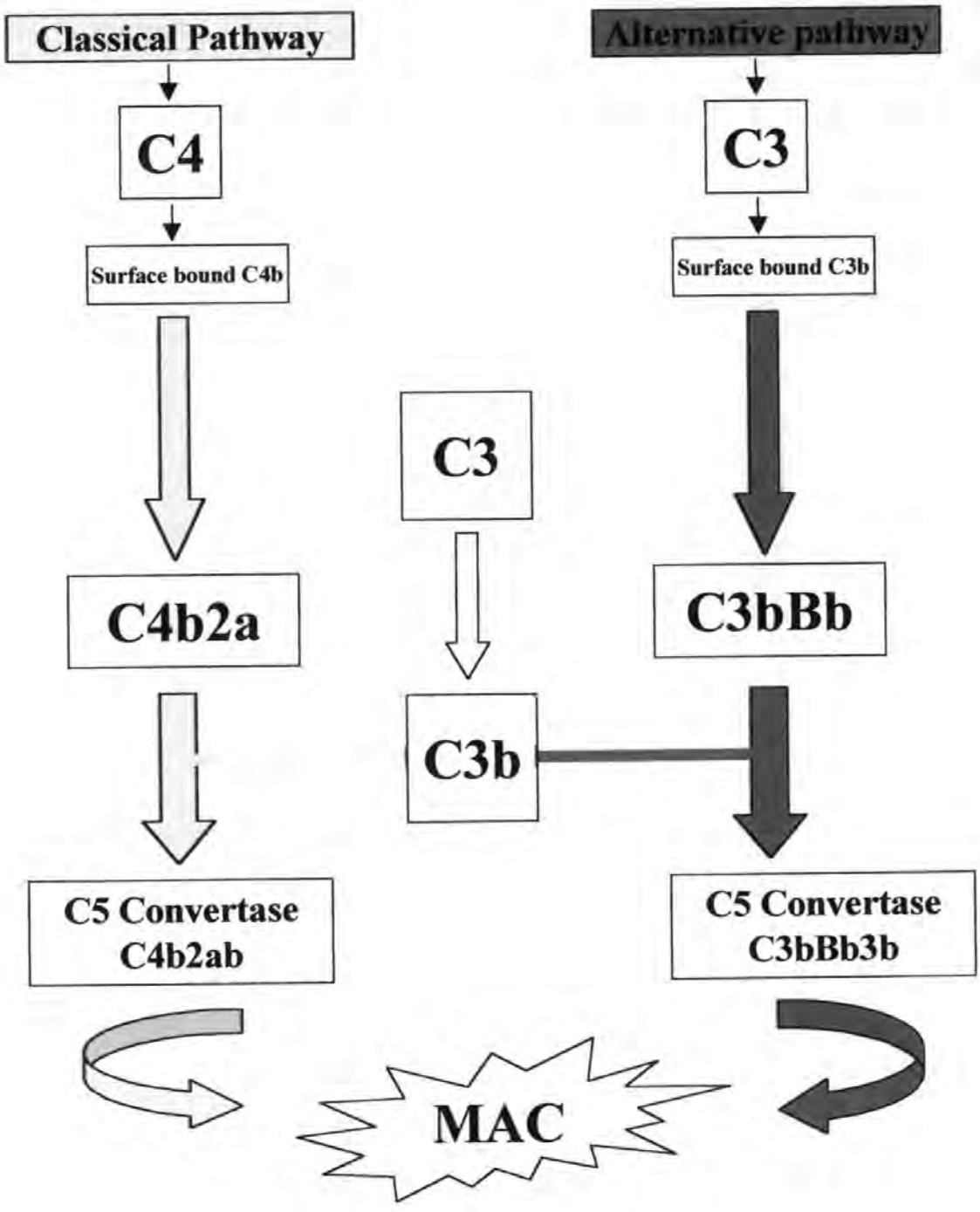


Figure 1.4. A simplified overview of the classical and alternative pathways of complement activation, which leads to the formation of the membrane attack complex (MAC). Diagram redrawn with modification from Roitt *et al* 1996. The complement proteins were first described in 1900 by Jules Bordet.

enzyme. The C3 convertase of the classical pathway is formed as a result of the binding of C4 with C2 proteins, known as C4b2a. The C3 convertase formed by the alternative pathway is as a result of C3 binding to FB proteins, and is known as C3bBb. Once C3 is cleaved by a convertase of either pathway, the C3a protein initiates fixation. The C3b protein is a C5 convertase and activates the terminal lytic sequence of complement by binding C5 with C9, which forms the membrane attack complex. The classical pathway links the adaptive response with the innate response, by its activation by antigen-antibody complexes (Schifferli *et al* 1986). The alternative pathway does not have such specificity and is activated when C3b binds to a membrane that does not contain a complement regulatory protein (Atkinson *et al* 1987) such as bacterial cell walls and other micro-organisms.

1.9 The specific or antigen mediated immune response

The adaptive or specific response is mediated by two main classes of receptor bearing cells that interact with MHC receptors, the lymphocytes and antibody producing B-cells. This response involves a two-stage process. An activation phase where host cells recognise a potential pathogen as 'foreign' and an effector phase where it is eliminated.

1.9.1 Lymphocytes

Lymphocytes are circulating white blood cells, that are grouped into two main classes the T-cells and B-cells. They exist as virgin cells before they encounter an antigen. After a lymphocyte has bound to an antigen it divides (clonal expansion). Generally this occurs when the lymphocyte migrates back to lymphoid tissue such as the spleen or lymph nodes. After an immune response during which Tc-cell and Th-cell clones will have developed, the majority of cells will die. A remaining population of cells, specific for the

antigen remain in the periphery, these are memory cells. If the antigen enters the body again a secondary response occurs which is greater in magnitude and response time due to the presence of these memory cells.

1.9.2 T-cells

T-cells are grouped into two main classes, although a third class is also recognised. The Th-cells (T-helper cells), which are $CD4^+$ and the Tc-cells (T-cytotoxic cells), which are $CD8^+$ are the classic T-cell sub-populations. The third group of T-cells which are generally $CD4^+$ hence are often regarded as a subset of Th-cells, also express CD25 and were previously known as T-suppresser cells. CD25 encodes the β chain of the IL-2 receptor and it is now clear that these cells function in a regulatory way be controlling the effects of IL-2 on T-cell populations, so are known as T-regulatory cells or $CD25^+$ $CD4^+$ T-cells. Th-cells are subdivided into Th1 and Th2 cells depending on the cytokines they produce and their transcriptional response (Mossmann & Coffman 1989). T-cells in a resting state, are designated Th0 (Rocken *et al* 1992). IL-2 and IL-12 induce a Th1 phenotype, whilst IL-2 and IL-4 work in synergy to induce a Th2 response (Romagnani 1994, 1997). Th1 cells produce IFNγ, TNFα and IL-2, and are associated with inflammatory and cell mediated responses. Th2 responses produce IL-4 and IL-10, and are associated with humoral and anti-inflammatory responses. Both subsets can produce IL-3, IL-13 and GM-CSF. Host T-cells can recognise foreign MHC molecules and proliferate in a direct manner or indirectly via antigen presentation. Antigen and foreign peptides displayed by APC with an MHC class II receptor bind to specific T-cells bearing a T-cell receptor (TCR) which recognises the antigen. Antigen is presented to Th-cells via the MHC class II molecule, which interacts with the T-cell receptor. This signal alone is not

sufficient to induce an immune response and a second or co-stimulatory receptor needs to be activated to induce T–cell stimulation.

1.9.3 The T-Cell Receptor (TCR)

The outline structure of the TCR was determined using X-ray diffraction studies and led to a proposed model similar to the structure of an antibody (Chothia *et al* 1988, Davis & Bjorkmann 1988, Boulot *et al* 1994). However, due to the technical difficulty in extracting large quantities of soluble TCR, it was crystallographic analysis of the β chain that led to the first three-dimensional view of the TCR, and confirmed previous models (Bentley *et al* 1995).

The TCR is a disulphide-linked glycoprotein that consists of either an α and β chain or γ and δ chain, linked by disulphide bonds. The TCR has immense diversity due to the arrangement of the genes encoding it. The genes are the V (variable), D (diversity) and J (joining). The α and γ loci have V and J segments only, whereas the β and δ chains contain V, D and J segments. Each chain consists of an external V and C domain, a transmembrane segment containing negatively charged amino acids and a cytoplasmic tail. The TCR subunits interact with the CD3 complex (Clevers *et al* 1988). The interaction between the TCR and MHC bearing APC results in clustering of the TCR subunits and phosphorylation of the CD3 complex. The CD3 complex consists of four polypeptide chains named, γ,δ,ε and ζ that span the cell membrane, and are all linked on chromosome 11. The γ, δ and ε chains interact with the TCR subunits in the extracellular region of the cell membrane, whereas the ζ chains form a heterodimer in the intracellular region and contain immunoreceptor tyrosine-based activation motifs (ITAMS). The binding of the TCR to an MHC receptor phosphorylates the ITAMS, due to the conformational change this induces. The subsequent signal transduction that leads to cytokine production is mediated by protein

kinase C isoforms, and results in transcription factor activation (Valge *et al* 1988, Sun *et al* 2000).

The C (constant) genes encode the transmembrane and intracellular motifs of the TCR, and are linked to the V gene. TCR diversity is the result of the random recombination of the V and D genes and the joining process, which is associated with the addition of nucleotide sequences (N-region diversification). Further diversity is acquired by the fact that the β chain contains two copies of each C, D, and J gene, adding to the possible number of recombination and joining variations. It has been estimated that the possible number of combinations for the α chain are 6.5×10^{12} and 4.4×10^{13} for the β chain (Hunkapiller & Hood 1990).

1.9.4 T-cell co-stimulation signals

During antigen presentation between an APC and a T-cell, the surface expression of molecules such as B7, LFA1 and CD40 on APC's, interact with their ligands on T-cells which include CD28, ICAM-1 and CD40L on $CD4^+$ Th-cells. Co-stimulation is thought to be involved in the activation of signal transduction pathways within the T-cell leading to its activation. Absence of a second signal causes anergy (Bretscher & Cohn 1970). Once activated the Th-cell produces numerous cytokines that in turn activate other immune cells. CD40 is a 50 KDa glycoprotein, which interacts with the 30-33 KDa cell surface molecule CD40L, which is transiently expressed on activated $CD4^+$ T-cells. CD40L is also expressed on mast cells, eosinophils and dendritic cells.

The CD40L – CD40 mediated signal pathway has been shown to be important in the functional interactions of T-cells with endothelial cells as cultured endothelial cells constitutively express a low level of CD40 antigen *in vitro* (Yellin *et al* 1995).

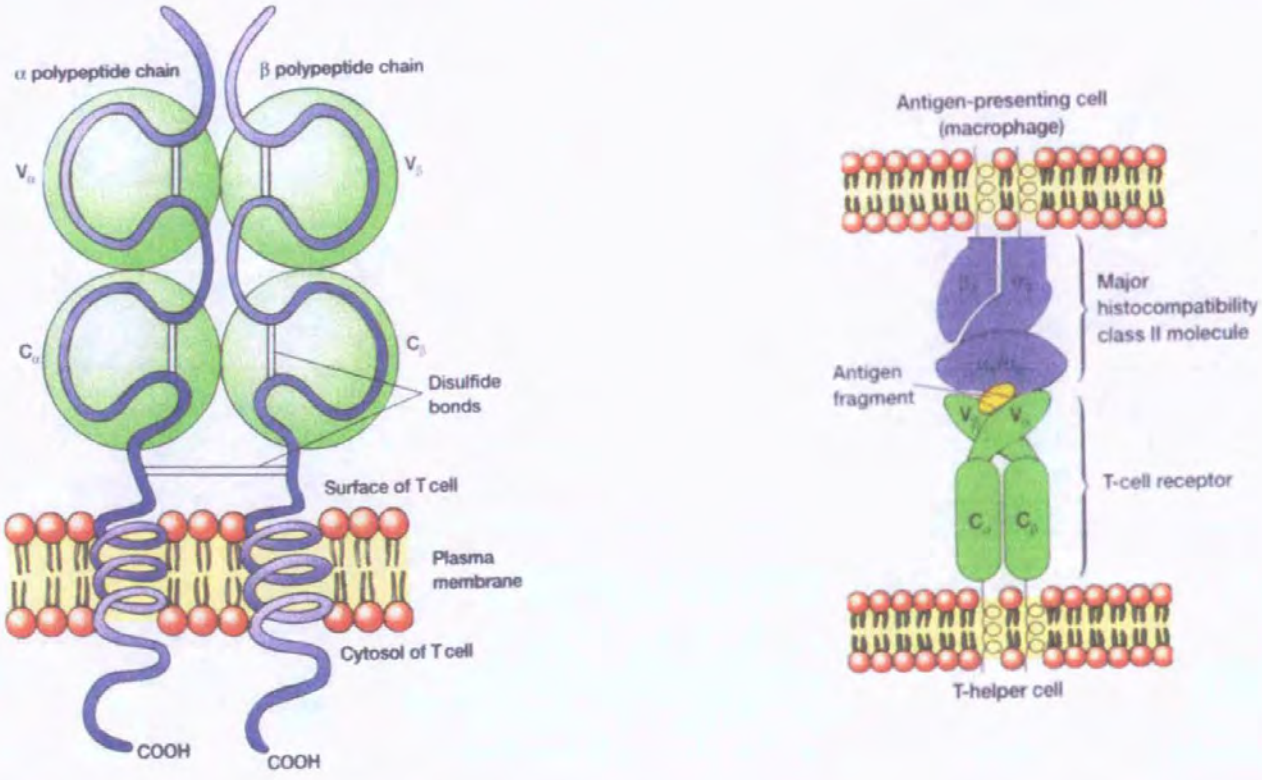


Figure 1.5. The structure of the TCR and its interaction with an APC. Picture reproduced with modification from Prescott *et al* 1996. The outline structure of the TCR was proposed in the late 1980's by X-ray diffraction analysis. However, its structure was not determined fully until 1995 using crystallographic analysis of the β chain (Bentley *et al* 1995).

It is thought that CD40 also functions as a signalling receptor in the development of T-cell mediated inflammatory responses (Karmann *et al* 1995). The same paper showed that CD40 ligand treated endothelial cells results in increased expression of VCAM-1, E-selectin and LFA-3. The hypothesis is that activated $CD4^+$ T-cells express CD40 ligand, which interacts with CD40 expressing endothelial cells to induce leukocyte adhesion molecules and with monocytes to induce cytokine production, contributing to local inflammation. T-cells produce IFN-γ and TNFα which also upregulate the expression of CD40 on endothelial cells CD40 stimulates human monocytes to release nitric oxide (generated by iNOS) and proinflammatory cytokines.

Co-stimulatory signals are also important in cell survival. CD40 inhibits Fas mediated apoptosis. Studies have shown sustained viability of monocytes and dendritic cells in culture when exposed to CD40L. Therefore, CD40 enhances the survival of APC and helps to sustain cell mediated immune responses.

In transplantation, studies have demonstrated that blockade of the CD40-CD40L pathway leads to improved graft survival (Larsen *et al* 1996). However, intragraft expression of T-cell cytokine transcripts IL-2, IL-4, IL-10 and IFN-γ in the early post-transplant period were unaffected by anti-CD40 MAbs, and that therefore, T-cells enter the graft early in the post-transplant period regardless of the CD40 pathway blockade (Larsen *et al* 1997). This indicates that the CD40-CD40L interactions occur downstream of the initial T-cell recognition. It has been shown that the CD40 – CD40L pathway can induce cytokine production via transcription factor activation (Hess *et al* 1995).

1.10 B-cells

B-cells express immunoglobulin on their cell surfaces. B-cells develop in the bone marrow and can be categorised into two types depending on whether or not they express

the cell surface marker CD5. CD5+ cells are a minority population, that develop in early life, and respond to common microbial pathogens, but only express a limited number of immunoglobulin types on their cell surface. The majority of B-cells in an adult are CD5- cells that have a greater diversity of immunoglobulin receptors on the cell surface.

B-cells can recognise foreign antigen directly, via the surface immunoglobulin binding to antigen, or indirectly via antigen presentation. B-cells receive antigen via interaction with Th-cells. Once an APC presents to a B-cell, it differentiates into a mature plasma cell. Plasma cells migrate to lymphoid tissue and actively secrete antibody.

1.10.1 Immunoglobulin (Ig).

The term 'antibody' was first used in 1890 by Emil Von Behring, who immunised rabbits with diphtheria toxin. Behring received the Noble Prize in 1901. B-cells that have been stimulated by recognition of an antigen secrete antibody. Hence, antibodies can be found as circulating forms, or expressed on the surface of B-cells. The surface expression of antibody by a B-cell is dependent on the activity of the CD79 molecule, which contains ITAMS and is involved in the signalling pathways resulting in antibody expression.

There are five main classes of antibody, IgG, IgA, IgM, IgD and IgE, which differ in their size, charge, amino acid composition and carbohydrate content. The general structure of an antibody was determined in the 1960's (Cohn & Porter 1964, Porter 1967ab Steiner & Porter 1967). The Nobel Prize was awarded to RR Porter in 1972 for his work on the structure of antibodies.

Antibody molecules contain two heavy chains and two light chains. The heavy chains differ between immunoglobulin classes. The heavy and light chains contain two separate regions, a C-terminal that is a constant region and shows no variability and an N-terminal region that is variable. The heavy chain does not vary as much as the light chain, and can

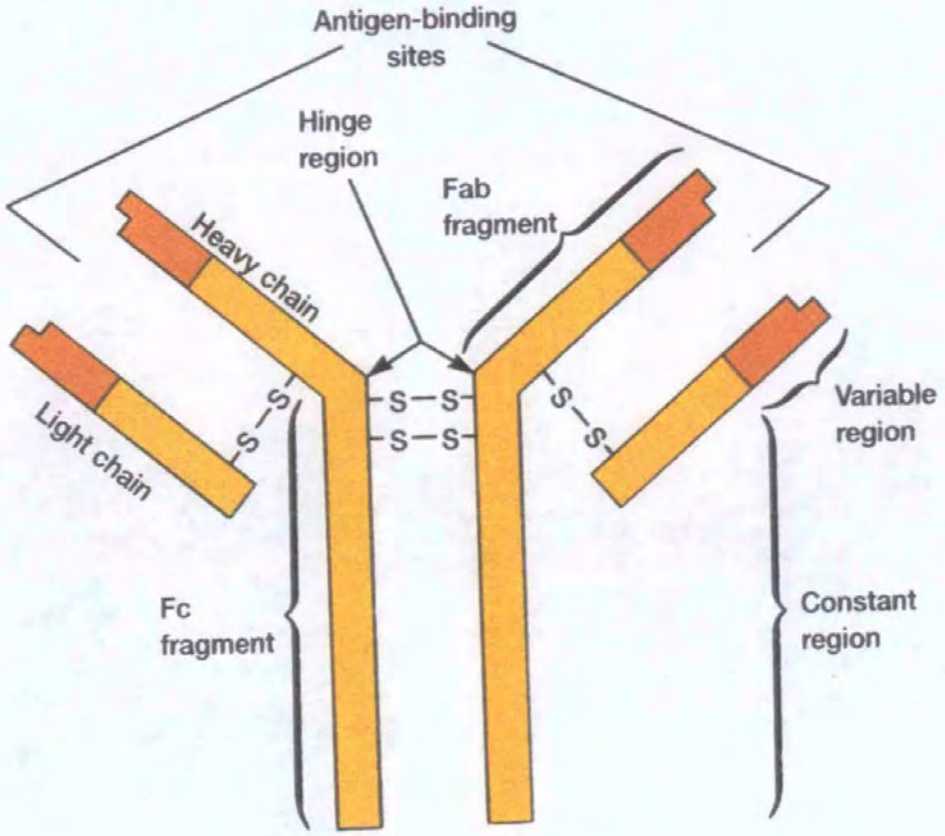


Figure 1.6. The basic structure of an antibody molecule comprising heavy and light chains with variable and constant regions. Picture reproduced with modification from Prescott *et al* 1996. The original structure of an antibody was first described in 1967 and led to the Nobel Prize in 1972 for RR Porter.

be of five main types depending on the Ig class (μ,γ,α,ε,δ). Heavy chains are generally 53 or more KDa in size. The light chains are usually 23KDa. The light chain contains two subunits the κ and λ chains, which are encoded by separate gene loci. IgG is the major serum Ig and is associated with stimulation of the classical pathway of complement activation. IgG can cross the placenta and can be divided into 4 subclasses (IgG 1-4). IgM is important in a primary response and fixes complement. IgA is found extensively in the mucous membranes and other sites of potential entry for a pathogen. Epithelial cells express a poly-Ig receptor that binds IgA dimers and transports them across the endothelium into tissues. IgD is only co-expressed with IgM and functions during B-cell maturation, but is not expressed in mature B-cells. IgE binds to mast cells and initiates the release of histamine after contact with an antigen. It is also found extensively at the mucousal surfaces. Ig diversity is achieved in a similar way to TCR diversity. Ig's are also subject to recombination between V, D and J genes and N-region diversification, but also have varied combinations of light and heavy chains and have somatic mutations within the V genes of individual B-cells. Estimates have suggested 400 possible combinations for the light chain and 4,800 combinations for the heavy chain, leading to 1,920,000 possible combinations of antibody structure (Nossal 1993).

1.10.2 Natural killer cells (NK cells)

NK cells are the third major subset of lymphocytes. NK cells are large granular lymphocytes. Cells expressing MHC class I molecules are protected from lysis by NK cells (Lao *et al* 1991). NK cells express immunoglobulin superfamily receptors on the cell surface known as killer inhibitory receptors (KIRs). Binding of 'self' MHC class I molecules to these KIRs inhibits NK cell activation (Long *et al* 1997). NK cells have been

shown to infiltrate organs during rejection, but depletion of NK cells in mice was shown to have little effect on preventing rejection (Manily & Sykes 1998).

1.11 Cytokines in the Immune response

Cytokines are soluble proteins that are secreted or expressed on the surface of many cell types. Cytokines bind to receptors on cell membranes and initiate signal transduction and second messenger pathways. Cytokines are released by many cell types during an immune response and function as a communication network between cells (Balkwill & Burke 1989). Cytokines are pleiotrophic and can act in synergy or as antagonists. They were first identified as soluble proteins released from lymphocytes that regulated the growth and differentiation of cells. Because they were thought to be produced by lymphocytes only they were initially known as lymphokines (Dumonde *et al* 1969). However as it became clear that these proteins were produced by many cell types the term 'cytokine' was developed (Cohn *et al* 1974). The term 'interleukin' was developed to describe cytokines that relay messages between leukocytes (Aarden *et al* 1979). However, as the pleiotrophic nature of many interleukins has become clear it is now common for the same protein to be called a cytokine, interleukin or lymphokine (e.g. IL-2). Cytokines can be grouped according to the cellular process they initiate, including the acute phase response (IL-1, IL-6, IL-8 and TNF-α), proliferation and differentiation of cells (IL-2, GM-CSF and G-CSF) and immunoglobulin class switching (IL-4 and IL-5).

Therefore cytokines can be grouped into 4 main classes

1. Mediators of natural immunity
2. Mediators of lymphocyte activation, growth, and differentiation.
3. Mediators of immature leukocyte growth and differentiation
4. Mediators of effector cell activation.

Cells contain receptors for cytokines that are expressed on the cell surfaces. Cytokine receptors can be grouped into classes depending on shared conserved sequences and properties. The major receptors are the class I receptors, class II receptors (IFN receptor family), TNF receptor family, IL-1 receptor family and chemokine receptors. After binding of a ligand to a receptor, cytokine receptors have a distinct and characteristic signal transduction response leading to cytokine production (Vilcek 1998).

The cytokine receptor superfamilies do not have intrinsic protein tyrosine kinases and are therefore associated with intracellular protein kinases. One particular set of kinases that are contained within the cytoplasm, receptor-bound and are involved in downstream actions after cytokine signalling are the Janus-associated kinases (JAK). JAK kinases phosphorylate tyrosine residues on signal transducers and activators of transcription (STATS), when a cytokine binds to its receptor. STATS dimerise to form heterodimers. STAT dimers then enter the nucleus and act as transcription factors binding to regulatory regions in cytokine genes causing the transcription of the gene. Different receptor families utilise different JAK-STAT pathways and many combine with other kinase pathways. The TNF receptor family do not use the JAK-STAT pathway, instead have their own intracellular receptor associated proteins (RIPs) (Hsu *et al* 1996).

Th1 cytokines such as IL-2 and IFNγ are important mediators of the immune response. IL-2 initiates the maturation of the precursors of Tc-cells into mature Tc-cells. IFNγ is produced by numerous cell types and induces the expression of MHC class II molecules on cell surfaces, further promoting immunogenicity. Th2 cytokines such as IL-4 are also important in the immune response. IL-4 is produced by active Th-cells, basophils and mast cells and is responsible for the proliferation and differentiation of B-cells into antibody secreting plasma cells. IL-4 is structurally similar to IL-13 another cytokine which acts as a T-cell stimulant, with 20% homology of their amino acid sequences (Minty 1993). Like

IL-2 expression of IL-4 is dependent on the binding of the NFAT transcription factor (Rincon & Flavell 1997). IL-4 has been suggested to function as a protective or anti-inflammatory cytokine. Expression of IL-4 leads to the up-regulation of other Th-2 dependent cytokines such as IL-10, as well as acting in an autocrine fashion to up-regulate its own expression. The protective nature of IL-4 has been demonstrated by its ability to induce the expression of anti-apoptosis proteins including Bcl-2 and anti-oxidant genes such as the heme oxygenase enzyme (Ke *et al* 2000). IL-4 induced into grafts via adenoviral transfer has lead to prolonged allograft survival in rats (Kato *et al* 2000a) and mice via the down-regulation of NFκB activity (Kato *et al* 2000b).

1.12 The Major Histocompatibility Complex (MHC)

The MHC in humans is coded for by a region on the short arm of chromosome 6 at position 6p21.3 (Trowsdale *et al* 1983, Morton *et al* 1984). The MHC region was first mapped in 1991 (Trowsdale 1991). The MHC region is subdivided into classes I, II and III. (Hood 1983, Campbell & Trowsdale 1993, Trowsdale 1993, 1995). Each class region contains many polymorphic alleles. The Class II region was cloned using yeast artificial chromosomes (YACs) in 1989 (Ragoussis *et al* 1989), with a more detailed YAC system incorporating the class I region in 1994 (Abderrallim *et al* 1994).

In humans, the MHC contains the human leukocyte antigens (HLA). The first HLA gene was identified in 1958 by Jean Dausset. The importance of associations with the HLA system and immune disease, such as autoimmunity and tumour resistance in chickens were first noted in the 1970's (Doherty & Zinkernagel 1974, Pazderka *et al* 1975, Bacon & Rose 1979). The MHC class I region contains the 'classical' HLA-A, B and C genes and the 'non-classical' HLA- E, F, G, and X genes (Koller *et al* 1988, Geraghty *et al* 1992). The MHC class II region contains the HLA-D genes, DR, DQ and DP (Trowsdale 1993, 1995).

The Class II region also contains genes for the LMP and TAP proteins (Glynne *et al* 1991). The MHC class III region contains genes for the complement proteins C2, C4 and factor B (Campbell & Porter 1983, Carroll *et al* 1985), the BAT6-BAT9 genes (Spies *et al* 1986) important HSP70 gene (Dunham 1987, Sargent 1989) and the TNF genes (Spies *et al* 1986, 1989).

The three-dimensional structure of the MHC was first described in the class II DR molecule (Brown *et al* 1993). Both MHC class I and II molecules consist of two glycoprotein chains which display small peptide fragments for T-cell recognition.

The HLA class I molecule is a dimer of a polymorphic heavy chain and a non-polymorphic light chain, known as the β-microglobulin chain (coded for on chromosome 15). Both the heavy and light chains of the class II molecule are encoded within the MHC. The DQ and DP loci are both polymorphic, whereas, the DR locus tends to be highly polymorphic in the heavy chain, but with a common shared light chain. The number of DR genes can vary amongst individuals. MHC class I antigens are expressed most, but not all nucleated cells (Daar *et al* 1984a). In a study of the distribution of HLA-A B and C antigens in normal tissue, weak expression was found in the endocrine system, including the Thyroid, Pituitary gland and Islet cells in pancreas and a high expression in the Respiratory and Cardiovascular systems. No expression of HLA class I antigens was found in the endothelium of the Cornea demonstrating that class I molecules are not ubiquitously expressed (Daar *et al* 1984a). MHC class I molecules are associated with interactions with $CD8^+$ Tc-cell response.

MHC class II molecules are highly expressed by lymphoid tissue including B-cells, dendritic cells and endothelial cells (Daar *et al* 1984b). Tissue distribution of class II shows a low expression in the endothelium of the brain and endocrine organs. In a similar fashion as class I, class II is not expressed by the Cornea (Daar *et al* 1984b).

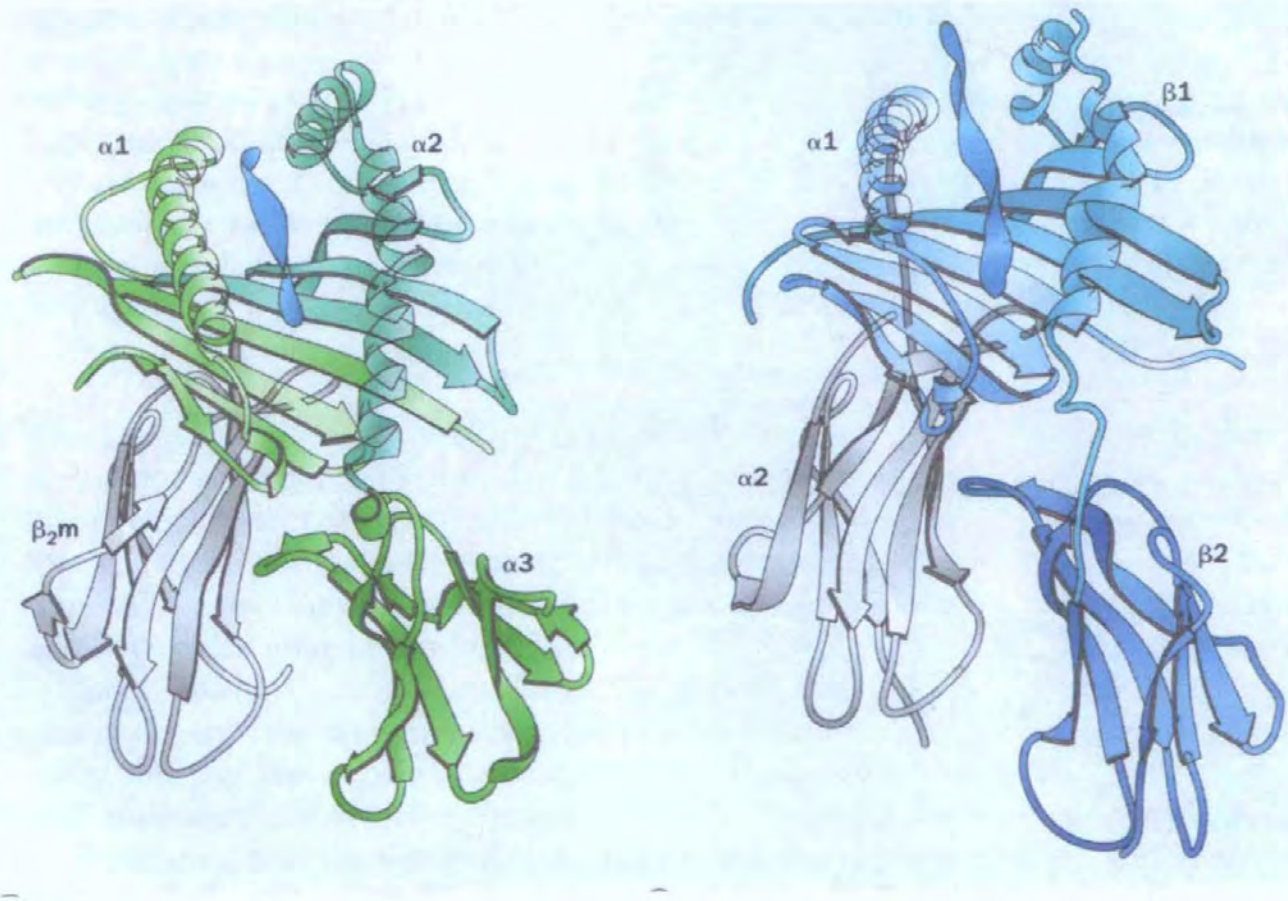


Figure 1.7. The structure of the MHC class I and II molecules. Picture reproduced with modification from Johnson & Feehally 1999. The 3D structure of the MHC was first determined in the class II DR molecule (Brown *et al* 1993).

However, during an active immune response, MHC class II molecules are upregulated on most cell surfaces (Pober *et al* 1996, Shackleton 1998).

1.12.1 HLA matching

Experiments with inbred mice strains in the 1930's demonstrated that mice of a certain strain would react to human serum proteins, whilst others would not, indicating the presence of an 'inherited antigenic difference' between the mice (Gorer 1936).

Tissue Typing was developed as a means of matching donor and recipients for HLA antigens in order to reduce the risk of rejection. It has been long established that HLA matched grafts survive for longer than poorly matched grafts (Batchelor & Joysey 1969, Oplez & Terasaki 1977). The traditional method was serology or cellular typing, however, DNA based typing utilising PCR technology has become the standard. Serological assays use test sera that is known to react with certain HLA class molecules to determine a persons HLA type. The idea stemmed from the fact that women are exposed to paternal HLA molecules during pregnancy. Some women react to these antigens and develop an antibody response to them. Sera were collected from many women and tested to see which HLA molecules it reacted against. A potential donors cells were than added to the different HLA containing sera, to see if the cells reacted to the sera.

Cellular typing was developed from the mixed lymphocyte reaction. A panel of cells homozygous for a HLA molecule is added to the donors lymphocytes to determine if there is a response. Cellular typing allowed the more specific identification of subtypes.

DNA based typing utilises PCR technology to amplify specific fragments of DNA. When the HLA genes were sequenced many new alleles and polymorphisms were identified. Commonly now, donor DNA will be screened with primers for different loci such as the DR or DQ region, then a sequence specific primer will be used to discriminate alleles.

The continual need to update and replenish stocks of sera and panel reactive cells led to the formation of international workshops for histocompatibility. This led to the use of the letter 'w' (from workshop) as an abbreviation for newly identified antigens. Later when reproducibility was accepted the 'w' would be dropped (i.e. DRw4, DR4).

The specificity of DNA based typing has made HLA nomenclature quite complex. A current gene, will have a number to represent the locus, a number to represent other structurally related genes and a number to represent its particular nucleotide sequence. For example the old name for the DR1 gene is currently DRB1*0101. The high degree of polymorphism in the MHC genes means that truly identical donors are rare in the population. With mismatches unavoidable for minor antigens, immunosuppression is always needed. Lower rejection rates are found with increasing HLA matches (Terasaki 1989, Beckingham *et al* 1994) and HLA-DR mismatches are associated with early and late rejection episodes (McKenna *et al* 1998). The first description of the benefits of matching HLA-DR antigens and outcome in renal transplantation was in 1978 (Ting & Morris 1978). The major loci that need to be matched are HLA-A, -B and -Cw of the Class I region and HLA -DR and -DQ of the class II region. The most important antigens to match are the DR and B antigens, with HLA-A antigens having less effect. Matching for the DR antigen is generally considered to have the largest effect on graft survival (Opelz *et al* 1993). Matching of HLA-A and HLA-B loci have been shown to influence graft rejection in males but not females (Opelz & Terasaki 1977). In comparison to perfectly matched grafts one mismatch is thought to effect graft loss by two-fold for A antigens, three-fold for B antigens and five-fold for DR antigens (UNOS 2002). Analysis of outcome in renal transplants found the best survival rates in patients with no HLA-A, -B or DR mismatches (000). The second most favourable match was one mismatch for HLA-A or –B or both loci, but without a mismatched HLA-DR allele (Morris *et al* 1999).

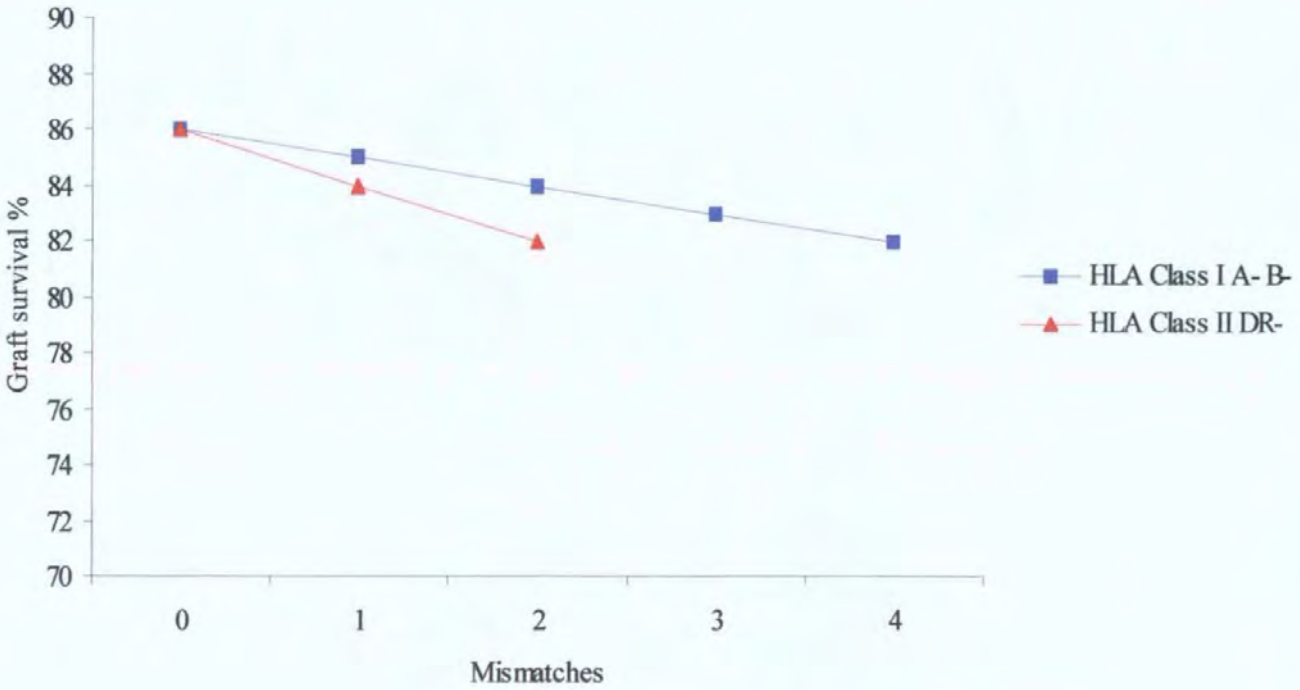


Figure 1.8. The influence of HLA matching on short term graft survival (Data from Hata *et al* 1996) demonstrating as has been known for some time the importance of HLA-DR matching on graft survival.

Current ideas are focusing on whether or not certain combinations of mis-matches may have a greater effect than others on long term survival. Matching alone does not guarantee a good outcome and factors such as ischaemic times are important (Morris *et al* 1999). The benefits of 0 HLA mismatches are lost in grafts with over 36 hours cold ischaemic times compared to grafts with 1 or more mismatches (71% Vs 72%) (Lee *et al* 2000). Also graft survival is higher in grafts with zero mismatches compared to grafts with one or more HLA-DR mismatch at one and five years only if the cold ischaemic time is less than 26 hours (Connolly *et al* 1996).

1.13 The Immunology of graft rejection

The first demonstration of the importance of an immune response to transplanted material was shown by Medawar and colleagues who showed that skin grafts from genetically disparate individuals (allografts) were rejected, whereas, grafts from the same individual (autografts) were tolerated (Gibson & Medawar 1943). The importance of this work was further developed in rabbits by the discovery of an immune memory to the grafts (Medawar 1944). The important components of the alloresponse to a graft are the immunoglobulin superfamily, adhesion molecules and cytokines (Halloran *et al* 1993).

1.13.1 'Self 'versus 'non-self '

It is estimated that everyday some 2 million new T-cells and 20 million new B-cells are produced (Scollay 1980, Osmond 1993). Antibodies to 'self' antigens such as keratin and DNA are common. How does the immune system achieve tolerance when there are some 55,000 proteins in the body and 10^{12} different B and T-cell idiotypes? (Matzinger 1994). 'Self' antigens have been the subject of much debate on how to classify. Early work centred on a dose response theory in that self peptides occurred at a concentration above a

certain threshold allowing them to be ignored (Jerne 1974). Amongst other ideas the notion of a positive selection theory was also proposed whereby the thymic cells positively select 'self' peptides and remove T-cells that may be reactive to them (Salaun *et al* 1990).

In terms of the T-cell 'self' is generally considered to be any peptide from an individuals own cells found complexed within the MHC (Matzinger 1994). 'non-self' is therefore anything from outside of the body, something that has caused much debate as the immune system will not respond to materials such as bone, silicone or many haptens, including some metals and food. Other complicating factors include privileged sites, areas where immune cells generally do not enter, such as the cornea, which are tolerated and freely transplantable. However, it was later demonstrated using components of the bacterial cell wall, that certain molecules are seen as more antigenic or foreign than others (Janeway 1989). There are four classes of structures to which the immune system reacts (1) visible self which the immune system is capable of reacting to, but is tolerant toward, (2) invisible self – structures to which the immune system has no response (3) visible non-self – foreign antigens to which the immune system normally responds and (4) invisible non-self-structures such as silicone, bone certain foods etc (Matzinger 1994). The function of the immune system is therefore to distinguish between these classes and make an appropriate response. Immunology is complex in its interpretation of how the immune system does this.

1.13.2 Tolerance

In 1945 Owen described an 'experiment of nature,' in which non-identical twin cattle who shared haemopoietic stem cells, were found to be tolerant to each others foreign cells. Later in 1953, Peter Medawar demonstrated tolerance by grafting skin from mice of non-identical strains, which had been made tolerant by injection of donor cells at birth

(Billingham *et al* 1953). These experiments were the basis of the 'clonal selection theory' proposed by MacFarlane Burnet in 1957. The theory states that a lymphocyte is selected by an antigen that it meets after birth and divides to form a clone. Self antigens or 'forbidden clones' in Burnets model are deleted from the T-cell repertoire, before birth. Lederberg in the 1959 modified the clonal selection theory to suggest that immature lymphocytes encounter antigen and develop to mature lymphocytes providing the antigen is not a self peptide. If the antigen is a self molecule the lymphocyte does not develop, therefore, there is 'clonal abortion' of autoreactive lymphocytes.

The first molecular evidence to explain tolerance came from the notion of second signal or co-stimulation requirements for T-cell activation (Bretscher & Cohn 1970, Lafferty & Cunningham 1975). The first signal is the interaction between the TCR and MHC bearing peptide APC. The mechanism proposed by the two groups was slightly different as the first signal in Bretscher and Cohns model switched the T-cell 'off' and destroyed it if a second signal was not received. In the Lafferty and Cunningham model absence of a second signal failed to activate the T-cell and led to anergy. These observations lead to a theory of tolerance based on the fact that an 'antigen specific' T-cell was supplying the second signal not the APC, which has no ability to distinguish self from non-self and would routinely express self peptides. T-cells in the periphery should not be reactive to self peptides according to the theories of clonal deletion and so therefore, any T-cell which binds to a peptide displayed in an APC should only have a TCR capable of recognising 'non-self peptides'. If the co-stimulatory molecules interact, this T-cell will replicate and become an active helper cell or cytotoxic killer cell.

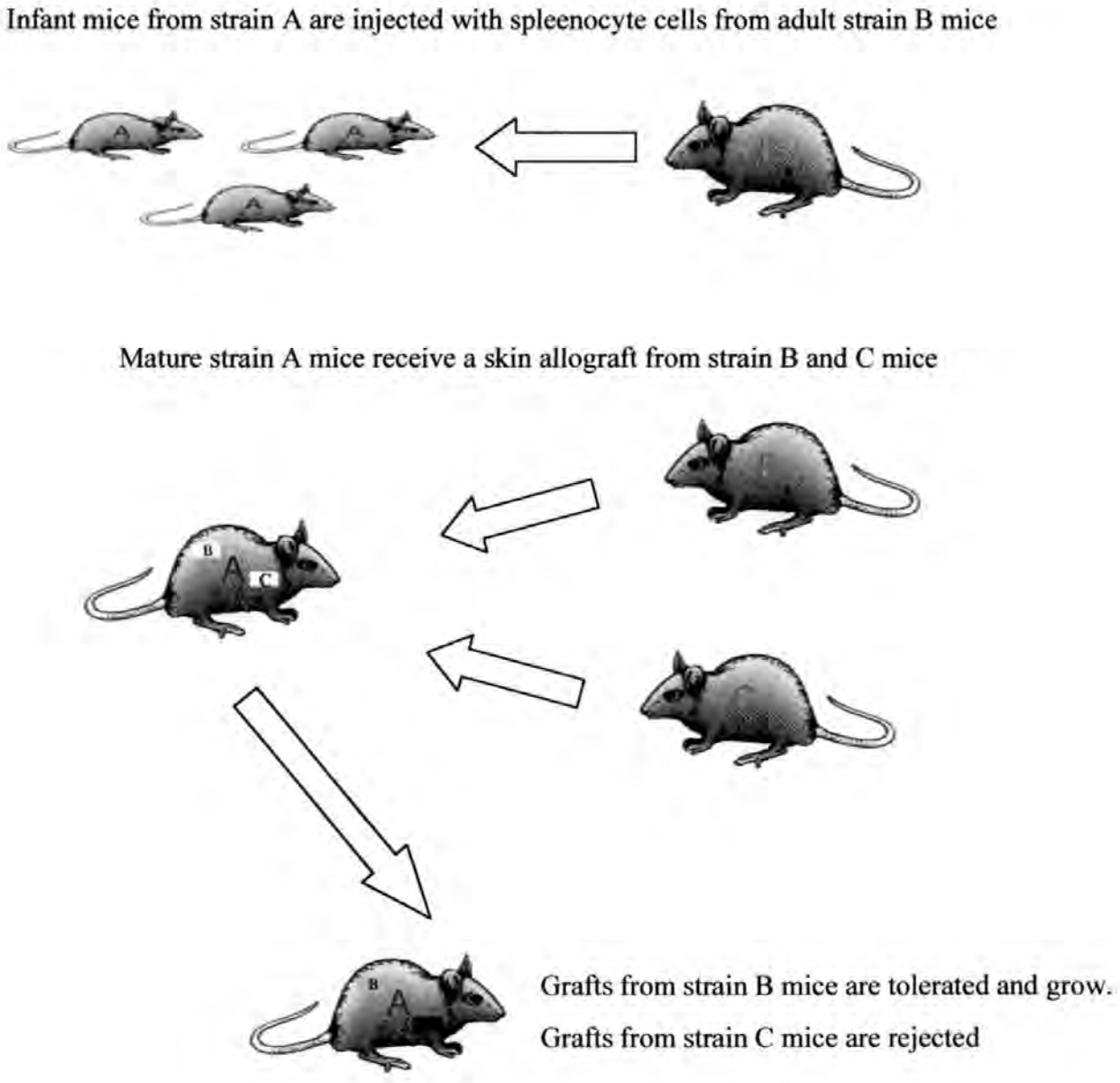


Figure 1.9. An overview of the classic experiment carried out by Peter Medawar and colleagues in 1953 who grafted skin from mice of different strains to demonstrate that tolerance could be induced by exposure of foreign antigen to the immune system during early development. The results of this experiment led MacFarlane Burnet to propose the 'clonal selection theory' in 1957 which explains the deletion of 'self' antigens in thymus during early life. Both Burnet and Medawar received the Nobel Prize.

1.14 Immunosuppression

The stages of T-cell activation are firstly stimulation of the TCR via binding to an APC or direct recognition of a foreign peptide. This interaction activates calcineurin, which dephosphorylates the nuclear factor of activated T-cells (NFAT) transcription factor. Provided the T-cell receives and appropriate second signal from a co-stimulatory molecule, NFAT will enter the nucleus and bind to the IL-2 promoter. IL-2 can work in an autocrine fashion as well as binding to its receptor on other T-cells. Binding of IL-2 to its receptor sends the T-cell into the cell cycle hence causing their proliferation and activation. All current immunosuppressive drugs target T-cells (Denton *et al* 1999). The mechanisms for this are via preventing clonal expansion, IL-2 cytokine production or both.

1.14.1 Calcineurin inhibitors

Cyclosporin was first described as an immunosuppressive agent in 1976 and was used in trials where it was found to increase one-year graft survival (Borel *et al* 1976). Cyclosporin binds to cyclophylin and inhibits calcineurin, thus preventing IL-2 production in T-cells. The problem with cyclosporin is its side effects. Cyclophylin is widely distributed in many tissues and cyclosporin is associated with acute nephrotoxicity and hypertension. The most damaging side effect of cyclosporine is its role in chronic nephropathy. Cyclosporin induces TGF-β expression, which is likely to cause the formation of interstitial fibrotic tissue associated with chronic graft nephropathy (Bennet *et al* 1996).

Tacrolimus was developed as a less toxic form of cyclosporin which also has the advantage of better oral adsorption (Spencer *et al* 1996). Tacrolimus binds to the FK506 binding protein that also blocks calcineurin activation and IL-2 production. Tacrolimus does not

induce TGF-β expression but has similar nephrotoxicity as cyclosporine due to an unknown mechanism. Patients treated with Tacrolimus have a 20% increase in diabetes, but the advantage over cyclosporin in terms of side effects, are less hypertension and hyperlipidaemia (Pirsch *et al* 1997).

1.14.2 Anti-proliferative agents

Azathioprine was developed as a derivative of 6-MP and has been used since the 1960's (Calne 1960). It works by preventing T-cell and B-cell proliferation by binding to DNA and inhibiting purine synthesis. This affects both DNA and RNA synthesis in the lymphocyte. The major disadvantage to this is the fact that this suppresses bone marrow. A newer version of azathioprine, mycophenolate mofetil is a more selective inhibitor of T-cell and B-cell purine synthesis and therefore has the advantage of not affecting bone marrow. A further anti-proliferative drug has been developed known as Sirolimus, which is a derivative of rapamycin. Sirolimus blocks the IL-2 induced entry of the T-cell into the cell cycle. It does this by blocking p70 kinase, which is important in cell cycle entry. Sirolimus is designed to be used with cyclosporine as it also binds to the FK506 receptor in the same fashion as Tacrolimus.

1.14.3 Anti-cytokine agents

Corticosteriods are non-specific anti-inflammatory agents that block inflammatory cytokine production by firstly binding to glucocorticoid response elements in cytokine genes and secondly by blocking the activation of the Nuclear Factor kappa B (NFκB) transcription factor (Scheinman *et al* 1995). The anti-IL-2 receptor antibodies, Daclizumab and Basiliximab have been shown to reduce the incidence of acute rejection

and may be used during induction immunosuppression (Nashan *et al* 1997, Vincenti *et al* 1998).

Patients are first given an induction immunosuppressive drug during the early post-transplant period to prevent an immune response to the graft. In many cases this is simply an increased dose of maintenance drugs, but can commonly include anti IL-2 receptor antibodies or the anti-CD3 antibody OTK3, which binds to the T-cell receptor down regulating its activity (Burt & Matuszewski 1997).

After the initial post-transplant period, patients are given a maintenance therapy, which is normally a combination of drugs given to reduce the side effects of any one drug alone. The usual combination therapy would likely be a calcineurin inhibitor such as cyclosporine or tacrolimus and an anti-proliferative agent such as azathioprine or mycophenolate mofetil.

1.15 New immunosuppressive strategies

The ideal situation in transplantation would be donor-specific tolerance, whereby the recipient maintains a normal immune response, but is tolerant to the graft. This is the aim of future immunosuppressive strategies, to immunosuppress or remove T-cells specific for donor antigens, but to leave an intact immune system. One potential way this could be achieved is in the selective blockade of co-stimulatory molecules (Sayegh & Turka 1998). Removal of co-stimulation causes T-cell anergy (Bretscher & Cohn 1970, Lafferty & Cunningham 1975), a situation that would be beneficial to induce in Th-cells specific for donor antigens (Denton *et al* 1999). Another potential target for future immunosuppressive strategies is the blockade of important transcription factors such as NFκB and AP1 (Mortellaro *et al* 1999).

Finally the advances that have been made in the use of gene therapy in transplantation (Wood 1997), have recently included adenoviral delivery of IL-4 and been shown to improve graft survival in rat models (Kato *et al* 2000a).

1.16 Rejection

1.16.1 Early graft loss

Early graft function is an important indication of long term graft survival (Ojo *et al* 1997). Delayed graft function (DGF) is normally characterised by the need for dialysis within the first week post-transplant. Studies have shown that DGF in combination with acute rejection reduced graft survival rates from 92% to 68% at one year (McLaren *et al* 1999). More factors are likely to influence short term survival graft function than long term. One year survival was found to be influenced by numerous factors such as sensitisation (9%) race (8%) transfusion (6%) donor age (6%) diabetes (3%) recipient age (3%) and cold ischaemia (1%) in a prospective study across many transplant centres in the US (Terasaki *et al* 1989).

Within the first 28 days post transplant, the major causes of graft loss are due to technical problems, acute rejection or acute graft thrombosis, usually of the renal vein (Hefty *et al* 1993). Renal vein thrombosis induced early graft loss accounts for 4-5% of all graft loss (Parrott 1995). Trials with low molecular weight heparin in paediatric transplants have shown reductions in the incidence of graft thrombosis, but have been coupled with high rates of postoperative haemorrhage (Parrot 1995). Reasons for this is currently unknown and explanations proposed include technical problems with surgery (size of renal vein) or non-anatomical factors such as the hydration-state of the recipient. The clinical signs are of graft tenderness; swelling, sudden allograft deterioration of function, anuria and diagnosis is usually confirmed by Doppler ultrasound scanning.

Other causes of early graft loss include urinary leak or obstruction by clot or lymphocele, and stenosis. Pre-formed antibodies in the recipient against HLA antigens from the donor cause Hyperacute rejection. This is normally due to a previous exposure to them (sensitisation) either from a previous transplant or a situation where exposure to foreign HLA types has occurred such as during pregnancy. This is now a rare event due to cross-matching before transplantation.

1.16.2 Acute rejection

Acute rejection occurs as result of the activation of recipient T-cells, which can directly recognise donor peptides in the donor MHC or indirectly via antigen presentation of donor peptides to Th-cells by recipient APCs. Passenger leukocytes are also thought to have the ability to sensitise the host as they have a high expression of MHC class II, and also express class I molecules, suggesting they can directly activate host T-cells (Fabre & Morris 1973). Data has also suggested that donor cells can initiate a recipient anti-donor response by the upregulation of MHC molecules (Pober *et al* 1996).

The clinical signs of a rejecting renal graft are the infiltration of lymphocytes into both the graft tubules and interstitium (tubulitis) and endothelium of small vessels (endothelialitis). In tubulitis, the infiltration occurs via the basement membrane where the highest degree of MHC expression is found (Hall 1991). Lymphocytes enter the endothelium via adhesion molecules. Adhesion molecules expressed on activated microvascular endothelial cells mediate peripheral recruitment of T-cells and their ligands expressed on differentiated T-cells. Adhesion molecules are grouped into three main classes, the selectins, integrins and the immunoglobulin superfamily (Bevilacqua 1993).

The selectins contain the molecules E-selectin (endothelial), P-selectin (platelet) and L-selectin (leukocyte) named after the cell type, which express them. They all share an N-

terminal lectin domain, which binds specific sugar groups. L-selectin is an important recirculation receptor, which also binds to the endothelium in inflammatory responses via its ligand, E-selectin. The expression of E-selectin is upregulated by IL-1 and TNF. Soluble e.g. shed levels of E-selectin are thought to represent activation of the endothelium as elevated levels have been found in septic shock patients (Newman *et al* 1993). P-selectin is stored in the granules of platelets and the weibel palade bodies of the endothelium and is released in response to the clotting cascade. The ligand for P-selectin is L-selectin. The selectins are primary adhesion receptors involved in the initial binding of leukocytes to the endothelium. This slows leukocyte passage and exposes endothelial cells to other surface molecules in the local environment. Selectins mediate the recruitment of Th1 but not Th2 cells to areas of inflammation (Austrup *et al* 1997).

The integrins are a family of α/β heterodimers, divided into three families, based on shared subunits $\beta1$, $\beta2$ and $\beta3$. The $\beta1$ family contains VLA 1-6; the $\beta2$ family contains LFA1, MAC1, p150 and p95. The $\beta3$ group contains VNR, gpIIb and gpIIIb, these are found on platelets and bind to the ligand Von Willebrand factor (VWF). Integrins represent the second level of adhesion.

The immunoglobulin superfamily contains the ICAM 1-3, LFA2-3, VCAM1, B7 and CD28 molecules. ICAM-1, VCAM-1 and E-selectin have been shown to increase in expression during ischaemic injury in the post-transplantation period and to be associated with the $CD4^+$ T-cell infiltration of the graft (Briscoe *et al* 1995). The same paper found that cell expression of E-selectin and ICAM-1 increase in cardiac biopsies prior to clinical and pathological rejection, whereas VCAM-1 is concurrent with active rejection episodes (Briscoe *et al* 1995). Endothelial cells constitutively express ICAM-1 and VCAM-1. Levels peak at around 12-18hrs during rejection episodes. Interactions between lymphocyte (LFA-1) molecules and ICAM-1 result in higher affinity firm adhesion of

leukocytes in rejection. VCAM-1 is a ligand for the integrin VLA-4 that is functionally expressed on activated lymphocytes, monocytes and NK cells.

The adhesion of the immunoglobulin superfamily represents the final category of firm adhesion. The sequential cellular and molecular kinetics of acute rejection has been studied in a rat model, over a 14-day period post transplantation (Nagano *et al* 1997).

1.16.3 Chronic rejection

Chronic rejection is often referred to as chronic graft nephropathy, and is the result of prolonged damage to the graft over time. Chronic rejection has no real basis of classification and is the subject of much debate, particularly as many of the symptoms of chronic rejection are seen in cyclosporine nephrotoxicity. In general the characteristics of chronic rejection are of patchy fibrosis within the graft, vessel wall thickening and narrowing of the luminar surface of the vessels leading to coagulation. There are a variable amount of lymphocyte infiltrates found in the graft during chronic rejection. Fibrosis is linked to TGF β expression in grafts. Correlation between TGF β and matrix deposition in needle biopsies from grafts with chronic nephropathy was found to correlate with collagen III and TGF β expression (Nicholson *et al* 1999). Biopsies studied one year after transplantation and after a 4 year follow up period showed a correlation between chronic graft nephropathy and TGF β mRNA expression (Hueso *et al* 2001). However, the same study showed that although the expression correlates with tubular necrosis, TGF β expression is also found in stable functioning grafts.

Cytokine Expression	**Peak**
TGF–β	Day 3
IL-6	Day 5
IL-2	Day 7
IL-1	Until day 7
TNF-α	Until day 7
RANTES	Until day 7
IFN-γ	Progressively until day 14
Adhesion Molecule Expression	**Peak**
E-Selectin	24 hours
ICAM-1	After day 3
LFA-1	Day 5
Other immune cells	**Peak**
Complement Protein C1	24 hours
Complement Protein C3	24 hours
MHC II	Consistently after day 1
Infiltrating $CD8^+$ Cells	Day 5
Infiltrating $CD4^+$ Cells	Day 5

Table 1.3. The sequential kinetics of acute rejection has been studied in rats, with differing peaks in the expression of cytokines and adhesion molecules. Adapted from Nagano *et al* 1997.

1.17 Histological analysis of rejection

The characteristic signs of rejection from histological studies are of an increased number of MHC class II positive, IL-2 receptor positive T-cell infiltrates in tubular basement membranes. Based on histology findings from rejecting biopsy samples a classification system of rejection was developed, known as the Banff criteria (Solez *et al* 1993). A biopsy is taken when elevated serum creatinine or loss of renal function suggests a rejection episode. For acute rejection the system is based on the presence of tubulitis and arteritis in the graft. Mild tubulitis and arteritis are associated with borderline rejection. Severe arteritis and associated necrosis with lymphocyte infiltration of the graft are the signs of severe acute rejection. Chronic rejection is identified by the presence of fibrosis in the interstitium of the graft and atrophy of graft tubules.

