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Ischaemia-Reperfusion-Induced Renal Injury: Pathogenesis & Modulation Implications for Transplantation

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This thesis is submitted to Trinity College for the degree of Doctor of Philosophy

2004

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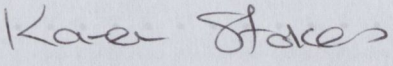
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Declaration

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Summary

Ischaemia-reperfusion is unavoidable during transplantation, but may promote allograft rejection. Preconditioning with a sub-lethal stress protects cells against a subsequent otherwise lethal stress, possibly through the induction of heat shock proteins. This thesis establishes a rat model of renal ischaemia-reperfusion injury and assesses the protective capability of several preconditioning methods. The most effective method was used to evaluate antigen expression and long-term protection.

Early post-ischaemic functional deterioration coincided with neutrophil infiltration and upregulation of intercellular adhesion molecule-1. Preconditioning with short ischaemic episodes did not confer immediate protection against ischaemia-reperfusion injury, but protection developed four hours after preconditioning. This was associated with heat shock protein 70 induction via a protein kinase C-dependent pathway. Since ischaemic preconditioning is invasive, the pharmaceutical inducer of heat shock protein 70, sodium arsenite, was used. Neither heat shock protein 70 expression nor protection against ischaemia-reperfusion injury were observed.

Hyperthermic preconditioning was employed next. This induced heat shock protein 70 and heat shock protein 60, and protected against ischaemia reperfusion-induced functional and morphological alterations. Leucocyte adhesion, alongside intercellular adhesion molecule-1 expression, remained at baseline, this possibly being the pathway through which injury was attenuated. Ischaemia-reperfusion-induced major histocompatability II expression was absent in preconditioned animals. Furthermore hyperthermic preconditioning abrogated morphological deterioration at 16 weeks reperfusion, suggesting that early protection by preconditioning may exert long-term benefits.

Finally, intravital videomicroscopy was performed to directly quantify leucocyte-endothelial interactions in a simple non-invasive model of interleukin-2-induced

inflammation. The target of many immunosuppressive drugs, interleukin-2 induces major histocompatibility complex expression. Adenosine, which has been implicated in preconditioning and reduces renal ischaemia-reperfusion injury, prevented interleukin-2-induced leucocyte adhesion and emigration, and L-selectin shedding. This method has been since used by our group to demonstrate that hyperthermic preconditioning reduces ischaemia-reperfusion and interleukin-2-induced leucocyte recruitment. These findings underscore an anti-inflammatory role for preconditioning, and suggest that heat shock protein 70 induction prior to transplantation may provide an effective means of reducing ischaemia-reperfusion injury, thereby improving graft survival, in particular in cadaveric transplantation.

List of Abbreviations

ARF:	acute renal failure
ATP:	adenosine 5′ triphosphate
BH_4:	tetrahydrobiopterin
BSA:	bovine serum albumin
C:	control group
Ca^{2+}:	calcium ions
cAMP:	cyclic 3′,5′ adenosine monophosphate
ChCl:	chelerythrine chloride
Cl^+:	chloride ion
CsA:	cyclosporine A
DAB:	diaminobenzidine
DGF:	delayed graft function
D_v:	venular diameter
eNOS:	endothelial nitric oxide synthase
ET-1:	endothelin-1
FITC:	fluorescein isothiocyanate
GFR:	glomerular filtration rate
GSH.Px:	glutathione peroxidase
H&E:	haematoxylin and eosin
HIR:	hyperthermia preconditioning + ischaemia-reperfusion
HLA:	human leucocyte antigen
H_2O_2:	hydrogen peroxide
HOCl:	hypochlorous acid
HSC70:	constitutive heat shock protein 70

HSE:	heat shock element
HSF:	heat shock factor
HSP:	heat shock protein
HTAB:	hexadecyltrimethyl ammonium bromide
ICAM:	intercellular adhesion molecule
IFN-γ:	interferon-γ
Ig:	immunoglobulin
I-κB:	inhibitory-κB
IL:	interleukin
IL-2R:	interleukin-2 receptor
iNOS:	inducible nitric oxide synthase
IR:	ischaemia-reperfusion
IPrec:	ischaemic preconditioning
K^+:	potassium ion
LFA:	lymphocyte function-associated antigen
LT:	leukotriene
Mac-1:	macrophage-1
MAdCAM-1:	mucosal addressin cellular adhesion molecule-1
MCP-1:	monocyte chemoattractant protein-1
MHC:	major histocompatibility complex
MPO:	myeloperoxidase
Na^+:	sodium ion
NADPH:	nicotinamide adenine dinucleotide phosphate
NF-κB:	nuclear factor-κB
NO:	nitric oxide
N_2O_3:	dinitrogen trioxide

NOS:	nitric oxide synthase
O_2^-:	superoxide
$ONOO^-$:	peroxynitrite
OCT:	optimal cutting temperature
OH^-:	hydroxyl radical
PAF:	platelet-activating factor
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate-buffered saline
PECAM-1:	platelet-endothelial cell adhesion molecule-1
PG:	prostaglandin
PGB:	phosphate-buffered saline, 20 mM glucose, 0.5% bovine serum albumin
PGI_2:	prostacyclin
PKC:	protein kinase C
PLA_2:	phospholipase A_2
PMSF:	phenylmethylsulphonyl fluoride
PRA:	panel reactive antibodies
PSGL-1:	P-selectin glycoprotein ligand-1
RANTES:	regulated on activation normal T-cell expressed and secreted
rIL-2:	recombinant interleukin-2
Aden+rIL-2:	adenosine pretreatment followed by recombinant interleukin-2
ROS:	reactive oxygen species
SA:	sodium arsenite
sLe:	sialyl Lewis
SOD:	superoxide dismutase
TBS:	Tris-buffered saline
TCR:	T-cell receptor

TNF:	tumour necrosis factor
Tx:	thromboxane
U/O:	urine output
VCAM:	vascular cell adhesion molecule-1
VLA-4:	very late antigen-4
V_{mean}:	mean red blood cell velocity
V_{rbc}:	centreline red blood cell velocity
V_{wbc}:	leucocyte rolling velocity
WSR:	wall shear rate
XO:	xanthine oxidase

CHAPTER 1

Introduction

1.1 BACKGROUND

The kidney is essential to live, playing a vital role in regulating the water and ionic composition of the blood, controlling blood pressure, regulating vitamin D metabolism and controlling red cell metabolism through the synthesis and release of erythropoietin (Guyton and Hall, 1997). A retrospective analysis of centres in Europe demonstrated that greater than 80% of patients with end-stage renal failure are treated by haemodialysis. However, this is associated with a high morbidity and mortality (approximately 15% per year) (Berthoux *et al.*, 1999). The alternative, and sometimes the necessity, is transplantation, and although even transplantation of marginal donor kidneys improves life expectancy over patients remaining on dialysis (Ojo *et al.*, 2001) long-term graft survival remains relatively short. The first kidney transplants in both animals and humans were performed in Vienna, by Ullmann in 1902. However, the surgical technique aspects of kidney transplantation were largely devised by Carrel between 1904 and 1910. This work resulted in a Nobel Prize in 1912, although a serious effort at transplantation did not begin until the 1950s. In this pre-immunosuppression era, the work of Murray *et al.* on transplantation between identical twins indicated that allograft failures were the result of rejection (Murray, 1982). Since the 1960s, increased availability of immunosuppressive drugs, in particular cyclosporine A (CsA) (Lu *et al.*, 1996), and more recently tacrolimus and anti-interleukin-2 receptor (IL-2R) antibodies (Light *et al.*, 2002), have prevented acute rejection and have prolonged allograft function and lifespan.

While the short-term results of organ transplantation have dramatically improved, five year graft survival rates remains low, at approximately 66% (Cecka, 2001). The main reason for late rejection is chronic rejection (Luke and Jordan, 2001). Chronic rejection

has been defined as a progressive functional deterioration, occurring months or years after grafting. The morphological manifestations of chronic rejection are persistent inflammation and graft arteriosclerosis (Paul, 1999). More specifically, vascular obliteration, glomerular sclerosis, tubular atrophy and interstitial fibrosis occur. In addition, in renal transplants some of these same features appear to relate to CsA nephrotoxicity, such as interstitial fibrosis and tubular atrophy (Yilmaz *et al.*, 1992a). The mechanisms leading to the process of chronic rejection are unclear (Joosten *et al.*, 2003). There are two main hypotheses to explain this phenomenon. First, the chronic rejection process is an immune response directed against the histo-incompatible allograft, an antigen-dependent process influenced by early immunologic injury and continuing host alloresponsiveness (Paul, 2001). It is argued that such changes are not seen in syngeneic transplants, even if immunosuppressed with CsA (Yilmaz *et al.*, 1992a). Second, alloantigen-independent factors contribute to the progressive changes. There is clear evidence that ischaemia time, surgical manipulation and perfusion and reperfusion injury have an influence on long-term outcome (Roodnat *et al.*, 2003). For example, functional and morphologic changes that resemble those of chronic rejection are also seen in response to ischaemia and subsequent reperfusion (IR) (Tullius *et al.*, 1994), albeit at a slower pace in the latter model. In fact recent studies suggest that CsA may not only attenuate immune responses to the alloantigens on the graft, but it may also act by reducing IR-induced injury to the organ during the transplantation process (Kouwenhoven *et al.*, 1999). It is, therefore, very likely that both immunological and non-immunological factors play important complimentary roles in the pathogenesis of chronic rejection (Kusaka *et al.*, 1999). An additional factor may include reduced renal mass (Azuma *et al.*, 1997). Experimentally and clinically, a low ratio of functioning renal parenchyma to body

weight may explain the influence of donor age, gender, and race on decreased long-term survival.

The most compelling evidence to date implicating ischaemia-reperfusion (IR) in chronic allograft rejection in humans is a clinical study by Land *et al.* (1994). This group demonstrated that administration (minutes before graft reperfusion) of superoxide dismutase (SOD), a short-acting agent which scavenges the oxygen free radical, superoxide (O_2^-), during the reperfusion phase, resulted in a significant reduction in both acute rejection and chronic rejection. This study suggests that decreasing IR injury decreases chronic rejection.

1.2 ISCHAEMIA-REPERFUSION (IR)

It is well established that prolonged organ ischaemia, i.e., interruption in the flow of oxygenated blood, causes tissue necrosis. The concept that subsequent restoration of oxygenated blood supply may be harmful (reperfusion injury) is more recent. This phenomenon has also been called the "oxygen paradox". Clearly, IR is an inevitable part of renal transplantation, with cessation of blood flow (ischaemia) during removal of the kidney from the donor and restoration (reperfusion) of oxygenated blood supply after placement of the organ in the recipient. However, it is likely that events initiated in the ischaemic period are promulgated during reperfusion, causing both local end-organ and systemic tissue injury. Several studies have demonstrated this phenomenon. Parks and Granger showed that three hours of ischaemia to the intestine caused relatively little injury to the intestinal mucosa, although substantial injury was seen after reperfusion (Parks and Granger, 1986). In fact, the injury observed after three hours of ischaemia and one hour of reperfusion was more severe than that generated after four hours of ischaemia alone. These data demonstrate that injury induced by IR is more severe than generated by ischaemia alone. Several studies have shown that reperfusion of ischaemic tissues with blood containing low levels of oxygen (anoxic or hypoxic) causes little injury (Perry and Wadhwa, 1988). These results indicate that some reaction initiated by the return of oxygenated blood to the ischaemic tissue is the cause of IR injury.

Although reperfusion of ischaemic tissues exacerbates the microvascular and parenchymal injury that occurs during an ischaemic insult, this phenomenon only occurs after relatively short periods of ischaemia. As the ischaemic period lengthens, the detrimental effect of oxygen deprivation to the tissues outweighs the effect of reperfusion, resulting in tissue

necrosis and eventual cell death. Under conditions of prolonged and/or severe ischaemia, restoration of blood flow will not significantly affect the ultimate viability of the tissue. Clinical conditions where the ischaemic period is not prolonged to the point of loss of viability occur in transplantation.

1.2.1 Role Of Reactive Oxygen Species In Ischaemia-Reperfusion Injury

In the vascular endothelium, under normal circumstances, hypoxanthine (a breakdown product of adenosine 5′ triphosphate (ATP) metabolism) is oxidised by the enzyme xanthine dehydrogenase to xanthine and uric acid. Under the anoxic conditions of ischaemia, there is a rise in the levels of hypoxanthine. During renal ischaemia, tissue levels of hypoxanthine rise rapidly to ten to three hundred times normal (Baker *et al.*, 1985). Anaerobic metabolism subsequent to ischaemia depletes the tissue of the energy transferring molecule ATP. This in turn leads to alterations in the function of the membrane ionic ATP-dependent pumps, favouring entry of calcium ions (Ca^{2+}), sodium ions (Na^{+}) and water into cells. Increased intracellular Ca^{2+} facilitates activation of a Ca^{2+}-dependent protease, converting xanthine dehydrogenase into xanthine oxidase (XO). The length of ischaemic time required for enzyme conversion varies between tissues, being approximately thirty minutes in the kidney (Weight *et al.*, 1996). After reperfusion, with oxygenated blood, the XO converts hypoxanthine and xanthine to uric acid and reactive oxygen species (ROS), particularly O_2^{-} and hydrogen peroxide (H_2O_2) (Figure 1.1). Superoxide cytotoxicity appears to depend largely on the subsequent production of the highly toxic hydroxyl radical (OH^{-}) through the iron-catalysed Haber-Weiss reaction. A role for iron in this process is supported by studies that demonstrate a protective effect with iron chelators (desferoxamine) or iron-binding proteins (transferrin) in models of IR

injury. Membrane-associated polyunsaturated fatty acids are attacked by $OH^{\cdot}$, resulting in lipid peroxidation. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentalization, resulting in cell lysis. Lipid peroxidation of cell membranes also releases arachidonic acid and lipid peroxyl free radicals. The former is metabolised by cyclo-oxygenase or lipoxygenase to release vasoactive eicosanoids (thromboxane A_2 (TxA_2), prostaglandins (PGs) and leukotrienes (LTs)), whilst lipid peroxyl free radicals promote further lipid peroxidation.

Evidence for the role of ROS in IR injury is based on studies using agents which either scavenge these molecules or inhibit their production. Experimental studies indicate that there is a burst of oxidant formation immediately after reperfusion, lasting two to five minutes. This burst is suppressed by treatment with the enzyme SOD, which scavenges $O_2^{\cdot}$. It has been demonstrated that SOD not only attenuates IR-induced microvascular injury (Granger *et al.*, 1989) but also post-ischaemic renal injury (Torras *et al.*, 1994). In fact levels of endogenous SOD are reduced following renal IR (Jolicoeur *et al.*, 2003).

There is also evidence to suggest a major role for other oxidants derived from $O_2^{\cdot}$ in IR injury. Catalase, an enzyme that catalyzes the disproportion of H_2O_2 to H_2O and oxygen has been shown to be protective in many models of IR (Suzuki *et al.*, 1991). In addition, non-enzymatic scavengers of $OH^{\cdot}$, including dimethyl sulfoxide, dimethyl thiourea and mannitol have been shown to diminish IR injury (Parks *et al.*, 1984). It has also been demonstrated that reduced glutathione and iron chelators protect against renal IR injury (Baud and Ardaillou, 1993).

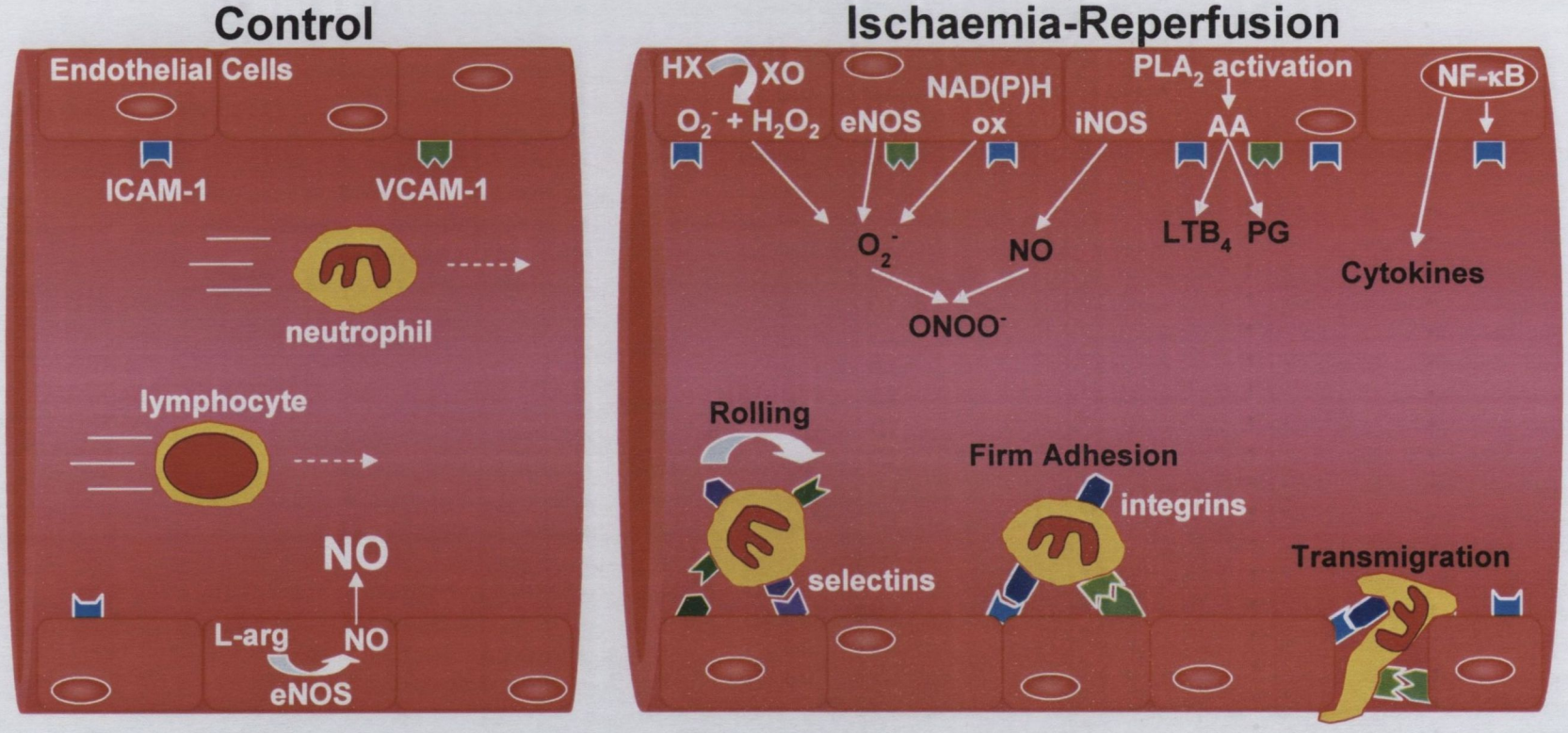


Figure 1.1 A schematic of microvascular inflammatory alterations following ischaemia-reperfusion. Under control conditions leucocytes rarely interact with the vessel wall, and basal nitric oxide release (NO) helps maintain an anti-inflammatory phenotype. However, following ischaemia-reperfusion, oxidants such as superoxide (O2-) are released from several sources. An overproduction of NO from iNOS in combination with O2- may lead to nitrosative stress. Other inflammatory mediators such as leukotrienes (LTB_4), prostaglandins (PG) and cytokines are released from endothelial cells and leucocytes. Adhesion molecules are upregulated leading to leucocyte recruitment (via rolling, firm adhesion and transmigration) which propagates the inflammatory changes, leading to tissue damage.

1.2.2 Role Of Xanthine Oxidase In Ischaemia-Reperfusion Injury

The XO inhibitors allopurinol, oxypurinol and pterin aldehyde have been used to assess the contribution of XO to IR injury. All of these inhibitors have been demonstrated to decrease parenchymal cell death and microvascular injury, indicating that XO may be an important source of the oxidants produced after IR (Granger *et al.*, 1986). In the kidney, XO appears to be the primary endothelial source of ROS following IR. Pre-treatment of animals with allopurinol before a renal IR insult, not only protects against oxidative stress and histological damage, but also improves post-ischaemic renal function (Rhoden *et al.*, 2000). Other evidence for the importance of XO in IR injury comes from studies using diet to deplete active XO. Administration of a tungsten-supplemented, molybdenum-deficient diet results in tungsten replacing molybdenum in the active site of XO, thereby inactivating XO. This also significantly attenuated IR injury (Smith *et al.*, 1989). Also direct intra-arterial injection of XO produces morphological changes identical with those seen after IR (Parks *et al.*, 1984).

1.2.3 Arachidonic Acid Metabolites

The post-ischaemic elevation of intracellular Ca^{2+} is thought to activate phospholipase A_2 (PLA_2) in endothelial cells. In the rat kidney there are multiple sites of PLA_2 activity, i.e. in the cytosolic, mitochondrial and microsomal fractions. When the kidney is exposed to forty five minutes of ischaemia and one hour of reperfusion, there are stable increases in PLA_2 activity in all fractions (Nakamura *et al.*, 1991). Activated PLA_2 splits off arachidonic acid from membrane phospholipids. The arachidonic acid is metabolised by several enzymes, initiating the arachidonic acid cascade which yields specific eicosanoids

(Figure 1.1). There are two pathways: one is catalysed by the enzyme cyclo-oxygenase and products include PGs (e.g. the vasodilatory prostacyclin or PGI_2) and the vasoconstrictive TxA_2; the second pathway is catalysed by the enzyme lipoxygenase and yields LTs (e.g. LTB_4).

Enzymatically-generated ROS added to media of glomerular cells cause their dose-dependent increase in the production of PGE_2, PGF_{2a} and subsequent production of the prostanoids 6-keto-PGF_{1a} and TxB_2 (stable breakdown products of prostacyclin and TxA_2 respectively). This is mainly due to the generation of H_2O_2, which causes an increased availability of arachidonic acid via the activation of PLA_2. The main consequence of ROS-induced eicosanoid synthesis in the kidney is an increase of the arteriolar resistances with a reduction of the glomerular ultrafiltration coefficient and an accumulation of cyclic 3′,5′-adenosine monophosphate (cAMP) within glomerular cells. Following IR in the kidney, the ratio of vasodilator to vasoconstrictor eicosanoids falls. This correlates with renal injury. For example, the ratio between PGI_2 and TxA_2 is important in the post-ischaemic kidney (Weight *et al.*, 2001). Indeed, the infusion of a synthetic analogue of the vasodilator PGI_2 prevents renal IR damage in the dog (Niemeyer *et al.*, 1986).

The bioactive lipid, platelet-activating factor (PAF), which has both vasodilatory and vasoconstrictive properties, is also formed from membrane phospholipids by the action of PLA_2. Its synthesis may be induced by ROS and it too may participate in the decrease of the glomerular ultrafiltration coefficient. Blocking the action of PAF prevents renal IR injury (Torras *et al.*, 1999). In fact, the antioxidant vitamin C attenuates the production of PAF and PAF-like lipids in post-ischaemic kidneys, resulting in a reduced leucocyte infiltration and improved function (Lloberas *et al.*, 2002).

1.2.4 Other Endothelium-Derived Substances

In addition to the arachidonic metabolites, the endothelium releases other vasodilatory (nitric oxide (NO)) and vasoconstrictive (endothelin-1 (ET-1)) substances. During inflammation the net result of endothelial cell stimulation is generally vasoconstriction, which, during IR, may exacerbate the ischaemia. The balance between ET-1 and NO is important in the homeostasis of cardiovascular and renal microvasculature. The kidney is approximately 10 times more sensitive to ET-1 than other vascular beds (Kaw *et al.*, 1993). Endothelin-induced vasoconstriction is Ca^{2+}-dependent. During ischaemia, the transcription of ET-1 is amplified and this leads to neutrophil infiltration via the upregulation of adhesion molecules involved in leucocyte-endothelial interactions (Espinosa *et al.*, 1996). Lopez-Farre (1991) found that the effects of ET-1 were mediated by PAF in the kidney. The use of a receptor antagonist against the vasoconstrictor endothelin A receptor improves short-term (Knoll *et al.*, 2001) and long-term (Forbes *et al.*, 2001) post-ischaemic function.

Conversely, NO promotes vasodilation. It is produced by the enzymatic oxidation of L-arginine to form L-citrulline. This reaction requires the co-factors nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH_4) and the enzyme nitric oxide synthase (NOS), which exists as several isotypes (Laroux FS *et al.*, 2001). Endothelial cells contain a constitutive form, endothelial NOS (eNOS) which produces what are generally thought to be protective levels of NO (Figure 1.1). In the kidney, tubular epithelium contains an eNOS-like enzyme also (Tracey WR *et al.*, 1994). During IR, an uncoupling of the enzyme from L-arginine may occur as a result of decreased BH_4 levels. This could actually lead to the production of O_2^-. In fact, pre-treatment of rats with

(6R)-5,6,7,8-tetrahydro-L-biopterin confers protection against post-ischaemic dysfunction in the kidney (Kakoki *et al.*, 2000). Inducible NOS (iNOS) is expressed in renal tubules and the interstitium and is activated during IR to produce high levels of NO. In fact, using a specific inhibitor, blocking iNOS attenuates post-ischaemic renal dysfunction, neutrophil infiltration and formation of the highly toxic peroxynitrite ($ONOO^-$) (Chatterjee *et al.*, 2003). Both ROS and NO can combine to produce not only $ONOO^-$ but other reactive nitrogen and oxygen species such as dinitrogen trioxide (N_2O_3) which can result in a combined oxidative and nitrosative stress. Thus SOD may have a dual role by inhibiting the O_2^--induced inactivation of protective NO and preventing the production of radicals such as $ONOO^-$, implicating an important role for the $NO:O_2^-$ ratio in determining the extent of injury following IR.

1.2.5 Role Of Leucocyte-Endothelial Interactions In The Pathogenesis Of Ischaemia-Reperfusion Injury

1.2.5.1 Role Of Polymorphonuclear Leucocytes In Ischaemia-Reperfusion Injury

In addition to the ischaemic tissues themselves, another major source of ROS after IR is the polymorphonuclear leucocyte or neutrophil. Neutrophils contain a NADPH oxidase that reduces molecular oxygen to O_2^-. This spontaneously dismutes to H_2O_2. Activated neutrophils also secrete the enzyme myeloperoxidase (MPO), which catalyses the formation of hypochlorous acid (HOCl) from the H_2O_2 and chloride ions (Cl^-). Hypochlorous acid is powerful oxidising and chlorinating agent, one hundred times more potent than H_2O_2. Hypochlorous acid reacts rapidly with primary amines to produce N-

chloro derivatives that are equivalent to H_2O_2 and HOCl in terms of oxidising capacity. The cytotoxicity associated with HOCl and N-chloroamines may be mediated through the oxidation of sulfhydryls, inactivation of haem proteins and cytochrome and degradation of amino acids and proteins (Grisham *et al.*, 1984).

Activated neutrophils also release a variety of enzymes, such as elastase and collagenase, which are injurious to the parenchyma and microvasculature. Investigators have examined neutrophil fluxes in tissue after IR, using both radio-labelled neutrophils and tissue measurements of enzymes specific to neutrophils, such as MPO (Kurtel *et al.*, 1992; Zimmerman *et al.*, 1990). In experimental studies of intestinal IR, ischaemia caused an increase in mucosal MPO activity, while reperfusion substantially increased this neutrophil infiltration. Kurtel *et al.* have shown a marked neutrophil infiltration of all tissue layers after IR (Kurtel *et al.*, 1992). Treatment with SOD or allopurinol attenuated this increase in neutrophil infiltration. Pre-treatment with either catalase or dimethyl sulfoxide also diminished neutrophil infiltration. This suggests that ROS play a role in recruiting neutrophils into post-ischaemic tissues (Zimmermann *et al.*, 1990). This accumulation of neutrophils after IR raises the issue as to whether neutrophil accumulation and activation is a cause or an effect of reperfusion injury. This has been answered in two ways. Firstly, the effect of neutrophil depletion, using a neutrophil antiserum was examined. This significantly reduced both microvascular injury and alterations in intestinal absorption after IR, indicating that neutrophils are primary mediators of IR-induced tissue injury (Hernandez *et al.*, 1987). The neutrophil membrane glycoprotein CD18 has been shown to play an important role in mediating neutrophil adhesion to the microvascular endothelium. Arfors *et al.* (1985) using monoclonal antibodies directed against the common functional epitope of CD18, inhibited neutrophil chemotaxis,

aggregation and adherence to endothelium. This monoclonal administration was just as effective as depleting neutrophils in attenuating IR tissue injury, indicating that neutrophil accumulation per se is not enough to promote neutrophil-mediated IR injury, rather adhesion to the microvasculature is an essential component for this inflammatory process to continue (Hernandez *et al.*, 1987).

Grangers group have examined in detail the effect of IR on neutrophil adhesion and migration through the endothelial barrier with intravital microscopy techniques (Granger *et al.*, 1989). This technique allows observation of leucocytes in vessels and in the interstitium by transillumination of the tissue *in vivo*. They demonstrated that sixty minutes of ischaemia increased leucocyte adhesion and transmigration. However, reperfusion for as little as ten minutes greatly exacerbated both adhesion and migration. Light and electron microscopy confirmed that over 85% of these leucocytes were neutrophils. Pre-treatment with a monoclonal antibody directed against CD18 significantly reduced this response. These observations indicate that IR promotes adhesion and migration of neutrophils and that this neutrophil-endothelial interaction is mediated by the leucocyte adhesion glycoprotein complex CD11/CD18. This neutrophil-endothelial interaction will be discussed in further detail in section 1.2.5.2.

Pre-treatment of animals with allopurinol and SOD was also shown to alter the adhesion and migration steps of neutrophil infiltration in mesenteric venules during IR (Granger *et al.*, 1989). Allopurinol, an XO inhibitor, decreased IR-induced leucocyte-endothelial interactions, but did not alter changes following ischaemia alone. This was in contrast to SOD which decreased both ischaemia- and IR-induced changes. However, SOD did not alter neutrophil adhesion to inert surfaces such as plastic, suggesting that O_2^--mediated

neutrophil adhesion is an endothelium-dependent process (Suzuki *et al.*, 1989). Overall these findings support the hypothesis that XO-derived oxidants initiate the adherence of leucocytes to microvascular endothelium induced by IR injury.

While the mechanism by which O_2^- mediates neutrophil-endothelial interactions is unclear, one possible mechanism is that O_2^- inactivates or inhibits anti-adhesive mediators on the endothelium. Nitric oxide, also known as endothelium-derived relaxing factor, is a biologically active substance released by endothelial cells, which is inactivated by O_2^-. It has been established that NO inhibits platelet aggregation and adherence to endothelium, and NO also inhibits neutrophil aggregation *in vitro*. Grangers' group demonstrated that inhibition of NO synthesis increases leucocyte adhesion and migration fifteen-fold *in vivo* (Arndt *et al.*, 1993). Others have demonstrated that administration of an NO donor inhibits IR-induced leucocyte adhesion and migration *in vivo* (Kurose *et al.*, 1994).

In addition to inactivating NO, oxidants can also interact with endothelial cell membranes to activate PLA_2 and consequently lead to the formation of powerful leucocyte chemoattractants such as LTB_4 and PAF as described in section 1.2.3. This is important because in order to adhere to the vascular endothelium, cells must be attracted to the inflamed vascular bed. Inhibition of PLA_2 has been shown to partially attenuate IR-induced intestinal injury (Otamiri *et al.*, 1987). Measurement of LTB_4 levels in tissues after ischaemia alone shows no change in tissue concentration, however following reperfusion there is an almost seven-fold increase (Mangino *et al.*, 1989). Inhibition of LTB_4 synthesis or blockade of LTB_4 receptors significantly reduces neutrophil tissue infiltration, suggesting an important role for this chemoattractant in mediating IR-induced neutrophil infiltration (Zimmerman *et al.*, 1990). Platelet-activating factor is also released

during reperfusion and may play a similar role. Intra-arterial injection of PAF mimics the changes of IR injury, increasing microvascular permeability and neutrophil infiltration of tissues (Kubes *et al.*, 1990), whereas the use of PAF-receptor blockade significantly reduces IR-induced tissue injury (Montrucchio *et al.*, 1993). It is known that exposure of cells to oxidants activates PLA_2, that reperfusion activates PLA_2, and that inhibition of PLA_2 attenuates IR-induced neutrophil infiltration of tissues and microvascular injury (Lewis *et al.*, 1988; Otamiri *et al.*, 1987). Overall this data suggests that ROS initiate a series of events including the activation of PLA_2 and subsequent generation of chemoattractants, such as PAF and LTB_4, resulting in leucocyte accumulation in the post-ischaemic tissue.

The above findings appear to be true for the kidney also. The concentrations of LTB_4 and PAF are increased in renal IR. It is likely that activated neutrophils play a role in the synthesis and actions of LTB_4 (Klausner *et al.*, 1989) and PAF. For instance, LTB_4 may induce renal injury directly or indirectly. Direct action involves the chemoattraction (Noiri *et al.*, 2000) and subsequent activation of neutrophils, stimulating these leucocytes to release TxA_2, PGE_2, PAF and ROS as well as cytotoxic proteases such as elastase and collagenases. Indirectly, LTB_4 may act via TxA_2 synthesis to enhance neutrophil transmigration and the passage of macromolecules through the endothelium, thereby reducing renal blood flow. In fact, Klausner *et al.* (1989) found decreased renal blood flow and increased LTB_4 and TxB_2 levels at five minutes of reperfusion. At twenty four hours of reperfusion morphological evidence of tissue damage, as well as organ dysfunction were present. Depletion of neutrophils or treatment with a leukotriene synthetase inhibitor to inhibit LTB_4 production prevented these changes.

Thromboxane causes vasoconstriction, reduction of renal blood flow and decreased glomerular filtration rate (GFR). It is also a permeability-promoting agent and may directly affect the vascular barrier of the reperfused kidney. Thromboxane can indirectly amplify reperfusion injury by promoting adhesion and diapedesis of neutrophils through the vascular barrier. Because TxB_2 levels were attenuated in neutropenic rats these cells must play a role in the synthesis of TxB_2, either directly or indirectly. Pre-treatment with a Tx synthetase inhibitor prevented post-ischaemic renal failure, thereby implying that it plays a role in renal IR injury (Garvin *et al.*, 1996). Torras *et al.* (1994) found that the protection conferred against renal IR injury by liposomal SOD was associated with the reduction of post-ischaemic TxB_2 levels, indicating that ROS may be responsible for PLA_2 activation in the kidney following IR.

In the post-ischaemic kidney, PAF levels peak within the first 15 min of reperfusion (Lloberas *et al.*, 2002). Blocking PAF protects the kidney in experimental models of warm ischaemia (Torras *et al.*, 1993; Torras *et al.*, 1999). The release of PAF from endothelial cells appears to play an important role in the recruitment of neutrophils to the post-ischaemic kidney. Kelly *et al.* (1996) demonstrated that a PAF antagonist not only attenuated post-ischaemic renal dysfunction but also reduced tissue MPO levels suggesting that neutrophil infiltration was decreased. Riera *et al.* (1997) showed that isolated kidneys subjected to cold IR sustained an injury only if the kidneys were perfused with neutrophils. This was associated with an increase in PAF production. Use of BN 52021 prevented both the increased PAF production and also the resulting injury. More recently Lloberas *et al.* (2002) demonstrated that pre-treatment with the antioxidant vitamin C attenuated the production of PAF and PAF-like lipids during early reperfusion of the kidney. This was not only associated with decreased oxidative responses, but

neutrophil infiltration (as measured by MPO levels) was reduced and tubular injury (as evidenced by morphology and dysfunction) was diminished, again supporting a role for ROS in the initiation of events leading to neutrophil accumulation and tissue injury in the post-ischaemic kidney.

However, unlike in other organs, an actual role for neutrophils in the pathogenesis of renal IR injury remains controversial. Lipid peroxidation in the first five minutes of reperfusion following renal ischaemia (Joannidis *et al.*, 1989) coincides with neutrophil accumulation in the inner stripe of the outer medulla but not elsewhere. This could reflect early activation and margination of neutrophils due to an initial formation of O_2^- anions by endothelial or epithelial cells and/or could coincide with neutrophil radical production, O_2^- via their NADPH oxidase system, HOCl (catalysed by MPO), or H_2O_2. This would suggest that neutrophils are unlikely to be the initiators of the tubular injury following renal IR, rather that early damage of these tubules may lead to release of chemotactic substances which attract the neutrophils to the area to cause more damage or maintain the level of injury. Conversely, neutrophil depletion has been shown to block tubular and capillary leakage. These processes involve LTB_4 and PAF and may be responsible for the reduced GFR observed during acute renal failure (ARF). Kelly *et al.* (1996) demonstrated that neutrophil depletion not only reduced post-ischaemic elevation of renal MPO levels, but that IR-induced functional and morphological deterioration were attenuated. Furthermore, Linas *et al.* (1992) demonstrated that the kidney is capable of activating neutrophils, and that activated neutrophils can mediate endothelial injury and organ dysfunction via an ROS and/protease-dependent mechanism. Several antibody studies support a role for neutrophils, or at least leucocytes, in the pathogenesis of renal IR injury. This will be discussed further in the next sections.

1.2.5.2 Adhesion Molecules

Cellular adhesion molecules (CAMs) support the initial infiltration of post-ischaemic tissue by neutrophils and later, by monocytes and lymphocytes. There are three main groups of adhesion molecules, which support the three main steps of recruitment: 1) selectins which support rolling and some adhesion, 2) integrins and 3) their ligands, the immunoglobulin (Ig) family, both of which may participate in rolling but predominantly participate in firm adhesion and transmigration of leucocytes (Ley and Sperandio, 2003) (Figure 1.1). Following IR, an initial rapid recruitment of neutrophils from the circulation occurs. This is mediated by P-selectin and L-selectin, PAF and integrins. Following this, a more sustained wave of leucocyte recruitment is seen, mediated by E-selectin, cytokines such as interleukin (IL)-8 and integrins. At later times, E-selectin declines but intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM-1) continue to rise. These events are likely to make the endothelial surface less adhesive to neutrophils and more so for monocytes and lymphocytes.

Adhesion of neutrophils to cultured endothelial cells is rapidly initiated by endothelial stimulators that increase cytosolic Ca^{2+}. Agents, including LTB_4 and H_2O_2 cause a rapid (1-2 min) fusion of Weibel-Palade bodies with the plasma membrane and release of von Willebrand factor as well as translocation of P-selectin to the surface of the endothelial cell. P-selectin is also stored in the α-granules of platelets and is similarly translocated to the surface of these cells within minutes of activation. This adhesion molecule is a member of the selectin family which mediates the rolling phenomenon that is a prerequisite for and precedes firm attachment of leucocytes to the vessel wall. Once released, P-selectin binds its ligands on the leucocyte surface. These include

tetrasaccharides such as sialyl Lewis X (SLe^x) and A (SLe^a), L-selectin, P-selectin ligand and P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leucocytes. The rapid release of P-selectin probably contributes to its function as an acute phase mediator. In the absence of a leucocyte response the P-selectin is rapidly internalized, within five to ten minutes post-stimulation. P-selectin expression has been shown to be increased on vascular endothelium following renal IR by several groups. This molecule has been observed almost immediately upon reperfusion (most likely reflecting mobilization of internal stores) (Zizzi *et al.*, 1997) and hours later (Okusa *et al.*, 2000). This second phase probably represents a transcription-dependent event although this type of upregulation has not been demonstrated to exist in humans to date. Interestingly, Singbartl *et al.*, (2001) found that platelet-derived P-selectin was primarily responsible for the role of this adhesion molecule in renal IR injury. In fact chimeric mice with P-selectin expressed only by the vascular endothelium but not by platelets were protected against IR-induced renal failure and neutrophil infiltration.

Another member of the selectin family, E-selectin, is not present in normal kidney but appears to be expressed by cytokine-activated venular and capillary endothelial cells. Synthesis and expression of this ligand are optimal after about four hours of cytokine treatment and then slowly decline to basal levels by twenty four to forty eight hours. E-selectin shares some of the same ligands as P-selectin, including SLe^x, SLe^a and L-selectin. Other ligands are E-selectin ligand and cutaneous lymphocyte antigen. Following renal IR, E-selectin expression peaks at six hours reperfusion, coinciding with peak neutrophil infiltration into the tissue, and preceding the transmigration of monocytes and lymphocytes. Takada *et al.* (1997) demonstrated that administration of a soluble form of PSGL-1 inhibited the infiltration of all leucocytes into the post-IR kidney and prevented

both the expression of the inflammatory products of the leucocytes and the induction of major histocompatibility complex (MHC) II. They showed that E-selectin expression was blocked by this treatment. However they did not directly measure the expression of P-selectin although they suggested that blocking this molecule was responsible for the prevention of neutrophil infiltration since these leucocytes were observed in the tissue before E-selectin expression increased in the untreated group.

The third member of the selectin family, L-selectin, is constitutively expressed on most circulating neutrophils and monocytes as well as other myeloid cells. Most B-cells and naive lymphocytes also express L-selectin, although only a subpopulation of memory T-cells and natural killer cells express it. It is rapidly shed from the surfaces of the leucocytes upon their activation. In addition to binding P- and E-selectin, L-selectin interacts with CD34, glycosylation-dependent cell adhesion molecule and mucosal addressin cellular adhesion molecule-1 (MAdCAM-1). Its role in renal IR injury is unknown. Brady *et al.* (1992) found that neutrophils, monocytes and lymphocytes bound in an L-selectin-dependent manner to bovine glomerular endothelial cells stimulated with tumour necrosis factor (TNF). However, Rabb *et al.* (1996) found that L-selectin gene knockout mice were not protected against renal IR-induced injury and increased neutrophil infiltration. While compensatory mechanisms could have been in place to override the role of L-selectin in these knockout mice, in the absence of further studies using antibodies or antisense oligonucleotides for instance, a possible role for L-selectin in renal IR injury cannot be discounted.

There are several integrin families although perhaps the most well characterized one is the β_2 integrin family. This contains lymphocyte function-associated antigen (LFA)-1,

macrophage-1 (Mac-1), p150,95 and $\alpha_D\beta_2$. These molecules possess a common β_2 CD18 subunit linked to one of the immunologically distinct α subunits, CD11a, CD11b and CD11c respectively. These adhesion molecules participate in many leucocyte interactions. The receptor LFA-1 is basally expressed on neutrophils and circulating lymphocytes and may undergo conformational changes to increase its avidity. It plays important roles in adhesion and emigration of neutrophils and as a co-stimulatory molecule on T-lymphocytes. This integrin can bind to members of the Ig family such as ICAM-1 and ICAM-2. Both Mac-1 and p150,95 are stored in granules of neutrophils, monocytes and natural killer cells, from which they are rapidly mobilised to the surface of these cells upon activation. Mac-1 binds ICAM-1, fibrinogen and C3bi. The ligands for p150,95 have not been completely defined but they may also include fibrinogen and C3bi. $\alpha_D\beta_2$ is expressed predominantly on macrophages and granulocytes in the red pulp of the spleen and binds to ICAM-3 and VCAM-1. Very little work has been performed to determine the role of integrins in renal IR injury. Kelly *et al.* (1994) used an anti-LFA-1 antibody to protect against the renal IR damage seen in their mouse model. Rabb *et al.* (1994) established a role for both LFA-1 and Mac-1 in this injury with the use of antibodies against these adhesion molecules. However this was only associated with a slight decrease in the neutrophil-endothelial interactions. Conversely there was no protection against renal IR injury in rabbits when CD18 was blocked using antibodies, indicating interspecies differences.

Most of the members of the Ig-superfamily of adhesion molecules can be found on endothelial cells. These include ICAM-1, ICAM-2, VCAM-1, platelet-endothelial cell adhesion molecule-1 (PECAM-1), MAdCAM-1. Ubiquitously distributed, ICAM-1 is basally expressed at low levels whereas ICAM-2 is constitutively expressed in moderate

quantities. Neutrophils may initially bind to vascular endothelium via P-selectin, become activated by PAF on the surface of these cells or other inflammatory mediators such as cytokines or LTB_4 and remain attached via β_2 integrin binding to ICAM-1, ICAM-2 and/or other ligands. The synthesis and expression of ICAM-1 continue to increase during IR, reaching a stable plateau of expression by about twenty four hours of reperfusion. This adhesion molecule is expressed in small amounts in the vascular component of the nephron, that is, endothelium and glomeruli, and to a much lesser extent in parietal cells of the Bowman's capsule and some interstitial cells in healthy human kidneys. Many studies have confirmed its role in the pathogenesis of renal IR injury.

Of the Ig adhesion molecules ICAM-1 has been most implicated in renal IR injury. Kelly *et al.* (1994) and Rabb *et al.* (1995) found that an antibody against ICAM-1 conferred protection against renal IR injury in the mouse. This effect was also seen in an isolated perfused kidney model by Linas *et al.* (1995). Kelly *et al.* (1996) went on to show that ICAM-1 knockout mice were also protected against this injury and the associated neutrophil infiltration. Neutrophil depletion had a similar effect. The use of an antibody against ICAM-1 with neutrophil depletion was not synergistic in their protection, thus ICAM-1 is probably a key mediator in the pathogenesis of renal IR injury through potentiation of neutrophil-endothelial interactions. Haller *et al.* (1996) and Chen (1999) showed that the use of antisense oligodeoxyribonucleotides for ICAM-1 were protective against ARF. The latter study showed that blocking the synthesis of ICAM-1 following renal IR was associated with the prevention of granulocyte and macrophage infiltration into the tissue, preserved function and attenuated histological damage. In agreement with results from CD18 antibody studies, there was no attenuation of renal IR injury in rabbits

when anti-ICAM-1 antibodies were used, supporting the existence of interspecies differences.

There are only a few studies in which other Igs have been demonstrated in renal IR. For example, PECAM-1 displays kinetics of induction similar to ICAM-1 and is diffusely distributed following renal IR (Dragun *et al.*, 2000). VCAM-1 is up-regulated more slowly compared with ICAM-1 and is primarily expressed on the vascular endothelium of elastic vessels by 24 h reperfusion of the kidney (Leonard *et al.*, 2002; Dragun *et al.*, 2000). While no definitive information as to the exact role of these adhesion molecules in the pathogenesis of renal IR injury has been published to date, VCAM-1 likely supports the infiltration of monocytes and lymphocytes into post-ischaemic tissue.

1.2.5.3 Accumulation Of Neutrophils Following Renal Ischaemia-Reperfusion

There is evidence both for and against the participation of neutrophils in the pathogenesis of renal IR injury, but more recent breakthroughs in leucocyte research support their role as mediators of the resulting tissue damage as mentioned in section 1.2.5.1. Apart from depletion studies, several studies to date have demonstrated the presence of neutrophils (Willinger *et al.*, 1992; Takada *et al.*, 1997; Okusa *et al.*, 2000) in the kidney following IR injury in a rat model of 45 min ischaemia, similar to the model in this thesis. Willinger *et al.* (1992) found that after ischaemia alone there was a doubling of neutrophil counts in the intraglomerular and inner medulla. After 2 h reperfusion neutrophils had accumulated throughout the kidney, in particular within the peritubular capillaries in the cortex and the inner stripe of the outer medulla. This accumulation of neutrophils may be indicative of

their role in both cell injury and post-ischaemic ARF as it is these areas that are the worst effected following IR.

1.2.5.4 Mechanical Disturbance By Neutrophils Following Renal Ischaemia-Reperfusion

Apart from the release of harmful substances neutrophils are thought to contribute to damage in renal IR injury by mechanical means, i.e. capillary plugging. Willinger *et al.* (1992) did not however find evidence of capillary plugging by the neutrophils. Klausner *et al.* (1989) found enhanced renal blood flow upon reperfusion in neutropenic rats compared with normal rats although evidence for capillary plugging was not sought. Paller *et al.* (1989) found no protection against ischaemic injury in neutropenic model of renal IR. However, Braide *et al.* (1986) found that infusion of an isolated rat kidney with leucocytes caused a sustained increase in vascular resistance and decreased GFR only during reduced flow, not at normal flow rates, implicating that the leucocytes might induce significant mechanical disturbance during an IR insult. Increased Ca^{2+} influx into erythrocytes during ischaemia can reduce their deformability thereby favouring reduced microvascular perfusion. Similarly for neutrophils, increased membrane rigidity may contribute to capillary plugging, a phenomenon that is not decreased by an antibody against the adhesion molecule CD11/18 in IR injury (Habazettl *et al.*, 1999). Free radicals may play a role in the development of this lack of deformability.

1.2.5.5 The Role Of Other Leucocytes In Renal Ischaemia-Reperfusion Injury

Lymphocytes are re-circulating cells that migrate from the blood into tissues, into the lymph and back into the bloodstream again. These cells mediate specific immune

responses. They enter the host compartments in various ways, including specialised vascular endothelium (high endothelial venules) in lymph nodes, tonsils and Peyer's patches, the spleen via its sinusoids and non-lymphoid organs through normal vessels. Macrophages are formed from monocytes that have transmigrated into the tissue where they have undergone structural and functional changes. They act in a similar manner to phagocytes, secreting cytokines such as IL-1 and IL-6 and participating in the activation of helper T-cells by processing and presenting antigens for destruction. As detailed above, physiological alterations such as ischaemia can initiate a cascade of events that may both cause and intensify interactions between circulating white blood cells and vascular endothelium, with subsequent diapedesis into the involved organ.

Lymphocytes, including T-cells and lymphokine-activated killer cells, can injure the endothelial cells probably through oxygen-independent mechanisms used by lymphocytes to lyse other cells. The adhesion molecule LFA-1 can undergo molecular activation on T lymphocytes, markedly increasing the affinity for its ligands, ICAM-1 and ICAM-2. T-cell-dependent cytotoxicity may be influenced by ICAM-1 and can be completely inhibited by an anti-ICAM-1 monoclonal antibody. Another Ig adhesion molecule, VCAM-1, is normally found in parietal epithelial cells of Bowman's capsule and a few cells in the proximal tubule but not on vessels. It is synthesised and expressed in response to IL-1 or TNF treatment. Both ICAM-1 and VCAM-1 appear to show some complementary distribution where ICAM-1 is constitutively expressed on capillaries and can be induced in tubules and VCAM-1 expression is vice versa. The VCAM-1 ligand on the leucocyte is the β_3-integrin, very late antigen-1 (VLA-4), expressed by lymphocytes and monocytes, and probably neutrophils. The T lymphocytes that migrate into inflammatory sites may be memory T-cells i.e. those that have been previously stimulated

by antigen. These memory T-cells are more adhesive than naive T-cells for cytokine-activated endothelial cells *in vitro* and uniquely appear to induce increased endothelial permeability by a contact-dependent pathway. Compared to naive cells, memory T-cells have diminished expression of L-selectin but increased expression of CD44. Perhaps both VLA-4 and CD44 can mediate the binding of T-cells to inflammatory venules. Transmigration of lymphocytes appears to involve the interaction of LFA-1 and ICAM-1 *in vitro*. Nevertheless the endothelial ICAM-1-lymphocyte LFA-1 pathway is more critical for neutrophils as people lacking the LFA-1 receptor exhibit lymphocyte infiltration at inflammatory sites, whereas their neutrophils fail to transmigrate. The β_3-integrins such as VLA-4 and extracellular matrix components e.g. laminin, collagen and fibronectin are also probably involved in leucocyte transmigration.

Lymphocytes and macrophages infiltrate post-ischaemic kidneys within the first few days of reperfusion. In the absence of an alloimmune response, this inflammation subsides for several months. However, a periglomerular and perivascular infiltrate consisting of macrophages and T-cells appears by 24 weeks reperfusion. At 52 weeks these slightly increase in numbers, coinciding with increased expression of both ICAM-1 and MHC II on endothelial cells of vessels, glomeruli and tubules (Tullius *et al.*, 1994). The expression of several chemokines was increased prior to these events and may have been responsible for eliciting the leucocyte homing to the post-ischaemic tissue (Azuma *et al.*, 1997). For example, by 24 weeks, monocyte chemoattractant protein-1 (MCP-1), which acts preferentially on eosinophils, monocytes and memory T-lymphocytes, increased intensely, just before the infiltration of macrophages was seen in the interstitium. After 32 weeks, the pattern of infiltration localised to the glomeruli. The expression of inflammatory mediators from these cells increased after their infiltration. Furthermore cytokines such as IL-1,

TNF-α and IL-6 all increased by 8 weeks of reperfusion when function began to deteriorate. In fact many cytokines can contribute to renal dysfunction by promoting morphological damage such as fibrosis and glomerulosclerosis. The blocking of E- and P-selectins or ICAM-1 prevents the early lymphocyte infiltration seen following renal IR and associated neutrophil infiltration in the rat (Takada *et al.*, 1997a; Dragun *et al.*, 1998) implicating either these adhesion receptors or neutrophil-mediated changes in the initial recruitment of lymphocytes at the site of the injury. The late functional and morphological changes observed in post-ischaemic kidneys are likewise abrogated by the pre-treatment of kidneys with anti-PSGL-1 or anti-ICAM-1 antibody, although this may reflect the prevention of damage early during reperfusion that would otherwise contribute to chronic deterioration of the organ (Takada *et al.*, 1997a; Dragun *et al.*, 1998).

1.2.6 Major Histocompatibility Complex Expression In Renal Ischaemia-Reperfusion

Nearly all vertebrate species studied have been shown to possess highly polymorphic cell surface molecules. These antigenic molecules are coded for by the human leucocyte antigen (HLA) genes as alleles of HLA-A, -B, -C (class I), and HLA-DR, -DQ, -DP (class II) loci. The HLA genes are also known as the MHC and the plasma-membrane proteins they code for are often referred to as MHC I and MHC II. The two classes of MHC proteins are found on different cells; class I are found on virtually every cell in the body except for erythrocytes; class II are found only in the surfaces of macrophages and a few other cell types, including B-cells. No two persons MHC genes are the same except for identical twins. The primary role of these molecules is to govern immune recognition by presenting self and non-self antigenic peptides to the immune system. MHC I have a high

affinity to endogenous, synthesised antigens such as viral antigens, whereas MHC II primarily presents peptides derived via endocytosis. Only when an antigen is bound to an MHC protein on an antigen-presenting cell e.g. macrophage to form an MHC-peptide complex can a T-cell recognize and bind to it. This triggers a transduction cascade leading to T-cell activation, differentiation and initiation of the antigen-specific immune response. The different subsets of T-cells require the peptide to be complexed to different MHC molecules. Cytotoxic and suppressor T-cells bind to MHC I-associated antigens, and are $CD8^+$; helper T-cells bind to MHC II-associated antigens and are $CD4^+$ (Krensky, 1997).

The binding of lymphocytes to MHC-bearing cells such as antigen-presenting cells is necessary for their activation and proliferation. This requires the interaction of the MHC-antigen complex with the T-cell receptor (TCR) complex which consists of the TCR, CD3 (a marker of T-cell phenotype) and either CD4 or CD8 (Figure 1.2). Signal transduction occurs after binding of the TCR to the antigen. It is regulated by the CD3 of the TCR-CD3 complex, not the TCR molecule itself. The CD4 and CD8 molecules produce a considerably stronger T-cell activation by binding the MHC proteins in conjunction with the TCR/CD3 complex than binding to the TCR/CD3 complex alone. The intracellular events supporting antigen binding and the subsequent T-cell proliferation also depend on the linking of accessory molecules such as ICAM-1 and LFA-3 on the antigen-presenting cell to LFA-1 and CD2 on the T-cell respectively. Activated T-cells increase their affinity to ICAM-1 via LFA-1 on their surface. In fact, cross-linking of the TCR/CD3 complex transiently converts the β2-integrin LFA-1 from low avidity to high avidity, providing a mechanism for regulating cellular adhesion and de-adhesion in an antigen-specific manner. This promotes a stronger adherence of these cells to vascular endothelium allowing them to transmigrate into the tissue. Use of anti-LFA-1 monoclonal antibody

decreases the T-cell response by inhibiting T-cell-directed adhesion and cytolysis of target cells. In addition, the VCAM-1 molecule mediates lymphocyte recirculation patterns and can also increase binding of the T-cell to MHC antigens, thereby contributing to antigen-dependent immunity.

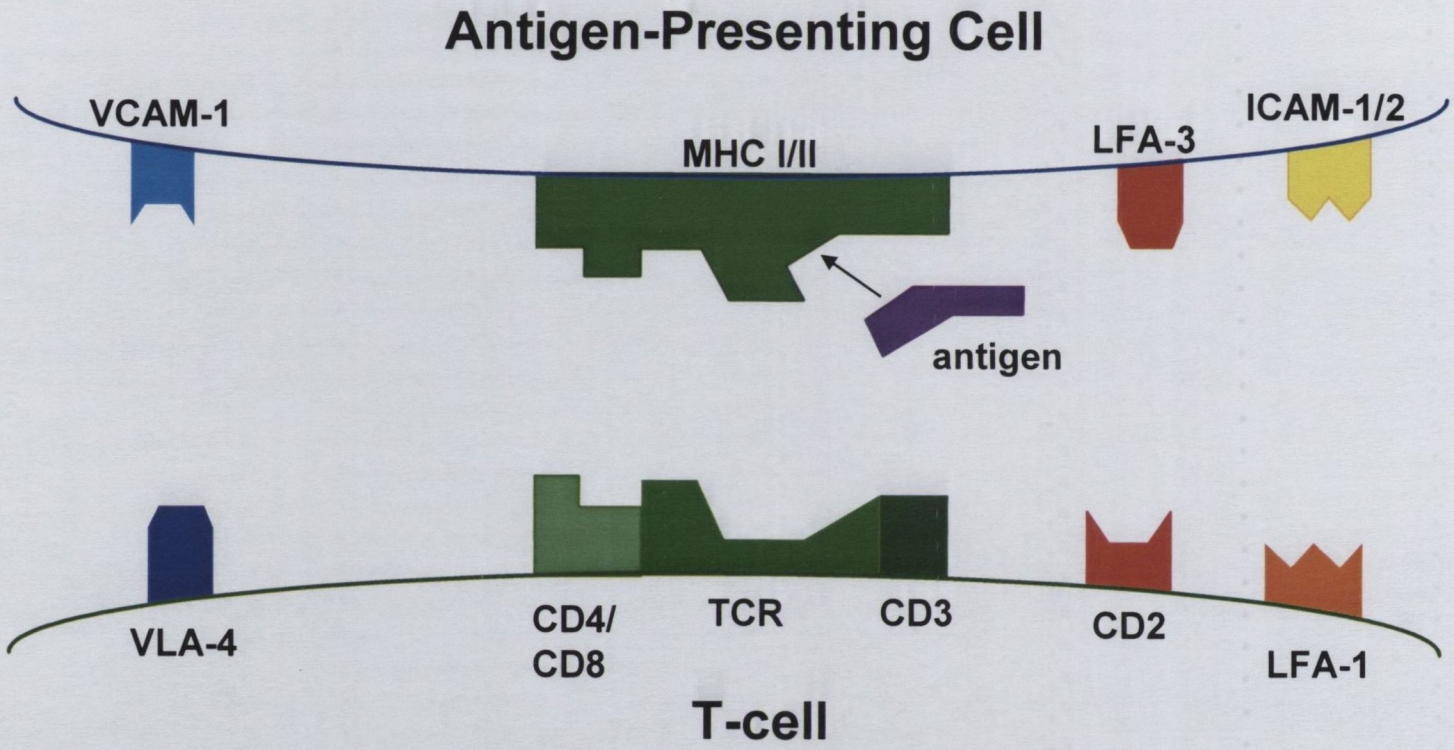


Figure 1.2 The T-cell receptor (TCR) complex and accessory molecules involved in T-cell interactions with antigen-presenting cells. The binding of the T-cell to the MHC-antigen complex leads to activation and proliferation of the T-cell. $CD4^+$ T-cells bind to MHC II whereas $CD8^+$ T-cells bind MHC I . These components of the TCR complex increase the resulting activation of the T-cell. Upon engagement of the TCR complex, the avidity of adhesion molecules such as LFA-1 on the surface of the T-cells may increase, thereby strengthening the association between the T-cell and the antigen presenting cell.

As mentioned before, there is also upregulation of MHC molecules following renal IR injury. Shoskes *et al.* (1990) showed that an ischaemic insult can result in increased expression of MHC antigens on renal tubular and interstitial cells in mice. Recovery from ischaemic injury also increased MHC antigen expression, especially MHC II, on the proximal tubular epithelium in rats (Shackleton *et al.*, 1990). This upregulation was seen by five days of reperfusion. Shoskes and Halloran (1991) found that the induction of MHC proteins following IR was dependent on the release of the T-cell/natural killer cell-derived cytokine interferon-γ (IFN-γ), and occurred following the infiltration of inflammatory cells in these kidneys. Takada *et al.* (1997b) showed that the increased expression of MHC II was associated with lymphocyte and macrophage infiltration into the tissue following upregulation of ICAM-1 and neutrophil infiltration in renal IR injury within the first week of reperfusion. They suggested that since this MHC antigen expression followed the expression of lymphocyte products such as IL-2, IFN-γ, TNF-α, some of these products were responsible. Furthermore, as the late inflammatory changes occur several months following the ischaemic episode, elevated expression of MHC II on endothelial cells of vessels, glomeruli and tubules again coincides with lymphocyte and macrophage accumulation (Tullius *et al.*, 1994; Azuma *et al.*, 1997). Although their role in IR injury is unknown, MHC molecules play a very important role in the immune response to allografted tissue.

1.3 ISCHAEMIA-REPERFUSION AND RENAL TRANSPLANTATION

The advent of organ transplantation and the fact that IR of the donor organ is unavoidable during the transplantation process has stimulated the investigation of IR injury in many organs. In the liver for example all transplanted organs undergo some degree of cell loss during cold ischaemia, while reperfusion reduces hepatocyte volume by a further 20% (Thurman *et al.*, 1988). Morphologically, reperfusion in this setting is associated with loss of sinusoidal cells, leucocyte adhesion, platelet adhesion and increased coagulation (Clavien *et al.*, 1992).

It has been well documented in the kidney that in addition to immunological factors, non-immunological factors contribute to graft loss. To this end, there is considerable evidence that post-transplant renal failure is in part a form of IR injury. In fact IR induces morphological (arteriosclerosis, glomerulosclerosis, tubular atrophy and interstitial fibrosis) and functional (proteinuria) changes comparable with allograft rejection albeit at a slower pace (Tullius *et al.*, 1994). Several inflammatory processes that have been implicated in the pathogenesis of renal IR injury may also contribute to organ damage and dysfunction following transplantation. These include the release of ROS such as O_2^- (Land *et al.*, 1994) and the induction of CAMs that support the infiltration of the grafted organ by leucocytes (Dragun *et al.*, 1998). Several of these events have been targeted for the development of therapies to reduce allograft rejection and are discussed in detail in section 1.3.6.

1.3.1 History Of Renal Transplantation

Allografts are grafts of genetically foreign tissue. It has only been in the past 50 years that the true complicity of the many pathways involved in the destruction and acute rejection of allografts has begun to be fully appreciated. The modern era of transplantation biology was opened by Medawar and Gibson during World War II, when they first described the immunological nature of skin transplant rejection in rabbits. During the 1940s and 1950s, a few attempts at renal transplantation were made in human patients; none of these kidneys functioned for more than a few days except in a single modified individual whose graft sustained him for five months. In 1954, the first successful renal transplant was performed by Dr. Joseph E. Murray between 23-year-old identical twins in Boston. The recipient died of unrelated causes eight years later. This and subsequent transplants between twins gave hope for the future of renal transplantation in humans. There followed a gradual appreciation of the importance of the immune responses and also the realization that they could be manipulated. This led to a series of transplants in Boston and Paris in patients that were immunosuppressed with total body X-irradiation. The first chemical immunosuppression experiments were then carried out in 1962. This breakthrough has lead to the availability of newer and more effective agents being used either alone or in combination (Light *et al.*, 2002). Current immunosuppression regimens employ an induction agent such as an anti-IL-2 antibody, and triple therapy which consists of a calcineurin inhibitor e.g., tacrolimus, an anti-metabolite such as mycophenolate mofetil and corticosteroids such as methylprednisolone. The new developments in this field including humanized monoclonal antibodies hold a lot of promise as they are more specific and less toxic than previously employed agents. In fact, some results suggest a

synergistic beneficial effect may exist between these antibodies and conventional therapy with CsA (Gassel *et al.*, 2000).

1.3.2 Histopathology Of Allograft Rejection

1.3.2.1 Acute Renal Allograft Rejection

There are two major histological patterns during acute rejection; tubular interstitial and vascular. The latter involves intravascular thrombosis, endothelial swelling and proliferation, and fibrinoid necrosis. There is considerable evidence that the microvasculature is the primary and most vital site of acute rejection (Shimizu *et al.*, 2000). After transplantation, there is an initial neutrophil infiltration, followed by macrophage and lymphocyte transmigration through the vascular endothelium of the graft and into the interstitium. The host lymphocytes recognize the donor antigens on antigen-presenting cells and proliferate and differentiate into specific killer and memory cells. During allograft rejection, these T-cells also secrete cytokines. Interleukin-2 is the primary cytokine involved in T-cell responses, allowing the T-cells to fuel their own proliferation. Other cytokines, in particular IFN-γ, amplify the response by inducing high-level expression of MHC II and adhesion molecules (e.g. ICAM-1, VCAM-1) on the graft. These molecules are key regulatory molecules in acute rejection episodes, participating in both recruitment and activation of T-cells. The cytokines can also promote accumulation of other cells into the site of inflammation, particularly macrophages, natural killer cells and platelets. In fact, vascular rejection is usually associated with high levels of macrophage infiltration and activation. The endpoint of acute rejection is irreversible microvascular thrombosis and subsequent necrosis of the transplanted organ.

1.3.2.2 Chronic Renal Allograft Rejection

Chronic rejection is a relentless and progressive process which is a major cause of long-term graft dysfunction and ultimate failure. It has been postulated that chronic graft dysfunction is mediated by both alloantigen mediated (MHC incompatibility) and independent (ischaemia) factors (Herrero-Fresneda *et al.*, 2003). The impact of the rejection process contributes to the increased difficulty of another transplant (Suthanthiran, 1997). The clinical picture of the chronically rejecting kidney shows progressive deterioration of renal allograft function i.e., increasing plasma levels of creatinine, hypertension, and proteinuria. Histologically, there are vascular, glomerular and tubulointerstitial changes. Intimal proliferation of the transplant arteries is present along with persistent inflammation, progressive glomerular sclerosis, interstitial and intimal fibrosis and tubular atrophy (Vuillemin *et al.*, 1997).

Neutrophils, lymphocytes, macrophages, eicosanoids and PAF are present in both acutely and chronically rejecting organs. The various populations of leucocytes show temporally different patterns of infiltration into various organ compartments during chronic rejection, with the most prominent cells being the monocyte/macrophage population. Neutrophils infiltrate grafts early on, in the first month or two. The lymphocytes appear to play a smaller role in chronic rejection when compared with acute rejection. Despite this, T-cells from recipients experiencing chronic rejection do show enhanced adherence to vascular endothelium, especially when the endothelium is activated and exhibits upregulation of CAMs such as E-selectin, ICAM-1 and VCAM-1 (Korczak-Kowalska *et al.*, 1997). In fact renal allograft rejection has been associated with parallel increases in VCAM-1 and serum creatinine. Circulating ICAM-1 levels are also increased during renal graft rejection.

When monocytes and lymphocytes were incubated with sections of rat kidneys undergoing chronic rejection, LFA-1 and ICAM-1 were identified as some of the accessory molecules involved in the interaction of these cells with the transplanted organ (Heemann *et al.*, 1994).

Both chemokines and another group of regulatory molecules, growth factors, are also involved in the molecular cascade in chronic rejection. They are secreted by inflammatory and/or tissue cells, particularly endothelial cells, in response to the persistent low-grade damage induced by the immune response. For example, the expression of regulated on activation normal T-cell expressed and secreted (RANTES) mRNA is induced in infiltrating mononuclear cells and tubular epithelium, and RANTES protein has been localised to these cells and to vascular endothelium during cell-mediated rejection. In a rat model of renal allograft chronic rejection, the early phase is characterised by upregulation of RANTES and ICAM-1. In fact, blocking RANTES not only reduces early infiltration and activation of mononuclear cells in renal allograft but the pace of chronic graft deterioration is slowed down (Song *et al.*, 2002). MCP-1 expression also elevated during the early stage of chronic rejection. It increases following maximal RANTES and ICAM-1 levels and corresponding with late monocyte and early lymphocyte infiltration (Azuma *et al.*, 1997).

1.3.3 The Role Of Acute Rejection Episodes In The Development Of Chronic Rejection

Despite having different mechanisms and histopathologies, it has been shown conclusively that the frequency and possibly the intensity of acute rejection episodes are more strongly

correlated to subsequent chronic rejection than any other clinical parameter (Sijpkens *et al.*, 2003). The occurrence of even one reversible acute rejection episode is associated with decreased short-term and long-term graft survival rates. The rate of graft loss due to chronic rejection 10 years following transplantation increases from 6% with no episodes of acute rejection to 14% when the first rejection episode occurs within the first three post-transplantation months to 55% of grafts experiencing chronic rejection when they undergo their first acute rejection episode after three months post-transplant (Sijpkens *et al.*, 2003). Furthermore, the number (Sumrani *et al.*, 1993) and severity (Van Saase *et al.*, 1995) of the acute rejection episodes increases the risk of chronic rejection occurring in the graft. Another consideration is the fact that each acute rejection episode reduces the functional mass of the graft. The remaining nephrons begin to hyperfiltrate and gradually undergo fibrosis. This may induce a vicious cycle with complete destruction of the graft at its endpoint (Heemann *et al.*, 1994). This is supported by the observation that changes seen in chronic rejection occur at a faster pace in reduced mass kidneys that are transplanted (Tullius *et al.*, 1994; Azuma *et al.*, 1997).