1.18 Cytokines in rejection

Numerous cytokines have been implemented as potential factors in transplant rejection (Mantovani *et al* 1998, Kaminski *et al* 1995, Cho *et al* 1998). Cytokine genes are polymorphic and certain polymorphisms have been linked to rejection (Abdallah *et al* 1999). The regulation of cytokine genes is likely to be important in many disease states as well as transplant rejection (Taniguchi 1988, Ferrara 1993, Dallman 1993). Polymorphisms are likely to be important in both the donor and recipient, as well as the matching combination between the two. Certain polymorphisms are functional and effect protein production resulting in high or low production individuals, for example IL-6 and TNFα (Fishman *et al* 1998, Bouma *et al* 1996). A high producer donor in combination with a low producer recipient may be less of a risk factor for graft success compared with a high producer donor and high producer recipient. Cytokine genes are polymorphic for both the receptor and protein itself. In a study of recipient cytokine receptor polymorphisms no

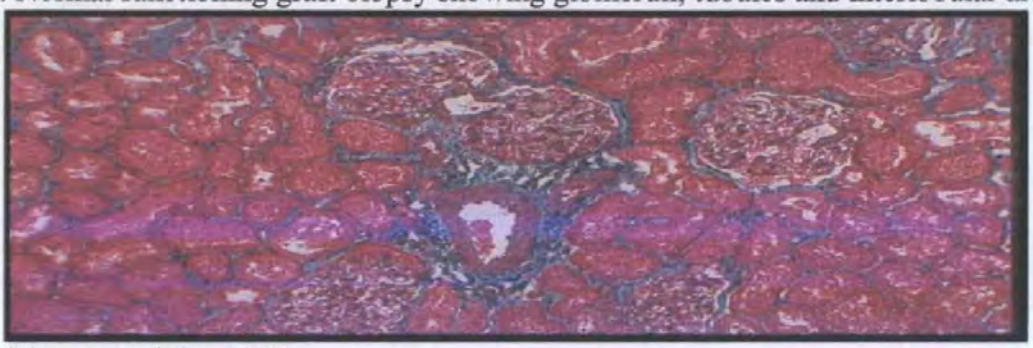
Category	Features
Borderline	No intimal arteritis and mild tublitis. <4 mononuclear cells/tubular section
Type IA	Significant interstitual inflammation and moderate tubulitis. >4 mononuclear cells/tubular section
Type IB	Significant interstitual inflammation and severe tubulitis >10 mononuclear cells/tubular section
Type IIA	Mild to moderate arteritis found in at least one arterial cross section.
Type IIB	Severe arteritis associated with loss of greater than 25% of the luminal area
Type III	Severe arteritis and necrosis of smooth muscle cells associated with lymphocyte inflammation of the vessel
Antibody mediated	Anti-donor antibody present in graft.

Table 1.4. Categories of the Banff criteria and associated histological findings used for the diagnosis of acute rejection episodes.

Category	Features
Grade I	Mild fibrosis of the interstitium affecting < 25% of the cortical area and mild atrophy of tubules affecting <25% of tubules
Grade II	Moderate fibrosis of the interstitium affecting 25-50% of the cortical area and moderate tubular atrophy affecting 25-50% of tubules
Grade III	Severe interstitial fibrosis affecting >50% of the cortical area with severe atrophy of tublues affecting >50% of tubules

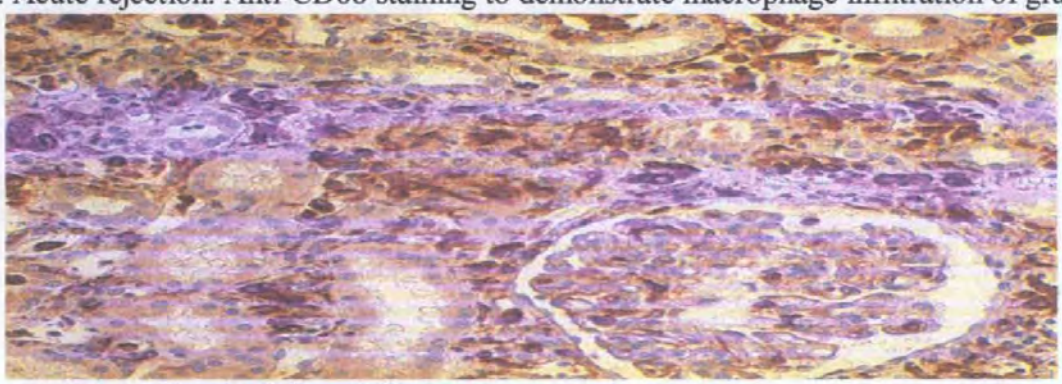
Table 1.5. Categories of the Banff criteria and associated histological findings used for the diagnosis of chronic rejection and chronic graft nephropathy.

A. Normal functioning graft biopsy showing glomeruli, tubules and interlobular artery.



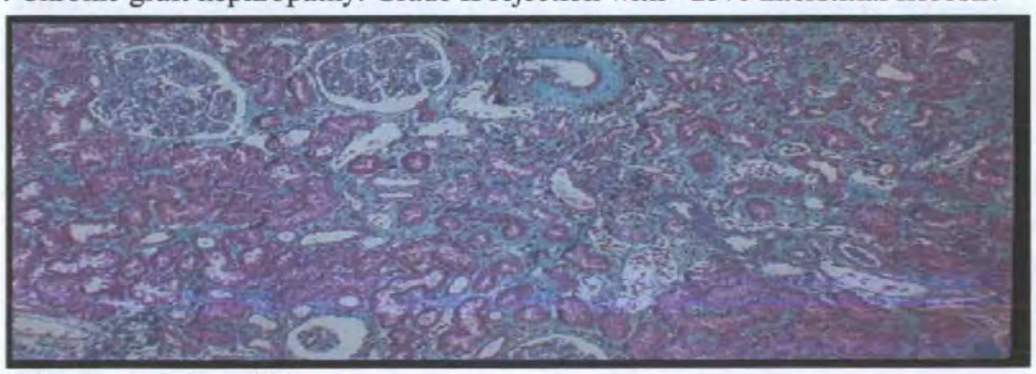
Trichrome staining x100

B. Acute rejection. Anti-CD68 staining to demonstrate macrophage infiltration of graft.



Immunoperoxidase staining x160

C. Chronic graft nephropathy. Grade II rejection with >25% interstitial fibrosis.



Trichrome staining x100.

Figure 1.10. Histological analysis of graft rejection showing a normal (A), acute rejecting (B) and chronic rejecting (C) biopsy.

association was found with 22 cytokine or cytokine receptor polymorphisms in 11 genes studied in 209 cadaveric transplant recipients, in particular TNFα and IL-10 (Marshall *et al* 2000). In donors, a cytokine polymorphism in the IL-6 gene was associated with incidence and severity of recipient acute rejection, recipient or donor-recipient matching for IL-6 had no effect (Marshall *et al* 2001). High and low producer status has also been studied in 29 donors for the TNFα, IL-6, TGFβ and IL-10 genes in relation to early graft function. The TNFα high producer status was found to correlate with graft rejection, the same trend was found in liver and heart grafts (Gandhi *et al* 2001). In recipients a study of 169 cadaveric transplants showed a correlation between high producer TNFα genotype and increased risk of rejection, more severe episodes and higher serum creatinine levels at one month post transplant (Poli *et al* 2001). However a similar study found a higher frequency of rejection in high IL-6 producers in 43 recipients but this was not significant. The same paper found also an increased frequency of rejection episodes in TNFα high producers but again not significantly so (Reviron *et al* 2001).

1.19 Vascular rejection/intragraft coagulation

1.19.1 The endothelium

The internal lining of the vascular system contains endothelial cells. It is estimated that in an average 70Kg adult the endothelium would occupy a surface area of more than $1000M^2$ and weigh in excess of 100g (Jaffe 1987). The earliest recognition of the importance of endothelial cells in injury was in Listers' 1865 paper on the role of the vessel wall in injury. The prime function of the endothelium is in the rapid exchanges between the blood plasma and the interstitial fluid. The endothelial cells participate in the regulation of vessel tone and permeability, haemostasis, fibrinolysis and the synthesis of growth factors. The endothelium produces many substances (see Table 1.8). Due to its

Polymorphism	Organ	recipient/Donor	Outcome	Author
IL-4 (-590)	Heart	Donor	rejection	Bijlsma *et al* 2002
VEGF(-1154)	Kidney	Recip	rejection	Shahbazi *et al* 2002
VEGF (-2578)	Kidney	Recip	rejection	Shahbazi *et al* 2002
TNFα (-308)	Kidney/Liver	Donor	rejection	Gandhi *et al* 2001
IL-10 (-1064)	Kidney	Recip	graft survival >15 years	Poli *et al* 2001
IL-6 (-174)	Kidney	Donor	rejection	Marshall *et al* 2001
IFNγ CA(n)	Kidney	Recip	rejection	Asderakis *et al* 2001
TNFα CA(n)	Kidney	Recip	rejection	Asano *et al* 1997

Table 1.6. Recent polymorphisms associated with rejection and graft survival in transplantation.

unique and crucial positioning the endothelium plays a vital role in the homeostatic maintenance of a non-thrombotic surface between the blood and tissue. Activation of the endothelium during an inflammatory response was first noted in the 1960's (Willms-Kretschmer *et al* 1967). The five main changes seen in the endothelial cell during activation are loss of vascular integrity, expression of adhesion molecules, a change in phenotype from anti-thrombotic to pro-thrombotic, cytokine production and HLA up-regulation (Hunt & Jurd 1998). The activation of endothelial cells occurs in a two-stage process. The first stage is the stimulation phase, which does not require *de novo* protein synthesis, so is a rapid response and results in the release of VWF and expression of P-selectin. The second phase is the activation phase that results in the transcription of genes including cytokines and further adhesion molecules (Mantovani *et al* 1997a, 1997b). In the endothelium of small arteries and arterioles the adhesion of lymphocytes, causes endothelialitis and destructive changes leading to graft destruction (Halloran *et al* 1993). The vascular endothelial cells participate in the process of allograft rejection by promoting both the recruitment and activation of alloreactive T-cells via three pathways (Rev by Briscoe *et al* 1998).

1. Endothelial cells mediate selective recruitment of $CD4^+$ T-cells, including the Th1 and Th2 subsets via chemokines and adhesion molecules.
2. Endothelial cells co-stimulate the production of effector cytokines by the Th cells.
3. Endothelial cells regulate T-cell apoptosis via signalling pathways e.g. CD40 -CD40L and Fas – FasL.

The hypothesis is that selective recruitment of the Th1 and Th2 cells to the site of inflammation is based on the differential expression of the adhesion molecule ligands and cytokine receptors (Austrup *et al* 1997). Under culture conditions, endothelial cells express

Connective tissue components	**Coagulation Components**
Laminin	Factor V
Fibronectin	Thrombomodulin
Vitronectin	Thromboplastin
Heparin	Von Willebrand Factor
Sulphate rich glucoproteins	Tissue Factor
Adhesion Molecules	**Vasoactive Substances**
E-Selectin	Endothelin
ICAM-1	Prostacyclins
VCAM-1	Nitric Oxide
	Angiotensin converting enzyme

Table 1.7. The major substances produced by endothelial cells

class I molecules but not class II MHC molecules. Endothelial cultures can activate $CD4^+$ T-cells only if they are pre-treated with IFN-γ to cause MHC class II expression.

The capacity of endothelial cells to activate alloreactive memory cells would suggest that graft endothelial cells could initiate rejection episodes (Pober *et al* 1996).

Endothelial cells express CD40 and CD40L which Influence T-cell activation and recruitment, and may regulate immune responses by expressing FasL and promoting T-cell apoptosis (Briscoe *et al* 1998). The leukocyte – endothelial interaction has been described as a multi-step paradigm in which a sequential process of leukocyte adhesion occurs. Firstly low affinity 'rolling' reactions followed by firm tight adhesion (Springer 1994). The three classes of adhesion molecules each play a vital role in this process of leukocyte adherence to the endothelium (Beekhuizen and Van de Gevel 1998). An activated endothelium may be vital in the early pathogenesis of many inflammatory diseases (Pober and Cotran 1990). The upregulation of MHC expression has been demonstrated in ischaemic conditions in animal models (Shackleton 1998). This follows early work by Thorsby (1975) who reported that cultured human endothelial cells could stimulate allogeneic T-cells to proliferate *in vitro*. This has lead to debate into whether graft endothelial cells are sufficiently competent APC's to initiate a host-anti-graft immune response (Pober *et al* 1996).

Numerous possible markers of a dysfunctional or activated endothelium have been suggested. There is however, considerable argument as to which represents the most ideal in relation to ease of measurement, reliability and specificity (Pearson 1993,Blann 1995).

1.19.2 Markers of endothelial dysfunction.

1.19.3 Von Willebrand factor (VWF).

VWF is a pro-coagulant product of the endothelium stored intracellularly in the Weibel palade body, a specific organelle of the endothelial cell, which are about 0.1μm wide and up to 4μm long (Weibel and Palade 1964). VWF has a molecular weight in the active form of 220-225 KDa. Transcriptional regulation of VWF production is in part regulated by the Oct-1 transcription factor (Schwachtgen *et al* 1998) and polymorphisms exist in coagulation factors suggesting a genetic component to vascular disease (Ferrer-Antunes 1998). VWF is produced by the α granules of platelets, but the majority of plasma levels originate from the endothelium (Wagner 1990). Raised levels are found in numerous inflammatory diseases where a damaged or dysfunctional endothelium is involved, including renal disease, and it is cited as a possible indirect marker of the development of atherosclerosis and/or thrombosis (Blann 1993, Lip and Blann 1996). Damaged endothelial cells release VWF and increased levels have been found during injury responses (Reinders *et al* 1987). Normal plasma levels are in the range of 5-10μg/ml. VWF is secreted via two distinct pathways. The first is a constitutive pathway, where the VWF molecules are composed mainly of small multimers and dimers. The second pathway of secretion is the regulated release from the Weibel-palade body. The stored pool of VWF tends to be of a higher molecular weight, which are more active in platelet aggregation (Sporn *et al* 1986). Numerous secretagoges including, PMA, thrombin, histamine, fibrin and the complement proteins C5b-9 can induce the secretion of VWF (Giddings & Shall 1987). VWF release is coupled to Ca^{2+} influx and an acidic intracellular environment. It has been shown that VWF levels are increased during exercise or adrenaline intake (Prentice *et al* 1972), suggesting a mass release from the Weibel-palade bodies. However,

it has also been shown that the release from storage pools does not affect the overall distribution or synthesis of VWF (Mayadas *et al* 1989).

VWF is involved in the aggregation and adhesion of platelets following endothelial injury. The mass platelet clumping associated with the condition thrombotic thrombocytopenic purpura is linked to the overproduction of large VWF multimers. The main function of VWF is to protect protein factor VIII from inactivation (Koppelman *et al* 1996). VWF is normally found in excess (x100) of factor VIII, which is degraded by protein C when not bound to VWF. Patients with VWF syndrome, who do not produce the molecule, have clotting abnormalities. Raised levels of VWF are thought to increase the risk of thrombus formation as VWF cross-links platelets and mediates adhesion of platelets to the sub-endothelia, which is exposed by damage to the endothelium (Blann 1998). The initiating stimulus for VWF release or synthesis in patients with vascular disease is currently uncertain, but is suggested that factors such as oxygen free radicals or hypoxia may be important (Lip and Blann 1997). During ischaemia/reperfusion injury in the rat, plasma VWF levels were elevated after surgery, but not significantly from sham operated rats and did not correlate with the degree of intestinal injury (Abu-Ziden *et al* 1999). As a marker of endothelial activation in transplant injury, VWF in xenotransplantation has been shown to be upregulated in pigs, but they did not receive any immunosuppression (Brouland *et al* 1999). Cyclosporine is cytotoxic to endothelial cells and has been shown to increase VWF production (Yussim *et al* 1994). Also in transplant recipients with CMV infection levels of VWF were found to be higher than in patients without the virus (Kas-Deelen *et al* 2000). In rejection, increased glomerular deposits of VWF were found in chronic rejecting grafts but less in acute rejecting grafts in primates (Lagoo *et al* 2000).

Endothelial cell activation during an immune response is a likely to influence the development of vascular damage and coagulation. Endothelial damage results in an altered

coagulant state, with down-regulated thrombomodulin and antithrombin III and up-regulated tissue factor and VWF factor expression. These alterations in endothelial function have been found to correlate with fibrin deposition in rejecting cardiac allografts (Salom *et al* 1998) and are thought to produce a pro-coagulant environment (Ogawa *et al* 1990). At least 13 coagulation proteins and nearly as many inhibitors are involved in the formation of a blood clot (Esman 1993).

Endothelial cells participate in many key events in the coagulation cascade, including the propagation of the initial stimulus and interaction with fibrinogen and fibrin to modify the structure of the clot. Synthesis of the major coagulation and anticoagulant factors occurs mainly in the liver, but the endothelium also synthesises some factors. Damaged endothelial cells can initiate the coagulation cascade, which involves the conversion of prothrombin to thrombin, activation of platelets and fibrinogen. Fibrinogen is converted to fibrin, which interacts with the activated platelets forming a thrombus. Thrombomodulin regulates thrombin by converting it from a procoagulant to an anticoagulant form. It has been proposed that soluble levels in cell culture supernatants reflect damaged endothelium (Ishii *et al* 1991). The proposed mechanism is that the appearance of soluble thrombomodulin reflects a loss of cellular levels and hence the homeostasis of coagulopathy. Soluble thrombomodulin is specific to the endothelium and independent of inflammatory cytokines such as IL-1 and TNF.

Drugs used to treat coagulative disorders such as atherosclerotic disease are aimed at lowering fibrinogen levels (e.g. aspirin, beta-blockers). The blood contains three major anticoagulant protein systems that regulate coagulation. One involves antithrombin III and is enhanced by heparin. The second involves two vitamin K-dependent factors protein C and protein S, along with the endothelial derived thrombomodulin. The third involves tissue factor and its inhibitor TFPI. Monocytes can be envisioned as circulating loci for the

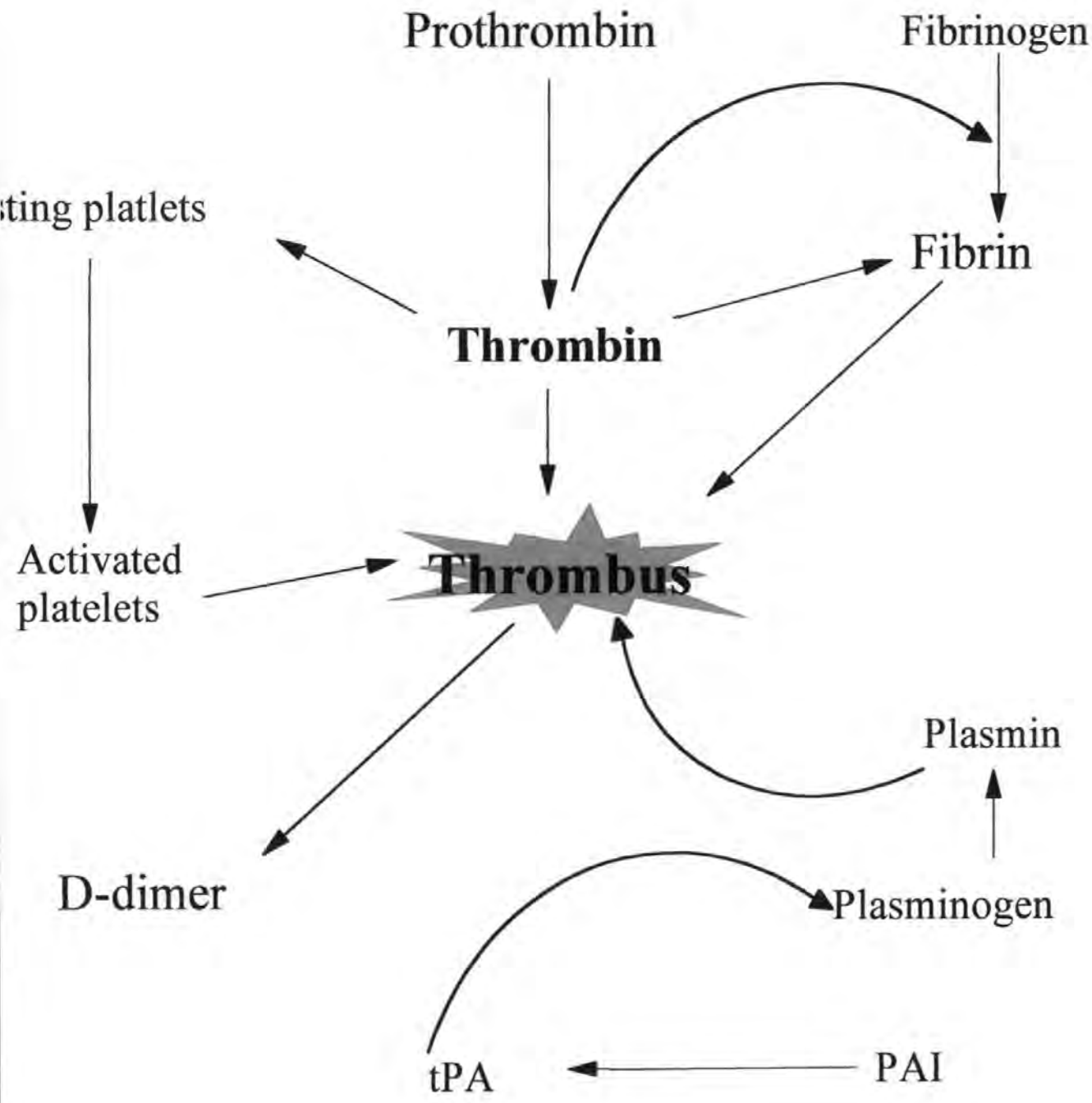


Figure 1.11. A basic overview of the coagulation cascade associated with vascular rejection. Adapted from Blann 1998

production of tissue factor in inflammation, atherosclerotic lesions and septic shock. Upregulation of endothelial adhesion molecules during inflammation leads to attachment of procoagulant monocytes to the endothelium. Platelets may then adhere to the bound neutrophils or monocytes via L-selectin and its ligand, P-selectin.

Anti-coagulant pathways may be down regulated by inflammation. Cytokines such as TNF and IL-1 can lead to the down regulation of thrombomodulin production in the endothelium. The thrombin receptor may be upregulated during inflammatory responses, as thrombin binding leads to mitogenic effects on fibroblasts, macrophages and smooth muscle cells. Thrombin activates endothelial cell release of vasoactive substances such as VWF, nitric oxide and prostacyclin. Thrombin also activates platelets. Thus, thrombin mediates coagulation and vascular tone in inflammatory processes. Thrombosis leads to the occlusion of vessels, causing progressive necrosis as a result of hypoxia.

1.20 Renal preservation and Ischaemic/Reperfusion injury

Preservation time includes the removal of the kidney from a donor, tissue typing, selection of a recipient, preparation of the recipient and surgical transplant of the graft. The use of donor kidneys within a restricted community can reduce preservation times, however, when transport to other areas is required, preservation times can exceed 60 hours and times of greater than 100 hours have been reported in the US (Marshall *et al* 1994). In the European transplant region the average cold ischaemia time is 22 hours (Offermann 1998). After removal from a suitable donor the kidney is preserved in one of two ways. Static storage involves the graft being immersed in cold preservation fluid and stored at 0-4^{0}C. Perfusion storage involves the kidney being continuously perfused with a solution at 4 - 10^{0}C. Generally studies have found no difference (Heil *et al* 1987) or better survival rates in static stored kidneys (Opelz & Terasaki 1982). Current preservation solutions are

designed to reduce stress to the graft and preserve organ integrity. Many exist including; Collins' type solution, citrate based solutions, sucrose based solutions, Bretschneiders's HTK solution and the University of Wisconsin solution. Storage in UW solution has been shown to have a less measurable effect on endothelial damage than HTK solution and Eurocollins solution as assessed by mitochondrial, ER and cytoskeletal fibre damage (Eberl *et al* 1999). Effects of ischaemic damage have been shown to reversible during rewarming, and when cells are replaced in culture media (Gerlach *et al* 1993). Cost effective calculation measurements have suggested the use of 10L of perfusion fluid per multiorgan donor (Muhlbacher *et al* 1999).

The ischaemic period consists of an intial normothermic ischaemia during organ removal (Warm ischaemia), a hypothermic preservation period during organ storage (Cold ischaemia), and finally revascularisation leads to reperfusion injury.

Ischaemia results in oxygen and nutrient depletion, continuing anaerobic metabolism, build-up of catabolites with the potential to precipitate cell death and obstruction of the vasculature with erythrocytes and leukocytes.

The organisms response to hypoxia and hypothermia has been well studied (Hochachka 1986). It has been shown that warm ischaemia is of greater importance than cold ischaemia in terms of damage, as the effect of 30 minutes warm ischaemia is more damaging to graft survival than an additional 24 hours of cold storage (Sacks *et al* 1973). Studies have also shown that warm ischaemic damage needs to be less than 30 minutes to be reversible (Florack *et al* 1986). Protection of the graft from warm ischaemia is to reduce the exposure time to a minimum by rapid cooling of the kidney using a double balloon catheter to begin *in situ* cooling of the graft in the donor.

Cold storage is recommended to be less than 24 hours (Abouna *et al* 1987), with an estimated 2% reduction in 5-year graft survival for every 12 hours cold ischaemic time

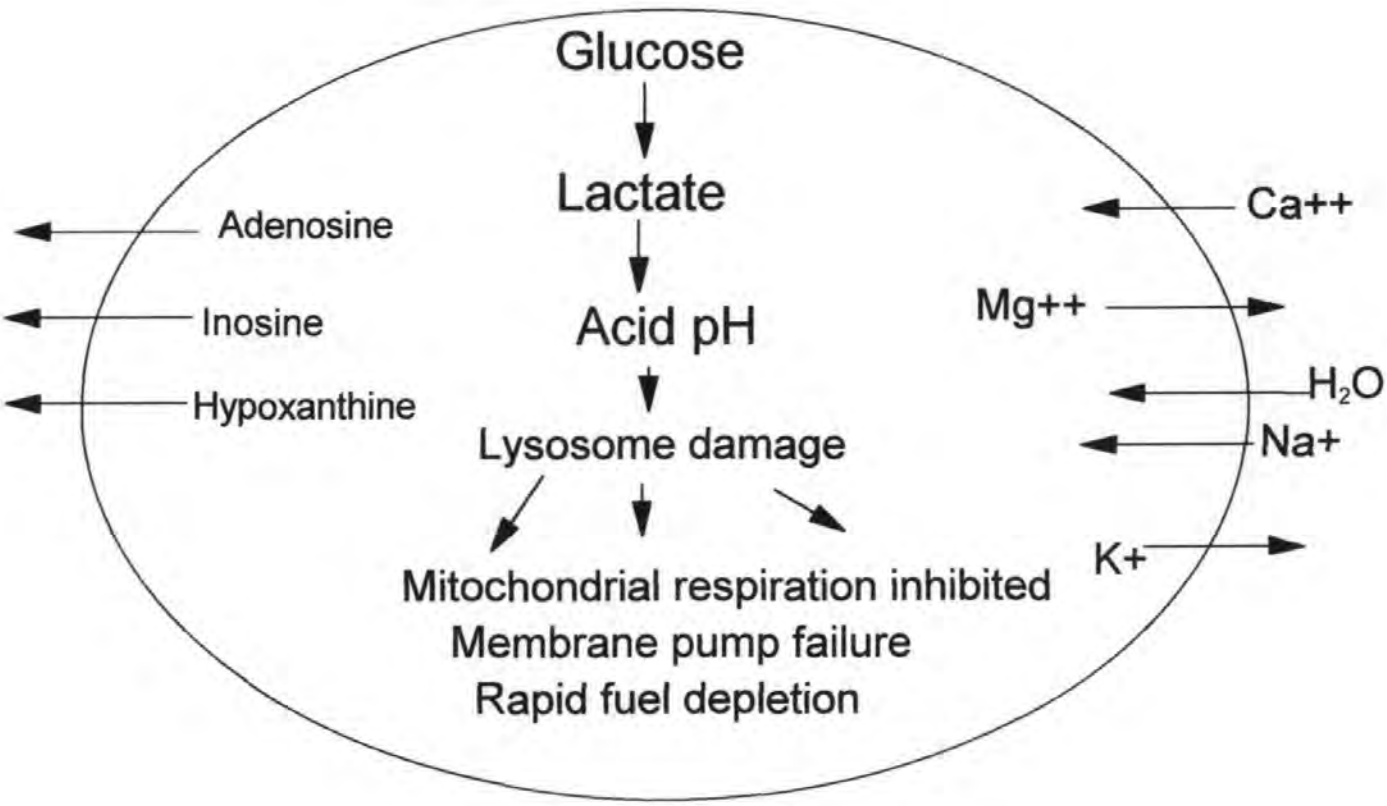


Figure 1.12. The effects of ischaemia on cellular metabolism. Adapted from Marshall *et al* 1994

(Held *et al* 1994). Studies have shown reduced long term survival of grafts that have experienced prolonged ischaemic periods (Naimark & Cole 1994). A recent study compared the incidence of delayed graft function and general graft function at one year in locally transplanted kidneys and kidneys shipped to other units in the US. The analysis was from data contained in the UNOS registry and compared 5446 pairs of cadaveric kidneys. The study found a significant increase in delayed graft function in shipped kidneys compared with locally transplanted kidneys, which was also found when adjusted for HLA matching (Mange *et al* 2001). The authors highlight increased CI times as a possible explanation, but also suggest units may tend to send damaged kidneys away (Mange *et al* 2001).

1.20.1 Hypoxia

The kidneys receive a large supply of oxygen, approximately 20-25% of the total cardiac output. The great majority (80%) is consumed by the medulla with the cortex less demanding of oxygen. Hypoxia is associated with a loss of ATP, which leads to the inability to function cellular transporters such as the Na^+ and K^+ ATP-ases. This results in the accumulation of calcium and sodium ions and hence cell swelling, due to the inability to regulate the electrolyte gradient in the cell. Hypoxia causes the upregulation of vasoconstrictors and smooth muscle mitogens such as PDGF-B, endothelin-1, VEGF, thrombospondin-1 and matrix remodelling factors such as collagenase IV, MMP-9, thrombospondin-1. Hypoxia inhibits vasodilatory or antimitogenic factors such as eNOS (Faller 1999). Hypoxia causes increased endothelial monolayer permeability, leukocyte-endothelial interactions and the prevalence of pro-coagulant over anti-coagulant properties, with increased polymorphonuclear cell adherence to endothelial cells (Arnould *et al* 1993). The activation of endothelial cells by hypoxia may be responsible for the microvascular

thrombosis seen after extensive periods of ischaemia. Hypoxia has been shown to induce apoptosis in endothelial cells (Matsushita *et al* 2000). Studies on endothelial cells have shown lactate and H2O2 release during hypoxia with peak production at 4 hours (Kondo *et al* 1996).

1.20.2 Hypothermia

Local cooling of the vasculature results in reduced blood flow due to an associated increase in blood viscosity. The formation of extracellular ice will cause damage to the intima of blood vessels and endothelial integrity when thawed. Endothelial damage and tissue oedema leads to poor reopening of the vasculature, a condition known as the "no reflow" phenomenon (Massberg & Messmer 1998). The endothelial cell monolayer has increased permeability, leukocyte-endothelial cell interactions are promoted and the procoagulant properties prevail over anticoagulant properties (Seigneur *et al* 1994). It has been shown that endothelial cells can tolerate low temperature with 79% cell survival at 4^0C for 48 hours (Hansen *et al* 1994). During cold stress, the plasma membrane is considered the primary site of damage, but in fibroblast, lymphocytes and granulocytes, disruption of lysosomes leading to mitochondrial damage was found to be the major lesion at slow cooling temperatures down to -40^0C (McGann *et al* 1988). In liver, aortic and coronary endothelial cells, susceptibility to cold injury was reduced if the cells were at a non proliferating stage of cell growth compared to proliferating cultures (Rauen *et al* 1994). Similar to the effects of hypoxia, cold ischaemia causes the inhibition of enzymes and active transport mechanisms resulting in the influx of Na^+, Ca^+, Cl^- and water, with the efflux of K^+ and Mg^{2+} resulting in cell swelling. Cryopreservation has also been shown to upregulate mRNA for mitogenic factors such as VEGF and PDGF (Liu *et al* 2000).

Sections from kidneys with increasing cold ischaemic times have shown a correlation with ICAM-1 expression, MHC class II expression and macrophage infiltration in both allografts and isografts of rats (Kouwenhoven *et al* 2001). Immunohistological analysis of donor biopsies from living or cadaveric sources showed a higher expression of ICAM-1, VCAM-1 and E-selectin in cadaveric donors compared with live donors, also higher degrees of tubular antigen expression in trauma related deaths correlating with rejection episodes (Koo *et al* 1999). T-cells cryopreserved at very low temperatures (-196^{0}C) have been shown to have a good viability when thawed. These T-cells retained their ability to produce cytokines such as IL-6, TNFα and IFN-γ, and had a significantly increased IL-2 production compared to control, freshly isolated T-cells (Wang *et al* 1998b). This may have implications for passenger cells in donor organs.

1.20.3 Reperfusion

ATP is reduced during hypoxia but is replaced during reoxygenation as is lipid peroxidation (Windischbauer *et al* 1994). Oxygen free radicals such as reactive oxygen intermediates (ROIS) and species (ROS) are central mediators of the cellular injury that occurs upon post ischaemic reperfusion. Critical to the signalling pathways found during hypoxia and reoxygenation are the intracellular thiol-redox proteases and antiproteases which are modified by oxidants and are involved in calcium mobilisation (Chakraborti & Chakraborti 1998). The intracellular redox state and the generation of oxygen free radicals and H2O2 activates protein tyrosine kinases which elevate intracellular calcium via flux from the intra- and extra- cellular space. H2O2 is an evolutionary conserved second messenger as it induces signal transduction activation in plants, as well as inducing transcriptional responses in higher vertebrates (Muller *et al* 1997).

Aerobic cells generate energy by the reduction of oxygen to water. The reduction of molecular oxygen produces intermediate molecules such as the superoxide anion radical, the hydroxyl radical and hydrogen peroxide. Lipid peroxidation also produces unstable intermediates. Excessive production of ROS such that the cells anti-oxidant enzymes systems cannot remove them leads to the condition known as oxidant stress (oxidative stress). Damage is caused by the fact that these ROS molecules have unpaired electrons so are free radicals. Free radicals have the ability to react with virtually any biological molecule. The major free radicals are superoxide, the hydroxyl radical, nitric oxide, and the lipid radicals. Other ROS's include H202, hypochlorous acid and peroxynitrite, but these have oxidising effects as opposed to free radicals. The lipid radicals are produced in a chain reaction when radicals react with polyunsaturated fats within a membrane forming fatty acid peroxyl radicals that can react with adjacent side chain molecules leading to lipid peroxidation and hence membrane damage. ROS cause the inactivation of nitric oxide ($NO^{\cdot}$).

1.21.1 Nitric Oxide

$NO^{\cdot}$ was discovered as a free radical produced when L-arginine is catalysed to L-citrulline in the presence of oxygen and NADPH by the $NO^{\cdot}$ synthase enzymes (Garthwaite *et al* 1988)

$$\textbf{L-arginine} \xrightarrow{O_2 \quad NADPH} \textbf{L-citrulline} + NO^{\cdot}$$

$NO^{\cdot}$ was initially thought to have a role in mainly physiological processes such as the control of blood pressure and respiration, but now is implicated in many events including platelet aggregation, blood brain barrier permeability and anti-bacterial/viral defences (Hurst & Dobbie 2001). In pathological states, high levels of $NO^{\cdot}$ can be found to be beneficial or detrimental. This has led to the conclusion that the relative amount of $NO^{\cdot}$ is a confusing measure and its level in relation to other free radicals and ROS is likely to influence the nature of a cellular response. There are three main $NO^{\cdot}$ synthases which have been identified in mammalian cells (Michel & Feron 1997). A constitutively expressed form identified in endothelial cells (eNOS), but also found in myocytes and blood platelets. An inducible form (iNOS) is found in many cell types and is induced by pro-inflammatory cytokines and mediators such as bradykinin and histamine. There is also a neurone-derived form of $NO^{\cdot}$ synthase (nNOS) that has been shown to also be produced by skeletal muscle (Michel & Feron 1997). $NO^{\cdot}$ stimulates the production of the second messenger, cGMP at normal levels. In neuronal cells binding of $NO^{\cdot}$ to soluble guanylyl cyclase receptors results in an increase in calcium influx to the cell. This activates nNOS and the accumulation of cGMP causing muscle relaxation. High levels of $NO^{\cdot}$ are cytotoxic, as it starves cells of ATP by inhibiting mitochondrial respiratory chain ATP production (Brown 1995). Glycolysis is also thought to be affected by high $NO^{\cdot}$ levels as it binds to the important glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme (Padgett & Whorton 1995) thus inhibiting its vital role in the glycolytic pathway. High levels of $NO^{\cdot}$ are also associated with deamination of DNA nucleotide bases and DNA fragmentation (Tamir *et al* 1996). $NO^{\cdot}$ reacts directly with protein prosthetic groups, and can interact with the superoxide radical to form peroxynitrite ($ONOO^{-}$) a powerful oxidant which can oxidise iron-sulphur clusters, zinc-fingers and protein thiol groups, resulting in cell toxicity (Christopherson & Bredt 1997). Knockout mice which are iNOS deficient show a

confusing pattern of inflammatory response, as noted by a reduction in neutrophil adherence to the endothelium and increased LPS-induced lung damage, but an increased rate of acute allograft rejection (Nathan 1997). In the absence of L-arginine eNOS can act as xanthine oxidase and produce superoxide and H202. This reaction is known as uncoupling.

1.31.2 Xanthine oxidase

Xanthine oxidase converts oxygen to H202, creating the superoxide anion. It is derived from xanthine oxidoreductase which catalyses the oxidation of xanthine. Superoxide production by xanthine oxidase is thought to inhibit $NO^{\cdot}$ leading to endothelial dysfunction (Cai & Harrison 2000)

1.31.3 NAD(P)H Oxidase

Increases in ROS during ischaemia and reperfusion may be the result of NADPH oxidase activity (Wei *et al* 1999). Two major NAD(P)H oxidases exist in humans. The neutrophil NAD(P)H oxidase and the vascular NAD(P)H oxidase. They are similar in structure, but differ in their output, with the vascular NAD(P)H oxidase thought to have one third the superoxide output of the neutrophil NAD(P)H oxidases (Griendling & Ushio-Fukai 1998). Vascular NAD(P)H oxidases release superoxide over minutes or hours compared with an instantaneous release in neutrophils.

The NAD(P)H oxidases are bound in the plasma membrane and contain 4 major subunits. The membrane spanning cytochrome b558 which is composed in turn of a large subunit known as gp91phox and a smaller subunit known as p22phox. The complex also has 2 cytosolic components, p47phox and p67phox, which regulate the activity of the

cytochrome subunits. The active complex is assembled and in part regulated by the small GTP-binding proteins rac2 and rap1A. The orientation of the enzyme complex would suggest the utilisation of intracellular NADH or NADPH and the removal of superoxide to the extracellular space. However, superoxide and H202 production is mainly intracellular (Griendling *et al* 2000).

The enzyme complex catalyses the reduction of oxygen using NADH or NADPH as the electron donor.

$$NAD(P)H + 2O_2 \longrightarrow NAD(P)^+ + H^+ + 2O_2^-$$

The major source of ROS in vascular tissue was thought to originate from xanthine oxidase, arachidonic acid and mitochondrial oxidases, but it is now thought that the NAD(P)H oxidases are the major source of ROS production in vascular tissue.

The NAD(P)H oxidases have been found to be important in physiological processes such as cell growth, migration and extracellular matrix modification (Rev by Griendling *et al* 2000). The role of NAD(P)H oxidases in the production of ROS has implicated them in diseases such as hypertension and arteriosclerosis (Cahilly *et al* 2000).

The NAD(P)H oxidase activity is upregulated by two mechanisms. Firstly, by the activation of a second messenger (such as calcium), and secondly by the direct upregulation of oxidase subunit mRNA. It has been shown that TNFα can induce the increased transcription of p22phox (De Keulenaer *et al* 1998).

Importantly reoxygenation after ischaemia and associated increases in lactate have been shown to cause the upregulation of NADH oxidase dependent superoxide production (Mohazzab *et al* 1997). The effect of upregulated oxidase activity is the increased

production of ROS which function as second messengers mediating further cellular responses. The majority of studies into the effect of polymorphisms of the NAD(P)H system are in vascular disease such as atherosclerosis. The p22phox subunit contains a C242T transition polymorphism that creates a histidine to tyrosine change altering the heme binding site in the gene (De Boer *et al* 1992). Individuals with the T allele have a less active NAD(P)H oxidase system and hence less superoxide production (Guzik *et al* 2000, Zalba *et al* 2001). It has been shown that control populations have a higher incidence of the T allele compared with patients with coronary artery disease (Inoue *et al* 1998). The polymorphism has also been linked to progression of the disease (Cahilly *et al* 2000). In a Japanese population with ischaemic heart disease the T allele was found to be statistically higher in patients with cerebrovascular disease, suggesting the polymorphism does not have a protective effect (Ito *et al* 2000).

Defences mechanisms exist in cells to breakdown ROS. Cells have protective enzymes such as catalase, superoxide dismutases (SOD) and glutathione peroxidase as well as anti-oxidants such as ascorbic acid and reduced glutathione (GSH). Endothelial cells have low catalase levels compared to other cells, but utilise GSH as a potent free radical scavenger. Reduced levels of GSH during ROS-induced injury are compensated for by an increase in superoxide dismutases. These also include the free radical scavengers such as vitamin E. Cells must maintain a balance between the production of ROS and their breakdown. Oxidative stress occurs as a result of an imbalance between these two systems.

ROS production can be induced by UV light, certain drugs and tissue hypoxia. ROS can react with any biological macromolecules including proteins, lipids and carbohydrates causing cellular dysfunction and ultimately cell death. Administration of SOD during surgery has been shown to reduce the production of free radicals (Land 1998). SOD does not transverse the endothelial and myocyte membranes due to its molecular weight of 32

KDa which suggests that free radical generation occurs at the cell surface of the endothelium or within the vascular lumen adjacent to the endothelium (Zweier 1998). In ischaemic tissue xanthine dehydrogenase is converted by a proteolytic cleavage to xanthine oxidase which reduces oxygen producing free radicals. During ischaemia elevated concentrations of the substrates xanthine and hypoxanthine may occur due to the breakdown of ATP. It has been shown in endothelial cells subjected to periods of hypoxia followed by reoxygenation that free radicals are produced (Zweier 1998). It is thought that xanthine oxidase was the major source and that iron mediated fenton reactions further catalysed the formation of the reactive hydroxyl radical from the enzyme derived superoxide and hydrogen peroxide. Clinical studies using more sophisticated methods are needed to confirm data indicating increased oxygen free radical formation in kidney transplant patients during the peri/post surgical phase (Illner & Land 1998).

1.22 The 'Injury response'

All types of post-transplant allograft destroying events such as hyperacute, acute and chronic rejection have been interpreted as the result of specific immunological phenomena, primarily brought about by cell mediated and/or antibody mediated tissue injuries. At the 1992 congress of the international transplantation society, it was suggested that initial nonspecific injury of an allograft mediated by postischaemic reperfusion injury contributes to the development of chronic graft failure. This idea lead to the formation of the hypothesis that, ischaemic/reperfusion injury mediates an inflammatory response that provokes an increased level of acute host immunological reactivity (Tilney & Guttmann 1997, Halloran *et al* 1997, Land 1998, Lu *et al* 1999). The hypothesis is the results of the initial finding that increased acute rejection in renal allografts which had experienced delayed function, was attributed to severe injury which made them 'more rejectable'

(Halloran *et al* 1988). This hypothesis is also an extension of theories that the immune system recognises potential danger and non-specific activation of the immune system (Matzinger 1994, Ibrahim *et al* 1995, Fearon & Lockersly 1996). The damage caused to the graft by surgical removal, transport and implantation are well known in terms of hypoxia, cold ischaemia and reperfusion, but how do these events influence rejection? One proposed answer is that rejection depends on the host T-cells recognising the donors foreign antigens. Injury results in inflammation, which leads to the more rapid infiltration and migration of recipient T-cells and APCs such as dendritic cells into the donor organ (Lu *et al* 1999). Many potential immunomodulatory mediators are released during ischaemic injury such as TGFβ, NO and IL-2 (Shackleton 1998). Dendritic cells migrate to lymph nodes and the spleen, where they can activate naive T-cells (Austyn 1996). Injury to the kidney whilst it is still in the donor may recruit donor dendritic cells into the graft which will leave the graft after transplantation and directly activate host T-cells by displaying foreign 'donor' MHC in the lymph. Similarly, recipient dendritic cells migrating into inflamed tissue could return to the lymph and activate T-cells indirectly by displaying donor peptides (Lu *et al* 1999).

The major cell type involved in the injury response leading to inflammation and initiation of rejection is the endothelial cell (Pober *et al* 1996). Many studies have shown that ischaemia leads to the upregulation of adhesion molecules on endothelial cell surfaces (Troulong *et al* 1996). The upregulation of adhesion molecules by endothelial cells has been shown to increase T-cell migration into tissues (Adams & Shaw 1994). It has also been shown in mice that the expression of endothelial E-selectin selectively recruits Th1 T-cell subsets into inflamed tissue (Austrup *et al* 1997).

It has also been shown that under ischaemic conditions endothelial cells have an upregulated MHC class II expression (Shackleton 1998). Endothelial cells also express CD40 (Yellin *et al* 1995), the co-stimulatory molecule involved in T-cell activation, hence, endothelial cell 'immunogenicity' is increased by the preservation process (Ono *et al* 1998).

Other evidence to back the injury hypothesis is pointed towards demonstrations that living unrelated donors have survival rates as good as well matched cadaveric donors (Terasaki *et al* 1995), also the benefits of good matching are lost in grafts with prolonged ischaemic times (Held *et al* 1994). Finally by the demonstrations that interventions such as the use of antibodies against adhesion molecules, or administration of anti-oxidants such as SOD improve outcome in animal models, is further indirect evidence for the role of injury in graft rejection. The problem with testing the injury hypothesis, is that the ideal test, is to compare grafts transplanted injury-free, of course this can not be done (Lu *et al* 1999).

1.23 The 'Heat shock' paradox

The demonstration that a prior heat shock can protect cells from an ischaemic insult has lead to a suggested 'heat shock paradox' protective response (DeMeester *et al* 2001). Protein biosynthesis is an essential part of homeostasis and requirement for cells to function. Formation of a protein molecule involves translocation, folding, assembly and degradation before the mature protein is formed. Stress can result in demands on cellular homeostasis resulting in misfolding and assembly of vital proteins leading to their mis-function. To maintain and re-establish protein synthesis during stress, cells possess chaperone proteins and proteases, known collectively as the heat shock proteins (Morimoto 1993). Heat shock proteins respond to environmental and physical stress and repair protein damage (Morimoto & Santoro 1998). Activation of the heat shock factor

(HSF1) transcription factor results in the up-regulation of the heat shock proteins, which in turn form an autoregulatory loop and further regulate the activity of HSF1 (Abravaya *et al* 1992). The activation of HSF1 requires phosphorylation and occurs via Ras and Rac GTP-protein dependent signalling pathways (Bornfeldt 2000). Studies have shown that mitogen activated protein kinase (MAPK) activity works as a repressor of HSF1 activation (Kim *et al* 1997).

The heat shock family of proteins is large and they are named on the basis of their molecular weight. Some of the more important larger proteins are hsp100, hsp90, hsp70, hsp60 and hsp40. The smaller proteins generally form complexes with the larger proteins and include hsp27 as well as some immunophilin molecules such as FKPB52.

Heat shock proteins such as hsp60, hsp70 and hsp90 recognise hydrophobic residues in polypeptide chains exposed during protein misfolding and bind as co-chaperone complexes utilising ATP to aid refolding of the protein (Hartl 1996). Chaperone proteins can attain concentrations of 1-5% of the total cell volume during stress (Morimoto & Santoro 1998).

Heat shock proteins are induced by ischaemia and may provide a degree of protection from ischaemia and hypothermia (Gowda *et al* 1998, Duquesnoy *et al* 1995). Hsp60 and hsp70 have been shown to act as a marker of preservation-induced injury in endothelial cells (Eberl *et al* 1999a, 1999b). The mechanism of heat shock activation during ischaemia is likely to be due to oxidative stress. Oxidative stress has been shown to activate and cause the binding of HSF1 to its consensus sequence (Tacchini *et al* 1995). As a protective response, tissue levels of ATP have been shown to be higher in rats treated with a heat shock inducer before hypothermic storage (Zhang *et al* 1996). Improvement in renal function, mortality and histological abnormalities are found in rats pre-treated with ischaemia to induce heat shock protein expression compared to rats without heat shock activation (Kelly *et al* 2001). This has led to the 'heat shock paradox' in that inducing the

activation of heat shock proteins by pre-treatment may protect during subsequent ischaemic insults. Recently hsp90 has been shown to influence phenotype and morphology in plants. Manipulation of hsp90 to reduce its function, led to several different phenotypes produced in response to environmental changes such as temperature variation (Queitsch *et al* 2002).

A similar situation is seen with an ischaemic pre-treatment. Animal studies have demonstrated that an initial ischaemic insult to cells can also protect them from subsequent exposure to ischaemia (Morgan *et al* 1999). Ischaemic adaptation in myocytes to repeated ischaemic/reperfusion results in a decreased production of oxygen free radicals when re-exposed to ischaemia, mediated by increased protective BCL-2 expression (Maulik *et al* 1999). Pre-treatment with mild hypothermia has also been shown to be protective of renal function in rats (Zager *et al* 1989). Ischaemic pre-treatment with H202 was shown to reduce TNFα induced IL-6, IL-8 and ICAM-1 and E-selectin expression in rats (Zahler *et al* 2000).

One proposed mechanism for this is in the fact that the heat shock response inhibits NFκB (Heneka *et al* 2000). The pro-inflammatory and pro-apoptosis nature of NFκB may be down-regulated by heat shock protein expression. It has been suggested that ischaemic pre-treatment acts by reducing subsequent MAPK activity on second ischaemic insult, at the level of reduced MKK phosphorylation (Park *et al* 2001). Reduced MAPK stimulation may lead to decreased NFκB activation. One study has suggested the recovery of cells from an ischaemic insult can be determined by the recovery of IκBα and increased expression of IκBα (Wong *et al* 1997). Attempts have been made to find drugs that can activate the HSF1 transcription factor and inhibit NFκB. These have included protease inhibitors and prostaglandins (Rossi *et al* 1997,1998) and anti-inflammatory drugs such as NSAIDS (Lee *et al* 1995).

However, NFκB has also been shown to have a protective role in ischaemic injury. For example NFκB activated by ischaemia has been shown to induce the expression of the 'protective' endothelial gene A20 (Ferran *et al* 1998). NFκB expression has been shown to have a protective anti/apoptosis function in liver grafts exposed to cold ischaemia injury (Takahashi *et al* 2001).

As information gathers about the potential damage caused by initial ischaemic/reperfusive injury to allografts in clinical transplantation, the pressure to reduce cold ischaemia times to a minimum has increased. However, many people are concerned that reducing cold ischaemia times will result in increased HLA mismatches due to the reduced time in which to match and prepare recipients and donors. Many believe and have produced data to show that purely immunological factors such as preformed cytotoxic antibodies, the number of previous graft failures in a patient and HLA mismatches have a much greater impact on graft survival than cold ischaemia time (Lange and Kuhlmann 1998). A combination of good matching and reduced ischaemic time in combination produces the better graft survival, than either alone (Connolly *et al* 1996).

1.24 Interventions/ treatments of ischaemic injury

The damage caused by ischaemia and reperfusion injury at a cellular level is only really detectable *in vitro* as it is technically difficult to determine graft viability *in vivo* (Southard 1989). The determination of successful interventions in clinical use therefore relies on outcome measures such as delayed graft function or the incidence of acute rejection episodes, which may be attributable to many other factors (e.g. recipient status). Preservation solutions have been designed to attempt to address the numerous problems of *in vitro* kidney storage. The first factor to overcome is the lack of an oxygen supply.

Attempts have been made to continuously perfuse the kidney with an oxygenated perfusate, but have not shown significantly improved graft function compared with static stored kidneys (Merion *et al* 1990). The potential of perfusion storage is in the use of higher risk donor organs to expand the donor pool (Tesi *et al* 1993). The idea of perfusion storage was to address the metabolic supply to the kidney. Cooling the kidney reduces the metabolic need and the addition of glucose, amino acids, fatty acids, ATP and adenosine to perfusion fluids has attempted to address this problem. Cellular pH is maintained in the range of 7.1-7.8 and cellular oedema is prevented by electrolyte balancing solutions. It has long been shown that the anion content of perfusion solutions is very important (Collins *et al* 1979). A major concern in kidney storage is free radical mediated tissue damage. Prevention of free-radical damage has been addressed by the addition of mannitol, an anti-oxidant to solutions. The addition of the anti-oxidant allopurinol, which inhibits the activity of xanthine oxidase has been found to improve creatinine clearance in canine heart beating donors, but had no effect on organs from non-heart beating donors (Hernandez *et al* 1999).

The addition of SOD to kidneys via viral gene delivery has recently been shown to reduce free radical production and cyclosporine induced nephrotoxicity in rats (Zhi *et al* 2001). Simple addition of lecithinized SOD, which binds with a higher affinity than recombinant SOD to cell membranes, to preservation solutions has also been shown to reduce neutrophil adhesion to endothelial cells during subsequent reperfusion in an endothelial cell culture (Koo *et al* 2001). Gaseous oxygen perfusion during cold storage has been found to be effective in rat livers if SOD is administered before treatment to reduce free radical tissue damage (Minor & Kotting 2000). Other antioxidants including trolox, deferoxamine, ascorbate and quinacrine have been added to solutions with varying degrees of success (McAnulty & Huang 1997, McAnulty & Waller 1999).

Other recent treatments have included the use of calcium channel blockers, direct energy sources such as ATP, allopurinol, free radical scavengers, vasoactive drugs, steroids and the use of protease inhibitors (Jung *et al* 1999).

As with its role in improving graft survival, adenoviral delivery of IL-4 has recently been shown to reduce ischaemia/reperfusion injury in rat models (Kato *et al* 2000b). A major cytokine target has been TNFα. One reason is that TNFα production leads to neutrophil infiltration, which has been shown to be prevented by administering an anti-TNFα antibody to rats during ischaemia (Donnahoo *et al* 1999b). Administration of anti-TNFα antibodies to rat lungs has also shown to prevented endothelial damage during ischaemia/reperfusion (Khimenko *et al* 1998).

Ischaemia/reperfusion in isolated rat hearts leads to NFκB activation, TNFα upregulation and ICAM-1 expression resulting in neutrophil infiltration and adherence to the endothelium. These events have been reduced by using an NFκB decoy oligonucleotide and TNFα inhibitor (Kupatt *et al* 1999). The role of SOD as a protective agent might be linked to the fact that the overexpression of manganese SOD suppresses NFκB activation and reduces TNFα mediated apoptosis (Manna *et al* 1998). SOD gene delivery has been shown in the liver to reduce both NFκB and AP1 expression, during ischaemia/reperfusion injury (Zwacka *et al* 1998). The advantage of blocking NFκB would be to prevent its inflammatory and apoptosis pathways, whilst preserving its anti-inflammatory properties. This has been achieved to some degree in rheumatoid synovium using adenoviral-mediated overexpression of IκBα directly into the inflamed joint (Bondeson *et al* 1999). A recent study has also demonstrated that blocking NFκB indirectly, by inhibiting guanylyl cyclase-A reduces the expression of P-selectin in myocardial ischaemia (Izumi *et al* 2001).

Further understanding of the nature of when NFκB activation is protective and anti-inflammatory, and when it is mediating an inflammatory response, may lead to its selective inhibition and useful intervention in the future treatment of ischaemia-reperfusion injury (Boyle *et al* 1999). There have also been advances in the use of stem cells in ischaemic injury, with a recent study demonstrating the regrowth of cardiac myocytes and endothelial cells in ischaemic coronary arteries of mice engrafted with adult stem cells (Jackson *et al* 2001).

1.25 The cellular response to stress

1.25.1 Signal transduction

Protein tyrosine phosphorylation was first described in 1980 (Hunter & Sefton 1980). Protein phosphorylation occurs as a result of phosphorylation by protein kinases, and removal of phosphates by protein phosphatases. Protein phosphorylation is crucial to many cellular events and is reflected by the fact that there are an estimated 2000 protein kinase genes in humans (Hunter 1995). Protein phosphorylation can occur via two main sets of kinases and associated phosphatases, the tyrosine kinases and the serine/theonine kinases. The process of phosphorylation involves the transfer of the terminal phosphate group in ATP to the hydroxyl group in serine, theonine or tyrosine residues on substrate proteins (Lodish *et al* 1995). The process of protein phosphorylation by kinases occurs when the stimulation of cell surface receptors leads to an elevation of intracellular second messenger molecules.

1.25.2 Second messengers

Kinases have regulatory domains bound to their active sites. Second messenger molecules bind to kinases and a conformational change leads to a release of the regulatory subunit and the enzymatic activity of the kinase is initiated (Lodish *et al* 1995).

The major second messenger molecules in humans are, cyclic AMP (cAMP), calcium, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG).

There is a great deal of cross-talk between these second messenger pathways with elevations in one leading to an accumulation of another. Calcium is stored intracelluarly in the ER and mitochondria. When calcium is released it binds to the cytosolic protein calmodulin. The calcium-calmodulin complex is involved in the activation of many kinases. The calcium-calmodulin complex inactivates cAMP, thus regulating certain cellular processes. Raised levels of intracellular calcium ions (due to release from the ER), is induced by IP3 hydrolysis of membrane lipids. Calcium also interacts with DAG to form a membrane associated complex that activates protein kinase C.

1.25.3 Receptor tyrosine kinases (RTK).

RTKs comprise an extracellular ligand binding receptor complex, a transmembrane spanning hydrophobic region, and an intracellular cytosolic domain containing protein tyrosine kinases. Binding to the receptor causes dimerisation and phosphorylation of tyrosine kinases (autophosphorylation).

RTKs stimulate the exchange of GTP to GDP by G-proteins such as the Ras and Rho families. Ligands for RTKs are protein/peptide hormones such as insulin and other growth factors. G-proteins exist in an inactive GDP-bound state. G-proteins can be activated by a number of different receptors, and G-protein families interact with each other. Ras is

activated by the guanine nucleotide exchange (GEF) factor Sos. Intracellular concentrations of GTP are higher than GDP, so GEFs catalyse the release of GDP allowing GTP to bind. There have been over 35 GEFs identified in humans (Bar-Sagi & Hall 2000). The GEF, Sos is recruited to the membrane by a number of mechanisms (Rev by Schlessinger 2000). The adapter protein Grb2 can form a complex with Sos by binding to its Src homology 3 (SH3) domains. The Grb2/Sos complex is translocated to the membrane when the SH2 domain of Grb2 binds to an activated RTK. The Sos/Grb2 complex can also translocate to the membrane by binding to the adapter protein Shc. The final way in which Sos can be recruited to RTKs is by binding to membrane docking proteins such as IRS1, which is phosphorylated by activated RTKs. Once at a RTK, Sos stimulates the exchange of GTP to GDP by Ras. Ras interacts with proteins such as Raf and phosphatidylinositol-3 (PI-3) kinase by binding to the N-terminus thus bringing them to the membrane. The Raf family of proteins are MAPK kinase kinases (MAPKKK) which phosphorylate serine residues, in particular MEK, leading to further pathway activation by including the serine/theonine kinases such as the MAPKs (Lopez-Llasaca 1998).

1.26 Mitogen activated protein kinase (MAPK) cascades

The function of signal transduction pathways is to relate signals across the cytoplasm to the nucleus to initiate an appropriate transcriptional response (Paul *et al* 1997, Woodgett *et al* 1996, Karin 1998). MAP kinase cascades function in higher eukaryotes as signal transducers from the cell surface to the nucleus where they phosphorylate a range of transcription factors including ELK1, c-Jun and CREB (Kyriakis 1998). Extracellular stimuli may bind to membrane receptors (growth factors, cytokines) or be a physical stress such as oxidation or osmotic shock. G-proteins activate PI3-K and then tyrosine phosphorylation via adapter proteins Shc, Grb2 and Sos (Lopez-Llasaca 1998). Redox

regulated pathways include the MAPK's, Phospholipase Cgamma (PLCgamma) and PI-3 kinase (Kamata & Hirata 1999). MAPK pathways are involved in cross talk between different pathways including the ceramide SMase activation of NFκB (Ballou *et al* 1996). MAPK's have been found to play an important role in a wide range of cellular functions including embryonic development, innate and acquired immunity and have recently been implicated in numerous disease processes including heart disease, stroke and diabetes (Kyriakis & Avruch 2001, Pearson *et al* 2001). MAPK activation leads to the phosphorylation and regulation of numerous cellular proteins, growth factor receptors, transcription factors, cytoskeletal proteins, phospholipases and protein kinases (Guan 1994, Treisman 1996). In renal injury the main cellular responses such as proliferation, growth arrest, hypertrophy, differentiation, and apoptosis have been linked to MAPK activation (Bonventre & Force 1998, Tian *et al* 2000, Wang *et al* 1998c).

The core of the MAPK pathway consists of three protein kinases that receive signals from the plasma membrane and other stimuli via GTP-binding proteins. The most upstream component of the cascade is the MAPKKK this phosphorylates the dual specificity MAPK kinase (MAPKK) which in turn activates the MAPK (Reiser *et al* 1999, Marshall 1994). Phosphorylation occurs on closely spaced threonine and tyrosine residues. In mammalian cells there are five conserved MAPK pathways which have been identified. These are the mitogenic extra-cellular regulated kinase (ERK) 1/2 cascade, the stress activated c-jun N-terminal kinase (JNK), the protein 38 (p38) cascades and the poorly understood ERK3 and ERK5 cascades. It has been suggested that MAP kinases can participate in the regulation of NFκB both in the cytoplasm and nucleus (Schulze-Osthoff *et al* 1997, Zechner *et al* 1998, Van den Berghe *et al* 1998). Both are activated in response to similar conditions such as hyperglycaemia (Yerneni *et al* 1999, Igarashi *et al* 1999), although the pathway by which is occurs is not clear (Wesselberg *et al* 1997). The role of MAPK activation during

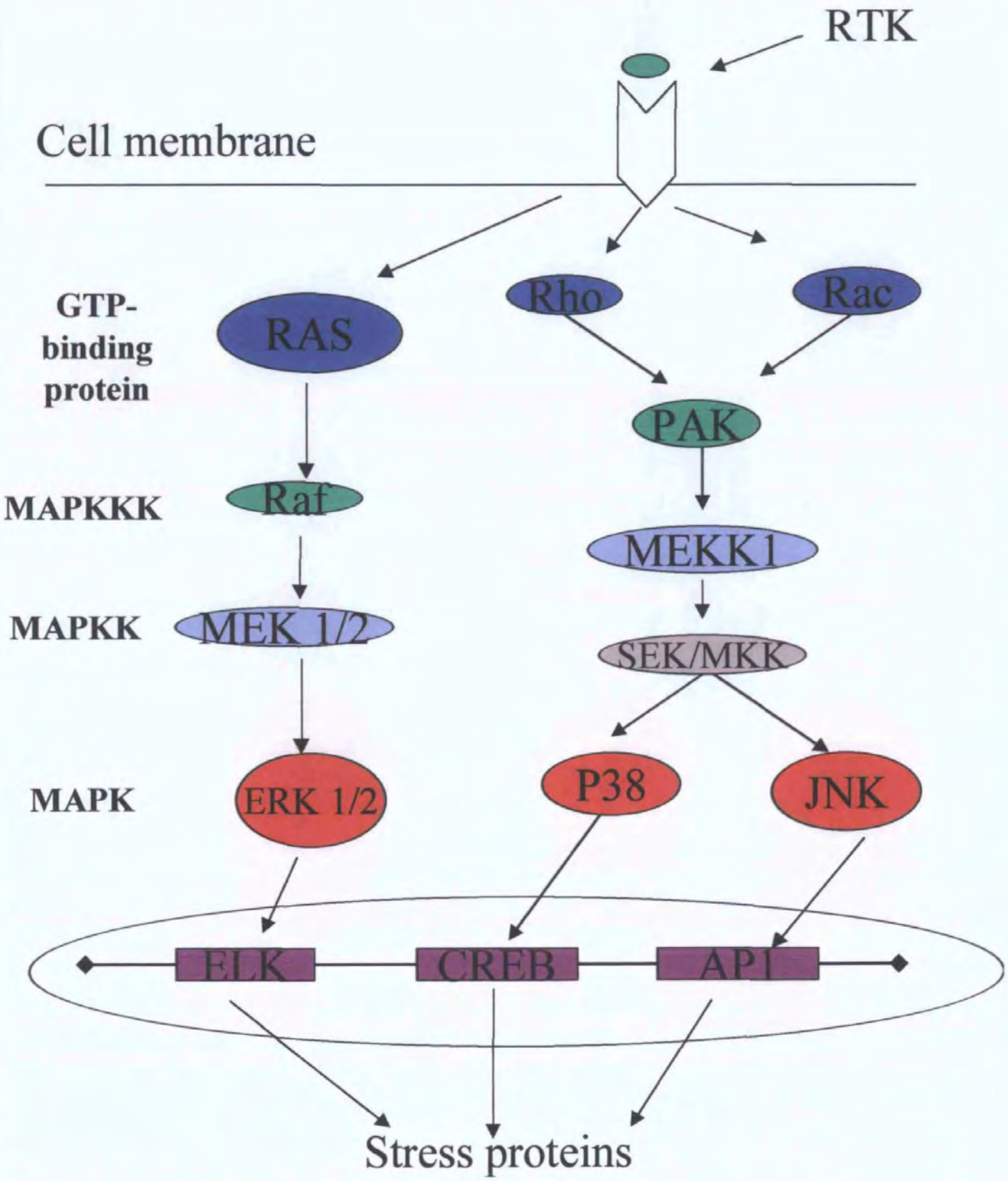


Figure 1.13. Basic overview of MAPK cascades resulting in the activation of transcription factors.

ischaemic injury is also unclear (Park *et al* 2001). The JNK MAPK cascade is known to respond to mainly stressful external stimuli including UV light, oxidative stress and inflammation (Guan 1994, Kyriakis *et al* 1994, Woodgett *et al* 1996). It is therefore often referred to as a stress activated protein kinase (SAPK). The p38 MAPK can be activated in a protein kinase C independent pathway (Igarashi *et al* 1999), suggesting that it can also respond to receptor independent stimuli. JNK activation in ischaemic/reperfusion injury has been shown in rat models (Onishi *et al* 1999) and most studies conclude that the JNK pathway is only activated by reperfusion (Bogoyevitch *et al* 1996, Bradham *et al* 1997). Severe hypoxia can activate both p38 and JNK proteins (Scott *et al* 1998), but inhibition of JNK has been linked to increased cellular apoptosis, whilst inhibition of p38 has no effect on cell death (Wang *et al* 1998c).

Hypoxia followed by reoxygenation has been shown to activate p38, JNK and ERK MAPK's (Scott *et al* 1998, Seko *et al* 1997). In perfused rat heart, ischaemia alone was found to activate the p38 and ERK kinases, but only reperfusion activated JNK (Bogoyevitch *et al* 1996). Warm and cold hypoxia followed by rewarming/reoxygenation is linked to apoptosis and inhibition of JNK during hypoxia has been shown to prevent apoptosis in hepatocytes (Crenesse *et al* 2000a, Crenesse *et al* 2000b). JNK induced apoptosis is via MEKK-1 regulated activation, as MEKK -/- cell lines do not activate JNK or undergo apoptosis when stressed with oxidative stress (Minamino *et al* 1999).

The production of an inflammatory response is likely to involve co-operative mechanisms between signal transduction pathways and transcription factors. Cytokine production may in part be regulated by MAPK activation (Kracht 2000). The p38 MAPK is involved in IL-8 expression during rewarming of epithelial cells (Gon *et al* 1998). P38 may be an upstream kinase in the activation of NFκB during myocardial ischaemia (Maulik *et al*

1998). Inhibition of NFκB and p38 have been associated with reduced expression of IL-1 beta, IL-6, TNFα and iNOS (Chandrasekar *et al* 1998).

In plants an MAPK type pathway has been identified and found to be activated by cold stress and drought (Jonak *et al* 1996). MAPK activation is also associated with osmotic shock, which is associated with Raf-1 and MEKK activation (Matsuda *et al* 1995).

Release of nitric oxide may be important in MAPK activation (Katori *et al* 1999) as stimulation with NO releasing compounds can result in p38 activation within 10 minutes (Huwiler & Pfeilschifter 1999).

1.17 Transcription Factors.

A gene is transcribed when an RNA polymerase is recruited to the genes promoter region. Mammalian cells contain three classes of RNA polymerases. Class I polymerases bind to promoter regions in the 5' end of a gene. Class II polymerases also bind to promoters in the 5' region and normally bind to TATA motifs, which are located about 30 base pairs upstream of the transcription start point. Some class III polymerase genes have TATA boxes, but most contain a regulatory region known as the A-B box in the coding region itself. RNA polymerases do not bind directly to DNA, but are recruited to the promoter by a set of proteins specific for each class of polymerase. The protein complex for class I polymerases are known as SL1. Class II polymerases are associated with the TFIID complex and class III with the TFIIIB complex. All three polymerase complexes contain the TATA-binding protein (TBP), which interacts with the TATA box (Rev by Cox & Sinclair 1998).

The interaction between the RNA polymerase, numerous general transcription factors, the polymerase specific transcription factor and numerous stabilising proteins at the gene promoter form the basal transcription complex. This complex is the basis for transcription

of the gene. In order to achieve tissue specific transcription of certain genes, numerous specific transcription factors exist that can be activated under certain circumstances and initiate the expression of a gene containing a binding site for the transcription factor. Transcription factors are transcriptional regulators that bind to regulatory elements in a gene which interact with the transcription initiation complex. Transcription factors are *trans*-acting as they are distinct from the gene it regulates. They bind to *cis*-elements which are contained within the gene it regulates. A transcription factor can either bind directly to a *cis*-acting sequence or interact with DNA-binding proteins. Transcription factors are group into 6 main classes depending on their effect on gene transcrition (from Semenza 1998).

1. Activators
2. Coactivators
3. Architectural factors (for protein structure)
4. Repressors and corepressors
5. Chromatin remodelling factors
6. Transcription elongation factors

Structurally transcription factors are of three main types. The largest group are the zinc finger proteins. Other types are either basic leucine zipper proteins or helix-loop-helix proteins. Mutations in the genes encoding transcription factors genes have been associated with numerous diseases including muscular atrophy, prostrate cancer and osteoporosis. Also, transcription factor genes contain nucleotide polymorphisms that are likely to effect their function (Shinohara *et al* 2001).

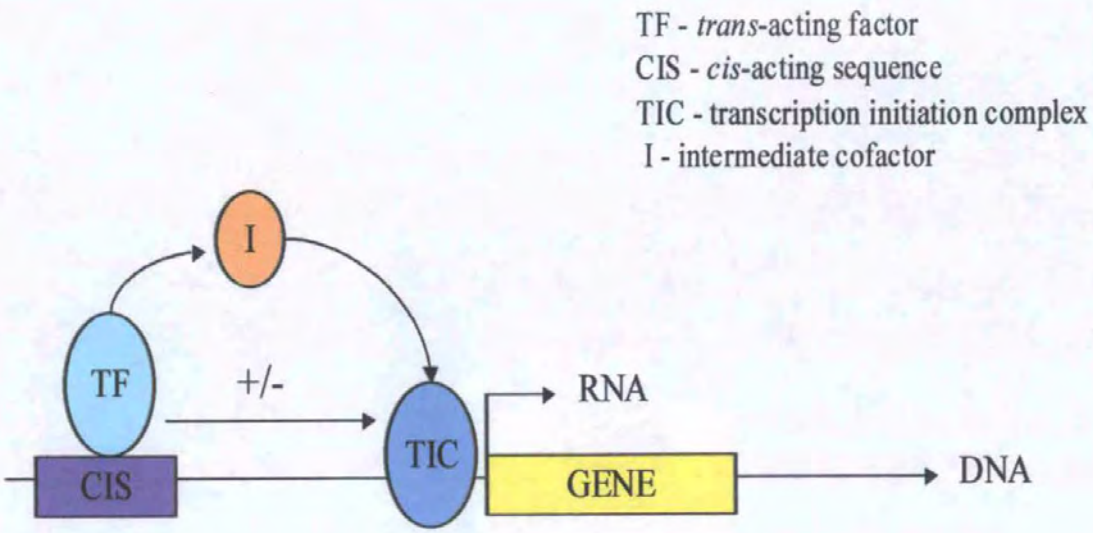


Figure 1.14. Cis-Trans interactions leading to gene transcription. Adapted from Semenza 1999

1.17.1 Nuclear factor kappa B (NFκB)

The NFκB transcription factor and its cytoplasmic inhibitor IκB are evolutionary conserved co-ordinating elements in the organisms response to situations of infection, stress and injury (Ghosh *et al* 1998). The activation of NFκB results in the increased expression of numerous proteins involved in the acute phase and inflammatory response. NFκB is found bound to its inhibtor complex, IκB in the cytoplasm of many cell types, including mature B-cells, plasma cells, macrophages and neurons. upon receiving an appropriate signal, the IκB/NFκB complex disociates and NFκB is released to translocate to the nucleus where it upregulates the transcription of specific genes. NFκB is involved in the expression of some 60 proinflammatory genes including IL-1, IL-6 and TNF α (Baeuerle 1998). The NFκB molecule also regulates the transcription of its inhibitor IκBα, forming an auotoregulatory pathway (Sun *et al* 1993). NFκB has been shown to be activated by UV light, heat shock, oxidative stress, cytokines and other agents (Ghosh *et al* 1998, Baldwin 1996). NFκB does not rely on *de novo* protein synthesis and therefore is able to transduce a quick message to the nucleus upon stimulation (Baeuerle & Baltimore 1988, Read *et al* 1994). The family of proteins to which NFκB belongs is known as the Rel proteins. Each contains an N-terminal 300 amino acid conserved sequence known as the Rel homology domain. This region is responsible for DNA binding, dimerisation and the interaction with IκB subunits. NFκB is a dimer of the rel family proteins. Rel protein members include Dif, Dorsal, Relish, v-rel, c-rel, p52, p65, p50 and rel-B. The most common NFκB dimer is the heterodimer formed between the p50 and p65 subunits, and is generally the protein referred to as NFκB (Ghosh *et al* 1998). Transgenic mice lacking RelA and c-Rel genes are found to have inhibited IL-4, IL-10 and IFN-γ expression, increased apoptosis and interfered cell cycle progression (Ferreira *et al* 1999). The NFκB

heterodimer or homodimer subunits, p50 and/or p65 have differing functions. A p65 antisense oligo and p50 oligo in combination was found to affect cell adhesion properties, but only cells treated with a p50 oligo maintained normal morphology (Narayanan *et al* 1993), the p65 subunit is likely to be involved in cell growth and regulation. Inhibition using a p65 anti-sense oligonucleotide has been found to reduce tumour regression in mice (Higgins *et al* 1993).

The crystal structure of the NFκB/IκBα complex has recently been discovered (Jacobs and Harrison 1998, Huxford *et al* 1998), which has revealed a detailed knowledge of the interactions between the subunits. Activation of NFκB is tightly regulated in the cytoplasm inhibitor IκB, which binds to NFκB and masks its nuclear localisation signal. NFκB is activated when IκBα is phosphorylated and dissociates, freeing the p65/p50 subunits to enter the nucleus (Rice & Ernst 1993). This leads to the proteolysis and degradation of IκBα at the proteosome (Henkel *et al* 1993, Lin *et al* 1995).

IκB is a member of a large family of proteins which contain a multiple region of homology known as the ankyrin-repeat motifs. These include IκBα, IκBβ, IκBε, IκBγ, and Bcl-3. This group also contains the Drosophila protein cactus. The IκBα is mainly involved in the interaction with NFκB, as it inhibits the nuclear translocation signal contained in the NFκB p65 subunit. IκBα phosphorylation can be induced by a wide variety of stimulants including UV light, glucose and oxidative stress (Du *et al* 1999).

NFκB mediated transcription can be initiated by cytokines such as IL-1 and TNF-α, which are also stimulated by NFκB, IL-6, IFN-γ and lymphotoxin, thus initiating an autoregulatory feedback loop. Recent studies however, have produced conflicting results as to the benefit of inhibiting this molecule in inflammatory disease (Satoh *et al* 1999, Steinle

et al 1999). Although targeting NFκB in systemic inflammatory diseases such as sepsis has reduced symptoms in animal models (Bohrer *et al* 1997).

Hypoxia and reoxygenation have been shown to activate NFκB (Koong *et al* 1994, Howard *et al* 1998, Li *et al* 1999) through redox mediated pathways and reactive oxygen intermediates (Daemen *et al* 1999, Onishi *et al* 1999). Some studies have found a lack of NFκB activation by oxidative stress in T-cells and epidermal cells (Brennan & O'Neill 1995). The hypoxic activation of NFκB is thought to be mediated by Ras signalling and to be independent of ERK 1 / 2 activation (Koong *et al* 1994). It may be that a combination of oxidative stress and cytokine production is needed for NFκB activation, as it was found that both hydrogen peroxide and TNFα were needed for NFκB activation in rat lung epithelial cells (Janssen-Heininger *et al* 1999). It has been suggested that NFκB is predominantly activated by the pro-oxidant state and AP-1 by the antioxidant state (Meyer *et al* 1994, Peng *et al* 1995). Hence, the response to hypoxia is predominantly AP-1 driven, whilst during reoxygenation its NFκB that contributes to inflammation (Rupec & Baeuerle 1995). Reperfusion has been shown to activate both AP-1 and NFκB with maximal mRNA activity after 60 minutes reperfusion (Bradham *et al* 1997). Endothelial cells exposed to H202 after reperfusion show upregulation of NFκB (Canty *et al* 1999) and anti-oxidants inhibit NFκB activity by scavenging hydroxyl radicals which act as a second messenger to NFκB (Shi *et al* 1999).

Little is known about the role of NFκB during cold ischaemia. NFκB expression has been shown to correlate with cold ischaemia in serial biopsies and expression at 120 minutes was found to correlate with post-reperfusion bile flow and sorbitol dehydrogenase activity (Ricciardi *et al* 2000). In the injury setting it has been found that inhibiting NFκB prevents

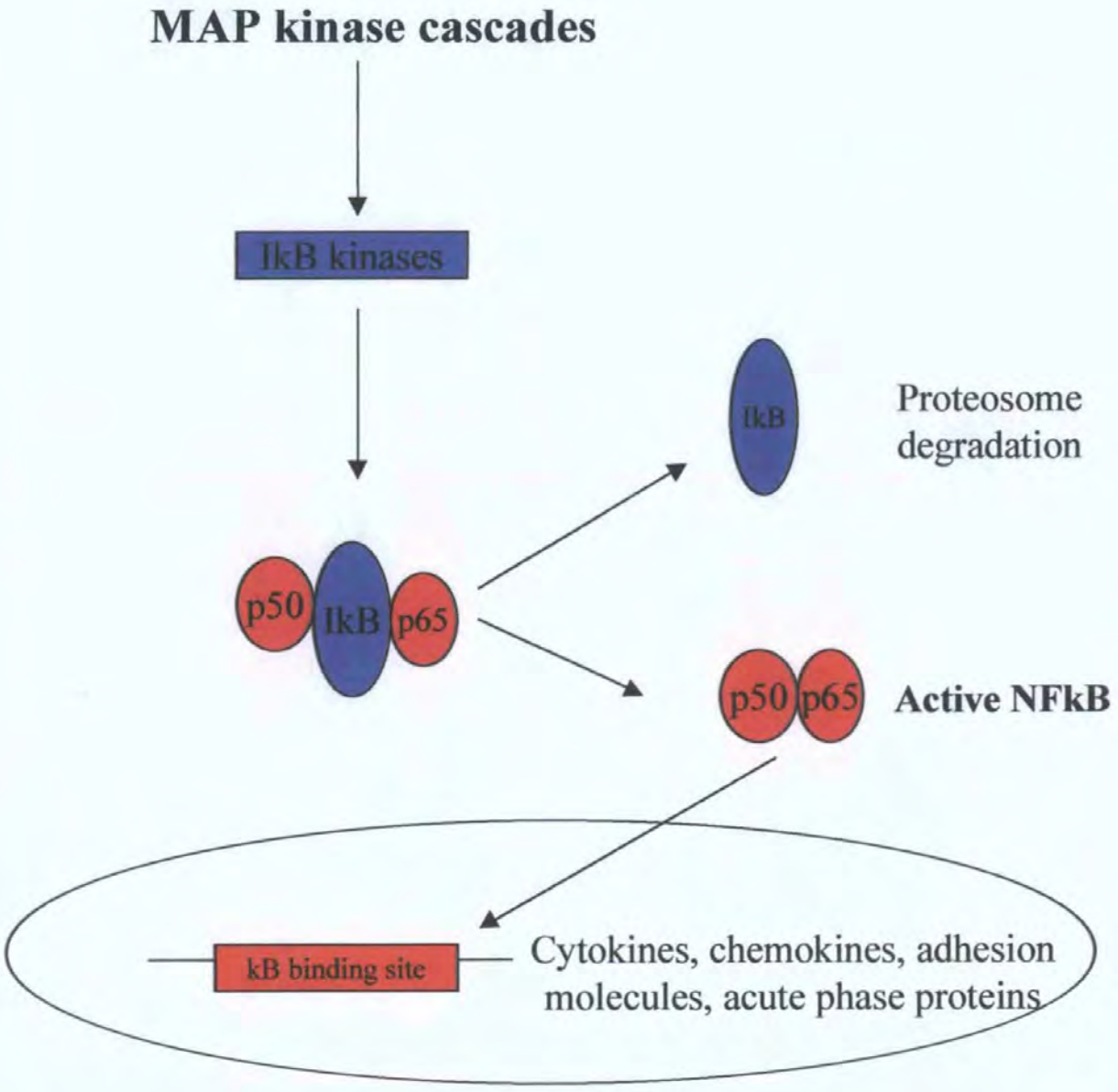


Figure 1.15. Activation of NFκB by degradation and proteolysis of IκBα, may be the result of MAPK activation.

LPS induced upregulation of HLA-DR, CD40 and CD1a in dendritic cells (Ardeshna *et al* 2000). Also that adhesion molecules are useful markers of graft damage (Troulong *et al* 1996) and are under NFκB control (Howard *et al* 1998).

1.17.2 Activator Protein 1 (AP1)

Ap1 is a multimeric transcription complex that consists of homodimers or heterodimers of the Jun, fos or AFT protein families. Typically AP1 is found as a heterodimer complex of c-Jun and c-fos. AP-1 is a vital transcription factor for leukocyte gene expression (Foletta *et al* 1998). MAPK pathways have been linked to AP-1 activation (Whitmarsh & Davis 1996). T-cell activation and IL-2 promoter activity are dependent on both NFκB and AP-1 costimulatory signalling (Jung *et al* 1995). Ap1 is activated by a similar set of stimuli as NFκB, particularly oxidative stress (Peng *et al* 1995, Jung *et al* 1995), TNFα (Kyriakis 1999) and post -ischaemic injury (Yeh *et al* 2000). Phorbol esters activate NFκB but not AP-1 (Tran-Thi *et al* 1995). Both transcription factors are vital for the production of inflammatory cytokines such as IL-8 (Lakshminarayanan *et al* 1998, Roebuck 1999). It is thought that NFκB is vital for IL-8 expression (Hsu *et al* 1999) but may be cell type specific and AP-1 binding is also required for gene transcription (Lakshminarayanan *et al* 1998). Treatment of cells with adenosine prevented TNFα production in rat hearts and blocked NFκB activation but not AP-1 activity (Li *et al* 2000). Hypoxia can induce the AP-1 transcription factor, however the level of AP-1 genes transcribed does not correlate with transcription factor activation. These include endothelin-1, platelet derived growth factor B, collagenase IV and c-Jun (Bandyopadhyay *et al* 1995). AP-1 components c-jun, junB and c-fos have been shown to be induced by ischaemia, but the study only reduced temperature to 33^{0}C (Kamme *et al* 1995). Recent

gene therapy for ischaemia/reperfusion and new immunosuppressive agents, have targeted NFκB and AP1 in mouse models (Zwacka *et al* 1998, Mortellaro *et al* 1999).

1.18 Cytokines during stress responses

1.18.1 Interleukin-6 (IL-6)

IL-6 is produced in response to injury of tissue or infection during graft rejection. IL-6 is a pleiotrophic cytokine involved in the regulation of immune responses, acute phase reactions, haematopoiesis and immunoglobulin production in B-cells. IL-6 deficient mice are severally defective in acute phase responses to infection or tissue damage showing that it is essential for localised inflammatory reactions but not systemic responses (Fattori *et al* 1994, Cuzzocrea *et al* 1999). IL-6 has a critical role in ICAM-1 expression (Nose 1993, Kukielka *et al* 1995). Myocytes show reperfusion dependent expression of IL-6 mRNA with peak expression after 1 hour of reperfusion (Gwechenberger *et al* 1999). IL-6 can be induced by ischaemia alone with 24 hours of ischaemia producing levels comparable with 1 hour ischaemia followed by 24 hours reperfusion, but IL-6 production is accelerated by reperfusion (Kukielka *et al* 1995). IL-6 has also been shown to be induced by hypoxia via NFκB activation (Muraoka *et al* 1997).

The role of IL-6 in neutrophil adherence via induction of ICAM-1 expression is demonstrated by the fact that neutrophils only adhere to isolated cardiac myocytes if the myocytes have been previously exposed to cytokines IL-1, TNF-α and IL-6 (Yamanchi-Takihawa *et al* 1995). These cytokines are likely to function in synergy, as IL-6 production is TNF-α dependent, and blockade of TNF-α by monoclonal antibody reduces IL-6 production (Yao *et al* 1997). IL-6 produced during reperfusion in coronary bypass grafting causes a higher percentage of neutrophil transendothelial migration compared to normoxic myocytes (78% Vs 26%) which is attenuated if an anti-IL-6 monoclonal antibody is

administered during reperfusion (Sawa *et al* 1998). The IL-6 promoter has several transcription factor binding sites including two AP1 sites and an NFκB site. The IL-6 gene and its receptor are regulated by a CEBP transcription factor binding site in the promoter/enhancer region (Chandrasekar *et al* 1999). NFκB is the primary inducer of IL-6 in cardiac myocytes, although binding of the NF-IL-6 transcription factor was also found during hypoxia (Matsui *et al* 1999). Studies have concluded that binding of NFκB to its site is sufficient for activation of the IL-6 gene, (Libermann & Baltimore 1990, Shimizu *et al* 1990, Muraoka *et al* 1997) but discrepancies between the abundance of activated NFκB and IL-6 mRNA levels are found, indicating that other transcription factors are necessary for IL-6 production (Patestos *et al* 1993). The explanation for the differences found in expression of IL-6, are that it exerts stimulant specific and tissue or cell type specific activation, especially during hypoxia (Gruss *et al* 1992, Fiebich *et al* 2000, Yan *et al* 1997a 1997b). Polymorphism of the IL-6 gene affects plasma levels of the protein (Fishman *et al* 1998). Several *in vivo* studies have implicated IL-6 as a possible marker of disease severity. IL-6 plasma levels are elevated during reperfusion in a rat model of hepatic ischaemia/reperfusion with maximal levels after 6 hours (McCurry *et al* 1993). In lung transplantation, IL-6 levels peak 4 hours after reperfusion and correlate with alveolar damage and low arterial/alveolar oxygen tension (Pham *et al* 1992). In liver transplantation, IL-6 levels have been shown to correlate with VWF during poor early graft function when measured in the first 50 ml of reperfused effluent (Basile *et al* 1999). However, IL-6 has also been shown to have a protective effect in rats pre-treated with recombinant IL-6 before a warm ischaemic period with reduced serum C-reactive protein (Carmargo *et al* 1997). But confusion still exists as LPS stimulated rats have reduced mRNA for TNFα and IL-6 and reduced apoptotic cells during reperfusion of preclamped renal vessels (Heeman *et al* 2000). *In vivo* during reperfusion of the aorta in aneurysm

repair, elevated levels of IL-6 are found during the operation and reperfusion correlating with endotoxin release and organ dysfunction (Holzheimer *et al* 1999). Also after coronary reperfusion IL-6 levels are increased systemically in the plasma (Seino *et al* 1995). IL-6 levels in the urine have been shown to correlate with progressive mesangial glomerulonephritis (Hirano 1998) indicating a role in kidney damage.

1.18.2 IL-8

IL-8 was first discovered in 1988 as an 'intercrine' cytokine with neutrophil attracting and inflammatory properties (Matsushima *et al* 1988). Mature neutrophils express IL-8 receptors at a much higher rate than T-cells. IL-8 regulates the expression of its receptors, as binding to the receptor causes the internalisation of the receptor before IL-8 is released. Internalised IL-8 causes the very rapid accumulation of intracellular calcium ions, within 2 seconds (Thelen *et al* 1988). IL-8 therefore initiates signal transduction pathways and IL-8 receptors are thought to be closely linked to protein kinase C activation and protein phosphorylation (Rev by Oppenheim *et al* 1991). The IL-8 gene has binding sites for NFκB, AP1 and AP2. IL-8 expression is thought to require the binding of transcription factors to two important *cis* elements in the IL-8 gene, an NFκB site and a C/EBP-like site (Oppenheim *et al* 1991). IL-8 is produced by a wide range of cell types including macrophages, T-cells, B-cells, endothelial cells, platelets and neutrophils. IL-8 also has chemoattractant properties for T-cells and has been shown to increase T-cell adherence to endothelial cells via up-regulated HLA class I expression on T-cell surface (Carveth *et al* 1989).

1.18.3 Tumour Necrosis Factor (TNFα)

TNFα was first identified to be the same as the macrophage secreted protein cachectin (Beutler *et al* 1985). TNFα is a proinflammatory cytokine produced by a variety of cell types including immune cells such as B cells, T cells and neutrophils and non immune cells such as smooth muscle and endothelial cells. TNFα synthesis can be induced by a wide range of stimuli, both biological and chemical. The synthesis of TNFα is tightly regulated so that under normal or quiescent circumstances TNFα production is small (Beutler *et al* 1995). Many human disease states are associated with increased TNFα production, including sepsis, autoimmunity and lymphoma and TNFα is known to be involved in autoimmune disease such as lupus nephritis (Jacob 1992) and the development of renal insufficiency syndromes (Meldrum & Donnahoo 1999, Donnahoo *et al* 1999a).

The TNFα gene is one of the immediate response genes induced by stress. TNFα does not rely on *de novo* protein synthesis and increased mRNA levels can be detected within 10 minutes of stimulation, because the factors necessary for TNFα synthesis pre-exist in the cell (Zhang & Tracey 1998). Inducers of TNFα include cytokines IL-1, IL-2 and IFN-γ, complement proteins and X-ray radiation. Suppressors of TNFα production include cytokines IFN-α, IL-4 and IL-10, glucocorticoids and cyclosporine A. IL-10 suppresses NFκB and TNFα activation in hepatocellular ischaemic injury (Yoshidome *et al* 1999a,b). TNFα has evolved as a mediator of host defence responses. In endothelial cells TNFα plays a vital role in the modulation of angiogenesis, cellular permeability, MHC class I expression, procoagulant activity, and the induction of IL-1, ICAM-1, VCAM -1, P- and E-Selectin. In the kidney increased TNFα levels can induce glomerular fibrin deposition, cellular infiltration and vasoconstriction leading to a reduced glomerular filtration rate

(Meldrum & Donnahoo 1999). The TNFα gene contains binding sites for NFκB, AP1 and NFAT amongst many other transcription factors. Its regulation is complex and the TNFα gene is highly polymorphic. Individual polymorphisms create differences in TNFα production within the population (Jacob *et al* 1992). The cell type specific regulation of TNFα activity is likely to be due to the differential use of regulatory elements in the TNFα promoter (Zhang & Tracey 1998) and polymorphic variations of the TNFα gene. TNFα is also involved in the release of IL-6 (Yao *et al* 1997, Kurokouchi *et al* 1998).

The MAPK pathways are also implicated in TNFα production via the activation of transcription factors important in TNFα synthesis (Donnahoo *et al* 1999).

In transplant rejection the infiltration of TNFα secreting macrophages correlates with acute rejection, elevated plasma TNFα and VWF production in the liver (Hoffmann *et al* 1993). Protein kinase C mediates TNFα expression leading to apoptosis under hypoxia in endothelial cells (Li *et al* 1999). TNF receptor cytoplasmic domains recruit Fas-associated death domain proteins that activate proteases and caspases (Natoli *et al* 1997).

TNFα and IL-1β have been shown to induce the expression IL-6, ICAM-1 and VCAM mRNA during hypoxia under the control of NFκB and AP-1 (Kurokouchi *et al* 1998, Kacimi *et al* 1998). Levels of TNFα production in bile effluent from liver grafts was found to be induced by both warm and cold ischaemia, and was reduced by cold perfusion of livers before warm ischaemia (Lutterova *et al* 2000). The TNFα microsatellite polymorphism has been associated with rejectors in kidney transplantation (Asano *et al* 1997).

1.19 Cell death

1.19.1 Apoptosis

Apoptosis is an organised process of cell death that functions to maintain a balance in normal cellular growth and development (Behrns *et al* 1999). It was first recognised in the 1970's as an organised cell disassembly, distinct from necrosis (Kerr *et al* 1972). Necrosis occurs during injury and is associated with profound inflammation of tissue and cells. Necrosis results in the breakdown of internal and plasma membranes resulting in the loss of cellular contents to the surrounding environment. This invokes an inflammatory response involving numerous cytokine secreting immune cells. Apoptosis is often found in mesangial cells during acute renal failure (Ueda *et al* 2000).

Apoptosis can be distinguished from necrosis as cell death in the absence of an inflammatory response. Apoptosis occurs in single cells and is associated with cell shrinkage, loss of contact, chromatin condensation and nuclear fragmentation (Allen *et al* 1997). Cell shrinkage is induced by loss of intracellular fluid by inhibition of membrane co-transporter systems. Biochemical changes in the plasma membrane cause phosphatidylserine to be exposed on the outer membrane surface. This is recognisable to phagocytes and parenchymal cells, which can engulf the apoptotic body. During formation of this apoptotic body chromatin in the cell nucleus condenses to form a mass and the DNA is fragmented.

Apoptosis can be induced *in vitro* by a range of stimuli including serum deprivation, oxidative stress and growth factor withdrawal (Hogg *et al* 1999). Cells undergo apoptosis when an appropriate signal activates a cascade of proteases known as cytosolic aspartate-specific proteases (caspases). Caspases exist as pro (inactive) proteases in the cytoplasm. There are approximately 14 caspase proteins in human cells. Caspases are highly conserved proteases that cut cytoplasmic and nuclear contents into apoptotic bodies. The

caspase cascade is induced when a ligand binds to a cell surface receptor. Cell receptors such as TNFα, TGFβ and Fas have intracellular death domains (DD) that interact with adapter proteins and activate the caspase cascade. Ligands that induce apoptosis must crosslink receptors and aggregate DD's. The orientation of DD's is vital in the recruitment of adapter proteins to activate the caspase cascade. Adapter proteins include the TNF receptor-associated death domain (TRAF) protein, apoptotic protease activating factor-1 (APAF-1) and apoptosis inducing factor (AIP) proteins. The major caspases in the cascade are caspases -8,9 and 10, which are known as initiators as they can directly activate the cascade. Caspases -3, 6 and 7 are known as effectors as they cleave proteins vital for cell survival. The third group of caspase proteins are involved in cytokine processing and cleavage. Caspase –1 cleaves IL-1β into its mature form, and caspase-3 cleaves IL-16 into its active and secretary form. Caspases –4, -5, -12, -13 and –14 are thought to be involved in cytokine processing. Receptor mediated apoptosis is the major way in which cellular apoptosis is induced, however, apoptosis can also be induced by loss of suppressive mechanisms. Cells contain many proteins that function to prevent apoptosis by inhibiting caspases or preventing activation of caspases. The Bcl-2 family of proteins regulates mitochondrial membrane permeability. This means they can function as pro or anti-apoptotic mediators depending on how they effect mitochondrial permeability. Cytochrome c, which is contained in the mitochondria, is released when the mitochondrial membrane is damaged (Reed 1997). Cytochrome c activates caspase –9 which in turn activates other caspases. Other proteins that inhibit apoptosis include the anti-oxidant glutathione and NFκB.

NFκB has been linked to the regulation of apoptosis in a cell type specific manner (Baichwal & Baeuerle 1997) NFκB knockout mice die of massive apoptosis in liver cells (Begg *et al* 1995). NFκB is thought to prevent apoptosis by blocking the activation of

caspase 8 (Chu *et al* 1997, Wang *et al* 1998a). NFκB mediated prevention of cell death is limited, as severe oxidative or cytokine induced stress causes cell death despite NFκB activation in a necrotic response (Li *et al* 1997). Hyperoxia in cell culture has been shown to induce cell death with an associated increase in NFκB activation, this was found to be independent of ERK 1 /2 MAPK activity (Kazzaz *et al* 1999). A potent stimulator of MAPK and SAPK known currently as haematopoietic progenitor kinase-1 (HPK1) is found to inhibit NFκB when mediating caspase cleavage and hence apoptosis (Arnold *et al* 2001). Inhibitor of apoptosis proteins (IAP) are under NFκB control (Erl *et al* 1999) and TNFα stimulation results in feedback between c-IAP2 activity and NFκB activity and anti-apoptosis (Chu *et al* 1997). Newly identified proteins involved in apoptosis such as Par-4 are found to inactivate protein kinase C and hence NFκB (Diaz-Meco *et al* 1999). NFκB anti-apoptotic effects are independent of *de novo* protein synthesis as shown in cells treated with cyclohexamide and IL-1β stimulated (Kajino *et al* 2000). In Cancer, dysfunctional NFκB activity has been shown in HeLa cells, breast carcinoma cells and malignant tumours (Rupec & Baeuerle 1995, Kurt *et al* 1998, Royds *et al* 1998). Caspase-3 cleaves and truncates IκBα resulting in its increased binding to NFκB and suppression of its activity, and hence apoptosis (Reuther & Baldwin 1999). During ischaemic injury reperfusion after 30 hours at 4^0C has been shown to induce apoptosis (Rauen *et al* 1999) as well as hypoxia and reperfusion, in animal models (Nogae *et al* 1998 Daemen *et al* 1999). In endothelial cells apoptosis can be induced in culture by the addition of cytokines such as TGFβ, oxidative stress or by serum deprivation (Hogg *et al* 1999). Signalling via TNFα and PKC pathways are vital in hypoxia-reoxygenation mediated apoptosis in endothelial cells (Li *et al* 1999). Also signals from MKK6 and p38/NFκB inhibit apoptosis in cardiac myocytes (Zechner *et al* 1998). In lymphocytes glucocorticoids such as dexamethasone can induce apoptosis (Fearnhead *et al* 1994, Cidlowski *et al* 1996). Dexamethasone reduces the

expression of NFκB (Tsao *et al* 1997). Heat shock proteins are likely to be involved in the regulation of apoptosis in stressed cells (Santoro 2000). The heat shock response is thought to inhibit NFκB (Heneka *et al* 2000). Hsp70 prevents the formation of the apoptosome by blocking the interaction of APAF-1 with caspase protein 9 (Morano & Thiele 1999).

1.20 Project overview

The aim of this project is to investigate the effects of hypothermia and reperfusion on endothelial cells. Preservation injury involves hypothermia, hypoxia and reperfusion. The effects of hypoxia are well documented in the literature, due to the fact that hypoxia plays a vital role in other disease processes such as angiogenesis during tumour growth and vascular disease (Rupec & Baeuerle 1995, Voelkel & Tuder 2000). The effects of hypothermia are less well documented as hypothermia in disease is a rare event. The aim of this project is therefore to focus on the effects of hypothermia at the cellular level and to try and extrapolate any findings into the clinical setting.

The injury sustained to graft cells during the procurement of organs for transplantation is thought to influence subsequent graft function. This has led to the formation of the 'injury hypothesis' that attempts to explain the role of ischaemic injury in graft function (Halloran *et al* 1998). However, data to support the injury hypothesis is not conclusive and many studies have found good graft survival rate in organs with longer ischaemic times, and argue that reducing ischaemic times may be detrimental to HLA-matching (Lange & Kuhlmann 1998). Also, recently studies have found a benefit from a pre-ischaemic insult to cells on preventing subsequent injury from ischaemia, leading to an apparent 'heat shock paradox' (Demeester *et al* 2001).

Endothelial cells are at a vital interchange between the vascular system and the organ from which they are found. Data has shown that endothelial cells up-regulate HLA class II molecules during ischaemia and may have the ability to activate host T-cells (Shackleton 1998, Pober *et al* 1996). Endothelial cells are also an important source of adhesion molecule expression as well as playing a vital role in the coagulation system. Renal vein thrombosis and vascular rejection can account for some 5% of early graft loss (Parrot 1995). One particular event that is likely to be important in the quality of graft cells is

apoptosis, which can be associated with inflammation (Daemen *et al* 1999). Previous studies have shown been conflicting as to the role of hypothermia in apoptosis (Hansen *et al* 1994, Kruman *et al* 1992). Cytokines are likely to be important mediators in the response to injury, as well as in the subsequent inflammatory response and host immune response. Recently cytokines such as IL-6 and TNFα have been shown to have polymorphisms which correlate with both protein production and rejection in kidney transplantation (Marshall *et al* 2001, Ghandi *et al* 2001). The aim of this project is to subject endothelial cells to hypothermia for a time course relevant to preservation times and determine the subsequent effect on cells. The outcome measures will be apoptosis, inflammatory markers such as TNFα and IL-6 production, as well as to try and elucidate the pathway by which this may occur. The transcription factor NFκB is likely to be involved in the regulation of both apoptosis and inflammatory cytokine production, although its role in hypothermia to date is unknown. The MAPK cascades are thought to play a possible role in NFκB activation, although there is also controversy as to how this may occur (Schulze-Osthoff *et al* 1997). There is great potential to use numerous inhibitors in cell culture to investigate pathways and to block the production of certain cytokines as well as the potential to add to preservation solutions.

Further to investigating these events in a cell culture system the aim of this project is to also link events to the actual graft itself. To achieve this, levels of hypoxic markers and inflammatory cytokines are going to be measured in preservation solutions surrounding kidney grafts during the cold ischaemic period. A non-invasive marker of graft damage would have a great use in a clinical setting (Hauet *et al* 2000).

The response of donor cells to cold ischaemia is likely to be different depending on the donor. This may in part be due to a polymorphism which the donor cells may have. In particular certain polymorphisms are linked to high or low producer status for certain

cytokines. The aim of this project is to also investigate functional polymorphisms in the IL-6 and TNFα genes of donor patients to determine if they correlate with rejection episodes. The role and importance of the donor in transplantation is becoming more evident. The role of oxidative stress in many disease processes is becoming more important as this area is more actively investigated. The NAD(P)H system is widely researched in the cardiovascular field and a functional polymorphism of the phox22 subunit has been shown to have a protective effect by reducing free radical production in heart disease (Inoue *et al* 1997). This polymorphism may play a protective role in transplant donors. The redox regulated transcription factor NFκB is involved in many disease processes and a recently discovered polymorphism in the NFκB gene, although not shown to be functional to date may be involved in the regulation of inflammatory responses in the donor kidney.

Finally the aim of this work on genetics in the donor is to try and correlate levels of cytokines found in the donor kidney with the functional polymorphism that they have. The IL-6 (-174) polymorphism is functional, but the role of IL-6 protein levels in early graft function is unclear, despite the fact that the polymorphism has been shown to correlate with rejection.

With the role of the donor being further appreciated as data accumulates, the aim of this project is to add to the 'injury hypothesis' by investigating the molecular events occurring during hypothermic and reperfusion stress in the donor organ during its procurement, storage and transplantation into the recipient.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Reagents

All reagents were analytical grade or equivalent.

Hydrochloric Acid, Magnesium Chloride, Orthoboric Acid, Potassium Chloride, Sodium Chloride, Sodium Dodecyl Sulphate (SDS), Sodium Fluoride and Tween 20 were purchased from BDH UK. Ammonium persulphate (APS), Aprotonin, Bromophenol blue, Dimethylsulfoxide (DMSO), Foramide, Glycerol, Glycine, 2-Mercaptoethanol, Phenyl-methyl-sulphonyl-fluoride (PMSF), Sodium Orthovanadate, Tetramethylethylenediamine (Temed), Triton x100, Tergitol (NP40) and Trizma Base (Tris) were purchased from sigma chemicals UK. Acrylamide and NN'-Methylene-Bis-Acrylamide (Bis) were purchased from Biorad UK. Chloroform, Ethanol and Methanol were purchased from Rathburns UK. Low melting point Agarose was purchased from Boehringer Mannheim Germany.

2.1.1 Water

Double distilled water (Millipore UK) was used for all stock reagent preparation. Sterile water (Baxter Healthcare UK) was used for PCR and primer dilutions.

2.1.2 Culture Media

Medium 199 supplemented with 10% Foetal Calf Serum (FCS), 5% Penicillin/Streptomycin (pen/strep) and 5% L-Glutamine (Life Technologies UK) for ACHN and ECV 304 cell lines. Medium 200 supplemented with Low serum growth Supplement (Totam Biologicals UK) and 5% Pen/Strep for primary endothelial culture. Cells were grown in a culture incubator (LEEC UK) maintained at 37^0C and 5% CO_2 (BOC Gases UK).

2.1.3 Tissue Culture Plastics

Sterile 5 and 10ml pipettes, 75 cm^2 cell culture flasks and 25ml plastic test tubes were purchased from Fahrenheit UK. All tissue culture was carried out in a class II microflow safety cabinet (Bioquell UK). Aseptic technique was observed at all times and cabinets swabbed with 70% industrial metholated spirit (IMS) before and after use.

2.2 Specialised reagents and kits

An electromobility shift assay system (EMSA) was obtained from Promega Life Sciences UK, containing oligonucleotides for NFκB, SP1, AP1 (see Table 2.) and a HeLa cell extract (5mg/ml total protein in 20mM HEPES, 0.1M KCL, 0.2 mM EDTA, 0.5mM PMSF, 0.5mM DTT and 20% glycerol). Taq polymerase and buffer were purchased from HT Biotechnology UK. Deoxynucleoside 5'-triphosphates (dNTP's), T4 polynucleotide kinase (T4 PNK) and γ^{32p} dATP radioactive isotopes were purchased from Amersham Pharmacia Biotech UK. Whatman 3MM filter paper (Whatman International UK) and ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK) were used for western blotting. Renal preservation solution (Baxter Healthcare UK) was obtained from Derriford Hospital, Plymouth.

2.3 Stock solutions

1. Soltran kidney perfusion solution pH 7.1

   Potassium citrate 8.6g/L, Sodium citrate 8.2g/L, Mannitol 33.8g/L, Magnesium sulphate 10g/L, Potassium 80mmol/L, Sodium 84mmol/L, Magnesium 41mmol/L, Citrate 54mmol/L, Sulphate 41mmol/L.

2. Tris/borate electrophoresis buffer (TBE)

   10x solution: 0.89mM Tris base, 0.89M Boric acid, 2mM EDTA pH 8.0

3. Tris-EDTA buffer (TE)

   10mM Tris-HCL pH 8.0, 1mM EDTA

4. Tris-Buffered Saline (TBS) Tween 20

   80ml 2.5M NaCl, 20ml 1M Tris, 1900ml H_2O, 2ml Tween-20

5. Western Blot Running buffer

   5x: 45g Tris-base, 216g Glycine, 15g SDS, 3L H_20

6. Western Blot Transfer buffer

   15.1g Tris-base, 72g Glycine, 5L H_20

7. Ethidium bromide

   10mg/ml Ethidium bromide diluted in H20

8. Xylene cyanol loading buffer

   0.25% w/v Xylene cyanol, 10% v/v Glycerol in 10x TBE

9. 10% (w/v) SDS

   10g SDS in 60ml H20 made to 100ml after dissolving

10. 10% (w/v) APS

    100mg APS in 100ml H20

11. Sample Loading buffer

    3ml H20, 1ml 0.5M Tris-HCL, pH 6.8, 1.6ml Glycerol, 1.6ml 10% SDS, 0.4ml β-mercaptoethanol, 0.5% w/v bromophenol blue

2.4 Autoclaving

All solutions and glassware were autoclaved at 121^{0}C and 15 p.s.i pressure for 30 minutes in a steam autoclave (Prior UK) before use.

2.5 Culture of endothelial cells

Primary cultures of Human Umbilical Vein Endothelial Cells (HUVECS) and Primary Human Aortic Endothelial Cells (HAEC) were obtained (Totam Biologicals, Northampton, UK). Cells were guaranteed to be of endothelial origin confirmed by positive immunohistochemical staining for VWF and the endothelial surface marker CD31 (Cascade Biologics, USA). To further confirm the cells were endothelial after long term culture, cell extracts were immunoblotted for VWF (see Fig 2.1).

Other cell lines were used as controls and to optimise experiments. These include a renal carcinoma cell line (ACHN) and a bladder carcinoma cell line (ECV 304) (both ECACC UK). Cells were cultured in Medium 200 (HUVEC, HAEC) supplemented with endothelial growth supplement, or Medium 199 (ACHN, ECV 304) with L-Glutamine (5%), Foetal calf serum (10%) and penicillin/streptomycin (5%) as previously described (Merrick *et al* 1997).

2.5.1 Routine for cell passage.

Cell growth was monitored using a Leica inverted microscope (Jencons Scientific UK). Confluent 75 cm^2 flasks were detached using 4ml Trypsin/EDTA solution (Life Tech UK). The flask was tapped gently to encourage the cells to detach from the flask, and the trypsin neutralised by adding an equal volume of FCS. Cells were harvested by removing the media and centrifuging at 1000rpm for 4 minutes. The pellet was resuspended in wash buffer (culture medium without supplements), and again pelleted at 1000rpm for 4 minutes. The resulting pellet was resuspended in complete media and cells counted by haemacytometer (Weber UK).

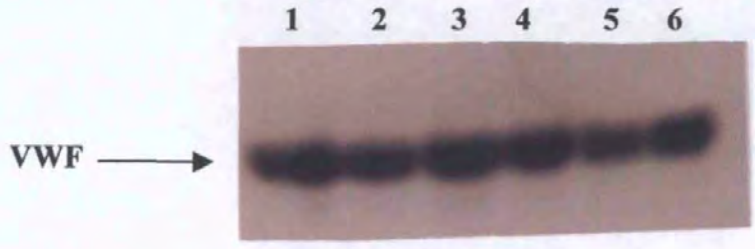


Figure 2.1. Positive Immunoblotting for VWF in 6 separate endothelial cell lines used as controls in different experiments. Blotting for VWF confirmed that cells used were endothelial. The picture also demonstrates equal protein loading using the Bradford assay to determine concentrations and accurate transfer of protein from gel to ECL membrane.

2.5.2 Neubauer haemacytometer cell counts

Cells were counted in a neubauer haemacytometer. To visualise cells, 50µl of cell suspension was mixed with 50µl of trypan blue. Cells exclude trypan blue reagent if they are viable, as it cannot transverse the cell membrane. However, if the cell membrane is damaged and hence the cell is dead trypan blue with enter the cell and stain it blue. The haemacytometer grid was covered with a cover slip, and the suspension added until the grid was covered. Cells were counted in sixteen squares. The area of the grid is $1.0mm^2$ and the depth is 0.1mm so the volume of cells in sixteen squares is calculated as n x 10^4 cells/ml. 10^6 were placed in a $75cm^2$ culture flask containing 20mls of complete media. Cells were grown to confluence in a 5% CO_2, 37^0C incubator (LEEC UK) with changes of media every 24 or 48 hours until confluence. The percentage cell viability was calculated as the number of cells excluding the Trypan blue dye divided by the total number of cells. Cell viability was normally found to be above 90%.

2.5.3 Cryopreservation of cells

To keep a stock of endothelial cells, certain cells were cryopreserved in liquid nitrogen for storage (BOC gases UK). After detaching cells and pelleting, cells were resuspended in 1ml of culture media supplemented with 10% DMSO and 30% FCS and placed in a plastic cryovial tube (Fahrenheit UK). Cells were frozen slowly at $^-20^0C$ for one hour, $^-80^0C$ overnight and finally transferred to the gas phase of liquid nitrogen in pods (BOC Gases UK).

2.5.4 Retrieval of cells from frozen storage

To retrieve cells from storage, 15-20 mls of media was pre-equilibrated in the incubator. Cells were removed from liquid nitrogen storage and rapidly thawed in a 37^0C water bath. Once thawed cells were transferred to a centrifuge tube and pelleted at 1000rpm for 4 minutes. The pellet was re-suspended in 5mls PBS to wash away potentially harmful DMSO. Cells were pelleted again at 1000rpm for 4 minutes and the pellet re-suspended in complete media. Cells were counted and viability tested, before being re-seeded in pre-equilibrated $75cm^2$ flasks.

2.6 Incubation of cells with growth factors, inhibitors and other agents.

Cells were stimulated with PMA (Sigma Aldrich Ltd, UK) at 5ng/ml for 18 hours as a positive control. PMA is an acute growth factor that is known to induce the expression of VWF, NFκB, AP1 and the MAPK cascades (Giddings & Shall 1987). To induce apoptosis cells were incubated with the anti-inflammatory glucocorticoid, Dexamethasone (Affiniti Research, UK) at 1mM for 12 hours in serum free media. A stock solution of 25mg/ml Dexamethasone suspended in DMSO was stored at -20^0C until use. To inhibit NFκB a cell permeable inhibitory oligonucleotide peptide (SN50) was used (Affiniti Research, UK). SN50 is a cell-permeable peptide that contains the nuclear localisation sequence residues 360-369 of the NFκB p50 subunit linked to the hydrophobic region of the kaposi fibroblast growth factor. The peptide was suspended in H20 at 100μg/ml and stored at -20^0C until use.

To inhibit MAPK's a MEK inhibitor (PD98059) for ERK 1 / 2 or a specific p38 inhibitor (SB203580) for p38 (Both Promega, UK). PD98059 was dissolved in DMSO at a stock concentration of 6.5mg/ml and stored at -20^0C until use.

SB203580 is a cell permeable inhibitor of the phosphorylation of p38 and the homologues p38α, p38β and p38β2. A stock solution of 10mM suspended in DMSO and stored at -20^{0}C until use. Cells were pre-treated with inhibitors in culture media for 1 hour before the experiment and fresh inhibitor was added to the preservation solution before cells were suspended in it and placed in hypothermia.

2.7 Incubation of cells for hypothermia, hypoxia and reperfusion experiments.

Confluent cell monolayers were washed twice in PBS and resuspended in renal preservation solution (Baxter Healthcare UK) with or without inhibitor. Cells were counted before and after cold storage experiments to correct for differences in cell number. Cells were placed in a LTD 20G 4^{0}C water bath (Grant Instruments, UK) for up to 72 hours. For reperfusion experiments an 'add back' protocol was used as previously described (Chan *et al* 1999). Before the end of the incubation period, half the preservation solution was removed and pre-warmed to 37^{0}C. The solution was then added back to the cells and the cells placed in a 37^{0}C water bath. For hypoxia experiments confluent cells were given fresh media and placed in a hypoxic incubator for the same time points. The incubator was equilibrated overnight before the experiment and maintained at <1% 0_2 and 37^{0}C (NuAire Inc, USA). At time points, cells were pelleted and supernatant collected and stored at –80^{0}C until use.

2.8 Preparation of whole cell and nuclear extracts

Nuclear extracts were prepared with modification as previously described (Dignam *et al* 1983). Cells were harvested and pelleted at 13000rpm for 30 seconds. Cell pellets were resuspended in Dignam buffer A containing 10mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10mM KCL, 0.5mM Dithiothereitol (DTT), 0.2% NP-40, 100mM

Aminoethyl Benzenesulfonyl Fluoride (AEBSF), 18.4mg/ml sodium orthovanadate (Na_3Vo_4), 42mg/ml sodium fluoride (NaF) and 2.2mg/ml aprotonin. The lysate was incubated on ice for 15 minutes, with vortexing at 5-minute intervals. Lysates were then centrifuged at 13000rpm for 15 minutes. Supernates contain cytoplasmic proteins. Nuclear pellets were then resuspended in Dignam buffer C containing 20mM HEPES, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.5mM DTT, 0.2mM EDTA, 100mM AEBSF, 18.4mg/ml Na_3Vo_4, 42mg/ml NaF and 2.2mg/ml aprotonin. The nuclear protein lysate was then incubated on ice for 15 minutes with vortexing and centrifuged at 13000 rpm for 15 minutes. The resulting supernate contained the nuclear proteins and was stored at -80^0C until use.

Whole cell extracts were prepared using two methods

Method one – Recommended by New England Biolabs, UK

Cells were lysed in SDS sample buffer containing 62.5mM Tris-HCL (pH 6.8), 2% SDS, 10% glycerol, 50mM DTT and 0.1% bromophenol blue. The suspension was sonicated for 15-20 seconds at 20,000cpm, and boiled for 10 minutes at 100^0C. The extract was cooled on ice and centrifuged for 10 minutes at 13000rpm. The resulting supernate contains a whole cell fraction. Whole cell proteins were stored at -80^0C until use.

Method two –

Cell pellets were re-suspended in 100µl of a lysis solution containing 0.1M Tris-HCL (pH 7.5), 5% NP40, 0.01M NaF, 0.01M $NaVo_4$ and 1µg/ml PMSF. The lysate was then placed on ice for 20 minutes. The suspension was then centrifuged for 15 minutes at 13000rpm. The resulting supernatant contained whole cell proteins that were removed and stored at -80^0C until use.

2.9 Determination of protein concentrations.

Protein concentrations for both whole cell and nuclear fractions were determined using a Bradford assay (Pierce UK). A standard curve was prepared using 2.0mg/ml bovine serum albumin (BSA) as the high standard and serially diluting to 0.125mg/ml 5µl of unknown protein was added to 1ml coomassive blue dye and incubated for 10 minutes at room temperature. The mixture was transferred to a cuvette and absorbance read against a blank containing the buffer cells were lysed in at 595nm on a Cecil 5500 spectrophotometer (Cecil, UK).

2.10 Preparation of DNA from cell lines

DNA was extracted from the cells by phenol/chloroform extraction. Cells were pelleted at 13000rpm for 30 seconds. Cells were lysed in 500µl DNA lysis buffer containing 1M Tris-HCL (pH 8.0), 1M NaCl, 10% SDS and 0.02% EDTA. Proteinase K was added to make 200µg/ml i.e. 5 µl of 20mg/ml stock added to 500µl lysate. The suspension was then incubated for a minimum of 1 hour at 37^0C and DNA extracted by adding 500µl of a 25:24:1 phenol/chloroform/isoamyl mixture and centrifuging at 13000 rpm for 10 minutes. After centrifugation the viscous top layer was removed and kept. 3.5M ammonium acetate (1/10 volume removed) and 2.5 volumes absolute alcohol were added. The DNA was allowed to precipitate overnight at –20 ^{0}C. A final centrifugation step at 13000rpm for 15 minutes pelleted the DNA. This was resuspended in double distilled water and 10u/ml DNase free RNase was added. DNA was diluted 1 in 10 with double distilled water and the quantity of DNA recovered was measured by its absorbance at 260nm versus a blank containing water only.

One optical density (OD) unit at 260nm is equivilant to 50μg/ml of DNA. Therefore absorbance at 260nm x 50 x 10 (the dilution factor used), is equal to the amount of DNA present in the sample (μg/ml).

2.10.1 DNA fragmentation analysis.

Apoptosis was measured by DNA fragmentation analysis. DNA extracts were separated on a 1.5% agarose gel. Gels were prepared by placing 1.5g of agarose in 100ml 0.5x TBE and boiled in a microwave to dissolve. Gels were stained with ethidium bromide (0.01% v/v) and run in 0.5X TBE buffer at 100V for 2 hours. Fragmentation was visualised using a UV transilluminator (UVP USA).

2.11 Electrophoretic mobility shift assay (EMSA)

The EMSA is a means of analysing the amount of a transcription factor present in a nuclear extract sample. A labelled oligonucleotide is incubated with the nuclear extract, and run through a gel. The electrophoretic mobility of the oligonucleotide will be less if a transcription factor has bound to it. Therefore a 'shift' away from the probe is an indication of the amount of the transcription factor present in the sample. The specificity of the probe can be demonstrated by adding an excess of the unlabelled oligonucleotide, which will compete for the transcription factor and remove the shifter band. Further the subunits of a transcription factor complex can be determined by adding an antibody, specific for the subunit, to the nuclear extract. The antibody will bind to the probe-transcrition factor complex and further reduce the electromobility of the complex and hence form a further 'shifted' band, known as a supershift.

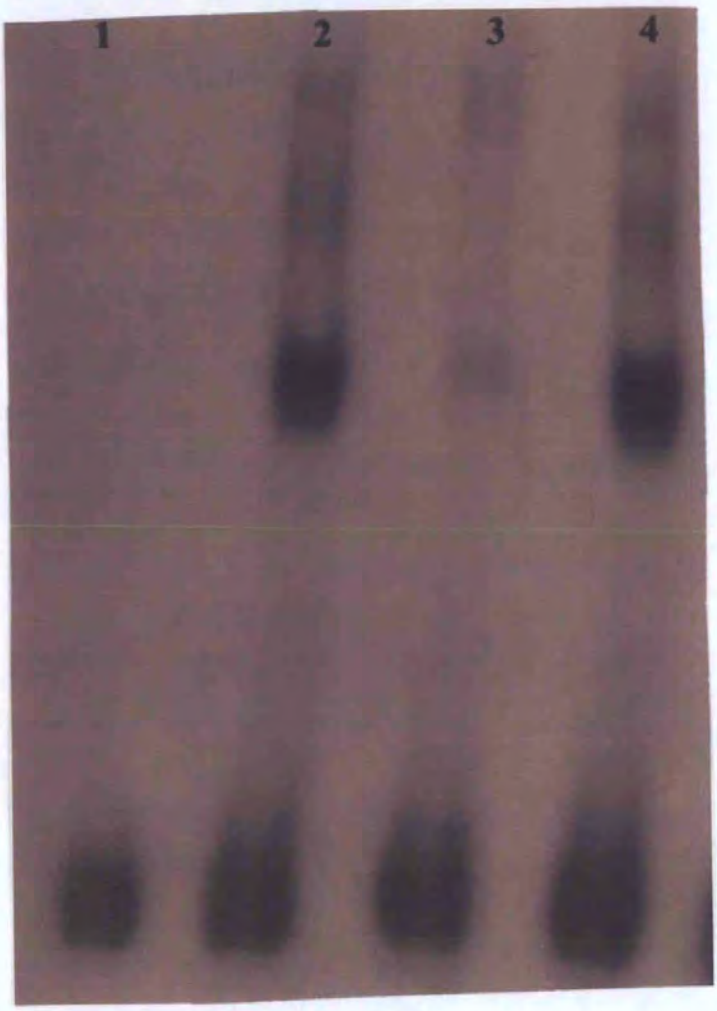


Figure 2.2. An example of the EMSA assay. Lane one - negative control, Lane Two - positive control, Lane three - positive control with competitive inhibition, Lane four positive control with non-specific inhibitor.