In 1997, Kokado *et al.* demonstrated that acute rejection in cadaveric donor renal transplantation has a major deleterious impact on long-term graft outcome, but acute rejection in living donor renal transplantation has no effect on long-term outcome. This finding suggests that the injury associated with brain death and preservation which accompanies cadaver donation (accounting for more than 90% of donors in Ireland and the UK (Belger *et al.*, 1997)), and is lacking in living donors, both related and unrelated, predisposes the graft to immunological recognition and rejection (Halloran *et al.*, 1997). Both leucocytes and the vascular endothelium participate in this immunological rejection.

1.3.4 The Influence Of Early Graft Function And Ischaemia-Reperfusion On Renal Allografts

Early graft function is one of the strongest predictors of ultimate graft survival suggesting that IR injury, which exerts an immediate impact on the reperfused graft, may play a role in transplant rejection. Opelz *et al.* (1978) and Sanfilippo *et al.* (1984) found in multi-centre studies that delayed graft function (DGF) decreased ultimate graft survival and increased patient death. Halloran *et al.* (1988a) found that the likelihood of graft loss increased by 2.86 times in patients with DGF. Many of these losses are within the first six months post-transplant (Prommool *et al.*, 2000). Shoskes *et al.* (1990b), in their studies with paediatric transplantation found similar results.

Grafts with long cold ischaemic time often exhibit poorer initial function than grafts with shorter cold ischaemic times. Age, panel-reactive antibodies (PRA), preoperative transfusions (Shoskes *et al.*, 1990b) and the warm ischaemia time and/or anastomosis time are other important factors. Using a score of one for each of the following factors, anastomosis time >45 min, highest PRA >80%, cold storage time >40 hr, donor age <4 years, Shoskes *et al.* (1990b) found there was a significantly higher score in the non-functioning versus the delayed functioning versus the early functioning grafts.

Transplantation necessitates IR when the organ is removed from its native bed and inserted into the recipient. Generally cold storage is a routine method of storage for an organ between removal from the donor and insertion into the recipient. In 1936, Bickford and Winton first showed the protective effect of hypothermic ischaemia over warm ischaemia in the kidney. This was confirmed by Wickham *et al.* (1974). Nowadays, in

order to minimize ischaemic damage, kidneys are rapidly cooled and flushed with a preservation solution. Cooling slows down cellular reactions and therefore decreases oxygen consumption. University of Wisconsin solution which has proven far better as a preservation solution than the previously used Collins solution, contains glutathione and adenosine to aid regeneration of ATP, lactobionate, phosphate and raffinose to prevent cellular swelling, and allopurinol to inhibit XO. Glutathione and raffinose also act as antioxidants (Weight *et al.*, 1996). Because of these developments, it is currently acceptable to transplant kidneys that have been subjected to long periods of cold ischaemia. However, cold storage time of more than 24 h is associated with increased DGF in both young and old recipients (Lee *et al.*, 2000a). In fact, if the cold ischaemic time is more than 48 h, the risk of delayed function increases more than twofold (Shoskes *et al.*, 1989). Furthermore, a recent study demonstrated that while immunological factors such as HLA mismatch exerted an initial negative impact on renal graft survival, the detrimental effect of cold ischaemia time of over 24 h was apparent over the long-term (Smits *et al.*, 2000). Lee *et al.* (2000b) demonstrated those cold storage times of longer than 36 h actually obviated the benefits of HLA matching on graft survival.

Anastomosis time has also been found to be a very strong predictor of graft function. It is often longer in transplants where there is a cadaveric donor rather than a living-related donor. Warm ischaemia time before the kidney is removed must also be considered. While multi-organ procurement techniques have decreased typical warm ischaemia time to almost zero, the increasing interest in non-heart-beating donors may make warm ischaemia times more common and so re-introduce this extra risk factor (Gonzalez-Segura *et al.*, 1998). Warm ischaemia time correlates with the number of reversible rejection episodes within the first year, possibly by increasing graft immunogenicity (Van Es *et al.*,

1983). Furthermore, both DGF and reduced graft survival are elevated in organs from non-heart-beating donors with warm ischaemia time above 45 min (Gonzalez-Segura *et al.*, 1998). Reducing ischaemia time significantly attenuated later vascular and glomerular changes whereas creatinine levels continued to rise following prolonged ischaemia (Yilmaz *et al.*, 1992b).

Many cadaver renal grafts suffer from reversible ARF in the immediate post-transplantation period because of injury sustained during harvesting and *ex-vivo* preservation techniques. The extent and rate of recovery from the resulting tubular epithelial damage may be important in the development of DGF. This initial delayed function increases the rate of both acute and chronic rejection and long-term graft loss (Halloran *et al.*, 1997). If there is no associated acute rejection, the risk of graft loss falls. This has been reiterated by many studies where graft survival drops significantly in the presence of both DGF and acute rejection episodes when compared with grafts undergoing either of these complications alone (Troppmann *et al.*, 1996). This may further explain why living unrelated kidney donors (e.g. spouses) have excellent graft survival despite extensive HLA mismatching. The lack the injury associated with brain death (warm ischaemia) and cold storage that is found in cadaveric grafts, may lead to higher graft survival rates from spousal donors because this injury predisposes organs to DGF as well as to immunological recognition and rejection. However, in the presence of both DGF and acute rejection even kidneys from living related donors experience lower graft survival rates than those that undergo either event alone (Senel *et al.*, 1998).

1.3.5 The Role Of Ischaemia-Reperfusion Injury In Allograft Rejection

Renal allograft IR is unavoidable in the course of graft retrieval and implantation. The IR injury occurs during the first hours post-transplantation. Clinical studies indicate that kidney grafts that manifest poor initial function have markedly worse survival rates than grafts that function immediately (Halloran *et al.*, 1988a). The initial cellular infiltration during rejection is antigen independent, and so therefore may be due to the IR injury (Tullius *et al.*, 1994). Following transplantation it has been elucidated that tubulointerstitial inflammation due to IR injury (as can be seen in Figure 1.3) synergizes with alloantigen-dependent development of vascular lesions, ultimately leading to chronic allograft nephropathy and graft loss (Herrero-Fresneda *et al.*, 2003). Furthermore, there is evidence to suggest that IR injury may actually increase graft immunogenicity, thereby increasing acute/chronic allograft rejection (Land *et al.*, 1994). Shackleton *et al.* (1990) first observed that ischaemia followed by both five and seven day recovery periods induced expression of class I and class II MHC antigens in an experimental setting, speculating that this may explain the link between IR injury and chronic rejection. Thus IR injury may render organs more susceptible to allospecific cell-mediated lysis.

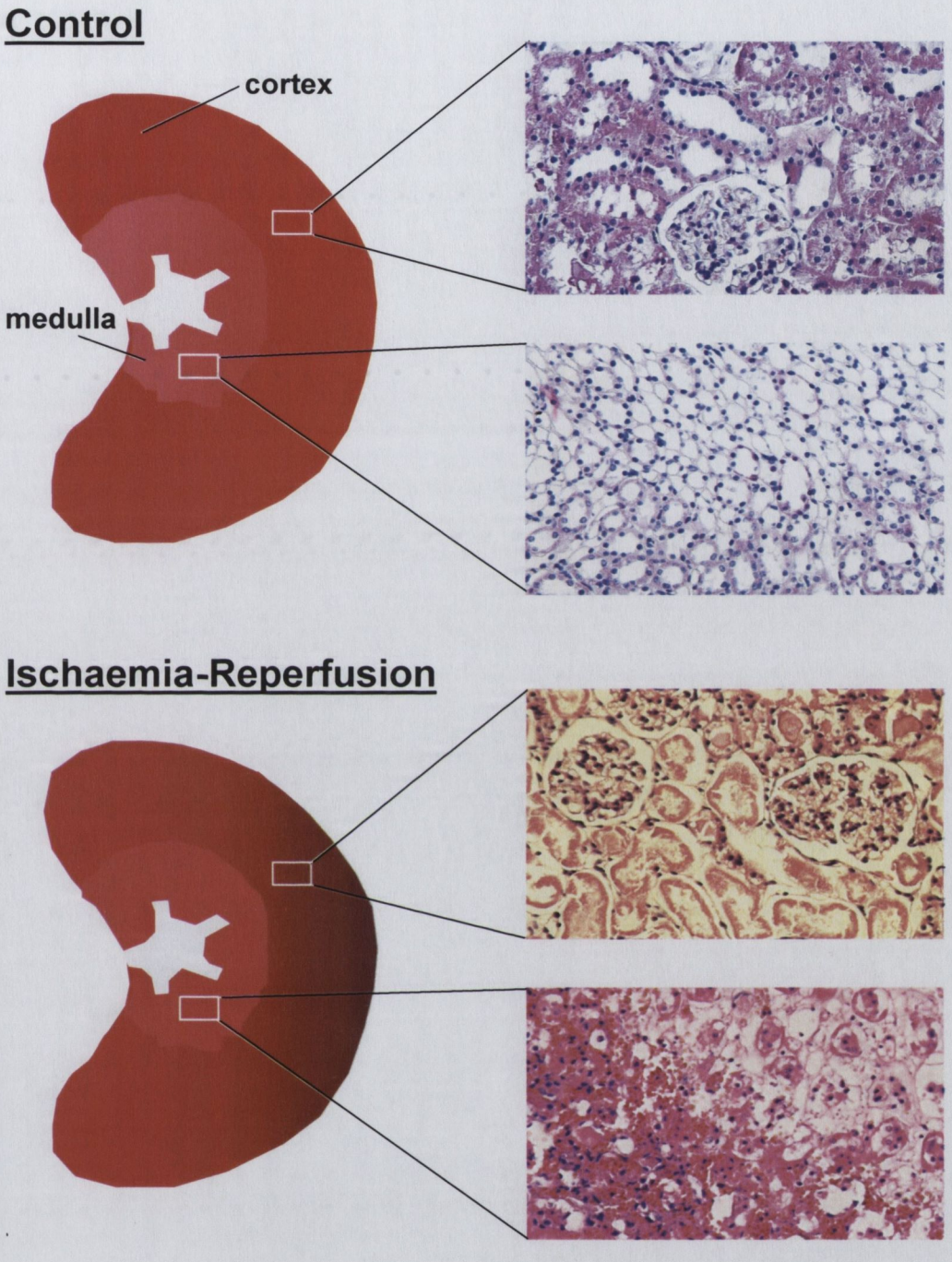


Figure 1.3 Histological representation of damage to the renal cortex and medulla following ischaemia-reperfusion. Under normal conditions, epithelial cells are in close proximity with each other forming an organized tubular network throughout the cortex (a) and medulla (b). However following IR, leucocytes infiltrate the kidney. The epithelium of the cortex deteriorates and detaches from the basement membrane (c). Furthermore, proximal tubules in the medulla are damaged and red blood cell congestion occurs (d).

1.3.5.1 Severity of Ischaemia-Reperfusion Injury

Yilmaz *et al.* (1992b) showed that reduction of ischaemia time from 60 min to 30 min significantly reduced both vascular and glomerular changes in rat renal allografts and also reduced the incidence of chronic rejection independently of intensity of immune response infiltration. Late changes comparable to those seen during chronic rejection have been found in human renal transplants between identical twins (Tilney, 1986). A study by Tullius *et al.* (1994b) found that experimental renal allografts that were re-transplanted into the donor strain at various post-transplant times, experienced reversal of rejection at early times, but there reached a critical point whereby rejection not only remained but was enhanced. This suggests that while earlier stages of chronic rejection are mediated by alloantigen-dependent events, and are reversible, later irreversible stages are probably antigen-independent.

1.3.5.2 Ischaemia-Reperfusion-Induced Leukocyte Recruitment

Tilney's group also demonstrated that the functional and morphological changes seen during chronic rejection in the rat were comparable to those caused by renal IR, albeit they occur at a slower pace and lower intensity following IR (Tullius *et al.*, 1994a). Furthermore, they showed that neutrophils were the initial cells entering the graft, followed by lymphocytes and macrophages during the initial acute rejection episode. Takada *et al.* (1997) also described this pattern of infiltration in renal IR injury, where neutrophil accumulation, was accompanied by increased E-selectin expression. The expression of ICAM-1 increased between one and three days of reperfusion. Lymphocyte infiltration began during the peak of ICAM-1 expression and was associated with

increased MHC II expression at day 5, in accordance with the findings by Shackleton *et al.* (1990). In the long-term, neutrophils and macrophages were also present in isografts at one year post-transplantation (Tullius *et al.*, 1994a), implicating a role for non-immunological factors in renal allograft rejection.

In contrast to these studies, Kouwenhoven *et al.* (2001a) recently demonstrated that the transplanting of kidneys into syngeneic hosts did not lead to any differences in renal function or morphology compared with uninephrectomised animals, with the exception of a greater infiltration of $CD4^+$ cells and macrophages, primarily in the glomeruli, along with a higher expression of ICAM-1 in the glomeruli and interstitium. They suggested that the actual act of transplantation and the associated IR insult were not important factors in the development of allograft rejection. However, this same group went on to demonstrate that allografts that had undergone ischaemia developed interstitial cell infiltration and tubulitis earlier than non-ischaemic allografts, suggesting a role for IR injury in the exacerbation of an immune-mediated injury, despite the absence of a discernable injury in ischaemic isografts (Kouwenhoven *et al.*, 2001b). Other recent work has demonstrated that the length of the ischaemic period is an important predictor of the development of chronic allograft nephropathy and subsequent loss of the graft (Nankivell *et al.*, 2001).

1.3.5.3 Ischaemia-Reperfusion and the Alloimmune Response: Role of ICAM-1 and IL-2

The IR-induced upregulation of ICAM-1 on the endothelium may provide an additional role for IR in the development of graft rejection. The microvascular endothelium is the first allogeneic barrier encountered by host blood cells and ICAM-1 is the receptor most intimately involved in T-cell adherence. These cells infiltrate the graft and are primary

immune cells involved in rejection. This suggests that ICAM-1 may be important not only in the initial stages of adherence of neutrophils to endothelium but also in strengthening the association between allospecific effector cells, such as T-cells, and the target cells of the graft.

The fact that both ischaemic and immunological factors contribute to DGF, which in turn predisposes to higher rates of rejection and graft loss suggests that these two factors may be interrelated. In fact Aprile *et al.* (1987) both suggested that ischaemic injury may predispose the graft to the development of the allograft response and thus rejection. Shoskes *et al.*, 1990a showed that ischaemic damage resulted in increased expression of MHC antigens on renal tubular and interstitial cells in mice. Recovery from ischaemic injury also increased MHC antigen, especially MHC II, expression on the proximal tubular epithelium in rats (Shackleton *et al.*, 1990). More recently, it was demonstrated that ischaemia enhances the expression of MHC II on both isograft and allograft endothelium and proximal tubular epithelium (Kouwenhoven *et al.*, 2001b). Blocking ICAM-1 expression using antisense oligonucleotides not only attenuated ICAM-1 upregulation and the associated neutrophil and monocyte infiltration but also reduced the MHC II induction and renal dysfunction in rat renal autografts (Dragun *et al.*, 1998). This suggests that ICAM-1 and inflammatory mediators from infiltrating neutrophils, monocytes and/or endothelial cells may promote T-lymphocyte infiltration. These T-cells in turn release cytokines such as IL-2 that are not only capable of promoting continued inflammation and expression of ICAM-1, but may also induce MHC II expression.

In agreement with this, peak MHC II induction and $CD4^+$ infiltration of kidneys occurs in parallel with maximum IL-2 levels, following renal IR or transplantation (Takada et al.

1997a; Nagano et al. 1997). In allografts once activated $CD4^+$ T cells bind to donor MHC II molecules, they release more IL-2 which leads to the clonal proliferation of alloreactive cells and stimulates $CD8^+$ T cell development into mature cytotoxic effector cells. Together with macrophages activated by IFN-γ, these leucocytes attack graft cells and may cause graft failure. Interestingly, expression of IL-2 occurs prior to and is significantly correlated with the subsequent development of acute renal allograft rejection (Oliveira G. et al. 1998).

A role for IR in rejection is underlined by the observation that increased levels of both ICAM-1 and MHC II are associated with acute rejection in human kidneys (Gonzalez-Posada *et al.*, 1996), thus IR may predispose tissue to rejection during transplantation by elevating both adhesion molecules and MHC expression (Kouwenhoven *et al.*, 2001b). The post-ischaemic induction of T cell cytokines such as IL-2 may participate in this inflammatory process through the induction of MHC and propagation of the response. Supporting this, another study found that strong expression of ICAM-1, VCAM-1 and MHC II on vascular endothelium and tubular epithelium alongside infiltrating macrophages and IL-2R-expressing-T-lymphocytes highly correlate with acute renal rejection in humans (Anderson, 1997). Furthermore, IR-induced adhesion molecule expression and subsequent leucocyte infiltration appear to participate in post-ischaemic tubular injury, and therefore subsequent renal dysfunction which could lead to DGF in a transplant setting, which would serve to confound the above inflammatory processes.

1.3.6 Role of Therapies Directed Against Ischaemia-Reperfusion-Associated Events In Transplantation

Development of therapies to counteract rejection is aimed at preventing graft rejection or intervening as early in the rejection process as possible in order to minimize damage, since it is likely that once the rejection has progressed to the chronic stage it cannot be reversed. Delayed graft function and acute rejection episodes negatively impact long-term graft survival and are correlated to chronic rejection. Therefore the rapidity of processes leading to progressive chronic obliterative transplant arteriosclerosis appears to be dominated by the number of repetitive endothelial injuries which may be brought about by events such as reperfusion injury and acute rejection episodes. With improved understanding of the pathophysiology of renal IR injury, strategies to decrease the rates of DGF, acute rejection episodes and possibly chronic rejection have been investigated clinically and experimentally. Preservation solutions have been designed to minimize ischaemic damage during kidney storage, prior to donor implantation. Components are added to decrease cell swelling, maintain calcium homeostasis, reduce free radical substrate formation, and to provide high energy substrates. Other agents that reduce inflammation, inhibit smooth muscle cell proliferation and prevent the generation of arteriosclerosis should also prove beneficial. Several therapies have been proposed to date.

1.3.6.1 Monoclonal Antibodies Against Adhesion Molecules And Antigens

Both polyclonal and monoclonal antibodies against cell-surface proteins have been used as induction therapy and/or anti-rejection drugs. However, some monoclonal antibodies can cause lysis of important subsets of leucocytes, can activate and desensitize leucocytes as a

consequence of signal transduction events invoked by specific antibody-antigen interactions, and can perturb other cellular functions through non-specific interactions with ligands. In fact studies using antibody therapies have provided somewhat mixed results. With these potential problems in mind, the valuable insights into mechanisms of injury afforded by the use of monoclonal antibodies must be interpreted cautiously (Rabb *et al.*, 1997).

Antibody therapies are proving somewhat more successful in some species than in others. Anti-ICAM-1 therapy, which is mildly effective in the mouse, increases renal graft survival and decreases allograft cell infiltration in the non-human primate (Cosimi *et al.*, 1990). This therapy also reverses pre-existing rejection caused by sub-therapeutic doses of CsA. In phase I trials in human renal transplantation an adequate serum level of anti-ICAM-1 monoclonal antibody is associated with a significantly lower incidence of rejection (Haug *et al.*, 1993), presumably by decreasing host T-cell infiltration of the graft without effecting the numbers of circulating lymphocytes. However, more recent data failed to find protection against renal dysfunction during acute rejection in humans (Salmela *et al.*, 1999). Species differences have also been observed with antibodies directed against other CAMs i.e. in cardiac graft models anti-VCAM-1 promotes only a modest increase in survival in the mouse, but improved outcome of these grafts by 5-6 days in the rat.

Monoclonal antibodies against LFA-1, a ligand for ICAM-1, are beneficial in promoting graft survival in non-human primate skin transplantation, mouse and rat cardiac grafts and thyroid grafts. Although initial studies suggested that anti-LFA-1 was ineffectual in the treatment of established acute kidney graft rejection in humans (Le Mauff *et al.*, 1991), a

more recent study has demonstrated that anti-LFA-1 antibody improved renal function and reduced acute rejection to levels found with anti-thymocyte globulin (Hourmant *et al.*, 1996).

Unexpected results with several antibodies have prompted further investigation as to the exact role of individual CAMs during allograft rejection. The improvement of graft survival in rat cardiac allografts receiving anti-VCAM-1 was independent of the number of graft-infiltrating cells, implicating a role for VCAM-1 in processes other than migration of cells into the graft. Use of an antibody against VLA-4, a ligand for VCAM-1, was also effective in rat heart transplantation.

The expression of both ICAM-1 and MHC increases on vascular endothelium early during acute rejection. Lymphocytes, stimulated via LFA-1/ICAM-1 adhere to endothelium and extravasate into the tissues or migrate to host lymph nodes where they impart their information to other cells. However, since ICAM-1 and LFA-1 do not function on the graft endothelial level alone but may be important as co-stimulatory molecules for host lymphocytes, combinational therapies of anti-LFA-1 and anti-ICAM-1 antibodies have been used in mouse cardiac grafts between fully incompatible strains. This has yielded indefinite graft survival. This tolerance was specific for the donor origin as recipients rejected skin grafts from a third strain but not from the donor strain. These two monoclonal antibodies were insufficient alone to prolong tolerance, and so may have acted synergistically to induce specific unresponsiveness. Antibodies against VLA-4 and LFA-1 have prevented or greatly delayed graft rejection in rat islet allografts.

Evidence that the extracellular matrix components may play a role as a signal for lymphocyte migration to specific organs including the allograft comes from studies where cardiac allografts were received by T-cell-deficient hosts. Antibodies to laminin were administered 30 min before lymphocyte injection and tissues were harvested 6 h later. Lymphocyte recovery from peripheral lymph nodes and grafts was significantly diminished in laminin antibody-treated animals compared to rats pre-treated with control serum alone. Other therapies include those using synthetic peptides that specifically block the interaction between integrins (not only on T-cells but also on other leucocytes) and their ligands (Bluestone J.A. *et al.*, 2000).

1.3.6.2 Anti-Oxidant Therapy

The reperfusion injury-induced free radicals might represent the initial step in the process of intragraft antigen recognition by allowing lymphocytes to immigrate into the graft and initiate processes of the biological cell cascade which ultimately leads to graft destruction. In chronic transplant failure, the reperfusion injury may play an additional role with ROS-mediated endothelial injury resulting in intima thickening caused by excess smooth muscle cell proliferation during the repair process.

The impact of IR on acute/chronic graft rejection has been further emphasized by a clinical study which demonstrated significant reductions in the incidence of acute and chronic rejection episodes with the use of SOD (Land *et al.*, 1994). The administration of human recombinant SOD to recipients of cadaveric renal allografts during surgery exerted a beneficial effect on both acute and chronic rejection events following transplantation (Land *et al.*, 1994). This was demonstrated by a significant reduction in first acute

rejection episodes as well as early irreversible rejection episodes. A significant improvement in the actual 4-year graft survival rate was also observed in the SOD-treated patients. While this supports many studies that have suggested that graft survival is increased in the absence of acute rejection episodes, treatment with SOD also prolonged the graft survival of those grafts that were subjected to acute rejection episodes. Thus it is likely that a combination of both reperfusion injury and acute rejection episodes leads to highest incidence of graft loss.

While its clinical use may be limited due to a relatively short half-life, several recent experimental studies have found that using modified versions of SOD with longer half-lives show promise. These include lectinized SOD, which binds with a high affinity to cell membranes, and which is capable of reducing microvascular endothelial CAM upregulation and leucocyte adhesion following cold hypoxia-reoxygenation. Furthermore, the lectinized SOD attenuated cell death due to cold storage (Koo *et al.*, 2001). When this modified SOD was used in vivo in renal allografts, early inflammatory responses mediated by neutrophils and macrophages was attenuated. However, while renal dysfunction and apoptosis were also reduced after 24 weeks reperfusion, lymphocyte infiltration and ICAM-1 and MHC II upregulation remained increased, i.e. unaltered by SOD treatment (Nakagawa *et al.*, 2002). This supports a role for both an early O_2^--dependent IR injury and an alloimmune O_2^--independent component in the development of chronic inflammatory responses in transplanted kidneys.

Mannitol has also been shown to improve early graft function when administered to the recipient just before reperfusion (Van Valenberg *et al.*, 1987). Adding this oxidant scavenger to the organ flush solution also reduces ARF in allografts (Salahi *et al.*, 1995).

1.3.6.3 Targeting Other Endothelium-Derived Injury Mediators

The use of an antagonist against the ET-1 receptor ET-A, improves not only post-ischaemic function (Knoll *et al.*, 2001), but also protects tubular and glomerular function following prolonged organ preservation in a renal transplant model (Hammad *et al.*, 2001), whereas the use of a NOS inhibitor increases renal allograft rejection and aggravates glomerular injury (Stojanovic *et al.*, 1996). Administration of a Tx antagonist improves allograft function and survival (Coffman *et al.*, 1989). Prostacyclin analogues have been shown to and prolong allograft survival in animal models (Redgrave *et al.*, 1992). However, evidence of a benefit in human transplantation remains to be shown.

1.3.6.4 Therapies Targeted Against Transcription Factors

Many immunosuppressive regimens focus on one of two pathways aimed at attenuating T-cell-mediated attack of the allograft: 1) the activation of the T-cell through the TCR pathway, and 2) co-stimulatory pathways that include other T-cell surface receptor interactions and the release of soluble factors such as IL-2. Many receptors (e.g. E-selectin, ICAM-1, VCAM-1, and various T-cell receptors), cytokines (e.g. IL-2, IFN-γ, TNF-α) and chemokines (MCP-1) involved in these processes are under transcriptional regulation, and therapies that modulate transcription factors may be of benefit. For example, the transcription factor, nuclear factor-κB (NF-κB) is essential for the induction of many of these immunoregulatory genes, including those encoding IL-2, IFN-γ, TNF-α, MHC I, MHC II, and CAMs such as L-selectin and ICAM-1. It may act synergistically with other transcription factors such as AP-1 to regulate some of these genes. NF-κB is normally kept in an inactive cytoplasmic complex with one of a family of inhibitory

proteins, inhibitory-κB (I-κB). Upon cell stimulation the I-κBs are degraded and NF-κB is released, whereby it migrates to the nucleus and activates its target genes. The use of NF-κB decoy oligodeoxynucleotides in a rat renal allograft model protects against early endothelial VCAM-1 expression and macrophage infiltration (Vos *et al.*, 2000). Interestingly, one of the mechanisms through which the immunosuppressive glucocorticoids act may be through induction of I-κB and therefore inhibition of NF-κB. Also with particular relevance to the field of transplantation, it has been demonstrated that CsA exerts at least some of its immunomodulatory effects through the inhibition of the calcineurin-dependent dephosphorylation of the transcription factor, nuclear factor of activated T-cell (Kung and Halloran, 2000). Inhibition of one or more of these transcription factors may prove effective in anti-rejection therapy.

1.3.6.5 Chimeric Antibodies Targeted Against Interleukin-2

Despite the interest in the prevention of inflammatory events at the transcriptional level, there has been a large amount of attention recently focused on blocking IL-2-mediated events during allograft rejection. This cytokine influences the adaptive immune response through the upregulation of MHC II expression and the promotion of clonal proliferation of alloreactive cells. Cyclosporine A, (CsA), an immunosuppressant widely used during transplantation limits the T lymphocyte-mediated response to the allografted tissue by inhibiting IL-2 synthesis. However, T cells appear to possess a CsA-resistant proliferative activity that is IL-2-dependent, suggesting that CsA does not completely block IL-2 activity (Geginat J., et al 2000). Chimeric antibodies have been recently developed to block the IL-2R subunit CD25, which is expressed only on T cells (both resting and activated) and is upregulated upon the engagement of the T-cell receptor with an antigen,

this being one of the earliest events upon T cell activation. The combination of CsA and these chimeric antibodies against CD25 achieves inhibition of IL-2 synthesis as well as the IL-2/IL-2R interaction (Madsen, M., Svendsen, U.G. 2000). This leads to reduction of acute rejection in human trials by as much as 30-40% (Ahsan, N. et al. 2002; Kahan, B. D. et al. 1999; Wiseman & Faulds 1999). Furthermore, since CsA possesses IL-2-independent anti-inflammatory properties, for example, the attenuation of ICAM-1 induction on renal tubular epithelium by TNF-α (Frishberg Y, Meyers CM, Kelly CJ. 1996), this combination therapy may be targeting both alloimmune-independent and alloimmune-dependent IL-2-mediated events during transplantation thereby improving graft outcome.

1.3.6.6 Other Pharmaceutical Agents

Calcium channel blockers have been shown to improve initial graft function and decrease the nephrotoxic effects of CsA. They act through a direct vasodilator effect on the proximal arterioles or through decreasing lipid peroxidation during reperfusion. Several prospective trials have shown improved function in the graft after treatment with diltiazem (a Ca^{2+} channel blocker) (Neumayer *et al.*, 1992).

Atrial natriuretic peptide had been effective in decreasing ischaemic renal injury *in vitro*, but clinical studies demonstrated little value (Gianello *et al.*, 1995). Other drugs include a new class of 21 aminosteroids, agents which prevent lipid peroxidation, and have been shown to be of benefit in the experimental setting (Stanley *et al.*, 1993).

1.4 THE HEAT SHOCK RESPONSE OR PRECONDITIONING

1.4.1 Discovery Of The Heat Shock Response

A physiological response is induced in cells from virtually all organisms, ranging from bacteria to mammals by exposure to elevated temperatures (Snoeckx *et al.*, 2001). This response, called the heat shock response, was first discovered in Drosophila by Ritossa in 1962. He found that exposure to heat caused chromosomal puffing in the salivary glands of this insect. Over ten years later, Tissieres *et al.* (1974) discovered that this phenomenon was associated with the induction of highly conserved proteins called heat shock proteins (HSPs). As the temperature of Drosophila rose between 23°C and 38°C cells stopped making most other proteins and instead produced far more HSPs than were normally expressed. The 70 kDa HSP (HSP70) was noted in particular. Temperatures inducing a heat shock response in cultured cells vary, depending on the organism from which the cells are derived. In Drosophila the response is seen at 35°C, humans and rodents at 41-43°C and in sheep at 45°C. In mammalian cells, the transient inhibition of normal protein synthesis is seen at about 2°C above the temperature inducing the synthesis of HSPs.

Knowledge of HSPs has rapidly expanded since then. It is now known that although many HSPs are constitutively expressed and active in normal cells where they serve important physiological functions, their synthesis is induced by a wide variety of stress-inducing agents apart from heat. These include oxidative injury such as IR, inflammation, fever, sodium arsenite (SA), amino acid analogues, heavy metals, alcohol and inhibitors of energy metabolism. For this reason the heat shock response is more appropriately known as the stress response, and HSPs as stress proteins. The induction of HSPs is rapid and

occurs below the toxicity thresholds of the inducing agents. Regardless of the type of stress, it appears that the common stimulus leading to HSP induction is protein damage. However, the actual pathway through which the cell senses protein damage and signals HSP expression has yet to be fully elucidated.

1.4.2 Classification And Role Of Heat Shock Proteins

The HSPs are members of a group of stress proteins which also includes glucose-regulated proteins. The HSPs are usually classified and named according to their apparent molecular weight. These include the low molecular weight HSPs e.g. HSP10, the HSP60 and HSP70 families and the high molecular weight family of HSP90. The role of the HSP is to ensure that protein assembly and folding is performed quickly and correctly. Thus it follows that a common factor to all stresses that induce HSPs is the denaturation of proteins. Most inducers of HSPs cause cellular proteins to lose their configuration, and therefore their biological function. In fact, the injection of denatured proteins into living cells actually causes induction of the stress response. Constitutive HSPs become unavailable once they bind proteins that are denaturing due to the stress, and so new HSPs, needed by the cell, are induced. The damaged proteins can be held by the HSPs, and their release delayed until they are properly folded. If some of the damaged proteins are irreparable, the HSPs are needed both to present the damaged protein to lysosomes for degradation and to help the successful synthesis of new fully functional and correctly folded proteins which are essential for the recovery of the cell. Stress proteins are also involved in the intracellular translocation of proteins. Their individual roles in the homeostasis of cells and organisms will be discussed in further detail in the following sections.

1.4.3 Heat Shock Protein 70 kDa Family

Perhaps the most conserved and best characterised HSP is HSP70. The HSP70 family includes the constitutive and inducible forms between 68 and 72 kDa. The constitutive member of the HSP70 family is HSC70 (or HSP68) and the inducible form, HSP70 (or HSP72). Confusingly, in rodents, HSC70 and HSP70 are often called HSP70 and HSP68 respectively (Pelham, 1986). For the sake of this thesis the former shall apply, i.e., the constitutive form shall be referred to as HSC70 and the inducible form as HSP70. Members of the HSP70 family bind damaged proteins, prevent premature folding and denaturation, and act as 'molecular chaperones' in other protein-protein interactions (Snoeckx *et al.*, 2001).

In growing cells, HSC70 comprises approximately 1% of total cell protein (Pelham, 1986). However, it is the inducible form, HSP70, which has been linked to the protection seen in many forms of preconditioning. Evidence for the role of HSP70 in thermotolerance comes from a study by Riabowol *et al.* (1988) where antibodies against HSP70 were microinjected into fibroblasts. This treatment prevented the translocation of HSP70 into the nucleus following mild heat shock. Coinciding with this was the abolition of subsequent thermotolerance. More specifically, the level of HSP70 in a tissue is proportional to the level of thermotolerance acquired.

Studies indicate that HSP70 can only recognize unfolded forms of a protein (Palleros *et al.*, 1991). Any folded protein that remains bound to HSP70 is probably non-functional (Flynn *et al.*, 1991). In unstressed cells, the interaction between HSC70 and immature proteins is transient and ATP-dependent. They are involved in the post-translational

import of proteins into the endoplasmic reticulum and mitochondria (Snoeckx *et al.*, 2001). The HSP70s coat the bound polypeptide chains as they emerge from ribosomes or membranes, preventing aggregation reactions and thus premature folding (Flynn *et al.*, 1991). Under stressed conditions the maturation of proteins undergoes difficulties and so they remain stably bound to the HSP70 escort or 'chaperone' (Figure 1.4).

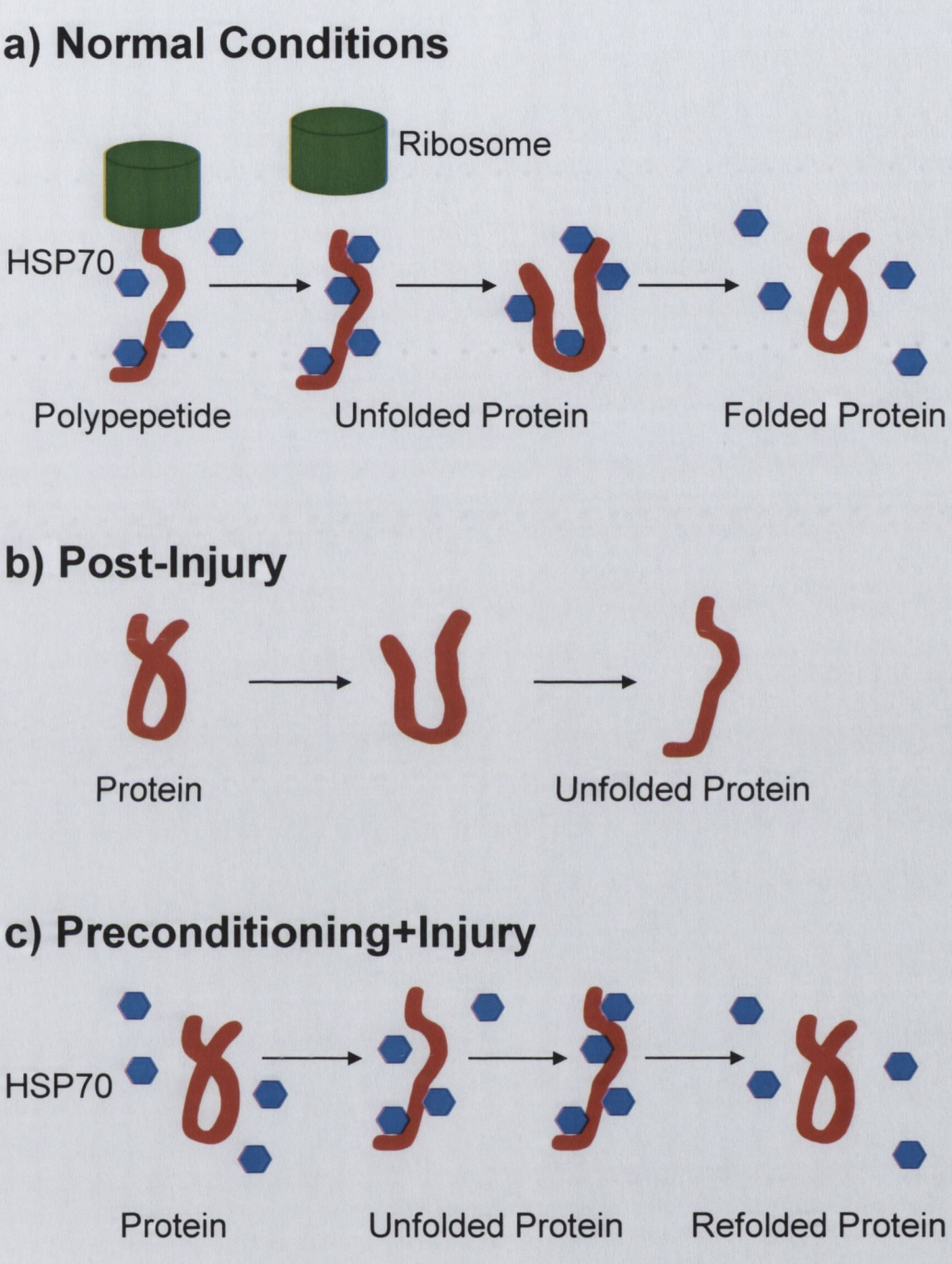


Figure 1.4 Chaperone role of HSP70. Under normal conditions (a), HSP70 binds peptide chains as they are forming, preventing them from folding prematurely or improperly. Once the protein is in the correct conformation the HSP70 releases from the protein. However, following injury (b), proteins becoming unfolded and cannot perform their function. Preconditioning induces HSPs (c) such as HSP70 which binds to unfolded proteins and holds them until they can be folded correctly or targeted for destruction, preventing a build-up of these damaged proteins in the cell.

1.4.4 Other Heat Shock Protein Families

Members of the HSP90 family play an important role in the regulation of other systems of proteins and cellular responses, such as cellular growth. They are mainly found in the cytoplasm and bind actin in a calcium-calmodulin dependent manner, transporting molecules through this interaction. Among its activities, HSP90 is known to interact with several protein-tyrosine kinases between the time of their synthesis and ultimate association with the plasma membrane (Pelham, 1986). In addition, HSP90 binds steroid hormone receptor, rendering it inactive. Once a hormone binds to its receptor, the HSP90 is released, allowing the receptor to become active.

Both HSP10 and HSP60 are found in the mitochondria of mammalian cells and chloroplasts of plant cells. They are essential to mitochondrial protein folding and assembly and refolding proteins imported from the cytoplasm. Folding proteins bind to and release from HSP60 many times during each folding event. This requires energy which is provided by the enzymatic splitting of ATP and the participation of HSP10, complexed to HSP60 (Snoeckx *et al.*, 2001).

Found in normal and cancer cells, HSP27 may also play a role in thermotolerance, as well as in drug resistance. Non-stressed cells display HSP27 predominantly within the perinuclear region near the Golgi apparatus, but following heat shock, the protein is predominantly localised in the nucleus. Other stresses e.g. SA do not stimulate entry of HSP27 into the nucleus of Chinese Hamster ovarian cells. In some tissues, HSP27 can be hormonally regulated. In fact this protein may co-operate with HSP90, HSP70 and HSP56, all of which are known to be associated with steroid receptors. Heat shock protein 27 is

also associated with actin and may be involved in the signal transduction pathway of several cell regulators, e.g. TNF, IL-1 (Ciocca *et al.*, 1993).

1.4.5 Regulation Of Heat Shock Protein Induction

Similar to IR, heat shock is a stress that disrupts metabolic processes and cell structures, causing cell death when a critical threshold is reached. Heat stress causes extensive damage to the cytoskeleton, leading to collapse of the threadlike intermediate filament network into large perinuclear aggregates, relocation of actin-containing fibres around the nucleus and disruption of microtubules and the mitotic spindle (Laszlo, 1992). Other cytological features of heat shock are mitochondrial swelling, loss of mitochondria and uncoupling of the oxidative phosphorylation pathway (Patriarca and Maresca, 1990; Funk *et al.*, 1999).

Characteristically, general protein synthesis is inhibited after extreme heat challenge as a result of phosphorylation of initiation factors such as eIF2α, which disrupts ribosomal assembly and inactivates cap-binding proteins (Clemens, 2001). In contrast, HSP genes are efficiently expressed after heat challenge. There are several possible reasons for this phenomenon. For example, HSP gene expression is facilitated by the absence of introns in several inducible genes, e.g. HSP70. In addition, alterations in mRNA splicing and heat-induced stabilization of mRNA are adaptive mechanisms to efficiently translate stress proteins, enabling these proteins to reach 15 to 20% of total intracellular protein within minutes after a physiological stress (Lindquist and Craig, 1988).

The transcriptional activation of stress genes occurs within minutes of changes in the cellular environment. This rapid response is facilitated by the cellular presence of the transcription factor, heat shock factor (HSF), and heat shock element (HSE), the conserved up-stream regulatory sequence in the promoter region of all stress-inducible HSP genes. HSF is present in four forms, HSF1, 2 and 4 in most mammals and the unique avian HSF3, which differentially mediate the activation of stress genes. During unstressed conditions, both the DNA-binding activity and the transcriptional activity of vertebrate HSF1 are under negative control. Experimental evidence suggests that HSF1 is maintained in an inactive monomeric state through association with either constitutive or inducible HSP70, as the HSF-HSP70 complex, suggesting that HSP70 may auto-regulate its own expression (Pirkkala *et al.*, 2001). Following a stressful stimulus, HSF is released from this complex, rapidly converts to a homotrimer and binds to HSE, initiating the up-regulation of HSP synthesis. Thus HSFs (other than HSF2 which is refractory to classical stressors) are activated rather than induced by cell stress or injury, both *in vitro* and *in vivo*. However, this DNA binding alone is not enough to cause transcription. Jurivich *et al.*, (1994) suggested that phosphorylation of the trimerised HSF is the other step leading to the high level of HSP70 transcription seen following stress. In fact HSF1 is hyper-phosphorylated in a *ras*-dependent manner by members of the mitogen-activated protein kinase subfamilies (ERK1, JNK/SAPK, and p38 protein kinase) during physiological stress (Knauf *et al.*, 1996). Conversely, induction of HSPs following hypothermia does not cause the hyperphosphorylation of HSF1 that is seen after hyperthermia, indicating that different stresses induce HSPs through distinct pathways (Cullen and Sarge, 1997).

The control of HSP synthesis may also involve the HSP-ADP complex, which binds avidly to denatured proteins. The high energy complex HSP-ATP in contrast has a low

affinity for proteins. Stresses that alter cellular metabolism and deplete ATP, thereby increase the concentration of ADP, leading to a depletion of the free HSP pool. This in turn may act as a signal to increase HSP synthesis. For example, Benjamin *et al.* (1992) showed that depletion of ATP was enough to induce DNA binding of HSF and therefore transcriptional activation of HSP70 when oxidative metabolism was impaired. In cardiomyocytes, ATP depletion caused by hypoxia occurs before the induction of HSP70 mRNA. Thus when the stress response is triggered during ischaemia, the subsequent synthesis of stress proteins probably contributes to the restoration of cellular homeostasis after reperfusion (Currie *et al.*, 1988).

1.4.6 Expression Of Heat Shock Proteins In The Kidney

Many HSPs are expressed in the kidney under normal unstressed conditions, although their distribution varies depending on the HSP (Beck *et al.*, 2000). For example, small HSPs display a distribution paralleling the corticopapillary osmotic gradient, with high amounts in the papilla and low levels in the cortex. In keeping with its localization within mitochondria, HSP60 is expressed in lower amounts in the inner medulla when compared with the cortex or outer medulla, where the numbers of mitochondria are higher. The majority of HSP90 is found in the distal convoluted tubule and in the cortical and medullary collecting duct. HSC70 has been observed in the epithelium of all zones of the rat kidney, as well as in glomerular podocytes and papillary interstitial cells. This ubiquitous distribution of HSC70 supports the view that non-stressed cells require assistance in protein folding, trafficking and controlled degradation.

Interestingly, the inducible HSP70 is also found in the normal kidney, in a similar pattern to the small HSPs, changing with alterations in medullary solute concentrations. This HSP is increased further by high extracellular solute concentrations and may reflect a role for this HSP in the adaptation of the medullary cells to these changes. However, many other stresses also induce HSP70. In 1991, Emami *et al.* showed that HSP70 was induced in the rat kidney in response to hyperthermia and ischaemia. They found that 15 min of heat stress induced HSP70 in the renal papilla within 1 h and in the medulla and cortex within 4 h, the expression peaking at 4-6 h after the heat shock and persisting for 10 days. Renal ischaemia induced HSP70 expression within 3 h of at least 15 min clamping. This induction reached maximum after 60 min ischaemia and persisted for 5 days. Again the expression was strongest in the renal papilla, followed by the cortex and then the medulla. Following both heat stress and ischaemia the HSP70 was localised to the soluble fraction of the homogenate rather than the membrane fraction. Recovery from renal IR depends on the length of time of the ischaemia. Reversible changes are seen in the renal cortex following 25-45 min of ischaemia. However 90 min of ischaemia results in irreversible damage (Sumpio *et al.*, 1984). Coinciding with this, Emami *et al.* (1991) showed that 90 min of renal pedicle clamping produced no increase in HSP70 levels in the kidney. Perhaps the induction of this HSP is important in the attenuation of damage, the repair process or both. Less HSP70 was found in the heart and more in the liver following heat stress than was seen in the kidney. Thus it seems that the amount of HSP70 induced is dependent on the location of the HSP70 within the body, the organ and the cell as well as on the insult.

Van Why *et al.* (1992) found that 45 min of ischaemia resulted in the induction of HSP70 mRNA in the rat kidney. This was observed within 15 min of reperfusion, peaked at 2-6 h

and fell by 24 h. The HSP70 protein expression increased progressively throughout 24 h. Since the mRNA expression had fallen by 24 h and Emami *et al.* (1991) had found a persistence of the HSP70 protein expression for 5 days, it is likely that slow degradation of the HSP rather than persistent protein synthesis was responsible for the prolonged expression. Schober *et al.* (1997) followed the time course and location of HSP70 induction following 60 min renal ischaemia and found that ischaemia caused an initial decrease in the level of the protein in the supernatant and an increase in the pellet fraction in the medulla suggesting that HSP70 migrates from the cytoplasm into the nucleus and/or associates with cytoskeletal structures immediately following ischaemia. The cytoplasmic HSP70 levels gradually increased in the cortex and outer medulla, but not in the inner medulla, unlike Emami *et al.* (1991).

Other HSPs are also induced in the kidney. Schober *et al.* (1997) also looked at the induction of HSP25 in the kidney following 60 min of ischaemia and found a similar pattern of expression as for HSP70. Brown and Rush (1984) showed that SA induced HSP90 in mammalian kidneys. Darasch *et al.* (1988) showed that both heat treatment and SA induced HSP90 in A6 kidney epithelial cells. The heavy metal cadmium is associated with dose-dependent nephrotoxicity and liver toxicity. The induction of HSP70, HSP90 and HSP110 precede the associated tissue damage (Goering *et al.*, 1993) and so may be markers of cell injury or indicators of cellular stress in these target organs.

1.4.7 Preconditioning: The Protective Role Of The Stress Response

The HSPs are ubiquitous in their synthesis. These proteins are not only involved in normal protein synthesis as well as in the recovery of already injured cells but they protect cells

against a subsequent stress. To this end, they constitute a universal cellular defence mechanism, whereby cells exposed to a sub-lethal stress are paradoxically protected against a subsequent otherwise lethal cellular stress (Snoeckx *et al.*, 2001). This phenomenon protects tissues from many stresses, and is called preconditioning. The preconditioning stress and the otherwise injurious stress can be caused by different stimuli, thus induction of HSPs associated with the acquisition of thermotolerance, or resistance to higher temperatures, also confers resistance to oxidative and other types of injury. However, not all types of stresses confer such cross-protection rather this depends on the type of stress, its intensity, and on the timing of the second injury (Lovis *et al.*, 1994).

1.4.7.1 The Role of Heat Shock Protein 70 (HSP70) in Preconditioning

Following a preconditioning stress, various HSPs may be induced on different cell types. The HSP70 most widely associated with preconditioning is HSP70. However, direct evidence demonstrating this has been lacking. Instead, HSP70 has been shown to be temporally related to the development of protection. For instance, the resistance of the cells to subsequent stress decays as the HSP70 expression decreases. Landry *et al.* (1982) showed that HSP70 induction peaked at 2-4 h following heat shock and was back at control levels by 8 h post-stress. The degree of thermotolerance to a subsequent lethal heat stress peaked at 4-8 hours, between the peak of the HSP70 induction and its return to control values. In 1993, Kim *et al.* showed a similar relationship between the appearance of the HSP70 and the acquisition of thermotolerance where the protein expression peaked at 2 h post-heat stress and the maximal protection seen against another heat stress followed at 6 h after the initial heat stress.

However, several more recent studies have addressed the potential for HSP70 to protect against injury more directly. For example, in an animal model of myocardial IR, transgenic mice expressing inducible human or rat HSP70 experienced reduced infarct size, preserved contractile function and decreased post-ischaemic release of creatine kinase (Marber *et al.*, 1995; Plumier *et al.*, 1995). More recently, findings from transfection studies where HSP70 has been introduced into renal tubular cells (Turman and Rosenfeld, 1999), coronary endothelial cells (Suzuki *et al.*, 1998) or whole organs e.g. heart (Suzuki *et al.*, 2000; Jayakumar *et al.*, 2001) have supported the concept that HSP70 can itself mediate protection against subsequent injury. In addition, disruption of HSF1 abolishes protection against apoptosis caused by subsequent heat shock (McMillan *et al.*, 1998).

Several forms of preconditioning have been extensively studied in models of IR injury, in particular in the heart. These include ischaemic preconditioning (IPrec), thermal preconditioning and pharmacological preconditioning. While both thermal and pharmacological preconditioning methods have been associated with HSP induction, IPrec appears to have both a HSP-independent and HSP-dependent phase in the heart, although the existence of similar pathways in other organs is still unclear. In addition, other mediators have been implicated in both phases of protection, including adenosine receptors, G proteins, alpha-adrenergic receptors, transient non-lethal increase in intracellular Ca^{2+}, K^{+} channels and other pathways (Millar *et al.*, 1996). Protein kinase C (PKC) activation and translocation appears to be a common denominator of these pathways and may also be involved in the induction of HSPs.

1.4.7.2 The Role of Protein Kinase C (PKC) in Preconditioning

The PKC family comprises of at least 12 serine/threonine kinases. These kinases can be divided into four main categories, conventional PKCs (α, β1, β2 and γ), which are Ca^{2+}-dependent and require diacylglycerol and phosphatidylserine for activation, novel PKCs (δ, ε, η, θ) which are Ca^{2+}-independent but require diacylglycerol and phosphatidylserine for activation, atypical PKCs (ζ, λ/ι), which are Ca^{2+}- and diacylglycerol-independent, but are phosphatidylserine-sensitive, and PKC-related kinases (PRKs). The activation of PKC in preconditioning was first implicated in ischaemic preconditioning (Baines *et al.,* 2001). The early experiments were in the heart where a PKC inhibitor was used to block the protective effect of ischaemic preconditioning and a PKC activator was found to mimic preconditioning. Both PKCε and PKCη were found to translocate from the soluble to the membranous fraction during ischaemic preconditioning, however it was established that PKCε and not PKCη was the important isoform that mediated the resulting protection against a subsequent IR insult. PKCε has since been implicated in many modes of preconditioning. In fact PKCε-deficient mice fail to exhibit cardioprotection against IR injury following preconditioning with either ischaemia or α-adrenergic receptor stimulation (Gray *et al.,* 2004). The role of this isoform in preconditioning is less well established in other organs, however, blocking PKC in rats prevents the development of ischaemic preconditioning-induced reduction of renal IR injury, suggesting that PKC also participates in preconditioning in the kidney (Lee and Emala, 2001). There is some evidence that PKC is also important in the induction of HSP70 expression. Kim *et al.* (1993) first demonstrated that blocking PKC prevented the transcription of both HSP27 and HSP70, suggesting that this was probably due to the inhibition of HSF phosphorylation. More recently, it was found that HSP27 and HSP70 reside in the PKCε

signalling complex in the myocardium, suggesting that this PKC isoform may in fact be important in modulating HSP induction (Ping *et al.,* 2001). While this has not been studied extensively, the inhibition of PKC blocks the protection afforded by hyperthermic preconditioning in models of cardiac IR injury (Yamashita *et al.,* 1997) and hypoxia-induced apoptosis in renal tubular cells (Meldrum *et al.,* 2001). This reduced protection is associated with decreased HSP70 induction by preconditioning, thus supporting a role for PKC in HSP-mediated preconditioning.

1.4.7.3 Inflammatory Mechanisms Targeted During Preconditioning

Regardless of the preconditioning stress used, the protection observed appears to be targeted against several aspects of the subsequent injury. With IR injury in mind, it has been demonstrated that several important steps in the inflammatory cascade, in particular, leucocyte-endothelial interactions and oxidative stress, can be attenuated by preconditioning. For example, preconditioning with heat shock prevented TNF-α-induced ICAM-1 upregulation on endothelial cells. This was associated with HSP70 expression. The protection appeared to be mediated by the inhibition of NF-kB, through the stabilization of I-κB (Kohn *et al.*, 1998). This may in turn prevent endothelial injury by leucocytes, as has been suggested by Wang *et al.*, (1995). They found that the induction of HSP70 on endothelial cells by sub-lethal heat or SA treatment prevented subsequent cell necrosis caused by neutrophils. These findings appear to be true in vivo also, inasmuch as neutrophil infiltration of post-ischaemic tissue as measured by MPO levels is reduced by preconditioning with hyperthermia (Javadpour *et al.*, 1998). In fact in the kidney, both MPO levels and renal dysfunction could be attenuated by preconditioning with hyperthermia. Blocking the associated induction of HSP70 using the bioflavanoid

quercetin prevented this protection, supporting a role for HSP70 in these responses (Kelly *et al.*, 2001). Furthermore, using intravital microscopy to directly observe leucocyte-endothelial interactions, induction of HSP70 by heat shock blocked IR-induced leucocyte recruitment in post-capillary venules of the rat mesentery (Chen *et al.*, 1997). Thus the stress response may prevent at least the early phase of IR injury mediated by neutrophil-endothelial interactions. Exactly how this is achieved is unclear however extensive work has focused on oxidant/antioxidant levels following HSP70 induction by preconditioning.

Ischaemia-reperfusion associated ROS can mediate membrane damage possibly resulting in membrane protein changes due to oxidation of thiol groups. Thus any mechanism that allows rapid repair of these proteins would have the potential to protect against the consequences of this disturbance of biomembrane characteristics and cellular ion homeostasis. Interestingly, HSP70 may play a far larger role than simply acting as a molecular chaperone. The protective role of HSPs against oxidant injury was suggested by a study where bacteria resistant to H_2O_2-induced injury spontaneously overproduced HSPs (Christman *et al.*, 1985). The induction of HSPs in the human macrophage actually inhibits the production of the O_2^- by these cells. In human monocytes, preconditioning with H_2O_2 is protective against subsequent exposure to H_2O_2 only when HSPs are induced (Wang *et al.*, 1996). HSP70 induction has also been associated with elevation of endogenous antioxidant activity. In fact inhibition of antioxidant defences induces production of HSPs .

Mitchell *et al.* (1983) first showed that heat stress was not only associated with the induction of thermotolerance in Chinese Hamster cells but also with increased levels of glutathione peroxidase (GSH.Px), a free radical scavenger that has been shown to protect

against renal IR injury (Baud and Ardaillou, 1993). Depletion of glutathione led to the absence of thermotolerance that normally followed this heat stress. In 1988, Currie *et al.* showed significant functional and ultrastructural protection in hearts exposed to IR 24 h after preconditioning with hyperthermia. This protection was associated with an accumulation of HSP70 and a twofold increase in levels of myocardial catalase enzymatic activity. Other antioxidants, SOD and GSH.Px levels were unchanged. Currie and Tanguay (1991) found this protection was not associated with increased levels of mRNA for catalase, but rather the heat shock may have altered the translational or post-translational processes. The same group found that blocking myocardial catalase activity prevented this heat shock-induced protection (Karmazyn *et al.*, 1990), suggesting that HSP induction was not key to the response.

Heat shock has also been associated with increased levels of the antioxidant SOD in mammalian cells. In 1988, Hass and Massaro found that Cu,ZnSOD was increased in lung tissue following heat shock. This occurred 2 h after the induction of HSPs 25, 70, 85 and 96 kDa. This suggests that while heat shock has an associated HSP induction and cytosolic antioxidant increase in the lung, these are regulated at different levels of gene expression. Liu *et al.* (1992) found activation of SOD in an isolated in situ pig heart following heat shock by 42°C warm blood perfusion. Increased SOD activity following heat stress also occurred in the swine liver (Schoeniger *et al.*, 1994). In 1995, Maulik *et al.* showed that pre-treatment of pigs with amphetamine (a sympathomimetic drug) increased the core temperature of the animals. This hyperthermia was associated with induction of the HSPs 27, 70 and 89 kDa in all organs studies including the heart, kidney and liver. Furthermore there was a concomitant induction of SOD and catalase in the heart. This pre-treatment protected against subsequent cardioplegic arrest injury in the isolated heart. Perhaps the

synthesis of HSPs is used as an antioxidant mechanism specifically by those cells producing high concentrations of ROS. Taken together, these studies support a role for HSP induction in tolerance against subsequent stress possibly via the elevation of the cells ability to metabolize free radicals.

During renal IR there is release of harmful ROS. While the use of SOD has proven effective in treating renal IR injury, increasing endogenous antioxidants by preconditioning may be more beneficial than the addition of exogenous antioxidants as the latter are restricted to the extracellular space, and thus may be less effective (Currie *et al.*, 1988). As can be seen from the above sections, HSPs are both constitutively expressed and can be induced in the kidney. Furthermore, several types of preconditioning stresses have been shown to protect the kidney from subsequent injury. Thus while it has not been extensively studied, there is great potential for preconditioning to protect the kidney against IR injury. This topic will be covered in detail in the coming chapters.

1.5 THESIS AIMS

Thus the aims of this thesis are: 1) to establish a model of renal IR injury, characterized by renal dysfunction and morphological damage, 2) to assess the efficacy of preconditioning with ischaemia and other stimuli to attenuate this renal IR injury, 3) to use the most effective and clinically applicable method to investigate whether this treatment is capable of preventing the upregulation of the adhesion molecule ICAM-1 and the antigens MHC I or MHC II, 4) to determine if long-term protection against renal IR injury can be achieved by preconditioning. Finally while establishing an intravital microscopy facility in our institution, a preliminary study was performed to assess if mechanisms of injury that can be inhibited by preconditioning may also attenuate injury caused by an inflammatory mediator known to be involved in the immune-mediated response to allografted tissue.

CHAPTER 2

Materials And Methods

2.1 RENAL ISCHAEMIA-REPERFUSION MODEL

2.1.1 Animal Preparation

All animal procedures were carried out under license from the Department of Health and Children. Animals were maintained on a purified chow pellet diet and water *ad libitum*. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 300-350 g were randomly placed into one of two groups; control and ischaemia-reperfusion (IR).

At the time of experimentation, anaesthesia was induced in a perspex chamber with 5% inhalational halothane (May & Baker Ltd., Dagenham, UK) in O_2, at a flow of 5000 L/min. Once the animal was under, it was taken out of the chamber, and the nose and mouth were placed at the opening of a double mask. Anaesthesia was maintained with 2% halothane in O_2, at a flow of 2000 L/min using a Fluovac system, with Fluosorber to remove excess vapour (Harvard Apparatus Ltd., Kent, UK). In all groups, the right internal jugular vein was cannulated for administration of saline (0.9% NaCl intravenous infusion BP; Baxter Healthcare Ltd., Norfolk, UK) and heparin (400 IU/kg body weight; Leo Laboratories Ltd., Bucks, UK). All animals were placed in a supine position and core temperature was monitored for the duration of the experiment by a rectal probe (Hewlett-Packard GMBH, Boeblingen, Germany). All animals received 1 ml saline per hour while under anaesthesia.

2.1.2 Experimental Model

A midline abdominal incision was made and the left kidney was mobilised in all groups. The left renal pedicle was ligated and a uninephrectomy performed. The right kidney was then mobilised (as depicted below in Figure 2.1). All animals received 400 IU heparin/kg body weight i.v. at this time to prevent blood clotting during ischaemia. The bladder was emptied by exerting pressure on the outside. In the IR group only, the right pedicle (renal artery, vein and nerve supply) was clamped using a microvascular clamp for 45 min. After the 45 min ischaemic period, the vascular clamp was removed (Klausner *et al.*, 1989; Weight *et al.*, 1998) and reperfusion was visually confirmed. In the control group the right kidney was mobilised after 45 min of sham ischaemia (where the right kidney was manipulated similarly to the IR group, but the pedicle was not actually clamped). The wound was closed with a 4/0 silk suture. A continuous suture was used on the abdominal wall and interrupted sutures on the skin. Animals in both control and IR groups were allowed to recover from the anaesthetic as kidneys reperfused for the time specified for each study (2 h, 6 h, 12 h, 18 h, 24 h, 5 d or 16 wk). At the end of the reperfusion period, animals were anaesthetised with inhalational halothane as detailed before (section 2.1.1), urine was collected (section 2.2.2), and blood was drawn from the heart to assess renal function (section 2.2.1). The right kidney was removed and weighed to determine the development of oedema (section 2.3). This kidney was then divided in two. In most cases, one piece was prepared for MPO assessment (section 2.4) and the other half was fixed in 10% formalin for H&E (section 2.5). Animals were then euthanased with an overdose of anaesthetic (Figure 2.1).

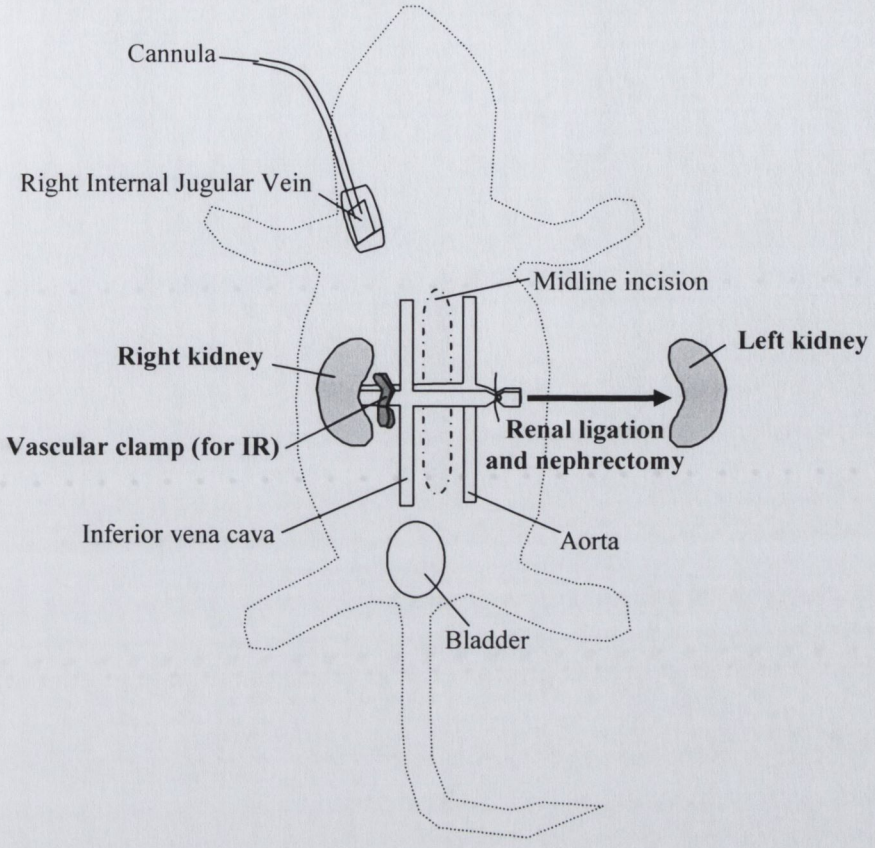


Figure 2.1 Rat model of renal IR injury, demonstrating cannulation of the right internal jugular vein, midline laparotomy, left nephrectomy and right renal IR.

2.1.3 Ischaemic Preconditioning Protocol

In a separate set of experiments (Chapter 4) ischaemic preconditioning (IPrec) was performed prior to the application of an IR insult as described above. All animals were anaesthetised using inhalational halothane (see section 2.1.1). The right internal jugular vein was cannulated for administration of 0.9% NaCl and heparin (400 IU/kg body weight). All animals were placed in a supine position and core temperature was monitored for the duration of the experiment by a rectal probe. All groups received 1 ml saline per hour while under anaesthesia.

A midline abdominal incision was made and the right kidney was mobilised. Animals underwent either sham preconditioning (sham-IPrec) or ischaemic preconditioning (IPrec). In the sham-IPrec animals the right kidney was manipulated at each time-point corresponding to the clamping or unclamping of the renal pedicle in the IPrec groups. IPrec was performed using two cycles of 5 min ischaemia. This was achieved by clamping the right renal pedicle with a microvascular clamp for 5 minutes following by removing the clamp and allowing reperfusion for 5 min. The pedicle was then clamped again for 5 min before the kidney was reperfused for 5 min, 2 h or 4 h prior to application of an IR insult (45 min ischaemia and 2 h reperfusion) as detailed in section 2.1.2. A separate group of IPrec animals underwent four cycles of preconditioning, where the kidney was subjected to four separate periods of 5 min ischaemia separated by 5 min reperfusion before undergoing and IR injury. The groups are listed in section 4.2.2. In the groups that were allowed to reperfuse for 2 h or 4 h between IPrec and IR, the abdominal wound was closed with a 4/0 silk suture at the end of the IPrec protocol. A continuous suture was used in the abdominal wall and interrupted sutures on the skin.

2.2 RENAL FUNCTION

2.2.1 Serum Creatinine, Urea, Potassium, Sodium And Chloride Levels

Venous blood was withdrawn from the right ventricle of the heart at the end of the reperfusion period. Serum creatinine, urea and ion concentrations were measured using a Beckman Synchron CX7 chemistry analyzer (Beckman Coulter, Inc., CA, USA). This caluclated creatinine and urea levels using an end-point colorimetric assay read at 520 nm and 340 nm respectively. Serum potassium (K^+), Na^+ and Cl^- ion measurements were based on an indirect ISE electrode-based assay. Ion levels from haemolysed samples were excluded from analysis.

2.2.2 Urine Output, Creatinine And Protein Levels

The bladder was emptied just prior to ischaemia or sham ischaemia by exerting pressure on the outside. In acute experiments, since only one kidney remained, urine from this kidney could be collected from the bladder rather than cannulating the ureter as has been previously described in a model where the control non-ischaemic kidney remained in situ (Chintala *et al.*, 1993). Thus urine output (U/O) was measured by piercing the previously emptied bladder with a 25G needle at the end of the reperfusion period and aspirating the urine into a syringe for measurement of volume. In the chronic renal IR model described in Chapter 7, rats were placed in metabolic chambers 24 h before harvesting of blood, urine and kidney tissue. These chambers enabled the collection of excreted urine. After 24 h, the total excreted volume of urine was determined using a graduated cylinder and U/O per hour was calculated. Urine creatinine and protein levels were read on a Beckman

Synchron CX7 chemistry analyzer. The urine creatinine assay was similar to that for serum creatinine levels (section 2.2.1), and urine protein measurements were based on an end-point colorimetric assay, read at 540 nm.

2.3 ORGAN OEDEMA

Excess perinephric tissue and the capsule were removed from both right and left kidneys after harvesting the organs, and their weights measured immediately. The difference between the two kidney weights was calculated and expressed as a percentage of the left control kidney (which had been removed at the beginning of each experiment). This percentage weight change was used to assess organ oedema (Klausner *et al.*, 1989).

2.4 MYELOPEROXIDASE ASSAY

Myeloperoxidase is a haem-containing enzyme found primarily in neutrophils, and so can be used as an indirect measure of neutrophil infiltration into the tissue assessed (Bradley *et al.*, 1982). A portion of the right kidney (approximately one third) was weighed and then homogenised in 10 ml of a solution containing 1.2 mM K_2HPO_4, 48.7 mM KH_2PO_4 and 13.7 mM hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, MO, USA) in distilled water immediately after the experiment. The homogenates were then stored at -80°C. To measure MPO activity, the samples were freeze-thawed twice and centrifuged at 2000 rpm for 10 min (model 5403; Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany). The supernatant was assayed spectrophotometrically for MPO activity by adding 0.1 ml of supernatant to 2.9 ml of freshly prepared solution containing 1.5 mM K_2HPO_4 and 60.9 mM KH_2PO_4 in 40 ml of distilled water, 5 ml of 53.6 μM dianisidine (Sigma Chemical Co.) in methanol and 5 ml of 0.006% H_2O_2 (20vol; Ricesteele, Dublin, Ireland) in distilled water. The change in absorbance was monitored every minute for 10 min, at a fixed wavelength of 460 nm using a scanning spectrophotometer (CPU 8720, UV/VIS; Philips, Eindhoven, Netherlands). One unit of MPO was defined as that degrading 1 micromole of peroxide/min at 25°C (Williams *et al.*, 1983) and calculated per gram of tissue using the following formula: (peak absorbance/10)/weight of tissue used/0.0113=MPO/gram of tissue.