Transcription Factor	Oligonucleotide Sequence
NFκB	5' AGTTGAGGGGACTTTCCCAGGC 3'
AP1	5' CGCTTGATGAGTCAGCCGGAA 3'
HSF1	5' GCCTCGATTGTTCGCGAAGTT 3'
SP1	5' ATTCGATCGGGGCGGGGCGAGC 3'

Table 2.1. Oligonucleotide sequences used for EMSA assays

Method

1μl of Oligonucleotide (1.75pmol) was labelled with [γ-^{32}P] ATP (20,000 Cpm) in a phosphorylation reaction containing T4 kinase (10u/μl), 10x T4 polynucleotide kinase buffer (700mM Tris-HCL pH 7.6, 100mM MgCl2 and 50mM DTT) and H_2O. The probe was incubated at 37^{0}C for 30 minutes. The reaction was stopped by the addition of 1μl 0.5M EDTA and placing on ice for 5 minutes. The reaction mixture was re-suspended in 89μl TE buffer. Nuclear protein (5-10μg) was mixed with a binding buffer (20% glycerol, 5mM MgCl2, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCL, 0.25mg/ml poly di-dC) at room temperature for 20 minutes. The probe (1μl) was added and the reaction allowed a further 20 minutes to develop. Protein-DNA complexes were resolved on a 5% native polyacrylamide gel containing 10x TBE, 2% bisacrylamide, 40% acrylamide, 80% glycerol, H_20 and 10% APS and TEMED as the catalysts. Gels were assembled in a Biorad protein II system (Biorad) and gels were run in 0.5x TBE for 3-4 hours at 100V. Following this gels were removed and exposed to autoradiography overnight.

Competition experiments to demonstrate the specificity of the probe were performed with a molar excess of unlabelled oligonucleotide or non-specific oligonucleotide. Competition probes were incubated with nuclear extracts for 20 minutes before the addition of labelled probes. For NFκB supershift experiments, 1μl of antibody to p65 subunit (pharminogen, UK) and/or p50 subunit (Affiniti) of NFκB were incubated with the nuclear protein and probe for 1 hour at 4°C before being resolved on the gel.

2.12 Immunoblotting (Western blotting)

Whole cell extracts (20μg) were resolved on 12% polyacrylamide gels, containing 30% acrylamide, 1.5M Tris-HCL (pH 8.8), 10% SDS and H_2O with a 4% stacking gel to compress the bands (30% acrylamide, 0.5M Tris-HCL pH 6.8, 10% SDS, H_20). Gels were run in western blot running buffer for 4-5 hours at 120V in the Protein II system. ECL membranes (Amersham International, UK) were soaked for 1 hour in transfer buffer before gels were removed. Gels were sandwiched between filter paper and ECL membranes and placed in the transfer buffer in a tank (Biorad). Gels were transferred overnight at 10mA. Following removal from the tank, membranes were washed in PBS. Gels were normally discarded at this point, but occasionally stained with gelcode blue stain reagent (Pierce) for one hour, which allows visualisation of protein bands. This was used to confirm all bands had transferred and that the transfer times and conditions were optimised. Further confirmation of accurate transfer was possible due to the fact that pre-stained molecular weight markers were run on each gel (Amersham UK). The proteins range from 220 (Myosin) to 14.3 (lysozyme) KDa and are coloured. The 'rainbow ladder' can be seen on the gel and ECL membrane and also allows the determination of protein size. Nonspecific sites were blocked using a blocking buffer (TBS-Tween 20, 5% non fat milk) for 1 hour with gentile agitation. Blots were then washed in 5minute steps for a total of 3 times in TBS-Tween 20. Blots were then incubated with primary antibodies. Primary antibodies were diluted in blocking buffer at a concentration dependent on the antibody. Membranes were incubated with primary antibody for 1-2 hours at room temperature with gentle agitation. Blots were then washed again for a total of 5 times in TBS-Tween 20. Careful washing at this stage is necessary to remove all unbound antibody to prevent 'background' on developed films. Blots were then incubated with a horseradish

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Antibody	Dilution	Host	Company
NFκB p65	1:500	Mouse	Pharmingen UK
NFκB p50	1:500	Mouse	Affiniti UK
IκBα	1:1000	Rabitt	New England Biolabs UK
P38	1:1000	Rabitt	New England Biolabs UK
JNK	1:1000	Rabitt	New England Biolabs UK
ERK	1:1000	Rabitt	New England Biolabs UK
BCL-2	1:1000	Mouse	Santa Cruise USA
HSP 70	1:1000	Goat	Santa Cruise USA
VWF	1:500	Mouse	Sigma UK

Table 2.2. Antibodies used for western blotting assays

peroxidase (HRP) conjugated secondary antibody (1:5000–1:10,000 dilution) which is specific for the primary antibody (e.g. Mouse IgG anti-human primary antibody, Anti-mouse IgG HRP conjugated secondary antibody). The fluorescence reaction was visualised by chemiluminescence using the supersignal detection system (Pierce, UK). Equal volumes of reagent 1 and 2 were incubated with the blot for 5 minutes. Excess fluid was removed and blots wrapped in saran wrap, before being exposed to film. An initial 2 minute exposure was used to determine optimal exposure time.

2.13 Autoradiography

All gels were transferred to Whatman 3MM paper, (Whatman Inc UK). If the gel was excessively wet, due to poor removal from the assembly apparatus it was dried using a vacuum heat source. Gels were wrapped in saran wrap and exposed to kodak XLS5 photographic X-ray film (XO graph image systems UK) overnight at -80^{0}C in a cronex intensifying screen. For western blotting fluorescence signals were extremely strong, so a minimal exposure time ranging from 30 seconds to 5 minutes were needed at room temperature.

Films were developed using X-ray developer, stop-bath solution and liquid fixer (Amersham UK) in a dark room.

2.14 Measurement of Lactate

Lactate was measured in supernatant by spectrophotometric analysis using an NAD reaction (Sigma). Vials containing NAD were reconstituted with 2ml Glycine, 4ml water and 0.1ml Lactate dehydrogenase enzyme. 100µl of sample was added to 2.9ml of this solution and incubated for 15 minutes at 37^{0}C. A standard curve was

constructed using a 4.4mmol/L lactate standard. Absorbance was read at 340nm within 10 minutes of the end of incubation.

2.15 Measurement of hydrogen peroxide (H202)

H202 was measured in culture supernatant by colourmetric analysis (R&D systems). A 25mM stock H202 solution was standardised at 240nm, where it has an absorbance of 1.08. A 1/250 dilution of this stock provided a 100μM standard that was serially diluted to make a standard curve. Reagent one (25mM ammonium iron sulphate, 2.5M H2S04) was added to 100 volumes reagent two (100mM sorbitol, 125 μM xylenol orange). 1 volume of standard or unknown was added to 10 volumes of reagent one/two mixture and incubated for 30 minutes at room temperature. Absorbance was measured at 560nm.

2.16 Enzyme Linked Immunoabsorbant Assay (ELISA)

Human IL-6, IL-8 and TNFα levels were measured in commercial kits from R&D systems UK. VWF levels were measured in a diagnostic ELISA kit from Baxter Healthcare Ltd UK. IL-4 was measured in a commercial kit from Pharminogen UK. All were measured in samples of cell culture supernate or renal preservation solution. The sandwich ELISA is performed in 96well microtiter trays coated in monoclonal antibody. The antibody is immobilised and binds to the free protein in the sample. A standard curve is constructed with each assay using recombinant standards. The samples and standards were added to the wells in the plate and incubated for 1-2 hours at room temperature. The plate is sealed to avoid contamination. The plate was then aspirated and washed for a total of 5 times in wash buffer containing TBS-Tween 20 with 0.02% thimerosal as a preservative. Washing removes any unbound and non-

specific proteins in the sample. The second phase is the conjugation reaction, where a labelled antibody binds to the immobilised antibody in the plate forming the 'sandwich'. A working detector is prepared within 15 minutes of use containing biotinylated monoclonal antibody and a 250x streptavidin-horseradish peroxidase conjugate and added to each well. The plate is sealed and incubated for 1 hour at room temperature. Plates were aspirated and washed 7 times with wash buffer. The second was phase removes unbound conjugate antibody. The final stage is the substrate reaction during which the peroxidase-conjugated antibody is oxidised in the presence of H202 and chromogen forming a blue/green colour, which is proportional to the amount of bound protein. The substrate solution (equal volumes hydrogen peroxide and tetramethylbenzidine) is prepared within 15 minutes of use and added to each well. The plate is incubated for 30 minutes at room temperature in the dark to allow the colour to develop. A stop solution (sulphuric acid) added to each well which turns the colour yellow and the absorbance can be read at 450nm with a correction at 570nm.

2.17 Polymerase chain reaction (PCR)

PCR allows the selective amplification of a region of DNA, such as a polymorphism by using primers designed to complement the region of interest (see Fig 2.3).

Primers are designed to flank the 5'-3' direction (forward) and 3'-5' direction (reverse) of the region of DNA of interest. Generally primers are around 20 bases long. There is a random base distribution and a guanosine and cytidine ratio of around 50%. The specificity of the primer can be checked using known sequences contained in databases such as on the Genbank webpage to prevent the binding to other regions in a gene containing a similar sequence creating artefacts and non-specific bands,

known as primer dimers. The primer and thermostable polymerase enzyme bind to the single strand of DNA and then synthesise a new strand by extending along the template region. Repeating cycles creates the selective amplification of a single fragment of target DNA that can easily be visualised by running out the PCR product on an agarose gel and staining with ethidium bromide. There are numerous ways to optimise the PCR reaction and conditions including optimising the Mg^{2+} concentration, number of cycles, dNTP concentration, annealing temperature and primer concentrations.

Primers used in this study were synthesised commercially by MWG Biotech Germany (Table 2.3.1 lists primers used). Primers are supplied at a scale of 0.05μmol and concentration made to 100pmol/μl with water. Primers are further diluted in sterile water to a concentration of 10pmol and stored in aliquots at $^{-}20^{0}$C until use.

2.17.1 5' end labelling of oligonucleotide

End labelling of the primers was achieved using the T4 'ready to go' polynucleotide kinase labelling system (Pharmacia Biotech, Sweden). 25 μl of sterile water was added to T4 PNK and incubated at room temperature for 5 minutes. 5μl (10pmol) of dilute primer was added and H_20 to make a 49 μl total volume. 1μl of γ^{32p} dATP (3,000Ci/mmol) was added and the sample mixed by vortexing and centrifuged at full speed for 10 seconds to collect the contents. The probe was incubated for 30 minutes at 37^{0}C and the reaction stopped by the addition of 5μl of 250mM EDTA and placing on ice for 5 minutes. Unincorporated nucleotides were removed using the 'Quick Precip^{TM,} system (Advanced Biotech Corps, USA). 5μl of 5M NaCl was added and 2μl Quick Precip and 160μl 100% ethanol before being centrifuged at 13000rpm for 3 minutes. The sample was vortexed and re-centrifuged at 13000rpm for 1 minute. The

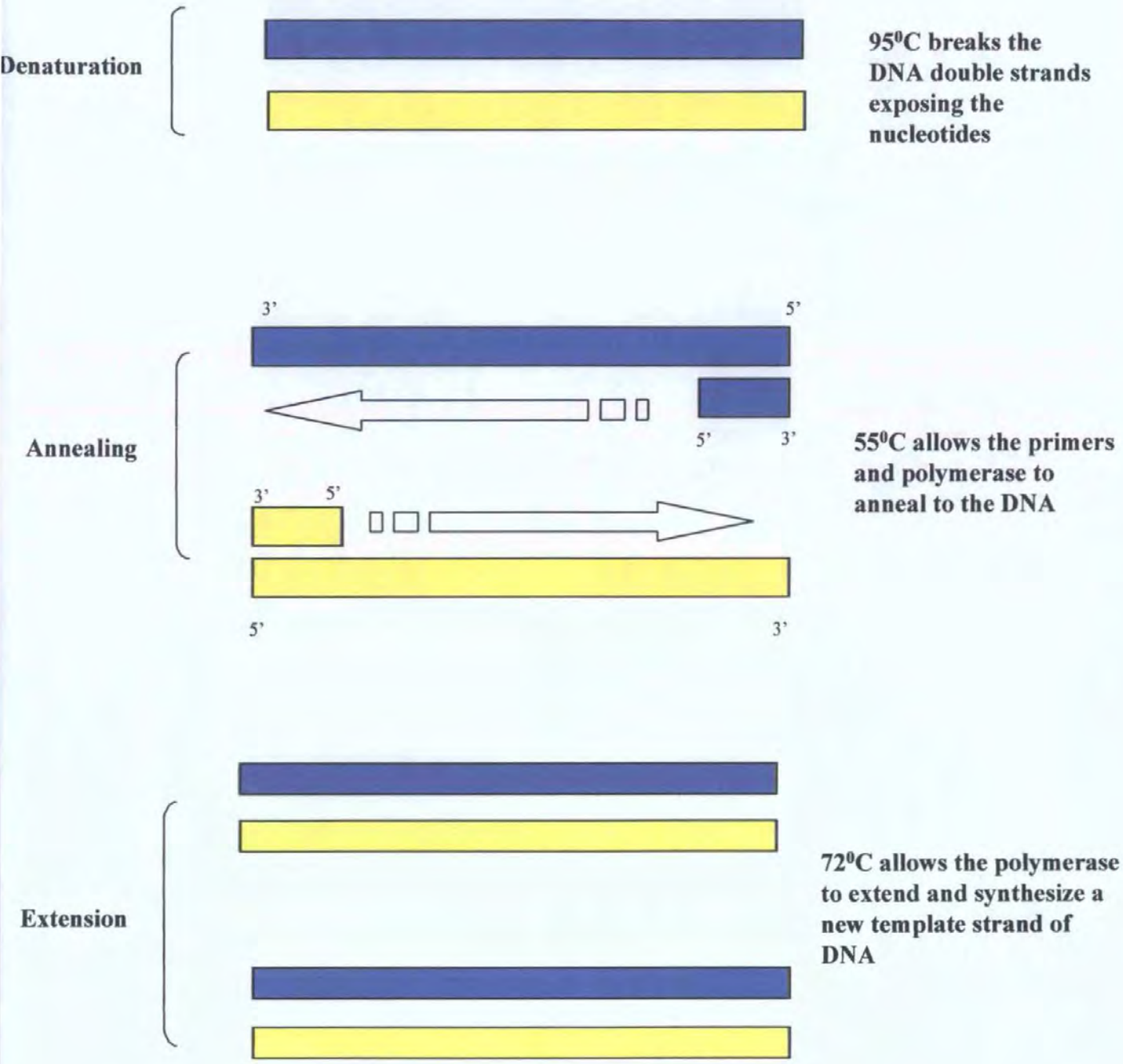


Figure 2.3. Basic overview of one PCR cycle. Typically 20-40 cycles are performed

Primer	Forward/Reverse	Sequence
NFκB	F	5' AGTTGAGGGGACTTTCCCAGGC 3'
	R	5' CAAGTAAGACTCTACGGAGTC 3'
TNFα	F	5' GCCTCTAGATTTCATCCAGCCACA 3'
	R	5' CCTCTCTCCCCTGCAACAACAA 3'
IL-6	F	5' TTGTCAAGACATGCCAAAGTGC 3'
	R	5' GGGAAAATCCCACATTTGATAA 3'
NAD(P)H	F	5' TGCTTGTGGGTAAACCAAGG 3'
	R	5'GGAAAAACACTGAGGTAAGTG 3'

Table 2.3.1 Oligonucleotide sequences used for PCR analysis

reaction was stopped by placing the sample on ice and the radioactive supernatant removed and disposed. The remaining pellet was washed in 70% ethanol and dried before being resuspended in 50µl H_20.

2.17.2 PCR Reactions

The following reaction was set up for NFκB: 100ng of genomic DNA was mixed with 3mM $MgCl_2$, 2µl 10x SuperTaq buffer (50mM Tris-HCL pH 9.0, 1.5mM $MgCl_2$, 250mM KCL, 1% Triton X-100, 0.1% w/v gelatin), 10pmol of forward primer, reverse primer and labelled forward primer, 200mM of each dNTP and 1 unit of Taq polymerase enzyme. H_20 was added to make a final reaction volume of 30µl.

The following reaction was set-up for TNFα: 100ng of genomic DNA mixed with 1.5mM $MgCl_2$, 2µl SuperTaq buffer, 10pmol of forward primer, reverse primer and labelled Forward primer,200mM of each dNTP and 1 unit of Taq polymerase enzyme. H_20 was added to make a final reaction volume of 30µl.

All PCR reactions were carried out in thin walled 0.2ml PCR strips (Anachem, UK). The cycles used are shown in Figs 2.3.2 and 2.3.3. The PCR reaction was carried out in a Techne 40/96 well thermocycler (Techne, UK).

PCR products were checked by running them through a 1.5% agarose gel for 30 minutes at 120V. Bands were visualised using a UV transilluminator.

If successful, PCR products were run on 6% polyacrylamide gels. Gels were constructed using a Biorad Sequigen GT 30x50cm electrophoresis system (Biorad). The system was cleaned thoroughly before use with warm water and 70% IMS. The assembled gel rig contained 0.4mm spacers and a 49 well comb. The gel was constructed using SequaGel reagents (National Diagnostics UK). 99ml of diluent, 15ml buffer, 36ml concentrate and 10ml foramide were mixed before the addition of

70µl TEMED and 10% APS. The gel mixed was inserted into the gel, avoiding air bubbles and allowed to polymerise overnight. Gels were pre-warmed to 40^0C in 1x TBE. PCR products were mixed with a stop solution containing 98% deionised foramide, 10mM EDTA (pH 8.0), 0.025% xylene cyanol and 0.025% bromophenol blue (Life Tech) and loaded to the gel using a sequencing pipette (Drummond Laboratories, USA). Gels were run for 3-5 hours at variable voltage in order to keep the temperature at 50^0C, for optimal resolution of the bands.

Gels were removed using a 10% methanol, 10% acetic acid solution, transferred to filter paper and dried on a gel dryer, before being exposed to film overnight.

2.17.3 Restriction Enzyme analysis.

The IL-6 C (-174)G polymorphism was amplified in the following PCR reaction. 100ng of genomic DNA was mixed with 3mM $MgCl_2$, 10pmol forward and reverse primers, 2µl 10x PCR buffer, 200mM of each dNTP and 1 unit of Taq polymerase. Water was added to make the final volume 20µl. PCR products were checked by running through a 1.5% agarose gel. If successful, a 10µg sample of the PCR product was digested with 10-20 units of the NlaIII restriction enzyme. The reaction volume was increased to a final volume of 30µl by the addition of enzyme buffer (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate and 1mM DTT). The reaction was allowed to proceed overnight at 37^0C.

The PCR reaction for the NAD(P)H phox 22 (-242) was: 100ng genomic DNA mixed with 1.5mM $MgCl_2$, 10pmol forward and reverse primers, 2µl 10x PCR buffer, 200mM of each dNTP and 1 unit of Taq polymerase. If the PCR was successful the PCR product was digested with RsaI restriction enzyme for 3 hours at 37^0C.

	PCR conditions	Cycles
NFκB	95^{0}C 5mins	Hotstart 1 cycle
	94^{0}C 30 secs	30 cycles
	53.5^{0}C 2 mins	
	72^{0}C 2 mins	
	72^{0}C 10 mins	1 cycle
TNFα	94^{0}C 5 mins	Hotstart 1 cycle
	94^{0}C 30 secs	30 cycles
	65^{0}C 2 mins	
	72^{0}C 2 mins	
	72^{0}C 10 mins	1 cycle

Table 2.3.2 PCR conditions used for microsatellite analysis of the NFκB and TNFα CA (n) repeat polymorphisms.

	PCR Conditions	Cycles
IL-6	95^{0}C 5mins	Hotstart 1 cycle
	94^{0}C 30 secs	30 cycles
	53.5^{0}C 2 mins	
	72^{0}C 2 mins	
	72^{0}C 10 mins	1 cycle
NAD(p)H	96^{0}C 2mins	Hotstart 1 cycle
	94^{0}C 30 secs	30 cycles
	56^{0}C 2 mins	
	72^{0}C 2 mins	

Table 2.3.3. PCR conditions used for analysis of IL-6 and NAD(P)H phox22 restriction site analysis

Digestion products were run on a 1.5% ethidium bromide stained agarose mini gel at 120v for 30 minutes. A molecular 100 base pair ladder was run with samples to check fragment sizes. Cut bands were visualised by using a UV transilluminator.

2.18 Data Analyses

Bands were quantified using a phosphorImager (BioRad) with multianalyst software, and expressed as optical density. By comparing to a control in each experiment a fold increase or decrease was obtained to account for differences in radioactivity, fluorescence and exposure times. Results are expressed as means $\pm$ SE. Control cells positive or negative are compared to stressed cells at different time points. This is a repeated measure, so ANOVA was used with Fisher's least significant difference test for planned comparisons between stressed and control cells. Two-sample comparisons were made by student's t tests, after an F-test to determine sample variance. Trends in data were compared by simple regression for two samples or multiple regression for more than two samples.

The frequency of alleles and genotypes in rejection and non-rejection donor DNA were compared by $\chi 2$ analysis and 2 X 2 contingency tables. All statistical tests were carried out using the *Statgraphics Plus* package (Statistical Graphics Corp, USA).

A p-value of less than 0.05 was considered significant in all statistical analysis.

2.19 Clinical samples

50 donor DNA samples were obtained from the Immunology department, Derriford Hospital, Plymouth. All donors had been typed and used in first cadaveric renal transplants. Full details of the patient clinical characteristics can be found in the appendix. All clinical data was blind to the investigator until after all samples were

genotyped. Seventeen preservation solutions were obtained from imported kidneys received for cadaveric transplantation at Derriford Hospital. Solutions were collected from fluid surrounding the kidney during its harvesting and transportation. Samples were collected at the time of kidney removal from storage by the surgeon performing the operation and placed in a sterile specimen jar. Samples were filtered to remove solid mass and frozen at -80^0C until use. A detailed description of the donors clinical characteristics can be found in the appendix. Details of ischaemic times remained blind to the investigator until all data was collected.

CHAPTER THREE: THE EFFECTS OF HYPOTHERMIA ON ENDOTHELIAL CELLS *IN VITRO*

3.1 Background

To investigate the effect of hypothermia on endothelial cells, HUVECS were re-suspended in renal preservation solution and placed at 4^0C for 72 hours. Total Lactate and H_2O_2 was measured as described in the methods. For comparison HUVECs were placed in a hypoxic incubator for the same time points and supernatant collected. To assess endothelial cell function VWF was measured by ELISA, during hypothermia. To determine the effect of hypothermia on apoptosis, cell viability was measured, DNA fragmentation was measured, BCL-2 and Hsp70 expression was measured.

3.2 Lactate

There was an increase in lactate production by HUVECS over time in hypothermia (Fig 3.1), reflecting a switch toward anaerobic metabolism. Control cells produced 1.52 ± 0.42 mmol/l over 72 hours. In cells exposed to hypothermia the production of lactate was significantly increased at 48 hours (6.32 ± 1.34 mmol/l $p=0.02$) and peaked at 72 hours (7.34 ± 1.82 mmol/l $p=0.03$). In cells placed in hypoxic conditions there was an increase in lactate compared to control cells at all time points (Fig 3.1) ($p<0.05$). There was a significant difference between the amounts of lactate produced during hypothermia and hypoxia at 6 hours (2.17 ± 0.1 mmol/l Vs 4.82 ± 0.1 mmol/l $p<0.05$) and 12 hours (2.53 ± 0.8 mmol/l Vs 7.3 ± 1.0 mmol/l $p<0.05$). However, after 12 hours cold storage there was no difference between the amounts of lactate produced between hypoxic and hypothermic cells.

3.3 Hydrogen peroxide

H202 is produced by endothelial cells, but there was no significant differences between control cells and hypothermic stored cells. Control cells produced 21.90 $\pm$ 0.32 μM. Peak levels during hypothermia were at 48 hours (22.73 $\pm$ 1.0 μM). During hypoxia levels peaked at 24.50 μM.

3.4 VWF

There was no significant increase in VWF levels during the 72 hour time period. Control cells produced 0.026 $\pm$ 0.003 IU/ml when cultured in standard conditions. PMA was a strong inducer of VWF production with levels rising to 0.45 $\pm$ 0.25 IU/ml (17.3 fold increase) when PMA was added to the culture. There was an initial increase in VWF after 5 minutes of hypothermic stress (3.9 fold increase), but this was not significant and levels did not increase further beyond this time point.

3.5 Cell viability

Cell viability remained above 95% until 48 hours when it dropped to 79 $\pm$ 3.5%. After 72 hours cold storage viability was reduced further to 61 $\pm$ 5%. During reperfusion, cell viability remained above 95% at all time points. Cell viability in dexamethasone treated cells was reduced to 37 $\pm$ 6%. ($p<0.01$ Vs control cells).

3.6 DNA Fragmentation

No fragmentation of DNA was observed during hypothermia. Dexamethasone was used as a positive control and it was found to cause shearing and fragmentation of DNA. Similarly no fragmentation was observed during reperfusion (Fig 3.4).

3.7 BCL-2 expression

BCL-2 was consistently expressed throughout the time course. There was no significant difference between the expression of BCL-2 at the time points. After 15 minutes of hypothermia levels were higher than control cells (1.23 $\pm$ 0.10 fold) and remained so through to 72 hours (1.48 $\pm$ 0.31 fold). There was a peak at 12 hours (2.0 $\pm$ 0.31 fold increase) but there was no significant difference from control cell expression.

3.8 Hsp70 expression

The highest expression of Hsp70 protein was in control cells (3.89 $\pm$ 1.32 OD units). There were no significant differences between the time points, but the expression of HSP70 was down-regulated during hypothermia. The highest expression during hypothermia was at 4 hours (3.33 $\pm$ 0.85 OD units), which was still 0.15 fold less than control cells. In contrast, Hsp70 expression was up-regulated during reperfusion, although not significantly there was an increase in optical density after both 30 minutes and 12 hours hypothermia followed by reperfusion.

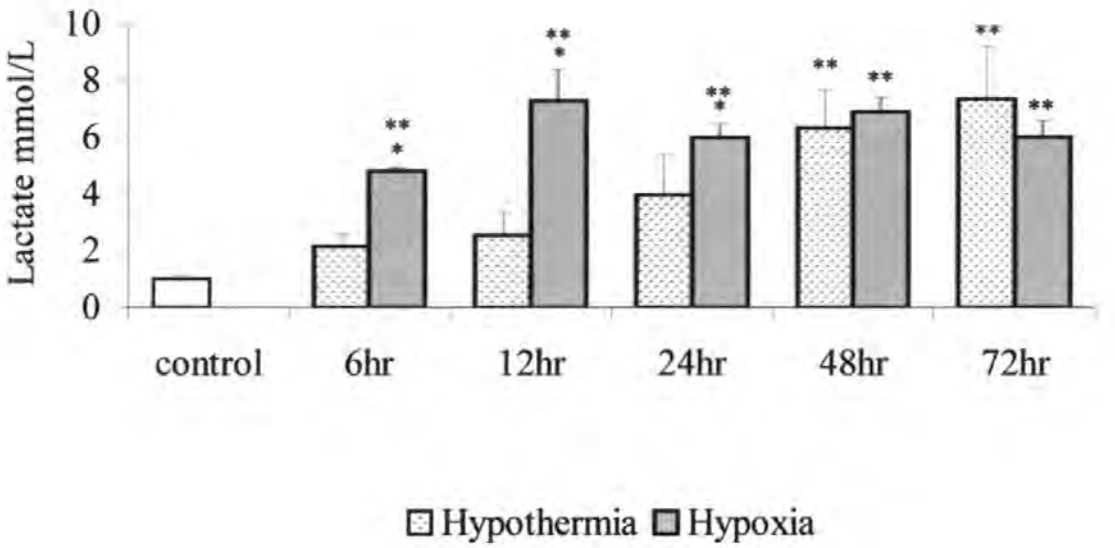


Figure 3.1. Lactate production by HUVECS during hypothermic and hypoxic stress. One asterisk indicates a significant difference between time points ($p<0.05$). Two asterisks indicate a significant difference from control cells ($p<0.05$). All experiments were duplicated.

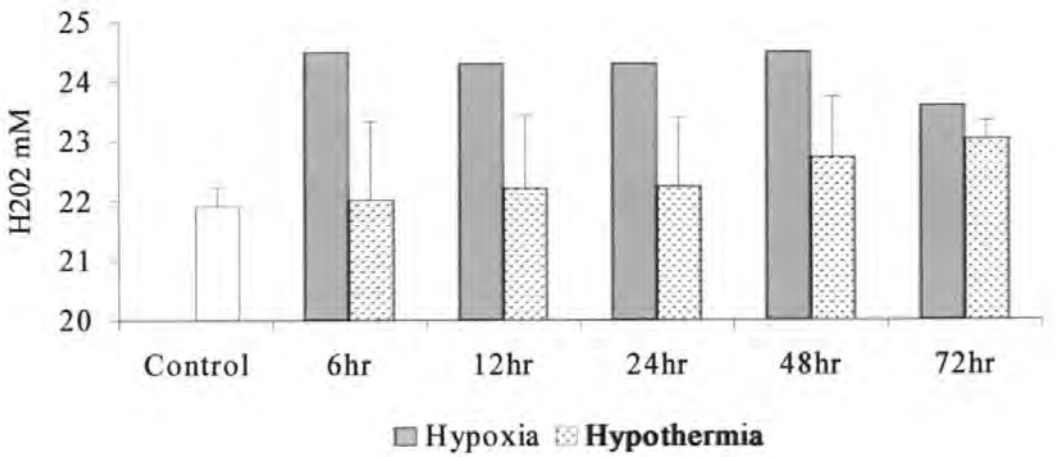


Figure 3.2. H2O2 production by HUVECS during hypothermic and hypoxic stress. H202 was measured by colourmetric analysis.

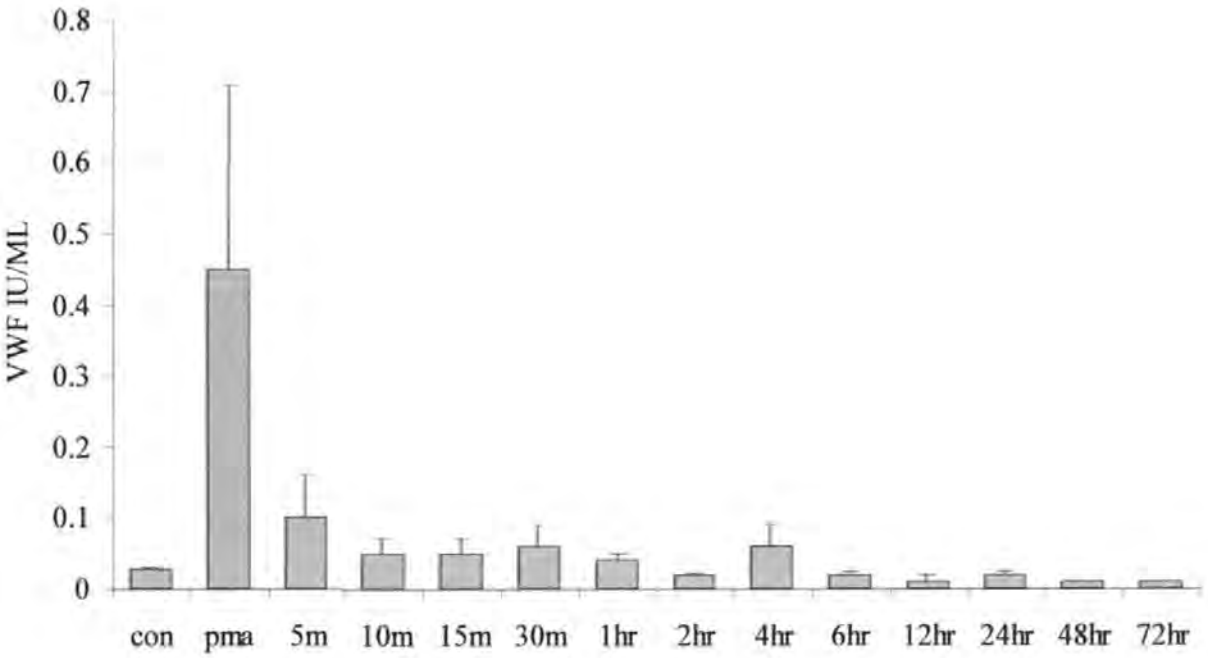
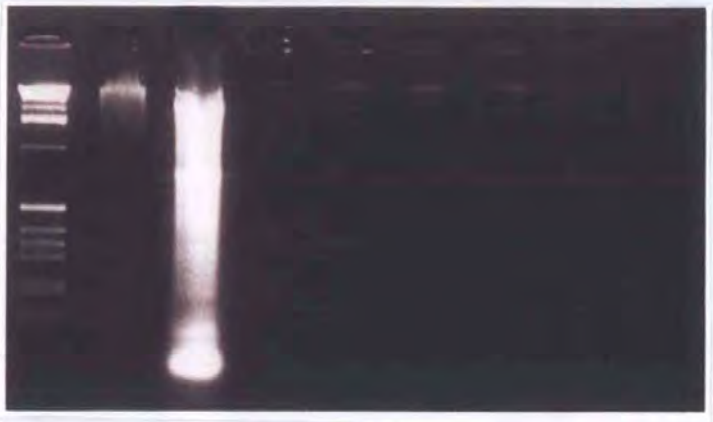


Figure 3.3. VWF production by HUVECS during hypothermic stress. Results are the mean of triplicate ELISA experiments. Although PMA was a strong stimulant of VWF synthesis there were no significant differences between samples.

Hypothermia 1 2 3 4 5 6 7 8 9



Lane 1: 1Kb ladder, Lane 2:Control cells, Lane 3: Dexamethasone treated cells, lane 4: PMA stimulated cells, Lane 5: 6 hours, Lane 6: 12 hours, Lane 7: 24 hours, Lane 8: 48 hours, Lane 9: 72 hours.

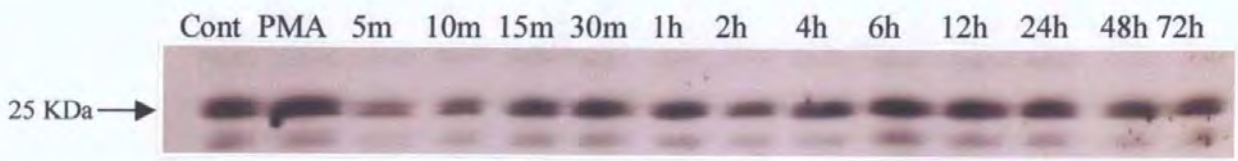
Reperfusion **1 2 3 4 5 6 7 8 9**

Lane 1: 1Kb ladder, Lane 2: Control cells, Lane 3: Dexamethasone treated cells, Lane 4: 30mins+15mins Lane 5: 30mins+30mins, Lane 6: 30mins+1hr lane 7: 12hr + 15mins Lane 8: 12hr +30mins Lane 8: 12hr +1hr.

Figure 3.4. DNA fragmentation was not seen during hypothermia or reperfusion in endothelial cells. Dexamethasone reduced cell viability to 37% and was associated with fragmentation and shearing of DNA.

BCL-2

A.



B.

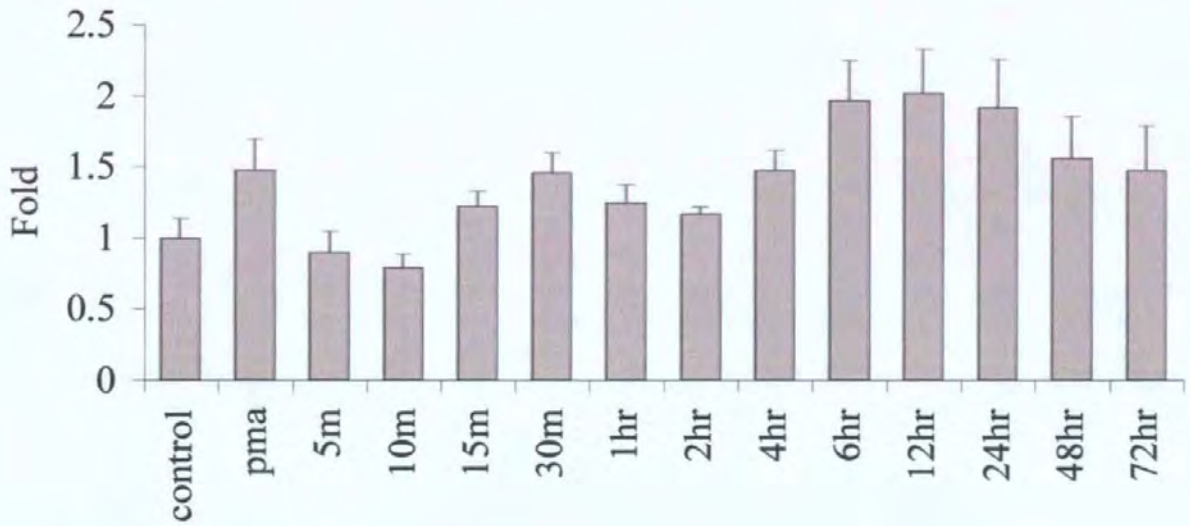
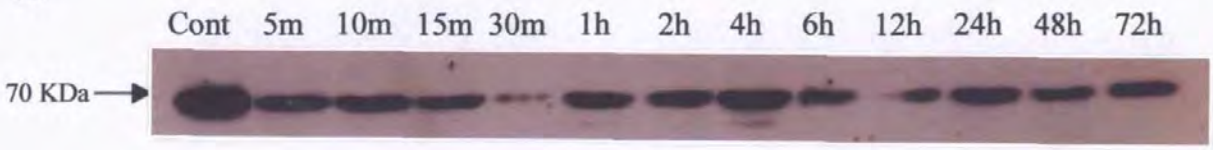


Figure 3.5. BCL-2 expression during hypothermic stress in endothelial cells. An example of a western blot for total BCL-2 is shown in A. The average optical density readings of four experiments is shown in B. There were no significant differences between time points by ANOVA.

Hsp70

A.



B.

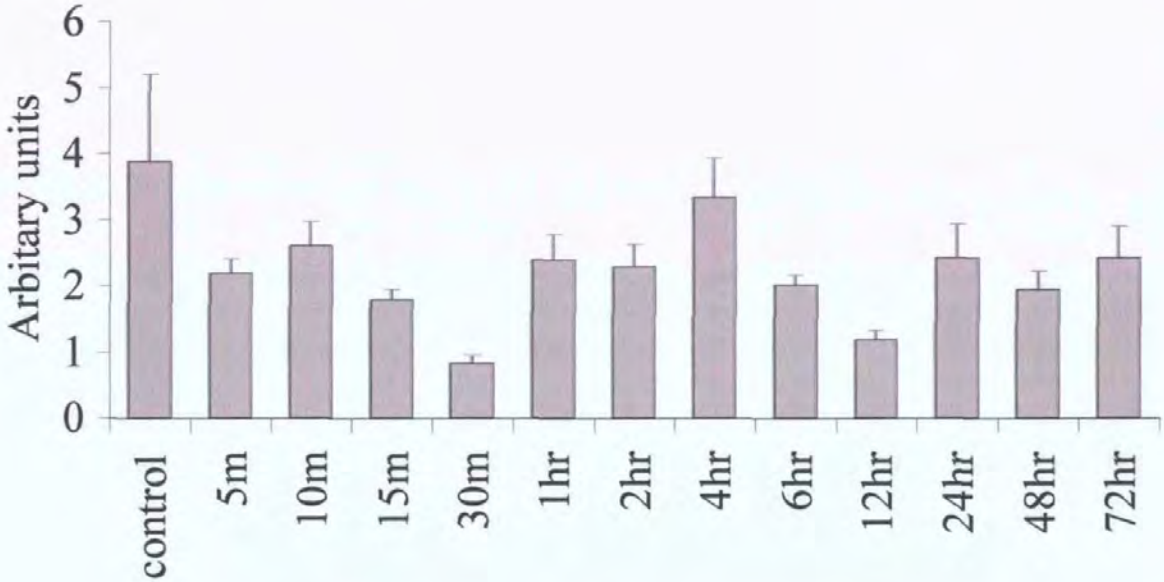


Figure 3.6. Hsp70 expression during hypothermia. An example of a western blot for total HSP70 is shown in A. The average optical density readings of three experiments is shown in B. A high expression of Hsp70 was found in control cells. There were no significant differences between time points by ANOVA.

3.9 Discussion

Placing endothelial cells in hypothermia for 72 hours in sealed tubes resulted in lactate formation in a similar amount to cells placed in a hypoxic environment for the same time point. Endothelial cells also produced H202 during hypothermia, but levels did not reach the levels found during hypoxia.

Endothelial cells appear to be resistant to hypothermic induced cell death (Hansen 1989). Placing cells at 4^0C for 72 hours reduced viability from 95% to 61%. However, there was no fragmentation of DNA noted during hypothermia. Dexamethasone treated cells had a reduced viability to 37 ± 6 % and a marked difference in DNA from these cells was seen in the fragmentation experiments.

Expression of the protective anti-apoptosis protein BCL-2 was not up-regulated during hypothermia, but levels were found to be similar to the expression in control cells. The protective protein hsp70 was highly expressed in control cells and cells experiencing hypothermia did not regain the same level of expression. Cells did not release VWF during hypothermic storage.

CHAPTER FOUR: THE EFFECT OF HYPOTHERMIA AND REPERFUSION ON TRANSCRIPTION FACTOR AND MAPK ACTIVATION

4.1 Background

The transcription factors NFκB, AP1 and HSF1 were measured in nuclear extracts by EMSA analysis. The three common MAPK pathways, p38, JNK and ERK 1 / 2 were investigated by immunoblotting with 'anti-active' antibodies. To investigate the effect of these pathways on NFκB activation, the specific p38 inhibitor SB203580 and the general MAPK inhibitor PD98059 were used.

4.2 NFκB

NFκB is activated by hypothermia, and translocates to the nucleus after 5 minutes of hypothermia (4.8 $\pm$ 1.1 fold increase over control cells $p<0.05$). Control cells have a low level of NFκB proteins in the nucleus using EMSA (Fig 4.1.1). PMA strongly stimulates NFκB activation. When HUVECS were treated for 18 hours with PMA, there was an 8.6 $\pm$ 1.8 fold increase in NFκB levels in the nucleus ($p<0.01$). There is a peak in NFκB in the nucleus at 24 hours (5.6 $\pm$ 2.0 fold $p<0.01$), which is comparable to, and not significantly different from the shift observed after 18 hours PMA stimulation ($p>0.05$). After 24 hours the activation remains significant at 48 hours (5.4 $\pm$ 1.1 fold increase $p<0.05$) and 72 hours (4.8 $\pm$ 1.0 fold increase $p<0.05$). Competition experiments were used to show the specificity of the NFκB band and supershift analysis demonstrates that the complex contained both p65 and p50 protein heterodimers. Reperfusion did not up-regulate NFκB after 30 minutes or 12 hours hypothermia ($p>0.05$) (Fig 4.1.5). The 'active' antibodies demonstrated the phosphorylation of IκBα (Fig 4.1.3). Inhibition of

NFκB with PD98059 inhibitor was associated with the inhibition of IκBα phosphorylation (Fig 4.7.1).

Western blotting of whole cell extracts suggested the expression of the p50 subunit of NFκB remains consistent in the cell, whereas the expression of the p65 subunit is less consistent (Fig 4.1.4).

4.3 AP1 activation

The AP1 transcription factor is activated by hypothermia (Fig 4.2.1) with peak activation after 12 hours (3.8 ± 0.2 fold increase $p<0.05$). There was a 2.6 ± 1.0 fold increase in AP1 activity after 48 hours ($p<0.05$). Reperfusion did not cause an increase in the expression of AP1 either after 30 minutes or 12 hours of hypothermia ($p >0.05$) (Fig 4.2.2). Competition experiments demonstrated the specificity of the AP1 band (Fig 4.2.3).

4.4 HSF 1

HSF1 was strongly expressed in control cells by EMSA (Fig 4.3.1). During hypothermia there was an initial drop in HSF1 expression until levels returned to control values after 30 minutes (Fig 4.3.1). There was no significant increase in HSF1 expression during hypothermia ($p>0.05$) or reperfusion ($p>0.05$) which was similar to what was found with the expression of hsp70 (Fig 3.6). Competition experiments demonstrated the specificity of the HSF1 band (Fig 4.3.2).

4.5 ERK 1 / 2

The ERK 1 /2 pathway was also activated by PMA stimulation (2.9 $\pm$ 0.4 fold increase p=0.005). During the initial 5 minutes of cold shock there was a 2.7 $\pm$ 0.08 fold increase in ERK phosphorylation ($p < 0.05$). However, the ERK 1 / 2 activation was sustained during the time course (Fig 4.4.1), with a second wave of peaks at 4 hours (2.99 $\pm$ 0.63 $p<0.05$), 6 hours (3.11 $\pm$ 0.65 $p<0.01$) and 12 hours (2.98 $\pm$ 0.26 p= 0.01). Activation of ERK 1 / 2 was significantly associated with NFκB (Fig 4.7.1) activation throughout the 72 hour time course ($p<0.05$ R^2= 0.33). Inhibition of ERK 1 /2 with the PD98059 inhibitor was associated with the reduced expression of NFκB ($p<0.05$) (Fig 4.7.2). Reperfusion did not effect ERK 1 /2 phosphorylation after 30 minutes or 12 hours hypothermia ($p>0.05$) (Fig 4.4.2).

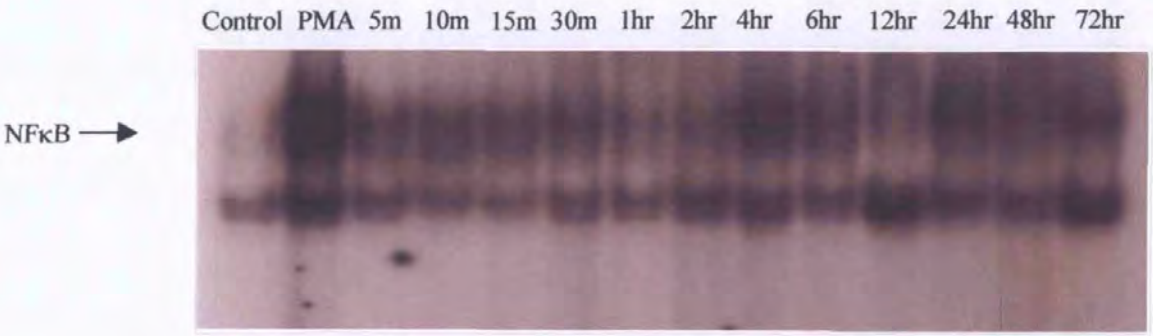
4.6 P38 MAPK

P38 MAPK was activated by hypothermia with peak phosphorylation after 5 minutes compared with control cells (13.6 $\pm$ 2.0 fold $p<0.05$). This was greater than during PMA stimulation (10.2 $\pm$ 0.9 fold). The initial stimulation of the p38 pathway was reduced over time (Fig 4.5.1). The initial activation of p38 during the first 30 minutes of hypothermia was significantly associated with NFκB activation (p=0.007 R^2=0.79). P38 activity was not increased by reperfusion after 30 minutes or 12 hours hypothermia ($p>0.05$). Inhibition of p38 with SB203580 was not associated with inhibition of NFκB (Fig 4.7.3).

4.7 JNK

The JNK pathway is not stimulated by hypothermia alone (Fig 4.6.1), but reperfusion back to 37^0C after 30 minutes of hypothermia causes the activation of this protein (Fig 4.6.2). Ten minutes of reperfusion caused a 4.8 $\pm$ 0.1 fold increase in JNK phosphorylation compared to cells that experience hypothermia alone ($p<0.05$). The JNK pathway remains activated up to 1 hour after reperfusion, where there is still a 4.9 $\pm$ 0.30 fold increase ($p<0.05$). However, reperfusion after 12 hours hypothermia did not up-regulated JNK activation ($p>0.05$) (Fig 4.6.2). JNK was not associated with NFκB activation ($p>0.05$).

A.



B.

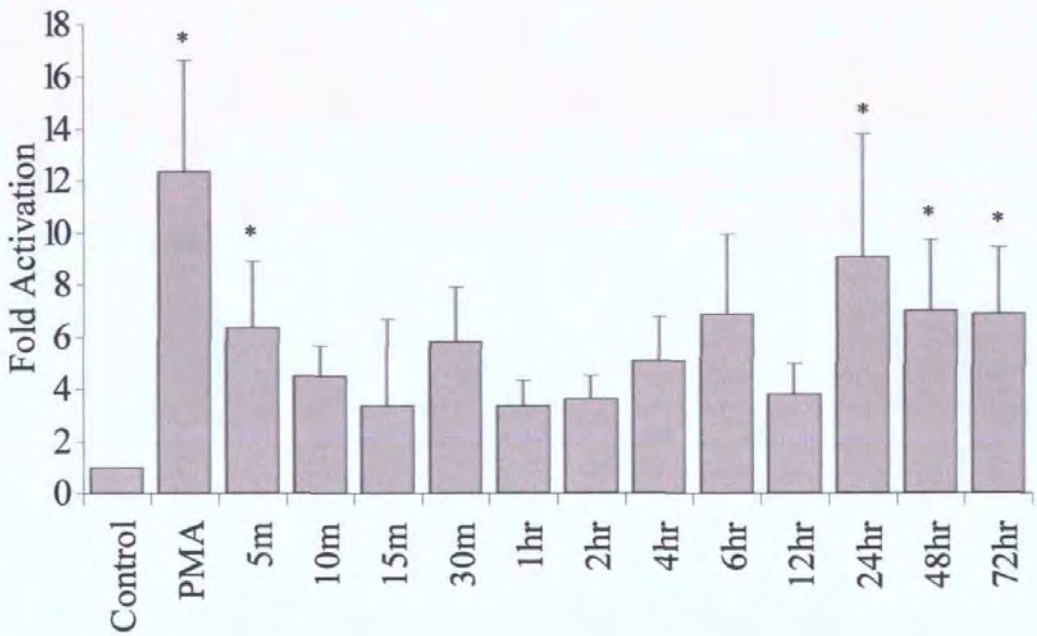


Figure 4.1.1. NFκB activation during hypothermic stress in endothelial cells. A typical EMSA assay for NFκB is shown in A. The fold activation from optical density readings are shown in B. Results are the mean of five experiments. Asterisks indicate a significant difference from control cells.

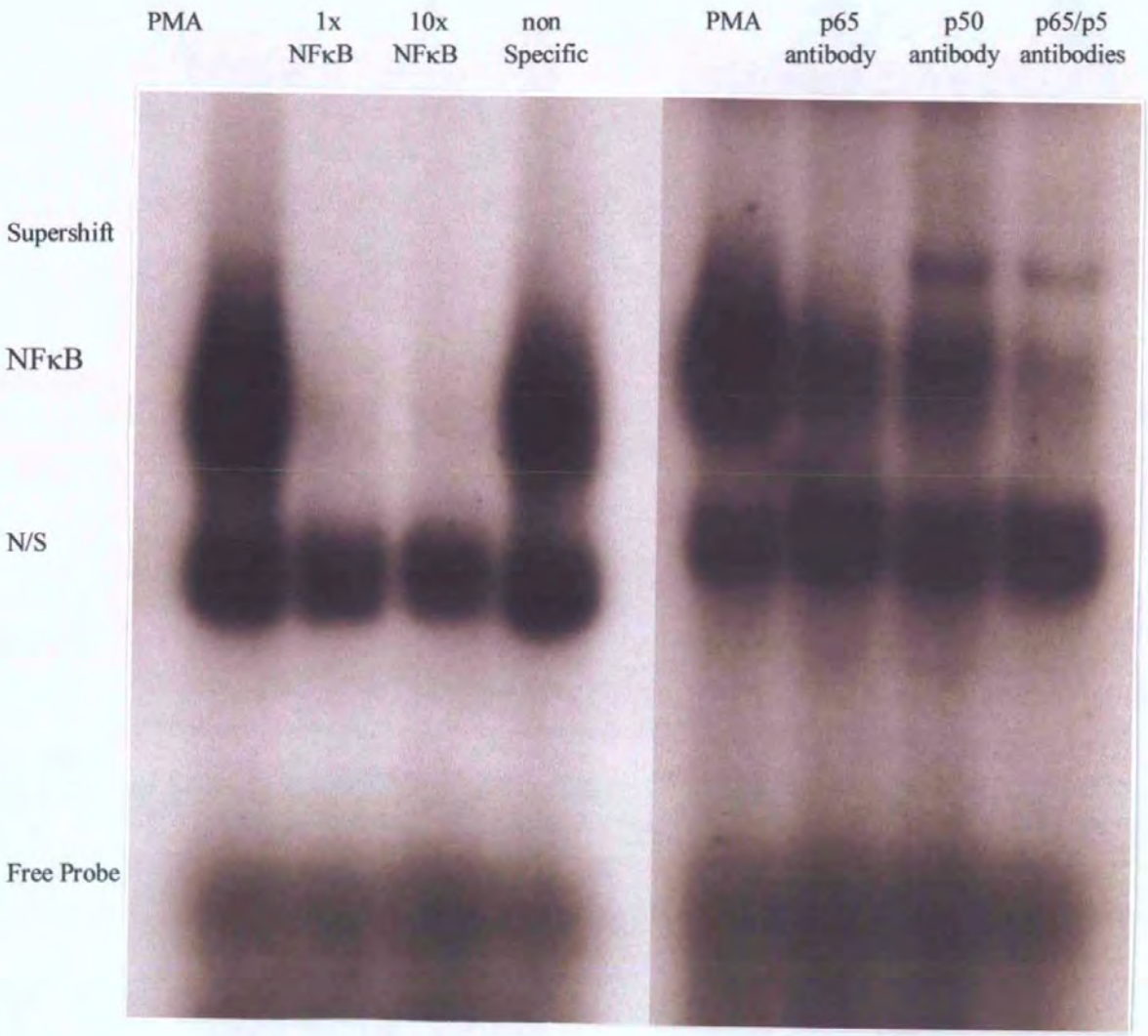


Figure 4.1.2 . Competition and supershift analysis to determine the specificity and subunits of the NFκB bands. Adding an excess of unlabelled oligo 'competes' for the NFκB probe and there is no shift from the free probe. Antibodies bind to the probe and increase the molecular weight of the complex causing a further 'supershift' away from the band.

A.

B.

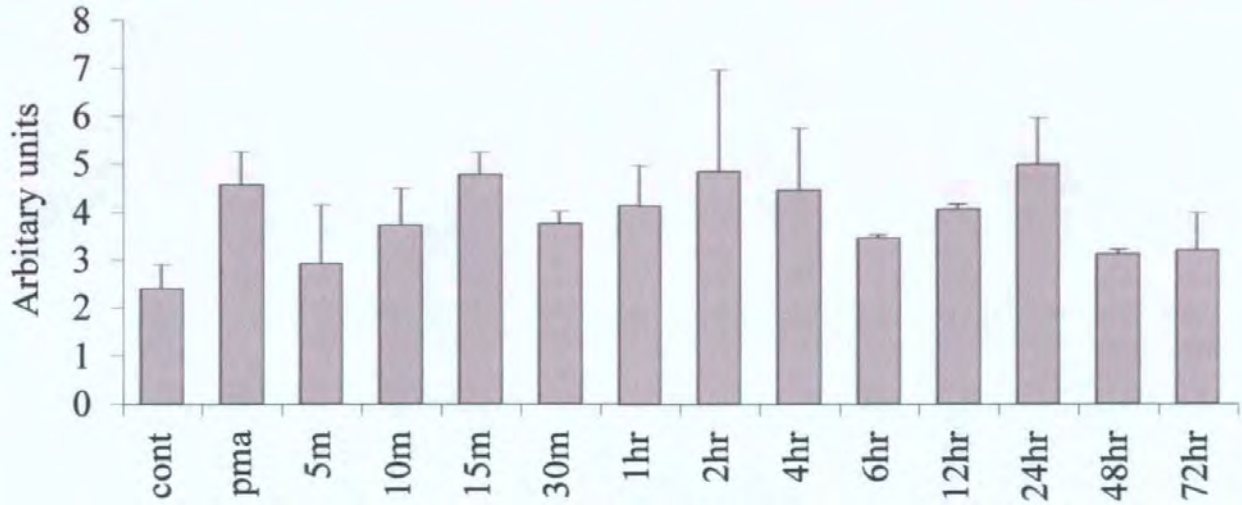
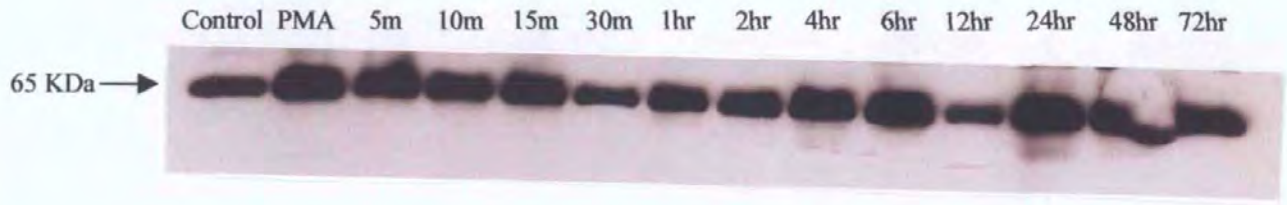


Figure 4.1.3. IκBα phosphorylation during hypothermic stress. A typical western blot is shown in A. Antibodies are specific for the phosphorylated form of the IκBα protein. Optical density readings are shown in B. Results are the average of duplicate experiments.

A.



B.

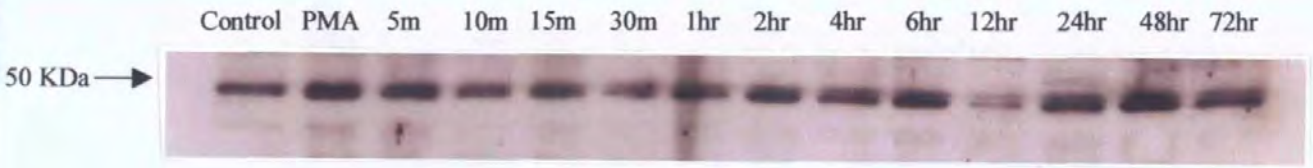
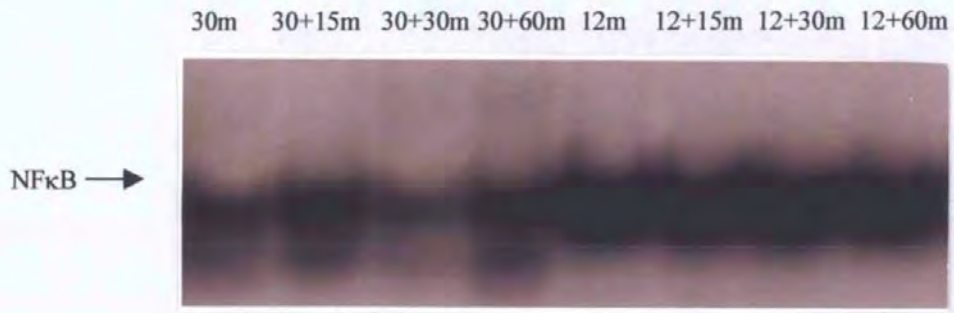


Figure 4.1.4. NFκB p50/p65 subunits. Whole cell extracts were western blotted for total p65 (A) or p50 (B) protein. The activation of NFκB appears to be associated with an increase in p65 expression, but little change in p50 expression in whole cell extracts. The experiment was only carried out once, so optical density readings are not shown.

A.



B.