2.5 TISSUE STAINING

2.5.1 ICAM-1 And MHC Antigen Expression

The expression of ICAM-1 and MHC II (Shackleton *et al.*, 1990) was assessed in the right kidneys of animals undergoing 2 h (ICAM-1) and 5 d (MHC II) of reperfusion after sham ischaemia (controls) or 45 min ischaemia (IR group). At the end of the experiment, a section of the right kidney was mounted in optimal cutting temperature (OCT) mounting medium (Tissue Tek, Miles Laboratories, Elkhart, Ind., USA), snap-frozen in liquid nitrogen and stored at -80°C. The expression of ICAM-1/MHC II was assessed on 6 μm thick tissue sections using a standard immunocytochemical technique as outlined on the data sheet for the StreptABComplex/HRP (code K377; Dako A/S, Glostrup, Denmark). All washes comprised of rinsing and incubating the sections with Tris-buffered saline (TBS) (25 mM Trizma, 150 μM NaCl) for 5 min. Sections of 6 μm thickness were cut from the frozen kidneys on a cryostat (Lahntechnik GmbH, Nassau/Lahn, Germany) and put onto poly-L-lysine (Sigma Chemical Co.)-coated slides. The sections were air dried at room temperature, fixed in acetone (BDH Laboratory Supplies Ltd., Poole, UK) for 10 min, and air dried again. Endogenous peroxidase and biotin activities were blocked using incubations with 0.6% H_2O_2 in methanol (Alkem Chemicals Ltd., Dublin, Ireland) for 5 min and a biotin blocking system (Dako A/S) respectively. Non-specific binding of antibody was blocked by incubating the sections with 20% normal rabbit serum (Dako A/S) in TBS for 20 min. The tissue sections were washed and incubated in the primary antibody (mouse anti-rat ICAM-1/MHC II (Serotec, Oxford, UK)) optimally diluted in TBS for 30 min (1:50 for ICAM-1 and 1:200 for MHC II). Sections were washed, and incubated with the secondary antibody optimally diluted 1:200 in TBS for 30 min. This

secondary antibody was a non-cross-reactive biotinylated rabbit anti-mouse Ig polyclonal (Dako A/S). Again the sections were washed, and then incubated for 20 min in a solution containing streptavidin complexed with biotinylated peroxidase (StreptABComplex/HRP; Dako A/S) in Tris. The sections were washed and placed in 1 mg/ml diaminobenzidine (DAB) (Dako A/S) in Tris and H_2O_2 for 4 min. This is a stable chromogen that can be used when peroxidase is the enzyme label. After three washes with distilled water, the sections were counterstained with haematoxylin (to stain nuclei), mounted in a neutral synthetic resin medium (BDH Laboratory Supplies Ltd., Poole, UK) and coverslipped.

The positive control was taken as a delegated tissue sample of rat kidney seen to contain ICAM-1/MHC II, and used in each run. Negative controls were performed using the secondary antibody alone.

Scoring was carried out by a blinded observer in a semi-quantitative manner. The vascular and glomerular endothelium and the tubular epithelium were scored. The following grading of 0-4 was used (as previously described by Nakai *et al.* (1994)):

0: no stain

1: weak staining, scant distribution

2: weak staining, widespread distribution

3: strong staining, scant distribution

4: strong staining, widespread distribution

2.5.2 Renal Morphological Assessment

Samples of renal tissue from control animals and animals exposed to ischaemia followed by 2 hours, 18 hours, 5 days or 16 weeks of reperfusion were fixed in 10% formalin and embedded in paraffin. Tissue sections were cut at a thickness of 4-5 μm and prepared for haematoxylin and eosin (H&E) staining in order to assess morphological damage following renal IR injury. Ten high-powered (600X) fields of view were examined in the renal cortex of each tissue. The total number of tubules (approximately 20-35) per field was counted and the percentage of degenerated (loss of nuclei and vacuolization of tubular epithelial cytoplasm) or completely destroyed tubules (loss of tubular epithelium and basement membrane) was calculated per field. The percentage of tubules with proteinaceous casts and the number of infiltrating leucocytes were determined in the same fields of view. Glomeruli were examined for degeneration, with attention paid to the capillary loops, basement membrane and nuclei as well as Bowman's space. The mean of each parameter within one animal was used for statistical analysis.

Masson's trichrome staining was carried out to identify the development of fibrosis in kidneys exposed to 45 min ischaemia followed by 16 wk reperfusion. Paraffin-embedded tissue sections were incubated at 60°C for 1 h, de-waxed and fixed in Bouin's fluid (a picric acid-based solution that precipitates proteins, rendering them insoluble; Sigma Chemical Co.) for 2-3 h. Following a wash with distilled water, the sections were treated with Celestine blue for 5 min, and washed again. Differentiation was carried out in acid alcohol. The sections were immersed in Harris haematoxylin followed by lithium carbonate. Once the nuclei were stained satisfactorily the slides were incubated in a cytoplasmic solution containing 0.1% w/v acid fuschin and 0.9% w/v Briebrich Scarlet in

100 ml 1% acetic acid for 10 min. The sections were washed and treated with 5% phosphomolybdic acid for 10 min and drained off. The final step consisted of a 7 min incubation in a solution containing 1% w/v light green in 4% acetic acid. Slides were then dehydrated through a graded series of alcohol (70%, 95% and absolute), cleared with xylene and mounted in a neutral synthetic resin medium (BDH Laboratory Supplies Ltd., Poole, UK) for examination. Fibrotic tissue appeared as diffuse green/grey staining usually in close proximity to evidence of tubular destruction. In addition muscle, collagen fibres, fibrin and erythrocytes could be identified with this stain.

2.6 HEAT SHOCK PROTEIN ASSESSMENT

2.6.1 Tissue Protein Measurement

Kidney, liver and lung tissue samples for assessment of HSP were taken from storage at -80°C and kept on ice at all times. In order to prevent endogenous proteases from breaking down the proteins to be assessed, 1 ml of freshly prepared 10 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co.) (980 µl cold PBS and 20 µl 0.5 M PMSF in methanol) was added to the samples immediately before homogenisation. The tissues were then centrifuged at 4100 g for 30 min at 4°C. Supernatants were drawn off and kept on ice. Protein content was measured using a modification of the spectrophotometric method of Lowry *et al.*, using bovine serum albumin (BSA) (Sigma Chemical Co.) as the protein standard. A stock solution of 30 µg/ml BSA in phosphate-buffered saline (PBS) (pH 7.2), was used to form a standard curve. A portion of each sample was diluted 1:1000 in PBS. Solutions were added to a 96 well plate as follows (all wells carried out in duplicate):

	Standard Curve							**Samples**
BSA conc (µg/ml)	0	5	10	15	20	25	30	Unknown
BSA stock (µl)	0	17	33	50	67	83	100	0
PBS (µl)	100	83	67	50	33	17	0	0
Sample (µl)	0	0	0	0	0	0	0	100
Reagent (µl)	100	100	100	100	100	100	100	100

The reagent, coomassie blue (Pierce Chemical Co., Rockford, IL, USA), was added to all wells last, immediately before reading the plate on a plate reader (Microplate autoreader, Bio-Tek Instruments Inc., Vermont, USA) at a wavelength of 570 nm. A standard curve was plotted using the mean absorbance value for each standard against the corresponding BSA concentration (Figure 2.2). Only standard curves with R^2 values of more than 0.98 were used, otherwise the protocol was repeated. Protein concentrations of the samples were calculated from the mean absorbance value for each sample and the equation for the standard curve, taking dilution into account. For samples where the absorbance was not on the straight part of the curve, the sample was either diluted further (if the absorbance value was too high) or diluted less (if the absorbance value was too low), and was run again with a new standard curve. All samples for each set of experiments were adjusted to the same protein concentration by dilution in PBS and then an aliquot was diluted 1:1 with laemmli buffer (20% 0.6 M Tris-HCl (pH 6.8), 175 mM sodium dodecyl sulphate, 20% glycerol, 150 μM bromophenol blue, 10% mercaptoethanol) and stored at -80°C for HSP protein assessment.

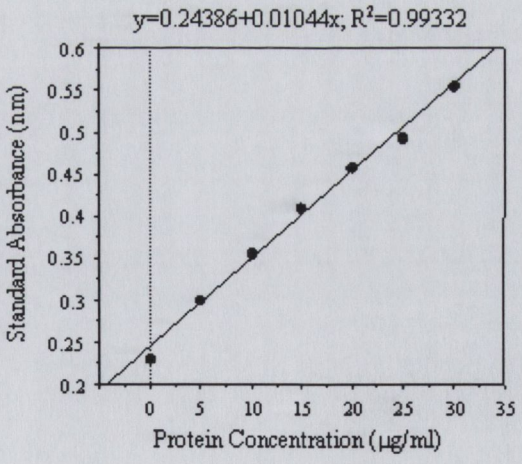


Figure 2.2 Example of a protein standard curve generated using BSA standards and coomassie blue reagent. Samples were analyzed spectrophotometrically at a wavelength of 570nm.

2.6.2 SDS-PAGE (Polyacrylamide Gel Electrophoresis)

Tissue HSP protein expression was assessed using western immunoblotting. Kidney, liver or lung samples in laemmli buffer were defrosted and denatured using a heating block (Dri-Block; Techne Ltd., Cambridge, UK) at 94°C for 10 min. The samples were then kept on ice until used. The proteins were separated by SDS-PAGE. A 12% separating gel (1.73 M acrylamide, 16.2 mM bisacrylamide, 20% 1.875 M Tris-HCl (pH 8.8), 3.5 mM sodium dodecyl sulphate, 0.04% temed, 2.94 mM freshly prepared ammonium persulphate) and a 5% stacking gel (717.4 mM acrylamide, 6.7 mM bisacrylamide, 20% 0.6 M Tris-HCl (pH 6.8), 34.7 mM sodium dodecyl sulphate, 0.1% temed, 6.6 mM freshly prepared ammonium persulphate) were used. A standard (containing broad range protein molecular weight markers (Promega Corp., Madison, WI, USA) and blank (PBS:laemmli buffer (1:1)) were run on each gel. 20 µl of the denatured samples were added to the remaining lanes. In the case of excess empty lanes, blanks were used. The gel was run for 1 h 30 min at 20 mA and 600 V using a gel electrophoresis box (Atto Corp., Tokyo, Japan) and power supply (Pharmacia Biotech Norden AB, Sollentuna, Sweden). Electrode buffer contained 49.5 mM Trizma, 380 mM glycine, and 3.5 mM sodium dodecyl sulphate. A duplicate gel that was subsequently stained for protein (see section 2.6.5) was run alongside the gel for HSP assessment in order to ensure equal loading was achieved.

2.6.3 Transfer

Before labelling, the gel was transferred to a nitrocellulose membrane (Sigma Chemical Co.) for 2 h 30 min at 400 mA and 600 V, or overnight at 70 mA, 600 V using a trans-blot electrophoresis transfer cell (Bio-Rad Laboratories, California, USA). Transfer buffer was

as follows: 20mM Trizma, 150 mM glycine, 1.04 mM sodium dodecyl sulphate, and 20% methanol in distilled H_2O.

2.6.4 Blocking And Labelling

To block non-specific binding of the monoclonal antibodies, the nitrocellulose membrane was incubated on a plate shaker for 1-2 h with 30 ml of freshly prepared blocking solution containing 0.05% Tween 20 (Merck-Schuchardt, Munich, Germany) and 5% Marvel dried milk in TBS (25 mM Trizma, 150 μM NaCl). The membrane was then washed three times with 50 ml TBS, 0.1% Tween for 10 min each time. The primary antibodies (mouse anti-human IgG1 monoclonal antibody specific for HSP27, HSP60, HSP70 or HSP90 (Stressgen, Victoria, British Columbia, Canada)) were optimally diluted at 1:1000 in 10 ml TBS, 0.05% Tween, 1% dried milk. The nitrocellulose membrane was incubated at room temperature for 2 h on a plate shaker (Diagnostic Products Corporation, California, USA), or overnight in the fridge with the monoclonal antibody solution. The membrane was then washed as before and incubated with the secondary antibody for 1-2 h on a plate shaker at room temperature. The secondary antibody, IgG1 anti-mouse conjugated to alkaline phosphatase (Serotec, Oxford, UK) was optimally diluted at 15:10,000 in 10 ml TBS, 0.05% Tween, 1% dried milk. The membrane was then washed as before and 10 ml of substrate solution (0.56 mM bromochloroindolyl phosphate/0.48 mM nitro blue tetrazolium (Sigma Chemical Co.)) was added to the nitrocellulose membrane. The membrane was gently shaken for 5-8 min (longer if the room temperature was cool) until the substrate slightly changed colour. The reaction was stopped by pouring off the substrate and running water over the membrane to wash it. The nitrocellulose membrane was allowed dry and was examined for bands corresponding to the molecular weight of

the appropriate HSP as indicated by molecular weight markers on the gel. If there were multiple bands per lane, suggesting non-specific binding of the antibodies, the gel was rerun.

2.6.5 Protein Stain

Once samples had been run on an SDS-PAGE gel, the gel was placed into 500 ml of coomassie blue stain, containing 0.25 g coomassie blue, 40% methanol and 10% acetic acid, for 30-60 min in a plate shaker. The gel was removed and background staining was reduced by placing the gel in a solution of 40% methanol and 10% acetic acid. This solution was changed several times until the background was reduced appropriately and the protein bands showed clearly. If the protein levels of the samples were correctly calculated and lanes were loaded properly, protein bands were similar for all samples regardless of the experimental group. In the event that this was not the case, the corresponding gel for labelling was not used, rather the protein concentrations were recalculated and the gel was rerun. An example of protein gel can be seen below in Figure 2.3.

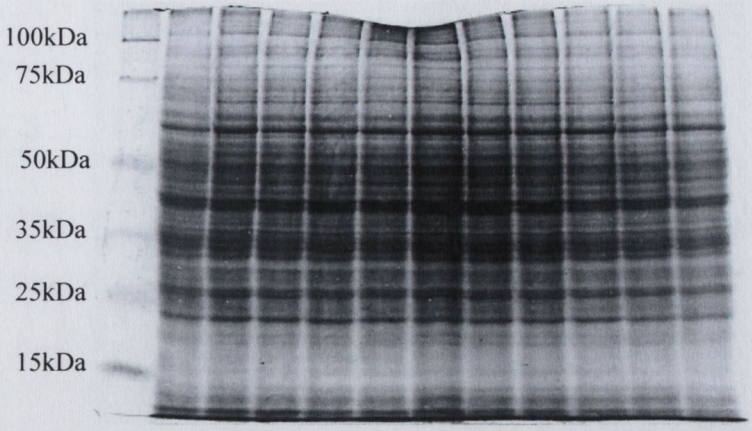


Figure 2.3 An example of a protein gel, which was run alongside gels for western immunoblotting to check for even sample protein content. The molecular weights of the standard protein markers are along the left side of the gel next to the relative protein bands.

2.7 INTRAVITAL MICROSCOPY

This technique is described in detail in section 8.2.

2.8 FLOW CYTOMETRICAL MEASUREMENT OF ADHESION MOLECULE EXPRESSION

Flow cytometry was used to measure CD18 and L-selectin expression on neutrophils. Samples of whole blood (approximately 300 μl) were drawn from the left internal jugular vein into heparinised tubes (20 IU/ml) at 15 min prior to recombinant IL02 administration and at 0, 2, 10, 30, 60, 90 and 120 min thereafter (section 8.2.4). The blood was divided into 5 tubes as detailed below. PBS containing 20 mM glucose (Sigma Chemical Co.) and 0.5% BSA (abbreviated to PGB here) was used for washing the cells and diluting the antibodies. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were used with the primary antibodies to detect CD18 and L-selectin (Serotec, Oxford, UK). Centrifugation steps were performed at 1600 rpm for 5 min. Red blood cells were lysed using lysis solution (Becton and Dickinson Immunocytometry Systems, San Jose, CA, USA). The pellets were resuspended in cold PGB and CD18 and L-selectin expression were assessed using flow cytometry (BD Biosciences Immunocytometry Systems, California, USA). The neutrophil population was identified by its forward and side scatter characteristics.

Tube #	1	2	3	4	5
Blood (µl)	50	50	50	50	50
PGB (µl)	5	5	5	0	0
Mouse anti-rat CD18 Ab (µl)	0	0	0	5	0
Hamster anti-rat CD62L Ab (µl)	0	0	0	0	5
Vortex; incubate 30 min @ RT; wash X2 with cold PGB					
FITC rabbit anti-mouse IgG rat adsorbed (µl)	0	5	0	5	0
FITC goat anti-hamster IgG (µl)	0	0	5	0	5
Vortex; incubate 30 min @ RT; wash X2 with cold PGB					
Lysis Solution (ml)	2	2	2	2	2
Incubate 13 min @ RT; wash X2 with cold PGB					

2.9 STATISTICAL ANALYSIS OF DATA

All data is expressed as mean±standard error of the mean. Unpaired and paired t-tests and analysis of variance with Scheffe post-hoc test were used for statistical analysis where appropriate. The scores for ICAM-1 and MHC II were entered into contingency tables and Chi-squared statistics were used. Statistical significance was set at $P<0.05$. The program Statview (SAS Institute Inc, Cary, NC, USA) was used for statistical analysis.

CHAPTER 3

Renal Ischaemia-Reperfusion (IR) Model

3.1 INTRODUCTION

Ischaemia followed by reperfusion (IR) occurs in a wide range of surgeries and disease processes. This chapter validates an IR model in the kidney, aiming to find the earliest reperfusion time-point that consistently demonstrated significant injury. This will be used in the preliminary assessment of the efficacy of various preconditioning strategies. The kidney was chosen because 1) it is a successful transplant organ, although there is still capacity for improvement of graft outcome; 2) both morphological and functional parameters in this organ can be easily studied. Following an IR insult to the kidney, renal structure quickly degenerates, as evidenced by severe tubular injury. If morphological changes are widespread throughout the kidney, renal function begins to deteriorate, as indicated by increased blood levels of solutes such as creatinine.

In order to design a renal IR model to study, several considerations were taken: 1) whether to remove the contralateral kidney or clamp bilaterally; 2) the duration of ischaemia; 3) the length of reperfusion. In this study, a uninephrectomy followed by contralateral single kidney IR was performed to more closely approximate the situation of renal transplantation. This also allowed the nephrectomised kidney to act as control for measurement of oedema. While a contralateral nephrectomy has been shown in some studies to improve both the functional and morphological recovery of the post-ischaemic kidney (Fried *et al.*, 1984), more recent data have shown that contralateral nephrectomy exacerbates post-ischaemic IR injury (Azuma *et al.*, 1997b). The length of ischaemia is of paramount importance in the resulting reperfusion injury. Several studies assessing post-ischaemic renal function or morphology have shown that 45 min ischaemia not only causes significant renal damage but is the longest period of warm ischaemia compatible

with survival in the rat (Jablonski *et al.*, 1983; Weight *et al.*, 1998). In agreement with these findings, a majority of studies employ 45 min ischaemia as the model of injury in the rat kidney (Takada *et al.*, 1997a; Klausner *et al.*, 1989), thus this chapter also uses 45 min ischaemia as the length of renal ischaemia. In contrast, reperfusion periods vary widely from study to study, and both functional and morphological deterioration have been demonstrated at several reperfusion times. Many studies do not address changes within the first twenty-four hours reperfusion, although substantial deterioration has already occurred within this time. Furthermore, data where earlier time-points have been examined have focused primarily on a single functional parameter or on morphology. Two hours reperfusion has been the earliest reperfusion time at which significant decline in function has been demonstrated. Thus for the purpose of this thesis, a reperfusion time-course was performed between two and twenty-four hours, and the earliest time-point at which significant functional alterations were noted was further analyzed for morphological injury and leucocyte infiltration.

Following an IR insult to the kidney, renal integrity rapidly deteriorates, characterized by severe tubular injury with relative sparing of the glomeruli. During reperfusion, tubular degeneration can be recognised morphologically by vacuolization of the epithelial cytoplasm, loss of nuclei, and separation of the epithelial cell from the basement membrane sloughing into the tubule lumen (Figure 1.3). Effected tubular epithelium may become oedematous, and/or undergo both necrosis and apoptosis. This damage to the tubular epithelium and consequential loss of function of these nephrons results in a compensatory elevation of filtration, reabsorption and secretion by non-injured nephrons to balance overall organ function. However this may lead to the formation of proteinaceous casts in these spared tubules. Medullary congestion is also seen. Leucocytes

infiltrate the renal vasculature, accumulating in the cortex and outer medulla in particular and releasing inflammatory mediators that can cause further damage (Willinger *et al.*, 1992).

If these morphological changes are widespread throughout the kidney, renal function begins to deteriorate, as evidenced by decreased GFR. This is reflected by increased serum creatinine levels (Chatterjee *et al.*, 2001; Lee and Emala, 2001; Jefayri *et al.*, 2000; Koo *et al.*, 1998). Several groups have also demonstrated increased serum urea levels following renal IR (Chatterjee *et al.*, 2001). The electrolyte balance in the body may be disturbed as reflected by decreased serum Na^+ levels (Jung *et al.*, 2000) and increased serum K^+ levels (Rabb *et al.*, 2000). Furthermore, a period of anuria often occurs following IR injury to kidneys (Rhoden *et al.*, 2001; Jung *et al.*, 2000) followed by a period of polyuria later. The present study uses renal functional parameters to identify an early time-point of renal dysfunction at which histology was performed to assess the extent of tissue damage.

Neutrophils appear to initiate the early response to IR injury in many organs through the release of oxidants and proteases which cause tissue injury (Mullane *et al.*, 1987). In the kidney, neutrophils are the first leucocyte population to enter post-ischaemic renal tissue within minutes of reperfusion and remaining for at least 24 h (Joannidis *et al.*, 1989; Klausner *et al.*, 1989; Willinger *et al.*, 1992; Okusa *et al.*, 2000). The tissue infiltration of these cells coincides with peak renal dysfunction (Takada *et al.*, 1997). Treatment either with monoclonal antibodies or with antisense oligodeoxynucleotides against one or more CAMs has proven very effective in animal models to not only attenuate leucocyte infiltration but also renal IR injury (Kelly *et al.*, 1996; Chen *et al.*, 1999; Tajra *et al.*, 1999; Singbartl *et al.*, 2000; Singbartl and Ley, 2000; Fuller *et al.*, 2001). Furthermore, depletion

of circulating neutrophils in mice using anti-neutrophil serum reduced the morphological alterations and renal dysfunction observed following renal IR (Kelly *et al.*, 1996). Of note, in human transplantation neutrophil infiltration of renal tissue is associated with prolonged ischaemic time and is accompanied by an increase in creatinine levels three and six months following transplantation (Koo *et al.*, 1998). Myeloperoxidase is a haem-containing enzyme that is present in abundance in neutrophils and also to a much lesser extent in monocytes. It has been used in a variety of inflammatory conditions as an indirect measure of neutrophil influx into the tissue (Bradley *et al.*, 1982; Williams *et al.*, 1983), and is employed in this study.

In order to assess the efficacy of preventative treatments against renal IR injury in future chapters, this study sought to find an early reperfusion time-point at which both functional and morphological damage were significantly deteriorated. Once an effective treatment is established, its protective impact against renal IR injury would be investigated in a long-term model of renal IR injury. Thus this study firstly investigates the time-course of IR-induced acute renal function over the first twenty-four hours of reperfusion and establishes an early time-point of renal dysfunction and oedema following IR. Secondly, the impact of renal IR on tissue neutrophil infiltration, U/O and renal morphology at this time-point is assessed.

3.2 METHODS

3.2.1 Animal Preparation

20 adult male Sprague-Dawley rats weighing 300-350 g were randomised into two groups; control and ischaemia-reperfusion (IR). These groups were subdivided into five reperfusion periods; 2 h, 6 h, 12 h, 18 h, or 24 h (n=2/subgroup). An additional animal was added to the Control-2h and IR-2h groups for MPO, urine output and histological assessment following initial analysis of the data.

3.2.2 Experimental Model

Animals underwent a left nephrectomy followed by sham IR (control group) or 45 min renal ischaemia (IR group) as described in Section 2.1. Animals in both control and IR groups were then allowed to recover from the anaesthetic as kidneys reperfused for 2 h, 6 h, 12 h, 18 h, or 24 h. At the end of the reperfusion period, animals were anaesthetised, urine was collected, and blood was drawn to assess renal function. The right kidney was removed and weighed to determine the development of oedema. This kidney was then halved. One piece was prepared for MPO assessment. The other half was fixed in 10% formalin for H&E. All methods are described in Chapter 2. Animals were then euthanased (Figure 3.1).

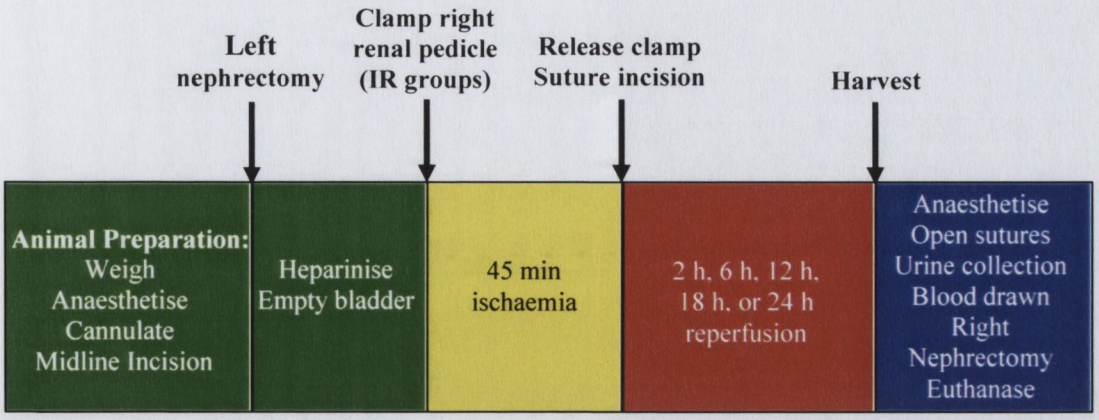


Figure 3.1 Timeline of the IR experiment where the right kidney was reperfused for different periods before harvesting and assessment of renal function. Animal preparation took approximately 15-20 min to complete.

3.2.3 Statistics

All data is expressed as mean±SEM. Unpaired t-tests were performed on all parameters to compare between groups.

3.3 RESULTS

3.3.1 Effect Of Ischaemia-Reperfusion On Renal Function

Ischaemia and subsequent reperfusion (IR groups) caused significant progressive renal functional impairment compared with controls. Figures 3.2 and 3.3 show both serum creatinine and urea levels increased significantly in all IR groups versus the corresponding control animals. These increases occurred as early as 2 h following release of the clamp. Serum creatinine levels increased from 66.0±4.00 μM in the Control-2h group to IR-2h levels of 117.0±1.00 μM ($P<0.01$) (Figure 3.2). These levels continued to rise, throughout the 24 h reperfusion period, with a 24 h serum creatinine value of 409.5±44.5 μM ($P<0.05$ versus control-24h). Similarly serum urea levels increased from 6.8±0.20 mM in the Control-2h group to 9.9±0.40 mM in IR-2h rats following 45 min ischaemia and 2 h reperfusion ($P<0.05$) as seen in Figure 3.3. Serum urea levels reached 55.3±4.60 mM by 24 h ($P<0.05$ versus control-24h). Serum K^+ levels were increased slightly at every reperfusion time-point examined compared with the respective control animals. However these elevations were not significant (Table 3.1). Serum Na^+ and Cl^- levels were not significantly altered by IR although both parameters were lower in the IR group compared with controls from the 12 h time-point onwards (Table 3.1).

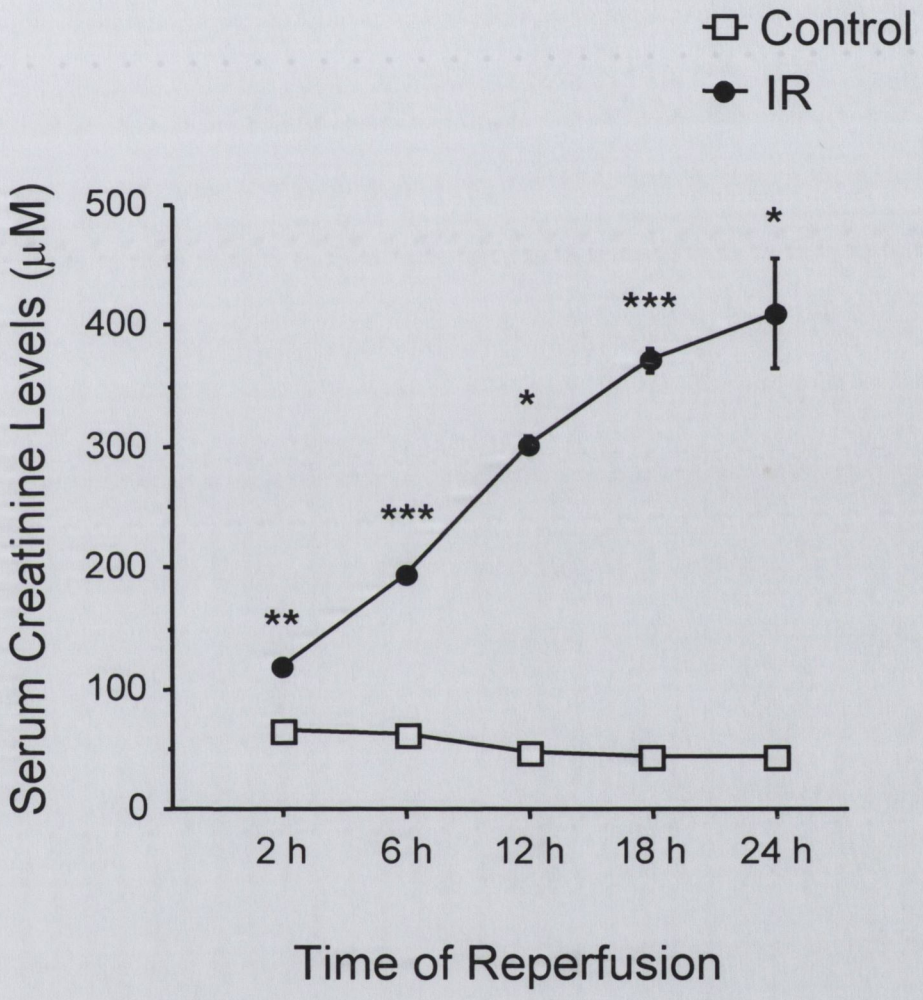


Figure 3.2 Serum creatinine levels measured at various reperfusion time-points following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney. Mean±SEM; n=2/group; unpaired t-test; *P<0.05 vs. control; ** P<0.01 vs. control; *** P<0.005 vs. control.

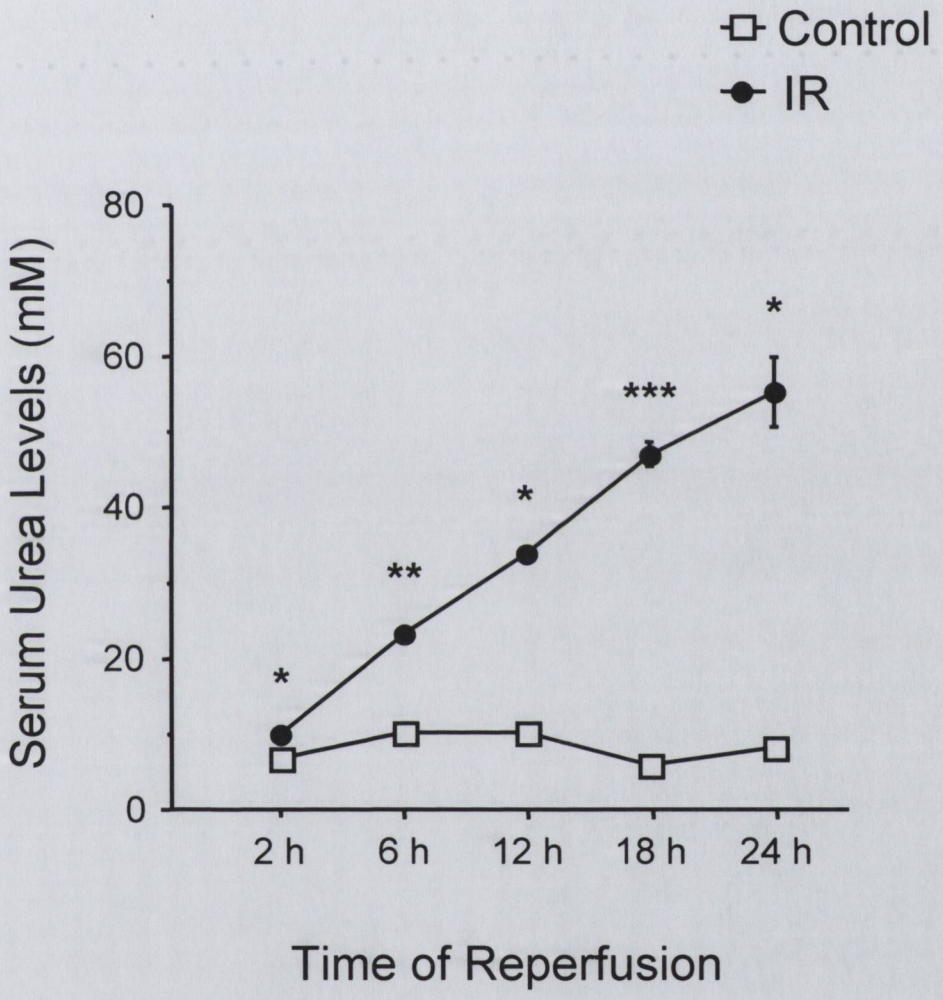


Figure 3.3 Serum urea levels measured at various reperfusion time-points following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney. Mean±SEM; n=2/group; unpaired t-test; *P<0.05 vs. control; ** P<0.01 vs. control; *** P<0.005 vs. control.

Table 3.1 Serum electrolyte levels (K^+, Na^+, Cl^-) measured at various reperfusion time-points following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney. Mean±SEM; n=2/group; unpaired t-test.

Electrolytes	Groups	Reperfusion Times				
		2h	6h	12h	18h	24h
Serum K^+ (mM)	Control	4.4±0.10	4.2±0.25	4.5±0.00	3.8±0.20	4.1±0.31
	IR	6.6±0.81	7.2±0.68	6.7±0.34	7.4±1.70	7.1±1.10
Serum Na^+ (mM)	Control	141±0.0	142±0.5	140±0.0	142±0.5	138±0.0
	IR	140±3.0	141±0.5	127±2.5	133±9.0	133±8.5
Serum Cl^- (mM)	Control	92±2.0	97±0.5	93±0.0	96±1.0	98±1.0
	IR	97±3.0	95±2.0	83±2.0	86±5.0	84±5.0

3.3.2 Effect Of Ischaemia-Reperfusion On Endothelial Integrity

Endothelial barrier function was compromised early in the reperfusion period following 45 min ischaemia, as evidenced by organ oedema (Figure 3.4). The percentage weight change between the left and right kidneys showed approximately equal weights of the two kidneys from each Control-2h animal (% difference = -1.7±4.71%). However in the IR-2h group the right kidney, subjected to IR injury, was 28.7±0.62% heavier than the left nephrectomised kidney ($P<0.05$ versus control-2h), showing that there was an accumulation of fluid in the injured organ (Figure 3.4). This appeared to somewhat resolve thereafter as although the % differences remained higher in the IR groups at all other time-points versus corresponding controls, these were lower than IR-2h levels. It should be

noted that the % weight difference in the control-18h group was elevated when compared with the 2 h timepoint. This was confirmed in chapter 6 and may have reflected an initial compensation by this kidney for the loss of the contralateral kidney. This appeared to resolve by 24 h.

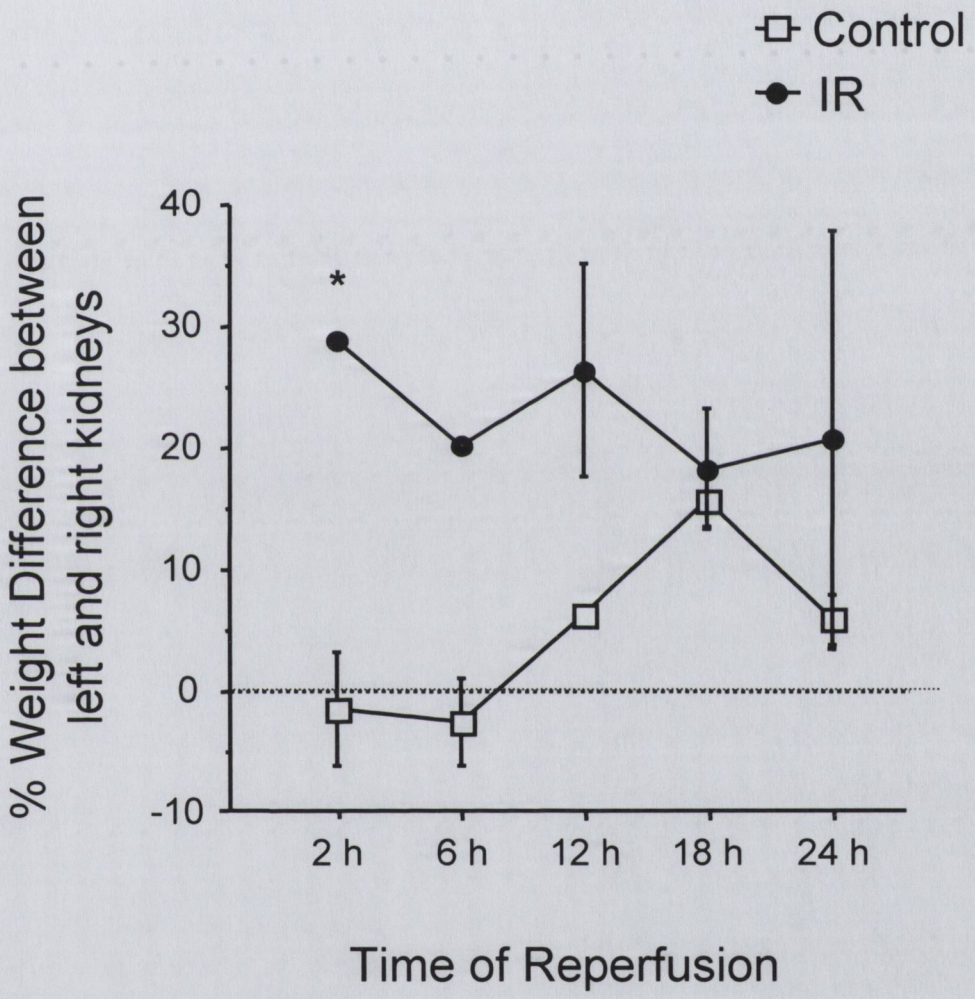


Figure 3.4 Indication of the present or absence of oedema in right kidneys exposed to sham ischaemia (control group) or 45 min ischaemia (IR group). The weight difference between the left baseline kidney that was removed at the beginning of the experiment and the right experimental kidney that underwent sham IR or IR was calculated as a percentage of the baseline kidney at each reperfusion time-point. Mean±SEM; n=2/group; unpaired t-test; *P<0.05 vs. control.

It was established that IR injury caused impaired renal function by 2 h of reperfusion which continued to worsen over 24 h of reperfusion. Renal oedema was present at 2 h of reperfusion only, and appeared to resolve by 6 h reperfusion. Thus, renal neutrophil infiltration, U/O and morphology were assessed during early injury at 2 h reperfusion. This allowed the use of an acute model where the animals were not required to be re-anaesthetized for injury assessment. Furthermore the stress of conscious recovery from the uninephrectomy and renal IR insult could be avoided in the animals.

3.3.3 Effect Of Ischaemia-Reperfusion On Kidney Neutrophil Infiltration (Renal Myeloperoxidase Levels)

Clamping the kidney for 45 min followed by a 2 h reperfusion period caused a significant increase in the MPO activity in the injured kidney (IR-2h group) (1.01±0.034 U/g) compared with sham-operated Control-2h kidneys (0.59±0.001 U/g) ($P<0.01$). This indicated that there was significant neutrophil infiltration of the tissue at this time (Figure 3.5).

3.3.4 Effect Of Ischaemia-Reperfusion On Urine Output

Urine output was assessed over the 2 h of sham-reperfusion in the Control-2h group and reperfusion in the IR-2h group. Urine output over these 2 h was significantly reduced in the IR-2h group (0.2±0.03 ml/2 h) versus the control-2h group (0.8±0.00 ml/2 h), $P<0.005$ (Figure 3.6).

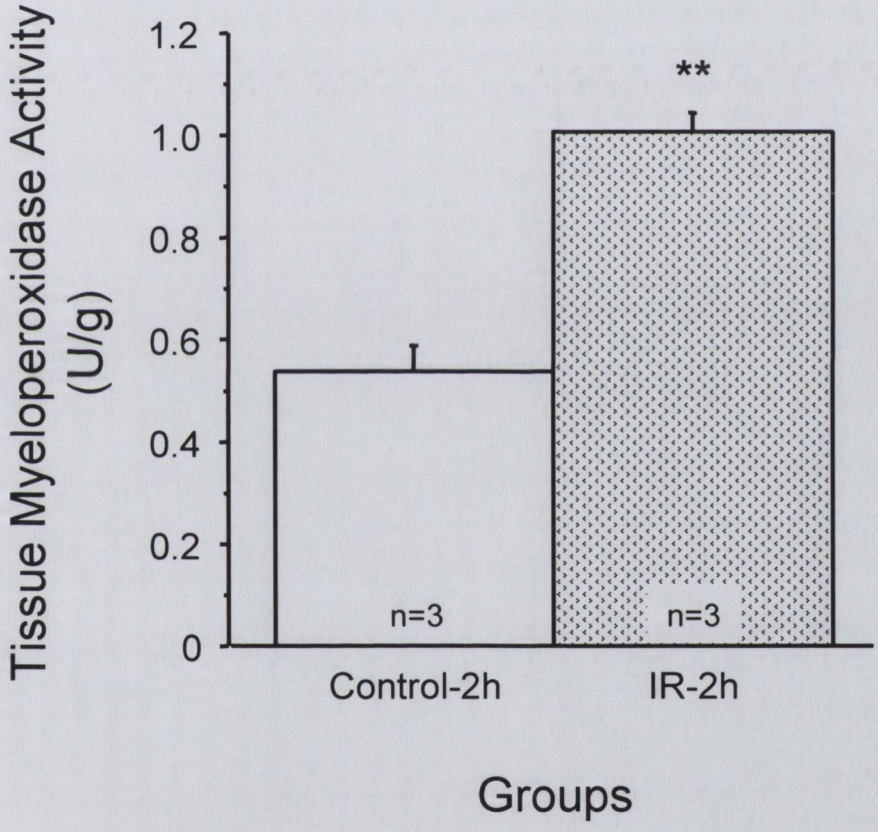


Figure 3.5 Tissue myeloperoxidase activity (an indicator of neutrophil infiltration) of the right kidney measured at two hours reperfusion following sham ischaemia (control-2h group) or 45 min ischaemia (IR-2h group). Mean±SEM; unpaired t-test; ** $P<0.01$ vs. control-2h.

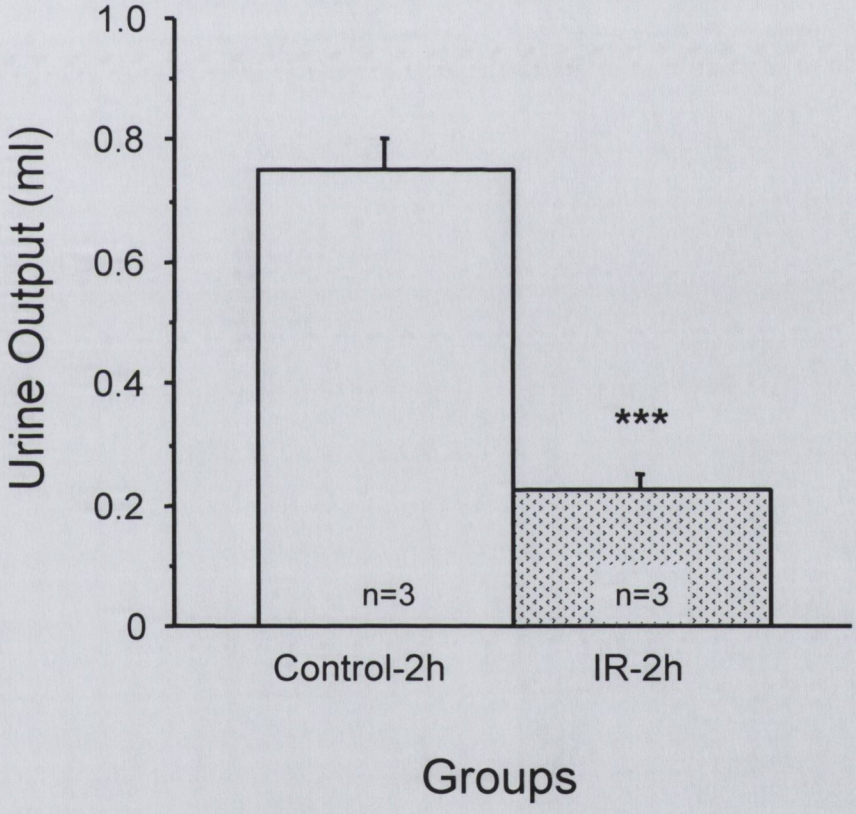


Figure 3.6 Urine output following sham ischaemia (control-2h group) or 45 min ischaemia (IR-2h group) of the right kidney. The bladder was emptied after a left nephrectomy, and the urine produced over the experimental (IR) period was collected at 2 h reperfusion. Mean±SEM; unpaired t-test; *** $P<0.005$ vs. control-2h.

3.3.5 Effect Of Ischaemia-Reperfusion On Renal Morphology

Figures 3.7a and 3.7b are representative images from the renal cortex after sham ischaemia and 45 min ischaemia respectively, both followed by 2 h reperfusion. In order to quantify differences in tubular morphology and proteinaceous cast formation, 10 fields of view in the renal cortex were examined in each animal, with an average of 33.8±0.86 tubules per field of view in the control group and 33.9±0.87 tubules per field of view in the IR-2h group.

3.3.5.1 Tubular morphology

Although 24.4±10.44% of the tubules in the Control-2h group demonstrated mild deterioration, tubular integrity was intact in these control animals, with close contact between tubules, and intact epithelium. However following IR, a significantly greater portion of the tubular epithelium exhibited degeneration (53.3±12.59%), with loss of nuclei and vacuolization of the cytoplasm ($P<0.05$ versus Control-2h). Furthermore complete destruction of some tubular epithelium with epithelial detachment from the basement membrane and loss of epithelial integrity was evident in the IR-2 h group ($P<0.01$ versus Control-2h) (Figure 3.8). In addition gaps had developed between tubules and tubular dilatation was evident.

3.3.5.2 Proteinaceous Cast Formation

In the less degenerated tubules of the IR-2h group, protein secretion was high and proteinaceous casts were present in 20.7±4.66% of tubules, compared with 1.8±0.54% of Control-2h tubules (Figure 3.9) ($P<0.05$).

3.3.5.3 Medulla Integrity

Medulla congestion was observed in kidneys exposed to IR-2h (Figure 3.7d) compared with sham-operated animals (Figure 3.7c). In addition, proteinaceous casts, which were largely absent in the Control-2h animals, were abundant in the inner medulla of IR-2 h kidneys.

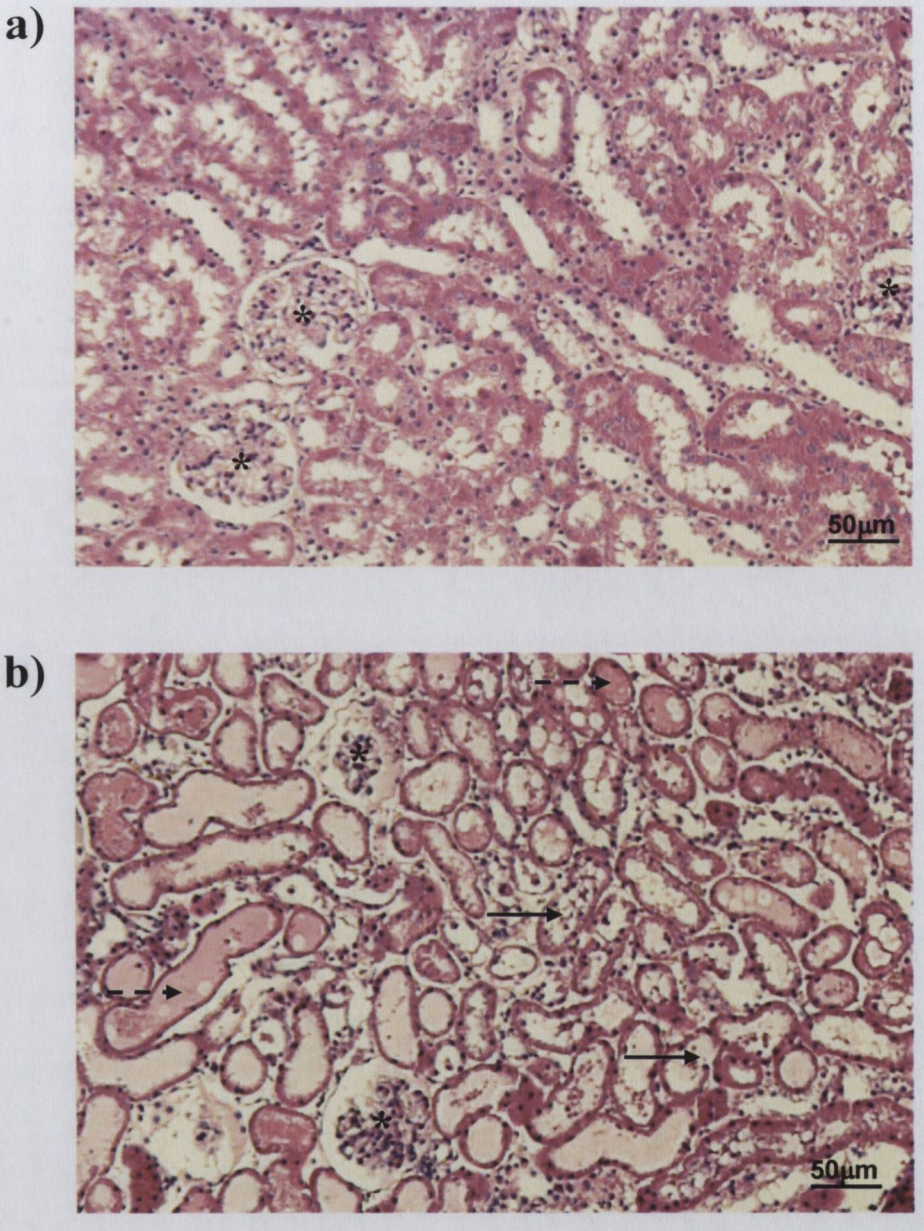
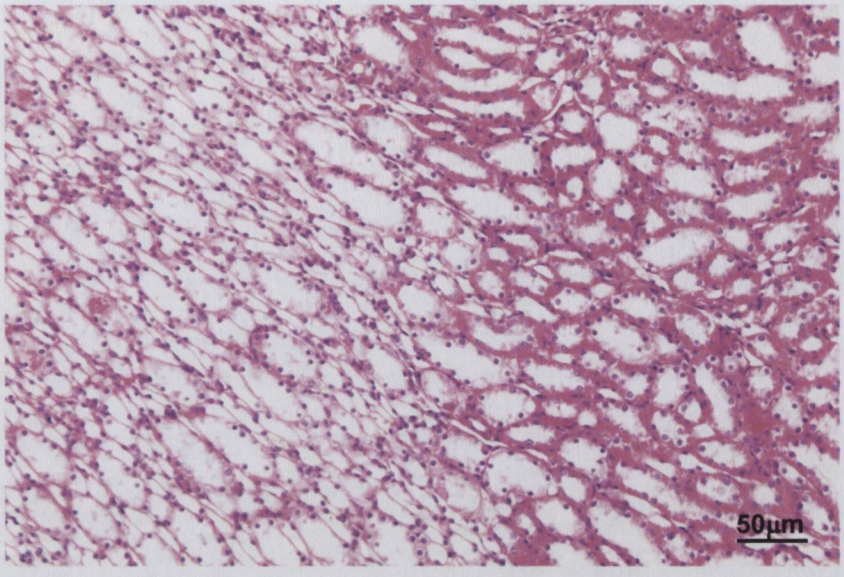


Figure 3.7a-b Representative images of renal cortex of the right kidney taken two hours following **a)** sham ischaemia (control-2 h group) or **b)** 45 min ischaemia (IR-2 h group). Tissues were stained with H&E. Note the degeneration of the tubular epithelium (solid arrows) and the development of proteinaceous casts (dashed arrows) in the IR-2 h group. Glomeruli are denoted by asterisks.

c)



d)

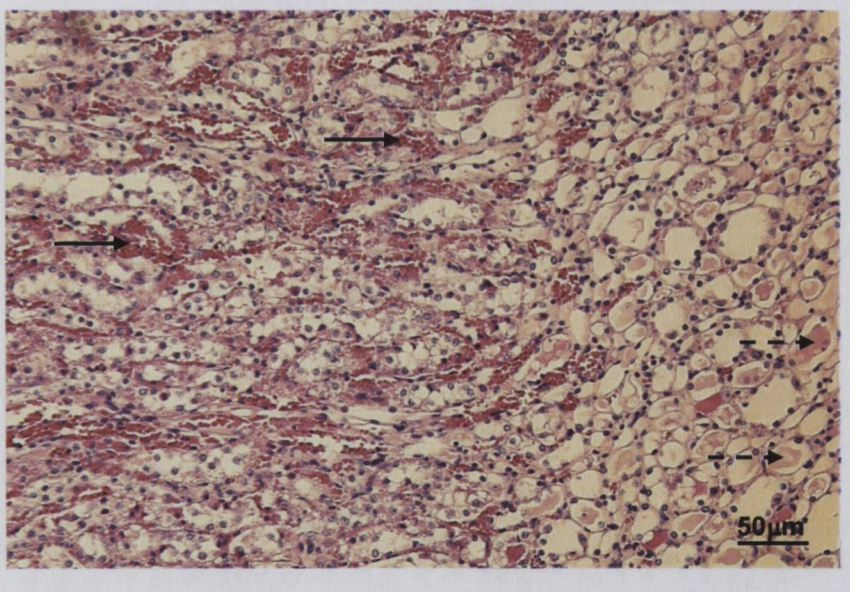


Figure 3.7c-d Representative images of renal medulla of the right kidney taken two hours following **c)** sham ischaemia (control-2 h group) or **d)** 45 min ischaemia (IR-2 h group). Tissues were stained with H&E. Note the red blood cell congestion (solid arrows) and the development of proteinaceous casts (dashed arrows) in the IR-2 h group.

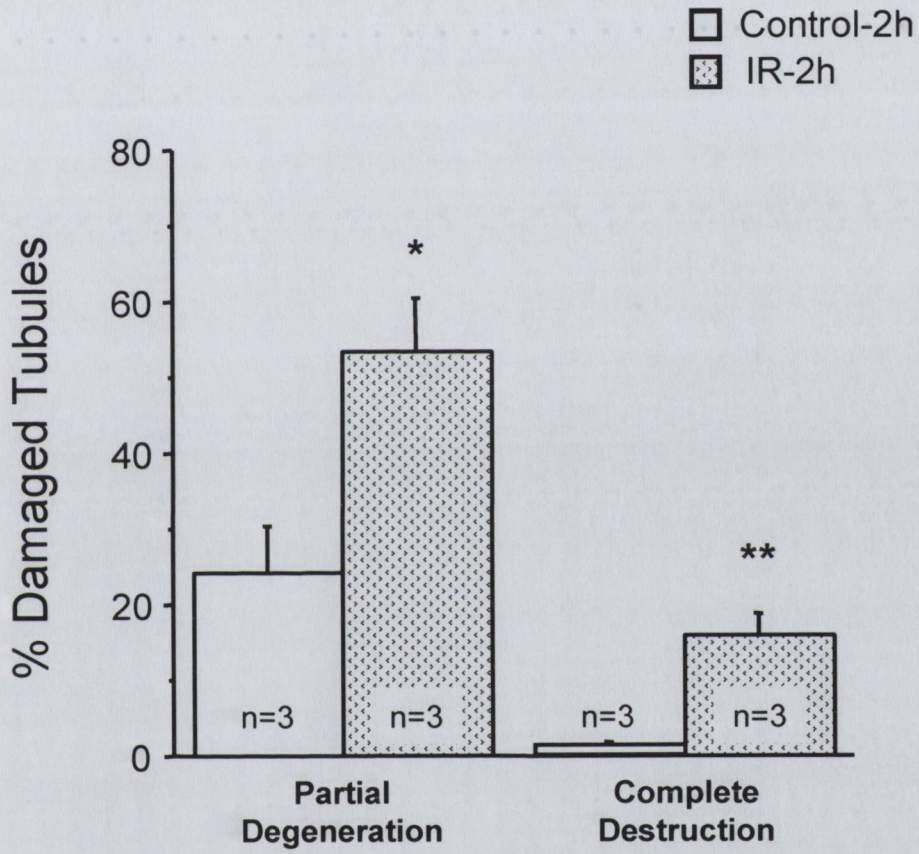


Figure 3.8 Quantification of tubular degeneration in the renal cortex of right kidneys that underwent sham ischaemia (control-2h group) or 45 min ischaemia (IR-2h group) followed by two hours reperfusion. Ten fields of view in H&E sections were assessed per kidney, and the mean for each animal used for statistics. Mean±SEM; unpaired t-test; * $P<0.05$ vs. control-2h; ** $P<0.01$ vs. control-2h.

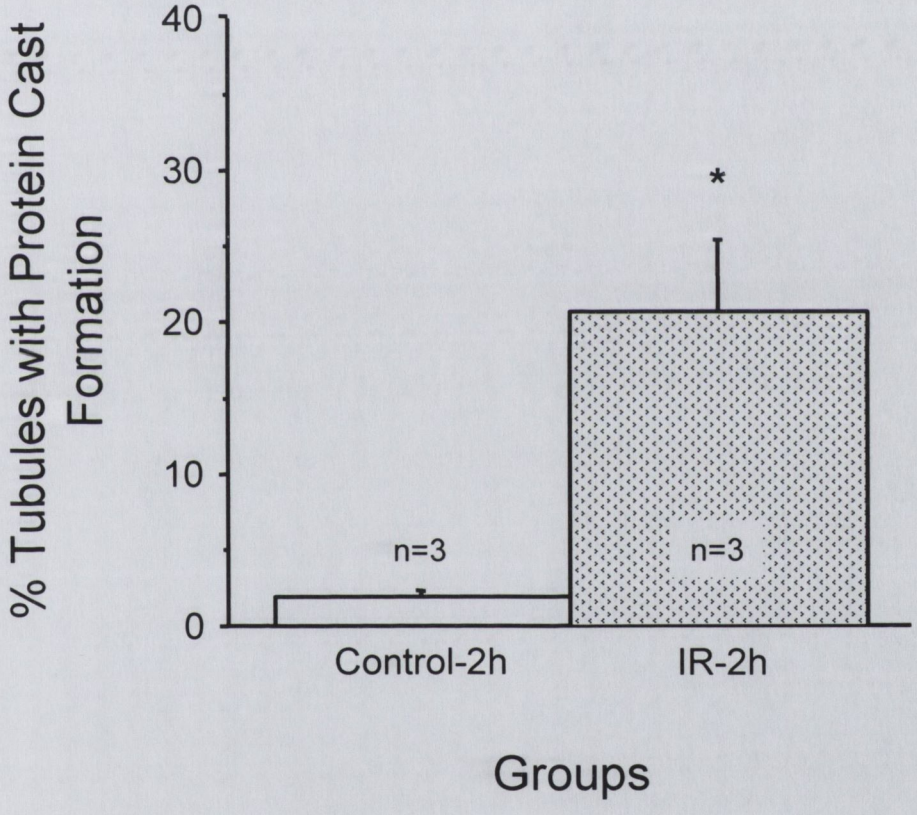


Figure 3.9 The percentage of tubules exhibiting proteinaceous cast formation in the renal cortex of right kidneys that underwent sham ischaemia (control-2h group) or 45 min ischaemia (IR-2h group) followed by two hours reperfusion. Ten fields of view in H&E sections were assessed per kidney, and the mean for each animal used for statistics. Mean±SEM; unpaired t-test; * $P<0.05$ vs. control-2h.

3.3.5.4 Leucocyte Infiltration

Leucocyte infiltration was significantly elevated in the cortex of kidneys subjected to IR-2h (72.6±5.81 leucocytes/field) compared with Control-2h kidneys (32.7±3.62 leucocytes/field). These appeared as cells with darker nuclei than those of the tubular epithelium, and were located primarily between tubules, near peritubular capillaries (Figure 3.10) ($P<0.005$ versus Control-2h). Some of these cells could be easily identified as neutrophils from the polymorphic appearance of their nuclei, thus supporting the MPO data. An example of some of these cells is shown in a histological section in Figure 3.10. Leucocyte accumulation was also found in the medulla.

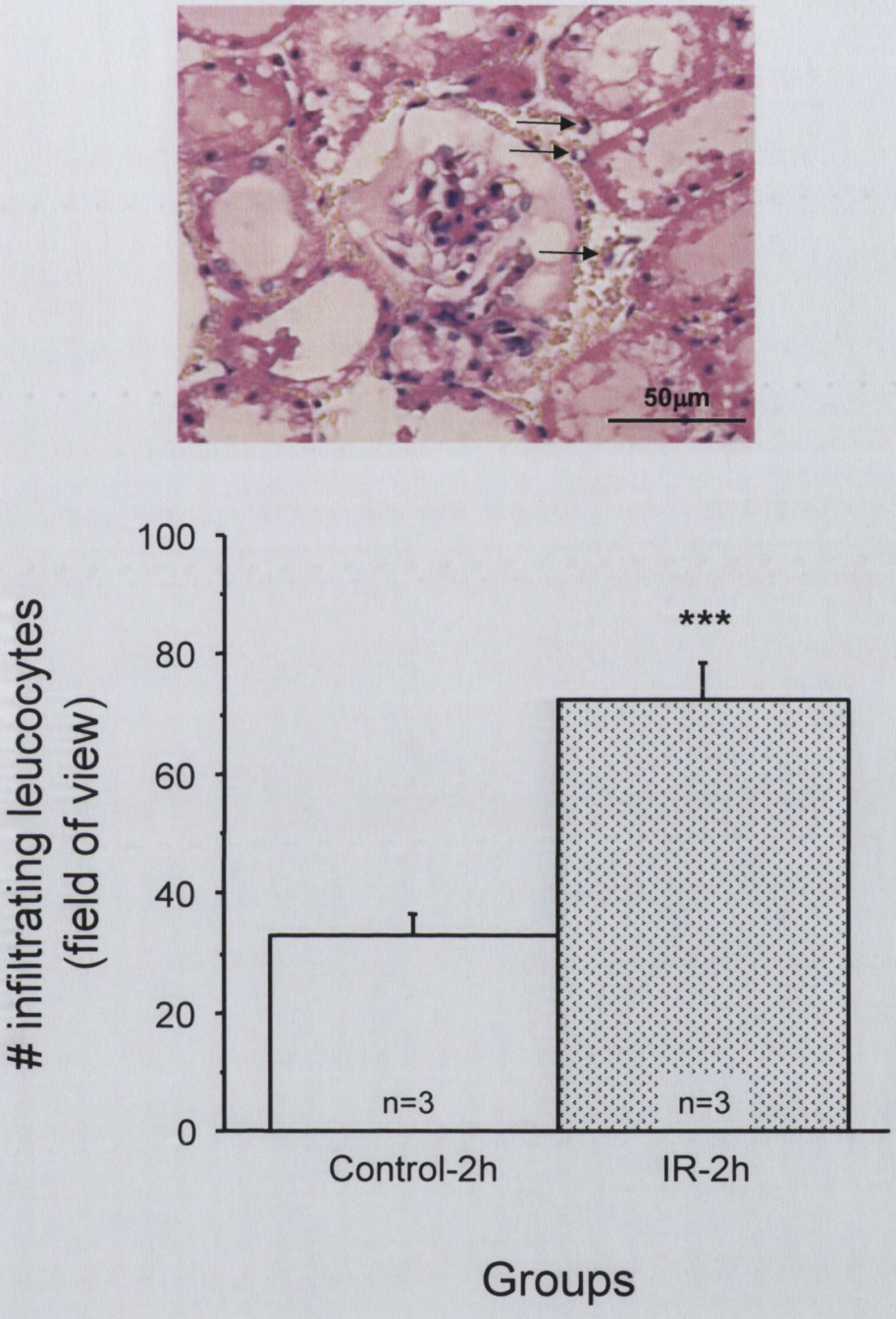


Figure 3.10 Leukocyte infiltration into the renal cortex of right kidneys that underwent sham ischaemia (control-2 h group) or 45 min ischaemia (IR-2 h group) followed by two hours reperfusion. Ten fields of view in H&E sections were assessed per kidney, and the mean for each animal used for statistics. Arrows on the histology image indicate neutrophils that have infiltrated into the right renal cortex of a kidney from the IR-2h group. Mean±SEM; unpaired t-test; *** $P<0.005$ vs. control-2 h.

3.4 Discussion

Renal IR injury has been implicated in DGF, increased allograft immunogenicity and subsequent allograft rejection. This model focuses on the effect of IR not only on renal function but the associated oedema, neutrophil infiltration and tissue damage. The results demonstrate that IR led to renal dysfunction within 2 h of reperfusion, at which time serum urea and creatinine levels were elevated. Renal function continued to deteriorate up to 24 h of reperfusion. Organ oedema was present in the IR-2h group, suggesting that the endothelial barrier function was compromised. However, this early increase in organ oedema was transient, decreasing after 2 h of reperfusion. Urine output was reduced at 2 h reperfusion also. Early endothelial activation at this time-point was indicated by the influx of leucocytes, in particular neutrophils, into the renal tissue. Alongside these alterations, tubular epithelial cell degeneration was evident in the renal cortex with loss of nuclei, deterioration of cell integrity, and the collapse of tubule-tubule contact. Some tubules that remained intact exhibited proteinaceous cast formation. In addition, medullary congestion was evident.

The aim of this chapter was to establish a renal model with an early reperfusion time-point at which both renal dysfunction and morphological injury could be detected using well established parameters, and which could be used to assess the efficacy of the pre-treatments presented in this thesis. In order to establish this model an ischaemic time of 45 min was chosen based on numerous reports that the resultant injury is consistent with significant impairment of both morphological and functional parameters. If the ischaemic time is reduced to 30 min ischaemia only mild alterations are observed, however, prolonging the ischaemic time to 60 min leads to an increased mortality (Jablonski *et al.*,

1983; Weight *et al.*, 1998). A contra-lateral nephrectomy was performed to allow for the determination of oedema, as well as more closely approximating a transplantation scenario. It has previously been demonstrated that 45 min renal ischaemia in the rat causes both functional and morphological injury that becomes apparent within as little as 2 h reperfusion. This continues to deteriorate over the first 24 h reperfusion (Williams *et al.*, 1997), after which time the tubular epithelium shows regeneration and renal function improves. Thus this chapter assessed several reperfusion time-points between 2 h and 24 h reperfusion, to establish the earliest time-point at which significant injury could be observed. In addition, a short reperfusion period would minimise the discomfort and recovery period for the animal. The chosen reperfusion time-point will be a starting point for assessing the efficacy of pre-treatments discussed in the following chapters with a view to observing injury in kidneys exposed to longer reperfusion times once the most suitable treatment has been established.

In the kidney, IR causes tubular deterioration which leads to functional impairment within 2 h of reperfusion and progressing for up to 24 h reperfusion (Williams *et al.*, 1997), as demonstrated in this study. Renal dysfunction is characterized by decreased GFR. A crude estimation of such as response can be made by measuring plasma creatinine levels. In many previous studies, increased serum creatinine and urea levels following renal IR injury or kidney transplantation have been used as a reliable marker for renal dysfunction (Chatterjee *et al.*, 2001; Lee and Emala, 2001; Jefayri *et al.*, 2000; Koo *et al.*, 1998) and previous data have revealed that the elevation of serum creatinine and urea usually peaks at approximately 24 h and then dissipates. In this study plasma creatinine levels were increased in rats that had undergone 45 min ischaemia followed by just 2 h reperfusion and continuing to increase for at least 24 h reperfusion. This was mirrored by plasma urea

levels. Serum electrolyte balance was slightly altered. This was likely to be due to the observed tubular damage, which decreases the ability of the tubular epithelium to reabsorb Na^+ and excrete K^+, both of which processes occur primarily in the proximal tubules in the renal cortex. Elevated fractional excretion of Na^+ may result (Jung *et al.*, 2000), and is perhaps which serum Na^+ levels are somewhat decreased by the 12 h time-point in this study. In addition, there is a reduced capacity to excrete K^+ thereby promoting hyperkalemia (Rabb *et al.*, 2000), as indicated by the rise in serum K^+ levels which peaked at 18 h reperfusion. Following warm ischaemic injury kidneys undergo a period of anuria followed later by a period of polyuria, although results by Chintala *et al.* (1993) disagree with this. However Rhoden *et al.* (2001) demonstrated that renal IR following uninephrectomy caused both elevated serum creatinine levels and decreased urinary volume, which were also observed here at 2 h following the restoration of blood flow.