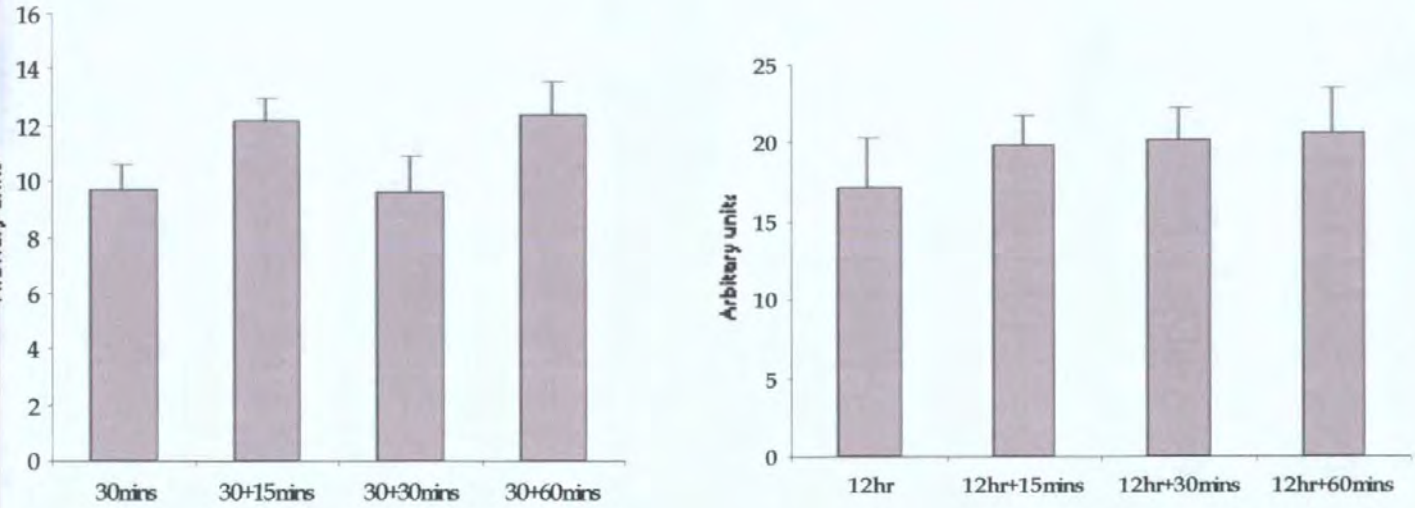
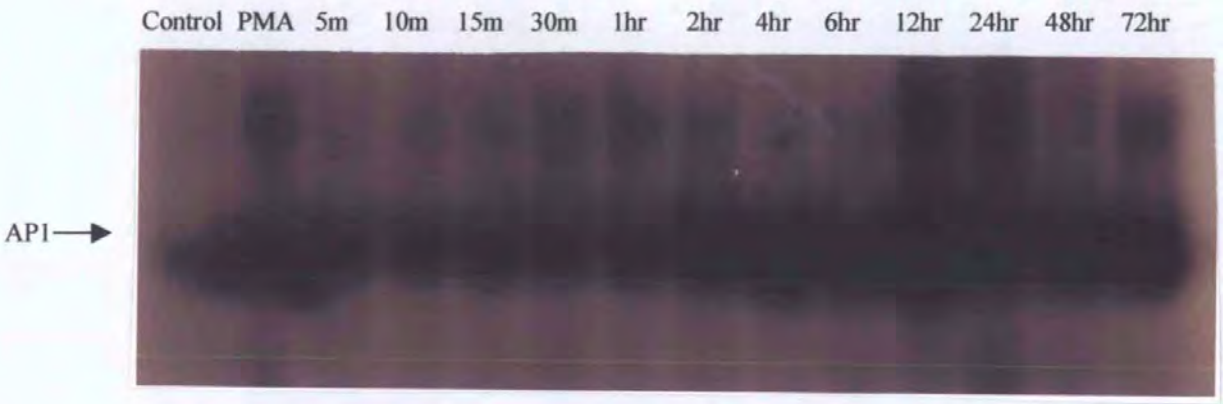


Figure 4.1.5. NFκB during reperfusion in endothelial cells. A typical EMSA is shown in A. Optical density readings are shown in B. Cells were returned to 37°C after 30 minutes or 12 hours of hypothermia. There was no significant up-regulation of NFκB during reperfusion.

A.



B.

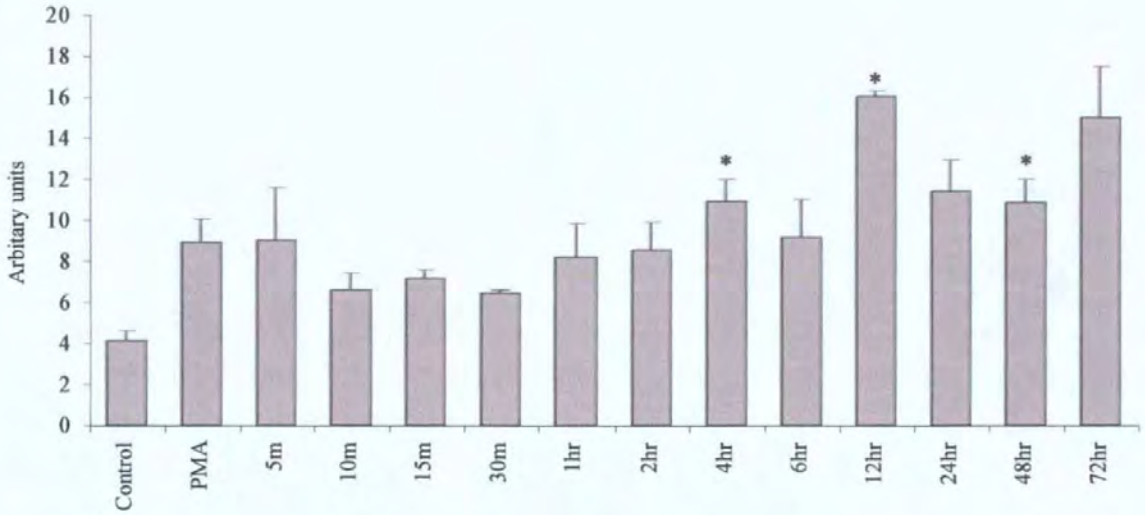
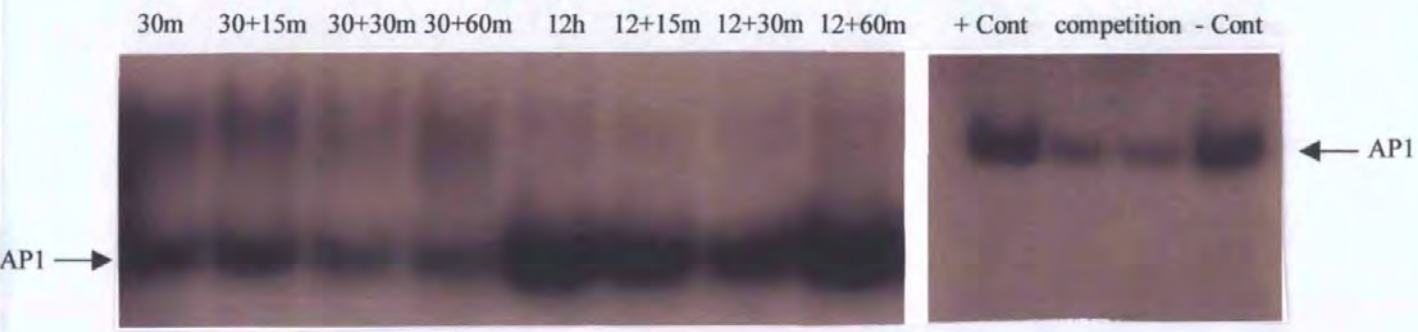
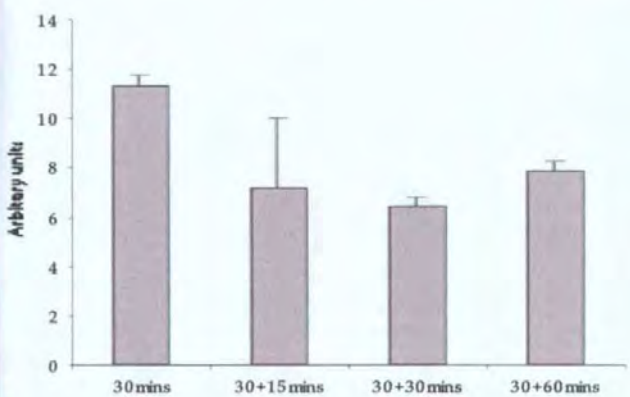


Figure 4.2.1. AP-1 activation during hypothermia in endothelial cells. A typical EMSA is shown in A. Optical density readings are shown in B. The results are the average of duplicate experiments. Asterisks indicate a significant increase from control cells.

A.



B.



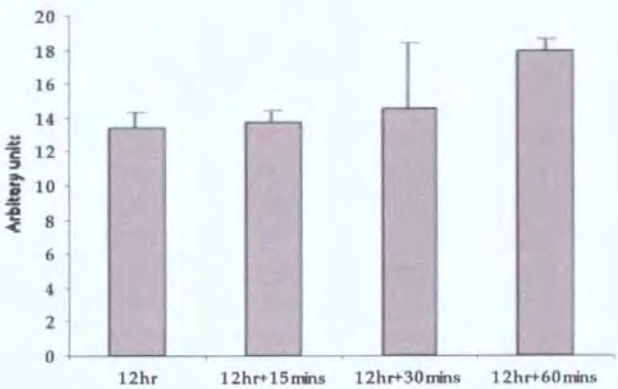
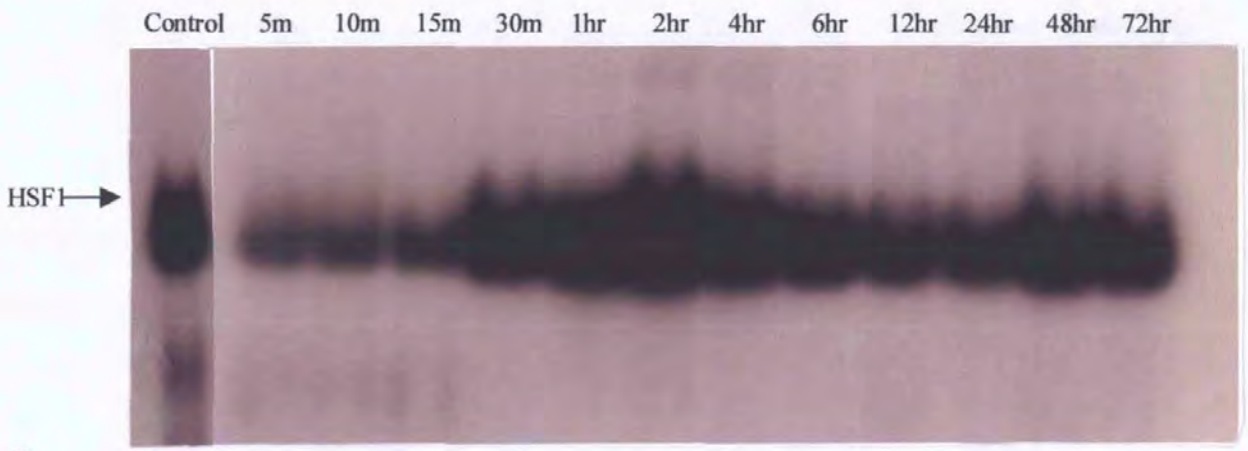


Figure 4.2.2. AP1 during rewarming in endothelial cells, and competition experiment to demonstrate the specificity of the AP1 band. A typical EMSA is shown in A, along with a competition experiment using an excess of unlabelled AP1 oligo to demonstrate the specificity of the band seen. Optical density readings of duplicate experiments are shown in B. There was no significant increase in AP1 activity after reperfusion.

A.



B.

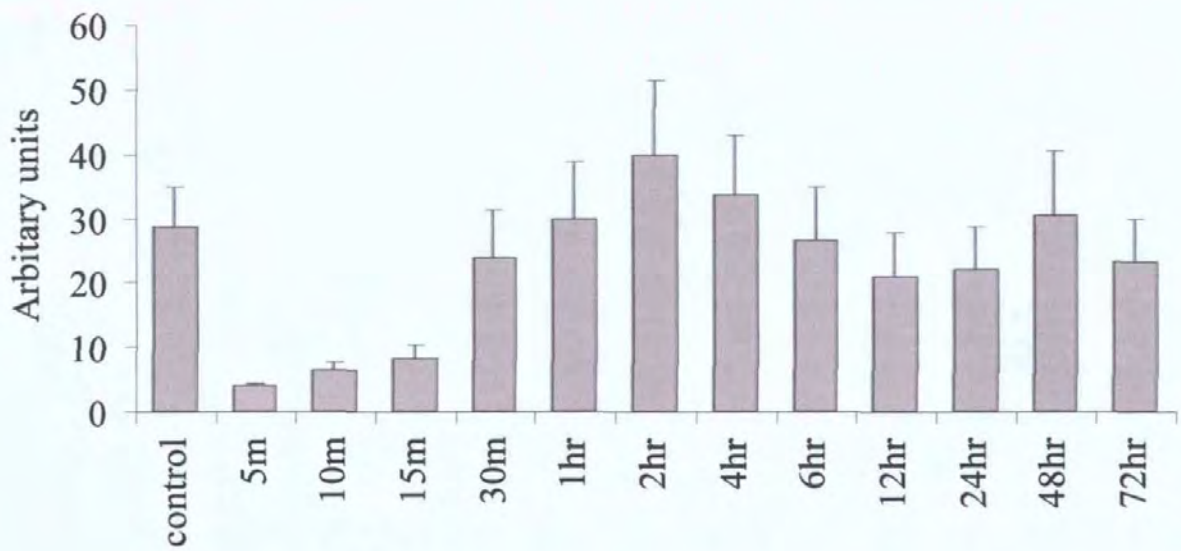
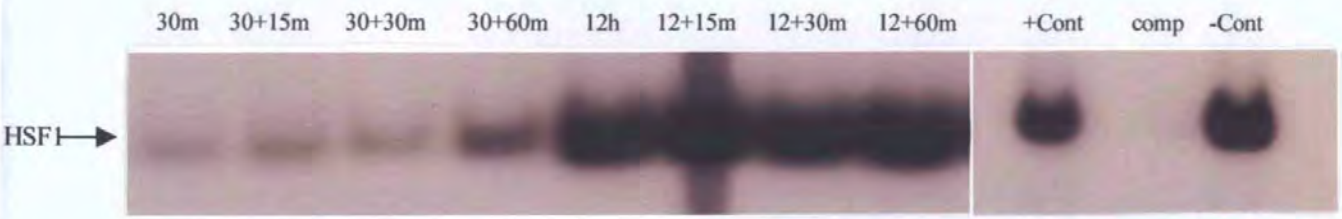
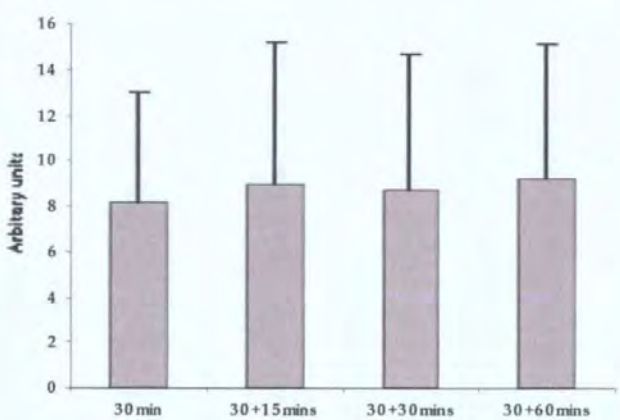


Figure 4.3.1. HSF1 activation during hypothermic stress in endothelial cells. A typical EMSA is shown in A. HSF1 activity was highest in control cells in a similar fashion to Hsp70 protein. Optical density readings are shown in B. Results are the average of three experiments. There were no significant differences in the expression of HSF1.

A.



B.



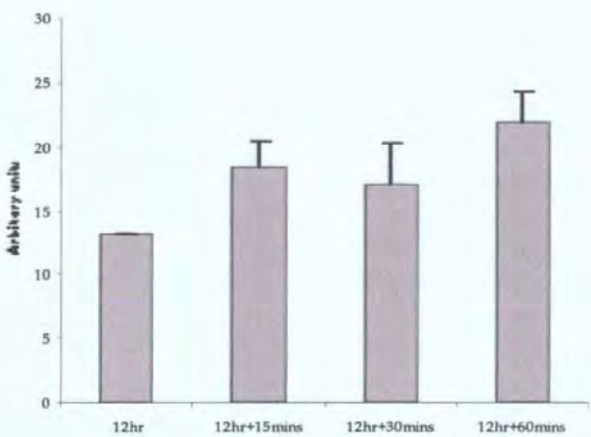
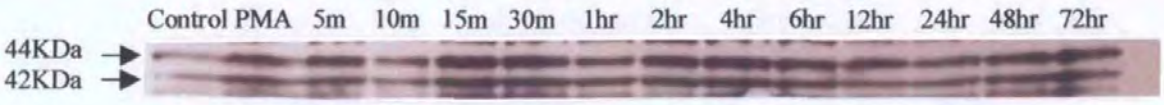


Figure 4.3.2. HSF1 during reperfusion in endothelial cells and competition experiments to demonstrate the specificity of the HSF1 probe. A typical EMSA is shown in A, also a competition experiment with an excess of unlabelled probe to demonstrate the specificity of the probe. Optical density readings of duplicate experiments are shown in B. There was no significant up-regulation of HSF1 by reperfusion.

A.



B.

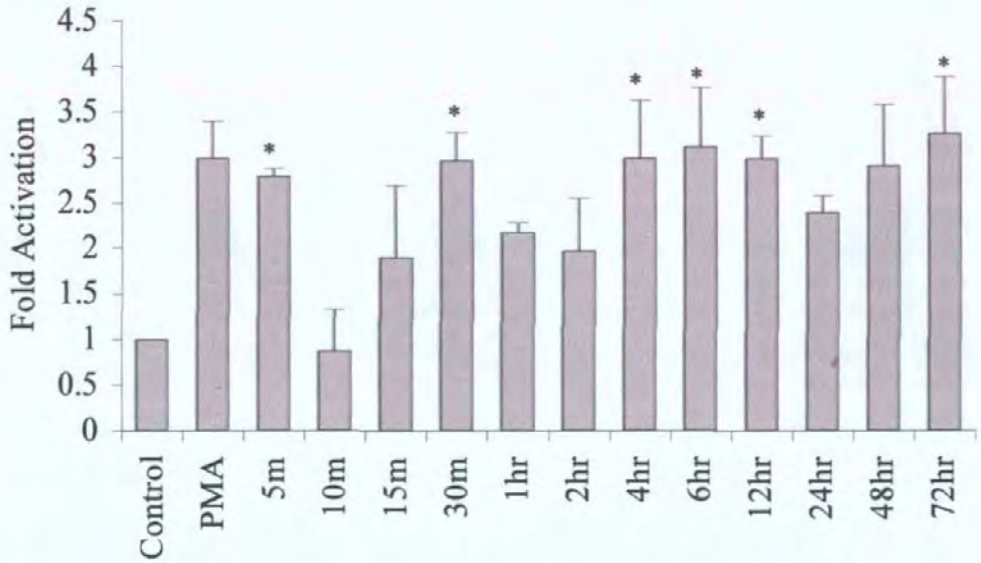
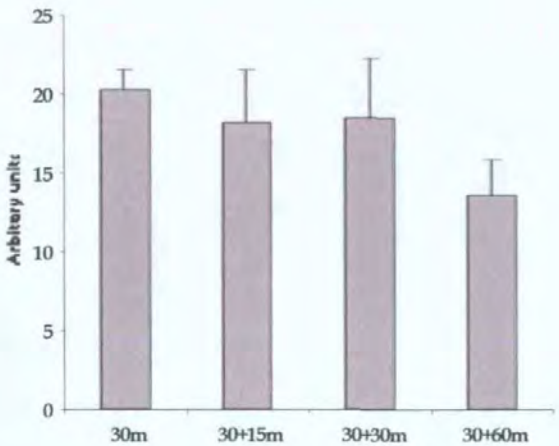


Figure 4.4.1. ERK 1 / 2 activation during hypothermic stress in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were repeated three times. Asterisks indicate a significant difference from control cells ($p<0.05$).

A.

B.



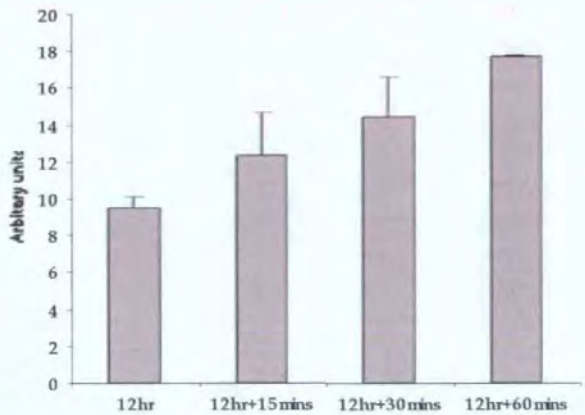
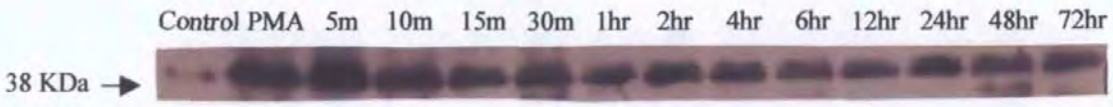


Figure 4.4.2. ERK 1 / 2 during reperfusion in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were duplicated. There was no significant up-regulation of ERK 1 / 2 by reperfusion.

A.



B.

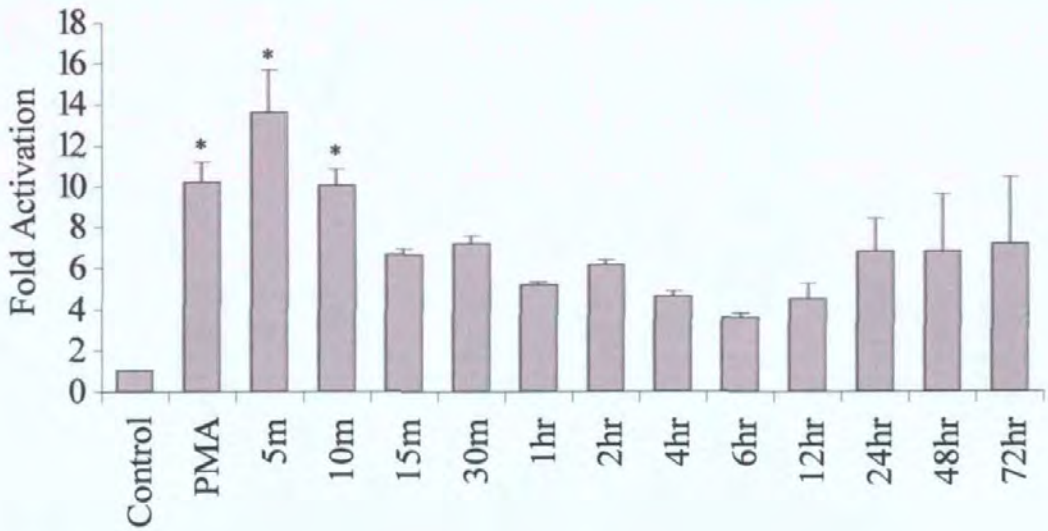
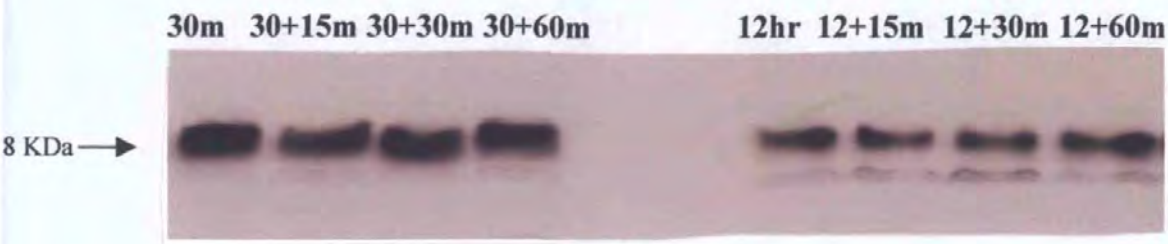


Figure 4.5.1. P38 activation during hypothermia in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were repeated three times. Asterisks indicate a significant difference from control cells ($p<0.05$).

A.



B.

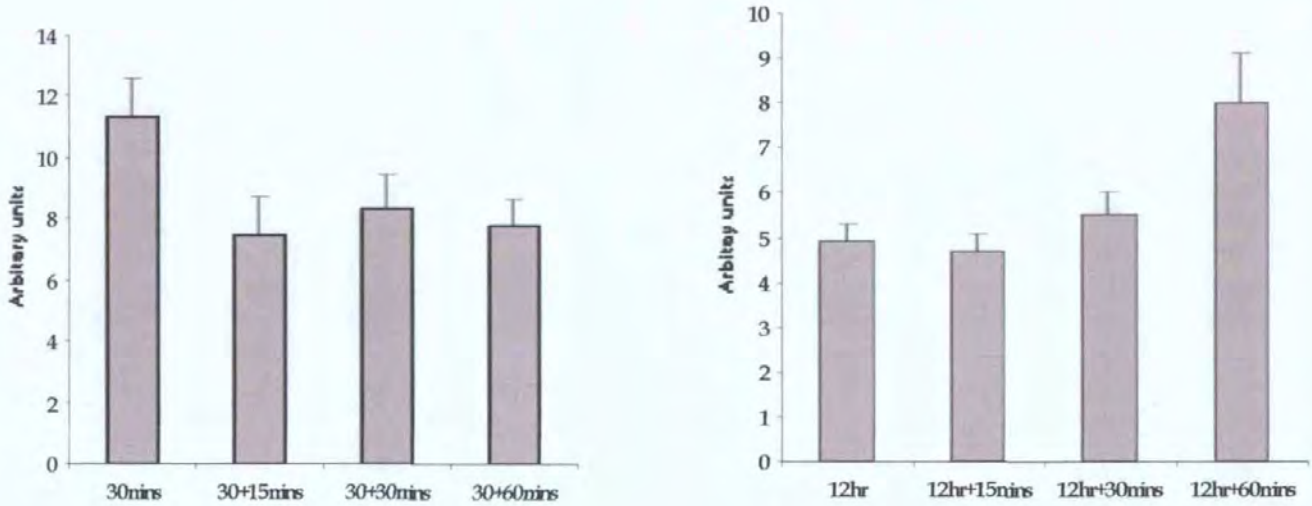
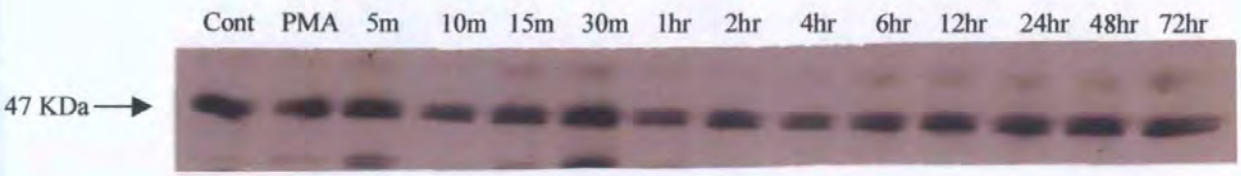


Figure 4.5.2. P38 during reperfusion in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were duplicated. There was no significant up-regulation of p38 by reperfusion.

A.



B.

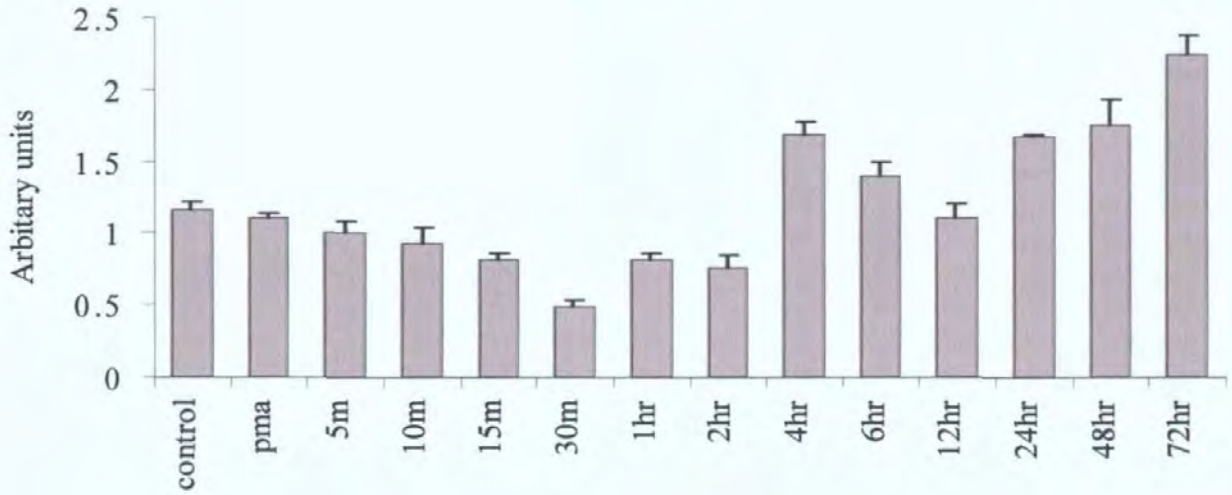
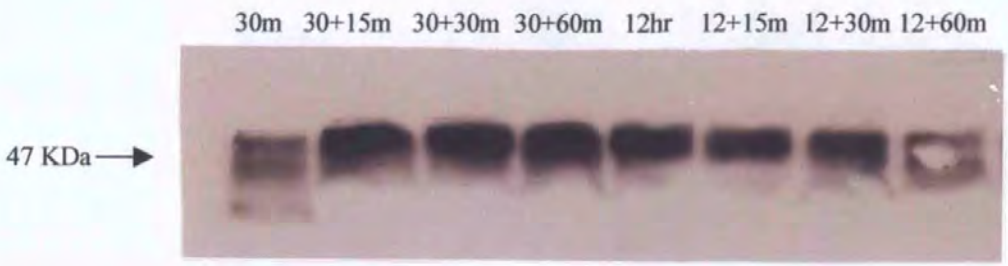


Figure 4.6.1. Lack of up-regulation of JNK during hypothermic stress in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were duplicated. Although there appeared to be an increase in JNK activity after 4 hours, there were no significant increases in JNK activity from control cells.

A.



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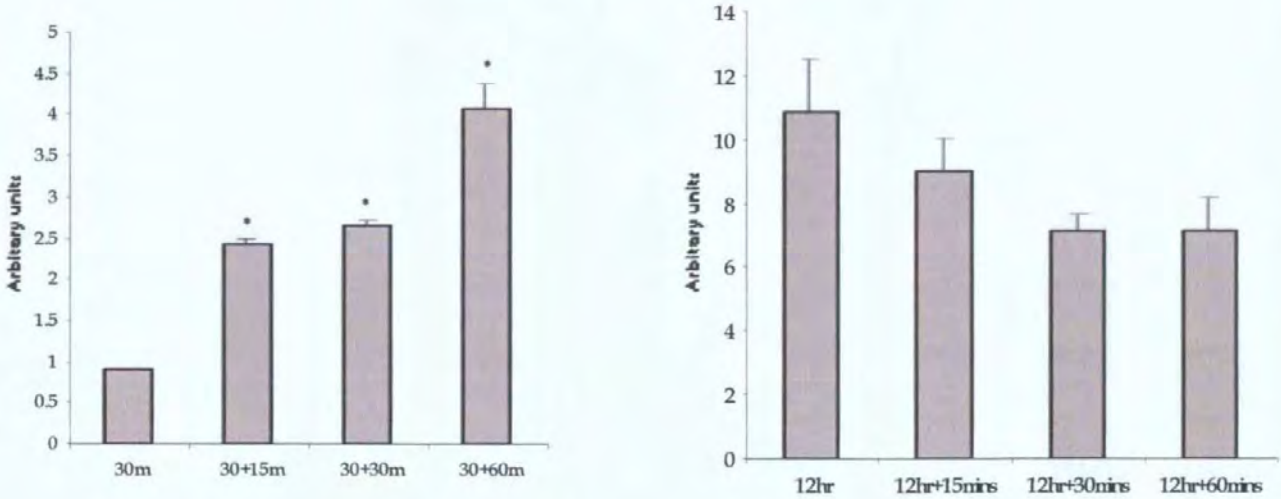
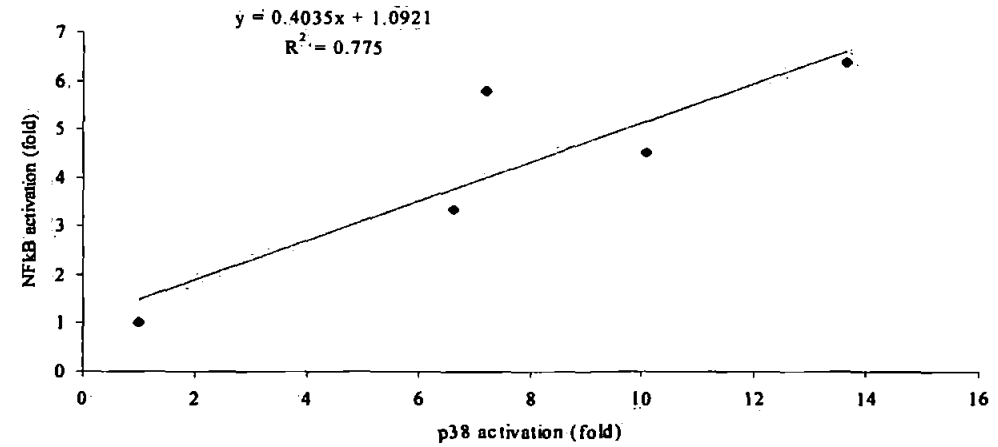


Figure 4.6.2. JNK during rewarming in endothelial cells. A typical western blot is shown in A. Antibodies only bind to the phosphorylated form of the protein. Optical density readings are shown in B. Experiments were duplicated. Asterisks indicate a significant increase ($p<0.05$).

A.



B.

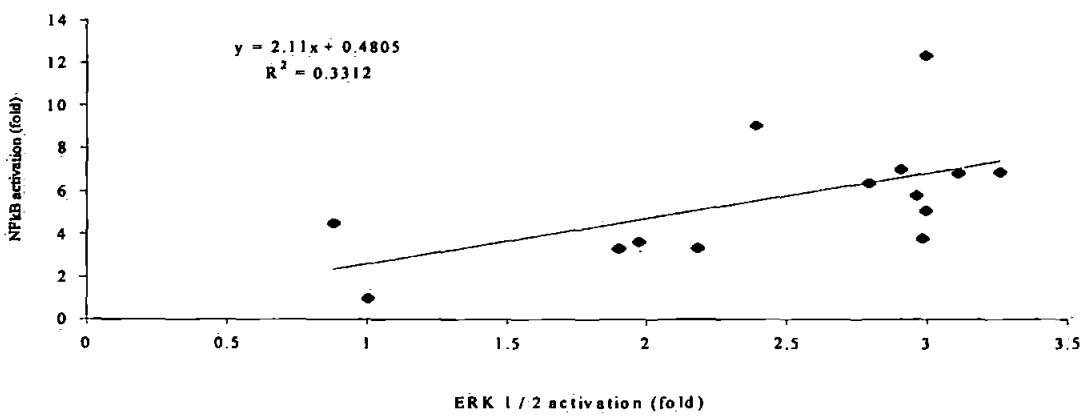
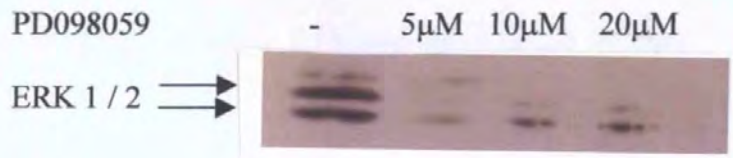


Figure 4.7.1. The relationship between NFκB and the MAPK's p38 and ERK 1 /2 during hypothermia. Regression analysis demonstrates the relationship between NFκB activation and MAPK activation during hypothermic stress. In the first 30 minutes there was relationship between NFκB and p38 ($p<0.05$) shown in A. Throughout the 72 hour time period studied there was a significant relationship between NFκB and ERK 1 / 2 ($p<0.05$) shown in B.

A.



B.

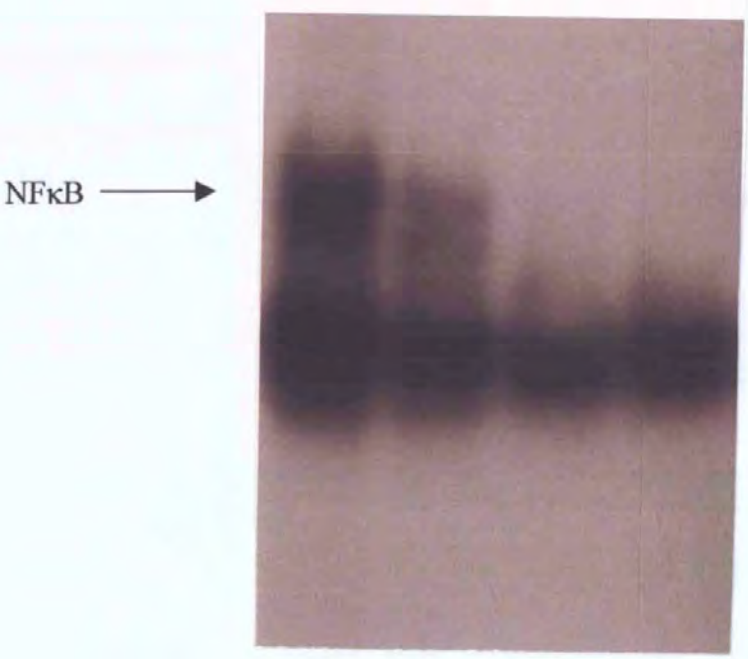


Figure 4.7.2. The effect of ERK 1 / 2 inhibition on NFκB activation in endothelial cells. ERK 1 /2 phosphorylation was blocked using the inhibitor PD98059 as shown in the western blot for ERK 1 / 2 in A. The effect of ERK 1 / 2 inhibition on NFκB can be seen by the EMSA shown in B. Inhibition of ERK 1 / 2 was associated with a significant reduction in NFκB activity ($p<0.05$).

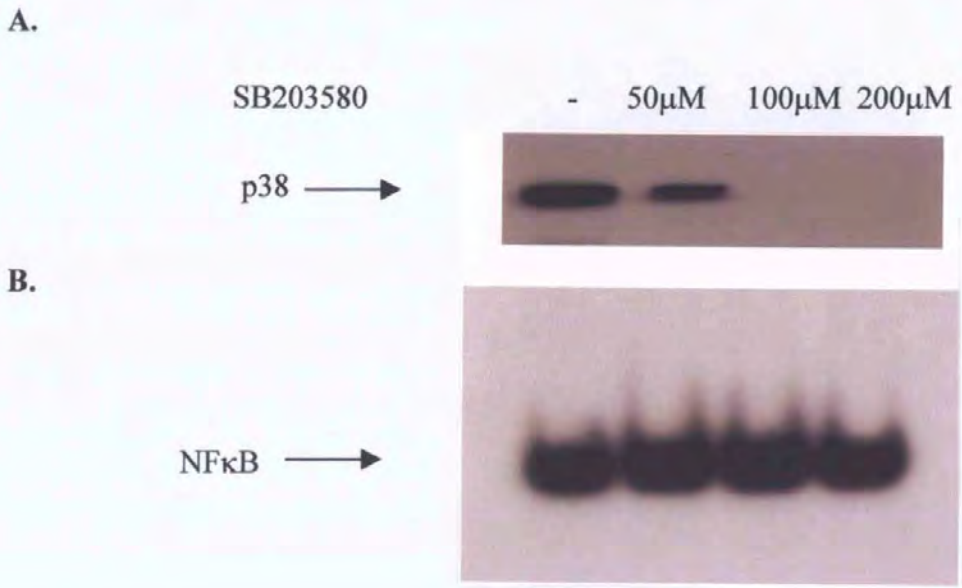


Figure 4.7.3. Inhibition of p38 selectively did not effect the mobility shift of NFκB. P38 phosphorylation was blocked using the inhibitor SB203589 as shown in the western blot for p38 shown in A. The effect of p38 inhibition on NFκB can be seen by the EMSA shown in B. Inhibition of p38 had no effect on the mobility shift for NFκB.

A.

B.

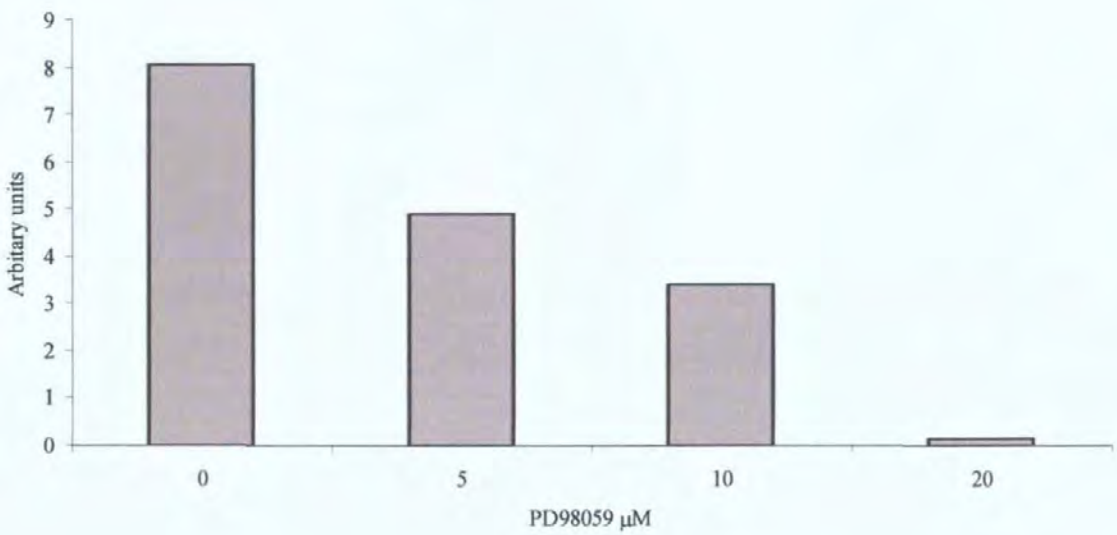


Figure 4.7.4. The inhibition of NFκB by ERK 1 /2 may be in part due the inhibition of IκBα. The inhibition of ERK 1/ 2 which was associated with the reduction in NFκB activity was found in this experiment to be associated with a reduction in phosphorylation IκBα. The western Blot for IκBα is shown in A, with the optical density shown in B. However, this experiment could not be repeated.

4.8 Discussion

The NFκB transcription factor was activated within minutes of hypothermia correlating with p38 and ERK 1 / 2 phosphorylation. A p38 inhibitor had no effect on NFκB activation, but a general MAPK inhibitor blocked NFκB translocation to the nucleus. This was associated with reduced IκBα phosphorylation. The JNK MAPK was not activated by hypothermia, but was strongly up regulated by reperfusion. The AP1 transcription factor was up regulated after 12 hours of hypothermia. Reperfusion had little effect on NFκB, AP1 and HSF1 transcription factors or the p38 and ERK 1 / 2 MAPKs. The HSF1 transcription factor was strongly expressed in control cells in a similar fashion to the Hsp70 protein.

CHAPTER FIVE: THE EFFECT OF HYPOTHERMIA AND REPERFUSION ON CYTOKINE PRODUCTION IN HUVECS

5.1 Background

The production of inflammatory cytokines IL-6, IL-8 and TNFα was investigated during hypothermia by ELISA. For comparison, cells were exposed to hypoxia for the same time points. To determine the role of NFκB in cytokine production the decoy oligonucleotide SN50 was used.

5.2 IL-6 production

At all hypothermic time points there was a reduction in IL-6 compared to control cells (Fig 5.2.1). However, at 12 hours there was a 15.6 $\pm$ 1.7 fold increase in IL-6 ($p=0.0004$). Hypoxia caused a large increase in IL-6 compared to control cells ($p<0.01$) (Fig 5.2.2). Reperfusion after 30 minutes hypothermia caused a large upregulation of IL-6 (Fig 5.2.2). 15 minutes of reperfusion caused an 18.1 $\pm$ 1.7 fold increase from 30 minutes of hypothermia alone ($p<0.01$), 30 minutes of reperfusion caused an 11.6 $\pm$ 0.5 fold increase ($p<0.05$) and 60 minutes of reperfusion caused a 13.0 +0.6 fold increase from 30 minutes of hypothermia alone ($p<0.01$). Reperfusion after 30 minutes hypothermia caused a higher amount of IL-6 than during hypoxic storage (299.28 $\pm$ 30.7 pg/ml and 266.5 $\pm$ 28.94 pg/ml respectively). The addition of SN50 caused a reduction in IL-6 at all time points compared with cells that experienced hypothermia alone (Fig 5.2.1). After 12 hours hypothermia this was associated with a significant reduction in IL-6 ($p<0.05$).

5.4 IL-8 production

Hypothermia caused the up-regulation of IL-8 at all time points (Fig 5.3.1), 6 hours (6.83 $\pm$ 4.8 Vs 190.3 $\pm$ 36.9 pg/ml p=<0.05), 12 hours (14.45 $\pm$ 7.0 Vs 343.2 $\pm$ 47.4 pg/ml p<0.05), 24 hours (75.1 $\pm$ 7.35 Vs 182.9 $\pm$ 22.2 pg/ml p<0.05) 48 hours (110.9 $\pm$ 49.1 Vs 286.4 $\pm$ 6.8 p<0.05) and 72 hours (66.8 $\pm$ 18.4 Vs 519.1 $\pm$ 70.45 pg/ml p<0.05).

The administration of SN50 during hypothermia reduced IL-8 expression at 12 hours (343.2 $\pm$ 47.4 Vs 45.0 $\pm$ 0.7 pg/ml p=<0.05). After 12 hours levels of IL-8 rose to levels similar to cells without the inhibitor (Fig 5.3.1). Reperfusion did not increase IL-8 levels after 30 minutes or 12 hours hypothermia (Fig 5.3.2). Hypoxia was a strong inducer of IL-8 expression at all time points (p<0.01).

5.5 TNFα production

Hypothermia caused a significant increase in TNFα levels after 6 hours (7.59 $\pm$ 0.3 Vs 5.14 $\pm$ 0.6 pg/ml p<0.05), 12 hours (8.94 $\pm$ 0.8 Vs 5.46 $\pm$ 0.4 pg/ml p<0.05) and 72 hours (8.04 $\pm$ 0.3 Vs 4.11 $\pm$ 0.2 pg/ml p=<0.01) of hypothermia. This was significantly reduced at 72 hours by the addition of SN50 inhibitor (6.17 $\pm$ 0.06 Vs 8.04 $\pm$ 0.3 pg/ml p=<0.05). Hypoxia caused the up-regulation of TNFα at all time points with a significant increase compare to control cells (p<0.01). Reperfusion caused a slight increase in TNFα production after 30 minutes and 12 hours.

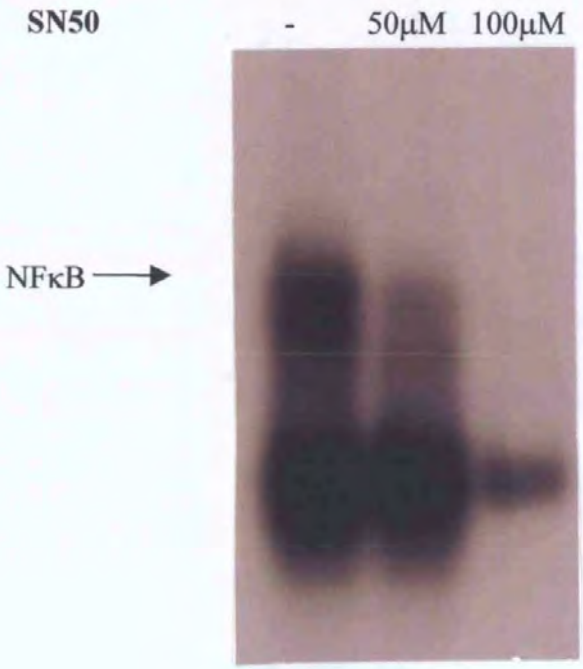


Figure 5.1. Inhibition of NFκB with the SN50 cell permeable inhibitor. Increasing concentrations of SN50 were found to block NFκB activation. An EMSA for NFκB is shown.

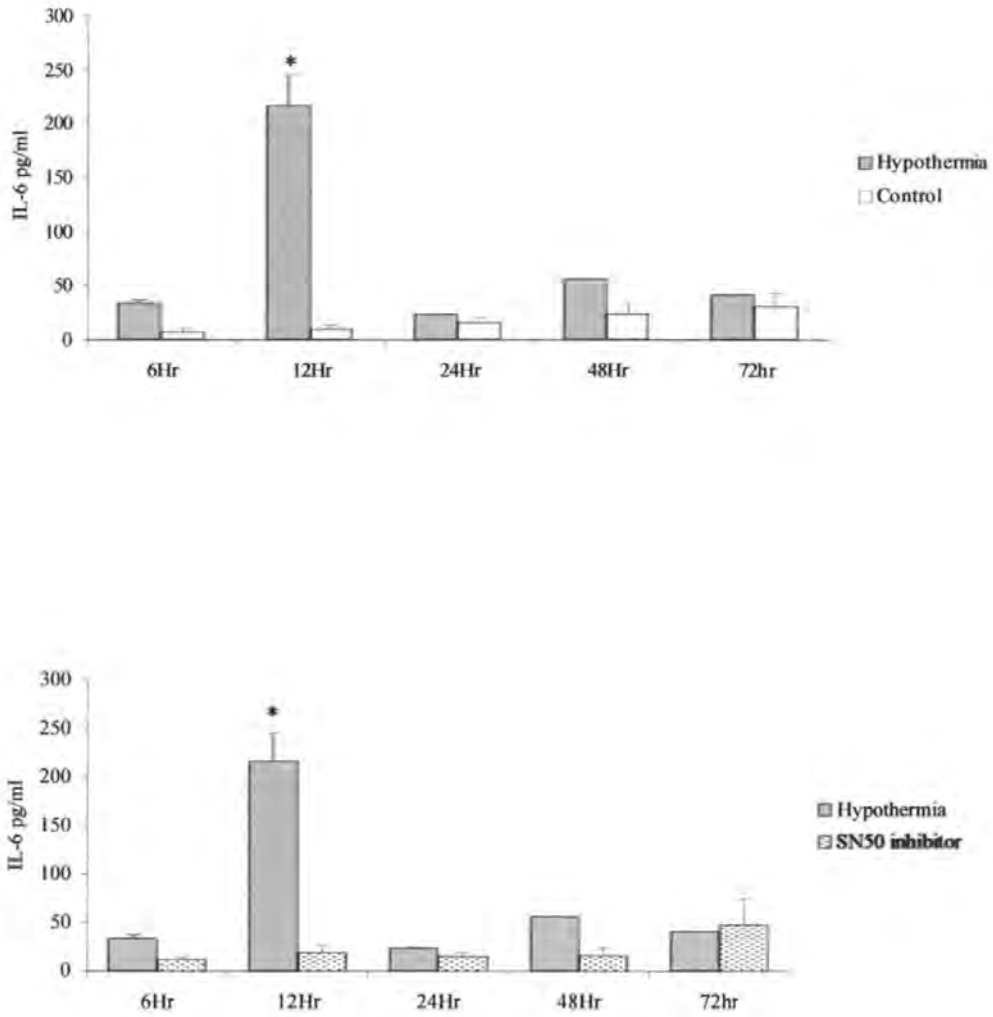
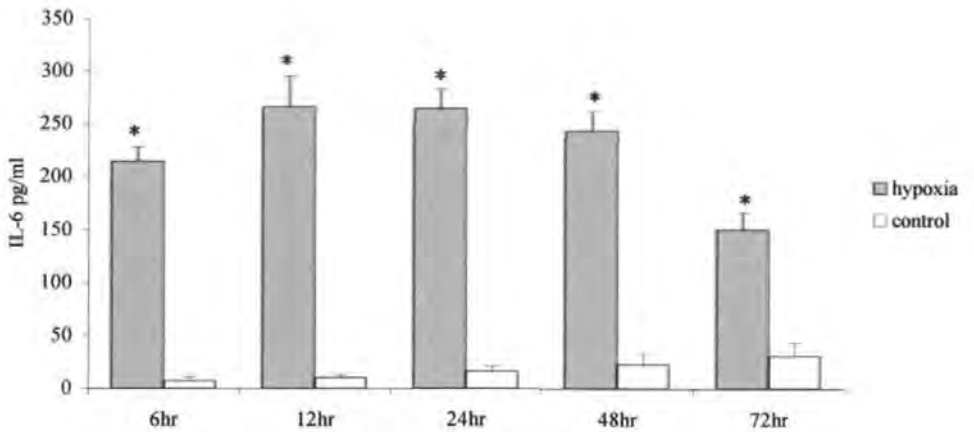
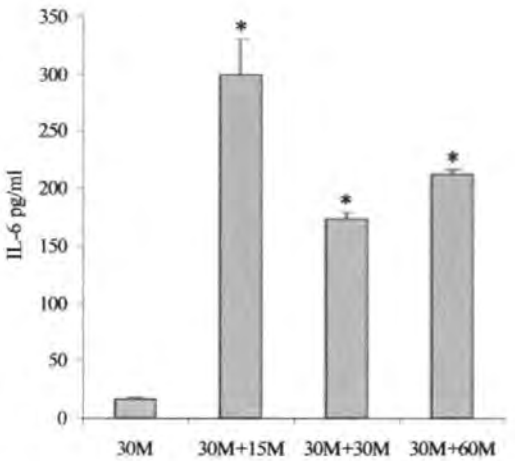


Figure 5.2.1. IL-6 production by HUVECS during hypothermia and reduction with SN50 inhibitor. IL-6 was measured by ELISA. Experiments were repeated three times. Asterisks indicate a significant difference between samples ($p<0.05$).





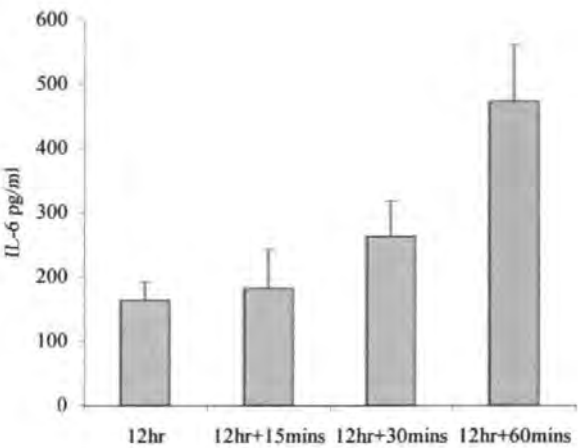


Figure 5.2.2. IL-6 production during hypoxic and reperfusion stress. IL-6 was measured by ELISA. Experiments were repeated at least twice. Asterisks indicate a significant difference between samples ($p<0.05$).

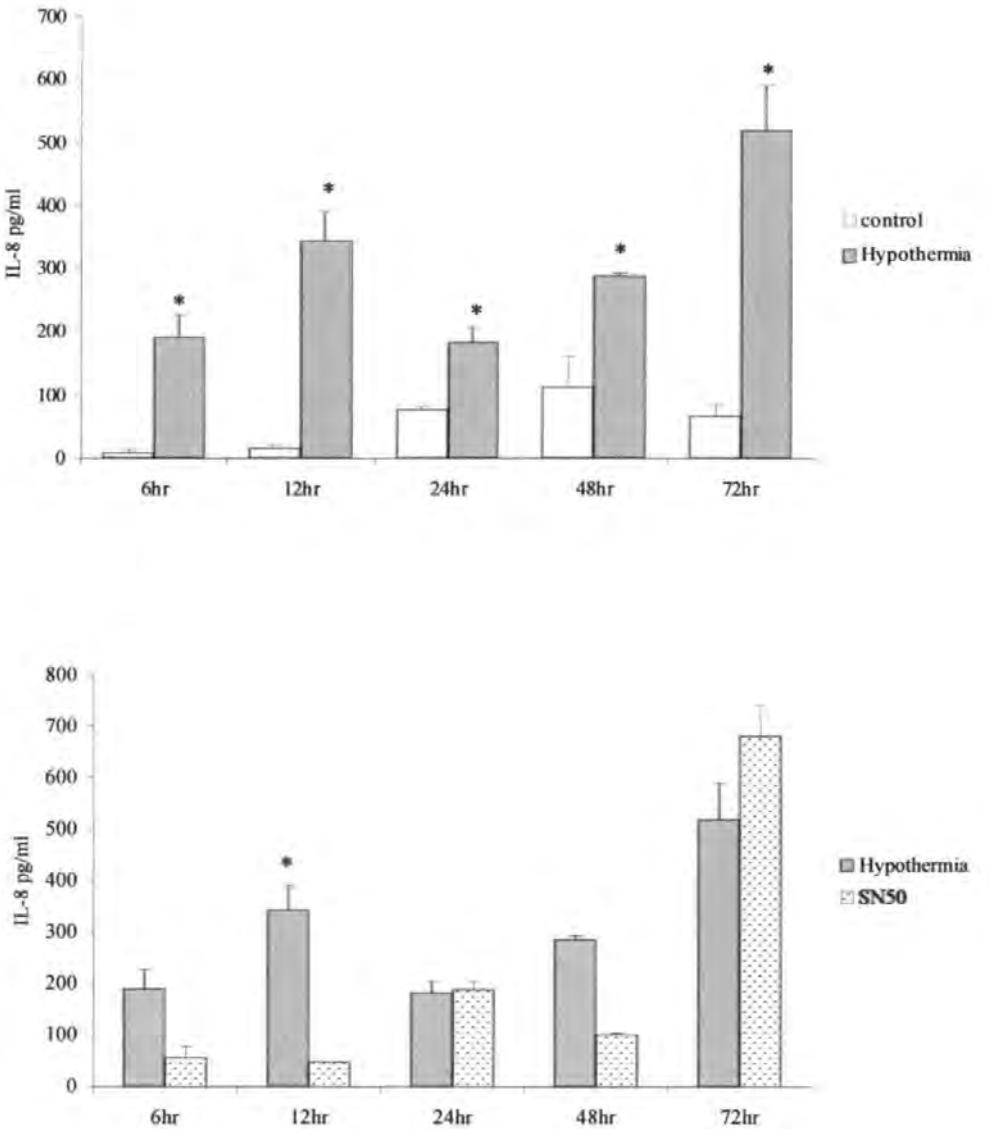
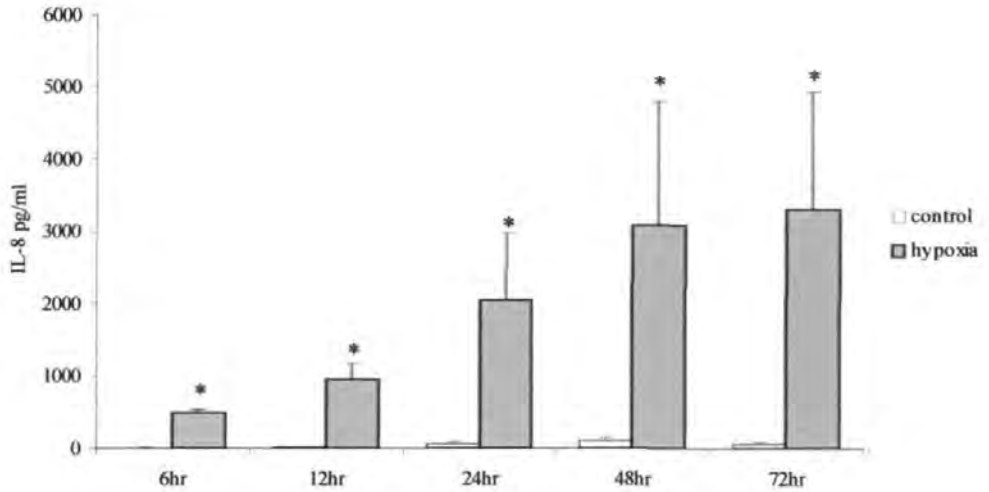
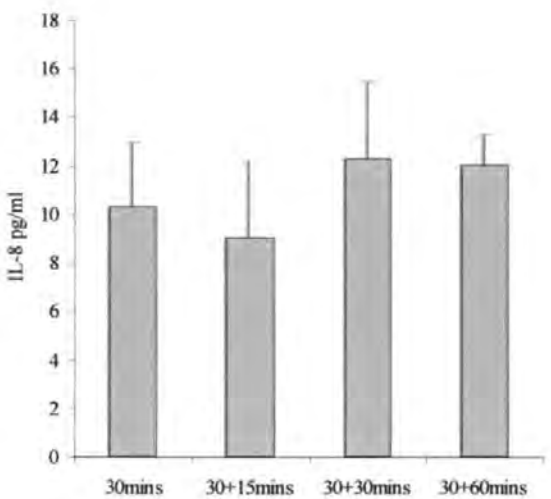


Figure 5.3.1 . IL-8 production during hypothermic stress and with the SN50 inhibitor. IL-8 was measured by ELISA. Experiments were duplicated. Asterisks indicate a significant difference between samples ($p<0.05$).





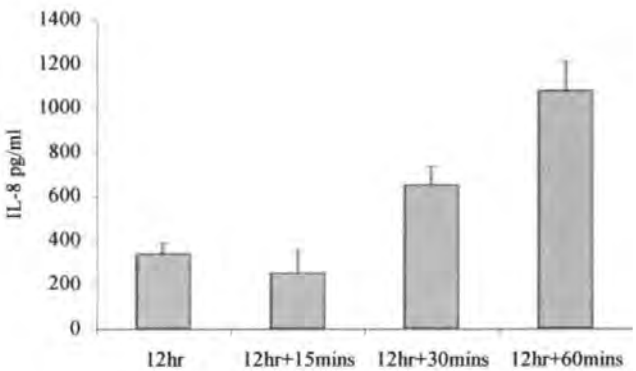


Figure 5.3.2 IL-8 production during hypoxic and reperfusion stress. IL-8 was measured by ELISA. Experiments were duplicated. Asterisks indicate a significant difference between samples ($p<0.05$).

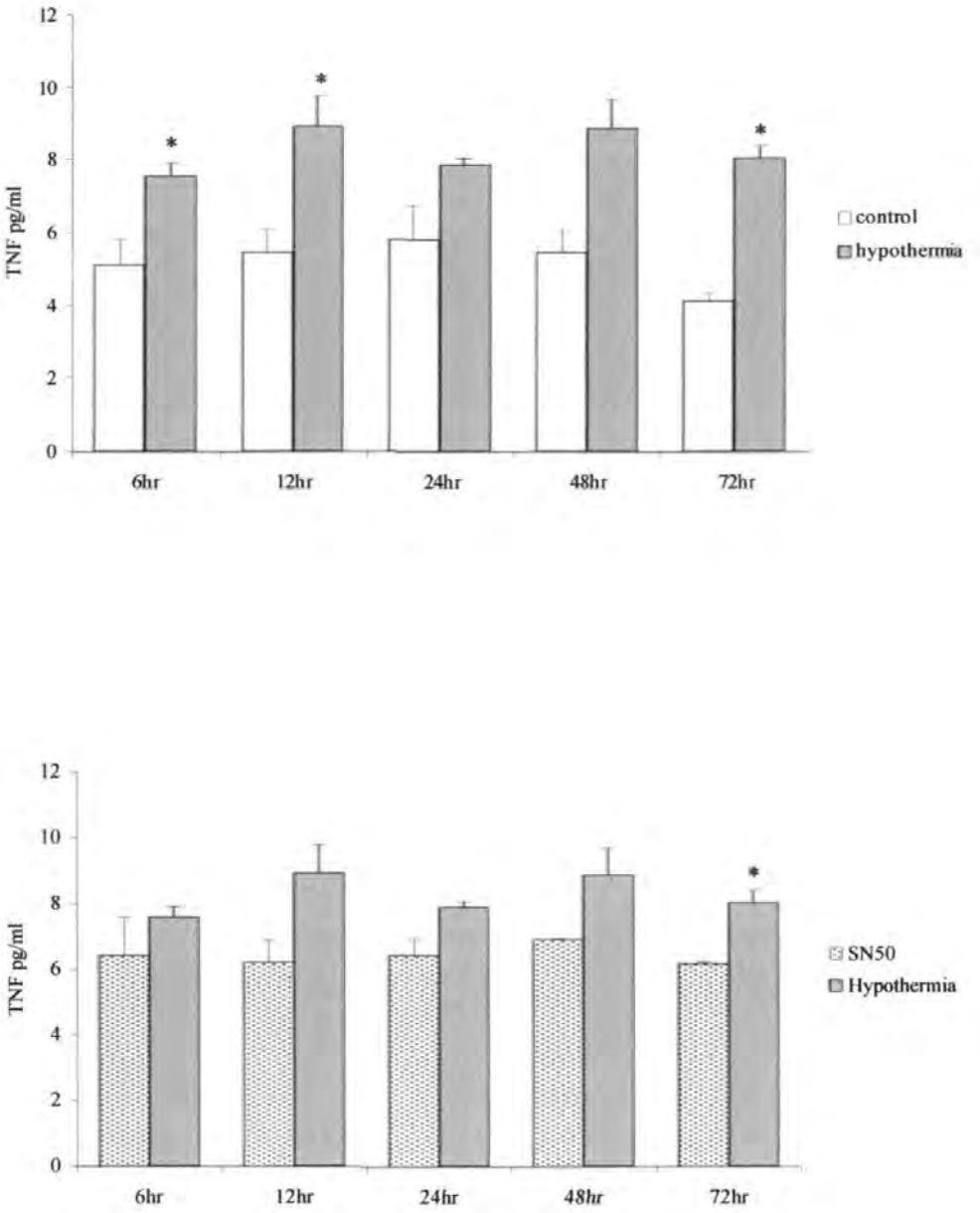


Figure 5.4.1. TNFα production during hypothermic stress and with the SN50 inhibitor. TNFα was measured by ELISA. Experiments were repeated three times. Asterisks indicate a significant difference between samples ($p<0.05$).

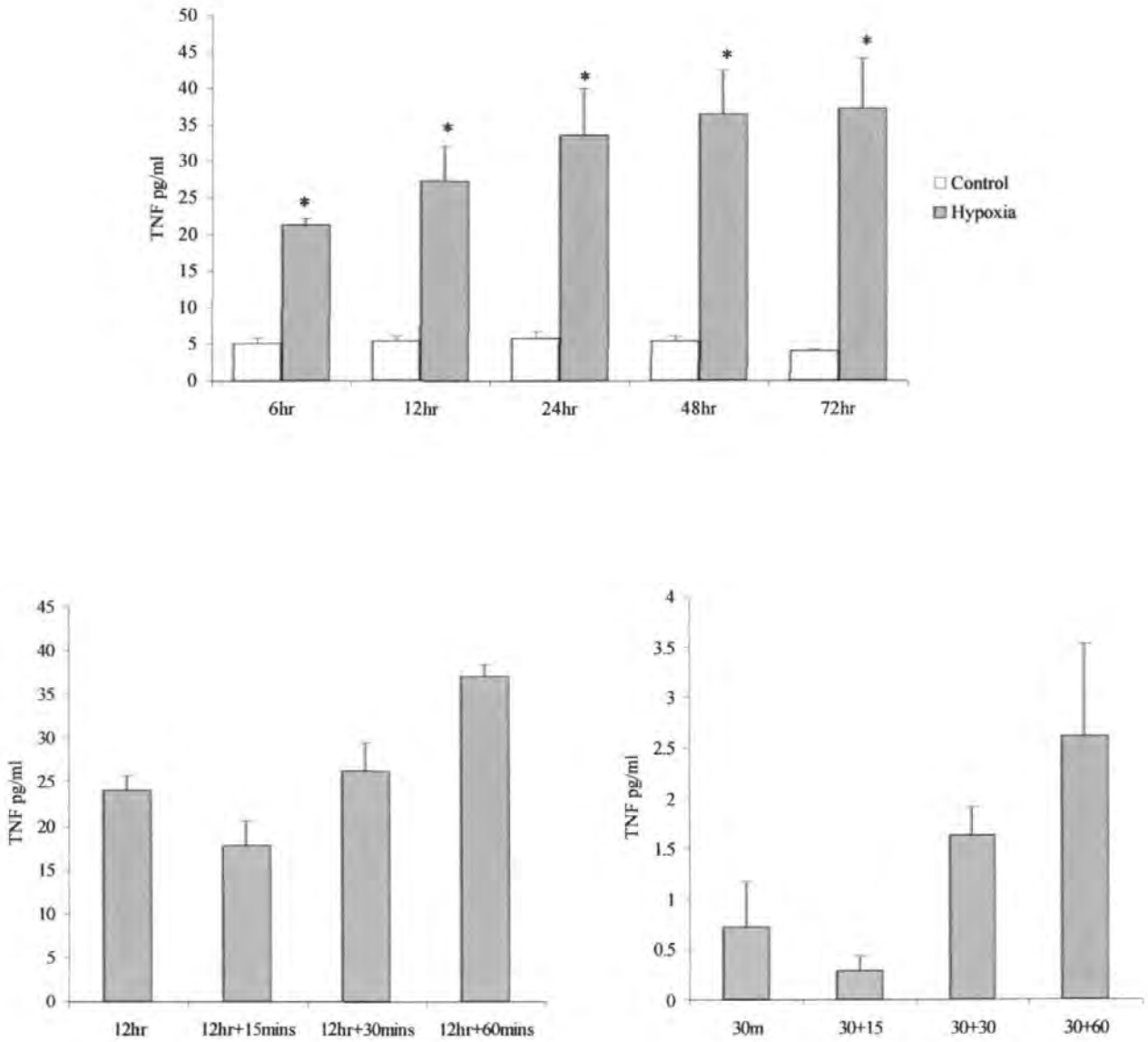


Figure 5.4.2 . TNFα production during hypoxic and reperfusion stress. TNFα was measured by ELISA. Experiments were duplicated. Asterisks indicate a significant difference between samples ($p<0.05$).

5.6 Discussion

Hypothermic stress caused a significant increase in IL-6, IL-8 and TNFα cytokine levels in endothelial cells. Each cytokine was significantly active after 12 hours of hypothermic storage. All three were largely up-regulated by hypoxia. Reperfusion after 30 minutes of hypothermia caused an increase in cytokine expression of IL-6, but not IL-8 or TNFα. Reperfusion after 12 hypothermia did not increase the production of any cytokine.

NFκB appears to play a role in the expression of all three cytokines, as blockade of NFκB with the SN50 oligonucleotide reduced the expression of all cytokines during hypothermia. However, Inhibition of NFκB was not associated with a reduced cytokine production at all time points. After 12 hours of cold stress inhibition of NFκB was associated with a reduction in both IL-6 and IL-8 levels in HUVECS.

CHAPTER SIX: ANALYSIS OF MARKERS OF GRAFT INJURY IN RENAL PRESERVATION SOLUTIONS

6.1 Background

Seventeen preservation solutions were collected from allografts received for transplantation at Plymouth. The inflammatory cytokines IL-6, IL-8 and TNFα along with the anti-inflammatory IL-4 were measured. Lactate was measured as a marker of hypoxia and VWF for endothelial dysfunction. Total protein was measured in the solutions and used to correct for differences in the volume collected.

The clinical characteristics of the donors are in the appendix. A summary is shown in table 6.1. There was a range in CI times from 13 hours 40 minutes to 37 hours 27 minutes (average 21 hours). WI times ranged from 20 minutes to 65 minutes (Average 34 minutes). Twelve samples were free from rejection and 5 experienced at least one rejection episode. Seven of the kidneys had delayed function and 10 had primary function.

Donor age (± SD)	45 ± 14.5
Male:Female Ratio	10:7
CI time (hours) ± SD	21 ± 7.0
WI time (minutes) ± SD	34 ± 15.7
Delayed function	7 of 17
Rejection episodes (0:1:2)	12:2:3

Table 6.1. Clinical characteristics of the donor kidneys from which solutions were collected (n=17). Full details are in the appendix.

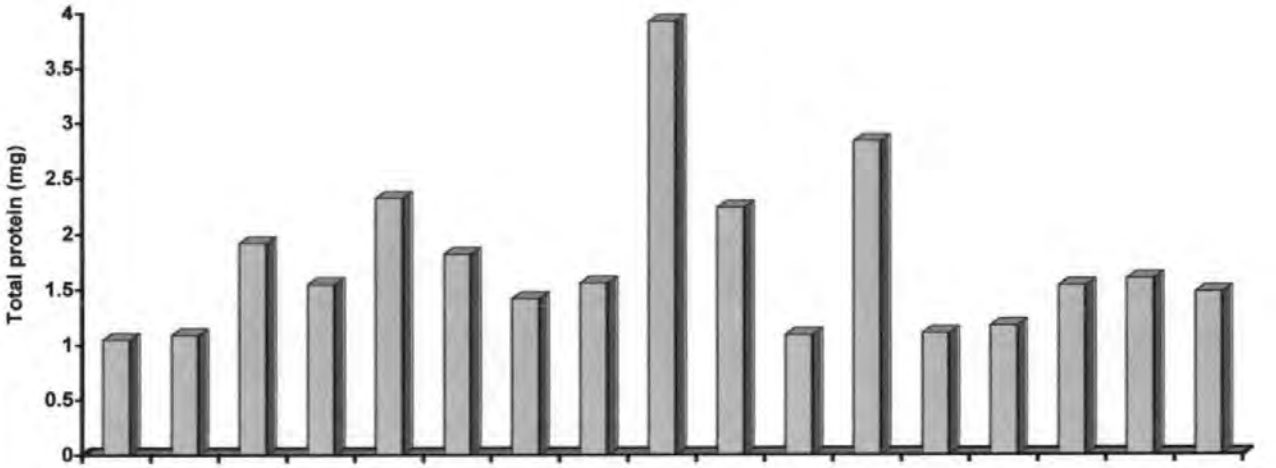


Figure 6.1. Total protein concentrations found in preservation solutions. Levels were measured by the Bradford assays to correct for differences in the volumes of samples collected.

6.2 IL-6

IL-6 levels were found to range from 54.73 pg/ml to 435.69 pg/ml (7.9 fold difference) in the 17 donors (Fig 6.1.1). There was no correlation between increased CI or WI times and IL-6 levels (Fig 6.1.2). In samples with < 24 hours CI time, the average level was 151.3 $\pm$ 31.9 pg/ml compared with 197.7 $\pm$ 22.6 pg/ml in kidneys with > 24 hours CI time (p=ns). There was a significant difference between IL-6 levels in kidneys that has experienced < 30 minutes WI time compared with > 30 minutes WI time, 108.22 $\pm$ 18.8 pg/ml Vs 210.6 $\pm$ 38.5 pg/ml (p=0.03). There was no difference in IL-6 levels between kidneys with primary or delayed function (6.1.4). The donors were genotyped for the C(-174)G polymorphism, and levels compared with genotype. Nine donors (53%) were GG homozygotes, 6 (35%) were GC heterozygotes and 2 (11%) were CC homozygotes.

There were no significant differences between the amounts of IL-6 in donors with the GG genotype compared with the GC or CC genotype (Fig 6.1.5). However the average in the GG genotype was higher than the CC genotype (196.17 $\pm$ 40.79 Vs 166.12 $\pm$ 6.23pg/ml p= ns)

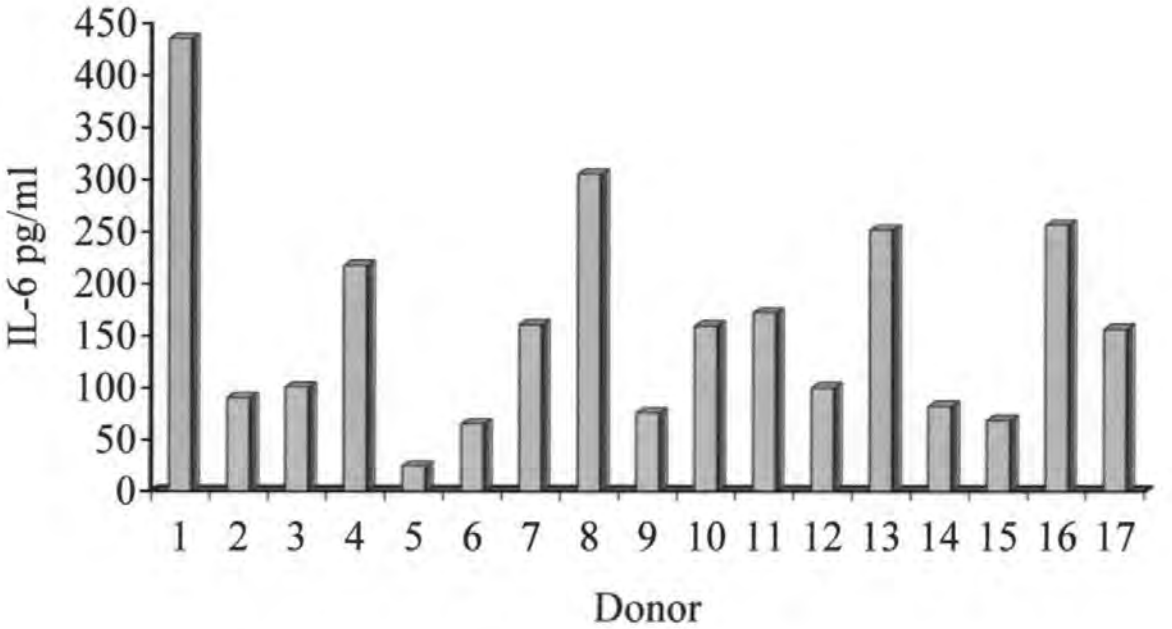
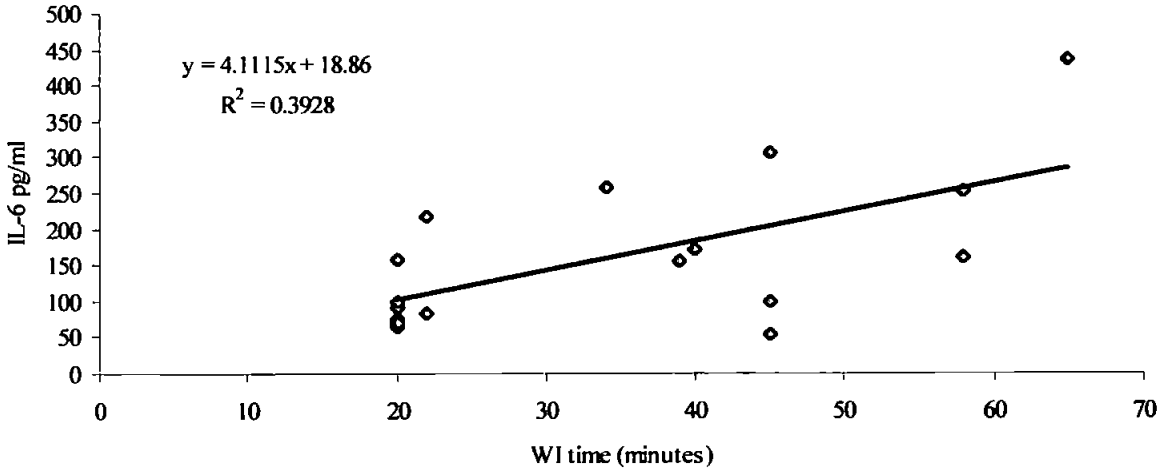


Figure 6.1.1. The Range in IL-6 levels found in donor preservation solutions. IL-6 was measured by ELISA and levels corrected for total protein. A range of 7.9 fold was found between samples.



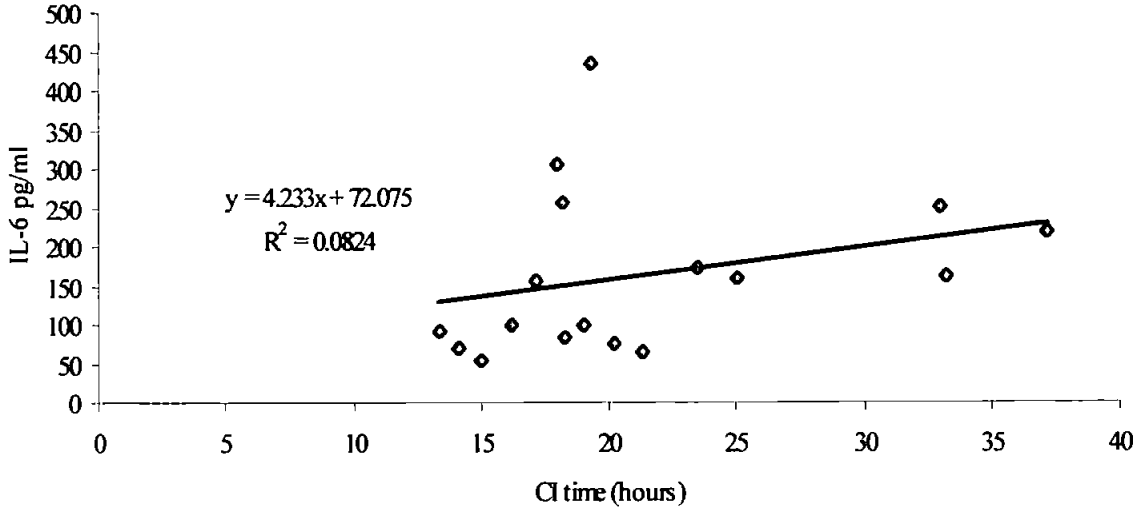
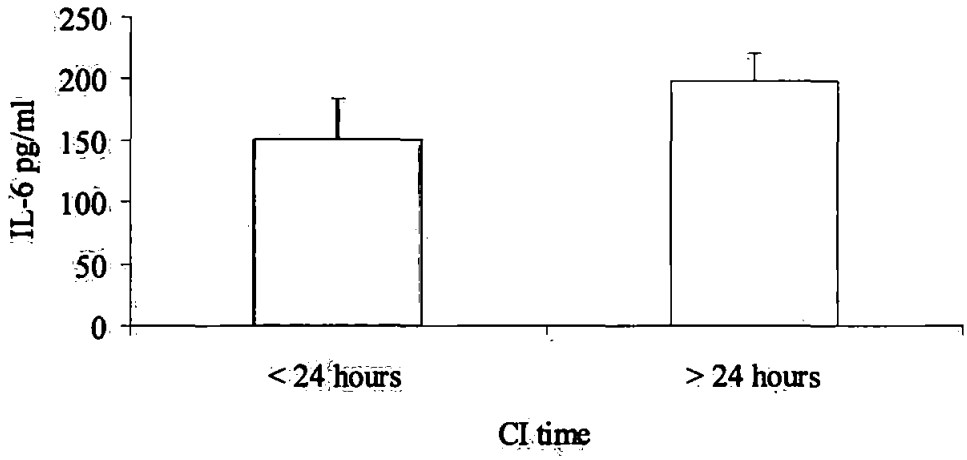


Figure 6.1.2. Increasing CI and WI times and IL-6 levels in 17 donor kidney preservation solutions. A positive correlation was found between increasing WI time and IL-6 levels.



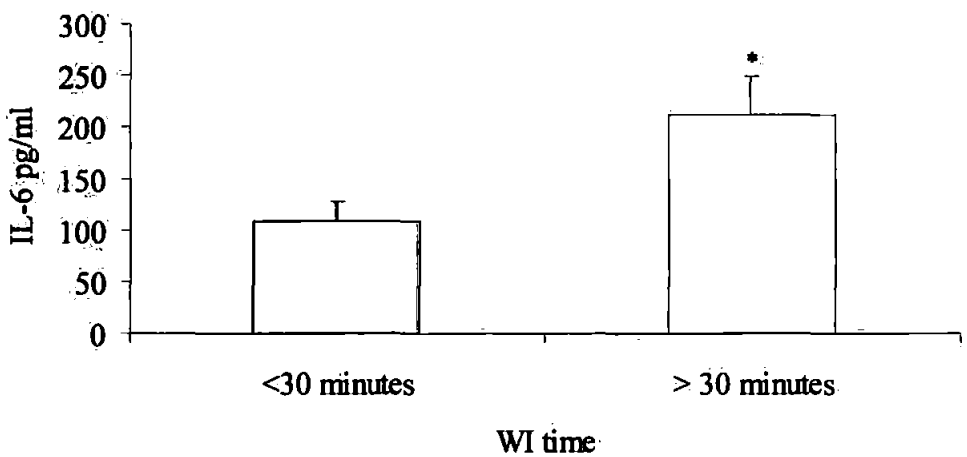
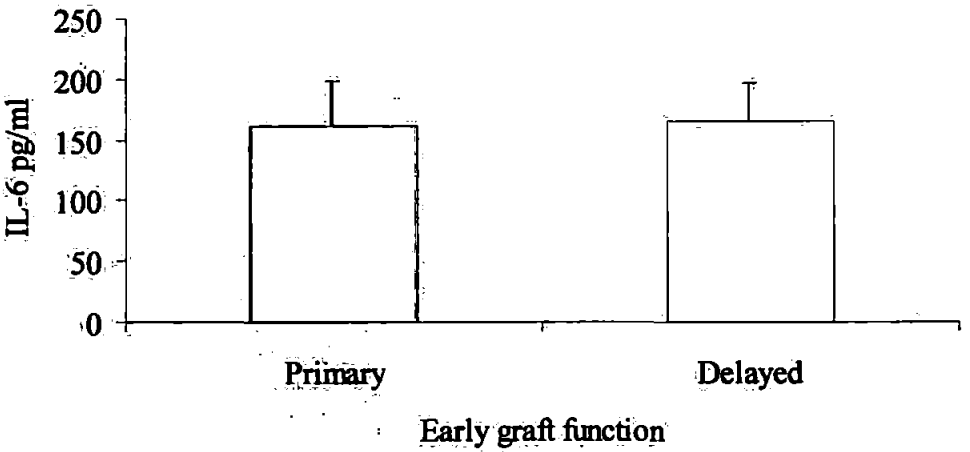


Figure 6.1.3 . IL-6 levels in preservation solutions from kidneys with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.



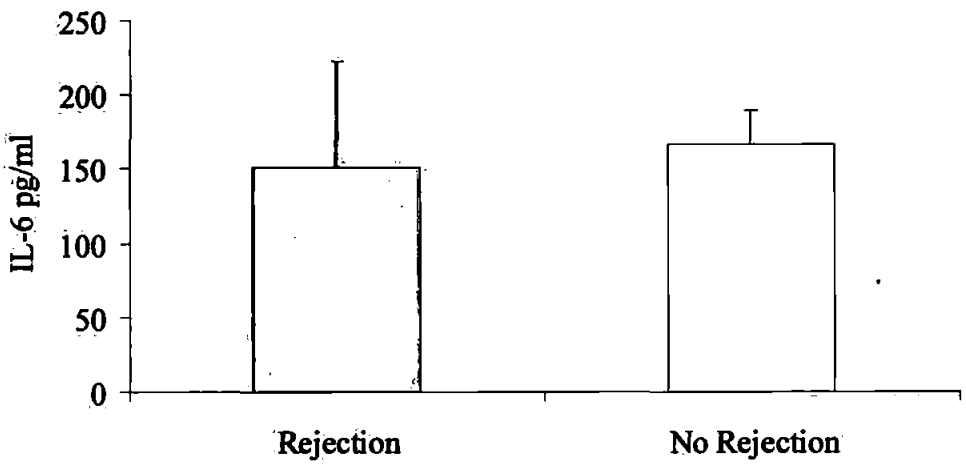


Figure 6.1.4 . IL-6 levels in relation to early graft function and rejection. Ten kidneys has primary function and 7 delayed. Five kidneys were rejected and 12 were rejection free.

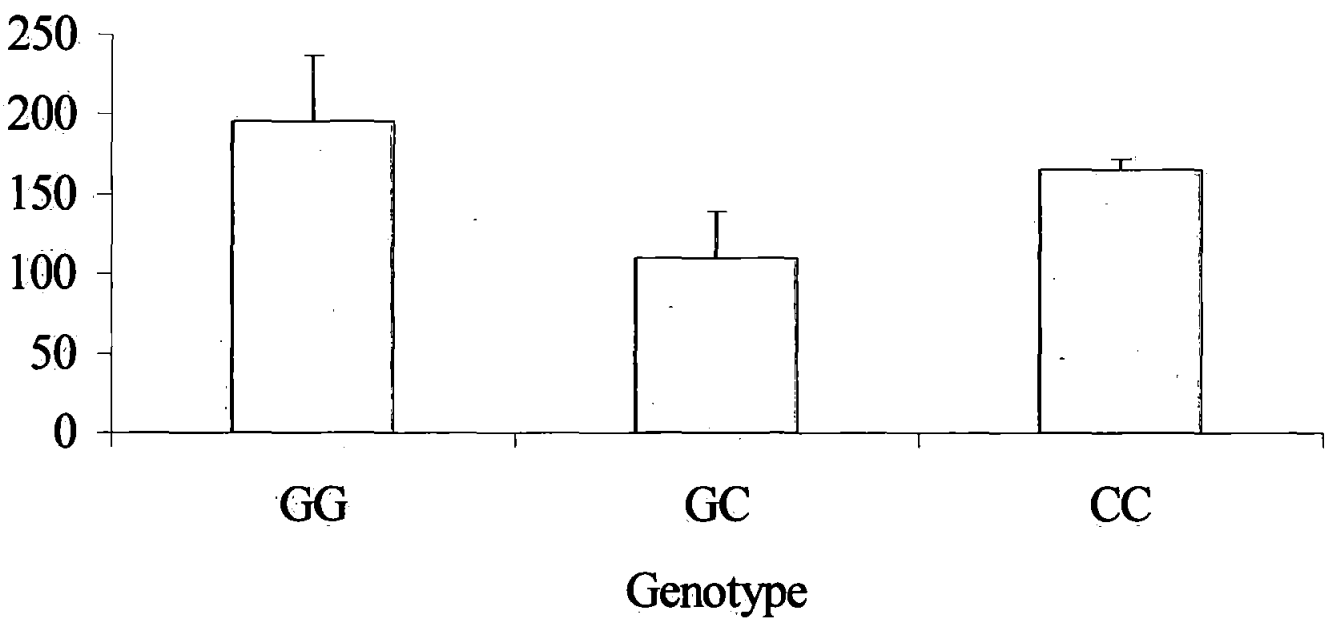


Figure 6.1.5. IL-6 levels in relation to genotype. Individuals with the G allele have higher IL-6 production than individuals with the C allele.

6.3 IL-8

Two donors had levels below the detection range of the ELISA. In the detectable levels, there was a range from 2.96 pg/ml to 119.3 pg/ml (40.3 fold difference). There was no association found between increased CI or WI times and graft function in relation to IL-8 levels (Fig 6.2.2). In kidneys with less than 24 hours CI time there was less IL-8 than kidneys with greater than 24 hours CI time (35.24 $\pm$ 11.4 pg/ml Vs 50.89 $\pm$ 14.5 pg/ml p=Ns). IL-8 was higher in kidneys with primary function (39.8 $\pm$14 Vs 37.5 $\pm$ 11.3 pg/ml), and in non-rejecting kidneys (45.7 $\pm$ 12.0 Vs 22.5 $\pm$ 11.0 pg/ml).

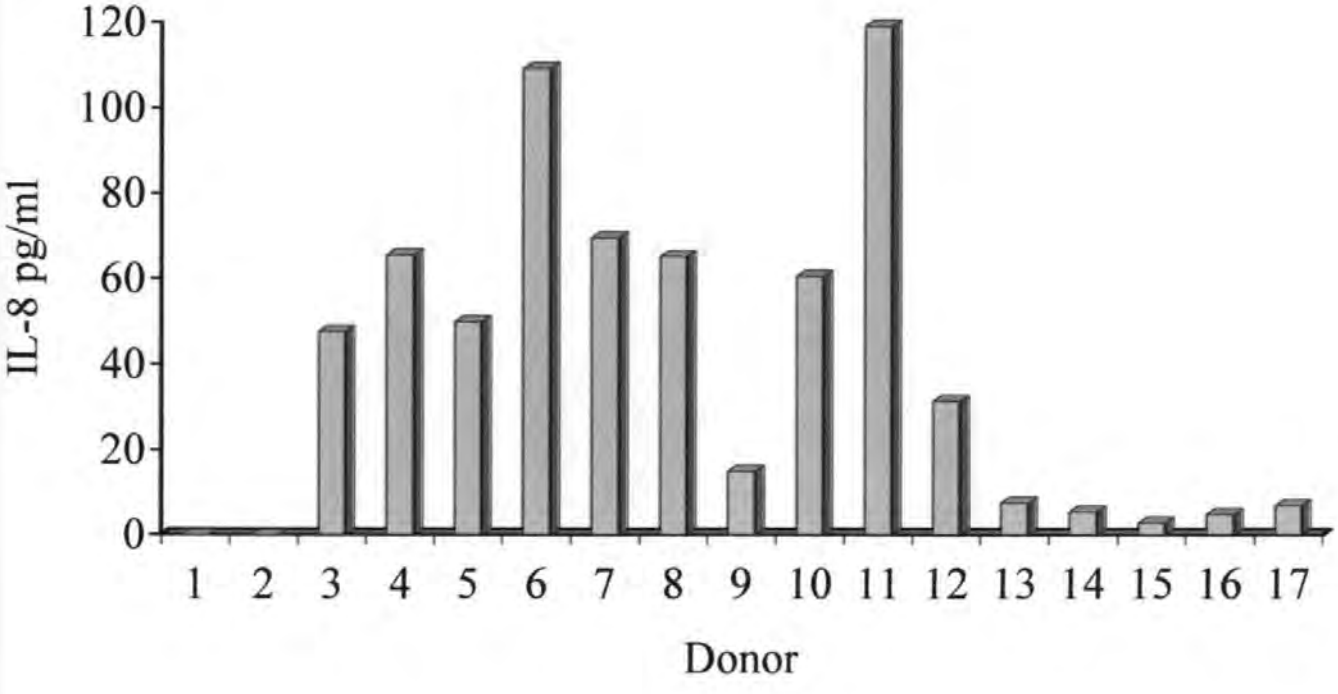
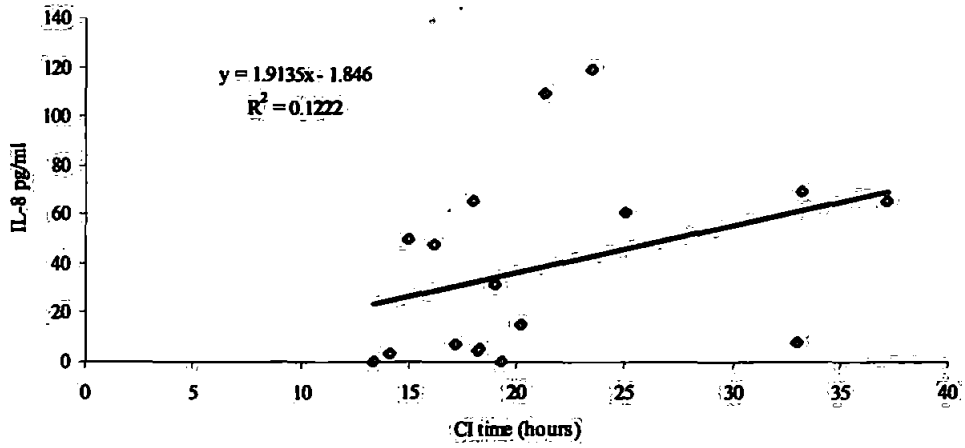


Figure 6.2.1. Range in IL-8 levels found in donor kidneys. IL-8 was measured by ELISA and levels corrected for total protein. A range of 40.3 fold was found between samples.



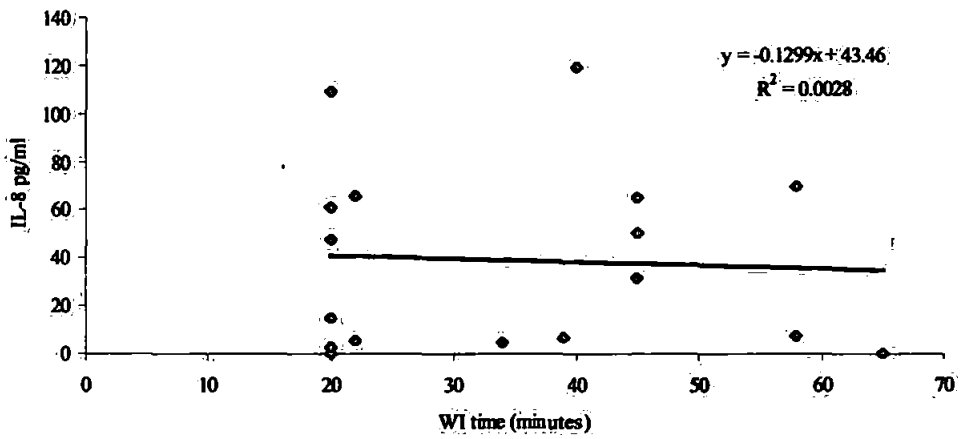


Figure 6.2.2. IL-8 levels with increasing ischaemic times. CI time ranged from 13 hours to 37 hours and WI times ranged from 20 to 65 minutes.

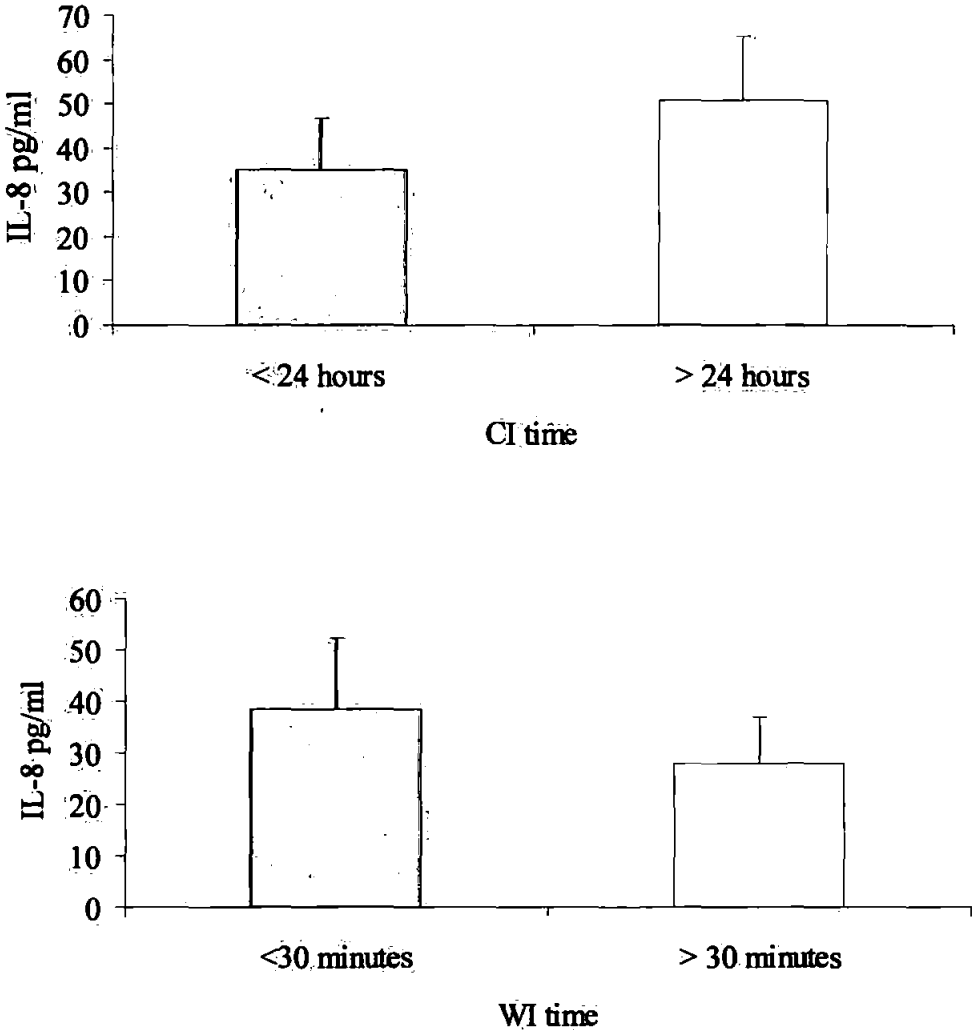


Figure 6.2.3. IL-8 levels in kidneys with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.

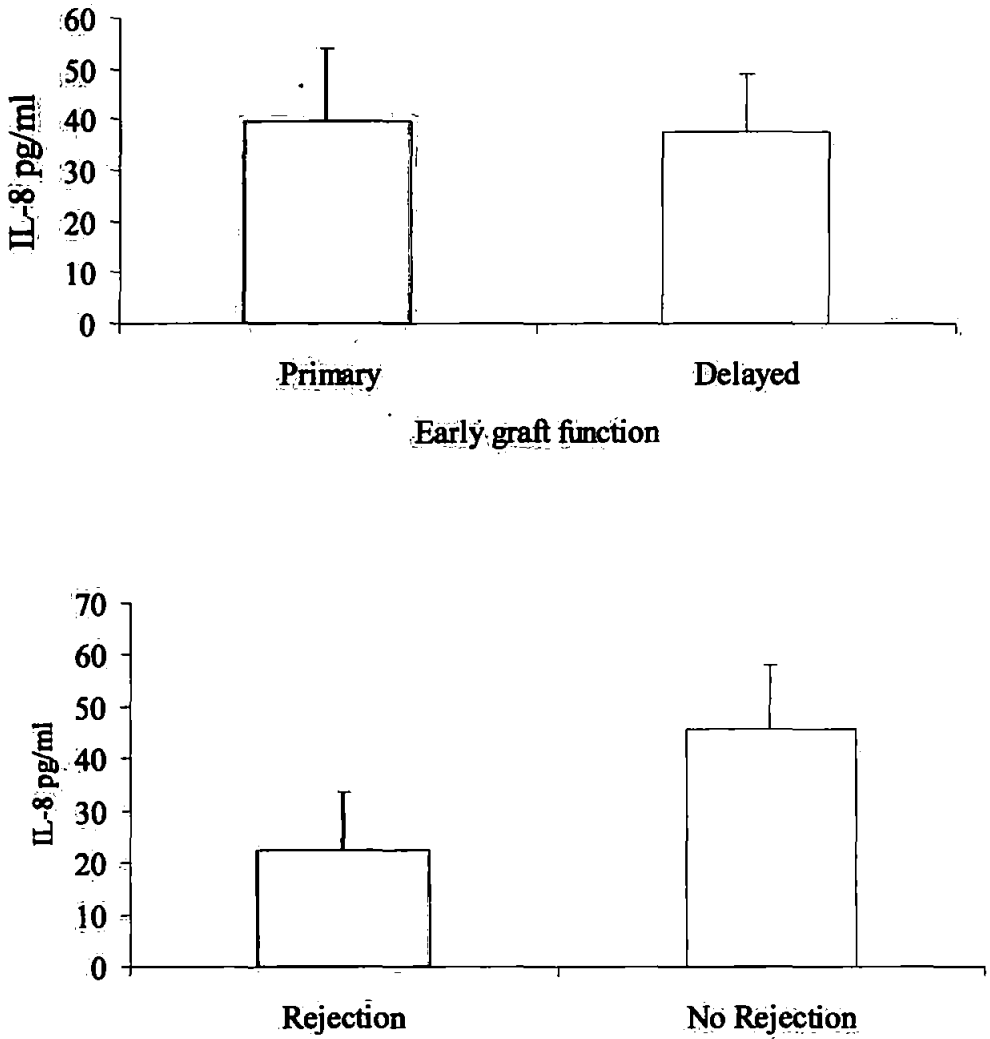


Figure 6.2.4. IL-8 levels in relation to early graft function and rejection. Ten kidneys had primary function and 7 experienced delayed function. Five kidneys were rejected and 12 were rejection free

6.4 TNFα

There was a range in TNFα levels in the donor samples from 1.76 pg/ml to 8.73 pg/ml (4.9 fold difference) (Fig 6.3.1). There were no significant differences between CI or WI times or Primary function, or rejection. TNFα levels were slightly higher in kidneys with > 24 hours CI time compared with < 24 hours CI time (5.44 $\pm$ 0.77 Vs 5.29 $\pm$ 0.58 pg/ml). Similarly in kidneys with > 30 minutes WI time compared with < 30 minutes WI time.(5.82 $\pm$ 0.6 Vs 4.76 $\pm$ 0.6 pg/ml). In the same fashion as IL-8, TNFα levels were higher in kidneys with primary function (5.87 $\pm$ 0.64 Vs 4.55 $\pm$ 0.61 pg/ml) and in non-rejection (5.59 $\pm$ 0.4 Vs 4.68 $\pm$ 1.1 pg/ml).

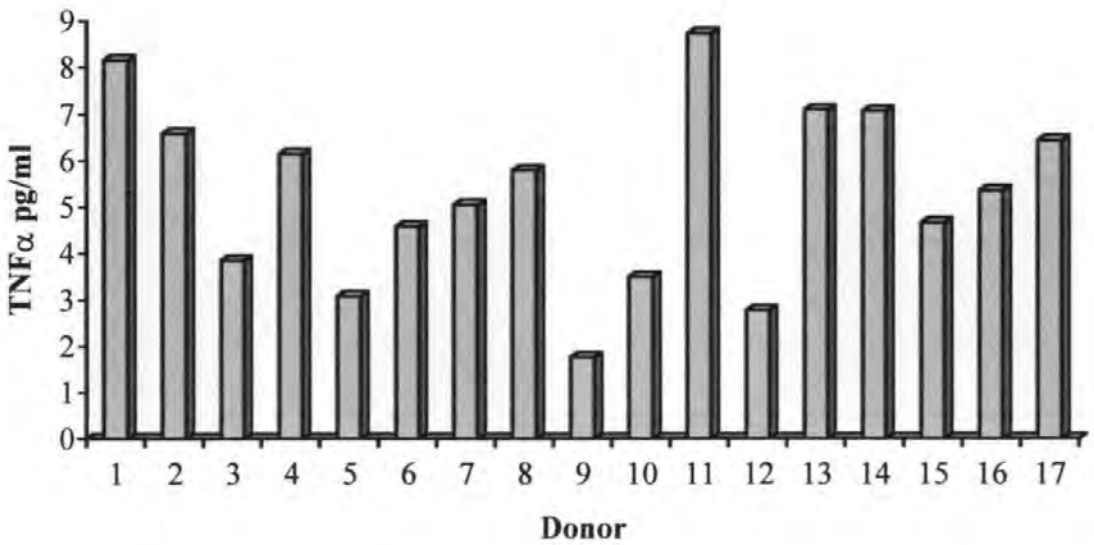


Figure 6.3.1. Range in TNFα levels found in donor kidneys. TNFα was measured by ELISA and levels corrected for total protein. A range of 4.9 fold was found between samples.

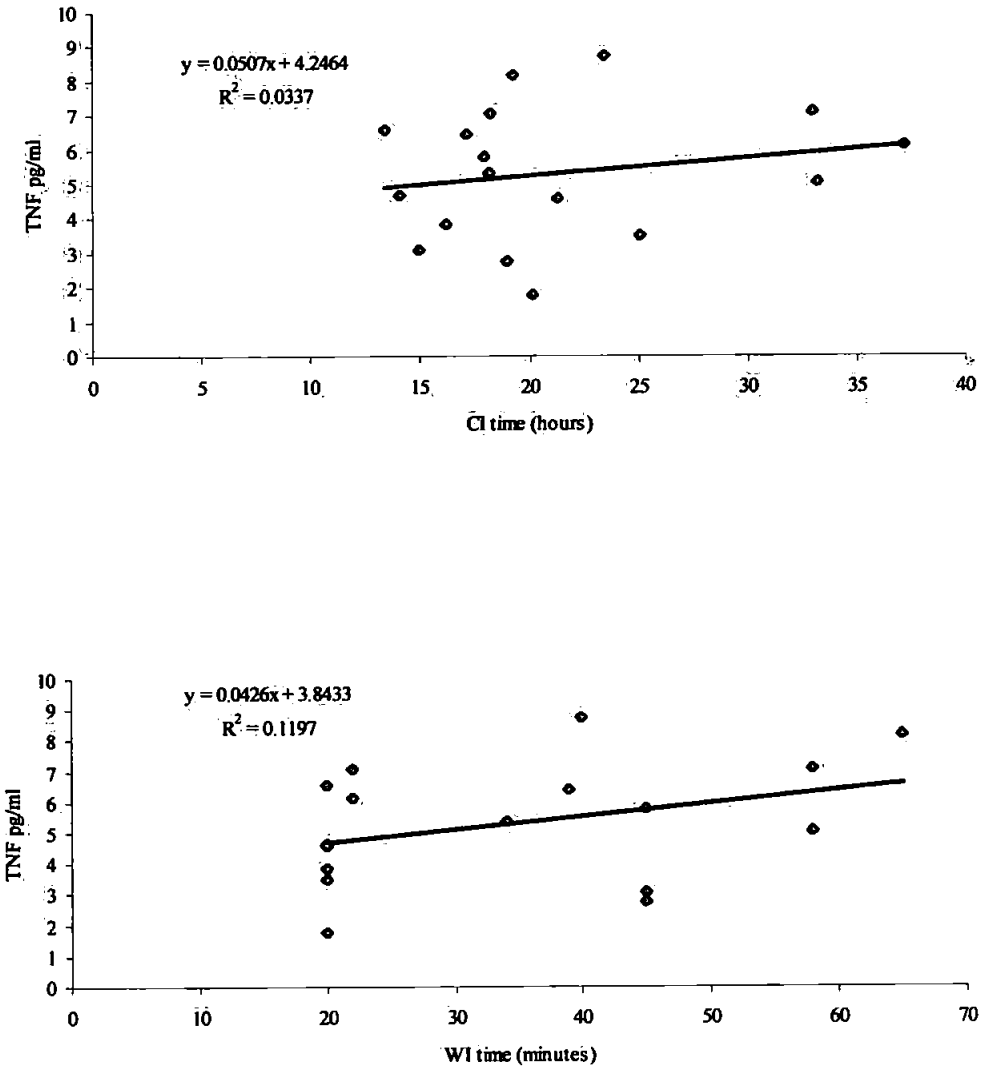


Figure 6.3.2. TNFα levels in relation to ischaemic times. CI times ranged from 13 hours to 37 hours and WI times ranged from 20 to 65 minutes.

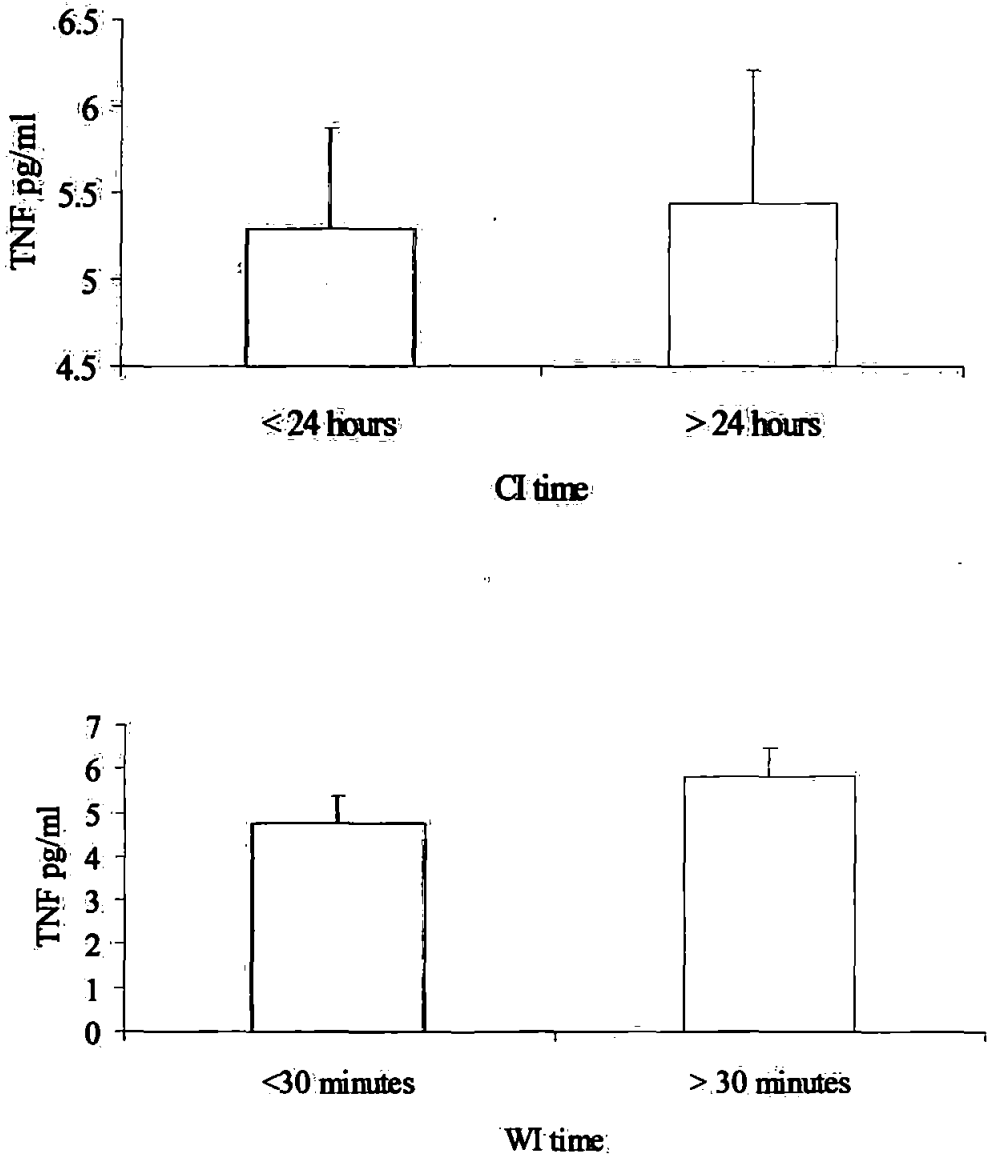


Figure 6.3.3. TNFα levels in solutions with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.

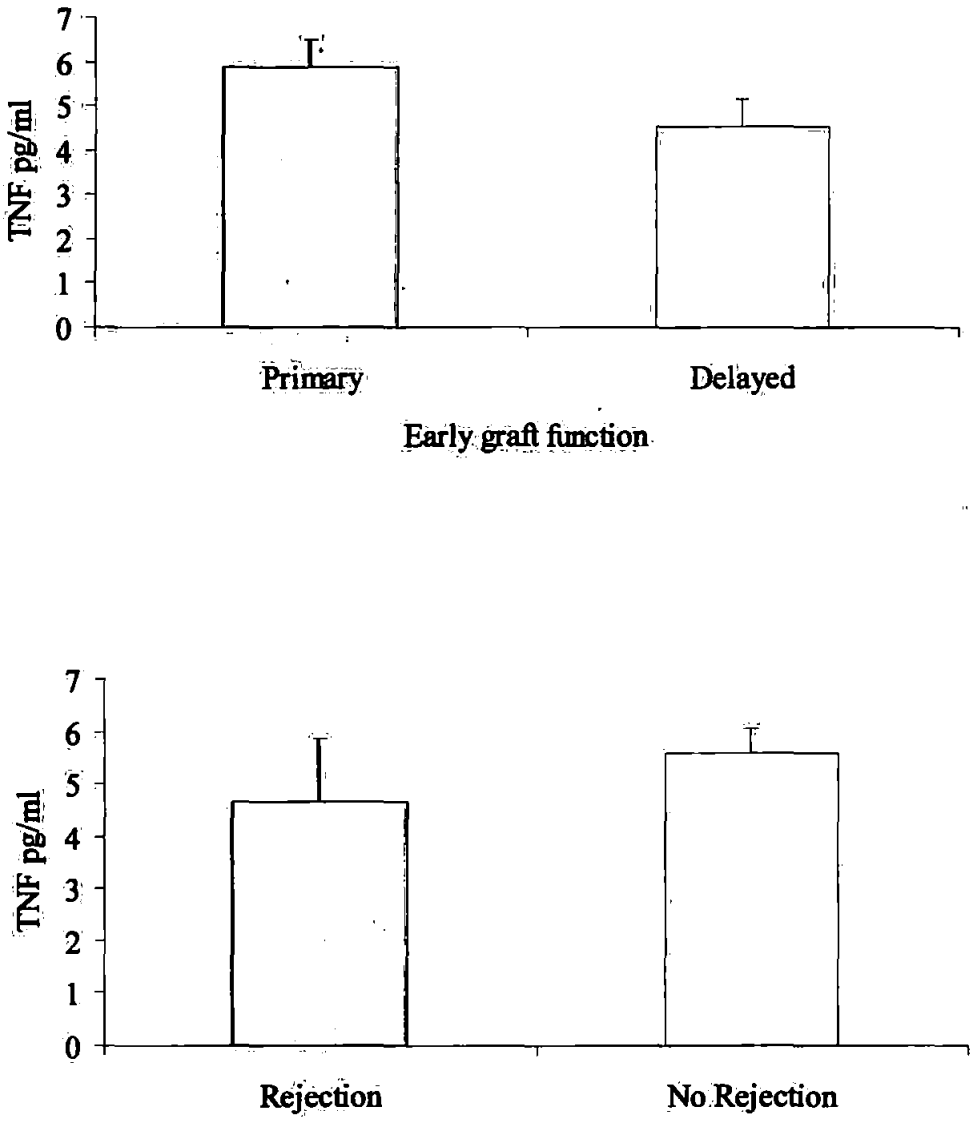


Figure 6.3.4. TNFα levels in relation to early graft function and rejection. Ten kidneys had primary function and 7 had delayed function. Five kidneys were rejected and 13 were rejection free

6.5 IL-4

The anti-inflammatory cytokine, IL-4 was found to range from 0.7 pg/ml to 82.15 pg/ml (117.3 fold difference). No association was found with ischaemic times. In grafts with primary function, there was a higher IL-4 level than in grafts with delayed function (21.28 $\pm$ 7.7 pg/ml Vs 9.65 $\pm$ 3.6 pg/ml p=NS). IL-4 levels were higher in kidneys with longer ischaemic times. Higher in kidneys with > 24 hours CI time (27.4 1.8 Vs 13.14 3.4 pg/ml) and > 30 minutes WI time (21.43 $\pm$ 8.8 Vs 10.94 $\pm$ 2.91 pg/ml). IL-4 levels were also higher in non-rejecting kidneys (20.63 $\pm$ 6.5 Vs 7.35 $\pm$ 1.7 pg/ml).

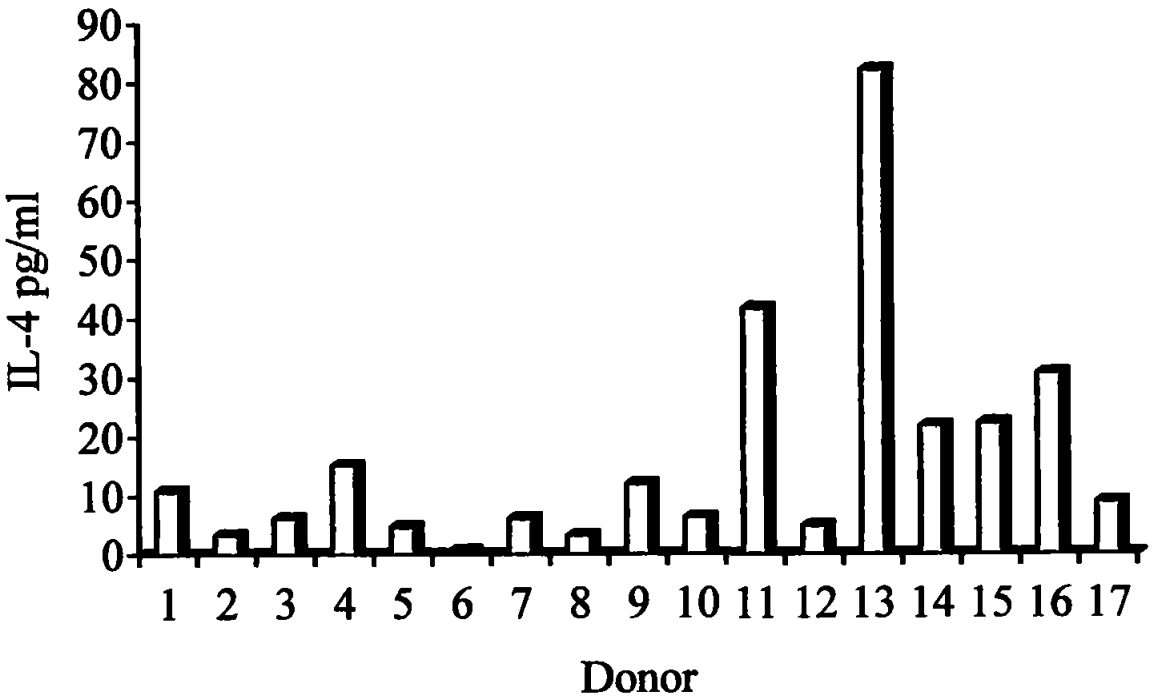
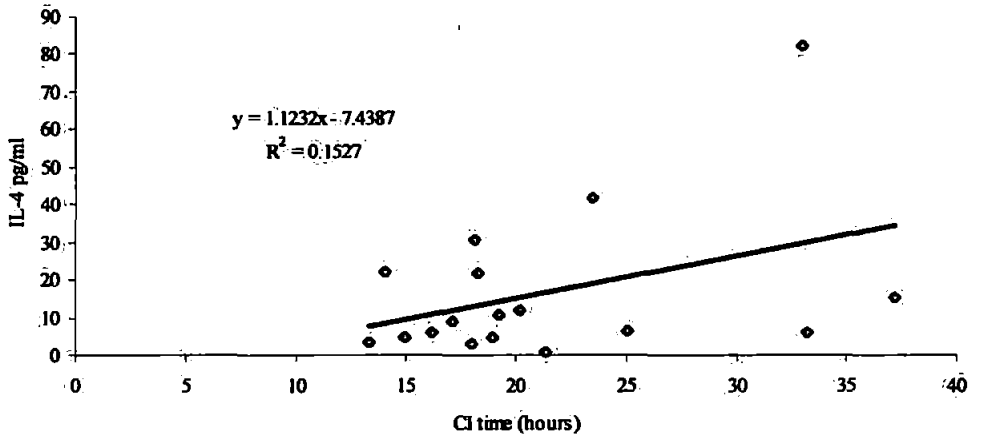


Figure 6.4.1. Range in IL-4 levels found in preservation solutions. IL-4 was measured by ELISA and levels corrected for total protein. A range of 117.3 fold was found between samples.