Since significant functional changes were present within 2 h reperfusion in our model, it was not surprising to see that morphological disturbances were also demonstrated in these kidneys. Following ischaemia, a majority of the morphological changes occur in the renal cortex, with relative sparing of the medulla (Weight *et al.*, 1998). Therefore, tubular degeneration was scored in such a way as to make quantitative analysis possible. Rather than giving scores of 0-4 according to the percentage of damaged tubules, absolute percentages were calculated in three categories: 1) tubular degeneration where there was loss of nuclei in some epithelium, and vacuolization of the epithelial cytoplasm and 2) complete destruction where there was loss of tubular epithelium and basement membrane 3) the formation of proteinaceous casts in in the tubule lumens. Significant degeneration (both partial and complete destruction) was observed after just 2 h reperfusion. Most of the tubules that still possessed good structure also contained proteinaceous casts, which

suggests that these tubules were compensating for the functional deterioration in surrounding tubules.

In many organs, IR has been shown to result in increased endothelial permeability which is normally associated with leak of fluids and plasma proteins, but not necessarily cells, across the endothelial barrier resulting in oedema. In this study, oedema was measured according to the method used by Klausner *et al.* (1989), who demonstrated oedema at 24 h after IR injury. They found the ischaemic kidney was 21% heavier than the previously excised normal kidney 24 h after IR. Here, a 28.7±0.62% weight difference in the IR group between left (removed prior to IR) and right kidneys was found, compared to -1.7±4.71% in the control animals by 2 h of reperfusion. While the values observed at 24 h reperfusion in this study were similar to those obtained by Klausner *et al.* (1989), these were variable and so not significant.

Oxygen free radicals can disrupt the endothelial integrity of the vessels, with consequent loss of intravascular fluid and blood cells into the previously ischaemic area, this possibly being the triggering event in reperfusion injury. In many organs, the most damaging effect of IR injury-induced free radical production is lipid peroxidation leading to the breakdown of cellular lipid membranes. This causes increased permeability of cell membranes of both endothelium (renal capillary) and epithelium (tubular), which could lead to disruption of vital transport functions and thus renal functional impairment (McCoy *et al.*, 1988; Joannidis *et al.*, 1989; Greene and Paller, 1991). While other reports contradict a role for lipid peroxidation in post-ischaemic renal injury (Gamelin and Zager, 1988), these studies may have missed the early event or focused on the least affected radical (Paller and Neumann, 1991). It is also likely that endothelial cells themselves contribute to the

dissolution of the endothelial barrier through the release of cytokines, especially IL-1, TNF-α and IFN-γ. These cytokines appear to alter the protease-anti-protease balances resulting in basement membrane protein degradation. This is particularly important in the kidney where there is a single layer of endothelial cells and a single layer of epithelial cells on the glomerular basement membrane while throughout the tubules a single layer of epithelium lies on a basement membrane. Once the endothelium and epithelium are disrupted the basement membrane is the remaining barrier. In fact, activated leucocytes can synthesize basement membrane degrading enzymes such as elastase and collagenase. Catalase, scavengers of hypochlorous acid, and inhibitors of metalloproteinases markedly reduce the degradation of glomerular basement membrane upon activation of neutrophils, suggesting there is activation of a latent metalloproteinase by a product of the MPO-H_2O_2-Cl^- system (Shah *et al.*, 1987). Increased MPO levels and significant leucocyte infiltration (some of which were identified as neutrophils) were found in the post-ischaemic kidney here, supporting a role for neutrophils in the IR-induced renal dysfunction.

Neutrophil-endothelial cell interactions are a prerequisite for the microvascular injury induced by IR. Enhanced neutrophil adhesion to endothelium has been clearly demonstrated in mesenteric post-capillary venules during reperfusion (Granger *et al.*, 1993). In a model similar to this, Willinger *et al.* (1992) demonstrated that neutrophils had accumulated in the cortex, inner and outer medulla, and particularly within the peritubular capillaries in the cortex and the inner stripe of the outer medulla after 2 h reperfusion. This is similar to the leucocyte infiltration demonstrated by histology in the present model. Our findings of increased MPO activity in renal tissue at 2 h reperfusion following 45 min ischaemia suggest that these leucocytes are neutrophils. Myeloperoxidase is a haem-containing enzyme found primarily in neutrophils (but also to a lesser extent in

monocytes) and so can be used as an indirect measure of neutrophil infiltration into the tissue (Bradley *et al.*, 1982; Williams *et al.*, 1983). Klausner *et al.* (1989) demonstrated that activated neutrophils are not only involved in IR-induced renal impairment, but also infiltrate the renal tissue following renal IR injury. Furthermore, recently Okusa *et al.* (2000) showed the elevation of MPO levels in the kidney following IR and confirmed histological findings by Willinger *et al.* (1992) that neutrophils were present in the outer medulla and cortex. Ysebaert *et al.* (2000) contradicted these findings by demonstrating that while MPO levels rise in the post-ischaemic kidney, very few neutrophils infiltrate the tissue and that in fact the MPO levels may have reflected the presence of macrophages instead. However, unlike neutrophils, macrophages have never been demonstrated in the kidney as early as 2 h after the tissue has been reperfused, rather neutrophils appear to be the first leucocytes entering the post-ischaemic kidney followed by monocytes and lymphocytes days later (Takada *et al.*, 1997).

Evidence that neutrophils are not only present in the kidney following IR, but actually participate in the post-ischaemic renal dysfunction comes from several different studies. First, the infiltration of the kidney by neutrophils coincides with peak renal dysfunction (Takada *et al.*, 1997). In fact blocking leucocyte infiltration by the administration of monoclonal antibodies or antisense oligodeoxynucleotides against one or more CAMs has proven very effective in renal IR to not only to attenuate leucocyte infiltration but also renal IR injury (Kelly *et al.*, 1996; Chen *et al.*, 1999; Tajra *et al.*, 1999; Singbartl *et al.*, 2000; Singbartl and Ley, 2000; Fuller *et al.*, 2001). Importantly, depletion of circulating neutrophils by anti-neutrophil serum not only attenuated the post-ischaemic elevation of kidney MPO levels but also reduced the morphological alterations and renal dysfunction observed following renal IR in mice (Kelly *et al.*, 1996). Similarly, neutrophils were the

initial cells entering rat renal isografts and allografts within 2 h and peaking with maximum serum creatinine and urea levels, followed by monocytes and lymphocytes during the first week of reperfusion, suggesting that IR plays an important role in initial injury to the graft (Dragun *et al.*, 2000). When assessed in a human transplantation setting, Koo *et al.* (1998) provided evidence for a role for neutrophils by demonstrating that neutrophil infiltration into cadaveric renal allografts significantly correlated with long ischaemia times. However this infiltration was not seen in living-related allografts where ischaemic time is limited. This suggests that initial neutrophil influx of post-ischaemic renal tissue could be an initial step in a cascade of events ultimately leading to graft rejection (Koo *et al.*, 1998). Furthermore, increased serum creatinine levels three and six months following transplantation were associated with this neutrophil infiltration of renal tissue, suggesting that neutrophils may play a role in renal dysfunction both early in reperfusion as in our model, and later under transplant conditions.

In conclusion, renal ischaemia and reperfusion causes a significant functional impairment within 2 h continuing to worsen over 24 h of reperfusion. Early on, at 2 h reperfusion, organ oedema was observed suggesting that the endothelial barrier function had been compromised. This was associated with increased MPO levels indicating neutrophil infiltration of the kidney. Furthermore, tubular deterioration was evident by 2 h reperfusion alongside leucocyte infiltration and medullary congestion. These parameters have been used by others to identify different aspects of IR injury and offer a good basis on which to evaluate prospective treatments. A model of uninephrectomy followed by 45 min ischaemia and 2 h reperfusion as described here is used in the subsequent chapters to assess the efficacy of different modes of preconditioning on the prevention of renal IR injury. Once a suitable preconditioning method has been found, other parameters pertinent

to the transplantation setting will be assessed with this treatment. These will include ICAM-1 and MHC expression in post-ischaemic tissue.

CHAPTER 4

Ischaemic Preconditioning Protects The Kidney Against Ischaemia-Reperfusion Injury

4.1 INTRODUCTION

Ischaemia-reperfusion, an unavoidable step during transplantation, may contribute to allograft rejection. Preconditioning, a process where a sub-lethal stress induces a transient resistance of the stressed cells to subsequent episodes of otherwise lethal stress, has received recent attention as a strategy to minimize the deleterious influence of IR on a grafted organ. This study assessed the ability of preconditioning with short periods of ischaemia, or IPrec, to protect against a subsequent renal IR injury using the model established in Chapter 2. In the myocardium, it has been established that two phases of protection exist following IPrec. The early phase of protection lasts up to one hour in rat hearts (Li *et al.*, 1992) and up to four hours in rabbits (Burckhartt *et al.*, 1995) and the delayed phase is evident approximately twenty four hours after preconditioning and can last up to three days (Baxter and Ferdinandy, 2001). Although IPrec is capable of protecting many other organs against subsequent injury (Zhang *et al.*, 2001), evidence for such bimodal protection in these organs is lacking.

While early studies failed to find a protective effect of IPrec on renal IR injury, more recent studies have be successful in showing the existence of this phenomenon in the kidney. In the first published studies using renal IPrec in rats, protection against IR was completely absent (Islam *et al.*, 1997). Similarly, IPrec in the porcine kidney (Behrends *et al.*, 2000) and dog kidney (Kosieradzki *et al.*, 2003) failed to protect against IR-induced renal dysfunction when the IPrec was applied immediately before the IR insult. However recently, Ogawa *et al.* (2001) demonstrated an early protection against IR-induced renal dysfunction 30 min following IPrec. In a study by Lee and Emala (2000) even more immediate protection was conferred against IR-induced elevation of serum creatinine by

IPrec in the rat kidney. Although other parameters of injury were not assessed, this IPrec-induced protection was found to be PKC- and G-protein-dependent (Lee and Emala, 2001). Jefayri *et al.* (2000) discovered that 4 cycles of IPrec in the kidney protected against subsequent IR-induced morphological alterations although functional parameters were unchanged by preconditioning. This was associated with increased release of NO. All of these studies employed different protocols and different numbers of preconditioning cycles to precondition the kidneys, this possibly explaining the contrasting positive and negative findings. However, the more clinically relevant possibility that protection could be extended if the recovery period between IPrec and the IR injury was prolonged was not investigated.

Turman *et al.* (1997a), using an *in vitro* model, demonstrated that arachidonic acid release from renal proximal tubular cells was attenuated during a subsequent hypoxic insult applied 24 h after preconditioning with hypoxia-reoxygenation. While no comparable animal study has been performed, this study does demonstrate that a long-lasting window of protection may exist in the kidney. Other promising data for the potential of IPrec to attenuate transplantation-associated IR injury, comes from studies where IPrec has also been shown to protect against cold preservation and reperfusion injury associated with transplantation in the heart (Kevelaitis *et al.*, 2001), intestine (Sola *et al.*, 2001), liver (Arai *et al.*, 2001), lung (Li *et al.*, 1998). Recently this has been extended to the kidney (Torras *et al.*, 2002) where IPrec followed by a 10 min recovery period, protected kidneys not only against warm IR injury but also against 5 h cold storage before transplantation into a syngeneic recipient.

There have been many mechanisms proposed through which IPrec protects against subsequent injury. It is likely that a chain of events takes place from receptor activation to effector proteins (e.g. HSPs and antioxidants). One possible effector protein, HSP70, has been widely implicated in mediating the more prolonged protection following IPrec in the heart. However, since it takes time to up-regulate the expression of HSPs this is unlikely to be the mechanism mediating early protection. Rather, different molecules such as antioxidants and other as of yet unidentified proteins may be the effectors during this early stress response as well as playing a role in prolonged preconditioning by acting in conjunction with HSPs. In the kidney, only a few mechanisms have been implicated in IPrec, including PKC, pertussis toxin-sensitive G-proteins and NO. All of these have been in models where immediate protection following the preconditioning is assessed. However, a role for HSP70 in renal IPrec has not been investigated. Interestingly, PKC has been implicated in both the early and delayed protection following IPrec in the heart (Mitchell *et al.*, 1995; Baines *et al.*, 2001) and in HSP70 induction.

In order to assess if the kidney can be preconditioned with ischaemia and if long-lasting protection against IR injury could be achieved earlier than previously thought by IPrec, the aim of this study was to evaluate the protective effects of IPrec with repeated bouts of short-term ischaemia on renal injury following 45 min ischaemia and 2 h reperfusion. The IR injury was administered immediately, 2 h or 4 h after IPrec. Renal dysfunction was evaluated using serum creatinine, urea and K^+ levels and U/O. Endothelial integrity was indirectly assessed by calculating oedema and MPO levels. The expression of HSP70 was measured in kidneys exposed to IPrec and/or IR. In addition the role of PKC in this model was investigated.

4.2 METHODS

4.2.1 Animal Preparation

Adult male Sprague-Dawley rats weighing 300-350 g were randomised into two treatments; control and IR. Control animals (n=12) underwent sham IPrec (sham-IPrec) followed by sham IR, as described in section 2.1.3. The animals that were to undergo IR were subdivided into six groups: one group was exposed to sham-IPrec (IR group; n=12) and the other five were subjected to different IPrec protocols as detailed below (n=3-5/group). IPrec is detailed in section 2.1.3. Thereafter kidneys in these latter six groups were made ischaemic for 45 min followed by 2 h reperfusion.

At the time of experimentation, animals were anaesthetised using inhalational halothane (as described in section 2.1.1). In all groups, the right internal jugular vein was cannulated for administration of saline and heparin. All animals were maintained in a supine position and core temperature was monitored for the duration of the experiment by rectal probe. All animals received 1 ml saline per hour while under anaesthesia.

4.2.2 Ischaemic Preconditioning (IPrec)

A midline incision was made and the right kidney was mobilized in all groups. At the time of experimentation, no positive data had been published on IPrec in the rat kidney. Thus the IPrec protocols chosen were based on several studies performed in the heart. After reviewing results from the first two protocols below, the reperfusion period between IPrec and the IR insult was extended. Several IPrec groups were investigated by exposing the

kidney to cycles of brief ischaemia and reperfusion as follows (after which all kidneys underwent IR injury as described in section 2.1.2):

IPrec0h+IR group (n=3): two cycles of 5 min ischaemia and 5 min reperfusion.

4XIPrec0h+IR group (n=3): (four cycles of 5 min ischaemia and 5 min reperfusion)+IR.

IPrec2h+IR group (n=4): (5 min ischaemia, 5 min reperfusion, 5 min ischaemia, 2 h reperfusion)+IR.

IPrec4h+IR group (n=9): (5 min ischaemia, 5 min reperfusion, 5 min ischaemia, 4 h reperfusion)+IR.

IPrec-ChCl+IR group (n=5): 5mg/kg chelerythrine chloride (ChCl) i.p. 15 min before (5 min ischaemia, 5 min reperfusion, 5 min ischaemia, 4 h reperfusion)+IR.

Since the period between sham-IPrec and IR injury did not influence the development of renal dysfunction, the results represent controls and IR groups with 4 h between these two manipulations.

In order to assess the role of PKC in IPrec, the potency and selectivity of several commonly used PKC inhibitors (staurosporine, H-7, polymixin B, calphostin C and chelerythrine chloride) that are well tolerated *in vivo* were considered. The fungal alkaloid, staurosporine, non-specifically inhibits serine-threonine kinases. The naphthalene sulphonamide, 1-(5-isoquinolinylsulfonyl)-2-methylpiperidine, or H-7, exerts inhibitory effects on other kinases. Polymixin B also inhibits calcium/calmodulin-dependent kinases. In addition to blocking PKC, calphostin C competitively inhibits diacylglycerol. Chelerythrine chloride (ChCl), a naturally occurring benzophenanthridine alkaloid, is a highly specific antagonist of PKC, and can inhibit PKCε, the isoform of PKC most widely

implicated in IPrec. It interacts with the catalytic domain of PKC, competitively inhibiting the phosphate acceptor. This serves to block the translocation of PKC, therefore preventing its activation. In contrast to staurosporine, ChCl is 100 times more potent inhibitor of PKC versus other kinases such as PKA and PKG. Furthermore this blocker has been used in many recent *in vivo* studies, in particular when addressing the role of PKC in preconditioning (Kukreja *et al.,* 1999; Lee & Emala, 2001). Therefore ChCl was chosen for this study. In the IPrec-ChCl+IR group, 5 mg/kg of the selective PKC inhibitor, ChCl, was administered i.p. in 0.5 ml saline 15 min prior to IPrec (in accordance with Lee and Emala (2001)) (see Figure 4.1 below).

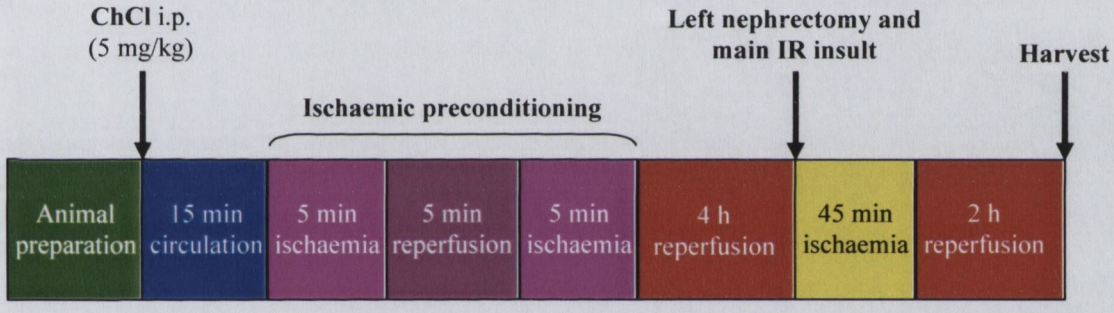


Figure 4.1 The time-course of ischaemic preconditioning followed by IR in the presence or absence of chelerythrine chloride (ChCl) administered 15 min prior to preconditioning.

4.2.3 Ischaemia-Reperfusion Insult

After 0 h, 2 h or 4 h recovery following preconditioning, the sutures in the midline incision were opened and the left kidney was mobilised in all groups. The left renal pedicle was ligated and a uninephrectomy performed. The right kidney was then mobilised

again. All animals received 400 IU heparin/kg body weight at this time to prevent blood clotting during ischaemia. The bladder was emptied. The control group underwent sham IR. The IR and IPrec groups underwent 45 min I followed by 2 h R as described in section 2.1.2. At the end of the reperfusion period the bladder was emptied to determine U/O, blood was drawn to assess renal function (see section 2.2) and the right kidney was removed to measure organ oedema and MPO levels as described in sections 2.3 and 2.4 respectively. Animals were then euthanised.

4.2.4 Heat Shock Protein 70 Expression

In order to determine whether preconditioning caused increased HSP70 expression, separate groups of animals were exposed to sham-IPrec, IPrec4h or IPrec-ChCl. Rather than subjecting these kidneys to IR as described above, the right kidneys were removed at the end of the preconditioning period (i.e. after 4 h reperfusion following short periods of ischaemia). These kidneys were snap-frozen for assessment of HSP70 expression as described in section 2.6. In addition, portions of right kidneys from control, IR and IPrec4h+IR animals were snap-frozen at the end of the full experiments for HSP70 analysis.

4.2.5 Statistics

All data is expressed as mean±SEM. ANOVA with Scheffe post-hoc test was performed on all parameters to compare between groups.

4.3 RESULTS

4.3.1 Effect Of Ischaemia-Reperfusion On Renal Function

Significant renal functional impairment was observed after 2 h reperfusion following IR. Serum creatinine levels increased from 69.2±3.17 μM in controls to IR levels of 119.3±8.79 μM ($P<0.001$) (Figure 4.2). Similarly serum urea levels increased from 7.6±0.44 mM in the control group to 13.9±1.34 mM in IR rats following 45 min ischaemia and 2 h reperfusion ($P<0.005$) as seen in Figure 4.3. Serum K^+ levels were significantly increased at 2 h reperfusion (8.7±0.97 mM) compared with the sham-operated control animals (4.6±0.22 mM) ($P<0.05$, Figure 4.4) (Note: In chapter 3, there was an increase in serum K^+ levels between the control-2h and IR-2h groups which was not significant, possibly due to the small number of animals per group. However, the significant findings in this chapter are confirmed in chapters 5 and 6). Serum levels of Na^+ and Cl^- were unchanged by IR (data not shown). Urine output was reduced from 1.2±0.20 ml in control animals to 0.5±0.17 ml in the IR group although this was not significant (Figure 4.5).

4.3.2 Effect Of Ischaemic Preconditioning On Ischaemia-Reperfusion-Induced Renal Dysfunction

4.3.2.1 IPrec0h+IR Group

In the IPrec0h+IR group, where animals were reperfused for 5 min following the IPrec episodes before the IR insult was applied, serum creatinine (103.2±5.49 μM) and urea (11.6±0.59 mM) levels remained at IR levels, significantly higher than values seen in the control group, $P<0.05$ for both (Figures 4.2 and 4.3 and Table 4.1). Serum K^+ levels in this

group were not significantly different to other groups, although were very close to IR levels (8.0±1.22 mM) (Figure 4.4). Serum Na^+ and Cl^- levels were unchanged by preconditioning (data not shown). Urine output remained at IR levels in the IPrec0h+IR group. This was not significant from controls (Figure 4.5).

4.3.2.2 4XIPrec0h+IR Group

Due to the lack of protection seen in the IPrec0h+IR group and a previous study by Lawson *et al.* (1993) in the heart, where the degree of protection was dependent on the number of ischaemic cycles used to precondition, the number of IPrec cycles was increased to four (4XIPrec0h+IR group). However, preliminary data from three animals demonstrated that renal function did not improve with this protocol. Instead, serum creatinine and urea levels were not only increased above controls but were significantly higher than values in both the IR and IPrec0h+IR groups (Table 4.1). Due to these results other functional data was not collected.

Table 4.1 Renal dysfunction (serum creatinine and urea levels) and oedema (% weight difference between left and right kidneys) measured at two hours reperfusion following sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney. Two separate IR groups were exposed to either 2 (IPrec0h+IR group) or 4 (4XIPrec0h+IR group) cycles of preconditioning (1 cycle=5 min ischaemia+5 min reperfusion) directly before the 45 min ischaemic insult. Mean±SEM; n=3/group; ANOVA with Scheffe post-hoc test.

Group	Serum Creatinine (μM)	Serum Urea (mM)	% Weight Change
Control (n=5)	70.3±6.61	7.6±0.83	1.5±2.52
IR (n=8)	111.9±3.71*	11.9±0.83**	17.5±4.08*
IPrec0h+IR (n=5)	103.2±5.49*	11.6±0.59*	16.8±4.56
4XIPrec0h+IR (n=3)	140.7±6.64**#^	18.4±0.12**#^	22.8±4.17*

* P<0.05 vs. Control; ** P<0.0005 vs. Control; # P<0.05 vs. IR; ^ P<0.005 vs. IPrec0h+IR

4.3.2.3 IPrec2h+IR Group

In contrast, recovery for 2 h after IPrec before the main IR insult conferred approximately 50% protection against IR-induced renal dysfunction, with serum creatinine (88.0±10.40 μM) and urea (10.1±0.26 mM) levels lying between control and IR values (Figures 4.2 and 4.3 respectively). Serum K^+ levels in the IPrec2h+IR group (6.4±0.64 mM) displayed a similar pattern, remaining between control and IR levels (Figure 4.4). There were no significant differences between renal functional parameters in the IPrec2h+IR group and controls or IR animals. Serum Na^+ or Cl^- levels were unaffected by IPrec (data not shown).

Urine output was similar to IR levels in the IPrec2h+IR group although these values were variable thus there was no significant difference versus controls (Figure 4.5).

4.3.2.4 IPrec4h+IR Group

Unlike the early preconditioning groups, there were significant differences between the serum creatinine and urea levels in the IPrec4h+IR group and the IR group. Serum creatinine was reduced from 119.3±8.79 μM in the IR group to 80.5±5.42 μM in the IPrec4h+IR animals, $P<0.05$ (Figure 4.2). Similarly, serum urea levels of IPrec4h+IR animals were almost equal to control levels at 8.6±0.62 mM, $P<0.05$ versus the IR group (Figure 4.3). Serum K^+ was also significantly attenuated in the IPrec4h+IR group (5.5±0.18 mM) versus IR ($P<0.05$, Figure 4.4). Urine output was 1.0±0.13 ml in the IPrec4h+IR group, between control and IR levels, although this was not significant compared with any other group (Figure 4.5). No significant differences could be seen between the control and IPrec4h+IR groups in any of the parameters measured.

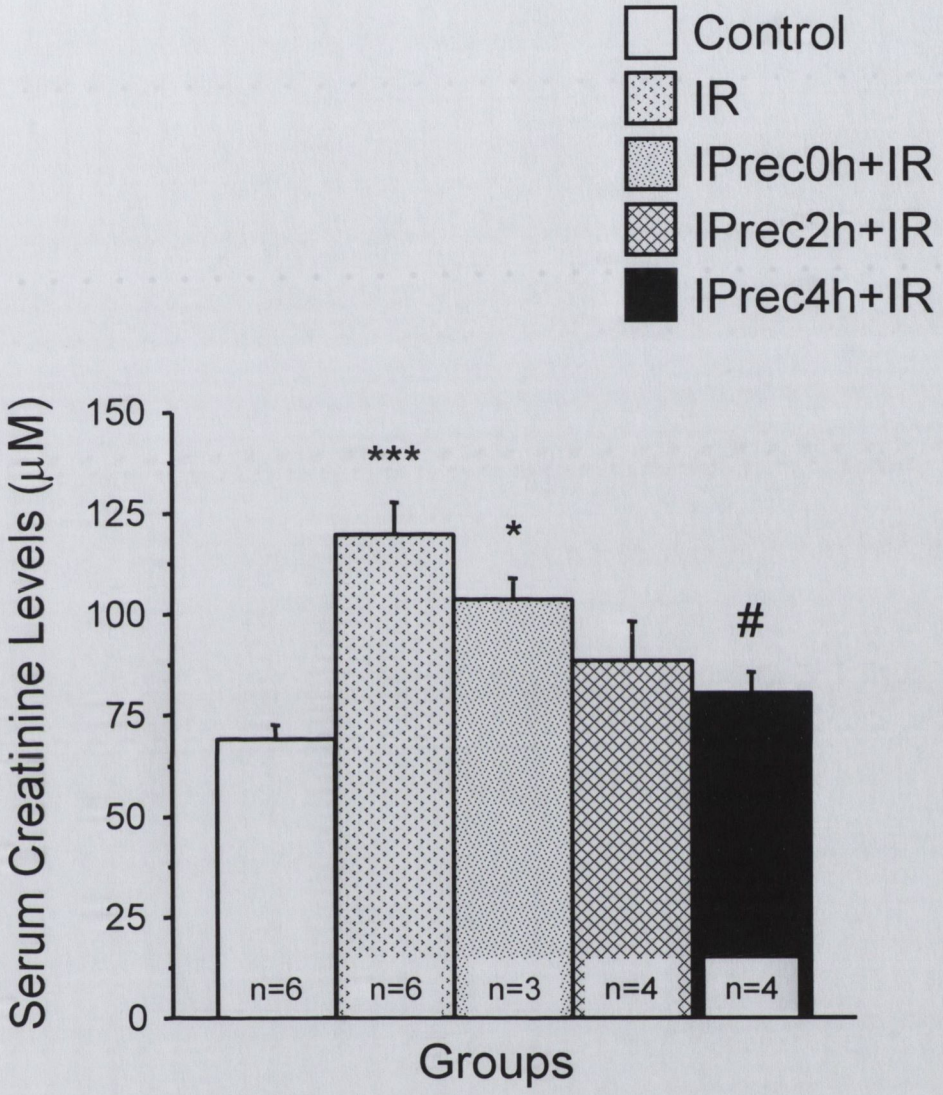


Figure 4.2 Serum creatinine levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in three IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 5 min (IPrec0h+IR group), 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test); *P<0.05 vs. control; *** P<0.005 vs. control; # P<0.05 vs. IR.

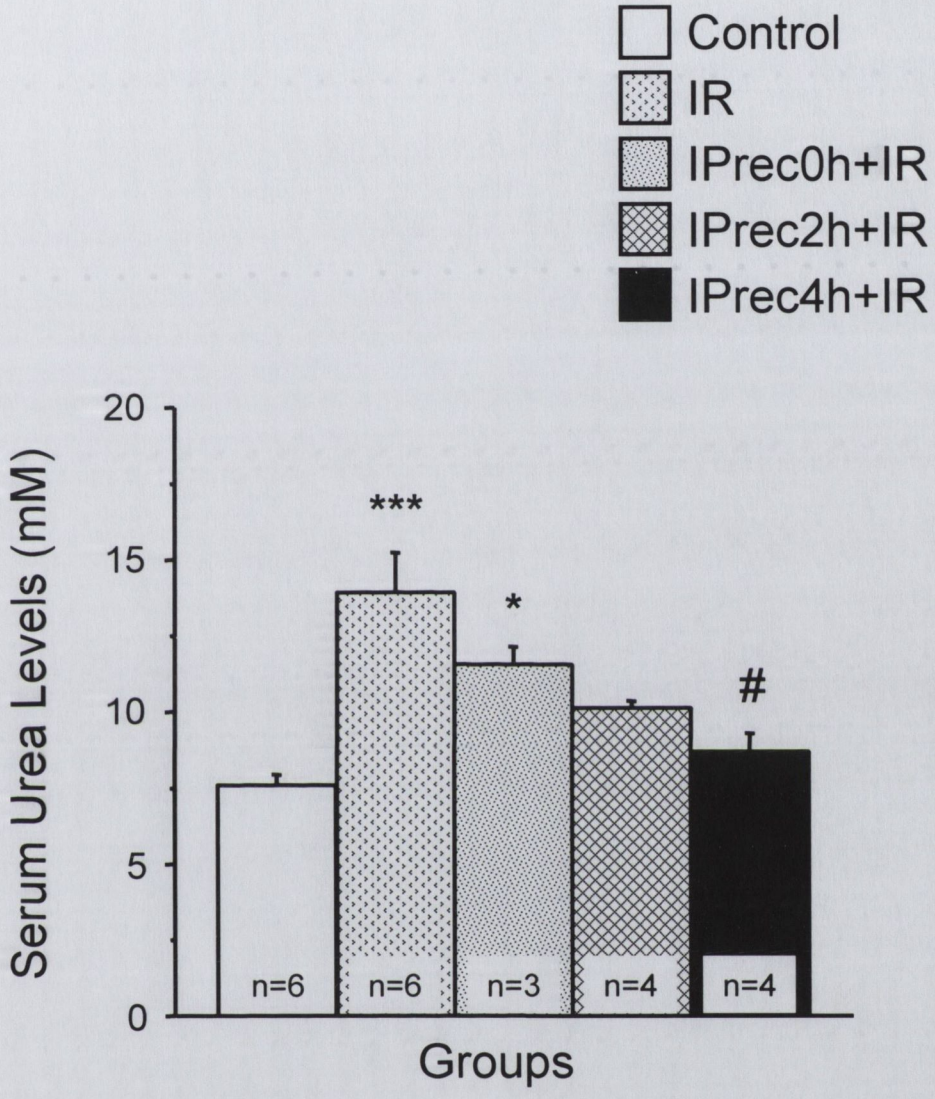


Figure 4.3 Serum urea levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in three IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 5 min (IPrec0h+IR group), 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test); *P<0.05 vs. control; *** P<0.005 vs. control; # P<0.05 vs. IR.

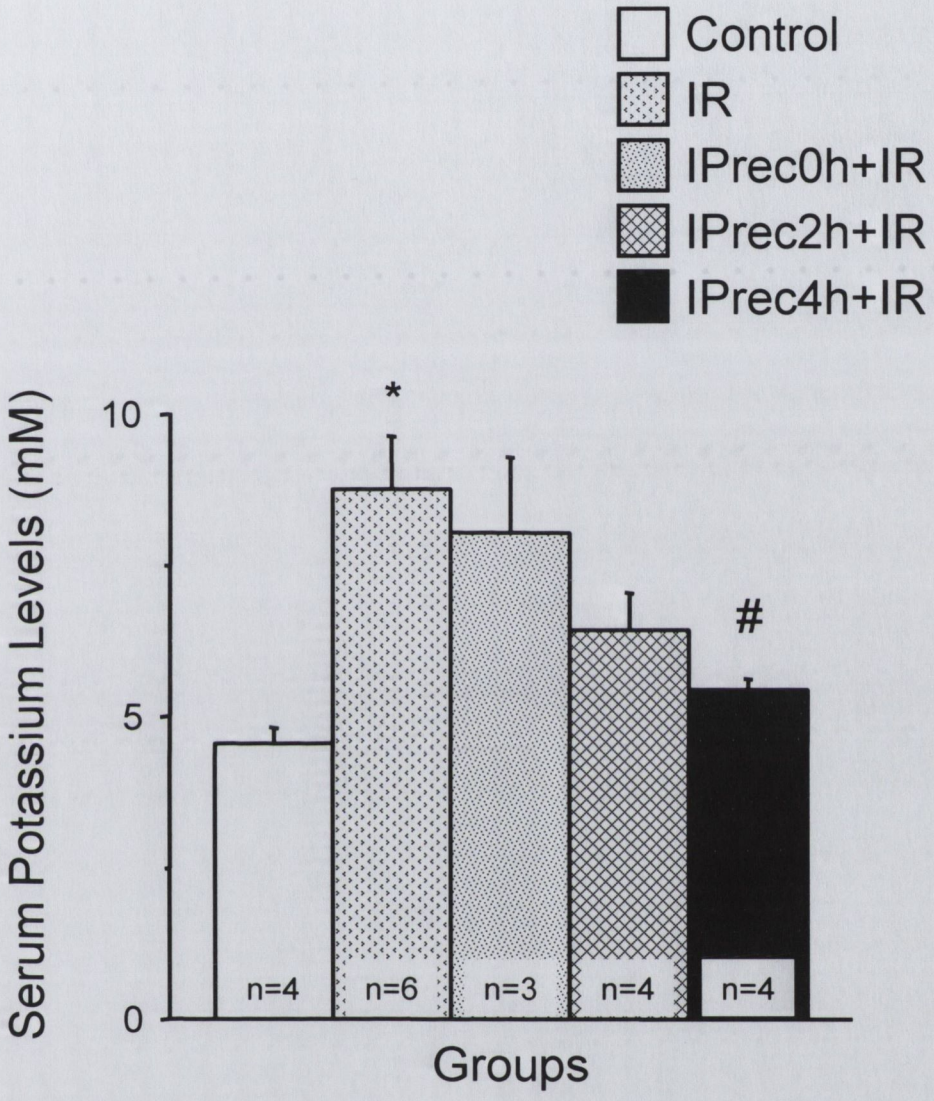


Figure 4.4 Serum potassium levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in three IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 5 min (IPrec0h+IR group), 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test); *$P<0.05$ vs. control; # $P<0.05$ vs. IR.

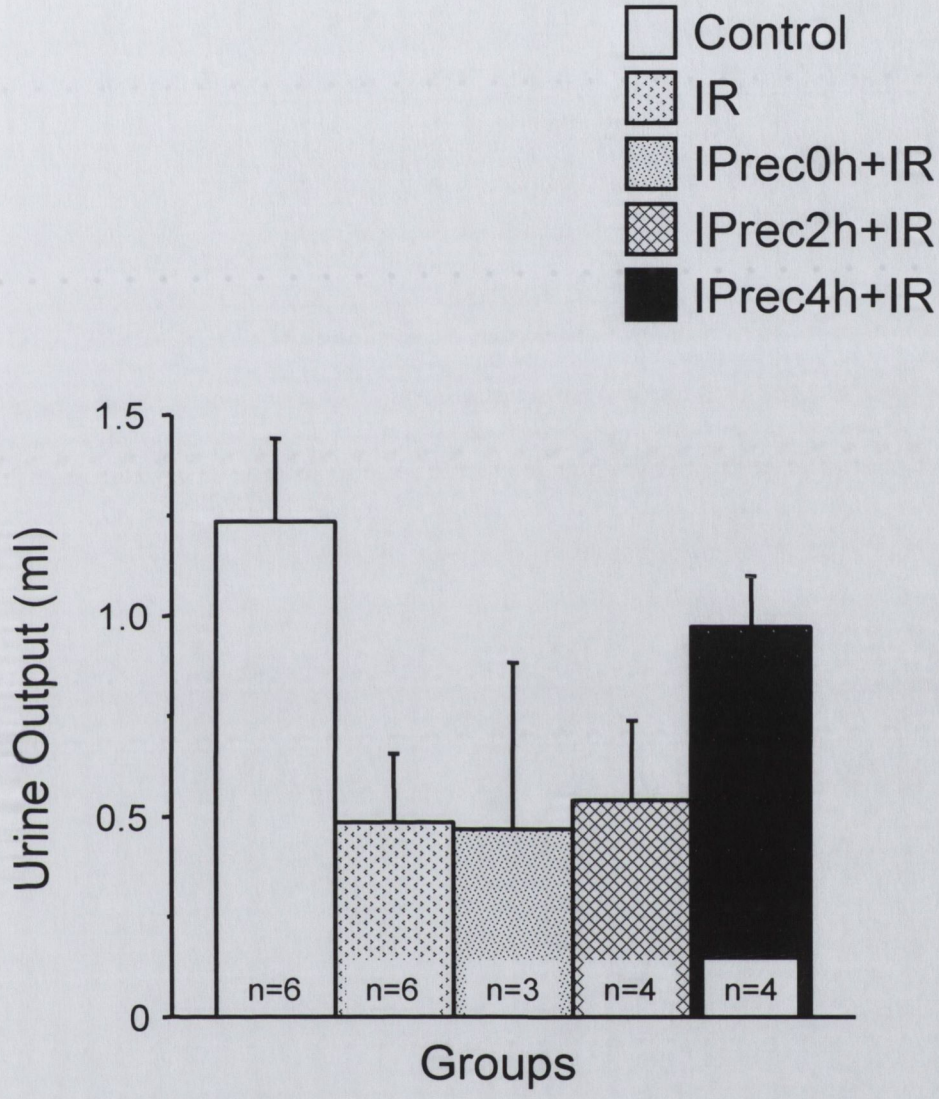


Figure 4.5 Urine output levels measured over 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in three IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 5 min (IPrec0h+IR group), 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test).

4.3.3 Effect Of Ischaemia-Reperfusion On Endothelial Integrity

Endothelial barrier function was compromised following the IR insult, as evidenced by organ oedema (Figure 4.6). The percentage weight change between the left and right kidneys showed approximately equal weights in the two kidneys from each control animal (% difference = -3.8±2.27%). However in the IR group the right kidney, subjected to an IR insult, was 15.5±1.81% heavier than the left nephrectomised kidney (P=0.0005 versus control), showing that there was an accumulation of fluid in the injured organ (Figure 4.6). Clamping the kidney for 45 min followed by a 2 h reperfusion period also caused a significant increase in the MPO activity in the injured kidney (1.2±0.11 U/g) compared with sham-operated control kidneys (0.5±0.05 U/g) (P<0.005), indicating significant neutrophil infiltration of the tissue (Figure 4.7).

4.3.4 Effect Of Ischaemic Preconditioning On Ischaemia-Reperfusion-Induced Endothelial Permeability And Neutrophil Accumulation

4.3.4.1 IPrec0h+IR Group

In the IPrec0h+IR group, organ oedema was evident to a similar degree as that found in IR animals, with a weight difference between the two kidneys of 16.8±4.56% (P<0.005 versus controls) (Figure 4.6 and Table 4.1). Due to the complete lack of protection against renal dysfunction in IPrec0h+IR animals, no MPO assay was carried out on these kidneys.

4.3.4.2 4XIPrec0h+IR Group

Similarly, increasing the number of preconditioning cycles from two in the IPrec0h+IR group to four in the 4XIPrec0h+IR group failed to attenuate the development of organ

oedema (Table 4.1). Taken together with the functional data in these animals, this group was not pursued further.

4.3.4.3 IPrec2h+IR Group

The IPrec2h+IR group demonstrated good protection against the development of organ oedema (% weight difference was -0.8±2.54%, $P<0.01$ versus IR and IPrec0h+IR groups) (Figure 4.6), despite only a 50% reduction in the renal dysfunction. However, contrary to these results, renal MPO levels (1.2±0.25 U/g) were comparable to IR levels, significantly elevated above controls values ($P<0.01$ versus controls) (Figure 4.7).

4.3.4.4 IPrec4h+IR Group

As would be expected from the functional data, preconditioning with brief periods of ischaemia followed by 4 h reperfusion prevented the oedema and increased MPO activity in the kidney seen following IR injury. There was a very small percentage difference between right and left kidneys of 0.7±1.61% in the IPrec4h+IR group, $P<0.05$ versus IR (Figure 4.6). The MPO activity was at control levels of 0.5±0.07 U/g in the IPrec4h+IR group, prevented from rising significantly to 1.2±0.10 U/g as seen in the IR or IPrec2h+IR groups ($P<0.005$ for both) (Figure 4.7).

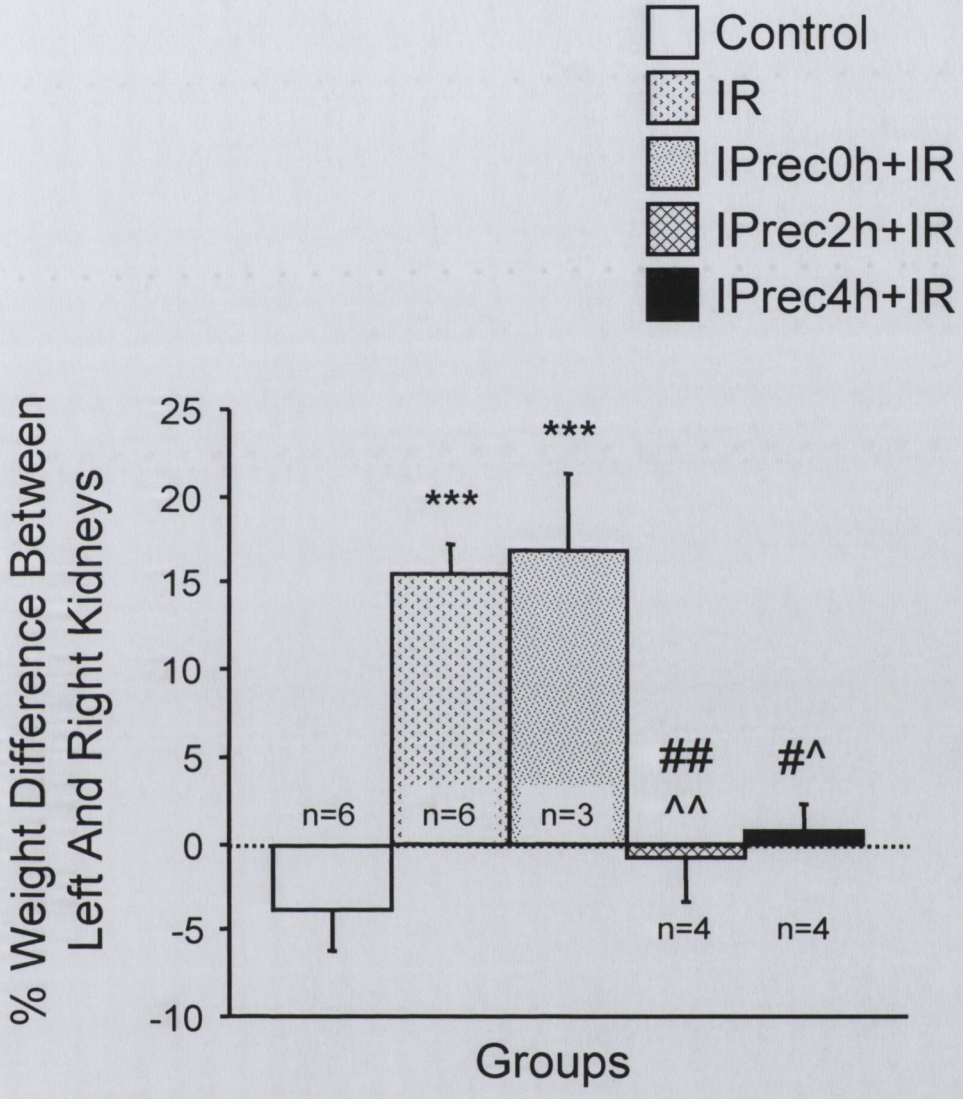


Figure 4.6 The % weight difference between right kidneys after 2 h reperfusion following sham ischaemia (control group) or 45 min ischaemia (IR groups) when compared with the left previously nephrectomised kidney, as an indicator of oedema. Kidneys in three separate IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 5 min (IPrec0h+IR group), 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test); *** $P<0.005$ vs. control; # $P<0.05$ vs. IR; ## $P<0.01$ vs. IR; ^ $P<0.05$ vs. IPrec0h+IR; ^^ $P<0.05$ vs. IPrec0h+IR.

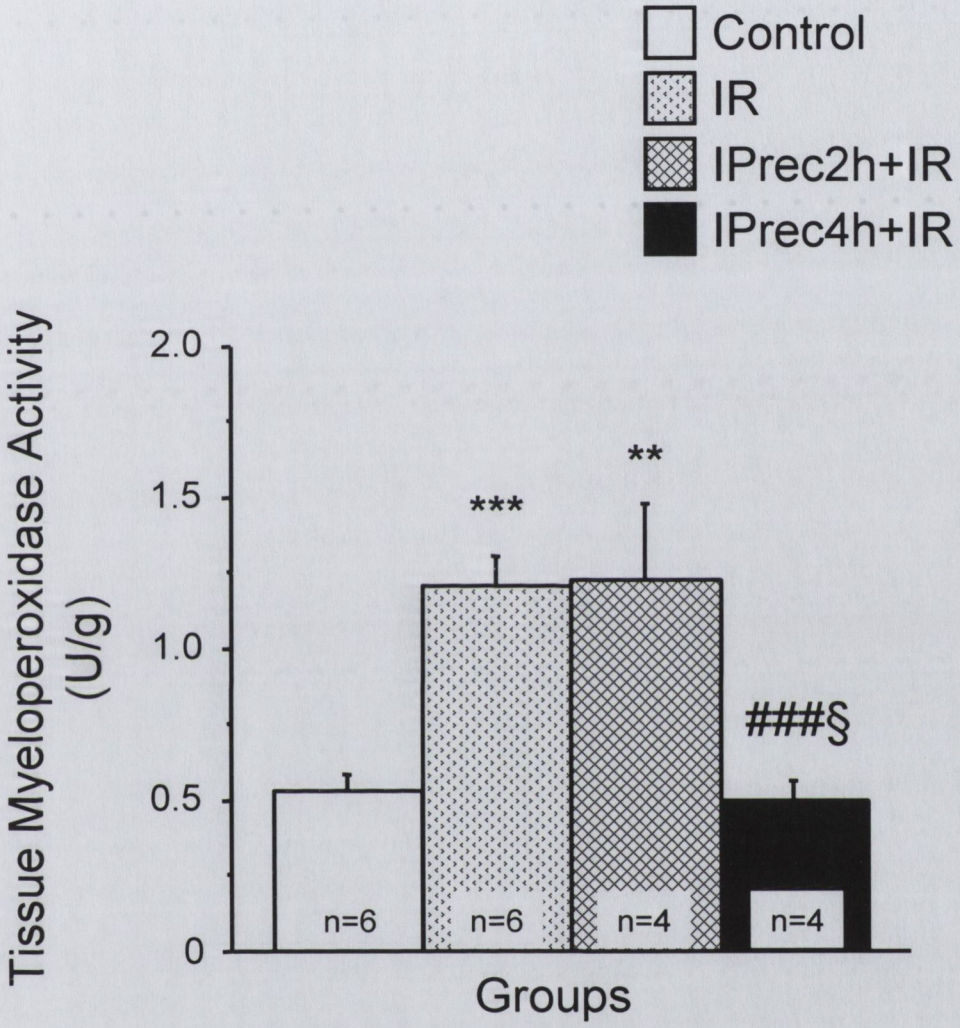


Figure 4.7 Renal myeloperoxidase levels at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia) and 2 h (IPrec2h+IR group) or 4 h (IPrec4h+IR group) reperfusion before undergoing the IR injury. Mean±SEM; ANOVA (Scheffe post-hoc test); ** $P<0.01$ vs. control; *** $P<0.005$ vs. control; ### $P<0.005$ vs. IR; § $P<0.05$ vs. IPrec2h+IR.

4.3.5 The Role Of Protein Kinase C In Ischaemic Preconditioning In The Kidney

Administration of 5 mg/kg ChCl, the selective PKC inhibitor, 15 min before IPrec4h with IR i.e. the IPrec-ChCl+IR group, negated the protective effect of preconditioning on the IR-induced renal dysfunction. Serum creatinine levels were elevated from 84.4±5.73 μMin the IPrec4h+IR group to 123.3±4.49 μM in the ChCl-treated animals ($P<0.0001$ versus control; $P<0.01$ versus IPrec4h+IR) (Figure 4.8) and serum urea increased from 8.6±0.62 mM to 15.5±1.61 mM ($P<0.0005$ versus control; $P<0.005$ versus IPrec4h+IR) (Figure 4.9). Similarly serum K^+ was raised significantly from 5.3±0.18 mM in IPrec4h+IR animals to 9.8±1.54 mM in the IPrec-ChCl+IR group ($P<0.05$), comparable to IR levels ($P<0.005$ versus control) (Figure 4.10). However, the prevention of organ oedema by IPrec was not altered by the treatment of animals with the PKC inhibitor. The % weight difference between the left control kidney and the right experimental kidney was small (3.8±5.20 %). This was significantly lower than levels observed in the IR group of 16.2±2.68 % ($P<0.05$) (Figure 4.11). There were no significant differences between MPO levels of 0.9±0.03 U/g observed in the IPrec-ChelCl+IR group and those in the IPrec4h+IR group or in the IR animals. Rather MPO in the ChelCl-treated animals fell between control and IR values (Figure 4.12).

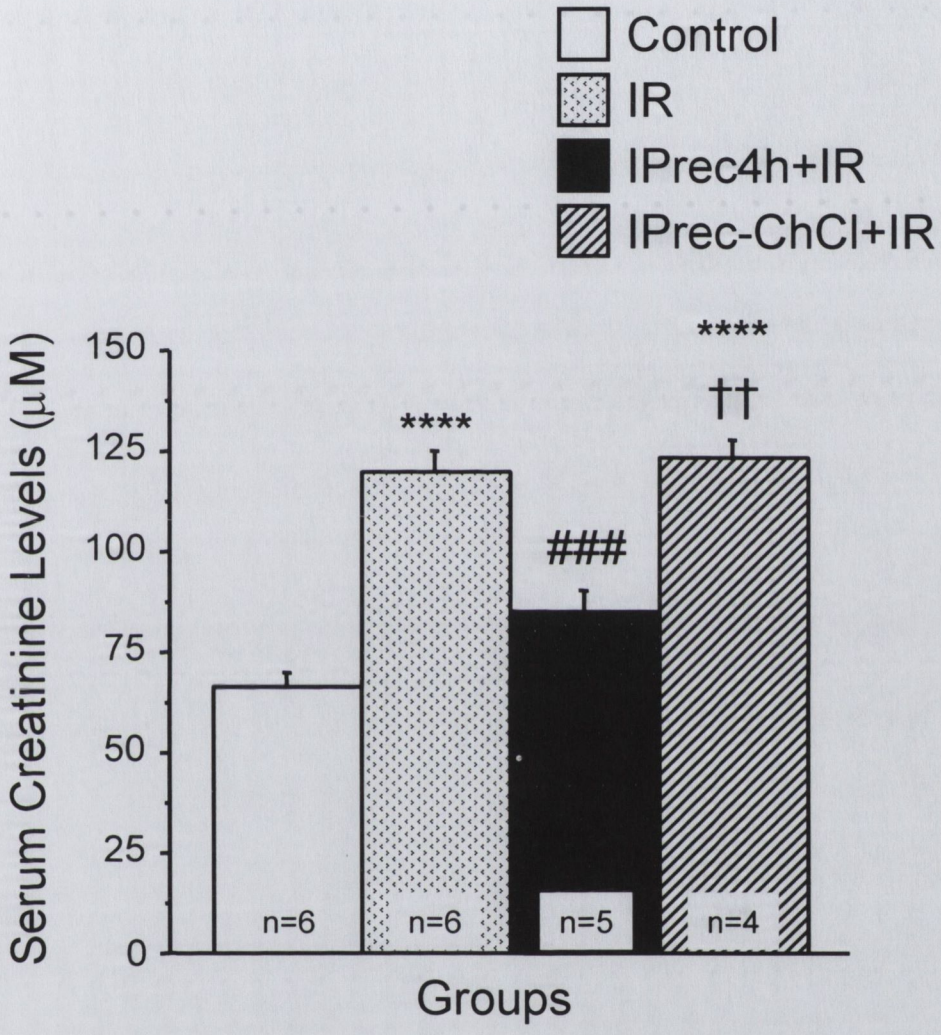


Figure 4.8 Serum creatinine levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h+IR and IPrec-ChCl+IR groups) before undergoing the IR injury. The IPrec-ChCl+IR group also received the PKC inhibitor chelerythrine chloride (5mg/kg i.p.) 15 min before preconditioning. Mean±SEM; ANOVA (Scheffe post-hoc test); **** $P<0.0001$ vs. control; ### $P<0.005$ vs. IR; †† $P<0.01$ vs. IPrec4h+IR.

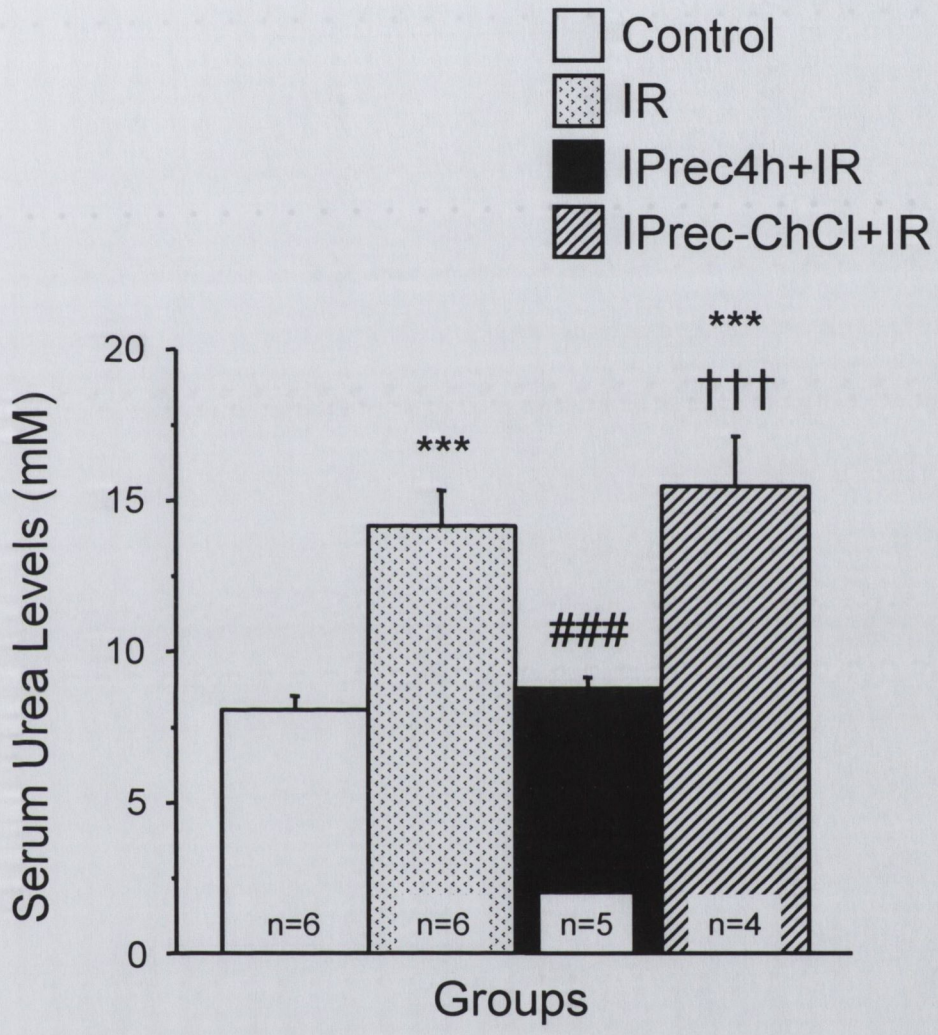


Figure 4.9 Serum urea levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h+IR and IPrec-ChCl+IR groups) before undergoing the IR injury. The IPrec-ChCl+IR group also received the PKC inhibitor chelerythrine chloride (5mg/kg i.p.) 15 min before preconditioning. Mean±SEM; ANOVA (Scheffe post-hoc test); *** $P<0.0005$ vs. control; ### $P<0.005$ vs. IR; ††† $P<0.005$ vs. IPrec4h+IR.

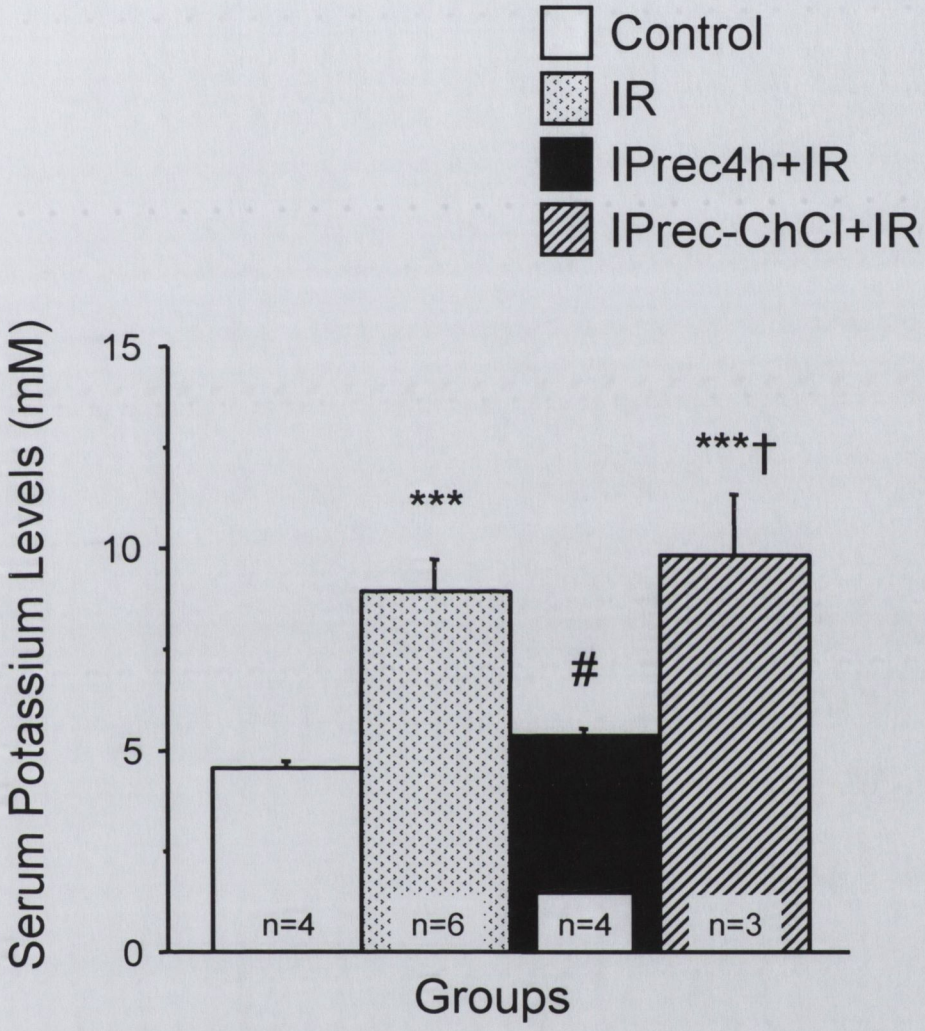


Figure 4.10 Serum potassium levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h+IR and IPrec-ChCl+IR groups) before undergoing the IR injury. The IPrec-ChCl+IR group also received the PKC inhibitor chelerythrine chloride (5mg/kg i.p.) 15 min before preconditioning. Mean±SEM; ANOVA (Scheffe post-hoc test); *** $P<0.0005$ vs. control; # $P<0.05$ vs. IR; † $P<0.05$ vs. IPrec4h+IR.

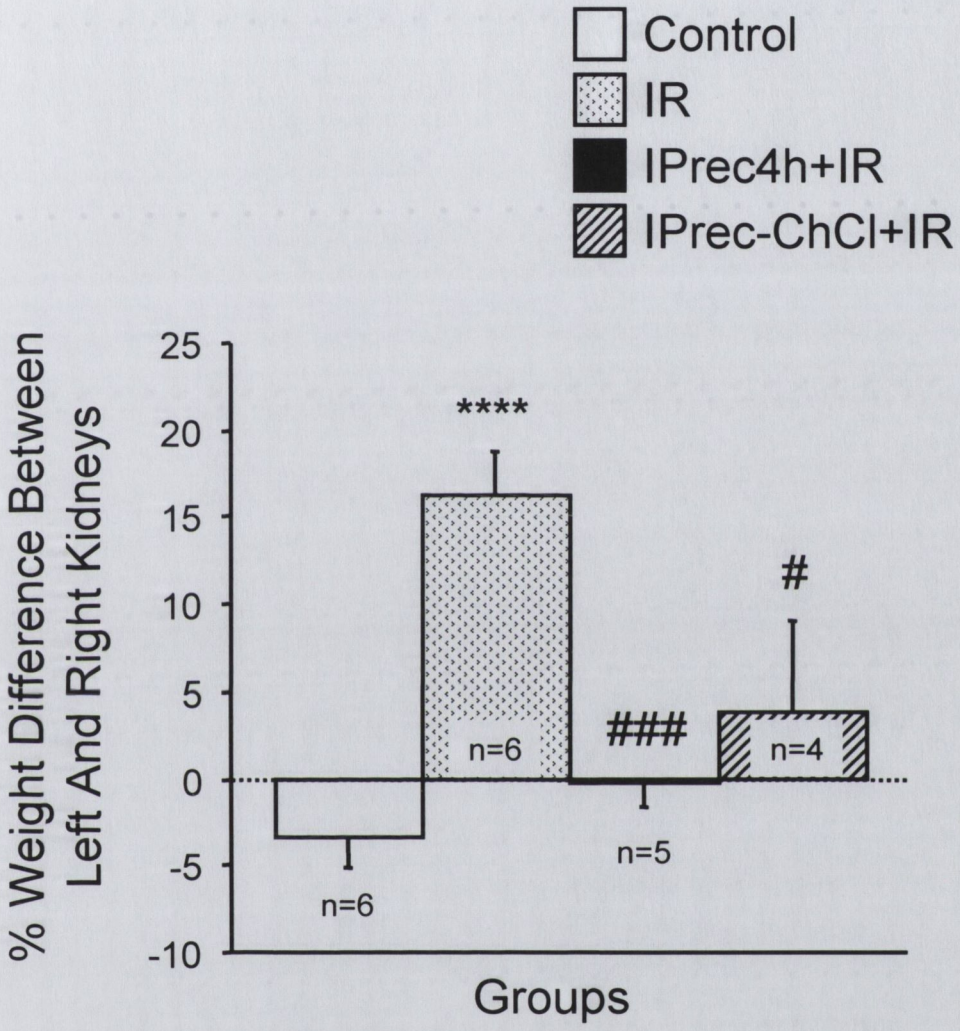


Figure 4.11 The % weight difference between right kidneys after 2 h reperfusion following sham ischaemia (control group) or 45 min ischaemia (IR groups) when compared with the left previously nephrectomised kidney, as an indicator of oedema. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h+IR and IPrec-ChCl+IR groups) before undergoing the IR injury. The IPrec-ChCl+IR group also received the PKC inhibitor chelerythrine chloride (5mg/kg i.p.) 15 min before preconditioning. Mean±SEM; ANOVA (Scheffe post-hoc test); **** $P<0.0001$ vs. control; # $P<0.05$ vs. IR; ### $P<0.005$ vs. IR.

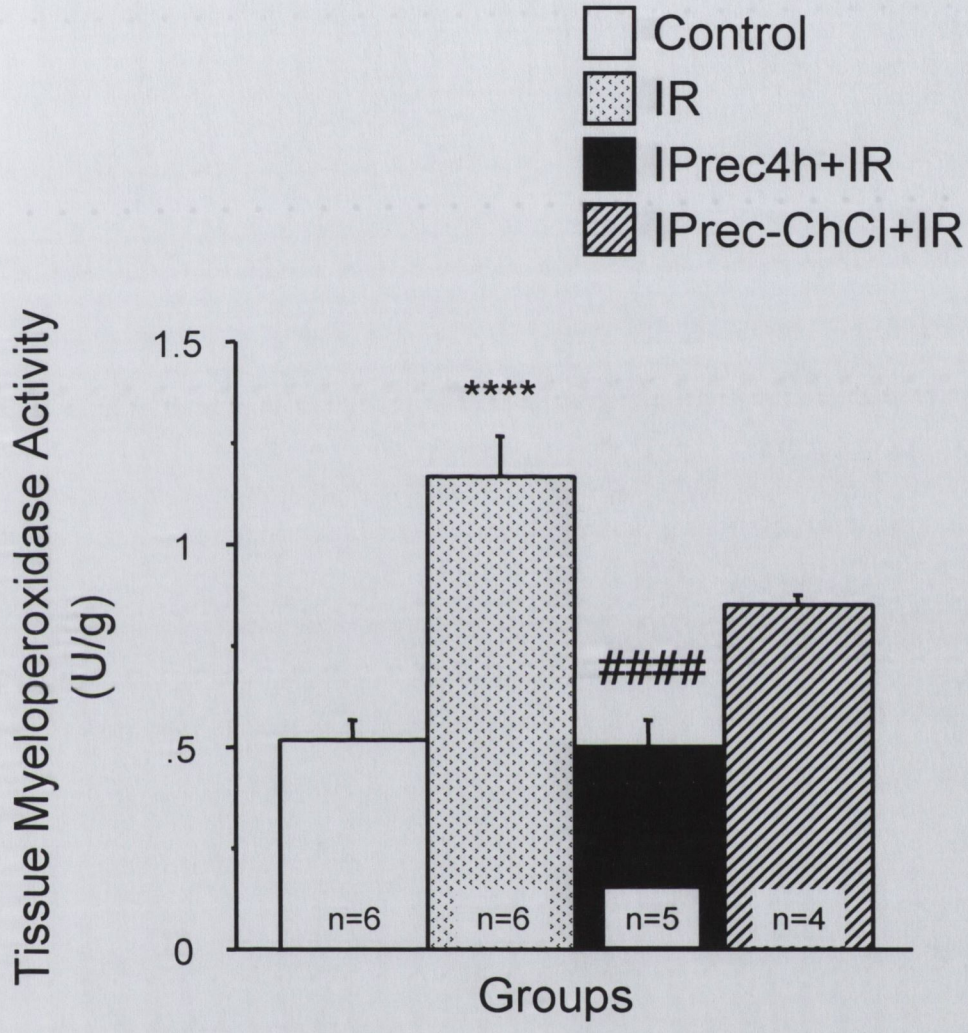


Figure 4.12 Renal myeloperoxidase levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney. Kidneys in two IR groups were pretreated with ischaemic preconditioning (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h+IR and IPrec-ChCl+IR groups) before undergoing the IR injury. The IPrec-ChCl+IR group also received the PKC inhibitor chelerythrine chloride (5mg/kg i.p.) 15 min before preconditioning. Mean±SEM; ANOVA (Scheffe post-hoc test); **** $P<0.0001$ vs. control; #### $P<0.0001$ vs. IR.

4.3.6 Heat Shock Protein 70 Expression

HSP70 expression was measured in kidneys from sham-IPrec, IPrec4h and IPrec-ChelCl groups where the right kidney was removed at the time the main IR insult would have been given, as well as control, IR and IPrec4h+IR animals where the right kidney was taken at the end of the full preconditioning and IR protocol (Figures 4.13a and 4.13b). HSP70 expression was induced above levels observed in control and sham-IPrec animals by IPrec followed by 4 h reperfusion (IPrec4h group). In addition, animals undergoing sham-IPrec followed by an IR insult (IR group) exhibited HSP70 induction, although these levels appeared to be slightly lower than those observed in the IPrec4h group (Figure 4.13a and 4.13b). HSP70 expression was elevated somewhat further in animals exposed to IPrec4h+IR (Figure 4.13a). Pre-treatment of animals undergoing IPrec4h with ChCl i.p. 15 min before preconditioning, demonstrated an attenuation of the induced HSP70 levels compared with IPrec4h kidneys (Figure 4.13b).