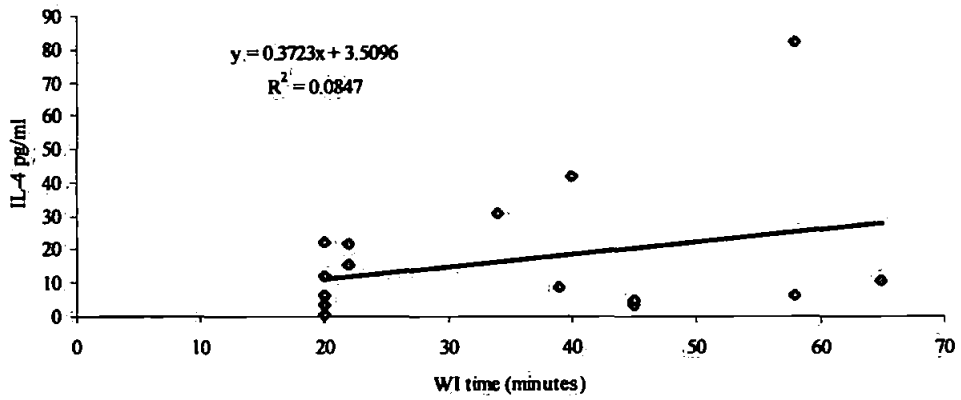


Figure 6.4.2. IL-4 levels with increasing ischaemic times. CI times ranged from 13 hours to 37 hours and WI times ranged from 20 to 65 minutes.

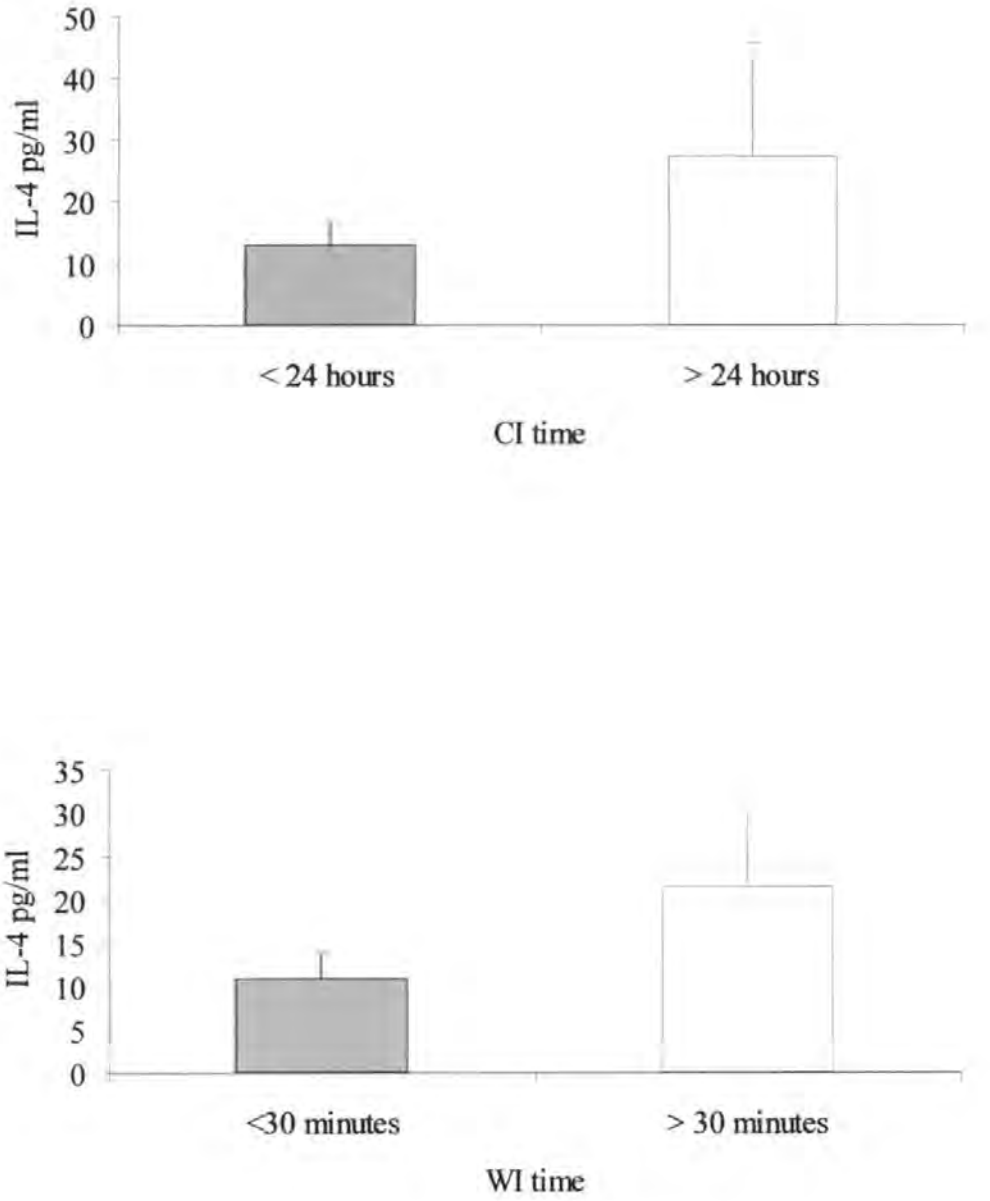


Figure 6.4.3. IL-4 levels in solutions with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.

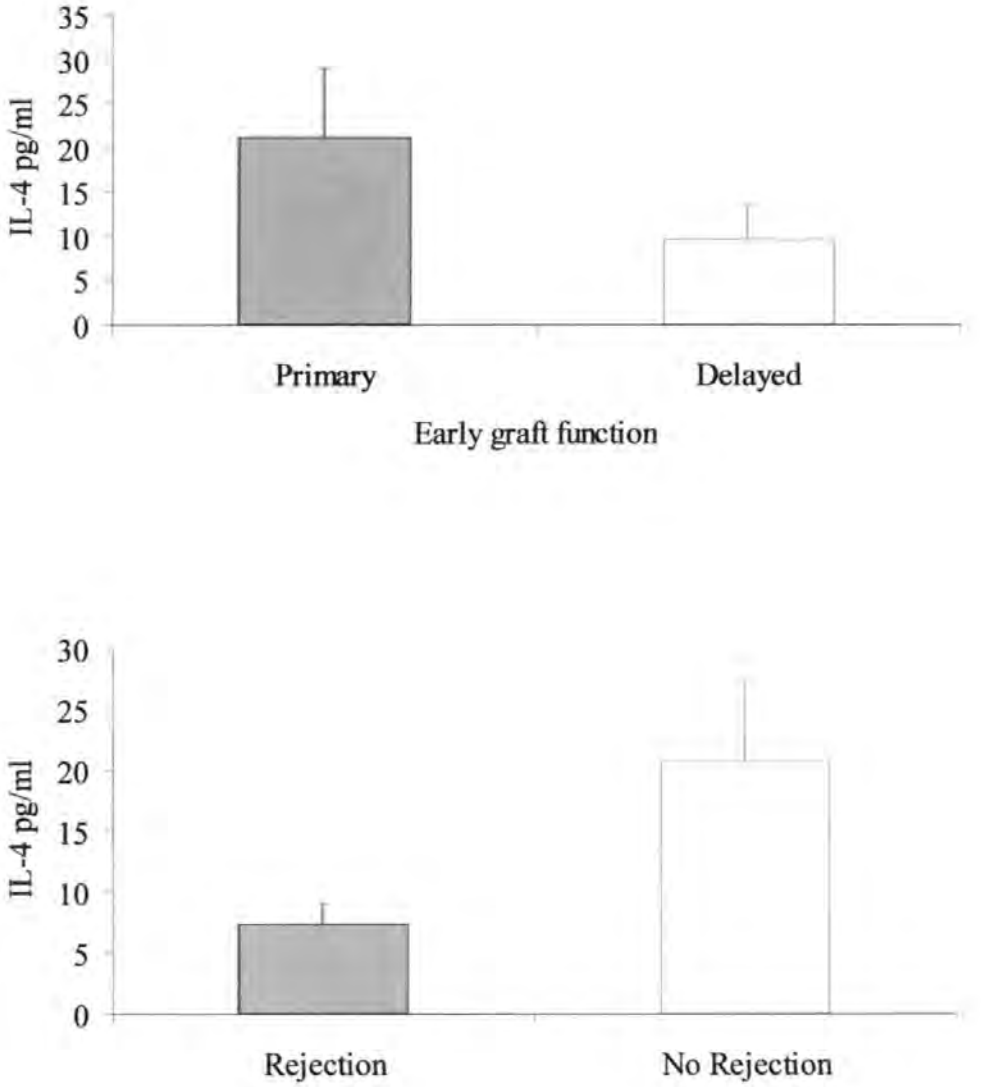


Figure 6.4.4. IL-4 levels in relation to early graft function and rejection. Ten kidneys had primary function and 7 had delayed function. Five kidneys were rejected and 13 were rejection free

6.6 VWF

VWF levels were found to range from 0.011 IU/ml to 0.265 IU/ml (26.5 fold difference). There was no correlation between increasing ischaemic times and VWF levels in the solutions. The average in solutions with < 24 hours CI time was 0.073 ± IU/ml compared with 0.108 ± 0.009 IU/ml in solutions with > 24 hours CI time (p=Ns). Levels were similar in solutions with < or > 30 minutes WI time (0.080 ± 0.02 IU/ml Vs 0.081 ± 0.01 IU/ml). Levels were slightly lower in kidneys with primary function (0.06 ± 0.06 IU/ml Vs 0.09 ± 0.03 IU/ml p=Ns), and in non-rejecting kidneys (0.07 ± 0.07 IU/ml Vs 0.10 ± 0.04 IU/ml p=Ns).

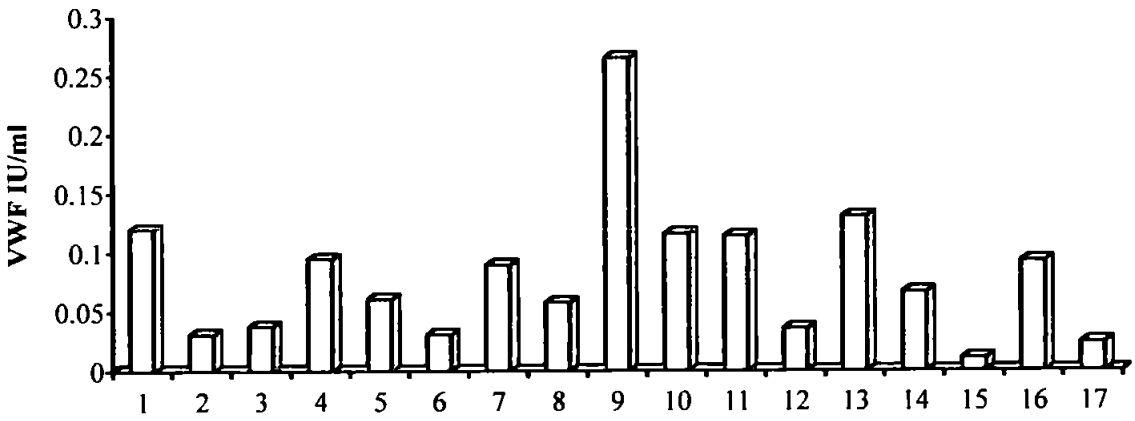


Figure 6.5.1. VWF levels in preservation solutions. VWF was measured by ELISA and levels corrected for total protein. A range of 26.5 fold was found between samples.

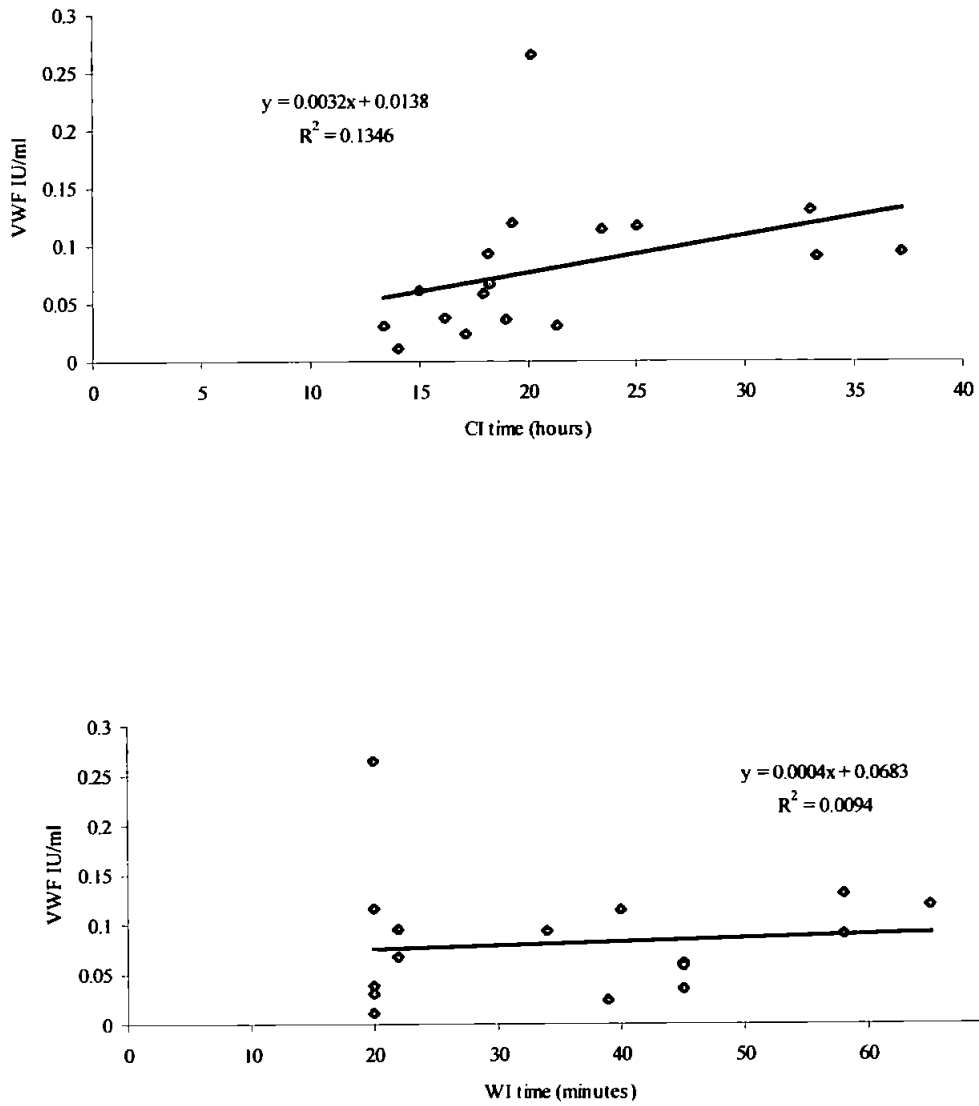


Figure 6.5.2. VWF levels in relation to ischaemic times. CI times ranged from 13 hours to 37 hours and WI times ranged from 20 to 65 minutes.

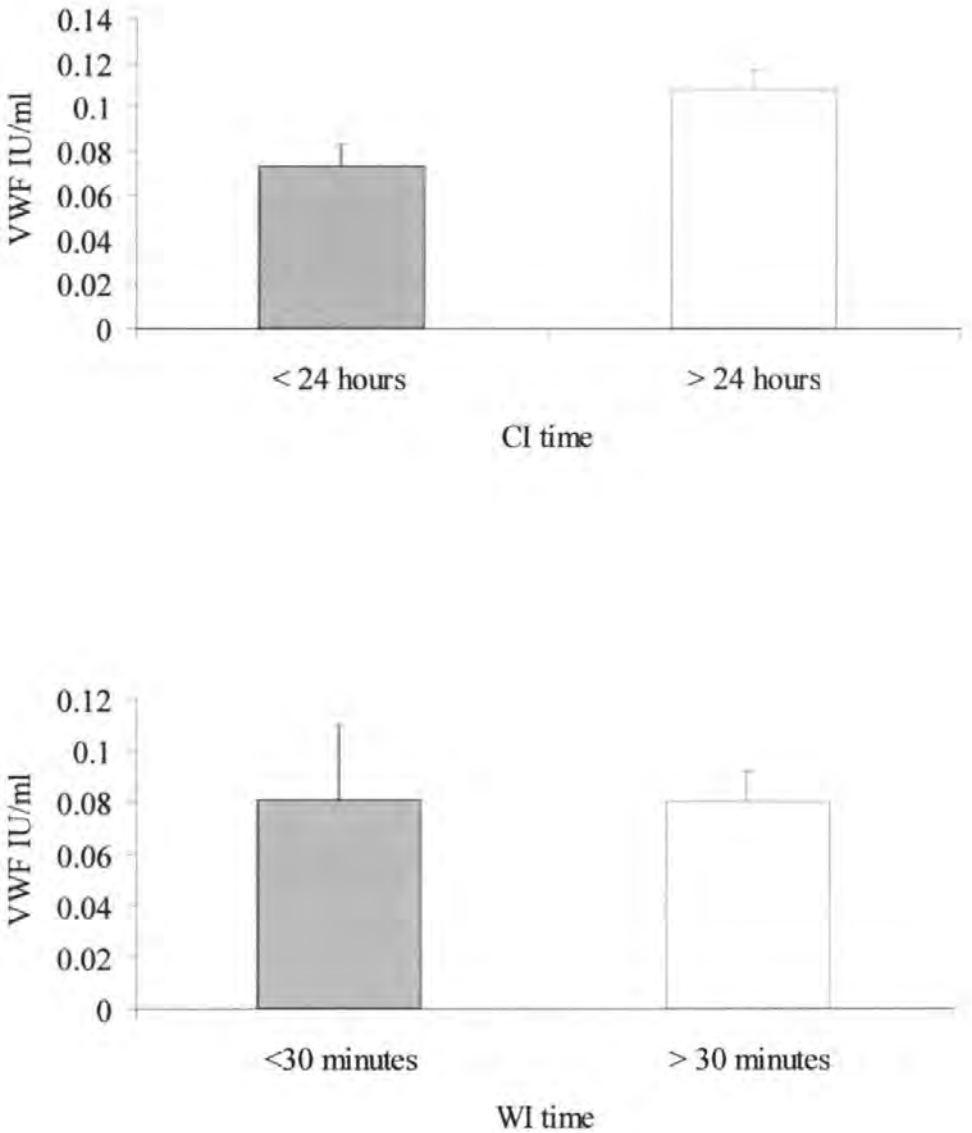


Figure 6.5.3. VWF levels in solutions with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.

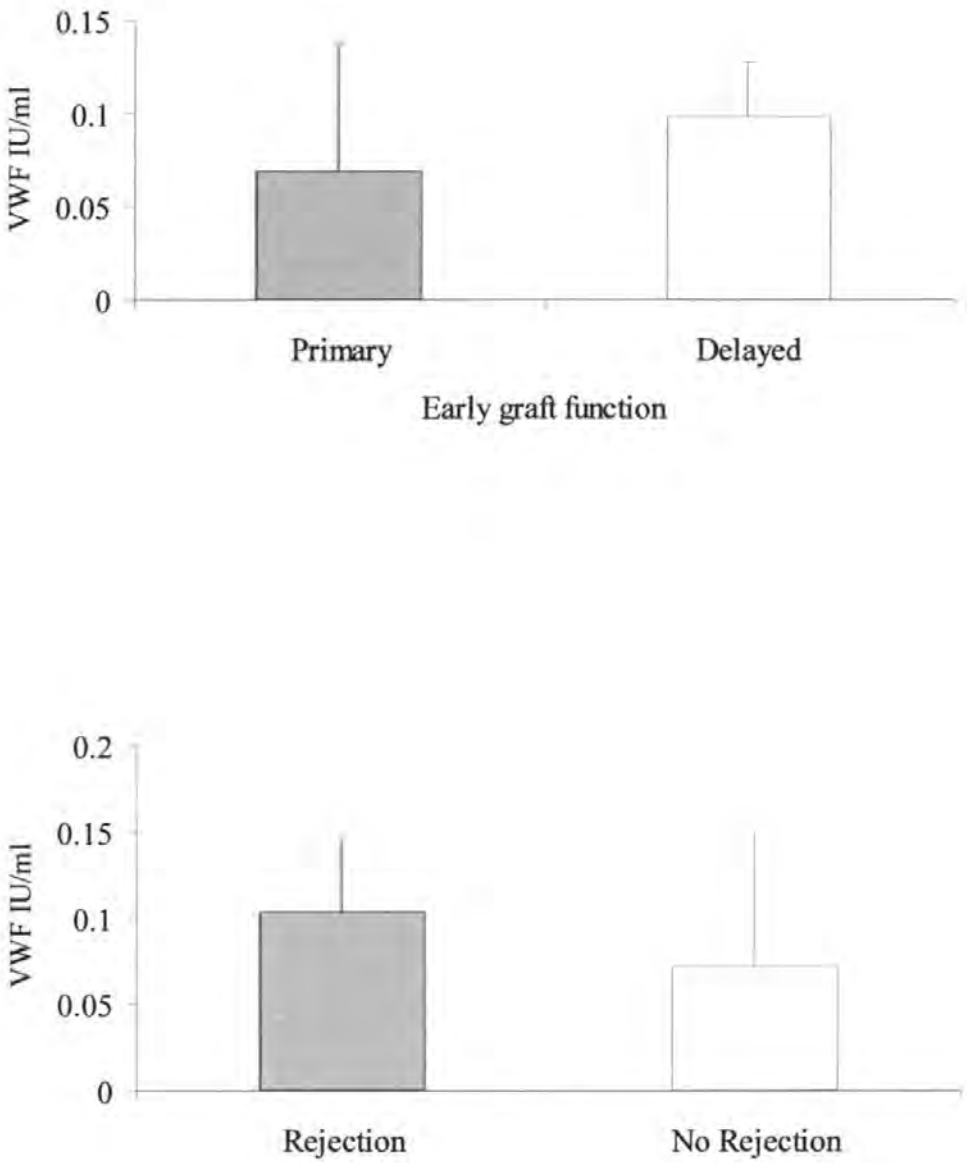


Figure 6.5.4. VWF levels in relation to primary graft function and rejection. Ten kidneys had primary function and 7 had delayed function. Five kidneys were rejected and 13 were rejection free

6.7 Lactate

There was a range in lactate levels between 1.02 and 17.50 mmol/L in the preservation solutions (17.1 fold difference). As a marker of hypoxia there was slightly higher levels in samples with > 24 hours CI time (7.36 $\pm$ 0.44 mmol/L Vs 6.90 $\pm$ 0.35 mmol/L p=Ns). Lactate was also higher in solutions with delayed function (8.45 $\pm$ 0.6 mmol/L Vs 5.99 $\pm$ 0.30 mmol/L p=Ns). Lactate was also higher in rejectors (9.19 $\pm$ 1.0 Vs 6.09 $\pm$ 0.27 mmol/l).

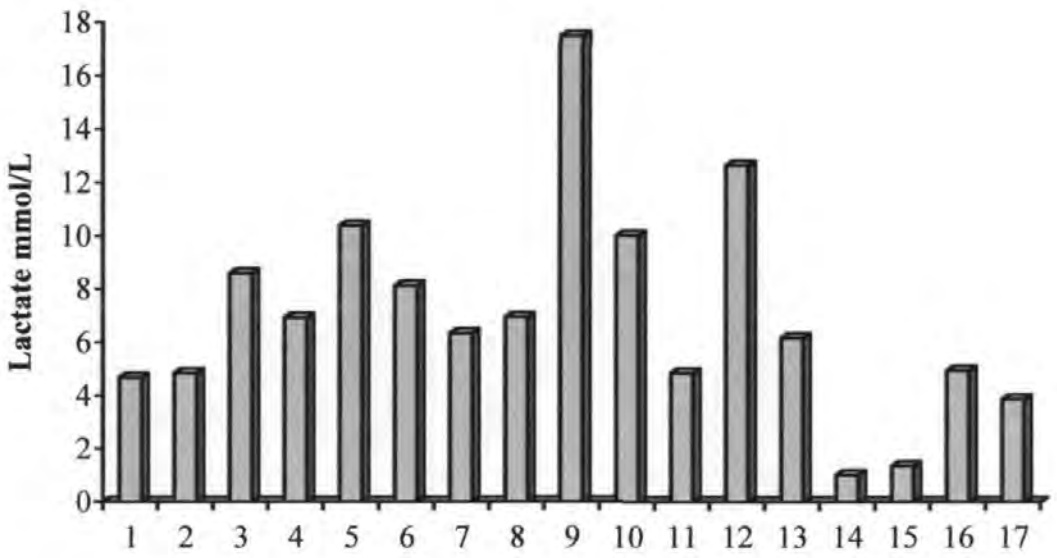


Figure 6.6.1. Lactate levels in preservation solutions. Lactate was measured by ELISA and levels corrected for total protein. A range of 17.1 fold was found between samples.

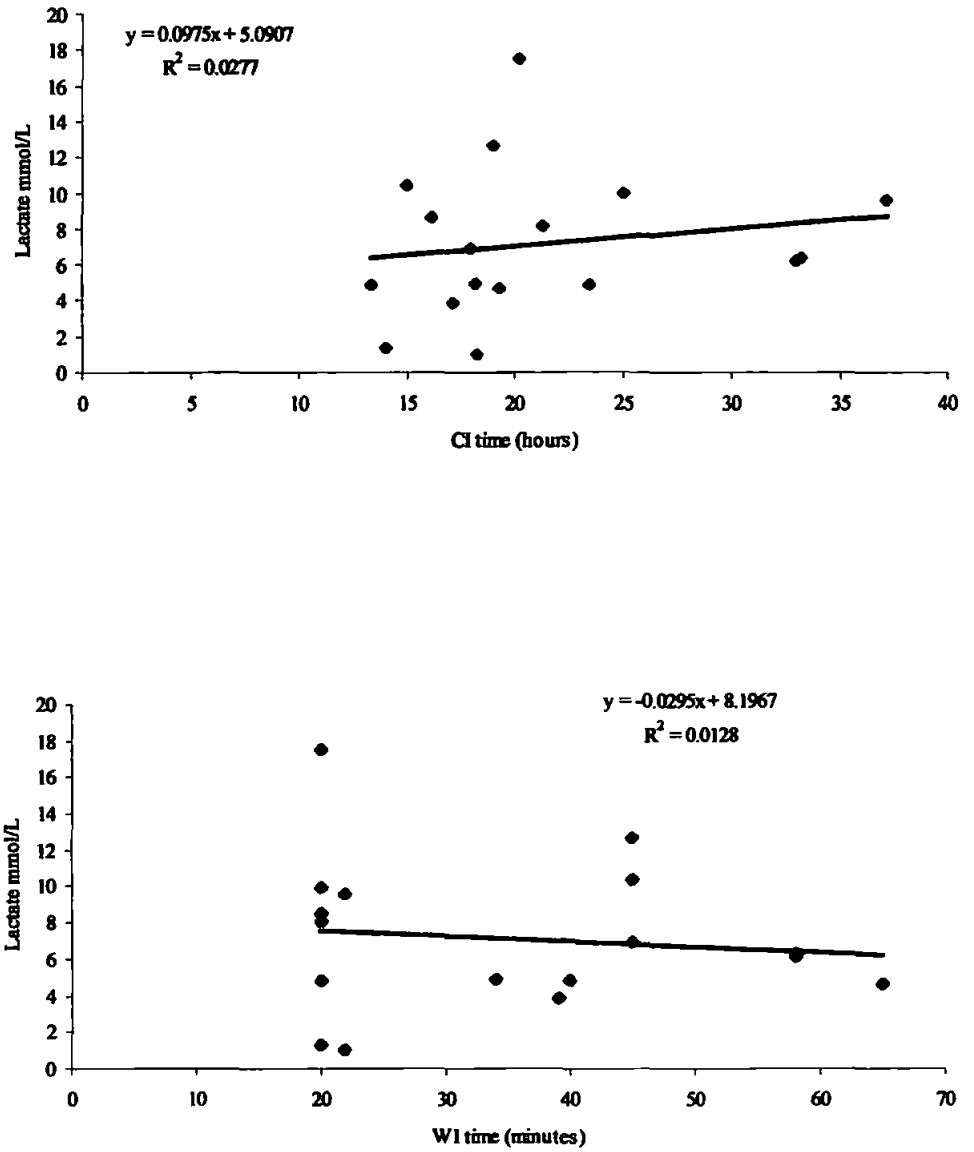


Figure 6.6.2. Lactate levels with increasing ischaemic times. CI times ranged from 13 hours to 37 hours and WI times ranged from 20 to 65 minutes.

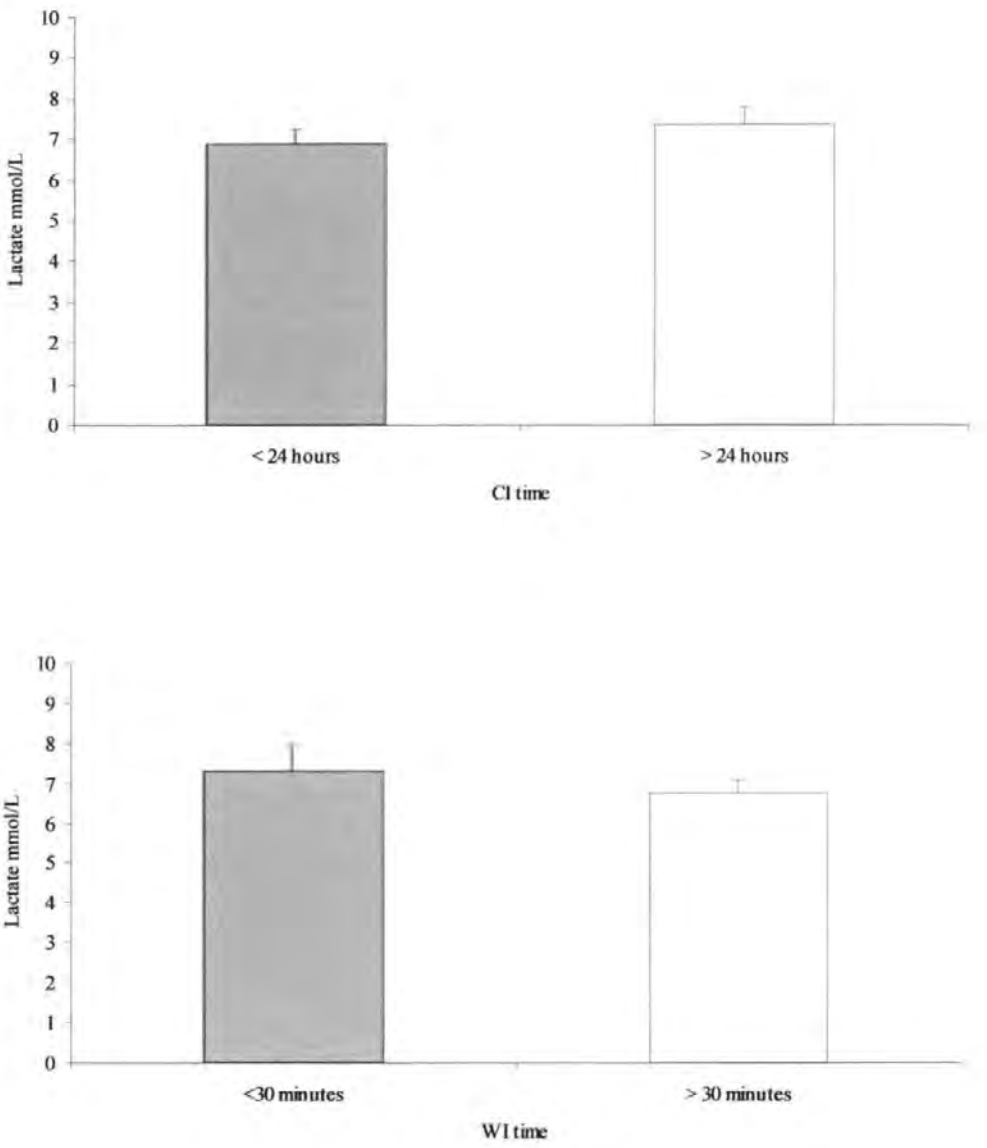


Figure 6.6.3. Lactate levels in solutions with less than (n=13) or greater than (n=4) 24 hours CI time and less than (n=8) or greater than (n=9) 30 minutes WI time.

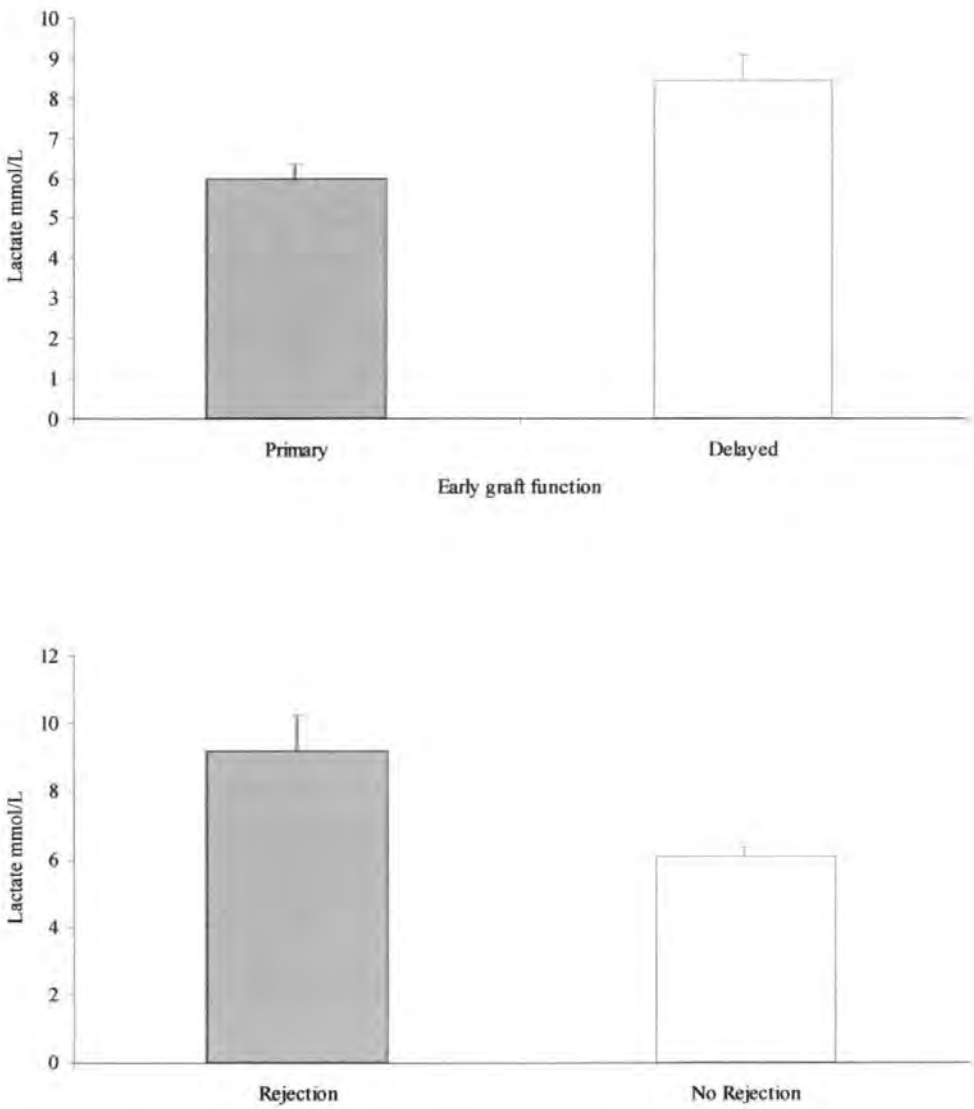


Figure 6.6.4. Lactate levels in relation to primary graft function and rejection. Ten kidneys had primary function and 7 had delayed function. Five kidneys were rejected and 13 were rejection free

6.8 Discussion

There was a range of levels found in the solutions. The largest fold difference between samples was found for IL-4 (117 fold). The lowest range was for TNFα (4.9 fold). Only two samples were undetectable and this was only for IL-8. It is difficult to compare the levels found in the solutions with any 'normal' level as cytokine levels in serum and plasma vary amongst individuals. During an inflammatory response cytokine levels are likely to be highly elevated (Land 1998, Mantovani *et al* 1998). According to the ELISA manufacturers recommendations the following are found.

Cytokine	**Serum level in normal healthy individuals**
IL-8	< 31.2pg/ml
IL-6	3.13 - 12.5pg/ml
TNFα	< 15.6pg/ml
IL-4	< 4 pg/ml

Eight samples for IL-8 were found to be in this range. No IL-6 samples were found to be in the range, the lowest was 54.73pg/ml. All samples were found to have TNFα levels below the range, with the highest 8.73pg/ml. Four samples for IL-4 were in the range with the highest 82.15pg/ml.

Normal blood plasma VWF are 5-10μg/ml. Levels of VWF have also been shown to be raised during infection or injury (Reinders *et al* 1987). Eight samples had VWF levels in this range with the highest value 26μg/ml.

Normal blood lactate levels are in the range of 1.0 ± 0.5 mmol/L, and levels above this are used as an indication of acid-base disturbances (Manji & Bion 1999). Only two samples were below this level and the highest was 12.65 mmol/L. Different preservation solutions have been suggested to effect cytokine production, with UW solution less damaging to endothelial cells than HTK solution and EuroCollins type solution (Eberl *et al* 1999). However, information on the types of solutions received was not available in this study.

The correlation between levels of IL-6 and the genotype of the donor revelaled that donors with the CC genotype had 1.1 fold less IL-6 than donors with the GG genotype (1.66 ± 6.2 pg/ml Vs 196.17 ± 40.79 pg/ml p=ns). In a previous study of healthy individuals a 1.6 fold difference between CC genotypes and GG genotypes was found in serum IL-6 levels (Fishmann *et al* 1998).

CHAPTER SEVEN: AN INVESTIGATION OF POLYMORPHISMS IN DONOR KIDNEYS

7.1 Background

Four polymorphisms were investigated in donor DNA samples. Two microsatellite CA(n) repeat polymorphisms in the TNFα and NFκB genes and two restriction enzyme sites in the IL-6 and NAD(P)H phox22 genes. Kidneys were separated according to whether they were subsequently rejected or not. The kidneys were then sub-grouped into less than three total mismatched alleles, one or more HLA-A mismatch and one or more HLA-B mismatch for further analysis. There were insufficient numbers of HLA-DR mismatched non-rejectors (n=4) to include analysis of HLA-DR matching. Full details of the donors can be found in the appendix. A brief description of clinical characteristics is shown in Table 7.1.

7.2 Microsatellite analysis

50 donor kidney DNA samples were used for microsatellite analysis, 4 were exported to other hospitals, 1 experienced surgical failure, and 2 were not used. Six donors had both kidneys removed and used. From this there was a total of 49 samples available for analysis. Of the 49 kidneys, 30 (61.2%) experienced a least one rejection episode and 19 (38%) were rejection free. Of the rejectors 18 (60%) had one episode, 7 (23%) had two episodes and 5 (16%) had three or more episodes.

7.2.1 HLA matching

41 (83%) of the donor-recipients had less than 3 mismatches. Of the rejectors 25 (83%) had less than 3 mismatches and 16 (84%) of non-rejectors had less than 3 mismatches. 38 (77%) had an HLA-A allele mismatch 21 (55%) in the rejection group and 17 (44%) in

the non-rejection group. Similarly 38 (77%) had an HLA-B mismatch, 25 (65%) in the rejection group and 13 (34%) in the non-rejection group. HLA-DR mismatch was found in 23 (46%) of patients with the majority 19 (82%) in the rejection group and only 4 (17%) in the non-rejection group.

	Donors (n=43)	Recipients (n=49)
Age ± SD	**47 ± 15.9**	**46 ± 12.9**
M:F Ratio	**21:22**	**33:16**
M-M	**19 (39%)**	
M-F	**6 (12%)**	
F-F	**9 (18%)**	
F-M	**15 (31%)**	
HLA-A Mismatch		
0	**11 (22.4%)**	
1	**32 (65.3%)**	
2	**6 (12.2%)**	
HLA-B Mismatch		
0	**11 (22.4%)**	
1	**29 (59.1%)**	
2	**9 (18.4%)**	
HLA-DR Mismatch		
0	**26 (53.1%)**	
1	**22 (44.9%)**	
2	**1 (2%)**	
Rejection episodes		
0		**19 (38.8%)**
1		**18 (36.7%)**
2		**7 (14.3%)**
>3		**5 (10.2%)**
Nephrectomy		**2 (4.1%)**

Table 7.1. Clinical details of donor and recipients used for microsatellite analysis. Full details are available in the appendix.

7.3 NFκB CA(n) microsatellite

In the total rejection group the most common allele was the 138 which was found in 24 (40%) donors compared with 11 (29%) in the non-rejection group (p=ns). The most common allele in the non-rejection group was the 126 allele which was present in 16 (42%) compared with 18 (30%) in the rejection group (p=ns). The most common genotype in the rejection group was the 126, 138 genotype, found in 8 (26%) donors and it was also the most common genotype in the non-rejection group with 3 (15%) (p=ns). No association was found between NFκB alleles or genotypes when comparing rejectors and non-rejectors (Table 7.2.1). In donors with <3 mismatches the most common allele in the rejection group was the 138 allele, which was found in 20 (40%) donors compared to 9 (28%) non-rejectors (p=ns). In the non-rejection group the most common allele was the 126 allele which was present in 14 (44%) donors compared to 14 (28%) rejectors (p=ns). The most common genotype in the rejection group was the 126,138 genotype which was 6 (24%) compared with 2 (12%) in the non-rejectors (p=ns). The most common genotype in the non-rejectors was the 126,126 which was found in 3 (18%) compared with 2 (8%) in the rejectors (p=ns). No association was found with any allele or genotype by comparing rejectors and non-rejectors with <3 HLA mismatches (Table 7.2.2). In donors with an HLA-A mismatch, the most common allele in the rejection group was the 138 allele which was found in 19 (45%) compared with 10 (29%) in the non-rejectors (p=ns). The most common allele in the non-rejectors was the 126 allele which was found in 15 (44%) donors compared with 11 (26%) in the rejection group (p=ns). The most common genotype in both the rejection group and non-rejection group was the 126,138 genotype which was found in 5 (24%) rejectors and 3 (18%) non-rejectors (p=ns). No association

was found between rejectors and non-rejectors with a mismatched HLA-A allele (Table 7.2.3). In donors with a mismatched HLA-B allele, the most common allele in the rejection group was the 138 allele, which was found in 21 (42%) compared with 7 (27%) in the non-rejection group (p=ns). In the non-rejection group the most common allele was found to be the 126 allele which was found in 11 (47%) donors compared with 16 (32%) in the rejection group (p=ns). The most common genotypes in both the rejection groups was the 126,138 which was found in 7 (28%) in the rejectors and 2 (15%) in the non-rejectors (p=ns). No association was found between NFκB alleles or genotypes in rejectors and non-rejectors with a mismatched HLA-B allele (Table 7.2.4).

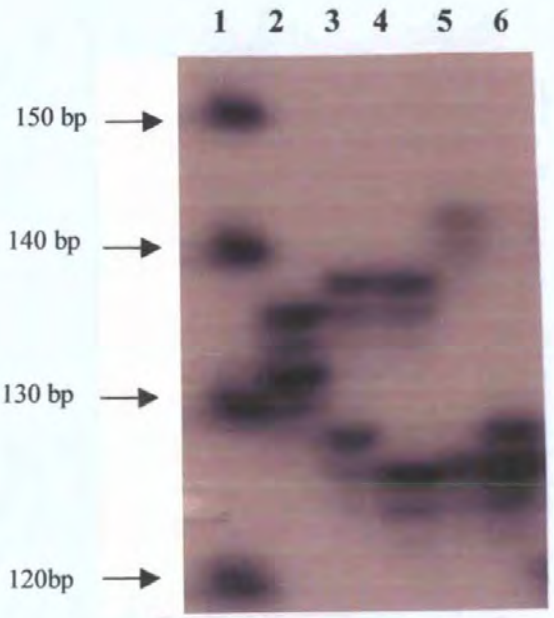


Figure 7.1. Representation of the NFκB CA(n) alleles found in donor DNA

Lane 1 – Base pair ladder

Lane 2 – 132/136

Lane 3 – 128/138

Lane 4 – 126/138

Lane 5 – 126/142

Lane 6 – 126

NFκB CA(n)	Rejection (n=30)	No Rejection (n= 19)	x^2	p value
Alleles				
126	30 (18)	42 (16)	0.72	ns
132	5 (3)	5 (2)	0.00	ns
136	6 (4)	5 (2)	0.00	ns
138	40 (24)	28.9 (11)	0.60	ns
x	18 (11)	18 (7)	0.00	ns
Haplotype				
126,126	10 (3)	15 (3)	0.30	ns
126,138	26 (8)	15 (3)	0.51	ns
136, 138	10 (3)	10 (2)	0.00	ns
138,138	13 (4)	10 (2)	0.07	ns
x,x	50 (15)	63 (12)	0.23	ns

Table7.2.1 The percentage frequency of NFκB alleles and haplotypes in transplanted kidneys which were rejected or not. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

NFκB CA(n)	Rejection (n=25)	No Rejection (n=16)	x^2	p value
Alleles				
126	28 (14)	43.7 (14)	1.03	ns
132	6 (3)	6.2 (2)	0.00	ns
136	8 (4)	3 (1)	0.72	ns
138	40 (20)	28.1 (9)	0.68	ns
x	18 (9)	18 (6)	0.01	ns
Haplotype				
126,126	8 (2)	18.7 (3)	0.81	ns
126, 138	24 (6)	12.5 (2)	0.57	ns
136,138	12 (3)	6.2 (1)	0.30	ns
138,138	16 (4)	12.5 (2)	0.07	ns
x,x	40 (10)	18 (6)	0.01	ns

Table 7.2.2 The percentage frequency of NFκB haplotypes in transplanted kidneys with less than 3 mismatched alleles. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

NFκB CA(n)	Rejection (n=21)	No Rejection (n=17)	x^2	p value
Alleles				
126	26.1 (11)	44.1 (15)	0.96	ns
130	4.7 (2)	5.8 (2)	0.04	ns
136	7 (3)	5.8 (2)	0.04	ns
138	45.2 (19)	29.4 (10)	0.91	ns
x	16.6 (7)	14.7 (5)	0.04	ns
Haplotype				
126,126	4 (1)	11.7 (2)	0.54	ns
126,138	23.8 (5)	17.6 (3)	0.39	ns
136, 138	14.2 (3)	5.8 (1)	0.58	ns
138,138	14.2 (3)	11.7 (2)	0.04	ns
x,x	42.8 (9)	52.9 (9)	0.14	ns

Table 7.2.3 The percentage frequency of NFκB alleles and haplotypes in transplanted kidneys with at least one mismatched HLA-A allele. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

NFκB CA(n)	Rejection (n= 25)	No Rejection (n=13)	x^2	p value
Alleles				
126	32 (16)	47.3 (11)	0.37	ns
136	8 (4)	7.6 (2)	0.00	ns
138	42 (21)	26.9 (7)	0.80	ns
x	18 (9)	23.0 (6)	0.18	ns
Haplotype				
126, 126	12 (3)	7.6 (1)	0.14	ns
126,138	28 (7)	15.3 (2)	0.48	ns
136, 138	12 (3)	7.6 (1)	0.14	ns
138,138	16 (4)	15.3 (2)	0.00	ns
x,x	32.0 (8)	53.8 (7)	0.71	ns

Table 7.2.4 The percentage frequency of NFκB haplotypes in transplanted kidneys with at least one mismatched HLA-B allele. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

7.2 TNFα CA(n) microsatellite

The most common allele in the rejection group was found to be the 2 allele which was present in 18 (30%) of rejectors compared with 6 (15%) in the non-rejection group (p=ns). The most common allele in the non-rejection group was the 10 allele which was present in 12 (31%) donors compared with 11 (18%) in the rejectors (p=ns). The most common genotype in the rejection group was the 6,7 genotype which was found in 3 (10%) of donors, compared to 0 (0%), in non-rejectors (p=ns). The most common genotype in the non-rejection group was the 6,10 genotype which was found in 4 (21%) donors, compared to 2 (6%) in rejectors (p=ns). No association was found with any allele or genotype by comparing rejectors and non-rejectors (Table 7.3.1). In donors with <3 mismatches the most common allele in the rejection group was the 2 allele, which was found in 14 (28%) donors compared to 5 (15%) non-rejectors (p=ns). In the non-rejection group the most common allele was the 10 allele which was present in 9 (28%) donors compared to 9 (18%) rejectors (p=ns). The most common genotype in both the rejection and non-rejection group was the 6,10 genotype which was 8% in the rejectors and 25% in the non-rejectors (p=ns). No association was found with any allele or genotype by comparing rejectors and non-rejectors with <3 HLA mismatches (Table 7.3.2).

In donors with an HLA-A mismatch, the most common allele in the rejection group was the 2 allele which was found in 13 (30%) compared to 4 (12%) in the non-rejectors (p=ns). The most common allele in the non-rejectors was the 10 allele which was found in 11 (32%) donors compared with 6 (14%) in the rejection group (p=ns). The most common genotype in the rejection group was the 6,7 genotype which was found in 3

(14%) donors compared with 0 (0%) in the non-rejectors (p=ns). In the non-rejection group the most common genotype was the 6,10 genotype which was found in 4 (23%) donors compared with 1 (4%) in the rejection group (p=ns). No association was found between rejectors and non-rejectors with a mismatched HLA-A allele (Table 7.3.3).

In donors with a mismatched HLA-B allele, the most common allele in the rejection group was the 2 allele, which was found in 14 (28%) compared with 4 (15%) in the non-rejection group (p=ns). In the non-rejection group the most common allele was found to be the 10 allele which was found in 11 (42%) donors compared with 8 (16%) in the rejection group (p=ns). The most common genotypes in the rejection group were the 7,10 which was found in 3 (12%) compared to 1 (7%) in the non-rejectors (p=ns) and the 6,7 which was found in 3 (12%) compared to 0 (0%) in the non-rejectors (p=ns). In the non-rejection group the most common genotype was found to be the 6,10 genotype which was found in 4 (30%) of non-rejectors compared with 2 (8%) in the rejection group (p=ns). No association was found between TNFα alleles or genotypes in rejectors and non-rejectors with a mismatched HLA-B allele (Table 7.3.4).

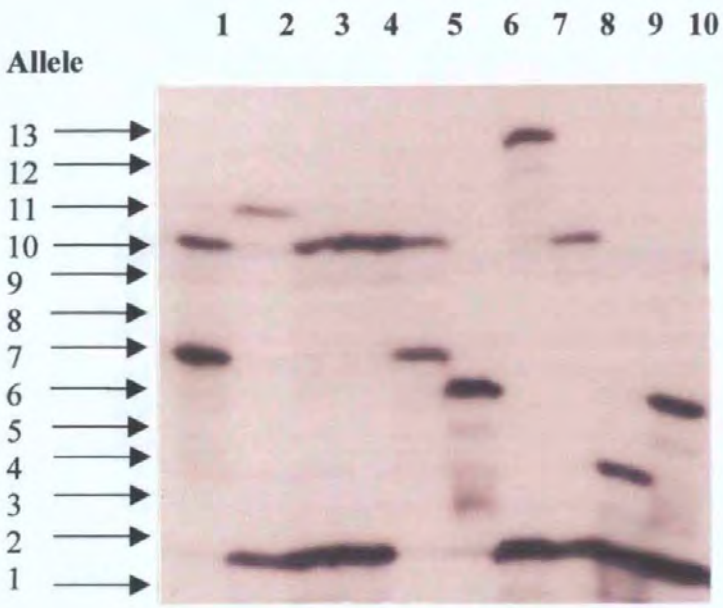


Figure 7.2. Representation of the 13 alleles found in the TNFα CA(n)

Lane 1 – 7/10

Lane 2 – 2/11

Lane 3 – 2/10

Lane 4 – 2/10

Lane 5 – 7/10

Lane 6 – 6/6

lane 7 – 2/12

Lane 8 – 2/10

Lane 9 – 2/4

Lane 10 – 2/6

TNFα	Rejection (n=30)	No Rejection (n=19)	x^2	p value
Allele				
2	30 (18)	15 (6)	1.59	ns
6	13 (8)	21 (8)	0.39	ns
7	13 (8)	5 (2)	0.24	ns
10	18 (11)	31 (12)	0.24	ns
x	25 (15)	26.3 (10)	0.01	ns
Haplotype				
2,2	3 (1)	0 (0)	0.63	ns
2,10	10 (3)	15 (3)	0.28	ns
6,7	10 (3)	0 (0)	1.83	ns
6,10	6 (2)	21 (4)	1.71	ns
10,10	3 (1)	5 (1)	0.10	ns
x,x	66.6 (20)	57.8 (11)	0.09	ns

Table 7.3.1 The percentage frequency of TNFα alleles and haplotypes in transplanted kidneys which were rejected or not. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

TNFα	Rejection (n=25)	No Rejection (n=16)	x^2	p value
Allele				
2	28 (14)	15 (5)	1.07	ns
6	16 (8)	25 (8)	0.67	ns
7	12 (6)	3 (1)	1.69	ns
10	18 (9)	28 (9)	0.74	ns
x	26 (13)	28.1 (9)	0.03	ns
Haplotype				
2,2	4 (1)	0 (0)	0.63	ns
2,10	4 (1)	12 (2)	0.88	ns
6,10	8 (2)	25 (4)	1.64	ns
7,10	12 (3)	0 (0)	1.84	ns
10,10	4 (1)	6 (1)	0.10	ns
x,x	68 (17)	56.2 (9)	0.13	ns

Table 7.3.2 The percentage frequency of TNFα alleles and haplotypes in transplanted kidneys with less than 3 mismatched alleles. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

TNFα	Rejection (n=21)	No Rejection (n=17)	x^2	p value
Allele				
2	30.9 (13)	11.7 (4)	2.59	ns
6	14.2 (6)	23.5 (8)	0.73	ns
7	16.6 (7)	5 (2)	1.67	ns
10	14.2 (6)	32.3 (11)	2.22	ns
x	23.8 (10)	26.4 (9)	0.04	ns
Haplotype				
2,2	4.7 (1)	0 (0)	0.79	ns
2,10	9.5 (2)	11.7 (2)	0.04	ns
6,10	4.7 (1)	23.5 (4)	2.20	ns
6,7	14.2 (3)	0 (0)	2.29	ns
7,10	9.5 (2)	5.8 (1)	0.15	ns
10,10	0 (0)	5.8 (1)	0.79	ns
x,x	57 (12)	52.9 (9)	0.02	ns

Table 7.3.3 The percentage frequency of TNFα alleles and haplotypes in transplanted kidneys with at least one mismatched HLA-A allele. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

TNFα	Rejection (n=25)	No Rejection (n=13)	x^2	p value
Allele				
2	28 (14)	15.3 (4)	0.96	ns
6	16 (8)	15.3 (4)	0.00	ns
7	16 (8)	3 (1)	1.98	ns
10	16 (8)	42.3 (11)	3.59	ns
x	24 (12)	23 (6)	0.00	ns
Haplotype				
2,10	12 (25)	15.3 (2)	0.07	ns
6,10	8 (2)	30.7 (4)	2.30	ns
6,7	12 (3)	0 (0)	1.50	ns
7,10	12 (3)	7 (1)	0.14	ns
10,10	0 (0)	7 (1)	1.83	ns
x,x	56 (14)	38.4 (5)	0.37	ns

Table 7.3.4 The percentage frequency of TNFα alleles and haplotypes in transplanted kidneys with at least one HLA-B mismatched allele. Only the most common types are shown. The actual number is shown in brackets. X represents other alleles or haplotype combinations.

7.4 Restriction digest analysis

A total of 67 donor DNA samples were available for restriction digest analysis. 49 samples were available from the previous microsatellite study, and the 17 DNA samples from the preservation solution donors were also available for analysis. From the previous study 49 samples had data available for analysis. From the 17 new samples, 1 had no data on HLA matching or acute rejection episodes so was excluded. This made a total of 65 donors.

7.4.1 HLA matching

A total of 35 (53.3%) of the donors had at least one rejection episode when transplanted. 30 (46.2%) had no rejection episodes. In the rejection group 28 (51.9%) had less than 3 mismatched alleles, compared with 26 (48.1%) in the non-rejection group. 25 (71.4%) of the rejectors had HLA-A mismatched recipients and this was found in 25 (83.3%) of the non-rejectors. HLA-B mismatches were found in 29 (58%) of rejected grafts compared with 21 (42%) in the non rejectors.

7.5 IL-6 G (-174) C

In both the rejection groups the most common allele was the G allele which was found in 39 (55.7%) rejectors compared with 32 (53.3%) in the non-rejection group (p=ns). The most common genotype in both the rejection group and non-rejection group was the GC which was found in 19 (54.3%) rejectors and 12 (40%) non-rejectors (p=ns). No association was found between IL-6 alleles or genotypes when comparing rejectors and non-rejectors (Table 7.4.1). In donors with <3 mismatches the most common allele in both the rejection groups was the G allele, which was found in 33 (60%) rejectors compared with 28 (53.8%) non-rejectors (p=ns). The most common genotype in both the rejection groups was the GC genotype which was 12 (42.8%) in the rejectors compared with 16 (38.5%) in the non-rejectors (p=ns). No association was found with any allele or genotype by comparing rejectors and non-rejectors with <3 HLA mismatches (Table 7.4.2). In donors with an HLA-A mismatch, the most common allele in the rejection group was the C allele, which was found in 27 (54%) compared with 24 (48%) in the non-rejectors (p=ns). The most common allele in the non-rejectors was the G allele which was found in 28 (53.8%) compared with 23 (46%) in the rejectors. The most common genotype in both the rejection group and non-rejection group was the GC genotype which was found in 27 (54%) rejectors and 10 (40%) non-rejectors (p=ns). No association was found between rejectors and non-rejectors with a mismatched HLA-A allele (Table 7.4.3). In donors with a mismatched HLA-B allele, the most common allele in the rejection group was the G allele, which was found in 32 (55.2%) compared with 20 (47.6%) in the non-rejection group (p=ns). In the non-rejection group the most common allele was found to be the C allele which was found in 22 (52.4%) donors compared with

26 (44.8%) in the rejection group (p=ns). The most common genotypes in both the rejection groups was the GC, which was found in 16 (55.2%) in the rejectors and 8 (38.1%) in the non-rejectors (p=ns). No association was found between IL-6 alleles or genotypes in rejectors and non-rejectors with a mismatched HLA-B allele (Table 7.4.4)

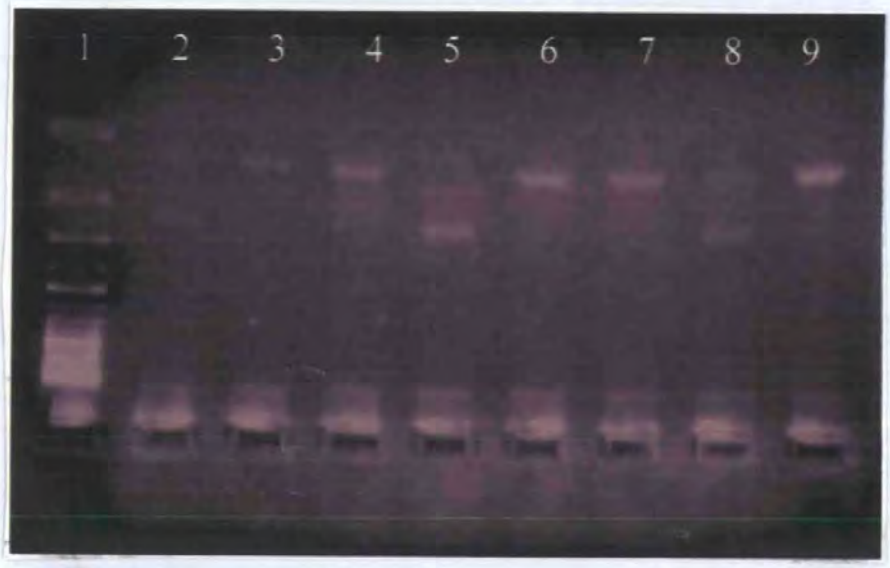


Figure 7.3. Representation of the IL-6 alleles created by the restriction enzyme nlaIII.