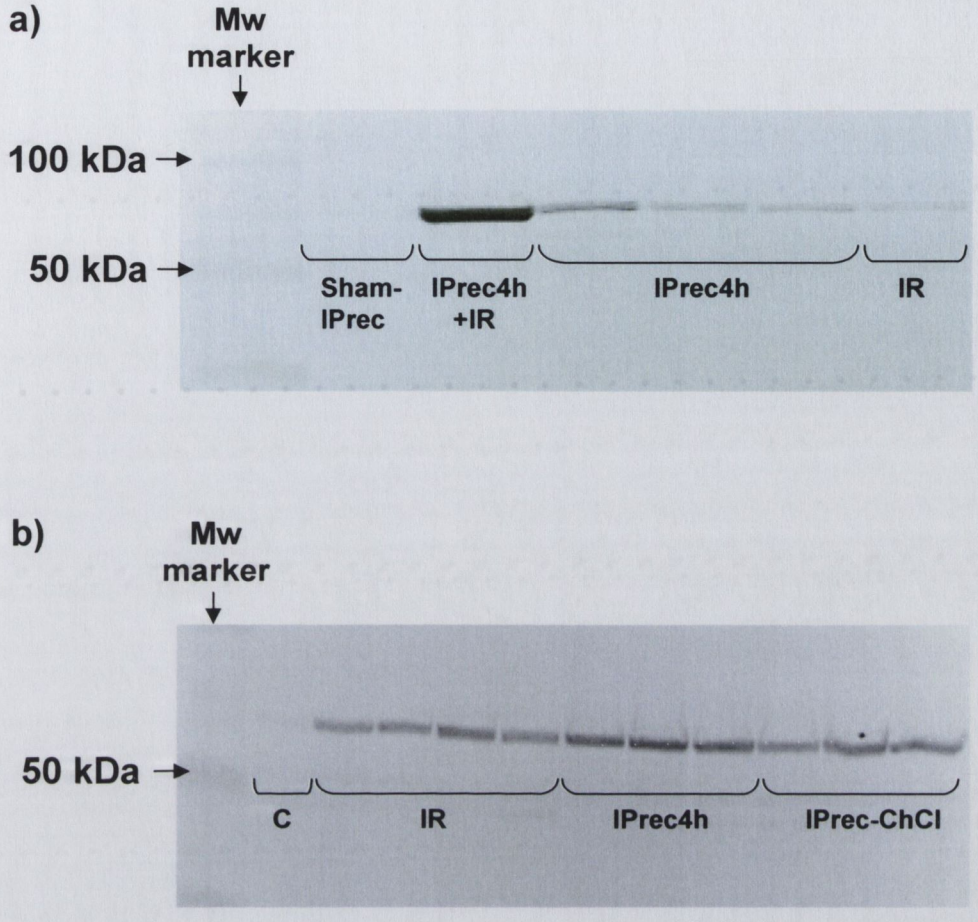


Figure 4.13 Representative western immunoblots for renal HSP70 levels measured before or after an ischaemia-reperfusion insult (each band represents a separate animal). Control animals underwent sham IR (C), and IR animals were subject to 45 min ischaemia and 2 h reperfusion of the right kidney. Separate groups of animals were pre-treated with IPrec (5 min ischaemia, 5 min reperfusion, 5 min ischaemia and 4 h reperfusion) (IPrec4h) after which right kidneys were immediately harvested (IPrec4h group) or were subject to IR (IPrec4h+IR group). The IPrec-ChCl group received the PKC inhibitor chelerythrine chloride (ChCl) (5mg/kg i.p.) 15 min before preconditioning, after which kidneys were harvested for HSP70 expression.

4.4 DISCUSSION

Due to its very nature, transplantation involves the stopping and restarting of blood flow to the grafted organ. However, the associated IR injury may itself contribute to allograft rejection. Preconditioning is whereby exposure to a sub-lethal stress paradoxically protects against a subsequent otherwise lethal stress. This study investigated a potential role for IPrec against IR-induced injury in the kidney. IR caused a significant renal functional impairment reflected by an increase in serum levels of creatinine, urea and K^+. Endothelial integrity was compromised as evidenced by an increase in neutrophil infiltration and marked organ oedema. Two cycles of 5 min ischaemia separated by 5 min reperfusion protected against this acute renal IR injury. However protection was not observed immediately, rather it took four hours after IPrec for this to develop at which time IR did not cause renal injury. The expression of HSP70 was induced within this time and was associated with the protection. Furthermore, both the IPrec-induced protection and HSP70 expression were partially inhibited by pre-treatment of the rats with the selective PKC inhibitor, ChCl.

The phenomenon of IPrec was first seen, although not fully appreciated, in 1971 by Maroko *et al.* It wasn't until 1986 that Murry *et al.* published a study recognizing the therapeutic potential of IPrec against myocardial IR injury in dogs. However, because of the conflicting results found between different IPrec protocols and species, the large body of the knowledge gained from animal studies is, to date, not directly applicable to the clinical arena. Despite this limitation, several studies have demonstrated that IPrec does exist in the human myocardium (Tomai *et al.*, 1999), justifying the investigation of the potential of this pre-treatment in other models. However, unlike the myocardium, a

bimodal pattern of protection has not been found to date in other organs following preconditioning. This study was designed to determine if the kidney could be preconditioned with ischaemia and to assess the ability of IPrec to confer long-lasting protection against subsequent IR injury in the kidney. To this end, a preconditioning protocol was decided based on studies in the heart, as no successful IPrec protocols had been published in the kidney at the time of experimentation. In the heart, a majority of groups have found that between one and three preconditioning cycles, each with between one and ten minutes of ischaemia and three to ten minutes of reperfusion as the preconditioning protocol, confer protection against subsequent IR injury. Taking into account this wide variety in protocols, the regime chosen for this study employed an average number of cycles as well as length of ischaemic and reperfusion episodes to precondition the kidney, i.e. two cycles of five minutes ischaemia and five minutes reperfusion.

Initial efforts to precondition the kidney with two IPrec cycles failed to attenuate renal IR injury. Previously Islam *et al.* (1997) had also found no protection against IR injury in the rat. This did not appear to be a species-specific finding as others published similar results in the pig (Behrends *et al.*, 2000) and the dog (Kosieradzki *et al.*, 2003). Due to an earlier study in the heart where the degree of protection was dependent on the number of ischaemic cycles used to precondition (Lawson *et al.*, 1993), the number of IPrec cycles in this model was increased to four (4XIPrec0h+IR group). However, if anything, this actually exacerbated the renal dysfunction caused by IR (Table 4.1). While it is not clear why this occurred, it may be simply a matter of inter-organ differences in the responses of the heart versus the kidney to cumulative cycles of preconditioning or perhaps the length of each cycle was too long to exert protection when applied four times before a 45 min

ischaemic insult. Therefore two cycles were used for the rest of the study. Islam *et al.* (1997) also did not observe an improvement in post-ischaemic renal function following four cycles of IPrec, although the length of the cycles were different and they allowed 30 min between IPrec and IR injury. However, more recently, there have been several reports that IPrec does exist in the rat kidney, and protects against subsequent IR injury. Furthermore, between one and four preconditioning cycles have been found to be effective, suggesting the number of cycles in the present study is not the reason for the absence of protection. The length of ischaemic episodes used to precondition the kidney here (i.e. five minutes) has also been used successfully in other studies (Riera *et al.*, 1999; Ogawa *et al.*, 2000). Likewise, in many of the studies 5 min appears to be a long enough reperfusion period to allow the preconditioning ischaemia to stimulate whatever mechanisms are responsible for the protection in other studies (Lee and Emala, 2000; Lee and Emala, 2001; Sugino *et al.*, 2001). Despite the above results, the most obvious explanation as to the discrepancy between these successful studies and findings here or in earlier renal IPrec studies remains the preconditioning protocols when considered in their entirety.

Other points of consideration are the injury parameters and experimental end-points used in studies where IPrec conferred protection against renal IR injury versus those where no amelioration of injury was found. Both Riera *et al.* (1999) and Lee and Emala (2000) demonstrated the existence of immediate protection by IPrec against subsequent IR injury in the kidney using serum creatinine levels as an indicator of renal dysfunction as was used here. Both of these groups used later reperfusion endpoints (i.e. 24 h up to 7 days reperfusion respectively) at which to assess injury following IR compared with that used in this study. However this is unlikely to be the explanation for the contrasting

observations. Others have found IPrec to prevent IR-induced renal dysfunction measured after just one hour reperfusion (Ogawa *et al.*, 2001). In fact, Sugino *et al.* (2001), using a protocol of two cycles of three minutes ischaemia and five minutes reperfusion, demonstrated that renal dysfunction was attenuated when measured at two hours reperfusion, the same end-point as this study. While most studies have focused on functional parameters, several studies have also shown that IPrec can attenuate morphological injury. Jefayri *et al.* (2000) observed immediate protection by IPrec against histological evidence of injury although renal dysfunction did not improve. Sugino *et al.* (2001) also found reduced morphological alterations after IPrec.

Despite the lack of protection found here if the IR injury was applied immediately following IPrec, increasing the recovery time between these two manipulations to two hours led to an approximately 50% reduction in IR-induced renal dysfunction as measured by serum creatinine, urea and K^+ levels. Furthermore the development of oedema was also prevented. However neutrophil infiltration into the post-ischaemic organ, was unchanged by IPrec two hours prior to IR injury. In the group where four hours of reperfusion was allowed between IPrec and IR (IPrec4h+IR group), there was complete attenuation not only of renal dysfunction as has been found in other IPrec studies, but also of oedema. A novel finding was that neutrophil infiltration of the kidney was also prevented. This is one of the earliest events that occurs following IR, and, surprisingly, has not been addressed in other IPrec studies in the kidney. There are several explanations as to why normal IR levels of MPO did not correspond to IR levels of dysfunction in the IPrec2h+IR group. First, the preconditioning in the IPrec2h+IR group may have caused a delay in the injury process, neutrophils had just infiltrated the tissue but dysfunction was only becoming apparent. Secondly, the preconditioning may had down-regulated an inflammatory

pathway induced by neutrophils after they get into the tissue, e.g. via production of antioxidants to counteract neutrophil-derived oxidants which could cause tissue damage. However, it appears that complete protection may require the prevention of neutrophil infiltration in the first place, which was evident in the IPrec4h+IR group. It has been previously demonstrated that *in vitro* preconditioning can attenuate IR-induced transcription factor activation, subsequent elevation of adhesion molecules on the vascular endothelium and the release of pro-inflammatory cytokines (Zahler *et al.*, 2000). This could act to prevent leucocyte infiltration, which would in turn reduce inflammatory mediator release and the resulting cascade of events that could eventually lead to the endothelial barrier being compromised culminating in oedema and tissue damage. This is supported in the IPrec4h+IR group by the attenuation not only of MPO levels and oedema, but also renal dysfunction that occurs following renal IR injury.

Emami *et al.* (1991) demonstrated that as little as 15 min of renal ischaemia induces HSP70 expression within 3 h of reperfusion. This induction was maximal after 60 min ischaemia and persisted for 5 days. Expression was strongest in the renal papilla, followed by the cortex and then the medulla. However, 15 min ischaemia is actually enough to cause mild morphological changes (Weight *et al.*, 1998), which would be undesirable as a preconditioning stimulus. Thus 15 min ischaemia was not used as the preconditioning regime here. Rather, two cycles of five minutes ischaemia separated by five minute reperfusion only conferred protection against subsequent renal IR injury when 4 h reperfusion had passed between the preconditioning protocol and the IR insult. This was enough time for HSP70 to be induced as measured by western immunoblotting. Thus this cytoprotectant protein was expressed by the time the kidney underwent 45 min of ischaemia and may have been involved in the subsequent reduction of MPO levels and

protection against renal dysfunction observed in preconditioned animals. In fact, it has been shown that leucocyte-endothelial interactions are attenuated in the microvasculature exposed to IR when hyperthermia was used to induce HSP70 prior to injury (Chen *et al.*, 1997) supporting the findings here that induction of HSP70 is associated with decreased neutrophil activity. Others have also shown that hyperthermia-induced attenuation of MPO levels in the post-ischaemic lung (Javadpour *et al.*, 1998) and leucocyte infiltration as well as oedema in the post-ischaemic liver (Terajima *et al.*, 2000) is associated with previous induction of HSP70 expression.

The activation of PKC has been shown to participate in IPrec in several organs. It appears to play a role in both the immediate and delayed protection observed in the heart (Mitchell *et al.*, 1995; Baines *et al.*, 2001), and has been implicated in IPrec in the kidney, albeit when the IPrec was performed immediately before IR (Lee and Emala, 2001). PKC has also been implicated in HSP70 induction following heat stress (Kim *et al.*, 1993). In the present study, ChCl, a commonly used potent selective PKC inhibitor, was used to block PKC activation during preconditioning with ischaemia. The inhibition of PKC activation attenuated the IPrec4h-induced protection against IR-induced renal dysfunction, but only partially reversed the reduction of oedema. Importantly, MPO levels were somewhat restored, although not fully. Perhaps IPrec was able to reduce MPO by attenuating adhesion molecule expression, in a PKC-dependent manner. This would be in agreement with Beauchamp *et al.* (1999) who demonstrated that the reduction of ICAM-1 levels on endothelial cells that had been preconditioned with hypoxia/reoxygenation could be blocked by inhibition of PKC.

Taken together, the results suggest that the IPrec mechanism is at least partially PKC-independent. A study by Fryer *et al.* (1999) in the heart, suggested that the mechanism of preconditioning differed depending on the number of ischaemic cycles used to precondition. They showed that ChCl completely inhibited protection conferred by one IPrec cycle but was only partially effective against three cycles of IPrec. However, this would be contradictory to findings by Lee and Emala (2001) in the kidney, where four cycles of preconditioning were used and ChCl (at the dose used here) fully prevented the protection found in the IPrec group. Interestingly, ChCl also partially blocked the HSP70 induction associated with IPrec. This supports a role for HSP70 in shielding the kidney from injury in this model and implicates PKC in part of the signal transduction pathway promoting induction of this protein. Perhaps IPrec activated one or more triggers such as adenosine, NO or oxygen free radicals which in turn activated more than one signalling pathway, PKC and perhaps tyrosine kinase leading to the induction of HSP70 and possibly other effector proteins. Alternatively, the dose of ChCl used in this study was too low to completely block the IPrec-induced protection and associated HSP70 induction, although this would be surprising as almost all studies that use this PKC inhibitor use the dose employed here (Kukreja *et al.*, 1999).

While HSP70 was one of the first proteins discovered to be associated with protection observed with the IPrec (Marber *et al.*, 1995), it is still uncertain as to its exact role, if any, in this protection in any organ (Baines *et al.,* 2001). However in the mouse, genetic overexpression of HSP70 confers protection against myocardial IR injury (Marber *et al.*, 1995; Hutter *et al.*, 1996). Similarly, in rat hearts transfected with HSP70 protection is observed against IR injury (Sakaguchi *et al.*, 2000; Suzuki *et al.*, 2000) or cold preservation injury (Jayakumar *et al.*, 2000), suggesting that HSP70 is capable of

protecting organs from injury either directly or indirectly, rather than simply being a marker of stress as has been suggested by others. Following a HSP-inducing stress HSF1, which normally exists as inactive monomers in the cytoplasm, is converted into homotrimers which translocate to the nucleus where they bind the consensus target sequence, the HSE, in the promoter region of the HSP genes. A study by Kim *et al.* (1993) suggested that simple binding of HSF to the HSE is not enough for transcriptional activation, but rather a modification to HSF, such as phosphorylation, may be required to stimulate transcription. They found that the PKC inhibitor, staurosporine, was capable of blocking both HSP27 and HSP70 transcription and suggested that this was probably due to the inhibition of HSF phosphorylation by this compound. This has been supported by other reports stating that transient activity of HSF is associated with phosphorylation. Recently Ping *et al.* (2001) further backed this argument by demonstrating that both HSP70 and HSP27 reside in the PKCε signalling complex in the myocardium. A role for PKC has been implicated in HSP70 induction (Kim *et al.*, 1993) and in both the early and late windows of IPrec (Mitchell *et al.*, 1995; Baines *et al.*, 2001). Furthermore, inhibiting PKC with ChCl not only blocks the protection offered by preconditioning with hyperthermia but also attenuates the associated induction of HSP70 in both an *in vivo* model of cardiac IR (Yamashita *et al.*, 1997) and an *in vitro* model of ischaemia-induced apoptosis in renal tubular cells (Meldrum *et al.*, 2001), thus supporting a role for PKC in the induction of HSP70.

The aim of this study was to assess the potential of IPrec to provide a long-lasting window of protection against subsequent IR injury in the kidney. It was found that IPrec is capable of protecting the kidney from subsequent IR-induced renal dysfunction and compromised endothelial integrity. However, contrary to findings by others, protection was not evident

immediately in the model described here rather it took 4 h for it to develop. This was associated with HSP70 induction, which appeared to be partly PKC-dependent. In light of the fact that a period long enough in which to induce HSP70 expression was required before protection was observed with this renal IPrec protocol, and the fact that IPrec is an invasive procedure, this limits its clinical value. Instead, further investigation includes assessing the ability of a pharmaceutical inducer of HSP70 to provide attenuate IR-induced renal injury in this model.

CHAPTER 5

The Effect Of Pharmacological Preconditioning With Sodium Arsenite On Renal Ischaemia-Reperfusion Injury

5.1 INTRODUCTION

Ischaemia-reperfusion injury is inevitable during transplantation but may actually contribute to the rejection process. Preconditioning is the phenomenon whereby a period of sub-lethal stress can protect against a period of subsequent otherwise lethal stress, for example IR. It was demonstrated in Chapter 4 that preconditioning with brief periods of sub-lethal ischaemia protected against subsequent IR-induced renal dysfunction, oedema and neutrophil infiltration. However, contrary to what has been demonstrated in other tissues, in particular in the heart, this protection was not evident immediately. Instead, it took several hours after the preconditioning stimulus for protection to develop. This was associated with HSP70 induction in the kidney 4 h following preconditioning. Due to the invasive nature of this procedure, it would not be clinically viable to subject a donor kidney to IPrec unless protection was evident immediately and lasted for several hours. However, there are numerous alternative means of inducing a stress response that are associated with HSP70 expression and which are capable of conferring protection against inflammatory conditions such as IR. Therefore, in light of the findings in Chapter 4, a non-invasive method of inducing the stress response or more particularly HSP70 expression was investigated here. The potential of the sodium salt of arsenous acid, sodium arsenite (SA), which is a chemical inducer of several HSPs including HSP70, to attenuate renal IR-induced injury was assessed in the rat model previously described in Chapter 3.

Sodium arsenite is a potent inducer of HSP70 in several organs including the kidney. Synthesis of a 74 kDa protein was induced in the rabbit kidney, heart and liver within an hour of an i.v. injection of SA. It was suggested that this was in fact HSP70 (Brown and Rush, 1984). Although HSP70 was not induced in the rabbit brain by SA, others have

since shown that i.p. administration of SA is capable of inducing HSP70 not only in the kidney, heart and liver of the rat but also in the brain (Yang *et al.*, 1998; Yang *et al.*, 1999). Furthermore, administration of SA via an i.v. route also caused increased expression of HSP70 in rat lungs (Ribeiro *et al.*, 1994), as well as the kidney. Renal HSP70 expression peaked between 6 and 24 h following SA injection and remained above baseline levels for over 72 h (Yang *et al.*, 2001). These studies suggest that HSP70 induction by SA may be species and organ specific and may depend on the route of administration. Arsenite is rapidly taken up by red blood cells and redistributed to other organs, in particular the kidney and liver (Haddad and Winchester, 1990). This may explain its ability to strongly induce HSP70 expression in these organs. SA is also capable of inducing the expression of other HSPs. Oral administration of SA results in the expression of HSP32 in the rat kidney and liver (Kitchin *et al.*, 1999). Furthermore, SA induces HSP expression in human cells. For example, human renal cells express HSP70 following treatment with SA, in addition to increased levels of HSP27, HSP60 and HSP90. In *in vitro* studies, human renal proximal tubules treated with SA for 4 h exhibited elevated HSP27, HSP60 and HSP70 expression, comparable to levels observed in cells treated with hyperthermia for 1 h (Somji, S., *et al.* 1999a; Somji, S., *et al.* 1999b; Somji, S., *et al.* 2000). In human endothelial cells incubated with SA, HSP70 is also induced to the same degree as in hyperthermia-treated endothelial cells (Wang *et al.*, 1995).

The induction of HSP70 expression by preconditioning with SA is associated with protection against a variety of inflammatory states, including IR (Tsuruma *et al.*, 1999) and sepsis (Hauser *et al.*, 2001). Ribeiro *et al.* (1994) and Villar *et al.* (1994) showed that not only did SA and hyperthermia protect rats against sepsis-induced mortality, but that this was protection was proportional to the induction of HSP70 in the lungs. In addition to

the HSP70 induction prior to the application of the injury in these models, several inflammatory pathways are disrupted by SA administration. For instance, SA-induced HSP70 expression has also been associated with reduced transcription factor activation following TNF-α treatment (Yoo *et al.*, 2000), and decreased neutrophil-induced endothelial cell necrosis (Wang *et al.*, 1995). IR-induced elevation of TNF-α and the post-ischaemic tissue infiltration of neutrophils were attenuated by i.v. administration of 6 mg/kg SA (Tsuruma *et al.*, 1999). Not only was HSP70 induced before IR, but levels of the anti-inflammatory cytokine, interleukin-10 were raised in plasma following the injury. Blocking HSP70 induction with the bioflavanoid quercetin prevented these protective responses (Tsuruma *et al.*, 1999). This suggested there was a causal relationship between HSP70 induction by SA and the subsequent anti-inflammatory effects. Others have demonstrated that mice transgenic for HSP70 expression (Marber *et al.*, 1995; Hutter *et al.*, 1996) or rats transfected with HSP70 (Sakaguchi *et al.*, 2000; Suzuki *et al.*, 2000) are protected against IR injury, strongly supporting the potential of HSP70 to mediate protection.

Thus this study addresses whether preconditioning with SA is as effective as IPrec at protecting against the model of IR-induced renal dysfunction and associated oedema described in Chapter 3. In addition, it was investigated whether the observed response was associated with altered HSP70 levels in the kidney at the time-point the IR insult commenced.

5.2 METHODS

5.2.1 Sodium Arsenite Preconditioning

Twenty-seven male Sprague-Dawley rats weighing 300-350 g were randomised into four groups:

Control (n=6): Saline i.v. 18 h before sham IR.

IR (n=10): Saline i.v. 18 h before IR.

SA-6+IR (n=7): 6 mg/kg sodium arsenite i.v. 18 h before IR.

SA-10+IR (n=4): 10 mg/kg sodium arsenite i.v. 18 h before IR.

In order to administer the saline or SA to these groups, animals were anaesthetised using inhalational halothane. Saline or SA was injected into the penile vein in a volume of 0.5 ml. The doses of SA and the route of administration were based on a study by Ribeiro *et al.* (1994) where 6 mg/kg SA via the penile vein induced HSP70 expression in the lung. This peaked between 6 and 24 h after injection and remained above control levels for 48 h. The maximum dose used in that study was 10 mg/kg, which induced a stronger expression of HSP70 but was associated with, 20% mortality. Also based on this study, saline administration was used in control animals here. Animals were allowed to recover for 18 h before undergoing sham ischaemia or ischaemia (Figure 5.1) (this recovery period permitted time for HSP70 induction and allowed for the lungs to be taken as positive controls for HSP70 induction based on Ribeiros findings). Since the work for this chapter was completed, Yang *et al.* (2001) demonstrated that 6 mg/kg SA injected via the penile vein also induced HSP70 expression in the rat kidney, which peaked between 6 and 24 h

following administration. Thus, in this chapter, animals underwent IR 18 h after receiving SA, at a time when renal HSP70 expression should have been maximal.

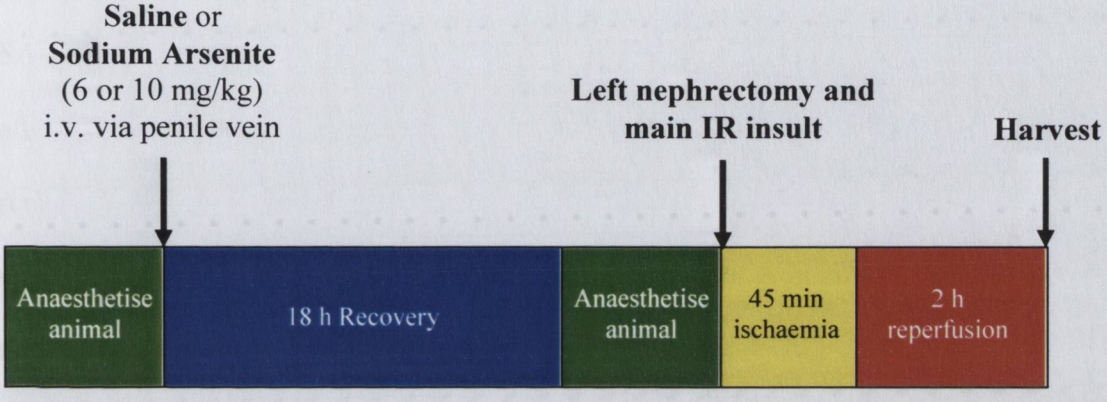


Figure 5.1 Time-course of preconditioning with sodium arsenite followed by a renal IR insult 18 h later.

5.2.2 Ischaemia-Reperfusion Injury

Following saline or SA administration, animals were allowed to recover for 17 h 30 min before being re-anaesthetized to undergo surgery (Figure 5.1). This allowed 30 min to prepare the animal such that the injury (ischaemia) could be applied exactly 18 h following SA administration. Briefly, the internal jugular vein was cannulated for administration of heparinised saline, and a left nephrectomy was performed in all four groups. The IR, SA-6+IR and SA-10+IR groups were subjected to 45 min ischaemia and 2 h reperfusion of the right kidney. In the control group the right kidney was mobilized (see section 2.1.2 for a detailed description). After 2 h of reperfusion or sham-reperfusion, urine was taken for measurement of U/O, blood was drawn for measurement of serum creatinine, urea and K^+ levels, the right kidney was harvested for assessment of oedema (as described in sections 2.2 and 2.3) and the animals were then sacrificed.

5.2.3 Heat Shock Protein 70 Expression

After establishing the effect of SA on renal IR-induced dysfunction and oedema, 12 animals were randomized into three groups and pre-treated with saline (SA-0), 6 mg/kg SA (SA-6) or 10 mg/kg SA (SA-10) as described above. Animals were re-anaesthetised after 17 h 45 min and the lungs, liver and right kidneys were removed. Excess tissue was removed and a piece of each organ was snap-frozen and stored at -80°C for subsequent assessment of HSP70 protein content using western immunoblotting as described in section 2.6.

5.2.4 Statistics

All data is expressed as mean±SEM. ANOVA with Scheffe post-hoc test was performed on all parameters to compare between groups.

5.3 RESULTS

5.3.1 Effect Of Ischaemia-Reperfusion On Renal Function

Ischaemia and subsequent reperfusion caused significant renal functional impairment compared with controls, as measured by serum creatinine, urea and K^+ levels and U/O. Figures 5.2 and 5.3 demonstrate that serum creatinine and urea levels increased significantly from control levels of 57.8±1.89 μM and 8.0±0.33 mM to IR values of 113.4±8.05 μM ($P<0.0001$ versus control) and 10.9±0.71 mM ($P<0.05$ versus control) respectively. Serum K^+ levels were elevated from 6.6±0.50 mM in controls to 9.0±0.66 mM by IR ($P<0.05$) (Figure 5.4). Urine output decreased from 1.3±0.10 ml in control animals to 0.2±0.07 ml in the IR group ($P<0.001$) (Figure 5.5).

5.3.2 Effect Of Sodium Arsenite Preconditioning On Ischaemia-Reperfusion-Induced Renal Dysfunction

Serum creatinine remained elevated in both SA-treated groups versus control levels ($P<0.0001$ for the SA-6+IR and SA-10+IR versus control), but comparable to levels in the IR group (Figure 5.2). While it appeared that serum urea and K^+ levels remained somewhat elevated above control values in both SA-treated IR groups, there were no significant differences between these levels and the control or IR groups (Figures 5.3 and 5.4). Urine output in the SA-6+IR group remained significantly depressed at 0.5±0.18 ml compared with control levels ($P<0.01$) (Figure 5.5). After dose of SA was increased to 10 mg/kg U/O was also significantly lower than levels in the control group ($P<0.05$).

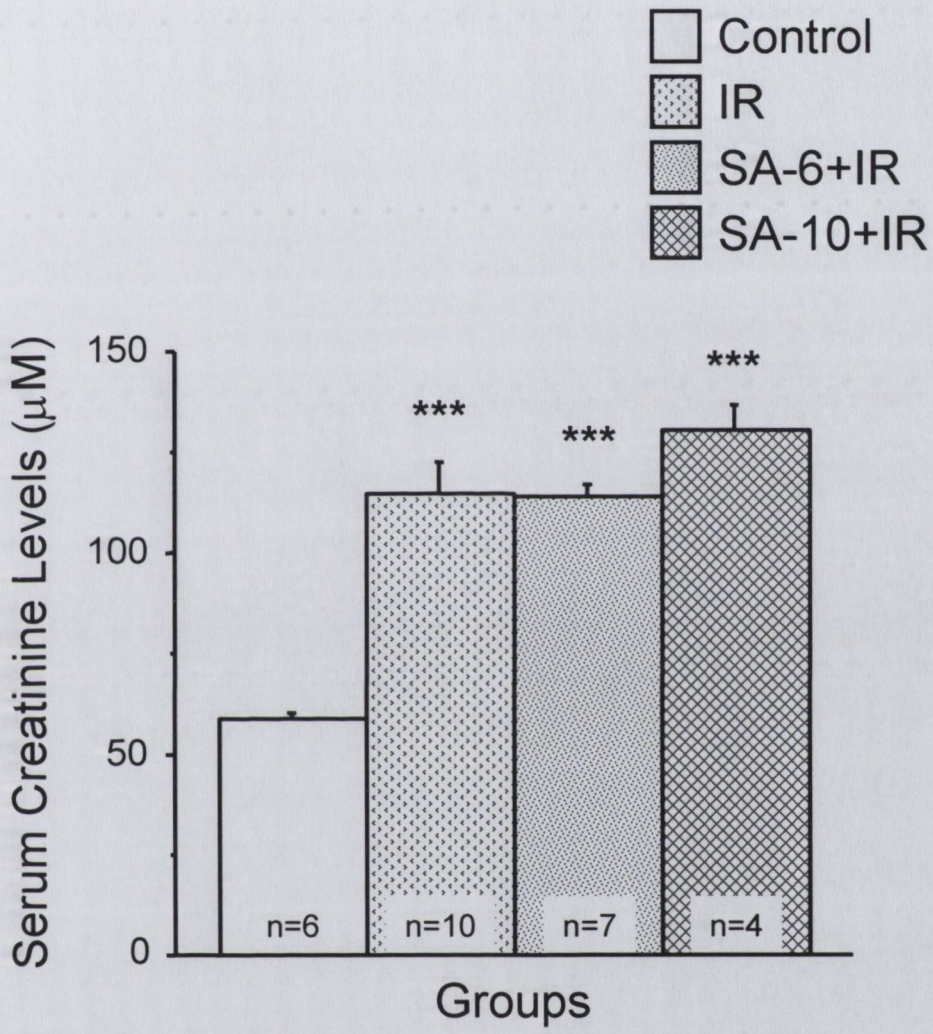


Figure 5.2 Serum creatinine levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney of saline-treated rats. Two separate IR groups received 6 mg/kg (SA-6+IR group) or 10 mg/kg (SA-10+IR group) sodium arsenite via the penile vein 18 h pre-ischaemia. Mean±SEM; ANOVA (Scheffe post-hoc test); *** $P<0.0001$ vs. control.

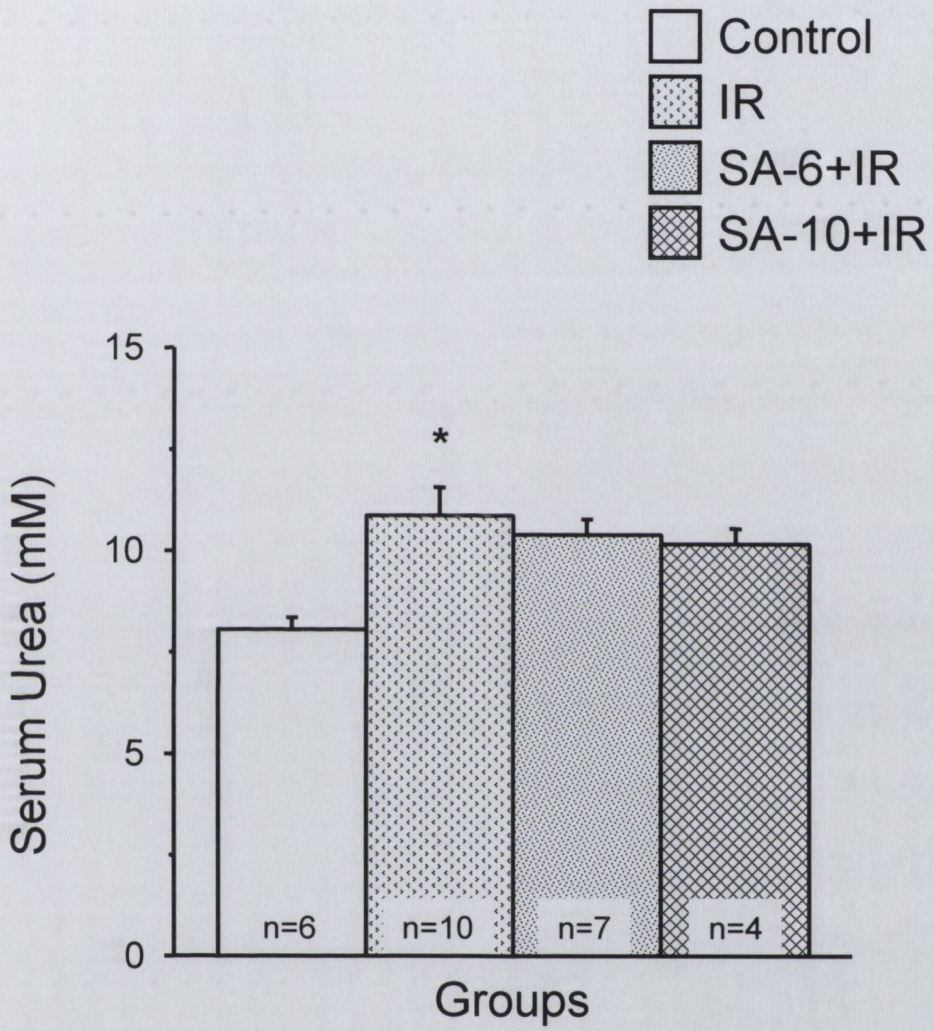


Figure 5.3 Serum urea levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney of saline-treated rats. Two separate IR groups received 6 mg/kg (SA-6+IR group) or 10 mg/kg (SA-10+IR group) sodium arsenite via the penile vein 18 h pre-ischaemia. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.05$ vs. control.

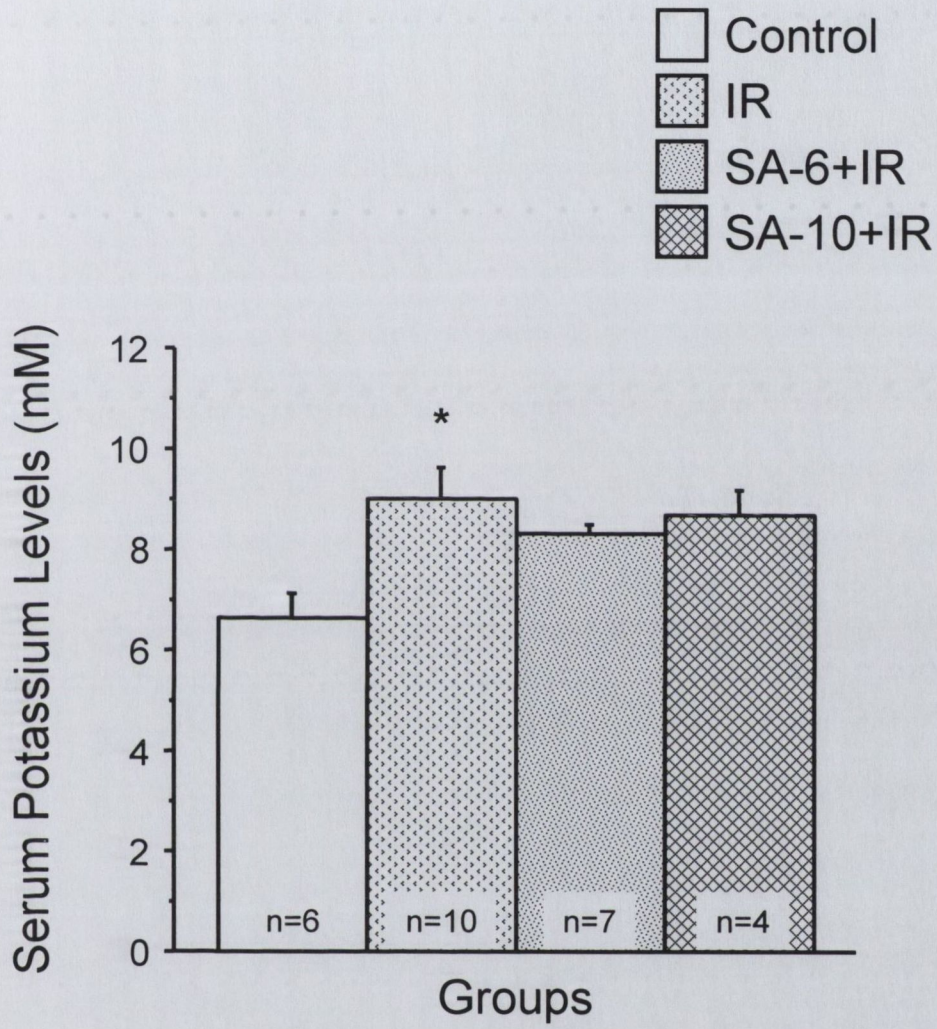


Figure 5.4 Serum potassium levels measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR groups) of the right kidney of saline-treated rats. Two separate IR groups received 6 mg/kg (SA-6+IR group) or 10 mg/kg (SA-10+IR group) sodium arsenite via the penile vein 18 h pre-ischaemia. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.05$ vs. control.

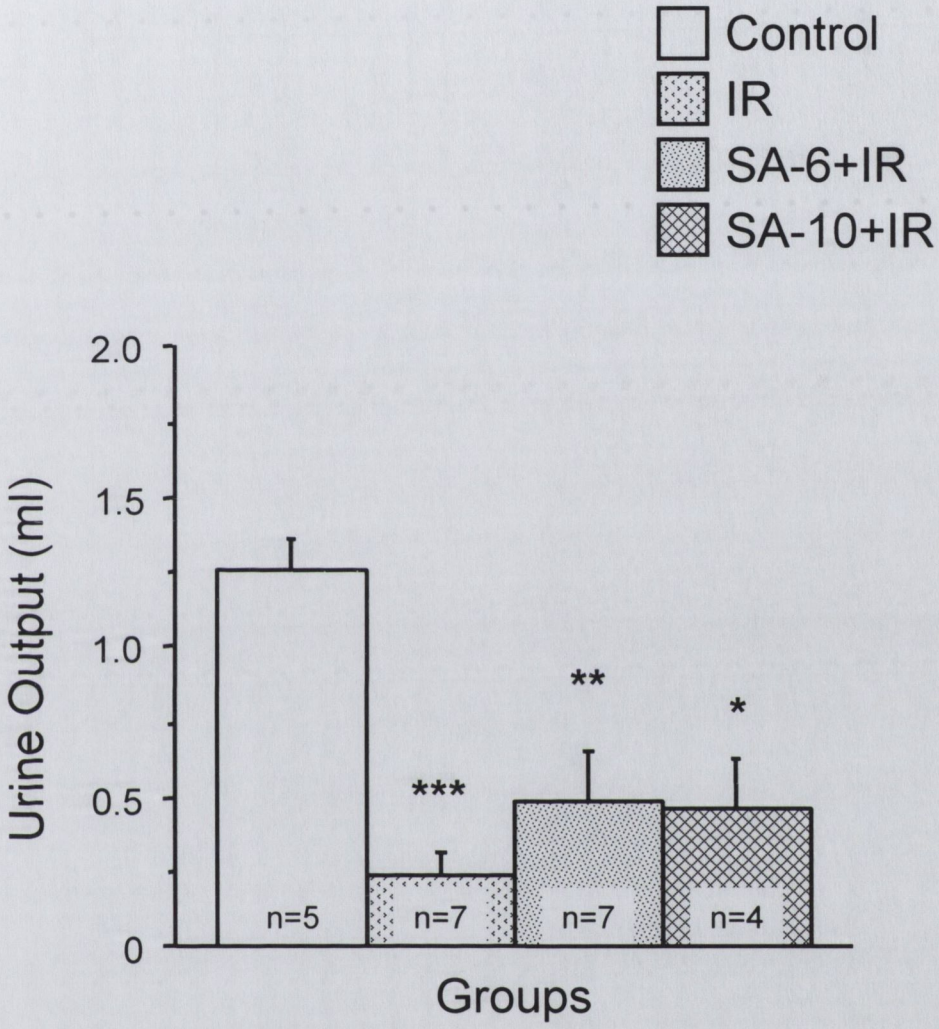


Figure 5.5 Urine output over 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney of saline-treated rats. Two separate IR groups received 6 mg/kg (SA-6+IR group) or 10 mg/kg (SA-10+IR group) sodium arsenite via the penile vein 18 h pre-ischaemia. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.05$ vs. control; ** $P<0.01$ vs. control; *** $P<0.001$ vs.control.

5.3.3 Effect Of Ischaemia-Reperfusion On Endothelial Permeability

Organ oedema was evident in kidneys exposed to IR when compared with sham-operated kidneys (Figure 5.6). The percentage weight difference between left and right kidneys was very low in the control group (% difference = 2.0±2.37 %). However in the IR group the right kidney was 32.5±4.15% heavier than the left kidney, showing that there was an accumulation of fluid in the organ subjected to IR ($P<0.05$ versus control).

5.3.4 Effect Of Sodium Arsenite Pre-Treatment On Ischaemia-Reperfusion-Induced Endothelial Permeability

Preconditioning with 6 mg/kg or 10 mg/kg SA failed to prevent oedema that developed following renal IR injury (Figure 5.6). The percentage weight differences between the left and right kidneys were significantly higher than those seen in control groups, at 35.2±8.12% in the SA-6+IR ($P<0.005$ versus control) and 36.6±3.61% in the SA-10+IR group ($P<0.01$ versus control).

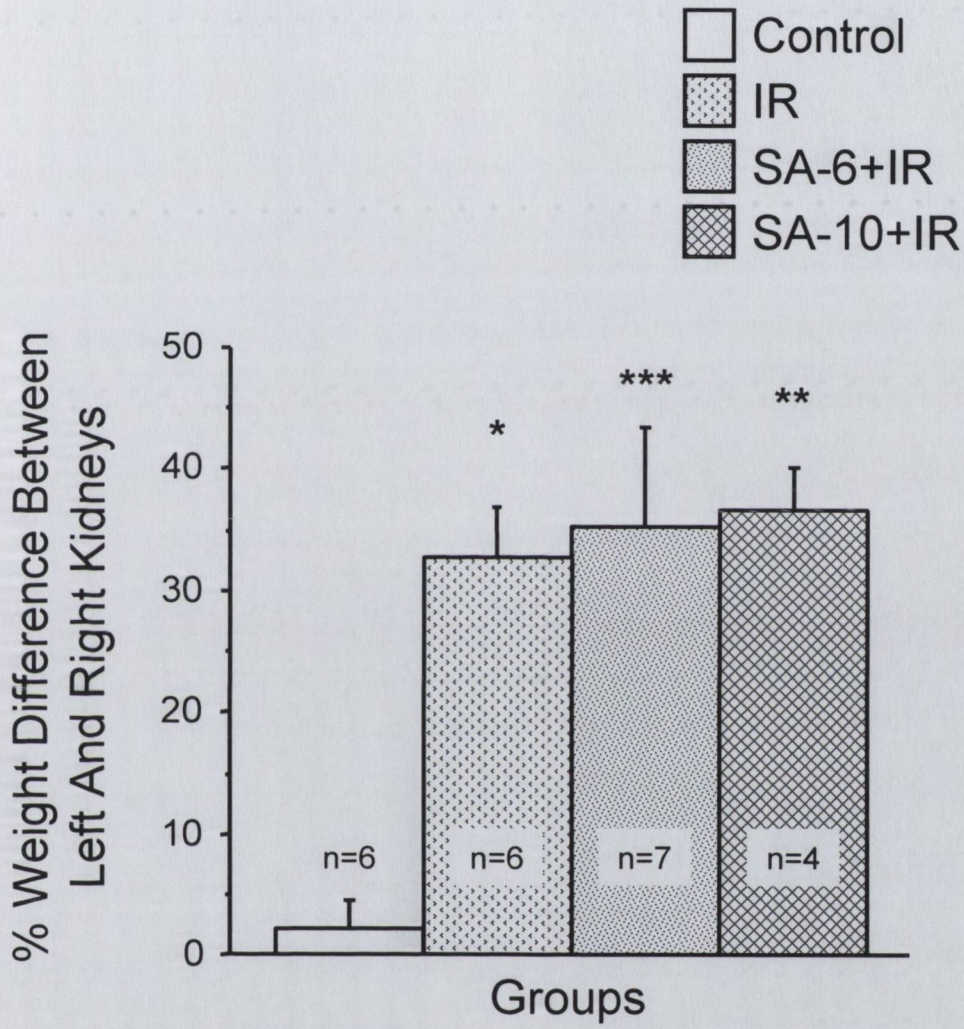


Figure 5.6 Renal oedema measured at 2 h reperfusion following left nephrectomy and sham ischaemia (control group) or 45 min ischaemia (IR group) of the right kidney of saline-treated rats. Two separate IR groups received 6 mg/kg (SA-6+IR group) or 10 mg/kg (SA-10+IR group) sodium arsenite via the penile vein 18 h pre-ischaemia. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.05$ vs. control; ** $P<0.01$ vs. control; *** $P<0.005$ vs. control.

5.3.5 Heat Shock Protein 70 Expression

Kidneys that were subjected to preconditioning with either of the doses of SA and removed before any IR insult exhibited no HSP70 expression (Figure 5.7a and 5.7b). A similar lack of response to SA was observed in the liver of these animals. In contrast to these observations, the lungs of these same animals exhibited a dose-dependent induction of HSP70 induction by SA compared with saline-treated animals (Figure 5.7a and 5.7b).

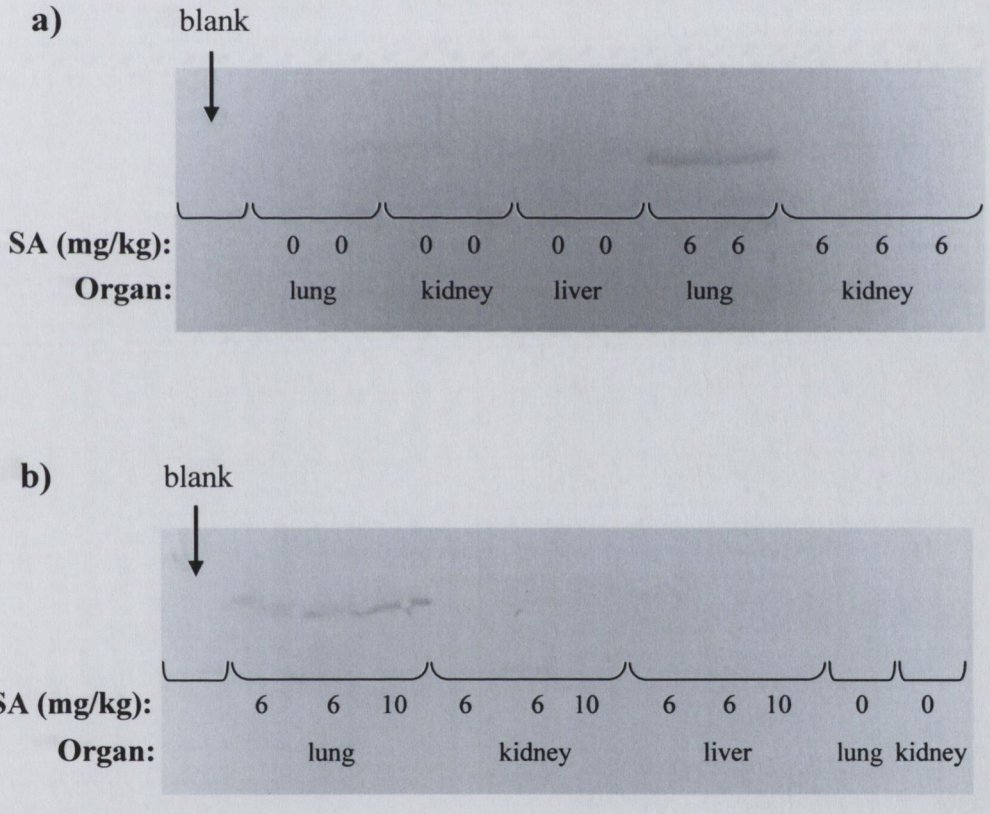


Figure 5.7a and b Representative western immunoblots for HSP70 expression in right kidneys, lungs and livers (each band represents a separate sample). Organs were removed 18 h after administration of 0 mg/kg, 6 mg/kg or 10 mg/kg sodium arsenite (SA) in saline via the penile vein for assessment of HSP70 induction.

5.4 Discussion

Preconditioning of an organ before transplantation provides an opportunity to prevent an inflammatory response to the inevitable subsequent IR insult. In chapter 4, preconditioning with short episodes of ischaemia protected against IR-induced renal dysfunction in association with the induction of the cytoprotectant protein HSP70. This study investigated the potential of a chemical inducer of HSP70 expression, SA, to similarly protect renal tissue against IR-induced injury. However, the administration of 6 mg/kg SA 18 h prior to ischaemia failed to confer any protection against renal IR-induced dysfunction or oedema. Furthermore, while HSP70 induction was observed in the lungs, implying that the SA was introduced into the circulation, neither the kidney nor the liver expressed any HSP70. This absence of HSP70 induction in the kidney may help explain the lack of protection against renal IR injury in animals preconditioned with SA. Raising the dose of SA to 10 mg/kg did not improve the renal dysfunction found following IR injury. A further increase of the SA dose was avoided as 10 mg/kg has previously been shown to be toxic to Sprague-Dawley rats (Ribeiro *et al.*, 1994).

As early as 1984, Brown and Rush (1984) reported that an i.v. injection of SA into rabbits resulted in synthesis of a 74kDa protein in the kidney, heart and liver similar to that induced by hyperthermia, suggesting that this was in fact HSP70. This protein was not induced in the brain, suggesting organ specificity. However, others have since shown that SA i.p. is capable of inducing HSP70 not only in the rat kidney, heart and liver but also in the rat brain (Yang *et al.* 1999; Yang *et al.* 1998) suggesting that the species or the route of administration may influence the induction of HSP70. Others have not only confirmed HSP70 induction in renal cells after treatment with SA, but it has been demonstrated that

SA induces other HSPs including HSP27, HSP32, HSP60 and HSP90. In the rat, oral administration of SA resulted in the expression of HSP32 in the kidney and liver (Kitchin *et al.*, 1999). In *in vitro* studies, human proximal tubules treated with SA for 4 h exhibited increased HSP27, HSP60 and HSP70 expression, similar to observations in cells treated with hyperthermia for 1 h (Somji, S., *et al.* 1999a; Somji, S., *et al.* 1999b; Somji, S., *et al.* 2000). Darasch *et al.* (1988) showed that both heat treatment and SA induced HSP90 in A6 kidney epithelial cells. From these studies, it would appear that kidney cells are quite susceptible to the induction of a stress response by SA.

While very few studies have incorporated SA as a protective agent *in vivo*, the protocol in the present study was based on two studies where induction of HSP70 by SA coincided with the attenuation of subsequent injury in Sprague-Dawley rats. In the small intestine, IR injury was prevented by the administration of 6 mg/kg SA i.v. 24 h prior to ischaemia (Tsuruma *et al.*, 1999). The accompanying neutrophil infiltration and pro-inflammatory mediator release were reduced by SA. This protection was associated with the induction of HSP70 expression in this organ. Ribeiro *et al.* (1994) also gave the non-lethal dose of 6 mg/kg SA i.v. via the penile vein. This resulted in the induction of HSP70 expression in the lungs which peaked between 9 h and 24 h and remained above control levels for 48 h following SA administration. When caecal ligation and puncture was performed 18 h following injection of SA, protection against sepsis-induced mortality was demonstrated. While 4 mg/kg SA was the minimum required dose to induce HSP70 in the lungs, increasing the dose of SA to 10 mg/kg resulted in an increase of HSP70 expression beyond that observed with 6 mg/kg. However at this highest dose, animals appeared lethargic and anorectic, which was associated with, 20% mortality. Thus 6 mg/kg SA was administered

i.v. 18 h prior to the renal IR insult in this chapter, with the hypothesis that HSP70 would be induced in the kidney.

However, HSP70 expression was not found in kidneys of SA-treated rats here. This was an unexpected finding, especially considering the subsequent publication of the study by Yang *et al.* (2001), where a similar injection of 6 mg/kg SA into the penile vein of Sprague-Dawley rats induced HSP70 expression in renal tubular epithelium. This resulted in a time-course of HSP70 expression comparable to that in the lungs in the study by Ribeiro *et al.* (1994). The contrasting observations of Yang *et al.* (2001) and this study cannot be explained by the preparation or route of administration of the SA, which were both similar in both studies, i.e. the SA was dissolved in saline and injected via the penile vein. In fact, the finding that HSP70 was induced in the lungs suggests that SA did enter the circulation. Sprague-Dawley rats were used both by Yang *et al.* (2001) and in this chapter, thus the differences in findings are not species- or strain-related. One discrepancy between the two studies is that Yang *et al.* used an antibody against HSP70 that appeared to stain for both the constitutive form of HSP70 as well as the inducible form. However since there was a very obvious increase in the levels of HSP70 following SA in that study, the inducible form of HSP70 was likely to have been expressed. The antibody used here was used in Ribeiros study in the rat lungs where a similar dose of SA induced HSP70 expression, as was seen in the current study. Furthermore, this antibody not only revealed HSP70 in the lungs in this study but also detected HSP70 induction in the kidney by IPrec (Chapter 4). Raising the dose of SA to 10 mg/kg, a potentially toxic dose enhanced HSP70 induction in the lungs beyond levels obtained with 6 mg/kg SA. However, a complete absence of HSP70 expression remained in both the kidney and liver with this higher dose of SA.

The lack of HSP70 expression in kidneys of rats treated with SA was an unexpected finding. However, in light of the fact that HSP70 is associated with SA-induced protection in other injury models (Ribeiro *et al.*, 1994; Tsuruma *et al.*, 1999), the corresponding absence of protection in the renal IR injury described here was not surprising. Renal dysfunction and oedema in SA-treated groups were comparable to saline-treated animals. Conversely, the recent study by Yang *et al.* (2001) in a rat kidney model of IR that is largely similar to the one used here demonstrated that pre-treatment of rats with 6 mg/kg SA i.v. 12 h before the IR insult protected against IR-induced apoptosis and renal dysfunction as well as histopathological damage. However, as discussed above, this protection was associated with HSP70 induction. While Yang *et al.* applied the ischaemic insult 12 h following SA administration whereas 18 h recovery was allowed in the present study, peak levels of HSP70 are observed in kidneys at least up to 24 h following SA in Yang's study, thus it is unlikely that the window of HSP70 induction had passed before IR was performed in our model.

Interestingly, it has been proposed that SA may also protect against inflammation in a HSP70-independent manner through the stabilization of IκBα, thereby preventing NF-κB activation (Hershko *et al.*, 2002). This would inhibit subsequent expression of inflammatory mediators and cell adhesion molecules which could promote tissue injury. However, this does not appear to be the case here. Rather it is likely that the findings in this chapter are in agreement with a majority of studies where SA-mediated protection against injuries such as sepsis and IR is in fact associated with the induction of HSPs. In rats, the protection conferred by SA administration 16 h before heatstroke-induced cerebral ischaemia was associated with HSP70 induction in the brain (Yang and Lin, 1999). It was demonstrated that HSP70 expression was also induced in the heart, liver and

kidney at this time. Induction of HSP70 on endothelial cells by sub-lethal heat or SA treatment prevents subsequent cell necrosis caused by neutrophils (Wang *et al.*, 1995). Tsuruma *et al.* (1999) demonstrated that pre-treatment with SA not only decreased intestinal IR-induced inflammatory cytokine production but also attenuated IR-induced elevation of neutrophil chemoattraction and MPO levels. In fact, blocking HSP70 induction using quercetin also prevented the reduction of IR injury by SA in that model. Thus the initiation of the heat shock response by SA may prevent at least the early phase of IR injury mediated by neutrophil-endothelial interactions, which may in turn attenuate subsequent steps in the inflammatory cascade which lead to tissue injury. Very little information is available as to the exact mechanism through which SA induces HSP expression. However, Chen *et al.* (2001) showed that SA increases HSF binding to HSE and induces HSF1 di- or trimerization leading to HSP70 induction, suggesting that SA induces HSP70 expression at the transcriptional level. This was inhibited by anti-oxidant treatment suggesting that SA acts as a pro-oxidant, an observation supported by Ozaki *et al.* (2000) who found that SA caused increased production of intracellular reactive oxygen species which in turn activated HSF1. Taken together, SA may act via an oxidant stress to promote HSP70 expression.

While the use of a pharmacological agent such as SA provided an attractive approach to induce HSP70, this study did not find protection at non-toxic levels of SA. In addition, SA exhibited organ specificity in its ability to induce HSP70 expression. Therefore, the use of such an agent would be limited in clinical practice. Interestingly, with relevance to the transplantation scenario, Tarumi *et al.* (1998) demonstrated that HSP70 induction by SA was associated with both reduced cold preservation injury and increased graft survival in a small bowel model of transplantation. Others have found that SA not only prevents renal

IR injury, but also attenuates kidney damage following CsA administration, an immunosuppressant commonly used in the transplantation arena. This was also associated with HSP70 induction. Perhaps stimulation of the stress response prior to organ implantation would not only help prevent DGF or acute rejection due to IR injury but may also protect the organ from CsA-mediated toxicity, either allowing a higher dose of immunosuppressants to be used or decreasing the level of immunosuppression required. Thus an alternative method of preconditioning, e.g. hyperthermia, that induces HSPs and exhibits consistency between organs and species would perhaps provide a more useful research approach in the search for a tool to prevent IR injury in human transplantation.

CHAPTER 6

The Effect of Hyperthermic Preconditioning in Renal Ischaemia-Reperfusion-Induced Renal Dysfunction and Associated ICAM-1 and MHC II Expression

6.1 INTRODUCTION

During transplantation lengthy ischaemic times may promote DGF thereby reducing long-term prognosis (Gonzalez-Segura *et al.*, 1998). IR injury may increase acute and/or chronic allograft rejection by impacting both alloimmune-independent inflammatory responses such as tissue leucocyte infiltration as well as alloimmune-dependent processes by increasing graft immunogenicity. Thus, this chapter not only evaluates renal morphology and function but also the effect of IR on kidney ICAM-1 and MHC II expression. Preconditioning induces a universal defence mechanism or stress response, whereby cells exposed to a minor stress, such as ischaemia or hyperthermia acquire protection from subsequent oxidative stress. In agreement with other studies, findings from chapters 4 (IPrec) and 5 (SA preconditioning) suggest that induction of HSPs (in particular HSP70) may mediate the protection conferred by preconditioning against subsequent renal IR injury. This chapter investigates the efficacy of the classical inducer of HSPs, namely hyperthermia, to protect the kidney against IR-induced dysfunction during the first 5 days of reperfusion. Furthermore, the impact of this method of preconditioning on IR-induced adhesion molecule expression and tissue antigenicity is assessed.

In the rat kidney, Emami *et al.* (1991) demonstrated that elevation of core body temperature to 42±0.5°C for just 15 min led to induction of HSP70, peaking at 4-6 h after the heat stress and persisting for 10 days. Although there have been reports to the contrary (Joannidis *et al.*, 1995; Turman and Rosenfeld, 1999), several studies have indicated that prior induction of HSP70 expression can not only protect against warm IR injury in the kidney (Kelly *et al.*, 2001), but also against damage acquired during transplantation of

renal isografts and allografts (Perdrizet *et al.*, 1989; Perdrizet *et al.*, 1993; Meldrum *et al.*, 2001; Redaelli *et al.*, 2001; Redaelli *et al.*, 2002). In a syngeneic renal transplant model, preconditioning with hyperthermia 6-8 h prior to 48 h cold ischaemia, significantly improved immediate post-IR function and increased recipient survival at 50 d reperfusion. This protection was associated with HSP70 induction in the transplanted kidney (Perdrizet *et al.*, 1989). Furthermore, when an alloimmune aspect was introduced in a model of porcine renal allografts, hyperthermic preconditioning 4-6 h before ischaemia also protected kidneys against initial renal dysfunction (measured by elevated serum creatinine levels) and improved early allograft survival versus non-preconditioned animals (Perdrizet *et al.*, 1993). This protection was also associated with increased HSP70 expression in these kidneys approximately 28 h after the heat stress, at the time of implantation. Taken together, these studies suggest that HSP70 induction by hyperthermia may protect against an IR insult as well as early allograft injury although it is not known if the latter effect is primarily due to an attenuation of IR-induced exacerbation of the alloimmune response to the grafted tissue. In addition to its protective effect against IR-induced organ dysfunction and post-transplant graft survival of the kidney, hyperthermic preconditioning has been shown to reduce other aspects of IR injury in several different organs. For example, preconditioning with hyperthermia attenuates elevated MPO levels in the lung, (indicating a reduction of neutrophil accumulation) (Javadpour *et al.*, 1998) as well as leucocyte infiltration and oedema in the liver (Terajima *et al.*, 2000) following IR. This protection was associated with prior induction of HSP70 expression by preconditioning, although the exact mechanism through which this protection occurred remains unclear.

The graft endothelium is the first allogeneic barrier encountered by the recipient's immune system upon transplantation of an organ. Transplantation of functional endothelial cells

into an ischaemic kidney protects against IR-induced renal dysfunction (Brodsky *et al.*, 2002), indicating that the vascular endothelium is one of the primary targets of a renal IR insult. The first indications of endothelial activation include the upregulation of adhesion molecules and subsequent leucocyte accumulation. The renal IR injury presented in Chapter 3 was associated with increased leucocyte counts in the renal cortex and medulla. This coincided with elevated MPO levels at 2 h reperfusion, suggesting that these leucocytes were neutrophils. This is in agreement with Takada *et al.* (1997) who demonstrated that neutrophil accumulation peaks during the first six hours of reperfusion in a model of renal IR similar to the one in this thesis. In fact peak neutrophil infiltration of post-ischaemic kidney has been shown to coincide with maximum renal dysfunction (Dragun *et al.*, 2000). This is followed by monocyte and lymphocyte accumulation within seven days of injury, which sustains the inflammatory response through the production of cytokines such as TNF-α, IFN-γ and IL-2 (Takada *et al.*, 1997). In addition, T-lymphocytes are the primary immune cells involved in rejection. One of the receptors most intimately involved in both early neutrophil and later T-cell adherence is ICAM-1, which is constitutively expressed on renal vascular endothelium. ICAM-1 is up-regulated following IR as well as during allograft rejection, in parallel with leucocyte infiltration of the renal tissue (Gonzalez-Posada *et al.*, 1996; Korczak-Kowalska *et al.*, 1997). This suggests that ICAM-1 may be important not only in the initial stages of adherence of neutrophils to endothelium but also in strengthening the association between allospecific effector cells, such as T-cells, and the target cells of the graft. Thus, in addition to causing end-organ injury, IR may favour rejection through the upregulation of ICAM-1 expression.

Another role for IR in allograft rejection may lie in its ability to elevate MHC expression. In an experimental setting, Shackleton *et al.* (1990) observed an increase in the expression of class I and class II MHC antigens at five days reperfusion in previously ischaemic rat kidneys. MHC induction follows the expression of several T-cell-derived cytokines including IL-2, IFN-γ and TNF-α during renal IR, some of which may be responsible for the expression of these antigens (Takada *et al.*, 1997). In fact, in both isografts and allografts, ischaemia enhances the expression of MHC II on the graft endothelium and proximal tubular epithelium (Kouwenhoven *et al.*, 2001b). Of note, increased levels of both ICAM-1 and MHC II are associated with acute rejection in human kidneys (Gonzalez-Posada *et al.*, 1996), thus IR may predispose tissue to rejection during transplantation by elevating both adhesion molecules and MHC expression (Kouwenhoven *et al.*, 2001b). The blocking of ICAM-1 expression using antisense oligonucleotides not only attenuates ICAM-1 upregulation and the associated neutrophil and monocyte infiltration but also reduces MHC II expression and renal dysfunction in rat renal autografts (Dragun *et al.*, 1998a). This suggests that ICAM-1 and inflammatory mediators from neutrophils, monocytes and/or endothelial cells may promote T-lymphocyte infiltration. These T-cells release cytokines that not only promote continued inflammation and expression of ICAM-1, but also induce MHC II expression. Furthermore, IR-induced adhesion molecule expression and subsequent leucocyte infiltration appear to participate in post-ischaemic tubular injury, and therefore subsequent renal dysfunction. These data indicate that the attenuation of adhesion molecule expression, and therefore leucocyte accumulation, may protect the kidney against post-ischaemic morphological and functional deterioration, as well as MHC expression.

Thus the initial aims of this study were to establish the time course of expression of several HSPs, including HSP70, following hyperthermia and to determine the efficacy of hyperthermic preconditioning in preventing IR-induced renal dysfunction, oedema and tubular damage at 2 h, 18 h and 5 days reperfusion. The impact of the IR insult not only on renal leucocyte counts and MPO levels, but also on the associated expression of ICAM-1 was then investigated at 2 h reperfusion. Furthermore, the influence of IR on leucocyte accumulation and MHC class II levels at 5 days of reperfusion was evaluated. The potential for HSP induction to attenuate leucocyte-endothelial interactions as well as ICAM-1 and MHC II expression was examined.

6.2 METHODS

6.2.1 Preconditioning With Hyperthermia

Male Sprague-Dawley rats weighing 300-350 g were randomized into normothermic animals and hyperthermic preconditioned animals. Both groups were anaesthetized with inhalational halothane (see section 2.1.1) and core body temperature was monitored using a rectal probe. The core body temperature of rats in the normothermic group was maintained at 37°C for 15 min with the use of an infrared lamp. Initially, the protocol for the hyperthermia group called for increasing the core body temperature to 42°C for 15 min in accordance with Emami *et al.* (1991). This was achieved by placing the animals in a water bath. The animals were induced and the anaesthetic hoses were attached with tape to the side of a water bath, such that the animals could be maintained under anaesthetic while lying partially immersed in the supine position on a plastic platform in the bath. However 42°C was associated with 100% mortality in the first three animals exposed to this temperature. Therefore, using the same method, the core body temperature of subsequent rats in this group was raised to 41±0.5°C for 15 min, which resulted in 0% mortality (the water temperature was 42±1.0°C. These animals were then towel dried. All animals were allowed to recover from anaesthesia for the times specified in sections 6.2.2 and 6.2.3.

6.2.2 Time Course Of Heat Shock Protein Expression

In order to establish a time course of HSP induction following preconditioning, 15 rats underwent a controlled normothermic period and 15 rats were exposed to hyperthermic preconditioning. As illustrated in Figure 6.1, three animals in each group were allowed to

recovery from anaesthesia for one of the following periods: 0 h, 6 h, 12 h, 18 h or 24 h, after which the animals were re-anaesthetized and the right kidneys were removed. These kidneys were snap-frozen in liquid nitrogen and stored at -80°C for assessment of HSP27, HSP60, HSP70 and HSP90 protein expression by western immunoblotting as described in section 2.5.

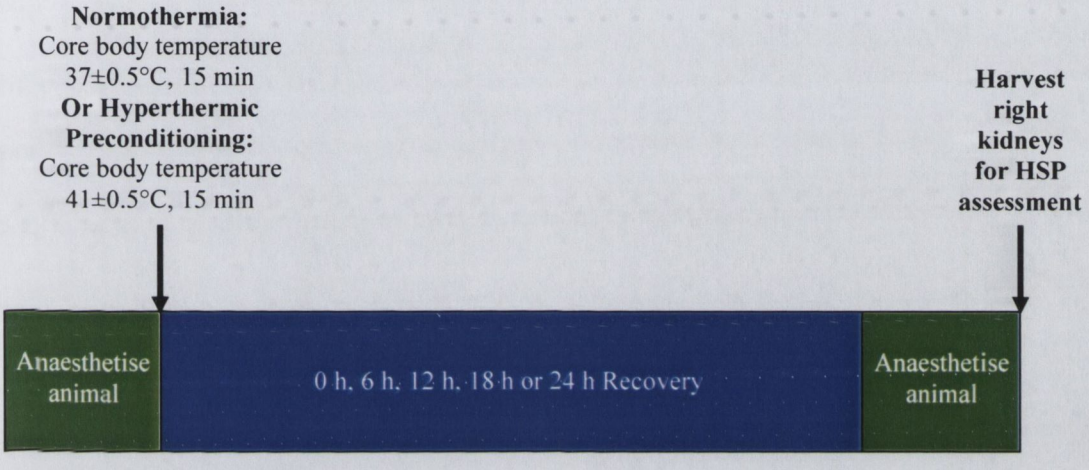


Figure 6.1 Timeline of preconditioning with hyperthermia to induce HSP expression. Animals were exposed to normothermia or hyperthermia for 15 min and allowed to recover for various periods before the kidneys were harvested to assess HSP expression.

6.2.3 Thermotolerance And Renal Ischaemia-Reperfusion

Sixty male Sprague-Dawley rats weighing 300-350 g were randomised into three groups:

Control (C): normothermia followed by sham IR.

Ischaemia-reperfusion (IR): normothermia followed by 45 min renal ischaemia with reperfusion.

Preconditioning and ischaemia-reperfusion (HIR): hyperthermic preconditioning followed by 45 min renal ischaemia with reperfusion.

Normothermia and hyperthermic preconditioning were performed as described in section 6.2.1, after which all animals were allowed to recover for 18 h before undergoing surgery. The C group underwent left nephrectomy followed by 45 min sham ischaemia of the right kidney as described in section 3.2. The IR and HIR groups were subjected to left nephrectomy followed by 45 min ischaemia of the right kidney as per section 3.2. All groups were subdivided into reperfusion times of 2 h, 18 h or 5 d reperfusion as described in Figure 6.2:

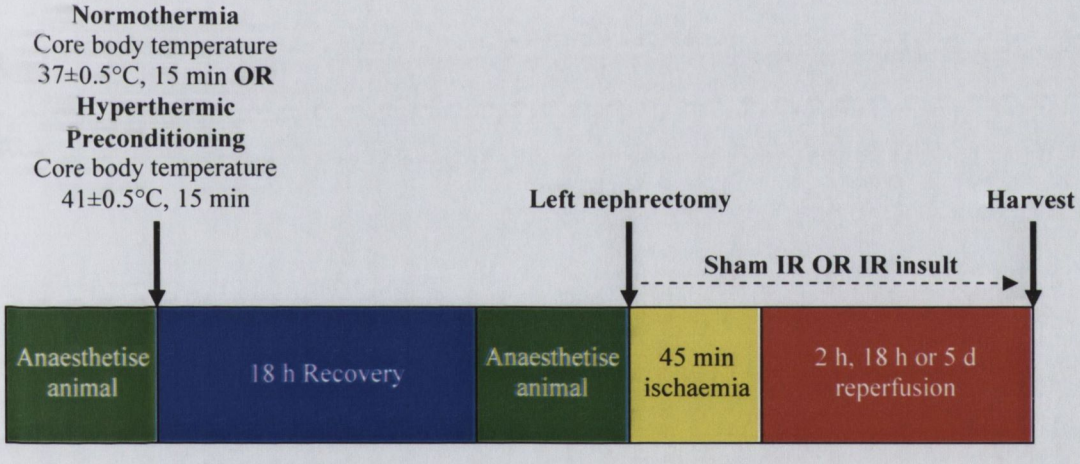


Figure 6.2 Timeline of preconditioning with hyperthermia followed by sham IR or IR injury.

In the groups allowed to reperfuse for 2 h (C-2h (n=8), IR-2h (n=8), HIR-2h (n=5)), blood, urine and the right kidney were taken for assessment of renal function, oedema, MPO activity, histology and ICAM-1 expression (detailed in sections 2.1-2.4) at the end of

reperfusion. In all 18 h (C-18h (n=7), IR-18h (n=7), HIR-18h (n=4)) and 5 d (C-5d (n=4), IR-5d (n=3), HIR-5d (n=3)) groups, blood was drawn for functional studies and the right kidneys were removed to assess oedema after 18 h and 5 d reperfusion respectively. In addition, a portion of the right kidneys harvested 5 d after the ischaemic insult was snap-frozen in OCT for assessment of MHC II expression by immunocytochemistry (described in section 2.4.1). This timepoint was chosen based on previous findings where MHC II expression takes 3-5 days to increase (Shackleton *et al.* 1990) and coincides with peak lymphocyte infiltration and the expression of T lymphocyte-derived cytokines (Takada *et al.* 1997). All animals were then euthanised.

6.2.4 Statistics

All data is expressed as mean±SEM. ANOVA with Scheffe post-hoc test was performed on all parameters to compare between groups.

6.3 RESULTS

6.3.1 Effect Of Hyperthermic Preconditioning On Renal HSP Expression

HSP27 was absent both in kidneys exposed to normothermia and in kidneys subjected to 41±0.5°C for 15 min and allowed to recover for 0, 6, 12, 18 or 24 h (Figure 6.3a). HSP60 was constitutively expressed in renal tissue of normothermic animals. This expression was similar in hyperthermia-treated animals at 0 h recovery, increasing by 6 h, and peaking at 12 h. Thereafter, HSP60 expression fell slightly but remained high for up to 24 h post-hyperthermia (Figure 6.3b). HSP70 expression was not observed in normothermic animals. Rats with 0 h recovery from hyperthermia exhibited negligible HSP70 expression. However expression was elevated at 6 h and 12 h. Thereafter HSP70 expression fell by 18 h, almost recovering to sham levels by 24 h following hyperthermia (Figure 6.3c). HSP90 was not expressed in any of the kidneys assessed (Figure 6.3d).

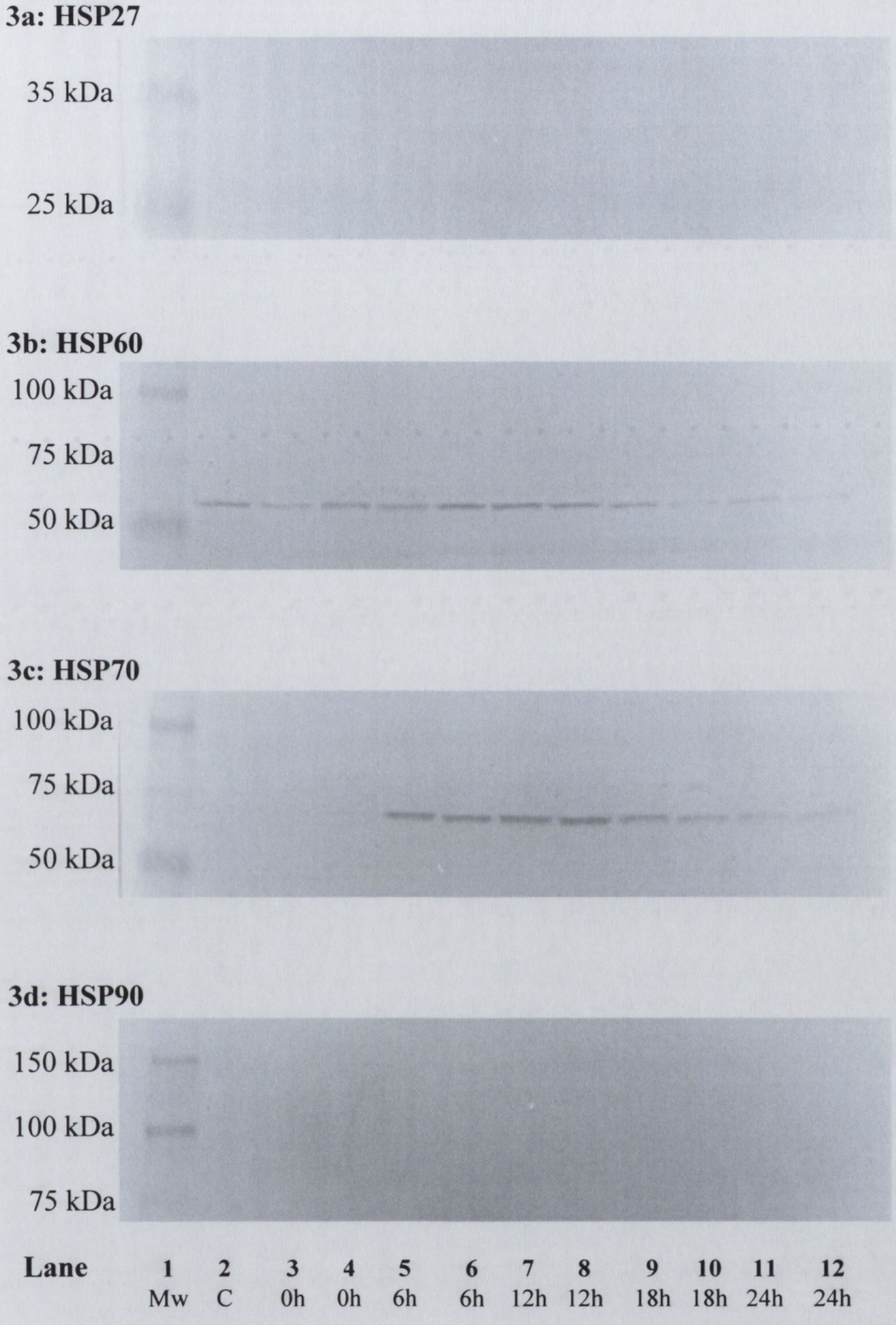


Figure 6.3 Western immunoblotting for a) HSP27, b) HSP60, c) HSP70 and d) HSP90 expression in the right kidneys of animals exposed to normothermia (C-lane 2) or 15 min hyperthermia followed by recovery for 0 h (lanes 3-4), 6 h (lanes 5-6), 12 h (lanes 7-8), 18 h (lanes 9-10) or 24 h (lanes 11-12). Molecular weight markers can be seen in lane 1.

In order to determine at what time-point the renal IR insult should be performed after hyperthermic preconditioning, the time-course of HSP induction was reviewed. Only a small amount of evidence exists for a role for HSP60 in protection conferred by hyperthermic preconditioning against subsequent injury (Tamaki T *et al.* 1997), and HSP27 and HSP90 were not expressed in the kidney at any time examined. However, it has been previously demonstrated that HSP70 expression is associated with protection against many types of insults after hyperthermic preconditioning. Thus, the time-course of HSP70 expression here was used to determine the most appropriate time-point following hyperthermic preconditioning at which IR should be performed. *In vitro* work by Landry *et al.* (1982) and Kim *et al.* (1993) showed that preconditioning with hyperthermia (heat shock) protected cells against a second otherwise lethal stress. This protection peaked between the maximum expression and recovery of normal levels of the HSP70 in the heat-shocked cells. More recently, an *in vivo* study in the rat heart demonstrated that preconditioning with hyperthermia conferred protection against subsequent IR injury only after the associated HSP70 induction had peaked, but before HSP70 expression had recovered to control levels (Yamashita *et al.*, 1997). Thus the IR insult in the current study was performed at 18 h post-hyperthermia. This was after peak HSP70 expression, when approximately half of maximal HSP70 expression remained.

6.3.2 Effect Of Ischaemia-Reperfusion On Renal Function

As previously demonstrated in chapter 3, IR led to significant renal dysfunction within 2 h reperfusion (section 6.3.2.1 below). This had deteriorated further by 18 h reperfusion 6.3.2.2. By 5 d reperfusion, renal function had partially recovered towards control levels 6.3.3.3.

6.3.2.1 Two Hours Reperfusion

Serum creatinine levels increased significantly from C-2h levels of 67.7±6.12 μM to IR-2h values of 127.8±5.78 μM ($P<0.0001$) (Figure 6.4). Serum urea was significantly elevated from 8.3±0.30 mM in C-2h rats to 12.0±0.73 mM in the IR-2h group ($P<0.0005$) (Figure 6.5). Similarly, K^+ levels rose by approximately 50% in the IR-2h group when compared with the C-2h rats ($P<0.005$) (Figure 6.6). Serum Na^+ levels remained unchanged after 2 h reperfusion (Figure 6.7). Urine output in the IR-2h group was less than half of the volume obtained in C-2 h animals, ($P<0.005$) (Figure 6.8).

6.3.2.2 Eighteen Hours Reperfusion

The C-18h group exhibited significantly lower serum creatinine ($P<0.05$) and K^+ ($P<0.005$) levels and elevated serum Na^+ levels ($P<0.01$) versus the C-2h animals. This was most likely due to an initial, but temporary, decrease in renal function while the right kidney tried to compensate for the loss of the left nephrectomised kidney, reaching equilibrium as reperfusion time increased. Furthermore the surgery and manipulation of the right kidney may have exerted a short-lived negative impact on the renal function in all groups. Following the IR insult, serum creatinine (Figure 6.4) and urea (Figure 6.5) levels continued to increase over time to 384.9±12.00 μM and 48.7±1.61 mM respectively in IR-18h group, more than three times the levels observed at 2 h reperfusion. By 18 h reperfusion, serum K^+ levels were approximately twofold greater than corresponding controls ($P<0.0001$) (Figure 6.6). Serum Na^+ levels were significantly decreased at 18 h reperfusion, from 141.0±0.44 mM in C-18h to 132.9±2.56 mM in IR-18h ($P<0.05$) (Figure 6.7).

6.3.2.3 Five Days Reperfusion

Five days after surgery, serum urea in the sham-operated C-5d group had been significantly reduced below levels in the C-2h and C-18h groups ($P<0.05$). Serum creatinine and K^+ levels in the C-5d animals were between C-2h and C-18h values ($P<0.05$ for serum K^+, C-2h versus C-5d). Serum Na^+ remained above C-2h levels in C-5d rats ($P<0.005$). These data most likely reflect stabilization of post-operative renal function in control kidneys. In the IR-5d group, where kidneys had been exposed to 45 ischaemia, serum creatinine (185.5±29.50 μM) and urea (24.3±3.07 mM) levels were approximately 50% of levels detected at 18 h ($P<0.0001$ for both parameters), remaining slightly higher than levels at 2 h reperfusion ($P<0.005$ for serum urea; NS for creatinine) (Figures 6.4 and 6.5). Serum K^+ levels of 7.2±1.97 mM in the IR-5d group were not significantly different from controls after 5 d reperfusion (Figure 6.6). Post-ischaemic serum Na^+ levels no longer differed from control values after 5 d reperfusion (Figure 6.7).

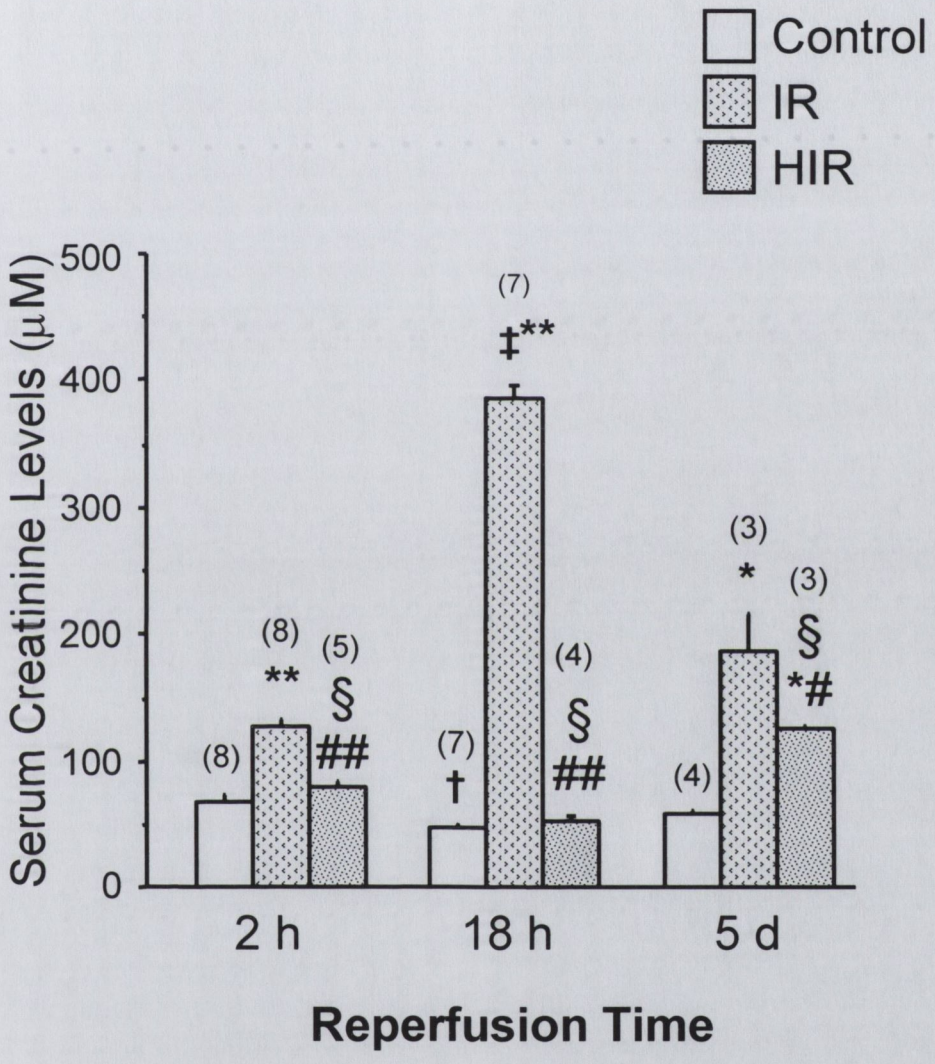


Figure 6.4 Serum creatinine levels measured at 2 h, 18 h and 5 d reperfusion following left nephrectomy and sham ischaemia (control groups) or 45 min ischaemia (IR groups) of the right kidney of normothermic rats. Separate IR groups were exposed to 15 min hyperthermia at 18 h pre-ischaemia (HIR groups). Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.05$ vs. C-2h; ‡ $P<0.0001$ vs. IR-2h and IR-5d; § $P<0.01$ vs. all HIR groups; * $P<0.01$ vs. respective C; ** $P<0.0001$ vs. respective C; # $P<0.05$ vs. respective IR; ## $P<0.001$ vs. respective IR.

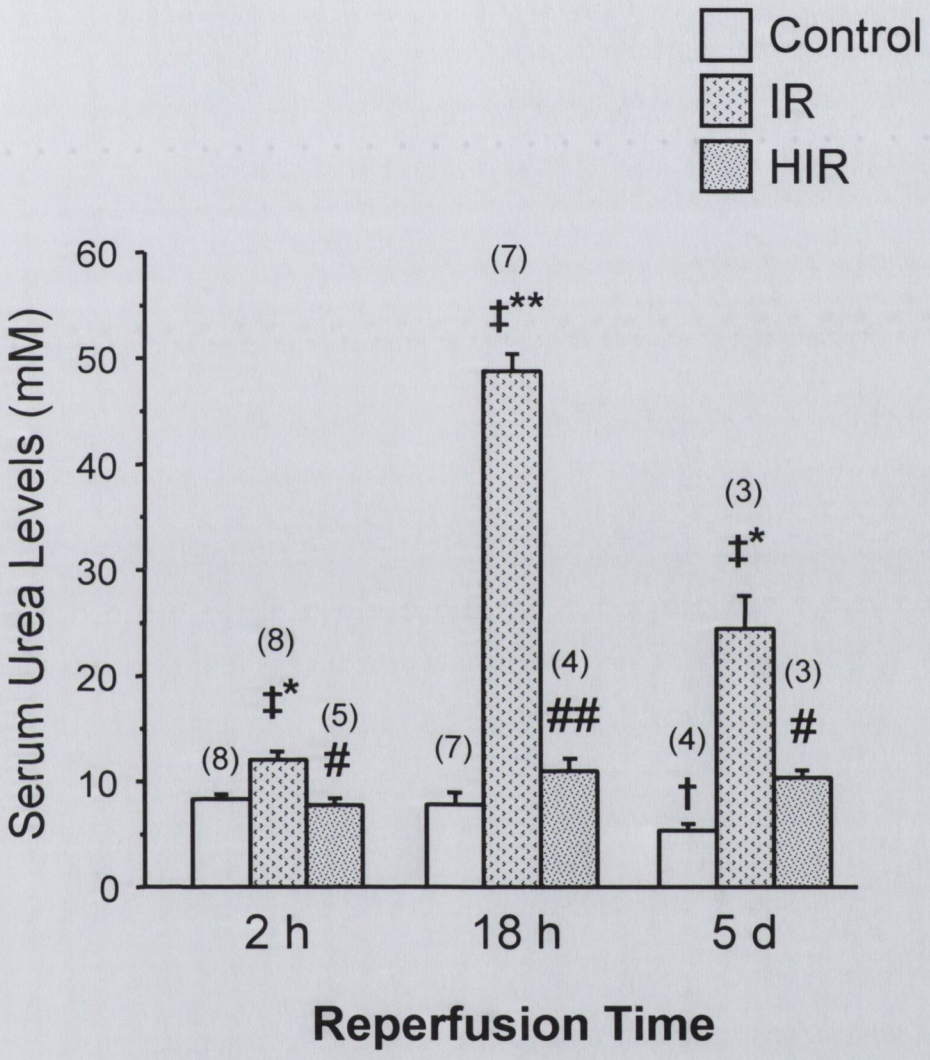


Figure 6.5 Serum urea levels measured at 2 h, 18 h and 5 d reperfusion following left nephrectomy and sham ischaemia (control groups) or 45 min ischaemia (IR groups) of the right kidney of normothermic rats. Separate IR groups were preconditioned with 15 min hyperthermia at 18 h pre-ischaemia (HIR groups). Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.05$ vs. C-2h and C-5d; ‡ $P<0.005$ vs. all IR groups; * $P<0.001$ vs. respective C; ** $P<0.0001$ vs. respective C; # $P<0.01$ vs. respective IR; ## $P<0.001$ vs. respective IR.

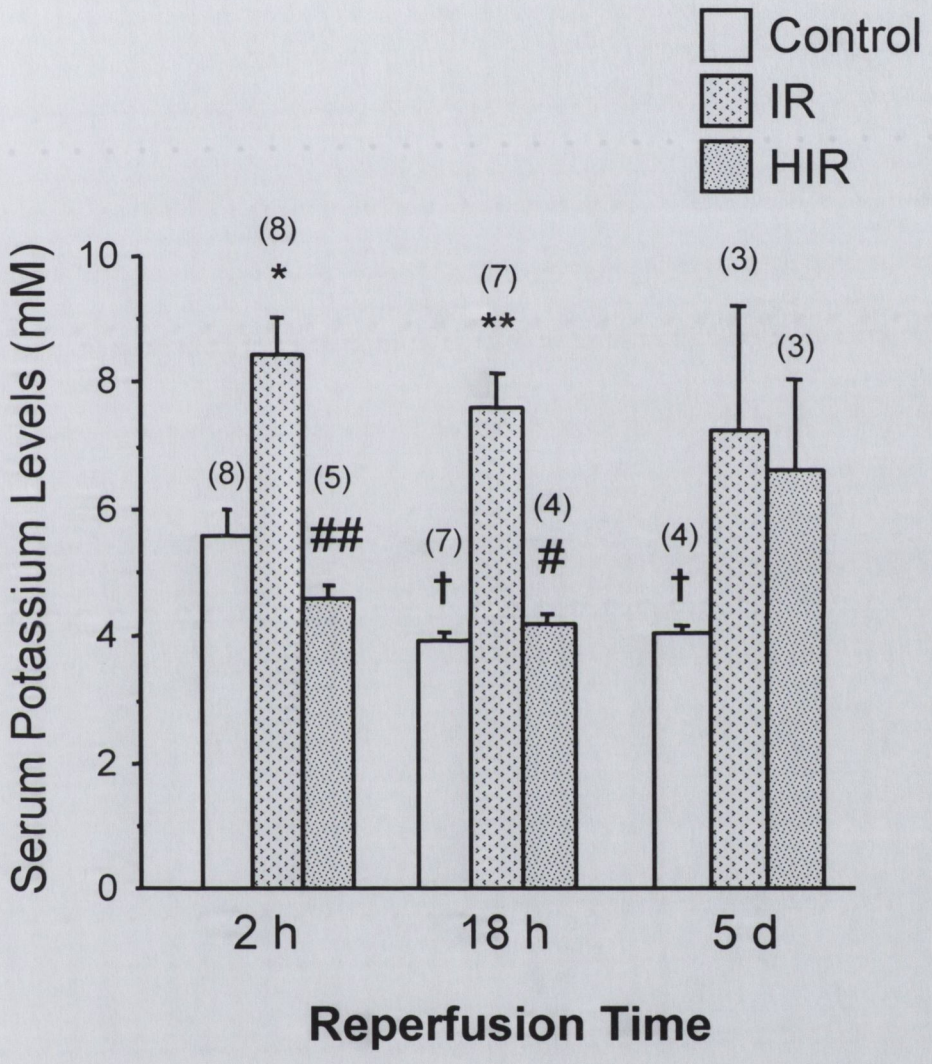


Figure 6.6 Serum potassium levels assessed at 2 h, 18 h and 5 d reperfusion following left nephrectomy and sham ischaemia (control groups) or 45 min ischaemia (IR groups) of the right kidney of normothermic rats. Separate IR groups were exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR groups). Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.01$ vs. C-2h; * $P<0.005$ vs. respective C; ** $P<0.0001$ vs. respective C; # $P<0.01$ vs. respective IR; ## $P<0.005$ vs. respective IR.

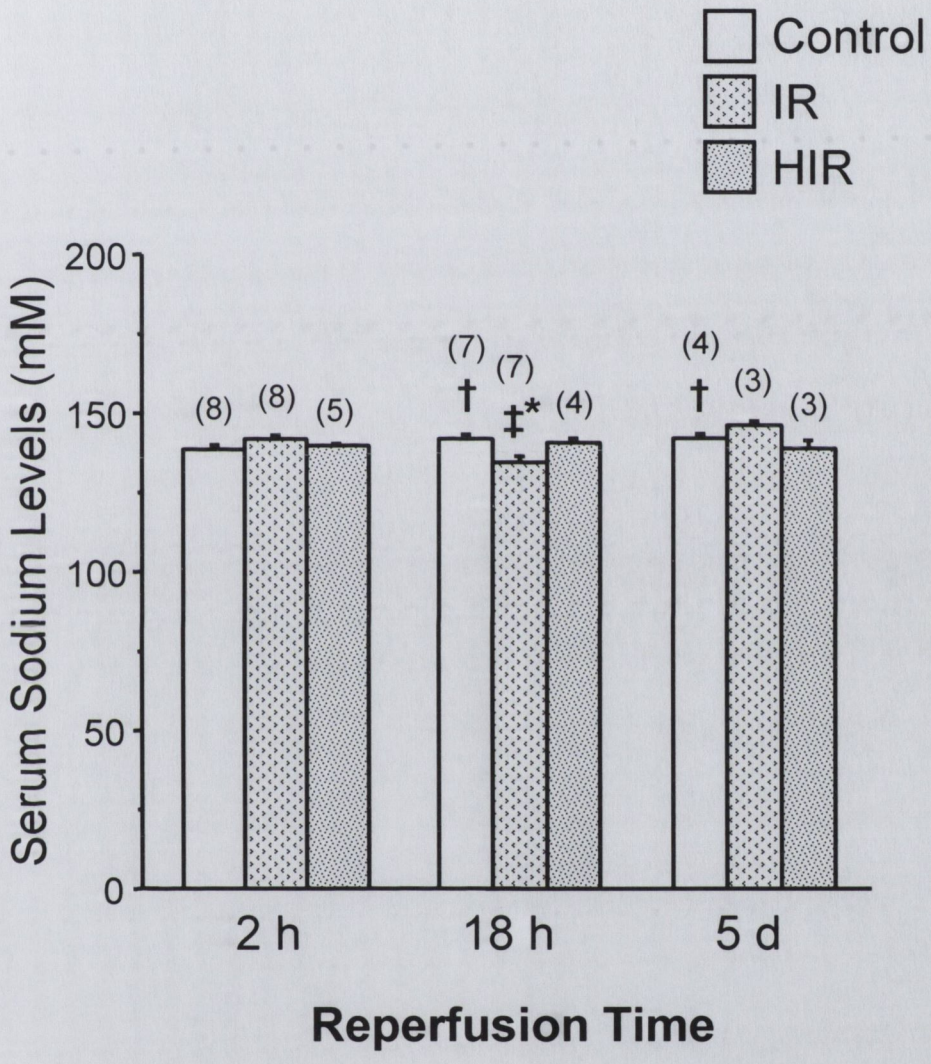


Figure 6.7 Serum sodium levels measured at 2 h, 18 h and 5 d reperfusion following left nephrectomy and sham ischaemia (control groups) or 45 min ischaemia (IR groups) of the right kidney of rats exposed to normothermia 18 h previously. Separate IR groups were preconditioned with 15 min hyperthermia 18 h before the IR insult (HIR groups). Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.01$ vs. C-2h; ‡ $P<0.005$ vs. IR-2h and IR-5d; * $P<0.05$ vs. respective C.

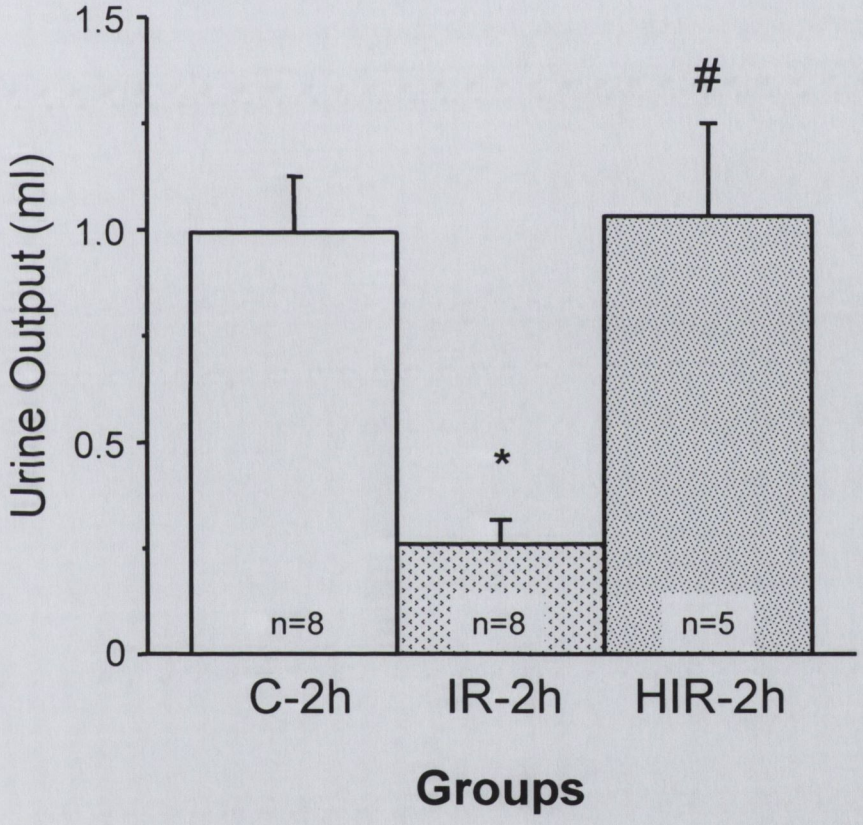


Figure 6.8 Urine output over 2 h reperfusion following left nephrectomy and sham ischaemia (C-2h group) or 45 min ischaemia (IR-2h group) of the right kidney of normothermic rats. A separate IR group was pre-treated with hyperthermia 18 h before ischaemia (HIR-2h group). Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.005$ vs. C-2h; # $P<0.005$ vs.IR-2h.

6.3.3 Effect Of Hyperthermic Preconditioning On Ischaemia-Reperfusion-Induced Renal Dysfunction

There were significant improvements in renal function observed between hyperthermia-preconditioned HIR groups and their corresponding IR groups at each reperfusion time-point.

6.3.3.1 Two Hours Reperfusion

Serum creatinine was reduced from 127.8±5.78 μM in the IR-2h group to 80.0±4.49 μM in the HIR-2h animals, $P<0.001$ (Figure 6.4). The urea levels in the serum of the HIR-2h animals (7.8±0.53 mM) was also significantly attenuated versus IR-2h ($P<0.0005$) (Figure 6.5). In addition, K^+ levels were decreased to control values by preconditioning with hyperthermia ($P=0.001$ versus IR-2h) (Figure 6.6). Not surprisingly, serum Na^+ levels in the HIR-2h group were not different from C-2h or IR-2h levels (Figure 6.7). Urine output was 1.0±0.21 ml in the HIR-2h group, more than twice that of the IR-2h animals ($P<0.005$) but similar to C-2h levels (Figure 6.8).

6.3.3.2 Eighteen Hours Reperfusion

Preconditioning with hyperthermia attenuated the IR-induced renal dysfunction observed at 18 h reperfusion. In the HIR-18h group, serum creatinine levels were 52.0±1.00 μM, significantly reduced compared with the IR-18h animals ($P<0.0001$; Figure 6.4). Serum urea levels in the HIR-18h animals (10.8±1.5 mM) were also significantly reduced versus values obtained in the IR-18h group ($P<0.0001$; Figure 6.5). Likewise, serum K^+ levels of 4.2±0.02 mM were significantly decreased in the preconditioned HIR-18h group versus corresponding IR-18h animals ($P<0.01$; Figure 6.6). Indeed, serum creatinine, urea and K^+

levels in the HIR-18h group were comparable to the corresponding control values in the C-18h group. Serum Na^+ levels in the HIR-18h rats fell between C-18h and IR-18h values (Figure 6.7).

6.3.3.3 Five Days Reperfusion

By 5 d reperfusion, serum creatinine was significantly decreased versus the IR-5d group to 124.3±2.60 μM in HIR-5d animals ($P<0.05$) (Figure 6.4). However these levels remained significantly elevated compared with C-5d values ($P<0.01$). Serum urea levels of 10.3±0.75 mM were also significantly attenuated in the HIR-5d when compared with the IR-5d levels ($P<0.01$) (Figure 6.5). While these levels continued to stay above control levels this was not significant. Serum K^+ (Figure 6.6) and Na^+ (Figure 6.7) levels in the HIR-5d group were not significantly different from either IR-5d or C-5d values.

6.3.4 Effect Of Ischaemia-Reperfusion On Endothelial Permeability

Endothelial permeability was higher in kidneys exposed to IR versus controls as evidenced by organ oedema and increased MPO activity.

6.3.4.1 Two Hours Reperfusion

Left nephrectomised kidneys and right kidneys that had undergone sham IR exhibited approximately equal weights in the C-2h group (% difference = -3.2±2.37%) (Figure 6.9). However in the IR-2h group, the right kidney was 33.6±5.07% heavier than the left nephrectomised kidney, demonstrating that there was an accumulation of fluid in the organ subjected to IR ($P<0.0001$ versus C-2h). Figure 6.10 shows that the MPO activity, used as

an indicator of neutrophil infiltration into the renal parenchyma, tripled from 0.4±0.06 U/g tissue in C-2h kidneys to 1.2±0.21 U/g tissue in the IR-2h group ($P<0.005$).

6.3.4.2 Eighteen Hours Reperfusion

The average percentage weight difference between left control and right experimental kidneys in the C-18h group was significantly higher than the value observed in the C-2h group ($P<0.05$). This was likely to have occurred as a result of the right kidney compensating for the loss of the left kidney as function stabilized. While the % weight difference between the two kidneys in the IR-18h animals was greater than that observed in C-18h animals, this was not significant. Furthermore, IR-18h values were slightly lower than levels observed in the IR-2h group (Figure 6.9). Myeloperoxidase levels were not measured at this time-point.

6.3.4.3 Five Days Reperfusion

The percentage weight difference between the left nephrectomised kidney and the right experimental kidney in the control rats at 5d remained significantly higher than levels observed in the C-2h group ($P<0.05$) but similar to the C-18h group. Kidneys that had been subjected to IR and allowed to reperfuse for 5 d exhibited extensive oedema (IR-5d group). The percentage weight difference between the left control kidney and right experimental kidney increased from 12.0±1.78% in C-5d animals to 114.7±11.57% in the IR-5d group ($P<0.0001$) (Figure 6.9). This was significantly greater than oedema values obtained at the two earlier reperfusion time-points examined ($P<0.0001$). Myeloperoxidase levels were not measured at 5 d reperfusion.

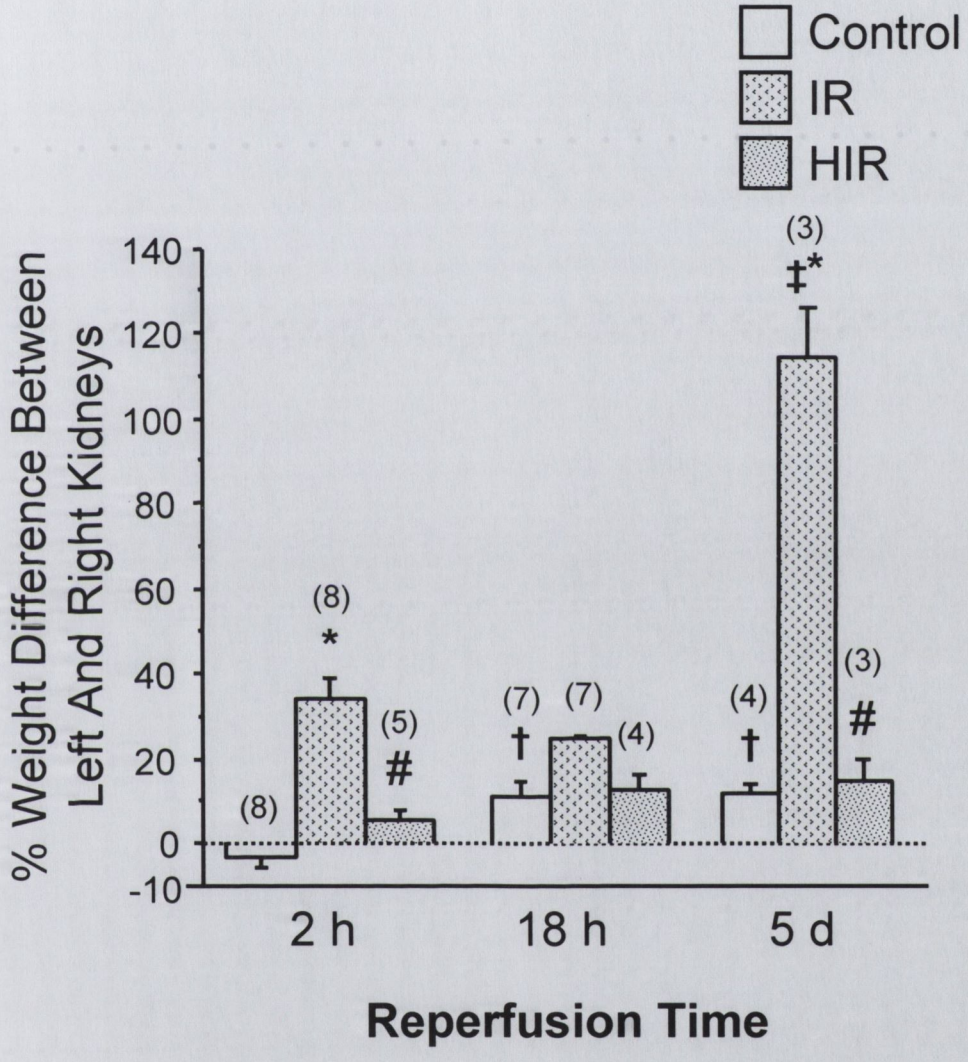


Figure 6.9 Renal oedema assessed at 2 h, 18 h and 5 d reperfusion following left nephrectomy and sham ischaemia (control groups) or 45 min ischaemia (IR groups) of the right kidney of rats exposed to normothermia 18 h previously. Separate IR groups were preconditioned with 15 min hyperthermia 18 h before the IR insult (HIR groups). Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.05$ vs. C-2h; ‡ $P<0.0001$ vs. IR-2h and IR-18h; * $P<0.0001$ vs. respective C; # $P<0.0001$ vs. respective IR.

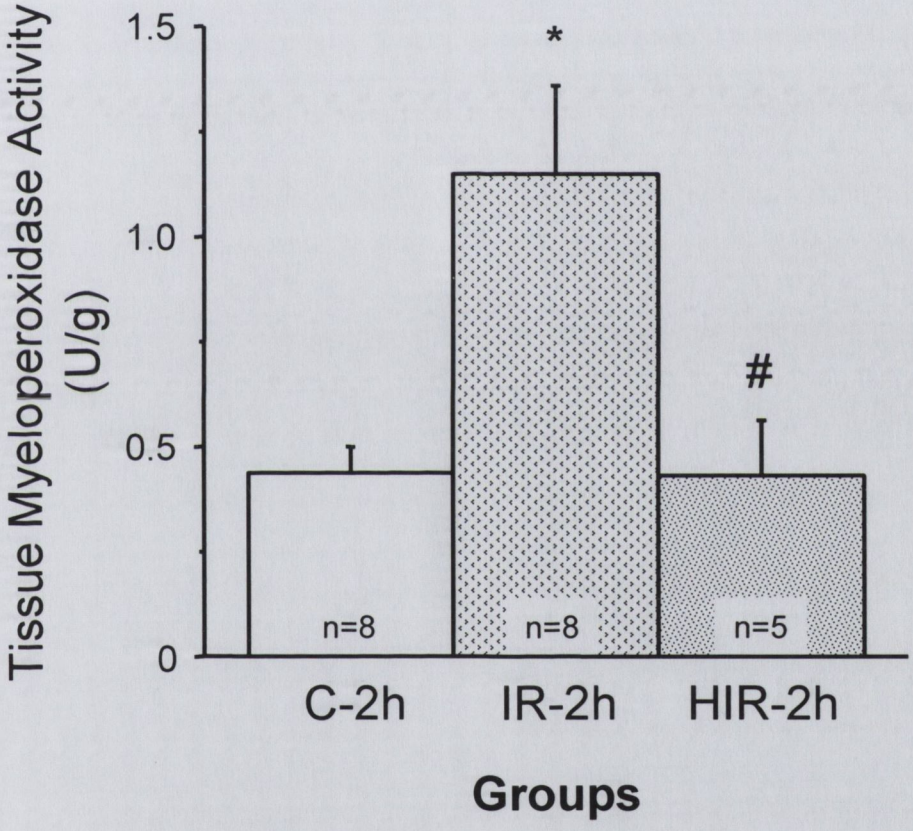


Figure 6.10 Tissue myeloperoxidase activity (an indicator of neutrophil influx) measured at 2 h reperfusion following left nephrectomy and sham ischaemia (C-2h group) or 45 min ischaemia (IR-2h group) of the right kidney of normothermic rats. A separate IR group was preconditioned with 15 min hyperthermia 18 h before the IR insult (HIR-2h group). Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.005$ vs. C-2h; # $P<0.01$ vs. IR-2h

6.3.5 Effect Of Hyperthermic Preconditioning On Ischaemia-Reperfusion-Induced Endothelial Permeability

Preconditioning with hyperthermia prevented the oedema and increased MPO activity in the kidney observed following IR injury.

6.3.5.1 Two Hours Reperfusion

There was only a small percentage difference of 5.4±2.32% between the weights of the right and left kidneys in the HIR-2h group (Figure 6.9), indicating that minimal oedema was evident. This was significantly lower than values in the corresponding IR-2h group ($P=0.0001$), but similar to C-2h values. The MPO activity was 0.4±0.13 U/g tissue in the HIR-2h group, comparable to C-2h levels. This was significantly lower than levels of 1.2±0.21 U/g tissue obtained in the IR-2h group, $P<0.005$ (Figure 6.10).

6.3.5.2 Eighteen Hours Reperfusion

The percentage weight differences between the left control kidney and right experimental kidney in the HIR-18h group were similar to those observed in the C-18h animals. While HIR-18h values were less than those seen in the IR-18h group, these were not significant (Figure 6.9).

6.3.5.3 Five Days Reperfusion

The development of oedema in the right kidney after 45 min ischaemia and 5 d reperfusion was significantly attenuated to control levels by hyperthermic preconditioning. The kidney weight difference of 114.7±11.57% in the IR-5d group was reduced to 14.6±5.23% in the HIR-5d group ($P<0.0001$) (Figure 6.9).

6.3.6 Effect Of Ischaemia-Reperfusion On Renal Morphology

Significant deteriorations in renal tubular integrity were observed following IR. The severity of these changes was reflected in the renal dysfunction found at the time-points examined, in that morphological destruction was worse at 18 h reperfusion than at 2 h, but the kidneys exhibited recovery by 5 d reperfusion. Leucocyte infiltration was observed at all time-points.

6.3.6.1 Two Hours Reperfusion

Figures 6.11a and 6.11b represent H&E sections of renal cortex from C-2h and IR-2h respectively. Figures 6.11d and 6.11e represent H&E sections of renal medulla from C-2h and IR-2h respectively. Samples from the C-2h group exhibited good renal morphology with intact tubular epithelium, normal tubular structure and low levels of protein secretion and leucocyte accumulation (Figure 6.11a and 6.11d) However, tubular integrity in kidneys exposed to IR-2h was reduced, with decreased contact between tubules. Loss of nuclei and vacuolization of the epithelium were observed in this group (Figure 6.11b). Red blood cell congestion was also evident in the medullas of kidneys within 2 h of reperfusion in the IR-2h group (Figure 6.11e). Ten fields of view were examined per kidney, with an average of 33.7±1.28 tubules/field counted in both the C-2h and IR-2h groups. Quantitatively, partial tubular degeneration was increased from C-2h levels of 26.9±4.75% to 58.7±8.94% in IR-2h kidneys (Figure 6.14). The percentage of tubules exhibiting total degeneration was elevated from 1.2±0.71% in C-2h animals to 14.0±3.74% in IR-2h kidneys ($P<0.05$) (Figure 6.15). Significant proteinaceous cast formation was observed in the IR-2h group ($P<0.0001$ versus C-2h) (Figure 6.16). This occurred predominantly in tubules that exhibited relatively normal morphology, indicating they were compensating

for the loss of surrounding tubules (Figure 6.11b). Leucocyte infiltration of the renal cortex and medulla was found in kidneys of IR-2h animals (Figures 6.11b and 6.11e). The leucocyte count in the IR-2h cortex was 73.8±3.38 cells/field, more than twice the levels in the C-2h kidneys ($P<0.0001$) (Figure 6.17).

6.3.6.2 Eighteen Hours Reperfusion

Figures 6.12a and 6.12b represent sections of renal cortex from C-18h and IR-18h animals respectively. Representative images of the medulla from C-18h and IR-18 groups can be seen in Figures 6.12d and 6.12e. Kidneys from the C-18h group exhibited normal morphology with good contact between tubules and intact tubular endothelium (Figure 6.12a), comparable to kidneys from C-2h animals (mean of 33.7±0.47 tubules/field were counted). However, complete deterioration of tubular epithelium occurred in the renal cortex of rats exposed to 45 min ischaemia followed by 18 h reperfusion (IR-18h group) (Figure 6.12b). Nuclei of a majority of the epithelial cells had been lost and many epithelial cells had detached from the basement membrane. Contact between tubules was compromised in this group (mean of 40.8±2.84 tubules/field were examined). Quantitatively, the percentage of partially degenerated tubules was significantly lower in the IR-18h group versus the C-18h and IR-2h groups ($P<0.05$) (Figure 6.14). However, this was far outweighed by the proportion of tubules that had completely deteriorated, which increased significantly from 4.8±1.18% in the C-18h group to 66.4±1.37% in the IR-18h animals ($P<0.0001$) (Figure 6.15), also significantly higher than levels observed at 2 h reperfusion following IR ($P<0.0001$). Proteinaceous casts had formed in 20.7±2.73% of tubules that had been exposed to IR-18h compared with 0.5±0.26% in the C-18h group ($P<0.005$) (Figure 6.16). The tubules with protein casts represented a majority of the tubules with normal morphology in the IR-18h group indicating that they were functional

and compensating for the large loss of surrounding tubules. Red blood cell congestion was found in the medulla of IR-18h kidneys. Loss of tubular integrity was also seen in the medulla of kidneys in the IR-18h group (Figure 6.12f). These alterations were not found in the medulla of C-18h animals (Figure 6.12e). The number of infiltrating leucocytes in the renal cortex after IR continued to rise during reperfusion such that by 18 h (IR-18h group) 101.1±2.30 cells/field had accumulated between tubules. This was three times the number observed in corresponding control (C-18h) animals ($P<0.0005$) (Figure 6.17), and significantly higher than levels observed at the 2 h reperfusion time-point ($P<0.001$).

6.3.6.3 Five Days Reperfusion

Figures 6.13a and 6.13b represent images of the cortex from kidneys of C-5d and IR-5d groups respectively. Figure 6.13d and 6.13e are H&E sections of renal medulla from C-5d and IR-5d groups respectively. Similar to the other control groups, the kidneys from the C-5d group demonstrated good tubular architecture, with intact epithelium (mean of 32.0±1.65 tubules/field were examined) (Figure 6.13a). Regeneration of tubular epithelium was noted throughout the kidneys of IR-5d animals (Figure 6.13b) when compared with the IR-18h group (Figure 6.12b). Epithelial cells were in close contact to the basement membranes, and nuclei and cytoplasm appeared intact in a large number of tubules in the IR-5d rats (mean of 27.8±1.50 tubules/field were counted). When these tissues were scored, 46.3±7.77% tubules in the IR-5d group were found to be partially degenerated versus 24.8±2.04% in the C-5d group ($P<0.05$) (Figure 6.14). The levels of complete deterioration were 6.4±1.73% in the C-5d group, which, while relatively small, were significantly higher than those observed in the C-2h group ($P<0.05$) (Figure 6.15). This was significantly elevated to 13.4±1.31% in the IR-5d group ($P<0.05$), although this significantly lower than levels of complete tubular deterioration noted in the IR-18h group

($P<0.0001$), suggesting that the kidney was recovering from the ischaemic insult. There were very few proteinaceous casts present in the renal cortex of either C-5d or IR-5d animals (Figures 6.13a, 6.13b and 6.16), less than in the corresponding groups at other reperfusion times examined. Leucocyte accumulation was significantly lower in the C-5d animals compared with C-18h levels ($P<0.01$). In the IR-5d group leucocyte infiltration was 55.7±2.91 cells/field. While this was significantly higher than C-5d values ($P<0.0001$), these numbers were significantly lower than those observed not only at 18 h reperfusion ($P<0.0001$) but also after 2 h reperfusion (Figure 6.17) ($P<0.01$). In addition, the interstitium surrounding the tubules appeared oedematous in the IR-5d group, as supported by the large weight difference between left and right kidneys in these animals. This was absent in the C-5d kidneys. The epithelium of the renal medulla in the IR-5d group (Figure 6.13e) exhibited relatively normal morphology, compared with the IR-18h group (Figure 6.12e), as regeneration occurred. However there were still some small areas of red blood cell congestion observed at 5 d reperfusion.

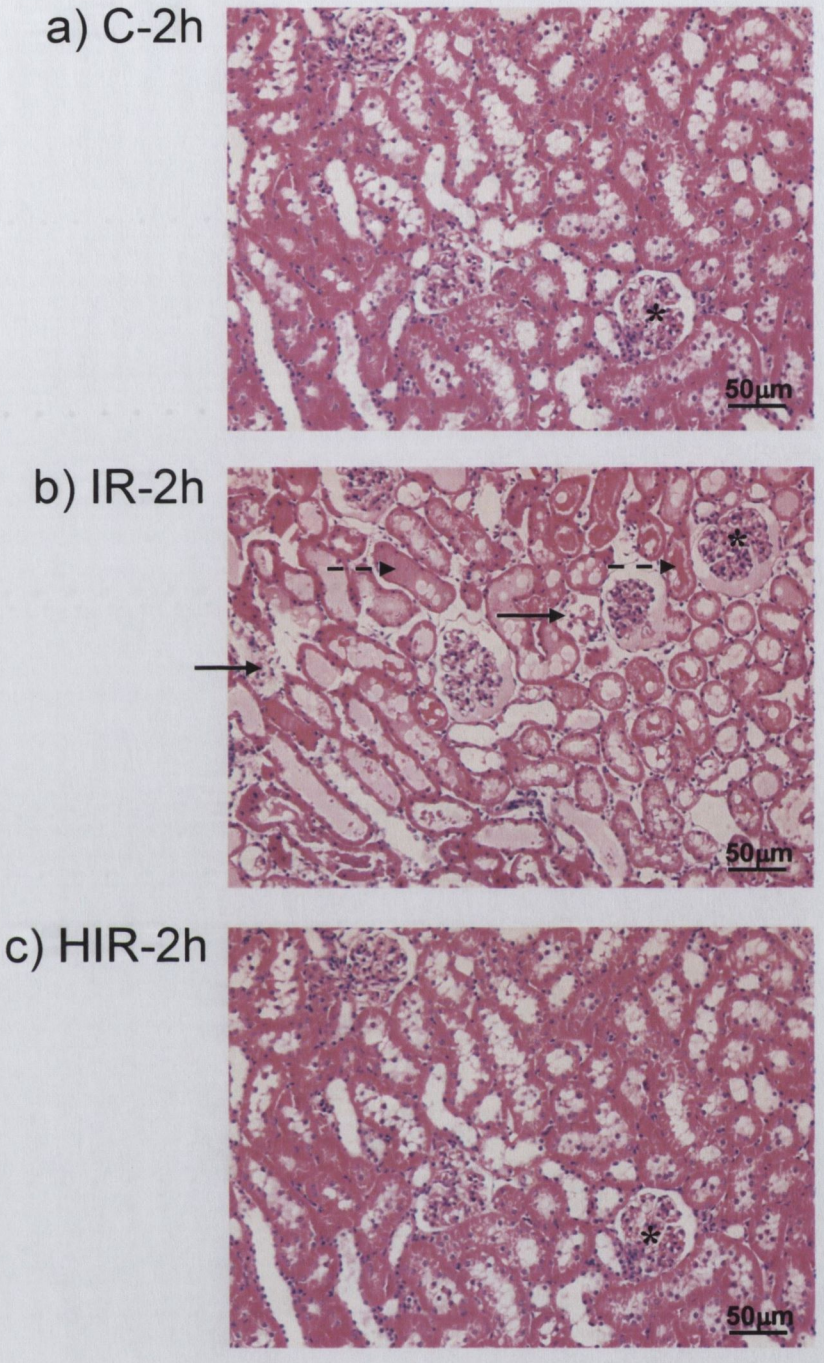


Figure 6.11a-c Representative images of renal cortex of the right kidney taken two hours following **a)** sham ischaemia (C-2h group) or **b)** 45 min ischaemia (IR-2h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-2h group **(c)**). Tissues were stained with H&E. Note the degeneration of the tubular epithelium (solid arrows) and the development of proteinaceous casts (dashed arrows) in the IR-2h group. Sample glomeruli are denoted by asterisks.

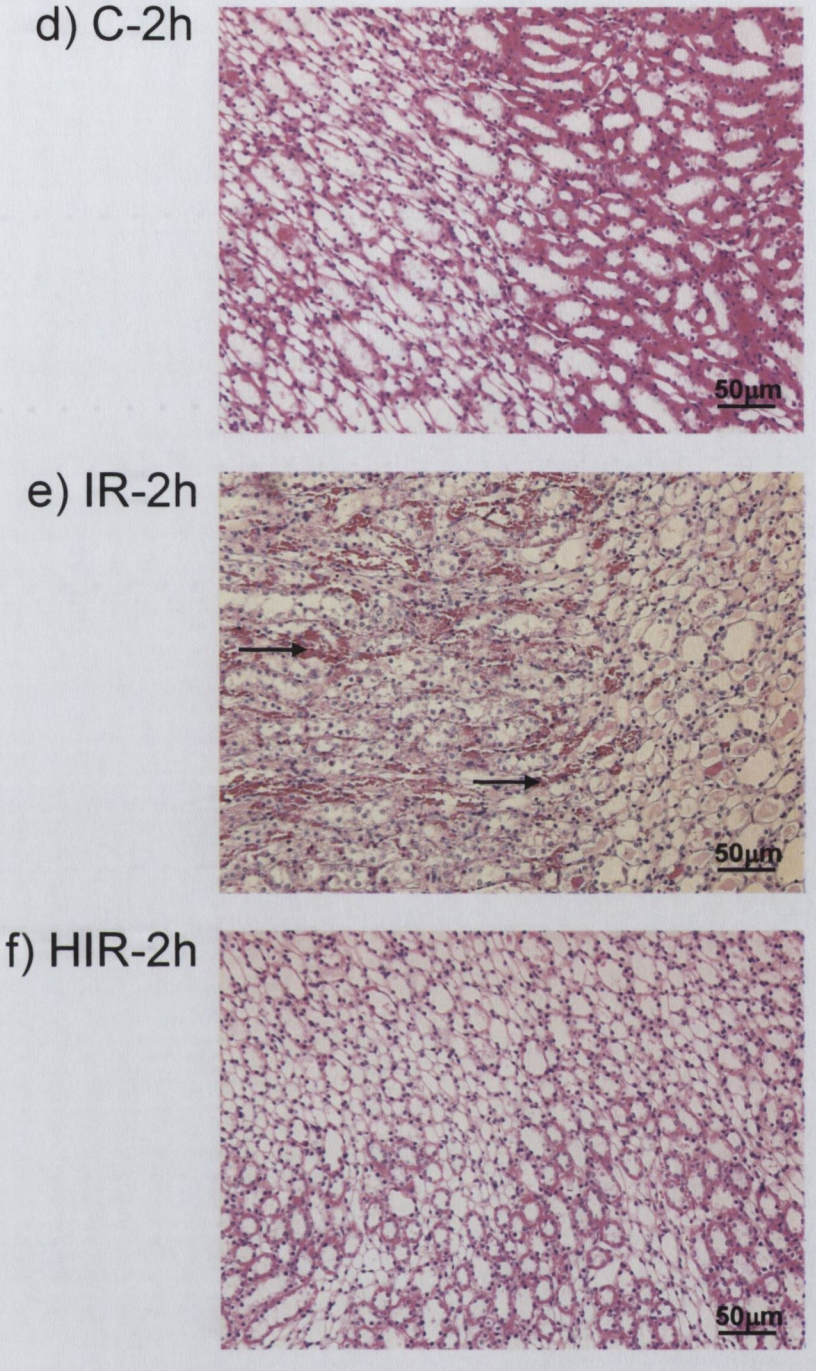


Figure 6.11d-f Representative images of renal medulla of the right kidney taken two hours following **d)** sham ischaemia (C-2h group) or **e)** 45 min ischaemia (IR-2h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-2h group **(f)**). Tissues were stained with H&E. Note the red blood cell congestion (solid arrows) in the IR-2h group.

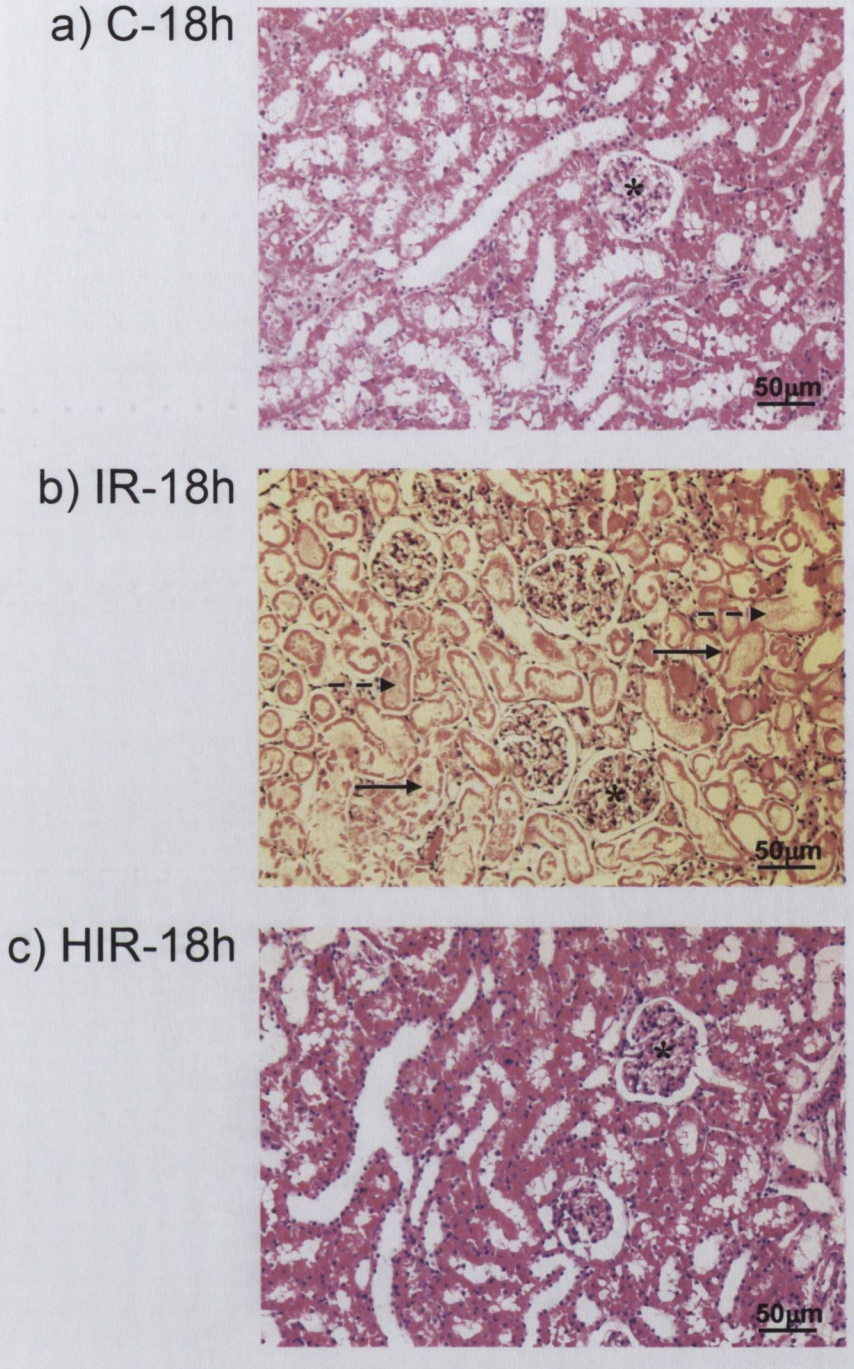


Figure 6.12a-c Representative images of renal cortex of the right kidney taken eighteen hours following **a)** sham ischaemia (C-18 h group) or **b)** 45 min ischaemia (IR-18 h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-18h group **(c)**). Tissues were stained with H&E. Note the degeneration of the tubular epithelium (solid arrows) and the development of proteinaceous casts (dashed arrows) in the IR-18h group. Sample glomeruli are denoted by asterisks.

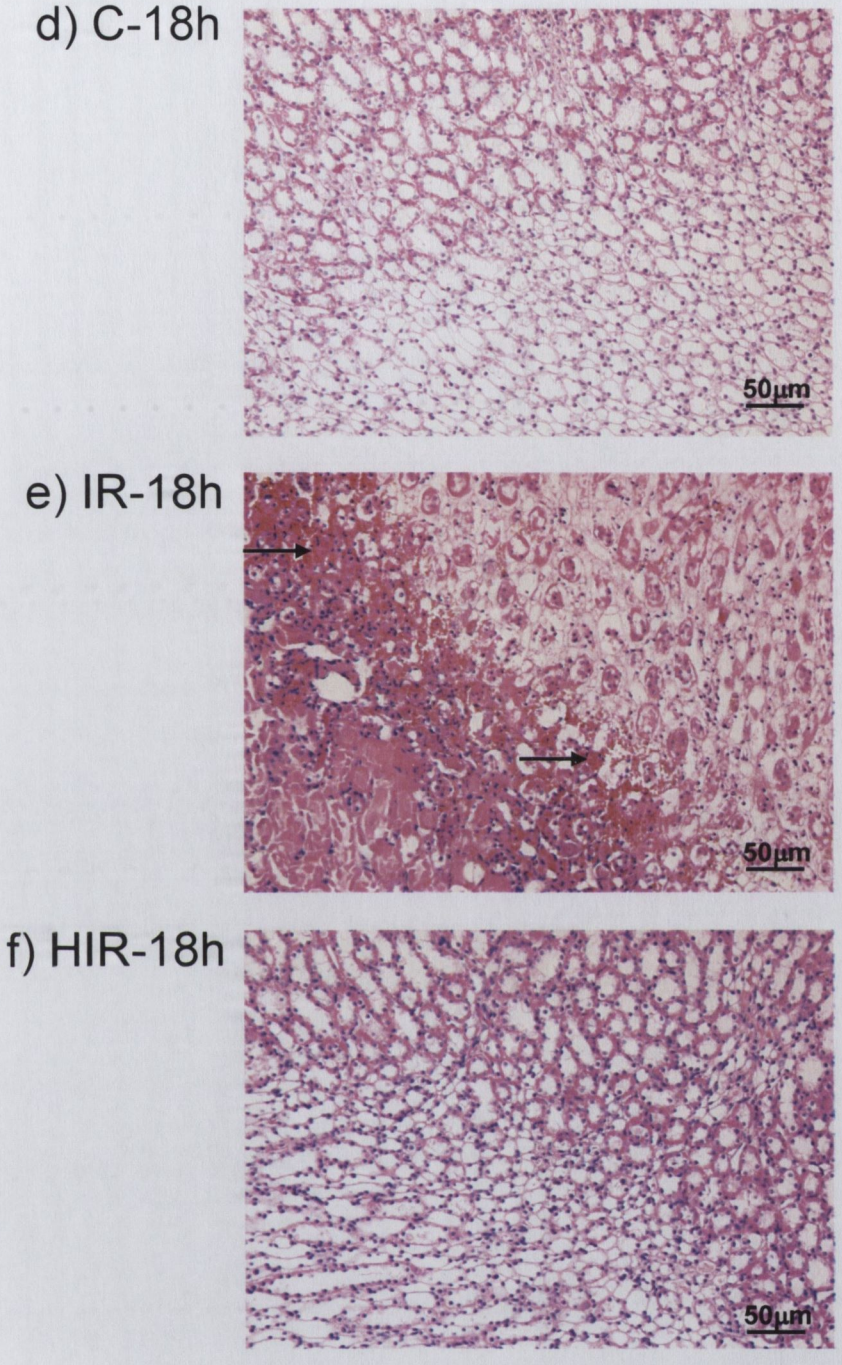


Figure 6.12d-f Representative images of renal medulla of the right kidney taken eighteen hours following **d)** sham ischaemia (C-18h group) or **e)** 45 min ischaemia (IR-18h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-18h group **(f)**). Tissues were stained with H&E. Note the red blood cell congestion (solid arrows) in the IR-18h group.

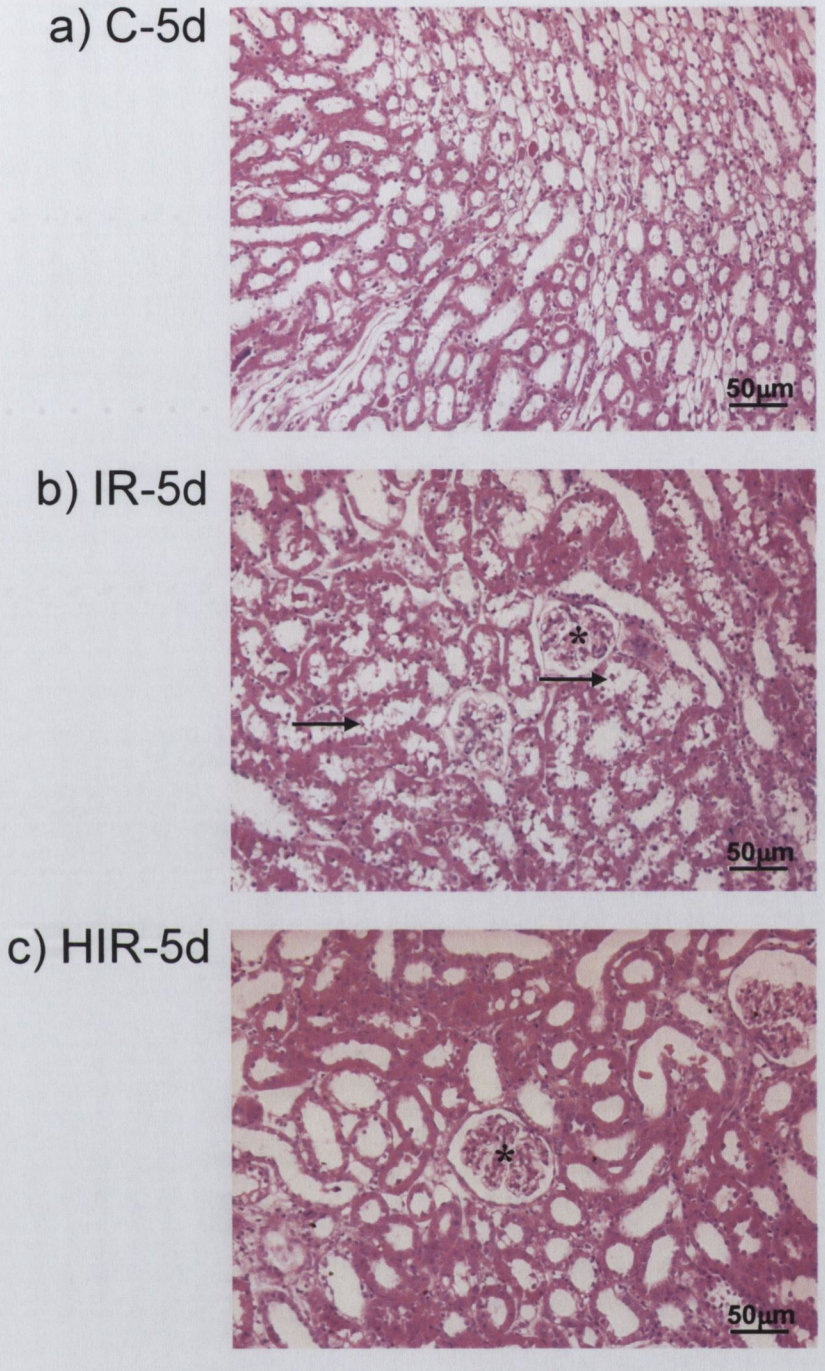


Figure 6.13a-c Representative images of renal cortex of the right kidney taken eighteen hours following **a)** sham ischaemia (C-5d group) or **b)** 45 min ischaemia (IR-5d group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-5d group **(c)**). Tissues were stained with H&E. Note the degeneration of the tubular epithelium (solid arrows) and the development of proteinaceous casts (dashed arrows) in the IR-5d group. Sample glomeruli are denoted by asterisks.