Lane 1 - 100 base pair ladder

Lane2 - GC heterozygote

Lane 3 - CC homozygote

Lane 4 - CC homozygote

Lane 5 - GG homozygote

Lane 6 – CC homozygote

Lane 7 – CC homozygote

Lane 8 – GC heterozygote

Lane 9 – CC homozygote

IL-6 C(-174)G	Rejection (n=35)	No Rejection (n=30)	x^2	p value
Allele				
G	55.7 (39)	53.3 (32)	0.02	ns
C	44.3 (31)	46.7 (28)	0.03	ns
Haplotypes				
GG	28.6 (10)	33.3 (10)	0.09	ns
GC	54.3 (19)	40 (12)	0.47	ns
CC	17.1 (6)	26.7 (8)	0.56	ns

Table 7.4.1 The percentage frequency of IL-6 alleles and haplotypes in transplanted kidneys which were rejected or not. The actual number is shown in brackets.

IL-6 C(-174)G	Rejection (n=28)	No Rejection (n=26)	x^2	p value
Allele				
G	**60 (33)**	**53.8 (28)**	**0.08**	**ns**
C	**40 (23)**	**46.2 (24)**	**0.11**	**ns**
Haplotypes				
GG	**39.3 (11)**	**34.6 (9)**	**0.06**	**ns**
GC	**42.8 (12)**	**38.5 (10)**	**0.05**	**ns**
CC	**17.9 (5)**	**26.9 (7)**	**0.41**	**ns**

Table 7.4.2 The percentage frequency of IL-6 alleles and haplotypes in transplanted kidneys with less than 3 mismatched alleles. The actual number is shown in brackets.

IL-6 C(-174)G	Rejection (n=25)	No Rejection (n=25)	x^2	p value
Allele				
G	**46 (23)**	**52 (26)**	**0.12**	**ns**
C	**54 (27)**	**48 (24)**	**0.12**	**ns**
Haplotypes				
GG	**16 (4)**	**32 (8)**	**1.08**	**ns**
GC	**60 (15)**	**40 (10)**	**0.67**	**ns**
CC	**24 (6)**	**28 (7)**	**0.06**	**ns**

Table 7.4.3 The percentage frequency of IL-6 alleles and haplotypes in transplanted kidneys with at least one HLA-A mismatch. The actual number is shown in brackets.

IL-6 C(-174)G	Rejection (n=29)	No Rejection (n=21)	x^2	p value
Allele				
G	55.2 (32)	47.6 (20)	0.18	ns
C	44.8 (26)	52.4 (22)	0.19	ns
Haplotypes				
GG	27.6 (8)	28.6 (6)	0.00	ns
GC	55.2 (16)	38.1 (8)	0.51	ns
CC	17.2 (5)	33.3 (7)	1.04	ns

Table 7.4.4 The percentage frequency of IL-6 alleles and haplotypes in transplanted kidneys with at least one HLA-B allele mismatch. The actual number is shown in brackets.

7.6 NAD(P)H C(-242)T

In the both the rejection groups the most common allele was the C allele which was found in 54 (77.1%) of rejectors and 43 (71.6%) in the non-rejection group (p=ns). The most common genotype in both the rejection group and non-rejection group was the CC which was found in 19 (54.3%) rejectors and 15 (50%) non-rejectors (p=ns). No association was found between NAD(P)H alleles or genotypes when comparing rejectors and non-rejectors (Table 7.5.1). In donors with <3 mismatches the most common allele in both the rejection groups was the C allele, which was found in 43 (76.8%) rejectors compared with 37 (71.2%) non-rejectors (p=ns). The most common genotype in both the rejection groups was the CC genotype which was 15 (53.6%) in the rejectors compared with 13 (50%) in the non-rejectors (p=ns). No association was found with any allele or genotype by comparing rejectors and non-rejectors with <3 HLA mismatches (Table 7.5.2). In donors with an HLA-A mismatch, the most common allele in both rejection groups was the C allele, which was found in 40 (80%) in rejectors and 37 (74%) in the non-rejectors (p=ns). The most common genotype in both the rejection group and non-rejection group was the CC genotype which was found in 15 (60%) rejectors and 13 (52%) non-rejectors (p=ns). No association was found between rejectors and non-rejectors with a mismatched HLA-A allele (Table 7.5.3).

In donors with a mismatched HLA-B allele, the most common allele in the both rejection groups was the C allele, which was found in 46 (79.3%) rejectors compared with 31 (73.8%) in the non-rejection group (p=ns). The most common genotypes in both the rejection groups was the CC, which was found in 17 (58.6%) in the rejectors and 11

(52.4%) in the non-rejectors (p=ns). No association was found between alleles or genotypes in rejectors and non-rejectors with a mismatched HLA-B allele (Table 7.5.4).

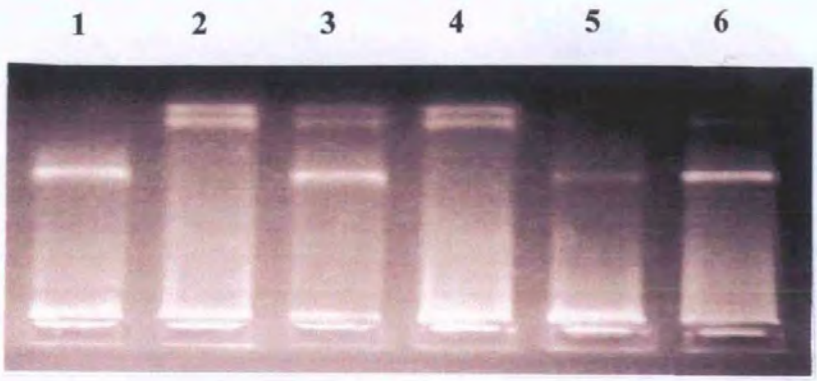


Figure 7.4. Alleles of the NAD(P)H p22phox subunit cut with the Rsa 1 restriction enzyme

Lane 1 – CC homozygote

Lane 2 – TT homozygote

Lane 3 – CT heterozygote

Lane 4 – TT homozygote

Lane 5 – CC homozygote

Lane 6 – CT heterozygote

NAD(P)phox C(242)T	Rejection (n=35)	No Rejection (n=30)	x^2	p value
Allele				
C	77.1 (54)	71.6 (43)	0.07	ns
T	22.8 (16)	28.3 (17)	0.30	ns
Haplotypes				
CC	54.3 (19)	50 (15)	0.04	ns
CT	45.7 (16)	43.3 (13)	0.01	ns
TT	0 (0)	6.6 (2)	2.25	ns

Table 7.5.1 The percentage frequency of NAD(P)H alleles and haplotypes in transplanted kidneys which were rejected or not. The actual number is shown in brackets.

NAD(P)H C(-242)T	Rejection (n=28)	No Rejection (n=26)	x^2	p value
Allele				
C	76.8 (43)	71.2 (37)	0.07	ns
T	23.2 (13)	28.8 (15)	0.26	ns
Haplotypes				
CC	53.6 (15)	50 (13)	0.02	ns
CT	46.4 (13)	42.3 (11)	0.04	ns
TT	0 (0)	7.7 (2)	2.07	ns

Table 7.5.2 The percentage frequency of NAD(P)H alleles and haplotypes in transplanted kidneys with less than 3 mismatched alleles. The actual number is shown in brackets.

NAD(P)H C(-242)T	Rejection (n=25)	No Rejection (n=25)	x^2	p value
Allele				
C	80 (40)	74 (37)	0.07	ns
T	20 (10)	26 (13)	0.32	ns
Haplotypes				
CC	60 (15)	52 (13)	0.09	ns
CT	40 (10)	44 (11)	0.03	ns
TT	0 (0)	4 (1)	0.98	ns

Table 7.5.3 The percentage frequency of NAD(P)H alleles and haplotypes in transplanted kidneys with at least one HLA-A mismatch. The actual number is shown in brackets.

NAD(P)H C(-242)T	Rejection (n=29)	No Rejection (n=21)	x^2	p value
Allele				
C	**79.3 (46)**	**73.8 (31)**	**0.05**	**ns**
T	**20.7 (12)**	**26.2 (11)**	**0.26**	**ns**
Haplotypes				
CC	**58.6 (17)**	**52.4 (11)**	**0.05**	**ns**
CT	**41.4 (12)**	**42.8 (9)**	**0.00**	**ns**
TT	**0 (0)**	**4.8 (1)**	**1.34**	**ns**

Table 7.5.4 The percentage frequency of NAD(P)H phox alleles and haplotypes in transplanted kidneys with at least one HLA-B allele mismatch. The actual number is shown in brackets.

7.8 Discussion

There was no association found between any of the polymorphisms studied and acute rejection episodes in the donor kidneys studied. This was independent of HLA matching as no associations were found with <3 mismatches or HLA-A or HLA-B mismatched kidneys. There were insufficient numbers of HLA-DR mismatched kidneys for analysis of this group. The observed and expected frequencies of genotypes confirmed Hardy-Weinberg equilibrium.

In this small number of donors, previous reports of an association between the IL-6 C allele and rejection in donors (Marshall *et al* 2001) could not be confirmed. Similarly, an association between the TNFα CA(n) microsatellite allele 9 (113bp) and rejection in recipients (Asano *et al* 1997) could not confirmed in donors. However, in kidneys with at least one mismatched HLA-B allele the frequency of the 10 allele was 42.3% in the non-rejection group compared with 16% in the rejection group (x^2 3.59 p=0.05 uncorrected). This allele should be investigated in a larger number of donors as a possible protective function in the rejection free group.

The NAD(P)H C(242)T polymorphism has not been previously investigated in transplant recipients or donors. The potentially protective TT genotype was found in 6.6% of non-rejectors compared with 0% in the rejection group (x^2 2.25 p=0.10). This should also be investigated in a larger number of donors.

CHAPTER EIGHT: DISCUSSION

This project has used endothelial cells as a model of kidney graft cells to determine the effects of hypothermia and rewarming associated with cold ischaemia and reperfusion on endothelial cells. Endothelial cells are thought to play a role in initiating an immune response (Pober & Cotran 1990, Pober *et al* 1996, Briscoe *et al* 1998). The endothelium is also a vital source of thrombotic proteins that may play a role in vascular rejection and graft coagulation (Ishii *et al* 1991, Esmon 1993, Hefty *et al* 1993, Lagoo *et al* 2000). Recent data has suggested that during injury or stress, endothelial cells may upregulate key immunogenic factors such as MHC class II molecules (Shackleton 1998, Eberl *et al* 1999b), hence ischaemic injury may increase graft immunogenicity (Ono *et al* 1998). Endothelial cells were stressed at 4^0C. The temperature was chosen as it is relevant to perfusion, static cold storage and machine perfusion storage (Marshall *et al* 1994, Massberg & Messmer 1998). Temperatures below 4^0C (nearing 0^0C) can result in the formation of intracellular ice, which is lethal to most cells (Mazur 1970, Mazur 1983, Karlsson *et al* 1993). The initial aim was to try and measure the effects that cold injury might have on immunogenicity and the prothrombotic process.

Hypoxia is associated with ischaemia and is known to cause the generation of ROI and ROS which are known to play a major role in many diseases and during stress (Hochachka 1986, Chen & Fang 1987, Ogawa *et al* 1990, Seigneur *et al* 1994, Faller 1999). Endothelial cells were rendered hypoxic to compare the effects of hypoxia with hypothermia. By using lactate as an indicator of a switch towards carbohydrate metabolism and hence hypoxia, it was shown that cells placed at 4^0C for 48 hours produce an equivalent amount of lactate as cells placed in an hypoxic incubator for the same amount of time. The role of oxidative stress in reperfusion injury is well known

(Wei 1999) however, the production of ROS during hypothermia is not well investigated. It is thought that hydrogen peroxide may function as a second messenger activating numerous cellular signal transduction pathways and redox regulated genes (Muller *et al* 1997). It was found that H202 was produced during hypothermia in endothelial cells, but did not reach the level found during hypoxia. This demonstrated that hypothermia is associated with hypoxia, but not ROI and that although oxidative stress is likely to be important in ischaemic injury, its role in hypothermia may not be so important. This may be due to the fact that the preservation solution used contained mannitol, which is an anti-oxidant and may have prevented the production of H202 by the cells. The next aim was to determine if this environment was actually damaging the cells or resulting in their death or loss of viability.

A marker of endothelial cell dysfunction is VWF (Blann 1993, Lip & Blann 1997). It is known that a release of VWF is associated with stress to endothelial cells (Sporn *et al* 1986, Pearson 1993). There was no sudden release of VWF in the endothelial cultures used in these experiments, suggesting that the cells although 'stressed' by a low temperature, that they would not normally experience *in vivo*, were not 'stressed enough' to release a classic marker of endothelial dysfunction. This may again in part be due to the fact that the cells were re-suspended in a preservation solution designed to try and enable cell survival, containing electrolytes to prevent osmotic damage and cell swelling. Further to the finding that the cells did not release VWF was the fact that cell viability remained above 95% for 48 hours of hypothermic stress. It has previously been shown that endothelial cells have a high viability after 48 hours of cold storage and the figure quoted in one study was 79% viable after 48 hours at 4^0C (Hansen *et al* 1994) which was

very similar to the percentage viability found in this study (82%). This data suggests that endothelial cells are resistant in some way to hypothermic-induced stress at 4^0C (Gerlach *et al* 1993, Hansen *et al* 1994). The analysis of DNA fragmentation as an indicator of cellular apoptosis also showed that cells were not killed by the hypothermic insult, as DNA fragmentation was not seen during the hypothermic storage period studied. Further there was a consistent expression of Hsp70 and a slight up-regulation of BCL-2 during the later stages of the time course investigated. Both of these proteins are linked to the inhibition of apoptosis via preventing of the formation of the apoptosome and inhibiting cytochrome-c release in the mitochondria (Badrichani *et al* 1999, Morano & Thiele 1999).

This data demonstrated that hypothermia was not a lethal stress to endothelial cells, so the next stage was to investigate the effect at the molecular level on the activation of signal transduction pathways and transcription factors.

The transcription factor NFκB has been widely investigated in numerous diseases since its discovery, but little is known about its response to hypothermia (Ricciardi *et al* 2000). It was found to be up-regulated within minutes of hypothermia. This rapid response is typical of the NFκB system (Baeuerle & Henkel 1994, Baldwin 1996) and further investigation demonstrated, as expected the phosphorylation of IκBα within minutes of hypothermia as well. This rapid response is likely to be mediated by a signal transduction pathway that would link the stress signal to the transcription factor activation.

MAPK cascades are thought to be involved in the phosphorylation of IκBα, but there is some debate in the literature as to their role in NFκB activation (Schulze-Osthoff *et al*

1997). The role of a kinase cascade is to transduce a message from the cell surface to the nucleus after receiving a stimulus. Both ERK 1 /2 and p38 MAPKs were found to be up-regulated within minutes of hypothermia and correlated with NFκB activation. This was particularly significant during the initial 30 minutes of hypothermia. The correlation between NFκB and p38 was found to be highly significant (R^2 0.77 $p<0.01$) and the relationship between NFκB and ERK 1 / 2 was significant throughout the 72 hour time course (R^2 0.33 $p<0.05$). The JNK SAPK was not up-regulated by hypothermia, with no difference found in phosphorylation between controls and cells stressed by hypothermia, indicating that this MAPK is not involved in the response to hypothermia.

Interestingly, the JNK pathway has been linked to apoptosis (Wang *et al* 1998a Tourniet *et al* 2000). The absence of JNK activation was concurrent with the absence of apoptosis in this model. Similarly, NFκB has been suggested to be an anti-apoptotic protein (Kajino *et al* 2000). Inhibition of NFκB with the SN50 inhibitory peptide did not result in cell death. Viability remained above 95% when NFκB was inhibited and no fragmentation of DNA was seen. However, this demonstrates that NFκB can be inhibited in endothelial cells without inducing cell death or affecting viability under hypothermic conditions.

In order to determine which of MAPK's are involved in NFκB activation, specific inhibitors were used. The PD98059 inhibitor was used to block ERK 1 /2, however this inhibitor blocks MEK, which is an upstream kinase involved in the activation of all MAPK's (Marshall 1994). The SB203589 inhibitor was used as it specifically blocks the p38 MAPK. Although the p38 MAPK was significantly associated with NFκB activation, treatment of cells with the SB203580 inhibitor was not found to prevent the activation of NFκB during the first 30 minutes of hypothermia. However, inhibition of ERK 1 / 2 with

PD98059 was found to prevent subsequent NFκB activation. As this inhibitor is working at the level of MEK, the data suggests that an interaction between MAPK's may be important in NFκB activation. The inhibition of NFκB with the PD98059 inhibitor was found to be associated with a reduction in IκBα phosphorylation.

Other important transcription factors were also found to be activated by hypothermic stress in endothelial cells. The HSF1 transcription factor was found to be expressed at a high rate in control cells in a similar fashion to Hsp70. Despite showing a peak after 2 hours of hypothermia, HSF1 did not reach the control levels seen during hypothermia. The HSF1 transcription factor is involved in the regulation of the heat shock proteins and the expression of Hsp70 followed a similar trend to the HSF1 transcription factor during hypothermia.

The transcription factor AP1 was also activated during hypothermia, and was found to peak after 12 hours. This was in contrast to the activation of NFκB within minutes, however, the AP1 transcription factor is formed as a heterodimer complex of c-jun and c-fos proteins and is not pre-formed in the cell as NFκB is.

Reperfusion was investigated by re-warming the cells back to 37^0C after hypothermia.

One of the main effects of reperfusion injury *in vivo* is the sudden availability of oxygen that results in the formation of ROIs. In the model in this study this was difficult to reproduce. Hypothermic experiments were carried out in sealed tubes, so for reperfusion experiments the lids were open to allow oxygen to enter.

A short time course of 30 minutes and a longer time of 12 hours were chosen and cells returned to 37^0C for up to 1 hour in order to investigate the effects of reperfusion. In a similar fashion to what was observed during hypothermia, endothelial cells had viability

above 95% throughout the time course studied. DNA fragmentation was also absent during the reperfusion experiments. There was no increase in either NFκB, AP1 or HSF1 transcription factor activity during reperfusion at both the time points studied. Reperfusion also had no effect on the ERK 1 / 2 and p38 MAPK's, however, JNK was largely up-regulated during reperfusion after 30 minutes of hypothermia. Reperfusion after 12 hours of hypothermia had less effect on JNK activity. JNK is thought to be activated predominantly by reperfusion or re-oxygenation (Bogoyeritch *et al* 1996, Laderoute & Webster 1997, Crenesse *et al* 2000a). This further suggests that the JNK MAPK is not involved in the response to hypothermia.

Reperfusion is likely to be an important factor in graft injury. This culture system offered the opportunity to study the effects of 're-warming' on endothelial cells, but was of limited use for studying the effects of hypothermia followed by re-warming and re-oxygenation as would be expected *in vivo*. Many of the pathways studied are redox sensitive and therefore the effects of reperfusion in relation to the production of free radicals is likely to important.

The downstream effects of the activation of NFκB were investigated by the production of cytokines. Previous studies have shown cytokine production during hypothermia is variable in respect to the levels found (Wang *et al* 1998b, Eberl *et al* 1999b). The cytokines studied were IL-6, IL-8 and TNFα. These were chosen as they all contain κB binding sites in their genes and are rapidly released in a stress responses, either due to the fact that they are preformed in cells or are quickly synthesized.

IL-6 was found to have a large peak in production after 12 hours of hypothermia, which was consistent with both NFκB and AP1 activation. The IL-6 gene contains both NFκB

and AP1 binding sites (Chandrasekar *et al* 1999). It has previously been shown that the co-binding of these two transcription factors is required for the synthesis of many cytokine genes including IL-6 and IL-8 (Matsui *et al* 1999, Oppenheim *et al* 1991). This peak at 12 hours was significantly reduced when the NFκB inhibitor was added before the experiment.

IL-8 was also produced during hypothermia and was significantly upregulated from control cells at all time points studied. The addition of SN50 significantly reduced IL-8 levels at 12 hours but did not reduce levels at the other time points. TNFα was also produced by the endothelial cells during hypothermia with an early peak at 6 hours which was maintained at 12 hours and a third peak at 72 hours was seen. Addition of SN50 significantly reduced the third peak at 72 hours. This data demonstrates that NFκB has a role to play in the expression of these cytokines during hypothermia, as its inhibition resulted in the reduced production of them. However, levels of cytokines were not blocked completely when inhibiting NFκB and it is likely that other factors are involved in the regulation of these cytokines. Reperfusion caused a large increase in IL-6 after 30 minutes of hypothermia, but not after 12 hours. IL-8 and TNFα levels were unaffected by reperfusion. Consistent with previous findings, hypoxia induced a large production of all three cytokines (Yan *et al* 1997ab, Li *et al* 1999).

Preservation solutions can easily be modified to contain such inhibitors if these or other cytokines are shown to have an effect on graft function. The next stage of this study was to try and determine firstly, if these cytokines are found in grafts and secondly if they have any effect on early graft function. The primary function of the graft is thought to be the most likely place that damage occurring to the graft during its procurement and

storage (Tilney *et al* 1997). Early graft function is known to be an important indication of the likely subsequent function of the graft (Ojo *et al* 1997, MacLaren *et al* 1999).

In the preservation solutions collected from donors, there was a large variation in levels of cytokines. The range was as high as a 117-fold difference between samples with IL-4 and 40-fold difference for IL-8. This suggests heterogeneity in the amount of cytokines produced by donor kidneys during storage. However, it is difficult to determine exactly where these cytokines have originated from, and what exactly induced their secretion. Land *et al* first demonstrated that high levels of IL-6 can be found in the donor before organ removal, which may be attributable to the donors death (Land *et al* 1998a). Perfusion during organ removal would be expected to 'flush' the kidney, removing any residual cytokines in the graft. The cytokines found in the solutions after static storage could be attributable to the ischaemic times or as a result of the donors death. Data was not available on the ventilation times and perfusion techniques used in these donors as the kidneys were imported from other Hospitals. It is possible that the cytokines are in the solutions as graft cells as a result of the donors death release them. However, their role as an indicator of subsequent graft function would be of great use irrespective of their origin.

Levels of IL-6 were found to correlate with increasing WI times. Similarly IL-6 levels from kidneys with greater then 30 minutes WI time were higher than from those with less than 30 minutes WI time. As shown with the hypoxia experiments in endothelial cells, IL-6 is largely up-regulated by hypoxia. Warm ischaemia is associated with hypoxia and is thought to be potentially more damaging than cold ischaemia if prolonged (Sacks *et al* 1973, Florack *et al* 1986).

IL-6 was also up-regulated by reperfusion in the culture system and has been shown to correlate with graft function when measured in the reperfused effluent in liver grafts (Basille *et al* 1999). There was a trend in the data with higher inflammatory IL-6, IL-8 and TNFα in grafts with delayed function and rejection and higher anti-inflammatory IL-4 in grafts with primary function and no-rejection episodes. Recent data has suggested a protective role for IL-4 in transplantation (Kato *et al* 2000ab). The fact that only 17 preservation solutions were available for analysis may have contributed to the lack of statistical significance.

One potential explanation for the range in cytokine levels found in the solutions may be due to certain polymorphisms which the donor may have in cytokine genes. The IL-6 C(–174)G polymorphism is linked to protein producer status (Fishman *et al* 1998). This polymorphism has recently been shown to correlate with rejection episodes in kidney transplant donors (Marshall *et al* 2001). A higher incidence of rejection in patients receiving a kidney from a donor who has the C allele, which is linked to lower production of IL-6 was found.

In the 67 donor samples studied there was no correlation found between either allele or genotype and rejection episode in theses donors. In the 17 preservation solutions there was a higher amount of IL-6 found in donors who have the G (high producer) allele than in those with the C allele. This did not reach significance, but raises the interesting point as to whether cytokine levels found in preservation solutions are as a result of polymorphisms which donors have. One explanation for the link between donor polymorphisms and transplant success is that cytokine producer status in the donor may influence the host immune response. However, another explanation may be that the

ability of the donor kidney to withstand the preservation process is linked to the production of certain cytokines, which may be protective or detrimental to graft function. A study of a large number of donor kidneys with a link between cytokine producer genotype, and cytokine levels in preservation solution or early perfused effluent would generate interesting data. The *in vitro* model has shown that inhibitor can be used to reduce cytokine production and these could easily be added to preservation solutions.

The microsatellite polymorphism of the TNFα gene has previously been shown to be associated with rejection in both kidney and heart transplant recipients (Asano *et al* 1997, Abdallah *et al* 1999). In our donor population there was no association found between TNFα microsatellite alleles or genotypes and rejection episodes. However, there was an increase in the frequency of the 2 allele in rejectors, which was most frequent when categorising donors with HLA-A mismatched alleles (x^2 2.59). The 6,10 genotype was not significantly different, but was higher in non-rejectors (x^2 3.04).

The number of donors in this study was relatively low, but if the same frequency was found with twice the number of donors, a significant difference would be found. Therefore, the TNFα microsatellite polymorphism should be investigated in a larger number of kidney transplant donors to determine if this allele and genotype are associated with rejection.

It is important that novel gene polymorphisms are studied to determine if there are any associations with rejection or graft survival. The NFκB gene contains a microsatellite polymorphism that has not previously been investigated in transplant donors or recipients. This polymorphism was not associated with rejection in the donors in this study. The NAD(P)H system is a major producer of the superoxide radical. The

polymorphism of the NAD(P)H oxidase p22 subunit has been shown to protect certain individuals from heart disease and is known to effect the heme binding site in the gene and is hence likely to effect the function of the subunit (Inoue *et al* 1998, de Boer *et al* 1992). This polymorphism has not been investigated in transplant patients or donors. There was no correlation found between this polymorphism and rejection in 67 donors. However, the potentially protective TT genotype was absent in the rejection group but present in 7% of the non-rejection group (x^2 2.07). This genotype should be investigated in a larger number of donors.

It is likely that numerous pathways are activated during ischaemic injury and this study has focused on one of potentially many. The difficulty is integrating a wealth of data on ischaemia and reperfusion into a model of graft injury that can be used to identify potential interventions or therapeutic targets. Events occurring to the donor during organ retrieval and the donors death, in combination with ischaemia lead to stress to graft cells. The first problem is that cells are likely to react in differing ways to this. For example, endothelial cells were resistant to death induced by cold, however, previous studies have demonstrated cold-induced apoptosis in tumour cells (Kruman *et al* 1992) and hepatocytes (Rauen *et al* 1999). The activation of stress pathways will lead to the production of inflammatory cytokines and stress proteins, which will work in an autocrine fashion to up-regulate adhesion molecules, co-stimulatory molecules and MHC class II molecules as well as to further induce cytokine production. The production of these cytokines and the response of the graft cells to stress will also be dependent of polymorphisms within important genes such as the cytokines or transcription factors that regulate them. This creates high/low producers and differing responses in individuals.

This creates a balance of many factors in combination that will affect graft function. Further to this there many factors in the recipient to take into account, such as polymorphism and HLA matching. The inflammatory and stress responses are evolutionary conserved responses that have evolved as a protective response to stress. Therefore, elements of an inflammatory reaction may have a protective function.

Conserved stress response mediators such as NFκB may therefore not be an ideal target for inhibition as binding sites are found in numerous genes (Pahl 1999). There is currently still debate as to whether it is pro- or anti-apoptotic and binding sites for both pro-inflammatory and anti-inflammatory proteins have been identified (Senftlebent & Karin 2002).

The aim of studying the effects of ischaemia and reperfusion injury are therefore to try and identify markers of graft damage which could be used to determine the quality of an organ. By studying the molecular biology of stress, a better understanding of the effects occurring at a cellular level during organ procurement may lead to interventions which could improve the quality of organs by reducing the injury caused by retrieval and storage.

In the shorter term, this information together with the increasing information from genetic associations with graft survival and rejection may lead to more tailored treatments for recipients. For example the amount of immunosuppression needed by a patient may be determined by his/her cytokine producer status, donors ischaemic times and cytokine genotypes in combination with information on the quality of the graft when it arrived for surgery. This would be of undoubted benefit to the patient as immunosuppression is associated with numerous long term and undesirable side-effects.

The ultimate aim would be to use non-invasive markers, such as IL-6 to gain information on the quality of organs to be able to predict graft outcome, tailor immunosuppression to the recipient or allow a wider organ pool by incorporating NHBD or marginal donors.

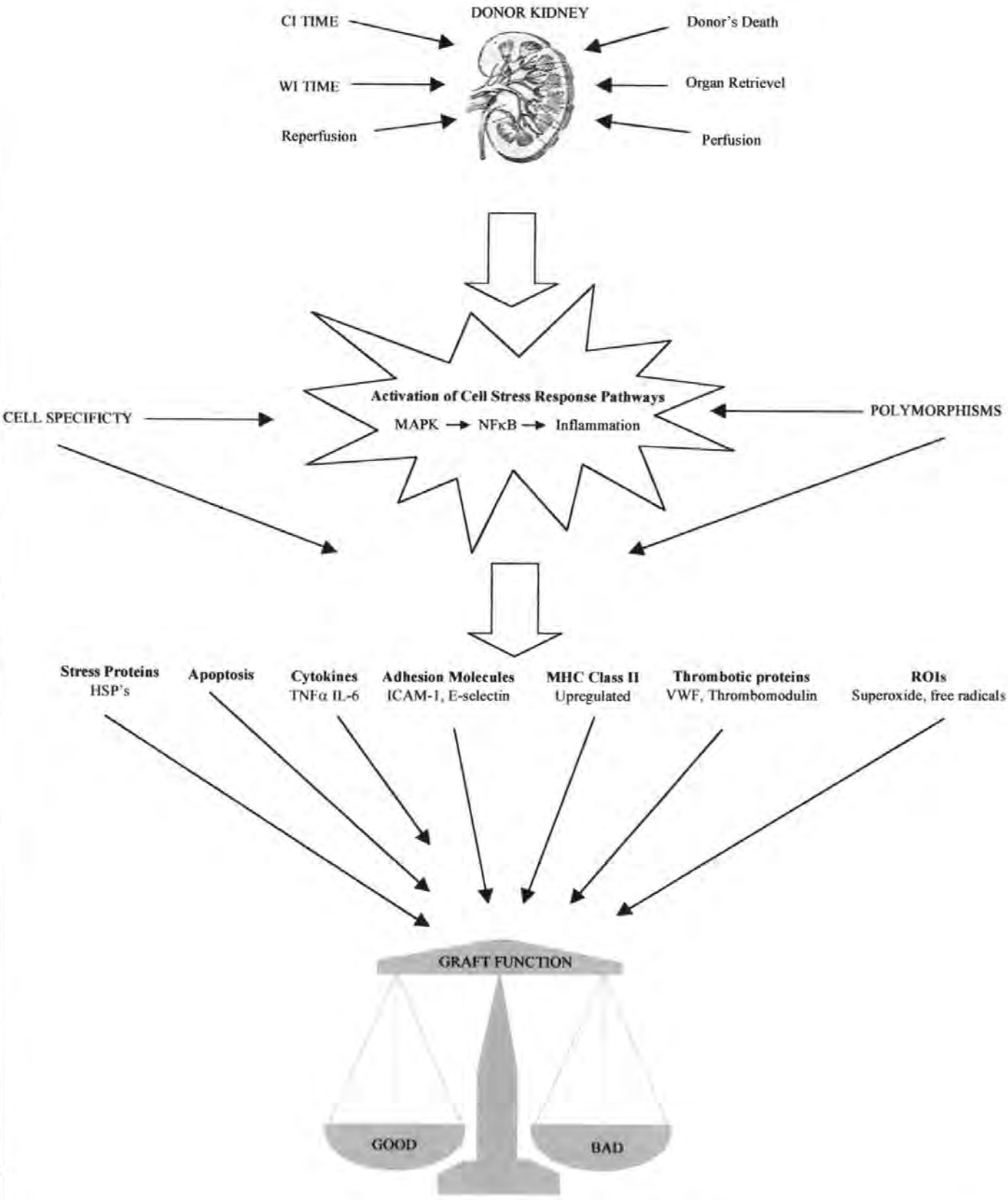


Figure 8.1. A basic overview of events during donor organ preservation that may effect graft outcome. The balance between successful organ retrieval and the detrimental effects of cell stress is dependent of many variables.

8.1 Conclusions from this thesis

NFκB is activated by hypothermic stress and the MAPK cascades ERK 1 / 2 and p38 are likely to be involved in this. ERK 1 /2 activation may be linked to IκBα phosphorylation. IL-6, IL-8 and TNFα are up-regulated during hypothermia and this can be reduced by inhibiting NFκB with an ODN. Hypothermia does not induce apoptosis in endothelial cells and cells maintain a high viability during hypothermia and reperfusion.

Cytokines are measurable in renal preservation solutions collected from static stored kidneys. IL-6 levels are elevated in kidneys that have experienced greater than 30 minutes warm ischaemia. Donors with the IL-6 high producer genotype have higher IL-6 levels in preservation solutions after static storage, regardless of ischaemia times.

Polymorphisms of the NAD(P)H oxidase phox22 subunit and NFκB CA(n) repeat polymorphism do not correlate with rejection or early graft function in donor kidneys.

8.2 Future work arising from this thesis

To collect a large number of preservation solutions from kidney grafts and to correlate protein levels of IL-6 with producer status. IL-6 may be a useful marker of graft injury, or predictor of rejection.

To use NFκB inhibitors in preservation solutions.

To further investigate the role of MAPK cascades in transcription factor activation

To determine why endothelial cells are resistant to hypothermic induced cell death and to determine if these survival pathways can be selectively switched on or off in other cell types to improve the longevity of cells during stress.

Date of sample	Cold ischaemic time	Warm ischaemic time	HLA match	Primary/delayed function	rejection episodes	Creatinine at 1 month
10/10/00	19 hours 30 mins	65 minutes	110	Primary	2 grade 2 vascular rejections	207
02/03/01 Durham	13 hours 40 mins	20 minutes	121	delayed	Nephrectomy due to renal artery thrombosis	
02/03/01	16 hours 20 mins	20 minutes	212	delayed	2 episodes treated.	752
23/03/01	37 hours 21 mins	22 minutes	110	primary	Nil	110
02/05/01	15 hours	45 minutes	000	primary	2 episodes. Nephrectomy due to severe vascular rejection	
18/05/01	21 hours 36 mins	20 minutes	110	primary	Nil	96
23/05/01	33 hours 27 mins	58 minutes	110	delayed	Nil	146
24/05/01	18 hours	45 minutes	010	delayed	Nil	135
31/05/01	20 hours 20 mins	20 mins	110	delayed	1 grade 2 rejection	244
05/06/01	25 hours 7 mins	20 mins	120	delayed	Nil	186
03/07/01	23 hours 46 mins	40 mins	110	primary	Nil	118
4/10/01	19 hours	45 mins	110	Primary	Nil	119
14/11/01	33 hours 2 mins	58 mins	000	Primary	Nil	
28/11/01	18 hours 30 mins	22 mins	000	Primary	Nil	
20/12/01	14 hours 40 mins	20 mins	110	Primary	Nil	
22/12/01	18 hours 19 mins	34 mins		delayed	Nil	
6/01/01	17 hours 19 mins	39 mins	101	Primary	Nil	

Appendix 1. Clinical details of donor kidneys from which preservation solutions were collected and subsequent graft function in the recipient. All donors and recipients were Caucasian. Empty boxes are where data was unavailable.

DONOR	DONOR AGE	RECIPIENT AGE	DONOR GENDER	RECIPIENT GENDER	HLA MATCH	No. OF REJECTION EPISODES
1	40	38	F	M	010	1
2	22	24	F	F	101	2
3	72	41	F	M	111	4
4	41	71	F	M	020	1
5	36	63 / 36	M	M / M	110 / 110	0 / 0
6	32	53	M	M	111	0
7	42	27	M	M	111	1
8	37	37	F	F	000	0
9	63	39	M	F	100	0
10	55	68	F	M	101	0
11	17	26	M	M	200	0
12	27	56	F	F	211	0
13	Exported					
14	Exported					
15	Not used					
16	54	54	M	M	111	1
17	67	30	F	F	100	0
18	26	53	F	M	111	3
19	50	51	F	M	112	3
20	Exported					
21	44	50	F	M	111	1
22	NA	20	M	F	110	0
23	22	39	M	M	110	0
24	NA	35 / 57	M	F / M	121 / 121	2 / 0
25	63	64	F	M	011	2
26	39	32	M	M	221	Neph
27	64	47	M	F	111	1
28	NA	41	M	M	000	0
29	NA	62	F	F	110	Neph
30	Not used		F			
31	47	59	F	M	220	1
32	43	45	M	F	000	1
33	18	48	F	F	111	2
34	67	52	F	M	221	1
35	65	63	M	M	201	1
36	42	45	M	M	110	2
37	52	58	M	M	110	0
38	68	59	F	F	121	3
39	NA	63	M	M	110	0
40	NA	63	F	M	120	0
41	NA	44	F	M	000	1
42	69	55	M	M	111	1
43	49	54 / 30	M	F / M	110 / 120	1 / 3
44	62	55	M	F	011	2
45	Exported					
46	67	37 / 49	F	M / M	110 / 110	0 / 1
47	50	44 / 34	F	M / F	011 / 011	2 / 1
48	58	27	F	F	000	1
49	32	34 / 58	M	M / M	110 / 111	0 / 1
50	51	38	M	M	220	0

Appendix 2. Clinical details of the 50 donor DNA samples received for use in genetic studies. Two numbers are given for donors which both kidneys were harvested. Some kidneys were exported to other Hospitals or not used for technical reasons. These were excluded from the study as subsequent information was not available. Only one donor was non-Caucasoid. All recipients were Caucasian.
Neph = Nephrectomy due to severe rejection. NA = Data not available.

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	0.11, 0.11, 0.03, 0.01, 0.00	0.05	1	0.02	-
PMA	0.46, 0.79, 0.46, 0.23, 0.23	0.43	8.6	0.10	<0.01
5mins	0.31, 0.46, 0.14, 0.14, 0.16	0.24	4.8	0.06	<0.05
10mins	0.19, 0.46, 0.16, 0.07, 0.09	0.19	3.8	0.06	-
15mins	0.13, 0.43, 0.13, 0.04, 0.06	0.15	3.0	0.06	-
30mins	0.28, 0.46, 0.14, 0.12, 0.15	0.23	4.6	0.06	<0.05
1hr	0.17, 0.32, 0.09, 0.06, 0.07	0.14	2.8	0.04	-
2hr	0.17, 0.38, 0.11, 0.06, 0.07	0.15	3.0	0.05	-
4hr	0.14, 0.64, 0.28, 0.04, 0.06	0.23	4.6	0.11	-
6hr	0.29, 0.48, 0.14, 0.16, 0.17	0.24	4.8	0.06	<0.05
12hr	0.18, 0.48, 0.07, 0.07, 0.11	0.18	3.6	0.07	-
24hr	0.39, 0.36, 0.20, 0.23, 0.26	0.28	5.6	0.03	<0.01
48hr	0.30, 0.59, 0.15, 0.15, 0.19	0.27	5.4	0.08	<0.05
72hr	0.16, 0.51, 0.26, 0.13, 0.17	0.24	4.8	0.04	<0.05

Appendix 3 NFκB optical density data taken from image analyser (p value vs control cells).

Reperfusion	Optical Density	Average	SE	P value
30mins	7.62, 8.96, 12.72	9.76	0.88	-
30+15	11.38, 10.58, 14.68	12.21	0.72	ns
30+30	5.12, 11.46, 12.28	9.62	1.30	ns
30+60	11.76, 8.93, 16.30	12.33	1.23	ns
12hr	22.38, 6.19, 23.16	17.24	3.19	-
12+15	22.87, 15.84, 23.88	19.86	2.03	ns
12+30	22.78, 13.13, 24.72	20.21	2.06	ns
12+60	22.35, 11.41, 28.31	20.69	2.85	ns

Appendix 4. NFκB reperfusion optical density data taken from image analyser (p value vs control cells).

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	1.69, 3.12	2.40	1	0.5	-
PMA	3.59, 5.55	4.57	1.9	0.69	ns
5mins	1.22, 4.67	2.94	1.22	1.21	ns
10mins	4.81, 2.65	3.73	1.55	0.76	ns
15mins	5.45, 4.13	4.79	1.99	0.46	ns
30mins	3.37, 4.13	3.75	1.56	0.26	ns
1hr	5.32, 2.97	4.14	1.72	0.83	ns
2hr	7.86, 1.86	4.86	2.02	2.12	ns
4hr	6.30, 2.64	4.47	1.86	1.29	ns
6hr	3.55, 3.38	3.46	1.49	0.06	ns
12hr	3.95, 4.21	4.08	1.70	0.09	ns
24hr	6.36, 3.64	5.0	2.08	0.96	ns
48hr	2.98, 3.28	3.13	1.30	0.1	ns
72hr	4.34, 2.07	3.20	1.33	0.8	ns

Appendix 5 . IκBα optical density data taken from image analyser (p value vs control cells).

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	3.65, 4.66	4.15	1	0.5	-
PMA	7.77, 10.09	8.93	2.15	1.16	-
5mins	6.57, 11.59	9.08	2.18	2.51	-
10mins	5.8, 7.43	6.61	1.59	0.81	-
15mins	6.78, 7.59	7.18	1.73	0.4	<0.05
30mins	6.39, 6.62	6.5	1.56	0.11	-
1hr	6.62, 9.81	8.21	1.97	1.59	-
2hr	9.93, 7.25	8.59	2.06	1.34	-
4hr	11.98, 10.01	10.99	2.64	0.98	<0.05
6hr	11.02, 7.46	9.24	2.22	1.78	-
12hr	16.37, 15.78	16.07	3.87	0.29	<0.01
24hr	12.95, 9.9	11.42	2.75	1.53	-
48hr	11.99, 9.86	10.92	2.63	1.06	<0.05
72hr	12.50, 17.57	15.03	3.62	2.53	-

Appendix 6 AP1 optical density data taken from image analyser (p value vs control cells).

Reperfusion	Optical Density	Average	SE	P value
30mins	10.66, 11.96	11.31	0.45	-
30+15	11.21, 3.17	7.19	2.84	ns
30+30	7.0, 5.89	6.44	0.39	ns
30+60	7.21, 8.49	7.85	0.45	ns
12hr	14.76, 12.05	13.40	0.38	
12+15	12.69, 14.78	13.73	0.37	ns
12+30	20.01, 9.12	14.56	0.49	ns
12+60	18.89, 17.12	18.00	1.13	ns

Appendix 7. AP1 reperfusion optical density data taken from image analyser

Control	IL-6 pg/ml	Average pg/ml	SE	P Value
6hr	2.45, 15.71, 3.89	7.35	3.63	-
12hr	9.47, 16.53, 5.21	10.40	2.85	-
24hr	24.90, 18.99, 6.14	16.67	4.79	-
48hr	18.34, 46.96, 4.58	23.29	10.80	-
72hr	29.65, 56.01, 6.62	30.76	12.35	-
Hypothermia				
6hr	25.58, 16.53, 16.35, 33.56	23.00	4.12	ns
12hr	118.78, 209.38, 111.44, 216.54	164.03	28.3	<0.01
24hr	18.51, 18.99, 15.25, 23.73	19.12	0.57	ns
48hr	36.1, 46.98, 31.93, 56.01	42.75	0.18	ns
72hr	40.85, 40.01, 41.69, 40.87	40.85	0.34	ns
SN50				
6hr	7.71, 15.7	11.70	2.82	ns
12hr	7.81, 30.0	18.90	7.84	<0.05
24hr	10.01, 20.70	15.35	3.77	ns
48hr	6.43, 25.70	16.06	6.81	ns
72hr	10.55, 85.30	47.92	26.42	ns

Appendix 8 IL-6 ELISA data, during hypothermia and with SN50 inhibitor (p values vs control)

Reperfusion	IL-6 pg/ml	Average	SE	P value
30mins	17.60, 13.26, 18.82, 16.46	16.53	1.19	-
30+15	273.50, 380.60, 236.21, 306.83,	299.28	30.7	<0.05*
30+30	202.94, 198.87, 176.93, 178.04,	173.00	6.81	<0.05*
30+60	221.45, 220.94, 203.24, 203.25,	212.22	5.18	<0.05*
12hr	118.78, 209.38, 111.44, 216.54	164.03	28.32	ns
12+15	268.43, 94.75	181.56	61.40	ns
12+30	340.83, 185.96	263.0	54.75	ns
12+60	725.12, 224.86	474.0	88.43	ns
Hypoxia				
6hr	211.60, 253.77, 194.43, 199.86	214.91	13.40	<0.01**
12hr	244.13, 352.01,223.62, 246.51	266.56	28.94	<0.01**
424hr	248.38, 312.51,226.98, 270.41	264.57	18.54	<0.01**
48hr	230.96, 295.72,207.43, 241.22	243.83	18.60	<0.01**
72hr	118.45, 123.29, 202.68, 156.32	150.18	16.80	<0.01**

Appendix 9 IL-6 raw data for reperfusion and hypoxia (*p value vs 30 mins hypothermia **pvalue vs control cells)

Control	IL-8 pg/ml	Average pg/ml	SE	P Value
6hr	0.00, 13.66	6.83	4.82	-
12hr	4.53, 24.38	14.45	7.01	-
24hr	64.73, 85.52	75.12	7.35	-
48hr	180.48, 41.50	110.90	49.13	-
72hr	92.88, 40.81	66.84	18.40	-
Hypothermia				
6hr	242.66, 138.10	190.30	36.96	<0.05
12hr	409.39, 275.20	343.20	47.44	<0.05
24hr	214.36, 151.50	182.92	22.22	<0.05
48hr	276.78, 296.04	286.41	6.80	<0.05
72hr	319.82, 718.38	519.10	70.45	<0.05
SN50				
6hr	25.91, 86.02	55.96	21.25	ns
12hr	46.02, 48.08	47.05	0.72	<0.05
24hr	166.30, 211.37	188.93	16.0	ns
48hr	104.97, 98.54	101.75	2.27	ns
72hr	766.80, 594.78	686.70	60.81	ns

Appendix 10 . IL-8 ELISA data during hypothermia and with SN50 inhibitor (p value Vs Control)

Reperfusion	IL-6 pg/ml	Average	SE	P value
30mins	6.52, 14.09	10.30	2.67	ns
30+15	4.56, 13.52	9.04	3.16	ns
30+30	7.84, 16.79	12.31	3.16	ns
30+60	10.32, 13.81	12.06	1.23	ns
12hr	409.39, 275.20	342.29	47.44	ns
12+15	400.81, 104.97	252.89	104.59	ns
12+30	542.50, 766.80	654.65	79.30	ns
12+60	1271.65, 891.33	1081.49	134.46	ns
Hypoxia				
6hr	413.20, 542.50	477.85	45.70	<0.01
12hr	636.24, 1271.65	953.84	224.6	<0.01
24hr	691.20, 3380.58	2035.89	950.8	<0.01
48hr	686.0, 5512.8	3099.40	1706.5	<0.01
72hr	1006.7, 5602.48	3304.59	1624.8	<0.01

Appendix 11. IL-8 reperfusion and hypoxia (p value vs control cells)

Control	TNFα pg/ml	Average pg/ml	SE	P Value
6hr	6.58, 4.95, 3.90	5.14	0.67	
12hr	5.06, 7.18, 4.14	5.46	0.63	-
24hr	5.06, 8.34, 4.14	5.84	0.90	-
48hr	5.88, 6.75, 3.79	5.47	0.62	-
72hr	3.71, 4.73, 3.90	4.11	0.22	-
Hypothermia				
6hr	6.81, 7.87, 8.10	7.59	0.34	<0.05
12hr	10.86, 8.34, 7.64	8.94	0.84	<0.05
24hr	7.59, 8.24, 7.87	7.90	0.16	ns
48hr	11.18, 7.61, 7.87	8.88	0.81	ns
72hr	8.86, 7.87, 7.41	8.04	0.37	<0.01
SN50				
6hr	8.10, 4.74	6.42	1.18	ns
12hr	7.18, 5.27	6.22	0.67	ns
24hr	7.18, 5.67	6.42	0.53	ns
48hr	6.95, 6.90	6.92	0.01	ns
72hr	6.27, 6.08	6.17	0.06	<0.05

Appendix 12. TNFα production during hypothermia and with SN50 inhibitor (p value Vs control).

Reperfusion	TNFα pg/ml	Average	SE	P value
30mins	1.36, 0.06	0.71	0.45	ns
30+15	0.48, 0.09	0.28	0.13	ns
30+30	1.25, 2.02	1.63	0.27	ns
30+60	1.72, 3.52	2.62	0.90	ns
12hr	27.35, 20.73	24.04	1.65	ns
12+15	21.81, 13.68	17.74	2.87	ns
12+30	30.73, 21.80	26.26	3.15	ns
12+60	38.98, 35.20	37.09	1.33	ns
Hypoxia				
6hr	413.20, 542.50	477.85	45.70	<0.01
12hr	636.24, 1271.65	953.84	224.6	<0.01
24hr	691.20, 3380.58	2035.89	950.8	<0.01
48hr	686.0, 5512.8	3099.40	1706.5	<0.01
72hr	1006.7, 5602.48	3304.59	1624.8	<0.01

Appendix 13. TNFα reperfusion and hypoxia (p value Vs control cells)

Time	Amount μM	Average	SE
Control	22.5, 21.40, 21.80	21.90	0.32
Hypothermia			
6hr	24.60, 20.36, 21.10	22.02	1.30
12hr	24.60, 20.50, 21.50	22.20	1.23
24hr	24.50, 20.83, 21.40	22.24	1.14
48hr	24.70, 21.40, 22.10	22.73	1.0
72hr	23.60, 22.83, 22.70	23.04	0.28
Hypoxia			
6hr	24.50		
12hr	24.30		
24hr	24.30		
48hr	24.50		
72hr	23.60		

Appendix 14. Hydrogen peroxide absorbances at 560nm

Time	Amount mmol/l	Average	SE	p value
Control	0.84, 0.91, 1.24	0.99	0.12	-
Hypothermia				
6hr	2.00, 1.56, 2.97	2.17	0.41	
12hr	1.74, 1.66, 4.19	2.53	0.83	
24hr	3.58, 1.74, 6.58	3.96	1.41	
48hr	6.42, 3.94, 8.61	6.32	1.38	
72hr	6.85, 4.45, 10.73	7.34	1.82	
Hypoxia				
6hr	4.71, 4.92	4.82	0.10	
12hr	8.40, 6.20	7.30	1.09	
24hr	5.57, 6.46	6.01	0.44	
48hr	6.35, 7.41	6.88	0.53	
72hr	5.47, 6.59	6.03	0.56	

Appendix 15 Lactate absorbances at 340nm

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	44.0, 33.89, 8.24	28.71	1.0	6.14	-
5mins	5.6, 3.78, 2.96	4.11	0.14	0.45	ns
10mins	10.2, 5.63, 3.40	6.41	0.22	1.15	ns
15mins	14.81, 8.03, 1.92	8.25	0.28	2.14	ns
30mins	44.41, 27.13, 0.41	23.98	0.83	7.38	ns
1hr	54.55, 32.63, 2.82	30.0	1.04	8.65	ns
2hr	69.98,46.97, 2.96	39.97	1.39	11.35	ns
4hr	61.21, 34.15, 5.33	33.56	1.16	9.31	ns
6hr	51.75, 26.29, 2.14	26.72	0.93	8.26	ns
12hr	42.07, 19.43, 1.33	20.94	0.72	6.80	ns
24hr	42.92, 19.0, 4.22	22.04	0.76	6.50	ns
48hr	61.72, 27.16, 2.80	30.56	1.06	9.86	ns
72hr	42.54, 23.83, 3.91	23.42	0.81	6.43	ns

Appendix 16. HSF1 optical density data

Reperfusion	Optical Density	Average	SE	P value
30mins	1.32, 15.03	8.17	4.84	-
30+15	0.19, 17.78	8.98	6.22	ns
30+30	0.37, 17.09	8.73	5.91	ns
30+60	0.84, 17.60	9.22	5.92	ns
12hr	13.21, 13.23	13.22	0.007	-
12+15	21.19, 15.56	18.37	1.99	ns
12+30	21.60, 12.56	17.08	3.19	ns
12+60	25.33, 18.70	22.01	2.34	ns

Appendix 17. HSF1 reperfusion optical density data

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	2.95, 0.48, 8.24	3.89	1.0	1.32	-
5mins	1.95, 1.66, 2.96	2.19	0.56	0.22	ns
10mins	3.04, 1.41, 3.40	2.61	0.67	0.35	ns
15mins	2.17, 1.29, 1.92	1.79	0.46	0.15	ns
30mins	0.96, 1.12, 0.41	0.83	0.21	0.12	ns
1hr	3.31, 1.09, 2.82	2.40	0.61	0.38	ns
2hr	2.79, 1.11, 2.96	2.28	0.58	0.34	ns
4hr	2.84, 1.83, 5.33	3.33	0.85	0.60	ns
6hr	1.54, 2.39, 2.14	2.02	0.51	0.14	ns
12hr	0.70, 1.57, 1.33	1.20	0.30	0.14	ns
24hr	1.63, 1.42, 4.22	2.42	0.62	0.51	ns
48hr	1.08, 1.94, 2.8	1.94	0.49	0.28	ns
72hr	1.0, 2.35, 3.91	2.42	0.62	0.48	ns

Appendix 18. Hsp70 optical density data.

Time	Mean Optical Density	Av	Fold	SE	p Value
Control	0.33, 0.78, 0.09, 0.36	0.39	1	0.14	-
PMA	0.65, 1.20,0.17, 0.32	0.58	1.48	0.22	ns
5mins	0.77, 0.06, 0.18, 0.48	0.37	0.90	0.15	ns
10mins	0.51, 0.07, 0.22, 0.47	0.31	0.79	0.10	ns
15mins	0.68, 0.60, 0.22, 0.44	0.48	1.23	0.10	ns
30mins	0.81, 0.80, 0.23, 0.45	0.57	1.46	0.14	ns
1hr	0.54, 0.80, 0.21, 0.44	0.49	1.25	0.12	ns
2hr	0.56, 0.47, 0.31, 0.53	0.46	1.17	0.05	ns
4hr	0.49, 1.01, 0.33, 0.51	0.58	1.48	0.14	ns
6hr	0.93, 1.50, 0.30, 0.36	0.77	1.97	0.28	ns
12hr	1.09, 1.52, 0.20, 0.35	0.79	2.02	0.31	ns
24hr	1.11, 1.53, 0.10, 0.26	0.75	1.92	0.34	ns
48hr	0.98, 1.29, 0.06, 0.12	0.61	1.56	0.30	ns
72hr	0.65, 1.46, 0.11, 0.13	0.58	1.48	0.31	ns

Appendix 19 . BCL-2 optical density readings.

Time	VWF IU/ml	Av	Fold	SE	p Value
Control	0.034, 0.023, 0.022	0.026	1	0.003	-
PMA	1.044, 0.196, 0.113	0.451	17.3	0.257	ns
5mins	0.262, 0.029, 0.015	0.102	3.9	0.06	ns
10mins	0.125, 0.027, 0.015	0.05	1.9	0.02	ns
15mins	0.103, 0.029,0.024	0.05	1.9	0.02	ns
30mins	0.146,0.029, 0.017	0.06	2.3	0.03	ns
1hr	0.087, 0.025, 0.02	0.04	1.53	0.01	ns
2hr	0.017, 0.027, 0.019	0.02	0.76	0.002	ns
4hr	0.15, 0.025, 0.019	0.06	2.3	0.03	ns
6hr	0.036, 0.024, 0.017	0.02	0.76	0.004	ns
12hr	0.004, 0.036, 0.016	0.01	0.38	0.008	ns
24hr	0.017, 0.036, 0.022	0.02	0.76	0.004	ns
48hr	0.014, 0.017, 0.022	0.01	0.38	0.002	ns
72hr	0.016, 0.017, 0.025	0.01	0.38	0.002	ns

Appendix 20. VWF ELISA levels during hypothermia

Time	Optical Density	Av	Fold	SE	p Value
Control	1.01, 1.03, 0.50	0.85	1	0	-
PMA	9.37, 11.10, 5.55	8.67	10.23	0.95	<0.05
5mins	11.67, 15.66, 7.83	11.72	13.66	2.05	<0.05
10mins	9.30, 10.84, 5.42	8.52	10.07	0.80	<0.05
15mins	6.45, 6.84, 3.42	5.57	6.65	0.27	ns
30mins	6.90, 7.52, 3.76	6.06	7.21	0.35	ns
1hr	5.15, 5.26, 2.63	4.34	5.20	0.10	ns
2hr	6.45, 5.94, 2.97	5.12	6.19	0.20	ns
4hr	4.94, 4.26, 2.13	3.77	4.60	0.30	ns
6hr	3.47, 3.84, 1.92	3.07	3.60	0.20	ns
12hr	3.78, 5.30, 2.65	3.91	4.50	0.75	ns
24hr	5.17, 8.48, 4.24	5.96	6.80	1.65	ns
48hr	4.12,9.62, 4.81	6.18	6.80	2.80	ns
72hr	4.10, 10.44, 5.22	6.58	7.20	3.20	ns

Appendix 20. p38 optical density readings (p value Vs control)

Reperfusion	Optical Density	Average	SE	P value
30mins	13.16, 9.44	11.30	1.31	-
30+15	9.29, 5.59	7.44	1.30	ns
30+30	9.87, 6.88	8.37	1.05	ns
30+60	8.98, 6.59	7.78	0.84	ns
12hr	5.47, 4.39	4.93	0.38	-
12+15	5.24, 4.18	4.71	0.37	ns
12+30	6.25, 4.84	5.54	0.49	ns
12+60	9.60, 6.39	7.99	1.13	ns

Appendix 21. p38 reperfusion optical density readings

Time	Optical Density	Av	Fold	SE	p Value
Control	1.07, 1.25	1.16	1	0.06	-
PMA	1.04, 1.17	1.10	0.94	0.04	ns
5mins	0.89, 1.12	1.00	0.86	0.08	ns
10mins	0.82, 1.05	0.93	0.80	0.106	ns
15mins	0.74, 0.89	0.81	0.69	0.05	ns
30mins	0.42, 0.57	0.49	0.42	0.05	ns
1hr	0.76, 0.88	0.82	0.70	0.04	ns
2hr	0.63, 0.89	0.76	0.65	0.09	ns
4hr	1.53, 1.84	1.68	1.44	0.10	ns
6hr	1.23, 1.56	1.39	1.19	0.11	ns
12hr	0.96, 1.27	1.11	0.95	0.10	ns
24hr	1.63, 1.71	1.67	1.43	0.02	ns
48hr	1.49, 2.02	1.75	1.50	0.18	ns
72hr	2.03, 2.45	2.24	1.93	0.14	ns

Appendix 22. JNK optical density readings

Reperfusion	Optical Density	Average	SE	P value
30mins	0.90, 0.91	0.9	0.003	-
30+15	2.36, 2.53	2.44	0.06	<0.05
30+30	2.58, 2.75	2.66	0.06	<0.05
30+60	3.65, 4.51	4.08	0.30	<0.05
12hr	13.16, 8.57	10.86	1.62	-
12+15	10.46, 7.56	9.01	1.02	ns
12+30	7.92, 6.34	7.13	0.55	ns
12+60	8.60, 5.67	7.13	1.03	ns

Appendix 23. JNK reperfusion optical density readings (p value Vs 30 minutes hypothermia)

Time	Optical Density	Av	Fold	SE	p Value
Control	2.74, 3.26, 1.56	2.52	1	0.21	-
PMA	7.07, 8.38, 6.06	7.17	2.99	0.41	<0.05
5mins	7.22, 9.19, 4.58	6.99	2.79	0.08	<0.05
10mins	4.64, 2.74, 0.21	2.53	0.89	0.45	ns
15mins	9.65, 3.86, 1.62	5.04	1.90	0.79	ns
30mins	8.86, 7.87, 3.66	6.79	2.96	0.31	ns
1hr	6.53, 6.61, 3.35	5.49	2.18	0.10	ns
2hr	8.50, 3.68, 2.66	4.94	1.97	0.58	<0.05
4hr	9.65,5.65, 5.84	7.04	2.99	0.63	<0.05
6hr	8.64,6.42, 6.61	7.22	3.11	0.65	<0.05
12hr	7.62,9.18, 5.48	7.42	2.98	0.26	<0.05
24hr	7.24, 6.94, 3.58	5.88	2.39	0.19	ns
48hr	9.56,4.05, 4.81	6.14	2.90	0.68	ns
72hr	10.11, 6.64, 6.36	7.70	3.26	0.62	<0.05

Appendix 24. ERK 1 /2 optical density readings (p value Vs control)

Reperfusion	Optical Density	Average	SE	P value
30mins	22.05, 18.39	20.22	1.29	-
30+15	22.82, 13.68	18.25	3.23	ns
30+30	23.76, 13.11	18.43	3.76	ns
30+60	16.73, 10.50	13.61	2.20	ns
12hr	10.34, 8.64	9.49	0.60	-
12+15	15.62, 9.17	12.39	2.28	ns
12+30	17.59, 11.34	14.46	2.20	ns
12+60	17.32, 18.14	17.73	0.14	ns

Appendix 25. ERK 1 /2 reperfusion optical density readings

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