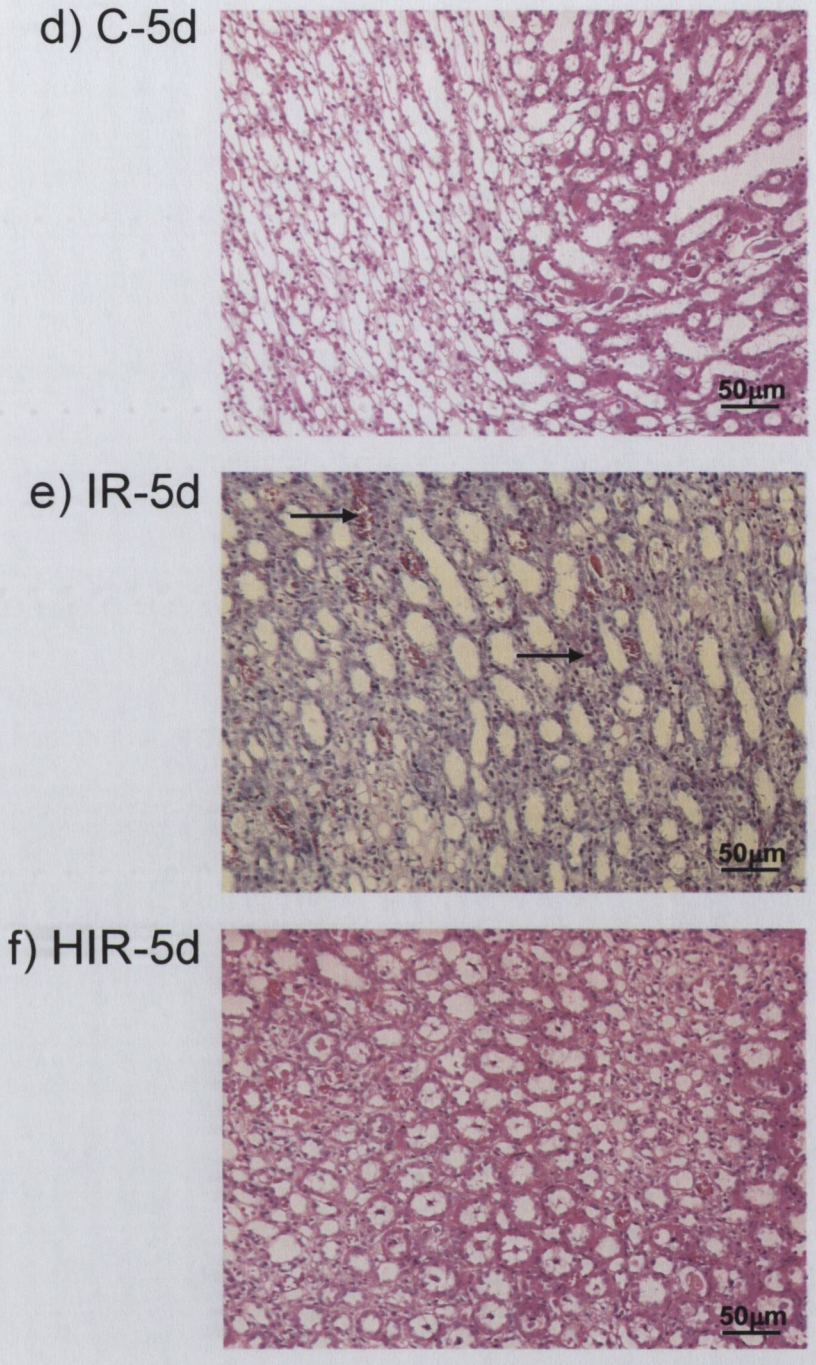


Figure 6.13d-f Representative images of renal medulla of the right kidney taken five days following **d)** sham ischaemia (C-5d group) or **e)** 45 min ischaemia (IR-5d group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-5d group **(f)**). Tissues were stained with H&E. Note the red blood cell congestion (solid arrows) in the IR-5d group.

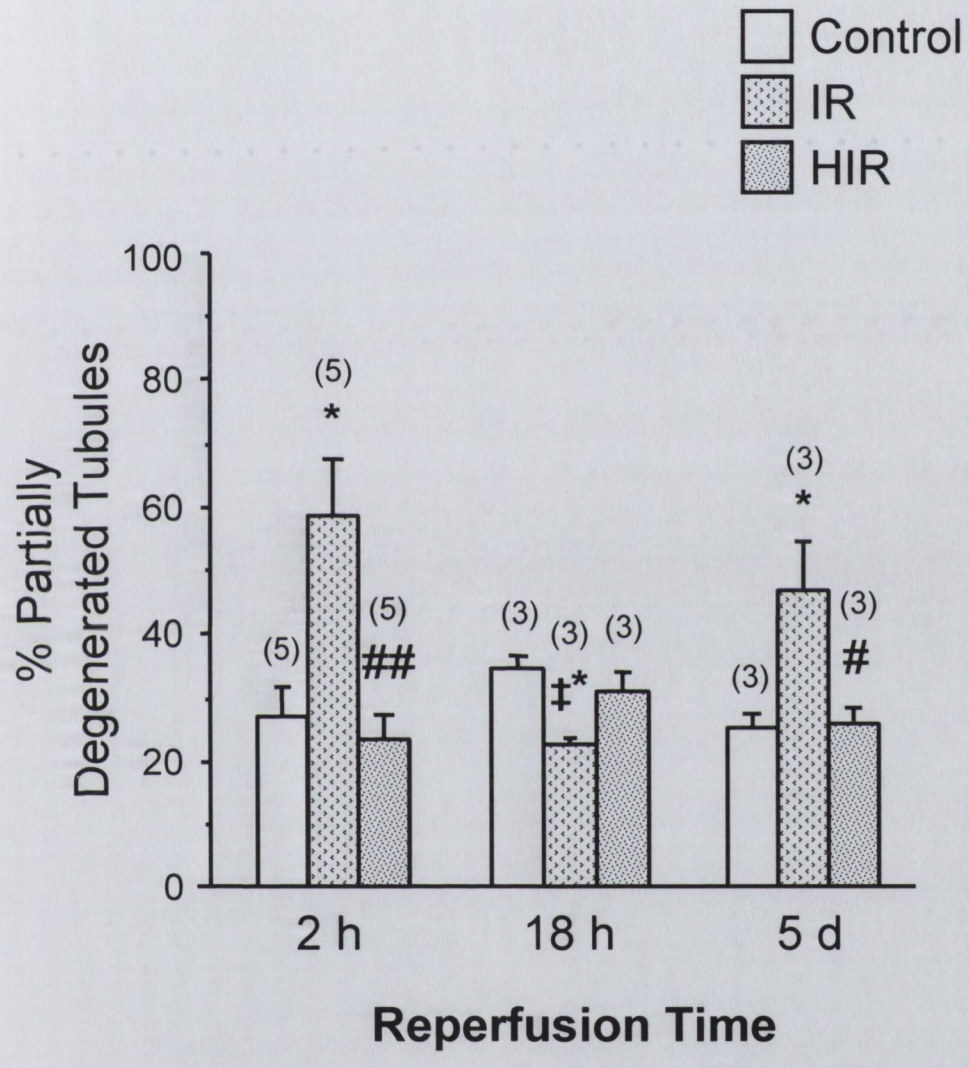


Figure 6.14 Quantification of partial tubular degeneration in the renal cortex of right kidneys that underwent sham ischaemia (control groups) or 45 min ischaemia (IR and HIR groups) followed by 2 h, 18 h and 5 d reperfusion. Rats were exposed to normothermia (control and IR groups) or were preconditioned with 15 min hyperthermia (HIR groups) 18 h prior to surgery. Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; ‡ $P<0.05$ vs. IR-2h; * $P<0.05$ vs. respective controls; # $P<0.05$ vs. IR-5d; ## $P<0.01$ vs. IR-2h.

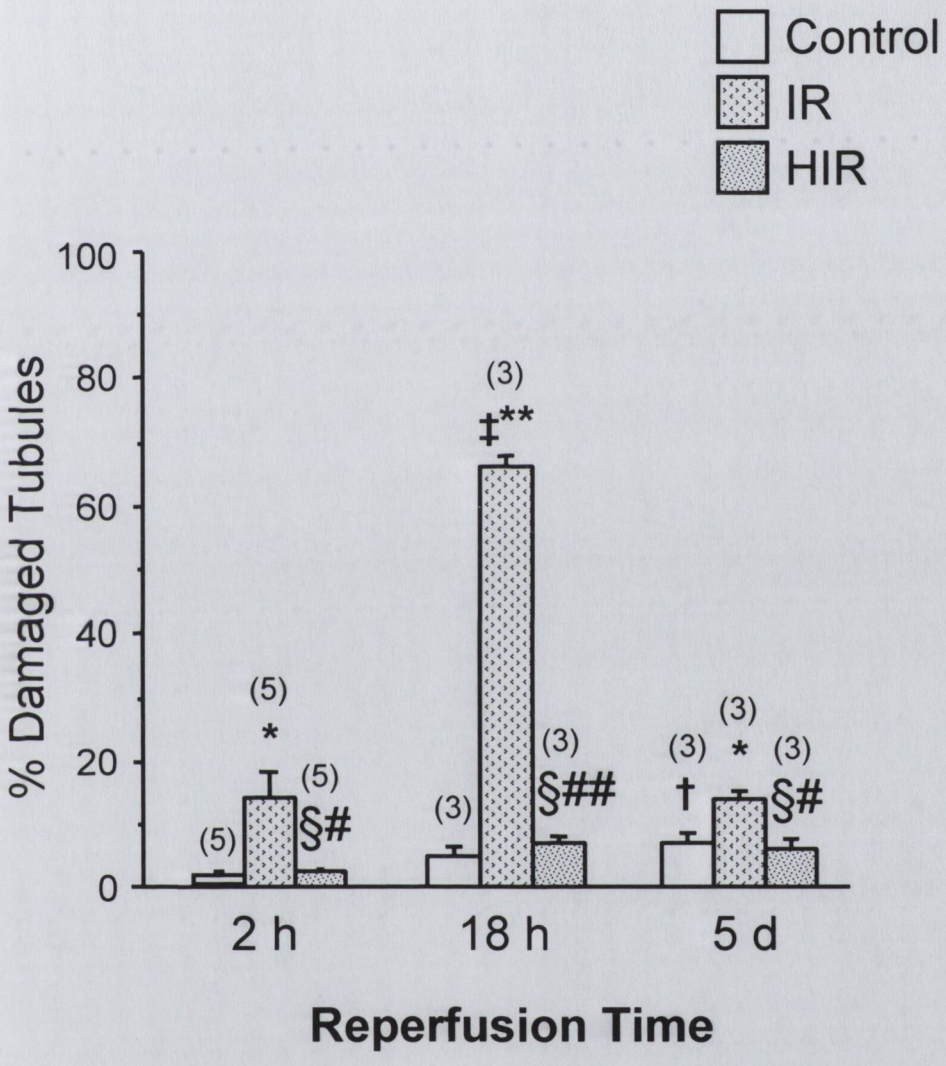


Figure 6.15 Quantification of complete tubular destruction in the renal cortex of right kidneys that underwent sham ischaemia (control groups) or 45 min ischaemia (IR and HIR groups) followed by 2 h, 18 h and 5 d reperfusion. Rats were exposed to normothermia (control and IR groups) or were preconditioned with 15 min hyperthermia (HIR groups) 18 h prior to surgery. Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.05$ vs. C-2h; ‡ $P<0.0001$ vs. IR-2h and IR-5d; § $P<0.05$ vs. HIR-2h; * $P<0.05$ vs. respective controls; ** $P<0.0001$ vs. C-18h; # $P<0.05$ vs. respective controls; ## $P<0.0001$ vs. C-18h.

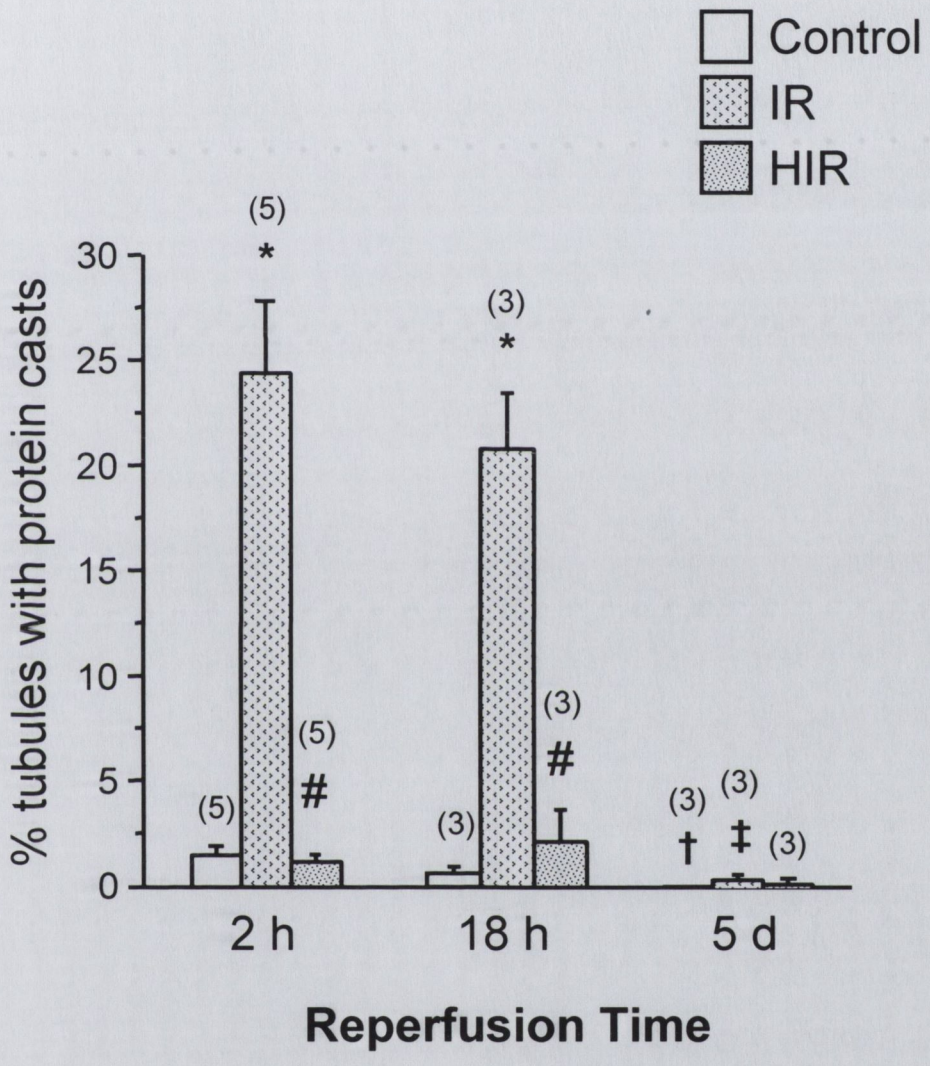


Figure 6.16 The percentage of tubules exhibiting proteinaceous cast formation in the renal cortex of right kidneys that underwent sham ischaemia (control groups) or 45 min ischaemia (IR and HIR groups) followed by 2 h, 18 h and 5 d reperfusion. Rats were exposed to normothermia (control and IR groups) or were preconditioned with 15 min hyperthermia (HIR groups) 18 h prior to surgery. Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.05$ vs. C-2h; ‡ $P<0.005$ vs. IR-2h and IR-18h; * $P<0.0001$ vs. respective control groups; # $P<0.005$ vs. respective IR groups.

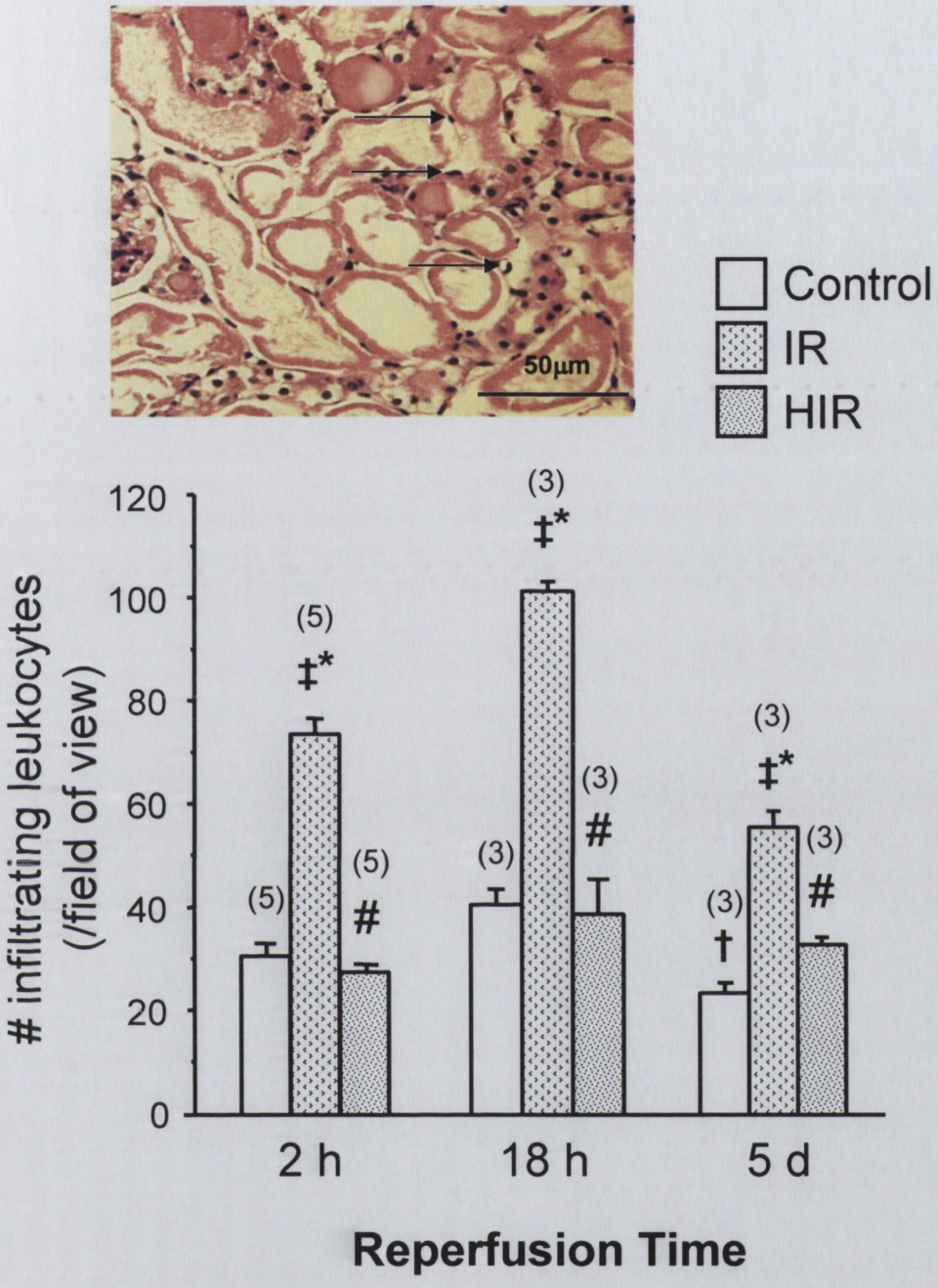


Figure 6.17 Leukocyte infiltration into the renal cortex of right kidneys that underwent sham ischaemia (control groups) or 45 min ischaemia (IR and HIR groups) followed by 2 h, 18 h and 5 d reperfusion. Rats were exposed to normothermia (control and IR groups) or were preconditioned with 15 min hyperthermia (HIR groups) 18 h prior to surgery. Histology represents the renal cortex of an IR-18h rat. Arrows indicate infiltrating leucocytes. Mean±SEM; ANOVA (Scheffe post-hoc test); n numbers are in parentheses; † $P<0.01$ vs. C-18h; ‡ $P<0.01$ vs. all other IR groups; * $P<0.0005$ vs. respective controls; # $P<0.0005$ vs. respective IR groups.

6.3.7 Effect Of Hyperthermic Preconditioning On Ischaemia-Reperfusion-Induced Morphological Damage

Pre-treatment of animals with hyperthermia protected the kidneys from renal IR-induced tubular deterioration, protein cast formation and leucocyte infiltration at all reperfusion times examined.

6.3.7.1 Two Hours Reperfusion

Figures 6.11c and 6.11f represent sections of renal cortex and renal medulla respectively from HIR-2h animals. It can be clearly seen that preconditioning attenuated the renal tubular epithelial and structural degeneration that was observed in the IR-2h group (Figures 6.11b and 6.11e). Only 23.4±3.58% of tubules were partially deteriorated in the HIR-2h animals compared with 58.7±8.94% in the IR-2h group ($P<0.01$) (Figure 6.14) (mean of 33.3±1.59 tubules/field were counted in the HIR-2h group). This was similar to control levels. Complete degeneration of tubules had been reduced from 14.0±3.74% in the IR-2h animals to 2.2±0.36% in preconditioned HIR-2h kidneys ($P<0.01$) (Figure 6.15). Furthermore the percentage of tubules with proteinaceous cast formation was decreased from 24.4±3.47% in the IR-2h animals to only 1.1±0.40% in kidneys preconditioned with hyperthermia ($P<0.0001$) (Figure 6.16). Leucocyte accumulation in the HIR-2h group was significantly reduced from IR-2h levels of 73.8±3.38 cells/field to 27.8±1.63 cells/field ($P<0.0001$) (Figure 6.17). All parameters in the HIR-2h group were comparable to values found in the C-2h group. Red cell congestion in the renal medulla was not as prevalent in the HIR-2h group (Figure 6.11f) versus the IR-2h animals (Figure 6.11e).

6.3.7.2 Eighteen Hours Reperfusion

Figures 6.12c and 6.12f are examples of renal cortex and medulla from HIR-18h animals. The most obvious difference between the IR-18h (Figure 6.12b) and HIR-18h (Figure 6.12c) kidneys was that tubular epithelium was intact, contained nuclei and had good contact with the basement membrane in the preconditioned group (an average of 35.9±2.95 tubules/field were counted in the HIR-18h group). Although partial tubular degeneration was slightly elevated in the HIR-18h group compared with the IR-18h group (Figure 6.14), this was far outweighed by the reduction of complete tubular deterioration from 66.4±1.37% in the IR-18h group to 6.9±1.11% in the HIR-18h group ($P<0.0001$) (Figure 6.15). However this was still significantly greater than levels observed in the HIR-2h group ($P<0.05$). The formation of proteinaceous casts was significantly decreased from 20.72±2.73% in kidneys exposed to IR-18h to only 2.1±1.50% in the preconditioned HIR-18h group ($P<0.005$) (Figure 6.16). In the HIR-18h group, 39.1±6.35 cells/field accumulated in the renal cortex. This was comparable to control levels, but significantly lower than counts in the IR-18h group ($P<0.0005$) (Figure 6.17). The red blood cell congestion of the medulla observed in the IR-18h group (Figure 6.12e) was also reduced in the HIR-18h animals by preconditioning with hyperthermia (Figure 6.12f).

6.3.7.3 Five Days Reperfusion

Figures 6.13c and 6.13f are examples of H&E staining of renal cortex and medulla respectively from HIR-5d kidneys. Tubular architecture has been preserved in these kidneys compared with the IR-5d animals that underwent normothermia followed by IR (Figure 6.13b). In fact there is a striking similarity between the HIR-5d and C-5d groups. Preconditioning with hyperthermia significantly attenuated the IR-induced partial tubular degeneration to 25.5±2.46%, approximately half of the levels observed in IR-5d kidneys

($P<0.05$) (Figure 6.14) (an average of 26.6±0.60 tubules/field were counted in the HIR-5d group). Complete deterioration of tubules was also decreased from 13.4±1.31% in the IR-5d group to 5.6±1.11% in the HIR-5d animals ($P<0.01$) (Figure 6.15), although it remained higher than levels found in the HIR-2h kidneys ($P<0.05$). There was no significant proteinaceous cast formation at this time-point in any of the groups (Figure 6.16). However leucocyte infiltration of the renal tissue was significantly reduced from 55.7±2.91 cells/field in IR-5d animals, to 32.8±1.89% cells/field in the preconditioned HIR-5d kidneys ($P<0.0005$) (Figure 6.17). The medulla of HIR-5d rats (Figure 6.13f) exhibited good structure and very little evidence of red blood cell congestion compared with IR-5d animals (Figure 6.13e).

6.3.8 Effect Of Ischaemia-Reperfusion On ICAM-1 Expression

Ischaemia-reperfusion resulted in a significant increase in ICAM-1 expression on the vascular endothelium after 2 h reperfusion (IR-2h group) compared with the low levels observed in the vessels from C-2h animals, $P<0.05$ (Figures 6.18a-6.18b and 6.18d). There was also appeared to be a low level of ICAM-1 induction on the tubular epithelium in the IR-2h kidneys compared with C-2h tissues (not significant).

6.3.9 Effect Of Hyperthermic Preconditioning On ICAM-1 Expression

Pre-treatment with hyperthermia in the HIR-2h group prevented the IR-induced expression of ICAM-1 on the vascular endothelium ($P<0.05$ versus IR-2h) (Figures 6.18c-d). In fact, ICAM-1 levels in the preconditioned HIR-2h kidneys were similar to the C-2h group. The

elevated ICAM-1 expression found on the tubular epithelium of the IR-2h group was also prevented by hyperthermic preconditioning.

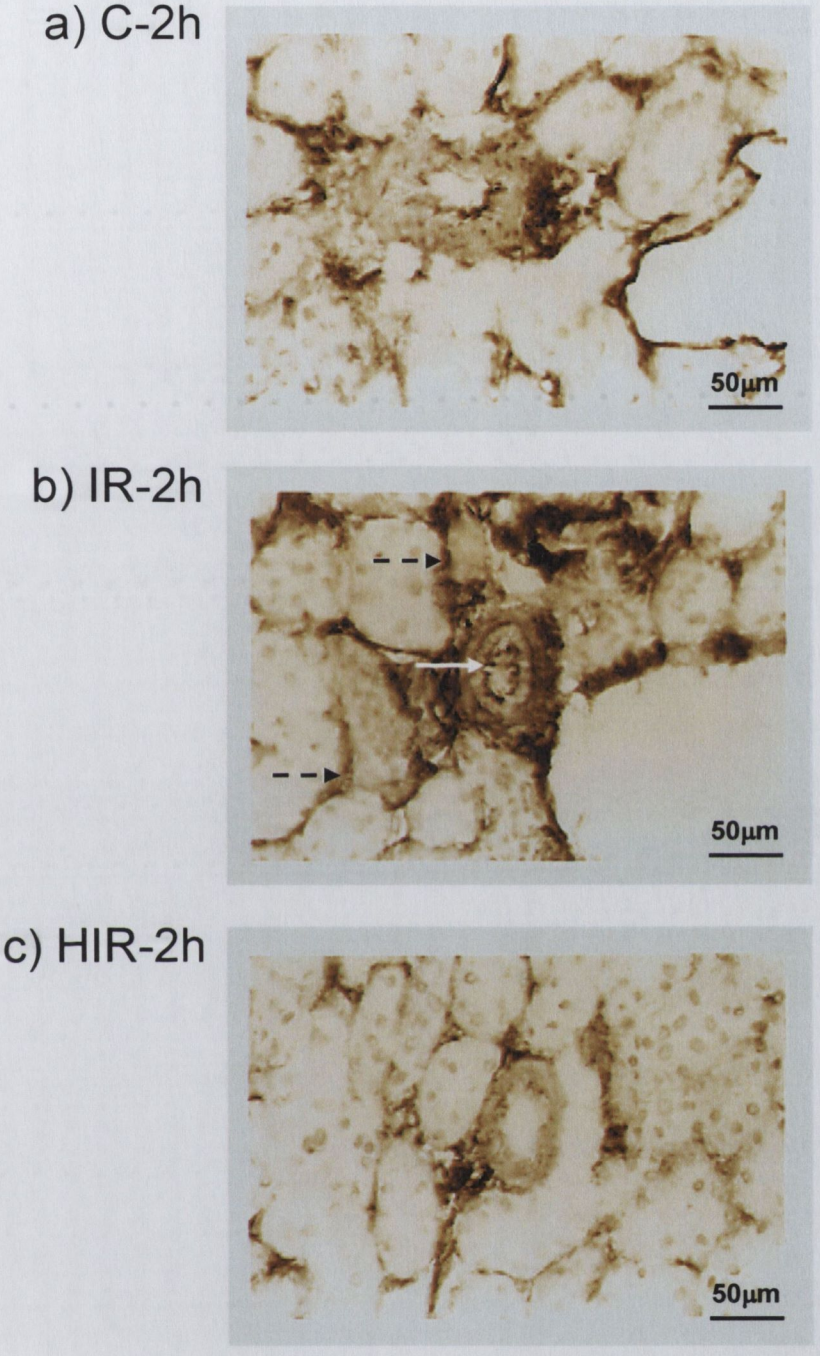


Figure 6.18a-c Representative images of ICAM-1 expression in the renal cortex of the right kidney taken two hours following **a)** sham ischaemia (C-2h group) or **b)** 45 min ischaemia (IR-2h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-2h group **(c)**). Note the staining of the vascular endothelium (solid arrows) and the tubular epithelium (dashed arrows) in the IR-2h group.

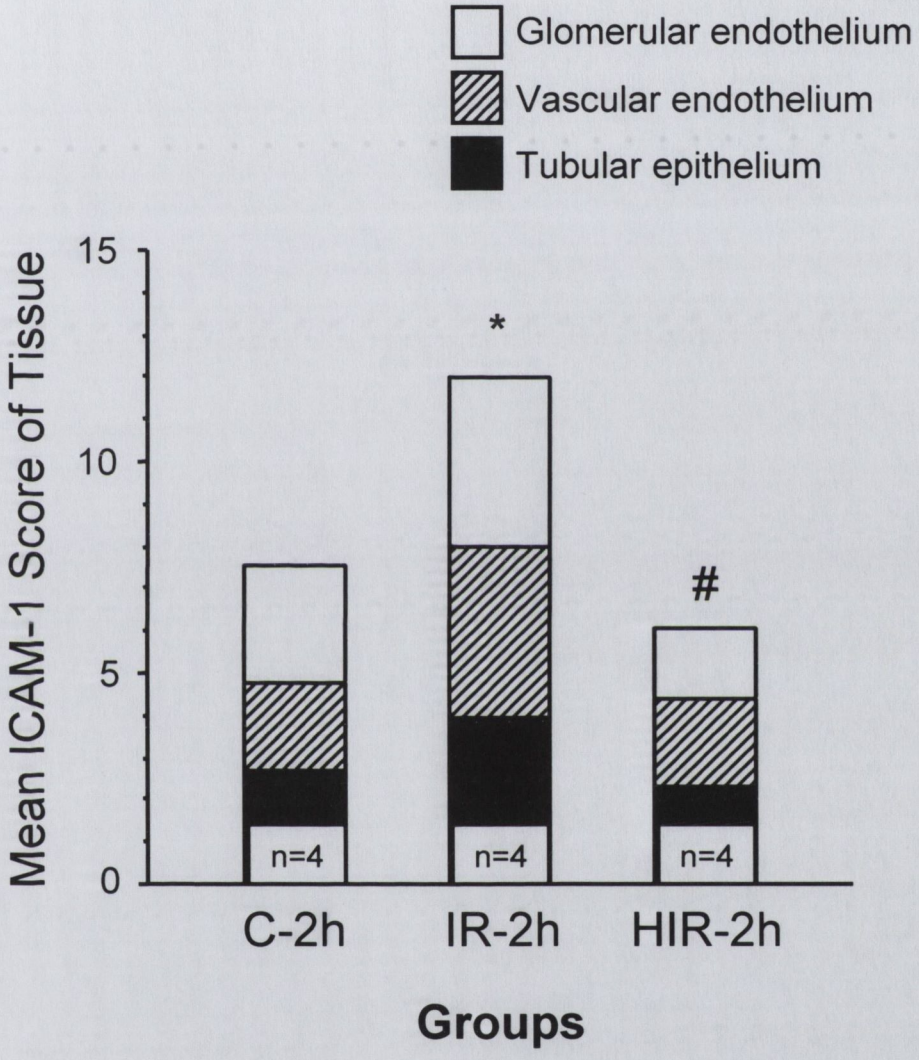


Figure 6.18d Semi-quantitative analysis of ICAM-1 expression on vascular and glomerular tissue and tubular epithelium of the right kidney taken two hours following sham ischaemia (C-2h group) or 45 min ischaemia (IR-2h group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-2h group). Mean±SEM; Chi-squared test; * $P<0.05$ vs. C-2h group for vascular endothelium; # $P<0.05$ vs. IR-2h group for vascular endothelium.

6.3.10 Effect Of Ischaemia-Reperfusion On MHC II Antigen Expression

MHC II was induced on tubular epithelium after 5 days of reperfusion (IR-5d group). This was significantly greater than the expression observed in sham-operated animals (C-5d group) ($P<0.05$) (Figures 6.19a-b, and 6.19d). MHC II expression was not induced on the vascular endothelium of IR-5d kidneys.

6.3.11 Effect Of Hyperthermic Preconditioning On MHC II Antigen Expression

Whole-body preconditioning with hyperthermia in the HIR-5d group (Figure 6.19c) significantly attenuated the induction of class II MHC antigen expression that had been observed in the non-preconditioned IR-5d group ($P<0.05$). Levels of MHC II in the HIR-5d group were similar to levels seen in C-5d animals (Figure 6.19d).

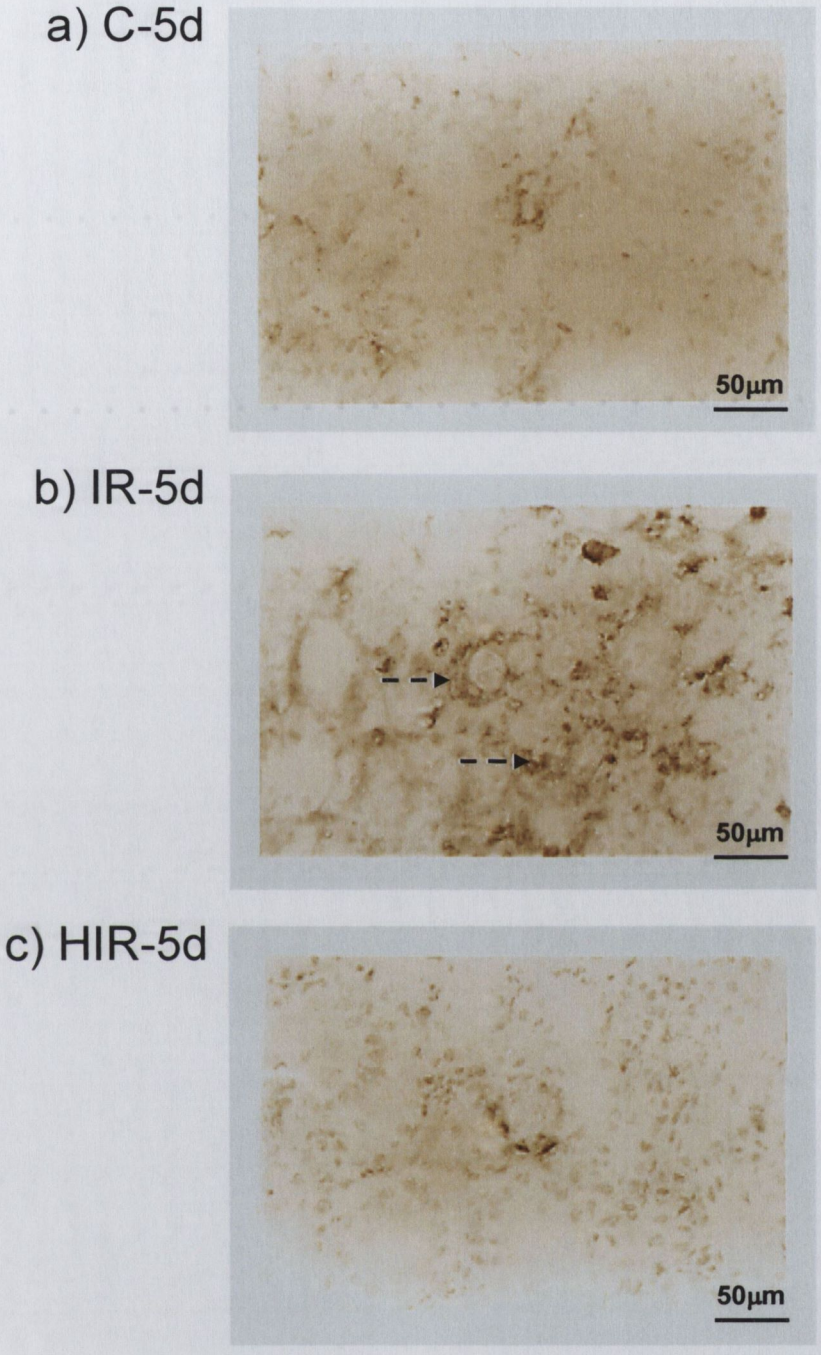


Figure 6.19a-c Representative images of MHC-II expression in the renal cortex of the right kidney taken five days following **a)** sham ischaemia (C-5d group) or **b)** 45 min ischaemia (IR-5d group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-5d group **(c)**). Note the staining of the tubular epithelium (arrows) in the IR-5d group.

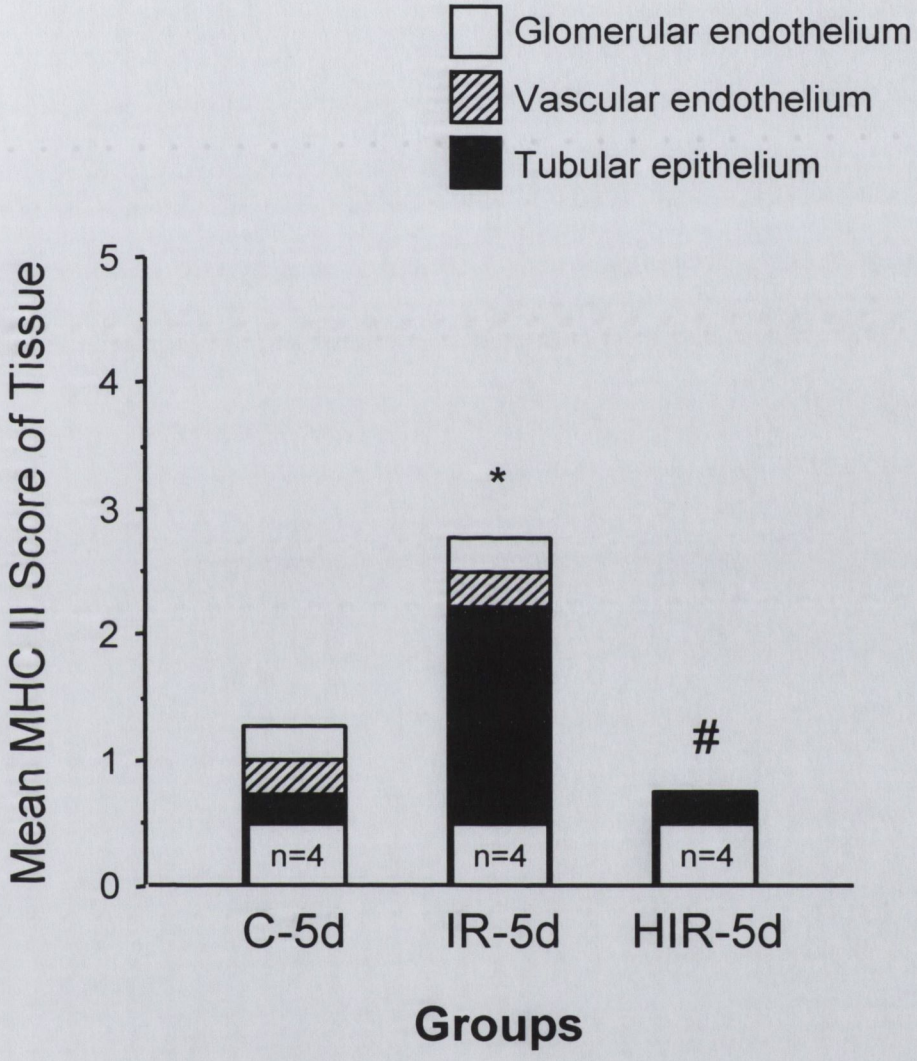


Figure 6.19d Semi-quantitative analysis of MHC II expression on vascular and glomerular tissue and tubular epithelium of the right kidney taken five days following sham ischaemia (C-5d group) or 45 min ischaemia (IR-5d group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-5d group). Mean±SEM; Chi-squared test; * $P<0.05$ vs. C-5d group for tubular epithelium; # $P<0.05$ vs. IR-5d group for tubular epithelium.

6.4 DISCUSSION

During transplantation, IR injury may not only promote organ dysfunction but may also augment alloimmune responses to the grafted tissue thereby predisposing the graft to allograft rejection. This study confirmed that 45 min of renal ischaemia resulted in damage to tubular epithelium, kidney dysfunction, tissue oedema and leucocyte accumulation within 2 h reperfusion. This was associated with elevated ICAM-1 expression and MPO levels. At 18 h reperfusion, mass tubular destruction was observed and renal function had deteriorated further. However by 5 d reperfusion, much of the tubular epithelium had regenerated and renal function had improved, although leucocytes and a large degree of oedema were present. This occurred alongside induction of MHC II on tubular epithelium, supporting suggestions that IR may increase allograft immunogenicity. Preconditioning is whereby a sub-lethal stress protects against a second otherwise lethal stress. Results from chapter 4 indicated that early post-ischaemic injury could be attenuated by preconditioning. This was associated with HSP70 induction. Here, the potential of preconditioning with hyperthermia to protect against renal IR injury for up to 5 d reperfusion was assessed. Hyperthermic preconditioning induced the expression of both HSP60 and HSP70 in the kidney. This was associated with the attenuation of IR-induced renal dysfunction, histological damage and oedema over the 5 d reperfusion period examined. Preconditioning with hyperthermia also reduced ICAM-1 expression at 2 h reperfusion and leucocyte infiltration at all time-points examined. Furthermore, the induction of MHC II expression by IR at 5 d reperfusion was blocked by pre-treatment with hyperthermia.

In the kidney, organ dysfunction is caused by the loss of tubular integrity which leads to decreased GFR. This can be crudely estimated by elevated serum creatinine and urea levels. In this study serum creatinine and urea levels were increased in rats that had undergone 45 min ischaemia followed by just 2 h reperfusion. This worsened by 18 h reperfusion. The obliteration of tubular epithelium in the renal cortex at 18 h reperfusion was also reflected in the perturbation of serum K^+ and Na^+ levels, since Na^+ reabsorption and K^+ secretion occur primarily in the proximal tubules of the renal cortex. However by 5 d reperfusion, regeneration of the tubular epithelium had occurred, and levels of tubular injury were comparable to levels observed at 2 h reperfusion. Renal function recovered accordingly. These findings are in agreement with several studies that have found that post-ischaemic renal alterations peak within 24 h reperfusion and recover thereafter, heading back towards control levels by 1 week reperfusion in the absence of alloantigen-dependent inflammation (Dragun *et al.*, 2000; Ysebaert *et al.*, 2000). Of note, the inclusion of control animals at each reperfusion time-point revealed that a brief period of mild renal dysfunction was present during the first few hours following surgery. This most likely reflected the loss of the left nephrectomised kidney, which appeared to be almost completely corrected for by the right kidney within 18 h reperfusion.

In many organ systems, leucocyte infiltration has been linked to the associated organ dysfunction observed following injury. Leucocytes interact with the vascular endothelium in order to enter the tissue. In fact the vascular endothelium is one of the first sites impacted by an IR insult. This results in increased expression of adhesion molecules such as ICAM-1, followed by leucocyte infiltration of the tissue. The renal vascular endothelium in the donor organ is also the first allogeneic barrier encountered by the circulating leucocytes of the host and it is here that the main attack occurs during allograft

rejection. In the model presented here, leucocyte infiltration exhibited a pattern similar to the dysfunctional and morphological changes, i.e. increased at 2 h, peaked at 18 h and fell back to 2 h levels after 5 d reperfusion. The fact that leucocytes infiltrated the post-ischaemic renal cortex very early during reperfusion, and that this coincided with elevated tissue MPO levels at this time-point, indicated that these leucocytes were likely to be neutrophils as has been previously found (Takada *et al.*, 1997; Dragun *et al.*, 2000). In fact Kelly *et al.* (1996) showed that depletion of neutrophils protected against renal IR-induced elevation of MPO. This was associated with reduced kidney dysfunction and less morphological deterioration. According to studies by Takada *et al.* (1997) and Dragun *et al.* (2000) neutrophil infiltration coincides with early organ dysfunction and subsides within 24-48 h reperfusion. Thereafter, monocytes and lymphocytes begin to enter the kidney, these most likely being the leucocyte populations observed at 5 days reperfusion here. Similar to functional changes, early inflammation subsides approximately a week after implantation in the absence of an alloimmune component during the transplant procedure (Dragun *et al.*, 2000). However in an allografted tissue, while the initial pattern of inflammation is similar to isografts there is a higher level of neutrophil accumulation. Furthermore, unlike the isografts which began to recover after 1 week of reperfusion, inflammation in allografts continues, followed by vascular and cellular rejection. In both isografts and allografts, early functional deterioration peaks at the time of maximum neutrophil infiltration suggesting these leucocytes play an important role during the initial response to injury.

The infiltration of leucocytes into an inflamed tissue is supported by endothelial adhesion molecules such as the adhesion glycoprotein ICAM-1 (Granger *et al.*, 1993), a member of the Ig gene superfamily which binds β2 integrin receptors, especially LFA-1, present on

leucocytes (Rothlein *et al.*, 1986). In agreement with this, the IR insult in our model also caused an upregulation of ICAM-1 expression on renal vascular endothelium and to a lesser extent, tubular epithelium, at 2 h reperfusion, coinciding with early leucocyte accumulation in the kidney. ICAM-1 appears to participate in the pathogenesis of post-ischaemic tissue injury and dysfunction by supporting tissue accumulation of leucocytes, which, along with the vascular endothelium, produce a wide array of pro-inflammatory mediators and harmful substances such as oxidants and arachidonic metabolites that propagate the response. In the kidney, several studies have shown such a role for ICAM-1 in experimental renal IR and isograft models using monoclonal antibodies directed against ICAM-1 (Kelly *et al.*, 1994; Rabb *et al.*, 1995) or one of its CD18 integrin ligands, LFA-1 (Rabb *et al.*, 1994; Kelly *et al.*, 1994; Ghielli *et al.*, 2000). Furthermore, administration of antisense oligodeoxynucleotides for ICAM-1 (Haller *et al.*, 1996; Dragun *et al.*, 1998a; Dragun *et al.*, 1998b; Chen *et al.*, 1999) or the use of ICAM-1 knockout mice (Kelly *et al.*, 1996) conferred protection against renal IR injury. Conversely, when Booster *et al.* (1995) performed a study in a canine model of renal auto-transplantation with prolonged ischaemia (which may be more applicable to transplantation), they found that an antibody against the ICAM-1 ligand, CD18, decreased leucocyte adhesion to vascular endothelium but did not improve post-transplant function or survival (Booster *et al.*, 1995). They suggested that leucocyte-dependent processes may not play as important a role during prolonged ischaemia such as that associated with cold storage during transplantation compared with short ischaemic insults employed in many rodent studies, although this has not been further investigated. Alternatively, other causes for the discrepancies between studies may include species-specific differences. As mentioned below, promising results using ICAM-1-blocking measures in several allograft models with more prolonged ischaemia suggest that the latter may be the case.

ICAM-1 is up-regulated on the renal vasculature during allograft rejection in humans and coincides with leucocyte infiltration of the graft (Anderson, 1997), suggesting that this adhesion molecule may be a promising target for therapeutic strategies in transplantation when an alloimmune component is present alongside the IR injury. Cosimi *et al.* (1990) found that treatment of primates with a monoclonal antibody directed against ICAM-1 reduced T-cell infiltration and attenuated the development of acute allograft rejection. The perfusion of kidneys with antisense oligonucleotides against ICAM-1 before grafting, prolonged survival in rat renal allografts (Stepkowski *et al.*, 1998). Promising results in humans were found in a Phase I trial of an anti-ICAM-1 antibody (Haug *et al.*, 1993). Conversely, Salmela *et al.* (1999) failed to achieve renal functional improvement during acute rejection in humans using a monoclonal antibody directed against ICAM-1. The infiltration of rat renal isografts and allografts by neutrophils and monocytes also coincides with staining for an ICAM-1 ligand, LFA-1, in the renal tissue (Dragun *et al.*, 2000). In contrast to results by Salmela *et al.* (1999), the administration of a monoclonal antibody directed against LFA-1 in human renal allografts provided protection against reduced graft function and acute rejection that was comparable to rabbit anti-thymocyte globulin (Hourmant *et al.*, 1996). Perhaps the discrepancy between these two results is due to the different regimes employed in these studies or to the specificity of the antibodies themselves. Therefore, while ICAM-1 appears to be important in renal IR injury and allograft rejection, and its induction correlates well with rejection episodes, its role in human transplantation is still unclear.

During reperfusion, both activated leucocytes and endothelial cells release many pro-inflammatory mediators, including oxidants, arachidonic acid metabolites and cytokines.

Cytokines e.g. TNF-α and IL-1, may promote the upregulation of CAMs and subsequent leucocyte interactions with the vessel wall, and are released into the circulation following renal IR. In addition, during reperfusion of a previously ischaemic kidney, the cytokines expressed in the tissue change as the type of leucocyte infiltrating the tissue changes. For example, in a model similar to the one used here, Takada *et al.* (1997) demonstrated that during peak lymphocyte infiltration (between 3 and 7 days reperfusion) mRNA of the T-cell-derived cytokines IL-2 and IFN-γ, both of which are capable of inducing MHC II expression, were expressed. As shown in this chapter, MHC II is expressed on renal tubular epithelium and vascular endothelium after 5 days of reperfusion. Shackleton *et al.* (1990) first observed that IR induced the expression of class I and II MHC antigens, and speculated that this may explain the link between IR injury and chronic rejection. While the role, if any, of MHC antigen expression in mediating IR injury is unknown, it is clear that the magnitude of the alloimmune response during transplantation is proportional to the amount of MHC antigen expression (Pfeffer *et al.*, 1987).

Taken together with previously published reports, the data in this chapter support a role for IR in mediating acute and chronic allograft rejection, both by activation of adhesion molecules on the vascular endothelium (Land *et al.*, 1994) and by increasing graft immunogenicity (Shackleton *et al.*, 1990). During rejection, it has been proposed that activated lymphoid and monocytic cells induce the expression of ICAM-1 via release of cytokines such as TNF-α, IL-2 and IFN-γ, which may also promote MHC induction. Interestingly, several studies have demonstrated that both ICAM-1 and class II MHC antigens are associated with acute cellular rejection in human renal allografts (Gonzalez-Posada *et al.*, 1996). Supporting this, another study found that strong expression of ICAM-1, VCAM-1 and MHC II on vascular endothelium and tubular epithelium alongside

infiltrating macrophages and IL-2R-expressing-T-lymphocytes highly correlate with acute renal rejection in humans (Anderson, 1997).

Heat shock proteins are constitutively expressed in normal cells, where they participate in folding, unfolding and translocation of intracellular proteins. However, under stressed conditions their expression in increased, and may confer protection against further stress (Villar *et al.*, 1993; Villar *et al.*, 1994). This study demonstrated that the expression of both HSP60 and HSP70 was increased following hyperthermic preconditioning, peaking by 12 hours and falling thereafter. This method of preconditioning protected the kidney against the subsequent IR insult. The exact mechanism through which HSP induction conferred protection is unknown, however the chaperone properties of HSP70 (a cytosolic and nuclear chaperone) and HSP60 (serves a chaperone function in the mitochondria) may be important (Snoeckx *et al.*, 2001). While evidence for a role for HSP60 in preconditioning is limited, many studies have supported a role for HSP70.

Emami *et al.* (1991) found that the elevation of core body temperature to 42±0.5°C, for 15 min, leads to induction of the cytoprotectant HSP70 in the rat kidney, peaking at 4-6 h after heat stress and persisting for 10 days. The 18 h delay between induction of thermotolerance and the IR insult in this study was based on previous studies which have demonstrated this to be the optimal timing for protection in several organs (Villar *et al.*, 1993; Villar *et al.*, 1994; Javadpour *et al.*, 1998) as well as studies suggesting that maximum protection against subsequent injury occurs when the insult is given after the peak expression of HSP70, when approximately half this expression is evident (Landry *et al.*, 1982; Kim *et al.*, 1993). In this study IR-induced renal functional impairment was significantly reduced by raising the animal's core body temperature to 41±0.5°C for 15

min, 18 h before IR injury. Induction of the stress response also attenuated the IR-induced oedema and morphological damage within the first 18 h reperfusion. Functional deterioration at 5 d reperfusion was also reduced in preconditioned compared with non-preconditioned IR animals, although serum creatinine levels were significantly higher than control levels. It is unlikely that this was due simply to a delay of the response to IR rather than an actual attenuation of post-ischaemic injury. If this was the case, it would be expected that the HIR group would exhibit some evidence of tubular injury (as detected morphologically) at 5 days reperfusion, before full regeneration had occurred. However, hyperthermic preconditioning completely attenuated morphological damage at 5 d reperfusion. Furthermore, other findings have shown that hyperthermic preconditioning can protect kidneys against post-ischaemic injury for up to 60 days. In the kidney, Perdrizet *et al.* (1989) were able to show that hyperthermic preconditioning not only improved survival in syngeneic grafts but also attenuated IR injury in allografts (Perdrizet *et al.*, 1993). They found that HSP70 induction was associated with improved renal function, as demonstrated by reduced serum creatinine levels. However, they did not further address the pathway through which this protection occurred. Although there has been very little attention paid to the role of HSP60 in the protection conferred by hyperthermic preconditioning against subsequent IR injury or allograft dysfunction, one study demonstrated that preconditioning animals for 30 min followed by recovery for 2 h improved post-transplant function of the heart. This was associated with increased cardiac expression of HSP60 (Tamaki T *et al.*, 1997). However, this group did not examine HSP70 expression in these animals therefore HSP70 could not be excluded as a mediator of protection in that model. The study presented in this chapter not only assessed the ability of hyperthermic preconditioning to attenuate IR injury over the first 5 d reperfusion, but also investigated potential mechanisms of protection.

It was found that in addition to protecting organ function, prior hyperthermia also decreased the expression of ICAM-1 and class II MHC antigen and reduced leucocyte infiltration following IR in the kidney. Since IR has been implicated in both acute and chronic allograft rejection (Land *et al.*, 1994) and both ICAM-1 and MHC II are associated with allograft rejection (Gonzalez-Posada *et al.*, 1996), the findings of this study support others that have suggested that IR predisposes the transplanted organ to acute cellular rejection by increasing ICAM-1 and class II expression. The most novel results here indicate that a potential mechanism through which preconditioning may preserve renal function following IR in this model and may in fact decrease acute rejection is through the inhibition of ICAM-1 and MHC II induction by IR. In fact, the initial absence of ICAM-1 and therefore attenuated leucocyte infiltration may be responsible for the subsequent lack of MHC II induction. The intimate relationship between ICAM-1 and MHC II was evident in a study by Dragun *et al.* (1998a) where ICAM-1 upregulation was blocked by antisense oligonucleotides in a rat renal autograft model. This treatment not only preserved renal function and decreased leucocyte infiltration due to IR but also prevented the associated upregulation of MHC II antigen, implicating that leucocyte-endothelial interactions play an important role in subsequent MHC II expression. Host lymphocytes can adhere to the graft endothelium via an LFA-1/ICAM-1 interaction and so the upregulation of ICAM-1 may be an important first step for these cells to interact with the graft. These lymphocytes then mediate the MHC-associated attack of the graft in part by promoting MHC expression on the graft cells via the release of cytokines such as TNF-α, IL-2 and IFN-γ. The findings here suggest that treatment of renal allografts prior to transplantation to prevent the upregulation of these two antigens may provide a novel approach to attenuate subsequent rejection.

Recently, Kelly *et al.* (2001) supported the observations here that hyperthermic preconditioning improved post-ischaemic renal function, elevated MPO levels and histological alterations after 48 h reperfusion. Furthermore, they found that this was associated with HSP22 and HSP70 mRNA expression. Both HSP mRNA expression and the associated protection were blocked by prior treatment with the bioflavanoid, quercetin, which has been shown to block HSP induction at both transcriptional and post-transcriptional levels, thereby supporting a role for HSPs in the observed protection. The reduction of MPO levels is in agreement with findings here and in other studies (Javadpour *et al.*, 1998), and reflected decreased leucocyte infiltration of the post-ischaemic organ. In fact, a study of leucocyte adhesion and emigration in mesenteric post-capillary venules following IR showed that leucocyte-endothelial interactions are significantly decreased by preconditioning with hyperthermia (Chen *et al.*, 1997). Although not addressed elsewhere, the attenuation of ICAM-1 expression observed in this chapter may explain the protection seen in the above reports.

While many studies disagree on whether induction of HSPs alone after preconditioning is enough to protect against subsequent injury, there has been substantial evidence suggesting that HSPs themselves are an important component of the protective mechanism induced by preconditioning. Studies using HSP70 transfection of renal tubular cells (Turman and Rosenfeld, 1999), coronary endothelial cells (Suzuki *et al.*, 1998) or whole organs e.g. heart (Suzuki *et al.*, 2000; Jayakumar *et al.*, 2001) have indicated that this HSP can itself mediate protection against subsequent injury. In fact the injection of fibroblasts with antibodies against HSP70 prevents the development of thermotolerance in these cells, supporting a role for HSP70 in mediating preconditioning (Riabowol *et al.*, 1988).

Furthermore, transgenic mice over-expressing human (Plumier *et al.*, 1995) or rat (Marber *et al.*, 1995) HSP70 exhibited reduced cardiac IR injury.

So exactly how do the HSPs confer this protection? Several pathways have been proposed. The chaperone role that HSPs play in normal cells may be more prominent following injury when many proteins are damaged or destroyed. The HSPs bind these proteins and hold them for repair or for subsequent degradation preventing them from causing further damage to the cell. However it is not widely accepted that this is enough. *In vitro* data has demonstrated that HSPs protect cells from ICAM-1 induction (Kohn *et al.*, 2002) and lysis by TNF-α (Kusher *et al.*, 1990). Expression of ICAM-1 is regulated by several cytokines, IL-1, TNF-α and IFN-γ (Dustin *et al.*, 1986), thus hyperthermic preconditioning may decrease ICAM-1 via a reduction of cytokine synthesis. Furthermore, since heat-treated cells retain most of their capacity to bind TNF-α, it is likely that heat shock alters cellular sensitivity to TNF-α by controlling protein synthesis of this cytokine.

Alternatively, hyperthermic preconditioning may act in a more direct manner by preventing ICAM-1 gene transcription. Hyperthermic pre-treatment to induce HSP70 expression has recently been shown to inhibit I-κB degradation and therefore subsequent translocation of the transcription factor NF-κB to the nucleus (Kohn *et al.*, 2002). This was associated with decreased TNF-α-induced ICAM-1 expression in vascular endothelial cells. Cytokines such as TNF-α, IL-2 and IFN-γ also promote the induction of MHC antigens through an NF-kB-dependent pathway. Other findings suggest that HSF1 may actually act as a transcriptional repressor, by binding to the proximal promotor and/or untranslated region of gene, preventing its transcription (Cahill *et al.*, 1996; Singh *et al.*, 2000). Thus it is conceivable that attenuation of MHC II expression by hyperthermic

preconditioning may be due to both decreased ICAM-1 expression which results in decreased leucocyte infiltration and cytokine release, and by reducing the actual transcription of MHC II. While the possibility remains for ICAM-1, the HSP70 expression may have fallen beyond levels that could actively protect against gene transcription by 5 d reperfusion, thus this is a less likely mechanism for prevention of MHC II induction.

Another potential pathway of protection in our model is suggested by a recent study that demonstrated that transfection of rat hearts with HSP70 was associated with increased activity of ecto-5'-nucleotidase, an enzyme involved in the synthesis of adenosine (Sakaguchi *et al.*, 2000). Adenosine is a purine nucleotide with potent anti-inflammatory properties including the attenuation of ICAM-1 expression and leucocyte-endothelial interactions. Furthermore this molecule has been implicated in IPrec in the heart. Although its role in IPrec in the kidney is doubtful (Lee and Emala, 2001), pre-treatment of animals with adenosine or adenosine receptor agonists protects against renal IR-induced dysfunction (Lee and Emala, 2000). These studies offer important insights into how IR-induced upregulation of ICAM-1 and MHC II in the kidney may be blocked in our model of hyperthermic preconditioning.

An additional leucocyte- and CAM-independent explanation for the role of HSP70 in the protection of renal integrity following IR comes from a study demonstrating that HSP70 interacts with cytoskeletal elements during the restoration of proximal tubule cell structure and polarity after renal ischaemia (Bidmon *et al.*, 2000). They found that HSP70-dependent stabilization of Na,K-ATPase occurred within the cytoskeletal fraction of the kidney following 45 min ischaemia and 18 h reperfusion. Therefore it is possible that HSP70 also acts directly to preserve renal integrity and function.

Several other studies suggest alternative pathways through which preconditioning may offer protection, including the induction of antioxidants. In fact, free radicals are produced by heat stress and can transduce the heat shock signal in mammalian cells. Thus a redox mechanism may be involved in the acquisition of this tolerance via the induction of both endogenous scavengers of reactive oxygen species (Karmazyn *et al.*, 1990; Yamashita *et al.*, 1998) such as catalase (Currie *et al.*, 1988), SOD (Hass and Massaro, 1989; Yamashita *et al.*, 1998), and GSH.Px (which themselves have been shown to protect against IR injury) as well as rescue proteins such as HSPs. While not measured here, it is plausible that hyperthermic preconditioning in the HIR group described here is acting through antioxidant induction thereby attenuating oxidant stress. This in turn blocks endothelial activation and ICAM-1 induction following IR. This may be relevant in renal IR injury as rats treated with SOD via adenovirus gene delivery exhibited improved function and morphology following 60 min renal ischaemia and two weeks reperfusion (Yin *et al.*, 2001). Furthermore, a clinical study of renal transplantation demonstrated significant reductions in the incidence of both acute and chronic rejection episodes with the use of SOD in the organ preservation solution (Land *et al.*, 1994). In fact, organ preservation solutions employed during transplantation contain several antioxidants. Conversely, some studies suggest that antioxidant enzymes induced by a preconditioning insult may not actually participate in HSP-mediated protection. For example, hyperthermia-induced overexpression of HSPs attenuates myocardial injury and oxidative injury independently of the associated increase in catalase activity (Chong *et al.*, 1998).

Performing whole body hyperthermia on humans as was carried out on rats in this study is obviously not a clinically viable method of preconditioning. However, HSP induction may

provide a promising approach to preventative treatment. Alternative methods of HSP induction include pharmacological inducers of these proteins. However, some of these substances e.g. amphetamines (Maulik *et al.*, 1995) may give rise to serious clinical problems themselves. Recent advances have highlighted the therapeutic potential of adenovirus gene delivery, which may overcome the problems associated with specificity and side effects that limit many current treatments. For example, Jayakumar *et al.* (2001) demonstrated that gene transfer of HSP70 conferred protection against subsequent IR injury in the heart. This raises the possibility that gene transfer of HSP70 at the time of organ retrieval may attenuate the IR injury associated with the transplantation process. A recent study by Lin *et al.* (2001) provided an alternative method to gene therapy. They exposed the right seventh intercostal nerve territory to local somatothermal stimulation which in turn induced HSP70 expression in the liver. This treatment protected the liver from subsequent IR-induced dysfunction, further underlining the potential of HSP70 induction in the prevention of IR injury. The results in this chapter suggest that HSP70 induction may attenuate IR-induced tissue deterioration and subsequent organ dysfunction by inhibiting renal leucocyte infiltration. This occurred via the reduction of ICAM-1 induction, which may also be at least partially responsible for the blocking of MHC II expression on the renal epithelium. Despite the mounting evidence for a protective effect of preconditioning against early IR injury and early allograft dysfunction, it remains to be seen whether protection extends beyond the immediate post-operative period. This is addressed in Chapter 7.

Chapter 7

The Impact Of Hyperthermic Preconditioning On Chronic Ischaemia-Reperfusion-Induced Injury In The Kidney

7.1 INTRODUCTION

Ischaemia and reperfusion is an integral part of the transplantation process. However, IR may not only cause injury to the transplanted organ but may predispose the organ to allograft rejection by activating the graft endothelium and increasing graft immunogenicity. As demonstrated in chapter 6, kidneys exposed to IR exhibit morphological damage that is manifested by impaired renal function. Furthermore, upregulation of ICAM-1 and class II MHC were observed in this model, features that are also present during renal allograft rejection. This may promote increased T-lymphocyte infiltration of the graft thereby strengthening the immune response to the transplanted tissue. Thus, the attenuation of IR injury during the transplant process may reduce the frequency and severity of allograft rejection accordingly. Preconditioning with hyperthermia attenuated the renal IR injury and associated ICAM-1 and MHC II induction in the model presented in chapter 6. This protection was associated with the induction of HSP70 and HSP60. However while several methods of preconditioning have proven protective against early post-ischaemic or post-transplant injury (Perdrizet *et al.*, 1993; Kelly *et al.*, 2001; Redaelli *et al.*, 2001), it is unknown whether this treatment has a prolonged benefit. Thus this chapter addresses the potential of hyperthermic preconditioning to attenuate chronic IR-induced changes in the rat kidney.

Although there has been a significant improvement in short-term graft survival following renal transplantation, the long-term prognosis has not demonstrated such marked progress. Several studies suggest the first year of graft survival is impacted most strongly by IR, which promotes early graft loss by causing DGF and acute rejection of grafted organs (Kouwenhoven *et al.*, 2001). However, other studies have suggested that IR also

influences long-term allograft outcome (Roodnat *et al.*, 2003). Prolonged ischaemia, as well as DGF and acute rejection may act synergistically to predispose allografts to chronic rejection and therefore reduced long-term graft survival. This is most likely to be due to the inflammatory response initiated by IR and propagated during subsequent acute rejection episodes. In fact, Terasaki *et al.* (1995) found that grafts from living donors, both related and non-related donors i.e. with or without a strong alloimmune component, exhibited superior survival rates over renal allografts from cadaveric donors which had longer ischaemic times, implicating IR injury in the graft outcome.

When the morphological and functional responses of renal isografts were compared to renal allografts with comparable ischaemic times and storage conditions, similar histological and functional deterioration as well as CAM expression were observed over the first week of reperfusion. This resolved over the second week in isografts whereas allografts that had received no immunosuppression began to show signs of cellular and vascular rejection. Immunosuppression attenuated the acute rejection episodes but not the IR-induced inflammatory responses in allografts (Dragun *et al.*, 2000). Taken together, this evidence suggests that early damage following implantation reflects IR injury rather than the alloimmune response to the graft. These early changes can be blocked by treatment of grafts with either antisense oligodeoxynucleotide directed against ICAM-1 (Dragun *et al.*, 1998a) or recombinant soluble PSGL-1 (Takada *et al.*, 1997a), both of which inhibit early leucocyte-endothelial cell interactions.

Chronic studies in renal IR models, isografts and allografts in the rat revealed that similar long-term morphological and functional changes occur following IR and in isografts, when compared with allografts, albeit these occur in a shorter time period in the latter

(Tullius *et al.*, 1994a). However, the kinetics of IR-induced injury varied significantly between studies depending on the ischaemic times and the models employed. Two groups who used a renal IR model with a similar ischaemic time to the one used in this thesis, i.e. 45 min ischaemia, found remarkably earlier histological and functional changes compared with Tullius *et al.* (1994a) who used 30 min warm ischaemia followed by 30 min cold ischaemia. Takada *et al.* (1997b) and Azuma *et al.* (1997b) demonstrated that 45 min of cold or warm ischaemia respectively led to progressive proteinuria which was evident within 16 weeks of reperfusion. At this time morphological deterioration was evident, with tubular atrophy and a moderate number of sclerotic glomeruli. Leucocytes infiltrated the post-ischaemic kidney alongside upregulation of ICAM-1. Both functional and morphological parameters continued to worsen as reperfusion time continued.

In a follow-up to the acute studies, the administration of recombinant soluble PSGL-1 during ischaemia and after three hours of reperfusion prevented the chronic responses to IR that were observed by Takada *et al.* (1997a). Similarly, in an isograft model where kidneys were exposed to one hour of warm ischaemia followed by 30 min cold ischaemia, Dragun *et al.* (1998b) showed that treatment of the donors with antisense oligodeoxynucleotide against ICAM-1 6 hours before removal of the kidney for transplantation attenuated the increases in serum creatinine concentrations and urine protein excretion that were observed at every reperfusion time-point examined (2, 4, 6, 8, 12, 16, 20 weeks). The accompanying histological damage, macrophage and lymphocyte infiltration and MHC II induction that were observed in non-treated isografts were all decreased by this treatment. Furthermore, there was a corresponding increase in graft survival. These studies suggest that early leucocyte-endothelial interactions, which are involved in the immediate post-ischaemic injury and acute rejection, may be important in

triggering the later functional, morphological and molecular changes that lead to rejection of allografts.

In chapter 6, it was found that preconditioning with hyperthermia to induce HSP70 in the kidney at the time of an IR insult protected against subsequent tubular injury and renal dysfunction over the first 5 days reperfusion. The IR-induced upregulation of ICAM-1 and tissue leucocyte infiltration were also decreased. Furthermore, MHC II induction was attenuated by this treatment. Whether reducing early IR-induced leucocyte-endothelial interactions and organ injury by preconditioning leads to prolonged protection against renal deterioration following IR, has not yet been investigated. Thus the aims of this chapter are to assess whether 45 min of ischaemia followed by 16 weeks of reperfusion results in significant functional and/or morphological injury in the kidney, as has been described by Azuma *et al.* (1997b) and Takada *et al.* (1997a), and to investigate the efficacy of preconditioning with hyperthermia to attenuate these IR-induced alterations. Renal IR was accomplished as described in chapter 2. Hyperthermic preconditioning was performed as detailed in chapter 6. Renal function was assessed by serum creatinine, urea, K^+, Na^+ and Cl^- levels, 24 h urine collection and urine protein levels. Oedema and hypertrophy were evaluated using the percentage change in kidney weight and body weight. Morphological assessment was carried out using H&E stained sections of the experimental kidneys. In addition Masson's trichrome staining was performed to determine if fibrosis was present in the renal tissue.

7.2 METHODS

7.2.1 Hyperthermic Preconditioning

Eighteen male Sprague-Dawley rats weighing 200-250 g were randomised into three group (n=6/group): C-16wk, IR-16wk and HIR-16wk. The C-16wk and IR-16wk groups were exposed to normothermia i.e. 37±0.5°C for 15 min, and the HIR-16wk group was preconditioned by raising the core body temperature of rats to 41±0.5°C for 15 min as described in section 6.2.1. Eighteen hours later, all animals were anaesthetized with inhalational halothane and underwent a left uninephrectomy (Figure 7.1). Samples of these kidneys were snap-frozen for subsequent assessment of HSP70 expression (section 2.5). C-16wk animals were then subjected to a sham IR protocol, while IR-16wk and HIR-16wk groups underwent 45 min ischaemia as per section 3.2.

7.2.2 Chronic Reperfusion Injury

After 15 weeks 6 days reperfusion, animals were placed in metabolic chambers to measure U/O (see section 2.1.2). Twenty-four hours later, animals were removed from the chambers. Observations were made at 16 wk reperfusion based on the findings by Azuma *et al.* (1997b) who found that chronic renal alterations began at 8 wk reperfusion with the development of proteinuria which continued to deteriorate thereafter. They also demonstrated that ICAM-1 expression increased at 8 wk, peaking at 16 wk and that morphological changes became apparent at 16 wk reperfusion. Each rat was weighed to determine the % body weight gain over the 16 week reperfusion period. Animals were

then anaesthetized, blood and urine were taken and the right kidney was harvested as outlined in section 2.2. The animals were then sacrificed.

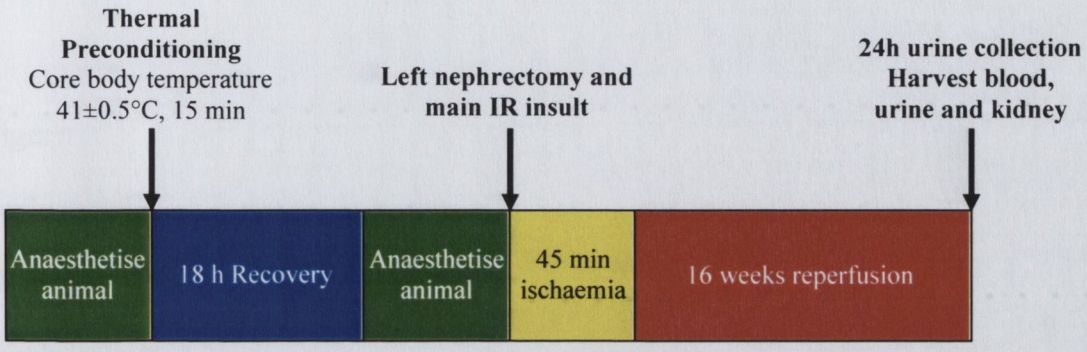


Figure 7.1 Diagram depicting the time-course of hyperthermic preconditioning followed by chronic renal IR injury.

Serum levels of creatinine, urea, K^+, Na^+ and Cl^-, and urine protein levels were measured (see section 2.1). Weight differences between the left kidneys removed before ischaemia, and the right kidneys removed after 16 weeks of sham IR (C-16wk group) or IR (IR-16wk and HIR-16wk groups) were calculated as described in section 2.2. A portion of the right kidney was fixed in 10% formalin for staining with H&E (for histological analysis of tubular structure, protein cast formation, numbers of infiltrating leucocytes) or with Masson's trichrome (for the assessment of fibrosis) (see section 2.4.2).

7.2.3 Statistics

All data is expressed as mean±SEM. ANOVA with Scheffe post-hoc test was performed on all parameters to compare between groups.

7.3 RESULTS

7.3.1 Effect Of Hyperthermic Preconditioning On HSP70 Expression

Figure 7.2 shows an example of a western immunoblot of kidney tissue from the left nephrectomy in non-preconditioned C16-wk and IR-16wk animals and preconditioned HIR-16wk animals. These demonstrated that preconditioning with hyperthermia (HIR-16wk group) induced the expression of HSP70 whereas both the C-16wk and IR-16wk groups (exposed to normothermia) did not express this protein at the time the IR insult was to be applied.

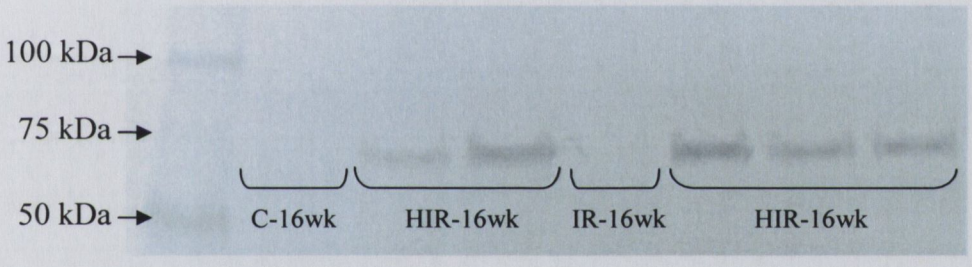


Figure 7.2 Western immunoblot for HSP70. Animals underwent normothermia (C-16wk and IR-16wk groups) or hyperthermia (HIR-16wk group) for 15 min 18 h prior to removal of the left kidneys. Protein was isolated from the left kidneys and a western immunoblot was performed to determine the absence or presence of HSP70 expression (represented by dark bands).

7.3.2 Long-term Influences of Renal IR on Kidney Function

Serum creatinine (Figure 7.3) and urea levels (Figure 7.4) were unchanged in rats exposed to renal ischaemia followed by 16 weeks of reperfusion (IR-16wk group) versus animals that underwent sham IR (C-16wk group). Serum electrolyte levels, namely K^+, Na^+ and Cl^- (Table 7.1) were comparable in the C-16wk and IR-16wk groups. Urine output (Figure 7.5) was similarly unaffected by IR. Urine protein levels were higher in the IR-16wk group compared with the C-16wk group, although this was not significant (Figure 7.6).

7.3.3 Effects Of Hyperthermic Preconditioning On Renal Function Following IR

Serum levels of creatinine (Figure 7.3) and urea (Figure 7.4) were unaltered by preconditioning with hyperthermia (the HIR-16wk group), compared with either the C-16wk or IR-16wk group. Serum K^+, Na^+ and Cl^- levels, in the HIR-16wk group were also similar to C-16wk and IR-16wk values (Table 7.1). Urine output remained unchanged by hyperthermic preconditioning versus the non-preconditioned two groups (Figure 7.5). Urine protein content in the HIR-16wk group was lower than levels in the IR-16wk, but comparable to the C-16wk group (Figure 7.6).

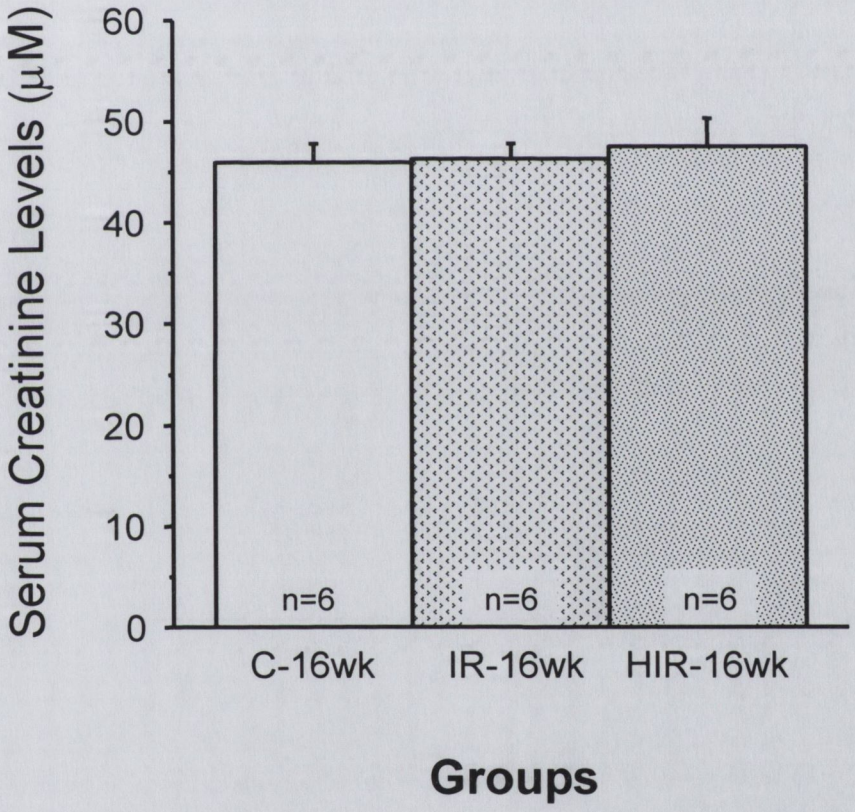


Figure 7.3 Serum creatinine levels measured at sixteen weeks reperfusion following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). Mean±SEM; ANOVA (Scheffe post-hoc test).

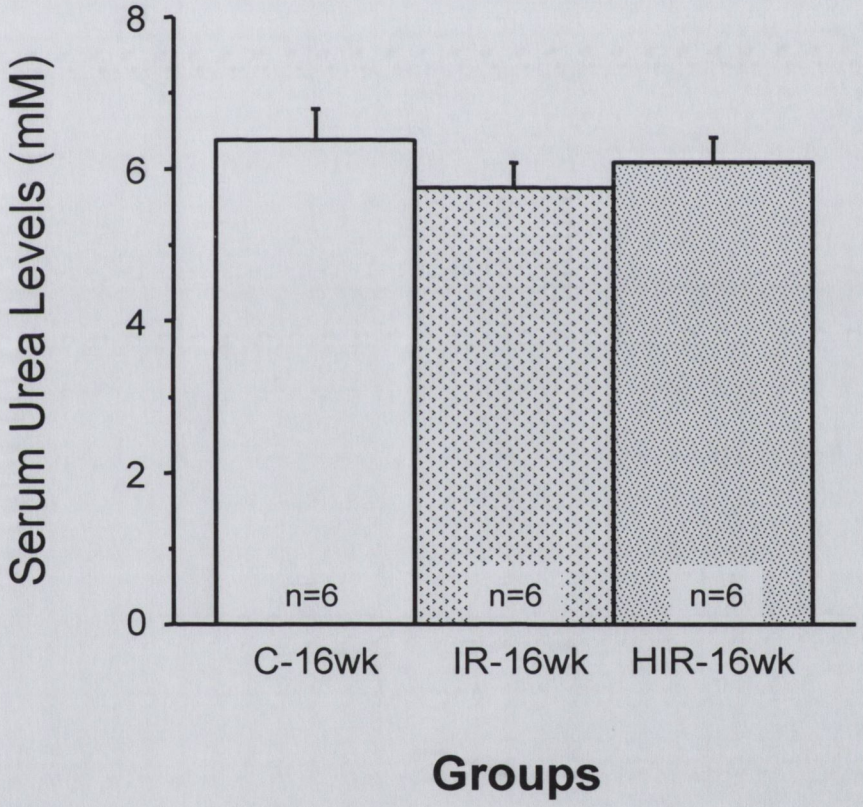


Figure 7.4 Serum urea levels measured at sixteen weeks reperfusion following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). Mean±SEM; ANOVA (Scheffe post-hoc test).

Table 7.1 Serum electrolyte levels (K^+, Na^+, Cl^-) measured at sixteen weeks reperfusion following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk and HIR-16wk groups) of the right kidney. The HIR-16wk group was preconditioned with 15 min hyperthermia 18 h prior to the IR insult. Mean±SEM; ANOVA (Scheffe post-hoc test); n=6/group.

Groups	K^+ (mM)	Na^+ (mM)	Cl^- (mM)
C-16wk	4.3±0.18	141.4±0.60	100.6±0.03
IR-16wk	3.9±0.14	142.7±0.33	102.3±0.62
HIR-wk	4.5±0.17	141.3±0.50	103.8±0.75

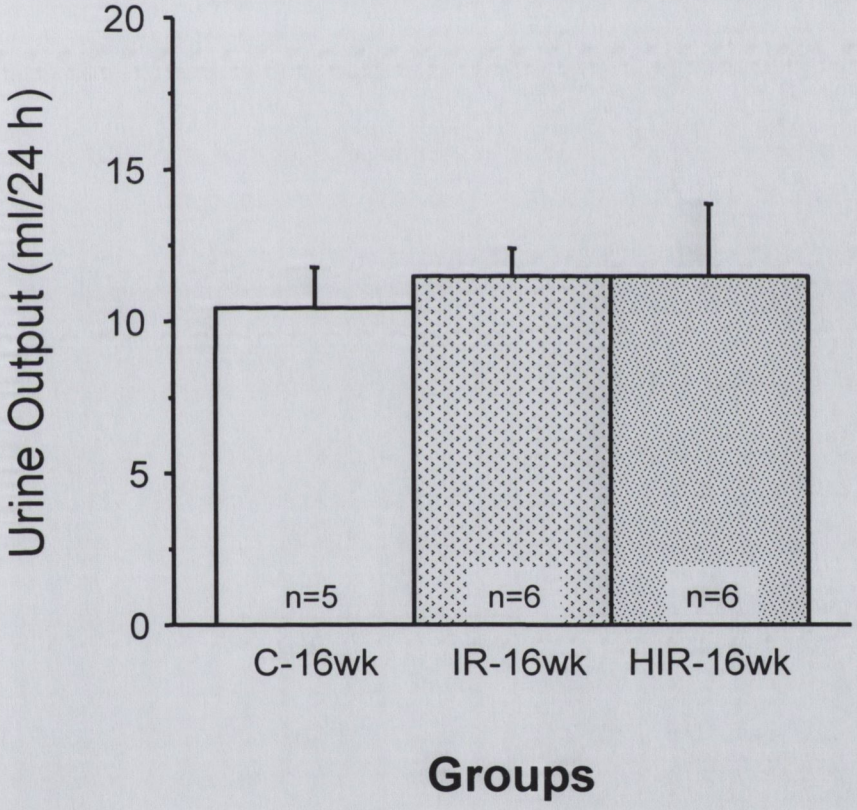


Figure 7.5 Urine output following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). After 15 wk 6 d reperfusion, animals were placed in metabolic cages and a 24 h urine collection was performed to determine urine output. Mean±SEM; ANOVA (Scheffe post-hoc test).

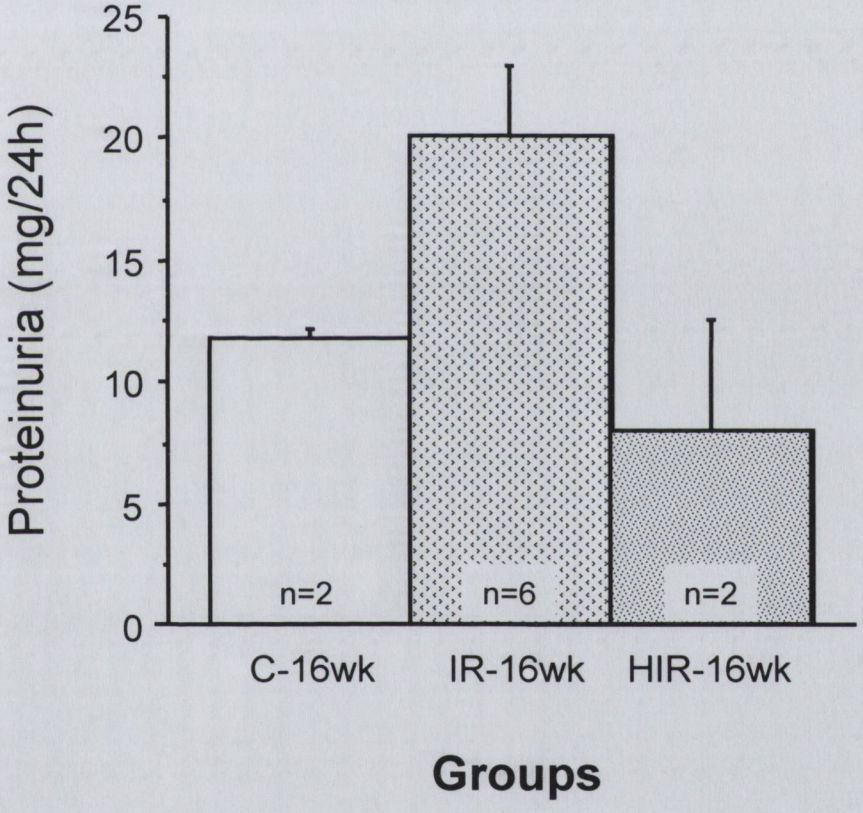


Figure 7.6 Urine protein levels following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). After 15 wk 6 d reperfusion, animals were placed in metabolic cages and a 24 h urine collection was carried out. The urine was then assessed for protein excretion over the 24 h. Mean±SEM; ANOVA (Scheffe post-hoc test).

7.3.4 Long-term Influences of Renal IR on Kidney Weight

There was no difference observed among the % weight changes between the left kidneys (removed before IR) and the right (experimental) kidneys in the C-16wk and IR-16wk groups (Figure 7.7). However, when the ratio of % kidney weight change:% body weight change was calculated, animals that underwent IR exhibited a two-fold greater ratio than that in C-16wk group ($P<0.005$) (Figure 7.8). This appeared to reflect a lower (non-significant) % body weight increase in the IR-16wk group compared with the C-16wk animals, indicating that the IR-16wk animals may have been ill (although their behaviour appeared normal) and that a more pronounced hypertrophy of the remaining right kidney was occurring in this group.

7.3.5 Effects Of Hyperthermic Preconditioning On Kidney Weight Following IR

The % increase in kidney weight between left (nephrectomised before IR) and right (experimental) kidney in the HIR-16wk group was similar to levels observed in both the C-16wk and IR-16wk animals (Figure 7.7). While % body weight change in the HIR-16wk group was comparable to the controls, the ratio of the % kidney weight change:% body weight change was significantly lower in the HIR-16wk group versus the IR-16wk group ($P<0.005$) (Figure 7.8).

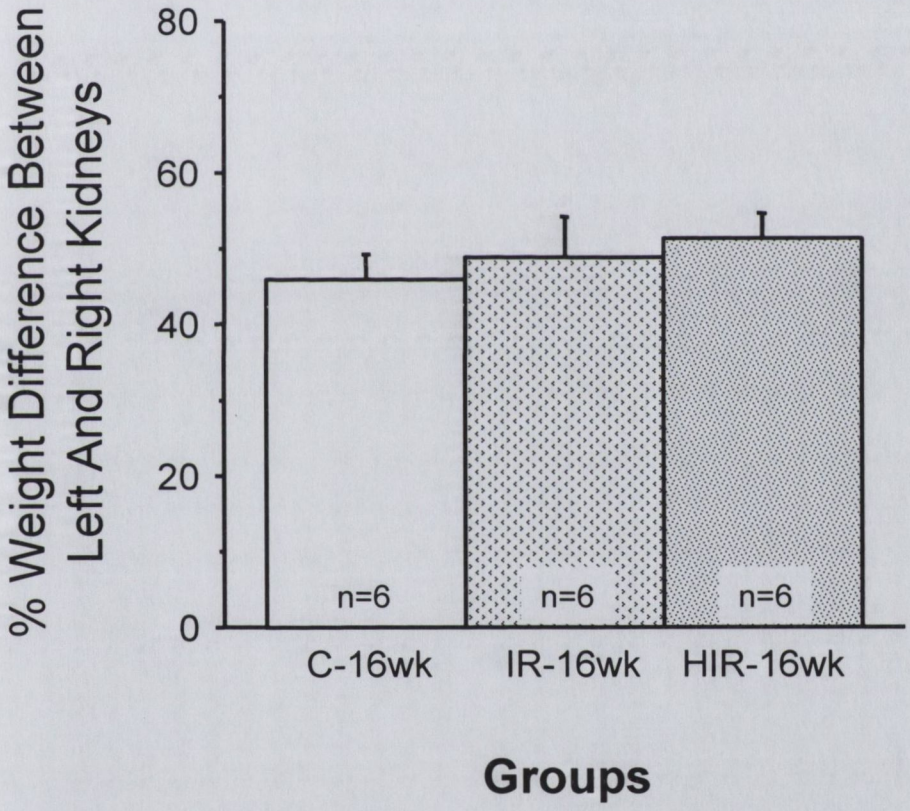


Figure 7.7 Renal oedema following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). The right kidney was harvested after sixteen weeks reperfusion and the weight difference between this and the previously nephrectomized left kidney was calculated as a percentage of the left kidney weight. Mean±SEM; ANOVA (Scheffe post-hoc test).

Groups	Body Weight Wk 0 (g)	Body Weight Wk 16 (g)	% Body Weight Change
C-16wk (n=6)	385±23.7	498.25±12.5	30±6.0
IR-16wk (n=6)	454.6±15.3	529.8±13.7	17±2.5
HIR-16wk (n=6)	418.6±18.0	546.6±21.4	32±8.1

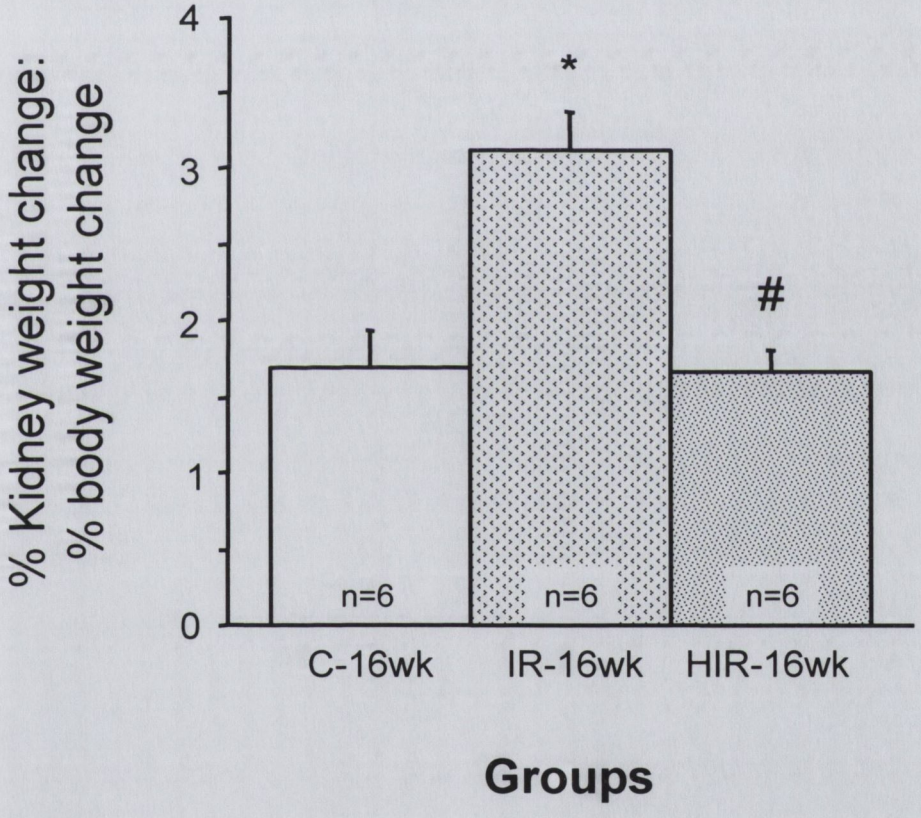


Figure 7.8 Table of body weight change and graph of the ratio of kidney weight change to body weight change at sixteen weeks reperfusion following left nephrectomy and sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk group) of the right kidney of normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to ischaemia (HIR-16wk group). The weight difference between the left nephrectomized kidney and the right experimental kidney was expressed as a % of the left kidney, and the ratio of this to the % body weight change over the same period was calculated. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.005$ vs. C-16wk; # $P<0.005$ vs. IR-16wk.

7.3.6 Long-term Influences of IR on Renal Morphology

Despite the lack of evidence of renal dysfunction, there were significant histological alterations evident between C-16wk (Figures 7.9a+d) and IR-16wk groups (Figures 7.9b+e) (an average of 21.4±0.60 tubules/field was counted in the C-16wk group versus 24.2±0.81 tubules/field in the IR-16wk group). While levels of complete tubular destruction were low in both of these groups, a significantly greater amount of partial tubular degeneration was observed in the cortex of kidneys exposed to 45 min ischaemia and 16wk reperfusion compared with corresponding control kidneys ($P<0.005$) (Figure 7.10). Despite only a low percent of tubules exhibiting proteinaceous cast formation in kidneys from IR-16wk animals, this was significantly higher than levels in C-16wk kidneys where no protein casts were observed ($P<0.01$) (Figure 7.11). Leucocyte infiltration was also significantly elevated in the cortex of IR-16wk kidneys compared with the sham-operated C-16wk group ($P<0.0001$) (Figure 7.12). Protein cast formation and a high level of red blood cell congestion were observed in the renal medulla of IR-16wk animals (Figure 7.9e) but were absent in C-16wk kidneys (Figure 7.9d). Small areas of fibrosis were beginning to develop in the cortex of kidneys from the IR-16wk group (Figure 7.13b), in particular near areas of dense cellular infiltrate. This was not observed in C-16wk kidneys (Figure 7.13a).

7.3.7 Effects Of Hyperthermic Preconditioning On IR-Induced Renal Morphological Alterations

In contrast to the histological deterioration found in kidneys exposed to IR without preconditioning, findings in kidneys from the HIR-16wk group (Figures 7.9c+f) were

comparable to C-16wk kidneys. Preconditioning with hyperthermia prior to IR (HIR-16wk group) attenuated tubular degeneration ($P<0.01$) (Figure 7.10) and protein cast formation ($P<0.01$) (Figure 7.11) versus the IR-16wk animals (a mean of 21.2±1.12 tubules/field was counted in the HIR-16wk group). In addition, the HIR-16wk group displayed significantly lower levels of leucocyte infiltration ($P<0.005$) compared with the non-preconditioned IR-16wk kidneys, but similar to levels observed in C-16wk animals (Figure 7.12). IR-induced alterations in renal medulla morphology were absent in animals preconditioned with hyperthermia, where red blood cell congestion and tubular deterioration not observed (Figure 7.9f). Furthermore, in contrast to the IR-16wk group, there was no evidence of fibrosis in the kidneys of HIR-16wk animals (Figure 7.13c). This was similar to observations in the C-16wk group (Figure 7.13a).

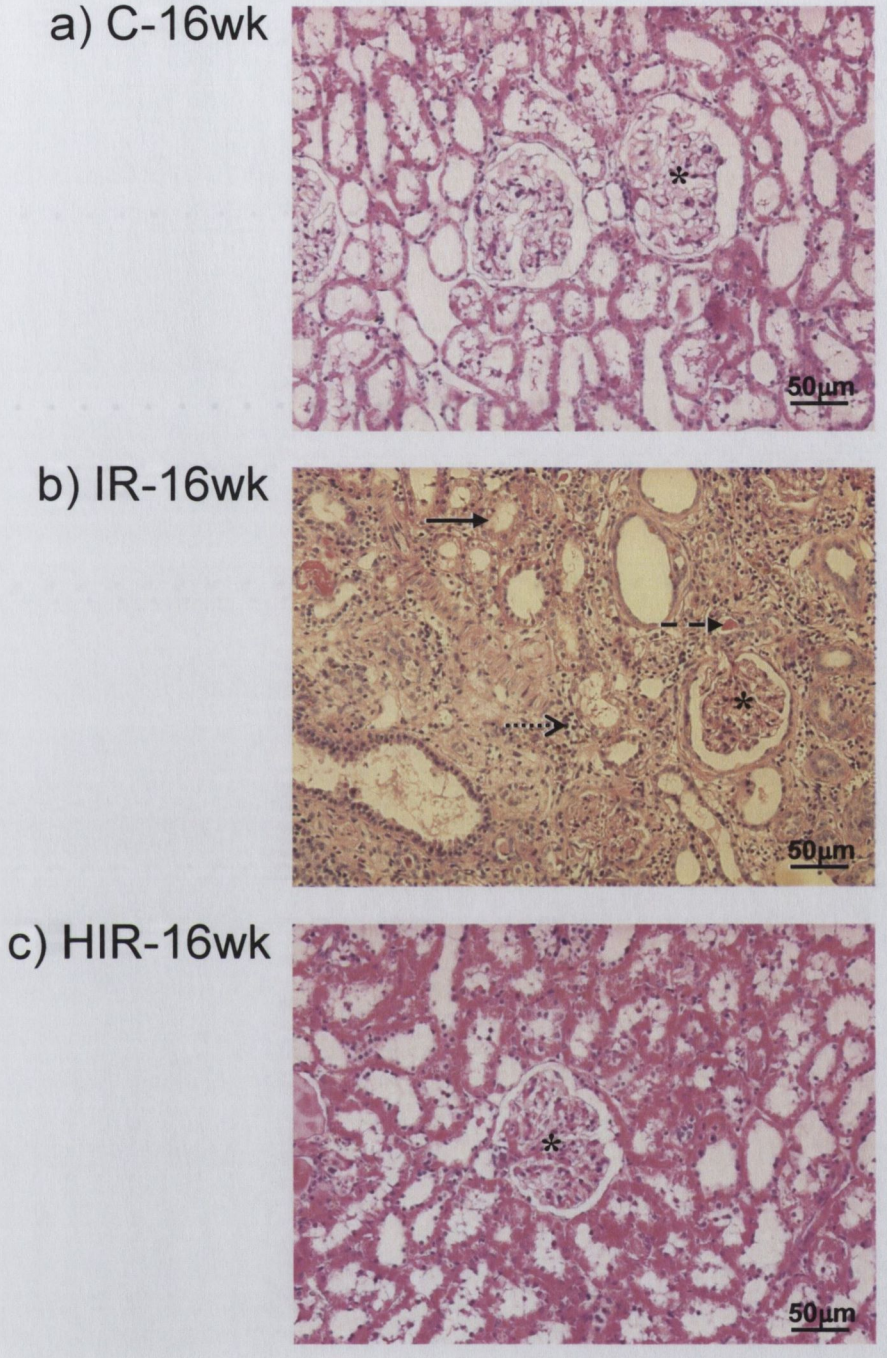


Figure 7.9a-c Representative images of H&E sections of renal cortex of the right kidney taken after sixteen weeks reperfusion following **a)** sham ischaemia (C-16wk group) or **b)** 45 min ischaemia (IR-16wk group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-16wk group **(c)**). Note the degeneration of the tubular epithelium (solid arrow), the development of proteinaceous casts (dashed arrow) and leucocyte infiltrate (dotted arrow) in the IR-16wk group. Sample glomeruli are denoted by asterisks.

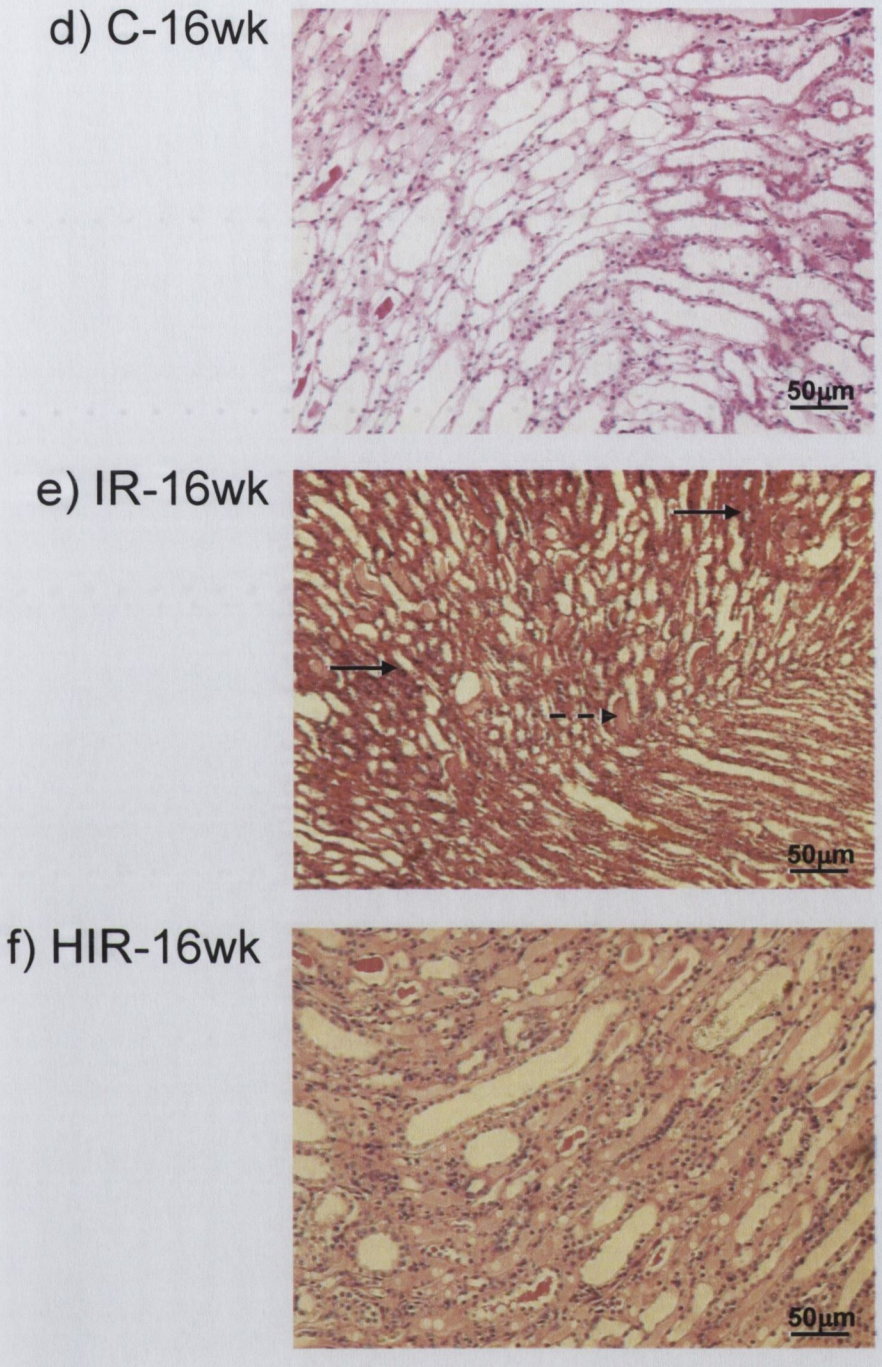


Figure 7.9d-f Representative images of H&E sections of renal medulla of the right kidney taken after sixteen weeks reperfusion following **d)** sham ischaemia (C-16wk group) or **e)** 45 min ischaemia (IR-16wk group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-16wk group **(f)**). Note the diffuse red blood cell congestion (solid arrows) and the development of proteinaceous casts (dashed arrow) in the IR-16wk group.

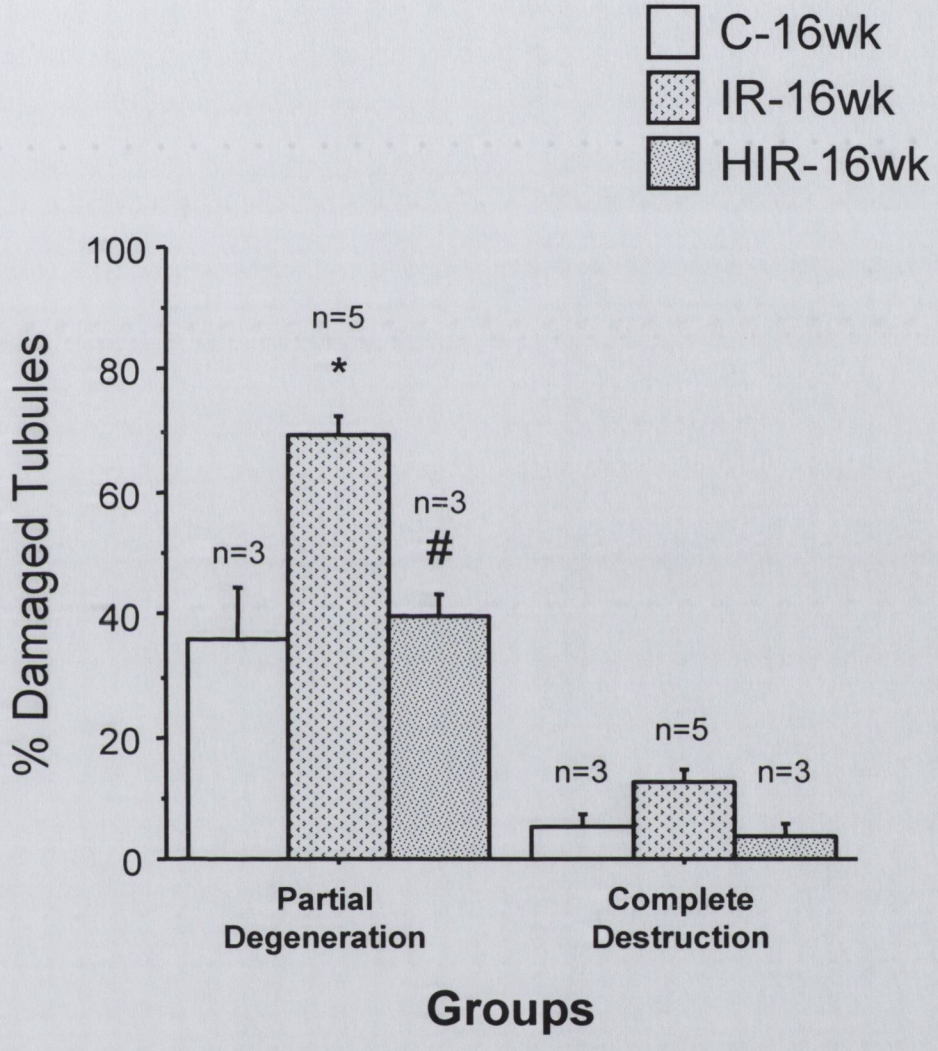


Figure 7.10 Quantification of tubular degeneration in the renal cortex of right kidneys that underwent sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk and HIR-16wk groups) followed by sixteen weeks reperfusion. Rats were exposed to normothermia (C-16wk and IR-16wk groups) or were preconditioned with 15 min hyperthermia (HIR-16wk group) 18 h prior to surgery. The % of tubules that were partially degenerated or completely destroyed was determined. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.005$ vs. C-16wk; # $P<0.01$ vs. IR-16wk

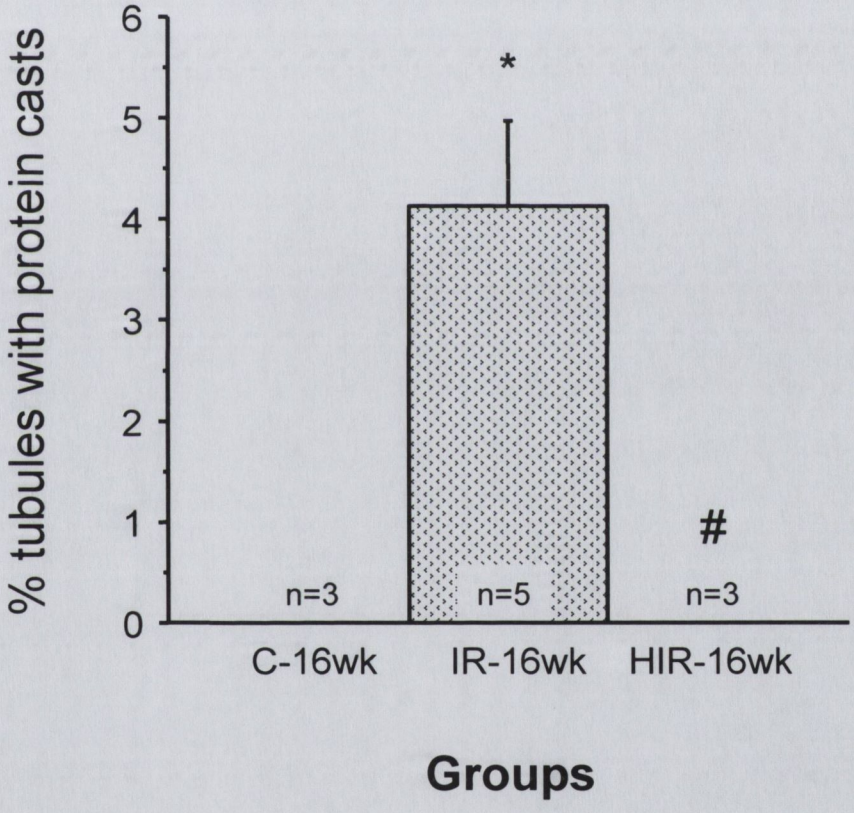


Figure 7.11 Quantification of renal tubular cast formation in the renal cortex of right kidneys that underwent sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk and HIR-16wk groups) followed by sixteen weeks reperfusion. Rats were exposed to normothermia (C-16wk and IR-16wk groups) or were preconditioned with 15 min hyperthermia (HIR-16wk group) 18 h prior to surgery. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.01$ vs. C-16wk; # $P<0.01$ vs. IR-16wk

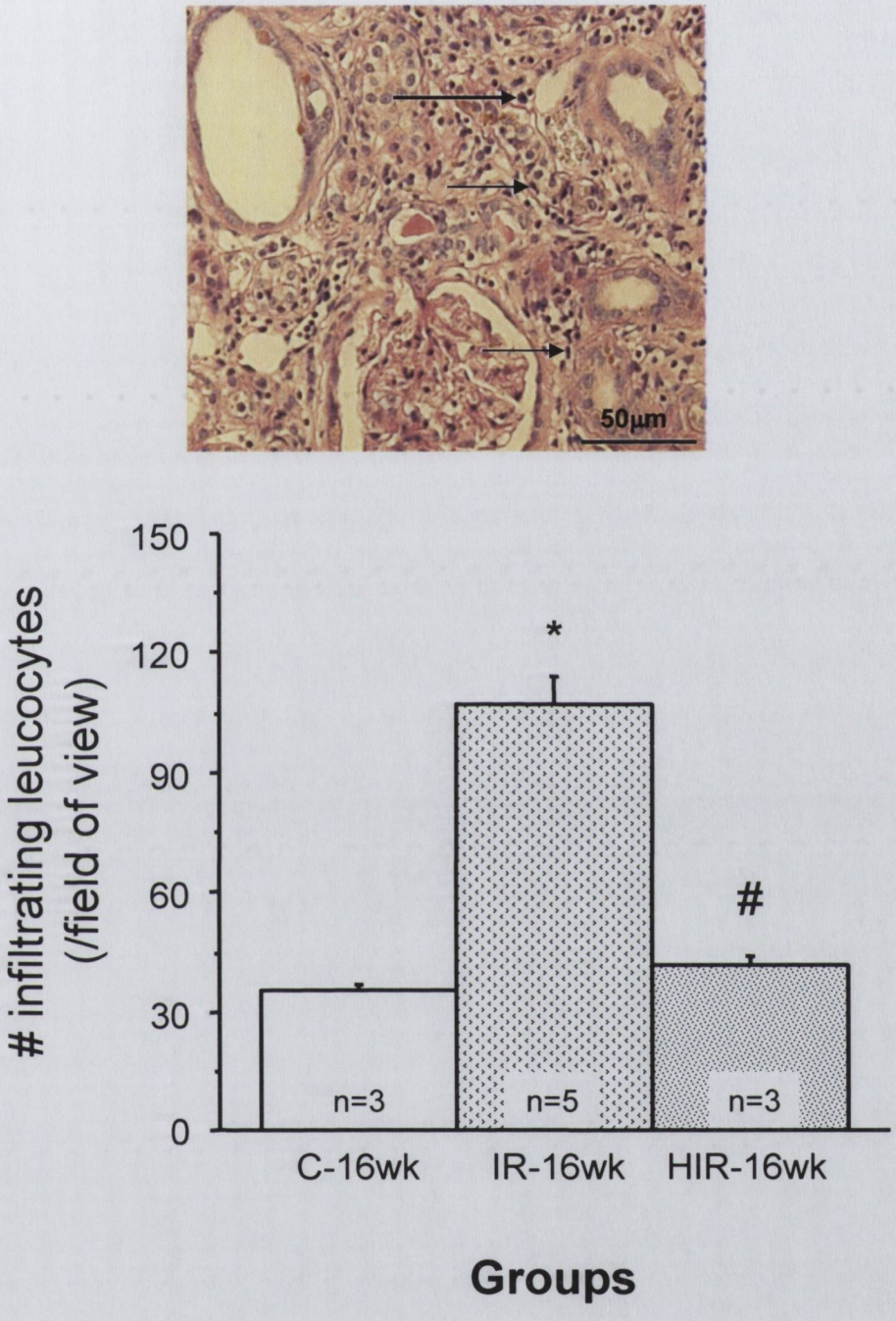


Figure 7.12 Quantification of renal leucocyte infiltration in the renal cortex of right kidneys that underwent sham ischaemia (C-16wk group) or 45 min ischaemia (IR-16wk and HIR-16wk groups) followed by sixteen weeks reperfusion. Rats were exposed to normothermia (C-16wk and IR-16wk groups) or were preconditioned with 15 min hyperthermia (HIR-16wk group) 18 h prior to surgery. The histology image represents the renal cortex of an IR-16 wk animal. Arrows indicate infiltrating leucocytes. Mean±SEM; ANOVA (Scheffe post-hoc test); * $P<0.0001$ vs. C-16wk; # $P<0.005$ vs. IR-16wk

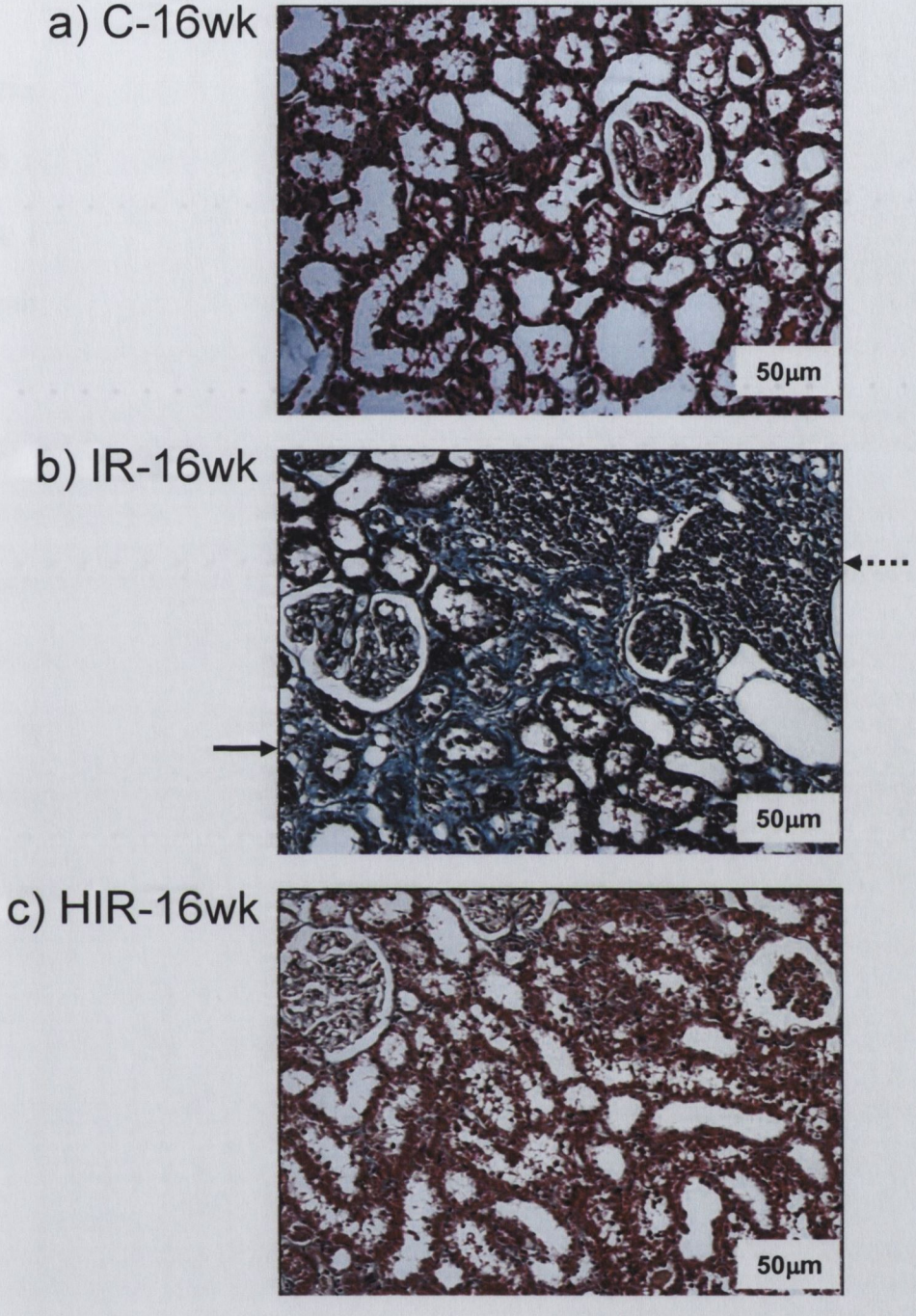


Figure 7.13a-c Representative images of Massons trichrome-stained sections of renal cortex of the right kidney taken after sixteen weeks reperfusion following **a)** sham ischaemia (C-16wk group) or **b)** 45 min ischaemia (IR-16wk group) in normothermic rats. A separate IR group was exposed to 15 min hyperthermia 18 h prior to the IR insult (HIR-16wk group **(c)**). Note the large area of fibrosis (blue/green staining opposite solid arrow) adjacent to dense cellular infiltrate (opposite dashed arrow) in the IR-16wk group.

7.4 DISCUSSION

Reperfusion of a previously ischaemic organ results in a series of pro-inflammatory events which result in organ dysfunction and leucocyte infiltration. Since IR is unavoidable during transplantation, this deleterious condition may contribute to acute and chronic allograft rejection. Preconditioning is a means of protecting against a potentially lethal stress by first exposing the tissue to a smaller sub-lethal stress. This is associated with the upregulation of highly conserved HSPs. In chapter 6, it was found that preconditioning with hyperthermia protected the kidney from subsequent IR-induced early renal dysfunction and leucocyte infiltration. Furthermore, hyperthermic preconditioning, which was associated with HSP70 induction in the kidney, attenuated IR-induced ICAM-1 and MHC II expression. The purpose of the present study was to assess whether this protection extends beyond the first week of reperfusion to attenuate chronic alterations in the post-ischaemic kidney. It was demonstrated that while mild proteinuria was apparent, renal function was otherwise unaltered by IR at 16 wk reperfusion. However, morphological changes were obvious. These included tubular deterioration, leucocyte infiltration and mild fibrosis. Preconditioning with hyperthermia prevented the IR-induced proteinuria, tissue damage and leucocyte accumulation as well as the early signs of fibrosis.

Although it has been well established in both the clinical and experimental arenas that renal IR injury is an antigen-independent risk factor for the development of chronic allograft failure, remarkably few studies have examined late changes following renal IR insult. Rather, the efficacy of many treatments have been only been tested over the immediate post-operative period. However, while several groups have demonstrated the apparent resolution of injury following this initial reperfusion period in acute models

(Dragun *et al.*, 2000), inflammatory responses appear again several months after IR, leading to progressive renal failure if left untreated (Azuma *et al.*, 1997b; Takada *et al.*, 1997a). In fact, Tullius *et al.* (1994a) found that kidneys exposed to IR undergo morphological and functional deterioration that is similar to inflammation in allografted kidneys, but that occurs at a slower pace. Other studies indicate that IR-induced injury may actually occur at a more comparable pace to the injury in allografts (Diamond *et al.*, 1992; Nadeau *et al.*, 1995; Azuma *et al.*, 1997b). Nonetheless, it is clear that both antigen-independent factors (i.e. IR) and antigen-dependent factors participate in the development of chronic rejection.

In the present study, the renal IR injury examined used 45 min ischaemia. This was based on several studies described in Chapter 2 and on results from Chapter 6. Due to the fact that IR-induced injury becomes apparent at significantly different reperfusion times depending on the ischaemic protocol employed (Tullius *et al.*, 1994a; Dragun *et al.*, 1998b), 16 wk was chosen for the reperfusion period in this chapter based on findings by other groups in models of 45 min ischaemia. Azuma *et al.* (1997b) performed 45 min warm ischaemia followed by a contralateral uninephrectomy and found that proteinuria developed at approximately 8 wk reperfusion and progressively worsened thereafter. The expression of IL-1 and TNF-α were increased between 8 and 16 wk. The expression of ICAM-1 was also increased at this time peaking at 16 wk at which time morphological alterations were found, with small numbers of infiltrating leucocytes, tubular atrophy and moderate numbers of sclerotic glomeruli. Takada *et al.* (1997a) found that 45 min ischaemia caused similar changes, although proteinuria was not evident until 16 wk reperfusion, and significant morphological alterations followed later. The delay was most likely due to the fact that the kidneys were perfused with ice-cold University of Wisconsin

organ preservation solution, which would have slowed down metabolism and exerted some protective effects. The expression of several cytokines was also increased during reperfusion, suggesting an inflammatory response was occurring. Thus this study used 16 wk as the reperfusion time-point at which to examine morphological and functional changes induced by warm IR and to assess the efficacy of hyperthermic preconditioning to reduce these responses.

A majority of studies in chronic models of renal IR use proteinuria as an accurate indicator of deteriorating renal function (Diamond *et al.*, 1992), rather than other measurements. Thus, this study assessed proteinuria as well as the functional parameters found to be disturbed during early reperfusion e.g., serum creatinine and urea levels. It was found that renal function had not been affected by IR to the same degree as other studies however proteinuria was beginning to appear at 16 wk reperfusion. Other functional parameters remained unchanged at this time. Although the differences between functional observations are difficult to explain, several experimental disparities exist between the findings in this chapter and the studies above. The models may exhibit interstrain differences as the other studies used Lewis rats. In fact protein levels in C-16wk animals were more than twice the levels in the corresponding controls of the other studies. In addition, the method of ischaemia (cold versus warm, clamp versus grafting) and the time of the contralateral nephrectomy (before versus after the IR injury) varied between all studies.

Even though the functional disturbances observed here were minor, the kidneys exposed to IR appeared to be undergoing the first signs of chronic injury. These were evident morphologically and were manifested as elevated leucocyte infiltration and extensive

tubular deterioration after 16 wk reperfusion. Both the fact that the degeneration of the tubular epithelium was partial, in that there was loss of some nuclei and vacuolization of the epithelial cells, rather than complete detachment of the epithelium from the basement membrane, and that the kidney has a large capacity to compensate for loss of functional mass, may explain why there was no apparent functional changes despite such a large degree of morphological alteration. A small amount of fibrosis was also present. The ratio of % kidney weight change: % body weight change was higher in IR-16wk animals compared with the remaining C-16wk kidney, suggesting that hypertrophy of the remaining kidney was more pronounced in the IR-16wk group. This indicates that the injured kidney had undergone adaptive responses to compensate for the damage that was observed in a majority of tubules.

The novel findings here come from the use of hyperthermic preconditioning in a chronic model of injury. It has been previously demonstrated that preconditioning with a short period of hyperthermia protected kidneys from IR-induced renal dysfunction and tubular deterioration for at least the first week of reperfusion (Chapter 6; Kelly *et al.*, 2001). The elevated serum creatinine levels associated with transplantation into syngeneic recipients were likewise reduced (Perdrizet *et al.*, 1989; Redaelli *et al.*, 2001). In fact survival up to day 50 of reperfusion was also enhanced by preconditioning. Furthermore, allografts demonstrated better function and 10 day survival following hyperthermic preconditioning. All of these studies showed that HSP70 expression was induced by the prior exposure to elevated temperatures. This study demonstrates that the early protection observed using hyperthermic preconditioning in Chapter 6 may exert a more prolonged influence on renal integrity, as animals that were similarly preconditioned exhibited significantly less

morphological damage after 16 wk reperfusion compared with their non-preconditioned counterparts.

The exact mechanisms through which hyperthermic preconditioning protects against acute injury are discussed in depth in Chapter 6. Whatever the exact pathway, it is likely that the chronic benefits of preconditioning reflect the attenuation of the initial injury responses to IR. Of particular importance during IR is the early leucocyte infiltration which has been implicated in the pathogenesis of IR injury in many organs. Several studies have shown that hyperthermic preconditioning reduces post-ischaemic leucocyte accumulation, as demonstrated by decreased leucocyte-endothelial interactions (Gang *et al.*, 1997) and lower MPO levels (Chapter 6; Javadpour *et al.*, 1998; Kelly *et al.*, 2001). A potential pathway through which this may be achieved was found in Chapter 6 where hyperthermic preconditioning inhibited the upregulation of ICAM-1, an adhesion molecule intimately involved in the adhesion of most leucocyte populations, including neutrophils and lymphocytes, to the vascular endothelium and their subsequent emigration into the interstitium. In addition, ICAM-1 may enhance lymphocyte interactions with MHC-associated antigens thereby strengthening alloimmune responses during transplantation.

This would be in agreement with findings by Dragun *et al.* (1998) who showed that the administration of antisense oligodeoxynucleotides against ICAM-1 to kidney donors 6 h prior to transplantation into a syngeneic recipient not only attenuated IR-induced renal dysfunction and tubular deterioration over the first week of reperfusion, but that this protection extended for up to 20 wk reperfusion. In addition, macrophage and lymphocyte accumulation in the post-ischaemic kidneys was decreased, alongside prevention of MHC II induction, suggesting that the blocking of early leucocyte-endothelial interactions has

the potential to protect against long-term organ injury possibly through the reduction of the initial inflammatory responses. Other studies have noted that inhibition of other adhesion molecules similarly prevented late IR-induced renal damage and dysfunction. For example Takada *et al.* (1997a) found that early post-ischaemic renal dysfunction and leucocyte infiltration were attenuated by the administration of recombinant soluble PSGL-1, which binds and blocks both P- and E-selectins. In addition, levels of several cytokines were reduced and MHC II induction was prevented by this treatment. This group went on to demonstrate that chronic IR-induced changes were also decreased by the administration of recombinant soluble PSGL-1 during ischaemia and after just three hours reperfusion (Takada *et al.*, 1997b). Importantly the employment of this treatment in allografts revealed that this early prevention of IR-induced injury acted synergistically with an immunosuppressive protocol to reduce both acute and chronic allograft rejection (Kusaka *et al.*, 1999). These findings support the widely accepted idea that leucocyte-interactions with the vascular endothelium in the immediate post-ischaemic period are of paramount importance in the ensuing inflammatory response, thereby contributing to subsequent organ damage and dysfunction.

Taken together these data indicate that the early benefit offered by hyperthermic preconditioning on post-ischaemic renal morphology and dysfunction, which was associated with reduced leucocyte infiltration and decreased ICAM-1 induction may be responsible for the long-term protection that was observed. Furthermore, when reviewed with findings in chapter 6 as well as those in transplantation studies (Perdrizet *et al.*, 1989; Perdrizet *et al.*, 1993; Redaelli *et al.*, 2001), the results here indicate that preconditioning to induce HSP70 expression may provide a promising therapeutic approach to planned surgeries such as transplantation. Importantly, the induction HSP70 expression does not

need to be sustained throughout the reperfusion period by artificial means, rather its expression at the time of IR appears to be enough to influence long-term outcome following a renal IR insult. With advances in genetic and pharmaceutical manipulation, it is plausible that induction of protective proteins prior to reperfusion of an organ during transplantation may be a simple means of prolonging the lifespan of allografts.

CHAPTER 8

Adenosine Protects Against Interleukin-2-Induced Leucocyte-Endothelial Interactions

8.1 INTRODUCTION

Both antigen-independent (i.e. IR injury) and antigen-dependent factors (i.e. allogenicity of the grafted tissue) impact the survival of grafts following transplantation. We have previously demonstrated that preconditioning to induce HSP70 expression exerts a protective effect against renal IR-induced dysfunction and morphological changes for at least 16 wk reperfusion. IR-induced MHC II expression was also found to be reduced by preconditioning suggesting that release of cytokines such as IL-2 may have been inhibited during reperfusion. Although the exact mechanism through which this protection occurred is unknown, the inhibition of both ICAM-1 upregulation and the associated leucocyte infiltration of the kidney by preconditioning may have been key factors.

IL-2 is a cytokine that has been implicated in allograft rejection and is expressed following renal IR injury (Nagano *et al.*, 1997; Takada *et al.*, 1997a). It influences the adaptive immune response through the upregulation of MHC II expression and the promotion of clonal proliferation of alloreactive cells. This cytokine is in fact one of the primary targets for immunosuppression during transplantation i.e. the well-established immunosuppressant CsA inhibits IL-2 synthesis and chimeric antibodies have been developed to block the IL-2R subunit CD25. However, IL-2 also impacts on the innate immune response by increasing microvascular macromolecular leak. Similar to IR injury, this occurs secondary to leucocyte (in particular neutrophil) interactions with the vascular endothelium (Edwards *et al.*, 1992). The exact mechanism of action of IL-2 in inducing the increased leucocyte rolling, adhesion and emigration is not fully understood, although it is known that IL-2 has little direct effect on either the endothelium or neutrophils.

Rather IL-2 can induce the production of several inflammatory mediators e.g. IL-1, TNF-α, PAF and the complement system (Lentsch *et al.*, 1999).

IL-2 is expressed prior to MHC II expression but after the initial leucocyte infiltration in a renal IR model similar to the one in this thesis. This suggests that the protective effect of preconditioning against MHC II induction may be through the indirect prevention of IL-2 production by inhibiting adhesion molecule expression and leucocyte infiltration. This would block subsequent inflammatory events that lead to cytokine release. However, hyperthermic preconditioning has also been demonstrated to attenuate IL-2-induced leucocyte recruitment in post-capillary venules (Gang *et al.*, 1996). Thus this preconditioning method may also exert a more direct impact on IL-2-mediated events. Taken together, these data suggest that the improved function and early survival found in allografts that have been preconditioned with hyperthermia (Perdrizet *et al.*, 1993) may be due to reduction of both IR injury and antigen-dependent events. Nevertheless, until the exact mechanism through which HSP70 induction protects against these responses is elucidated, some attention should be focused on treatments that are currently available and which may have similar benefits. One such alternative is adenosine.

Adenosine is an endogenous purine nucleotide that accumulates in tissue during ischaemia due to the inhibition of adenosine kinase. It differentially modulates physiological responses through its interaction with four known receptors, A1, A2a, A2b and A3 (Jacobson, 1998). In addition, adenosine exerts anti-inflammatory properties, including blocking oxidant release from adherent neutrophils and preventing IR-induced neutrophil-endothelial interactions and microvascular dysfunction following IR (Grisham *et al.*, 1989). Adenosine is also capable of reducing the production of cytokines such as TNF-α

during IR (Meldrum *et al.*, 1997) and can in fact activate cellular antioxidant defence systems.

One source of adenosine during inflammation comes from adenine nucleotides are released by a variety of cells including endothelium, platelets and leucocytes when injured. These nucleotides are rapidly converted to adenosine by ecto-nucleotidases (Cronstein *et al.*, 1986; Linden *et al.*, 2001). Interestingly the reduction of myocardial IR injury by transfection of rat hearts with HSP70 was associated with increased activity of ecto-5'-nucleotidase. Inhibition of ecto-5'-nucleotidase in turn blocked the protection granted by HSP70 transfection, suggesting that adenosine may be important in the reduction of injury by HSP70 (Sakaguchi *et al.*, 2000). Adenosine has also been implicated as a mediator of IPrec in the heart (Thornton *et al.*, 1992). In fact, adenosine has been used in humans to preconditioning hearts during cardiac bypass surgery. This results in improved post-operative cardiac function (Zarro *et al.*, 1998).

While adenosine does not appear to mediate IPrec in the kidney (Lee and Emala, 2001), adenosine and adenosine agonists can themselves attenuate renal IR injury through A1 or A2a receptor activation or A3 antagonism (Lee and Emala, 2000; Okusa *et al.*, 1999). Administration of an A2a receptor agonist was also associated with reduced P-selectin and ICAM-1 expression as well as decreased neutrophil infiltration (Okusa *et al.*, 2000), this possibly being the mechanism through which adenosine acted to attenuate renal dysfunction, similar to the findings in Chapter 6 with hyperthermic preconditioning. Adenosine is one of the components of preservation solutions used to store organs during transplantation. It is used to aid with the regeneration of ATP levels upon reperfusion. Perhaps more consideration should be given to exploiting its anti-inflammatory actions

with a view to reducing IR injury of the allograft. However whether adenosine is capable of altering IL-2-mediated responses, similar to hyperthermic preconditioning, remains to be seen.

Thus the aims of this study were firstly to establish a model of microvascular inflammation, using IL-2, a key player in the pathogenesis of allograft rejection, as the stimulating agent. This was assessed using the newly established intravital microscopy set-up in our laboratory, which enables the "real-time" monitoring of each step (rolling, firm adhesion and transmigration) involved in leucocyte interactions with the microvascular endothelium. The rat mesentery was used, as it requires very little manipulation to prepare for observation thereby minimizing inflammation due to handling. Furthermore observation of this tissue does not require the fluorescent labelling of leucocytes. The dose of recombinant IL-2 (rIL-2) was chosen based on other intravital microscopy studies, where $1X10^6$ U rIL-2 were administered as a bolus. In addition a two hour observation period was similarly used by other groups (Edwards *et al.*, 1992). Secondly, this study assesses the potential of adenosine to attenuate these interactions, and investigates whether this is due to alterations in the expression of adhesion receptors on the circulating leucocytes as measured by flow cytometry.

8.2 METHODS

8.2.1 Animal Preparation

All animal procedures were carried out under license from the Department of Health and Children. Ten male Sprague-Dawley rats (Charles River, UK) weighing 120-220 g were fasted for 24 h in order to minimize bowel movement during the experiment, and randomised into two groups (n=5/group): rIL-2 group and adenosine-treated rIL-2 group (Aden+rIL-2). All animals were anaesthetized with an i.p. injection of pentobarbitone sodium (50 mg/kg) (Rhone Merieux Ltd., UK). A rectal probe (Hewlett-Packard GMBH, Boeblingen, Germany) was used to monitor core temperature for the duration of the experiment. The left common carotid artery was cannulated to monitor the mean arterial pressure. The right jugular vein was cannulated for administration of heparin (Leo Laboratories Ltd., Bucks., UK), saline, rIL-2 and adenosine (Sigma Chemical Co.). Recombinant interleukin-2 was kindly donated by Mr. David Briscoe at Chiron, UK.

8.2.2 Intravital Microscopy

Intravital microscopy was used to assess leucocyte-endothelial interactions in post-capillary venules of rat mesentery. A tracheotomy was performed to facilitate breathing during the experiment. 400 IU/kg heparin was administered i.v. to each animal. The rat mesentery was prepared for microscopic observation as described by Granger *et al.* (1989). A midline laparotomy was performed and the animal was laid on its side. The small bowel and mesenteric vessels were exposed and laid over a coverslip on a Plexiglas viewing stage. The tissue was kept moist by gauze soaked in warm bicarbonate-buffered

saline (131.9 mM NaCl, 4.69 mM KCl, 1.98 mM $CaCl_2$, 1.16 mM $MgSO_4$, 20 mM $NaHCO_3$). Briefly, the mesenteric microcirculation was transilluminated with a 12 V, 100 W light source and observed through an intravital video microscope (Nikon Diaphot 300, Nikon, Tokyo, Japan) with a 40X objective lens. A video camera (CCD-100E; Mitsubishi Electric Corporation, Tokyo, Japan) mounted on the microscope transmitted the image onto a colour monitor (KV-M2150L, Sony, Tokyo, Japan). The images are recorded using a videocassette recorder (Philips, Eindhoven, Netherlands) for playback analysis (Figure 8.1).

Single, straight (of at least 150 μm in length), unbranched venules of 20 to 35 μm in diameter were studied. Venular diameter (D_v) is measured on-line using a video calipers (Microcirculation Research Institute, Texas A&M University, USA). Centreline red blood cell velocity V_{rbc} was measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute). Venular blood flow was calculated from the product of mean red blood cell velocity (V_{mean}=centreline velocity/1.6) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (WSR) was calculated based on the Newtonian definition $WSR=8(V_{mean}/D_v)$. Leucocyte rolling velocity (V_{wbc}) and the numbers of rolling, adherent and emigrated leucocytes were determined during playback videotaped images. Rolling leucocytes were defined as white blood cells that move at a velocity less than that of red blood cells in the same vessel. Leucocyte rolling velocity was determined as the average time required for an individual leucocyte to move along 100 μm of the microvessel (μm/sec) and was calculated from ten cells. The number of rolling leucocytes per 100 μm was calculated as the mean number of leucocytes rolling across as fixed point per second divided by the V_{wbc}. A leucocyte was defined as adherent to venular endothelium if it was stationary for 30 s or longer.

Leucocyte adhesion was determined within a 100 μm length of venule over the 10 min observation period. Emigrated leucocytes were expressed as number of leucocytes per high powered field in the tissue adjacent to the venule.

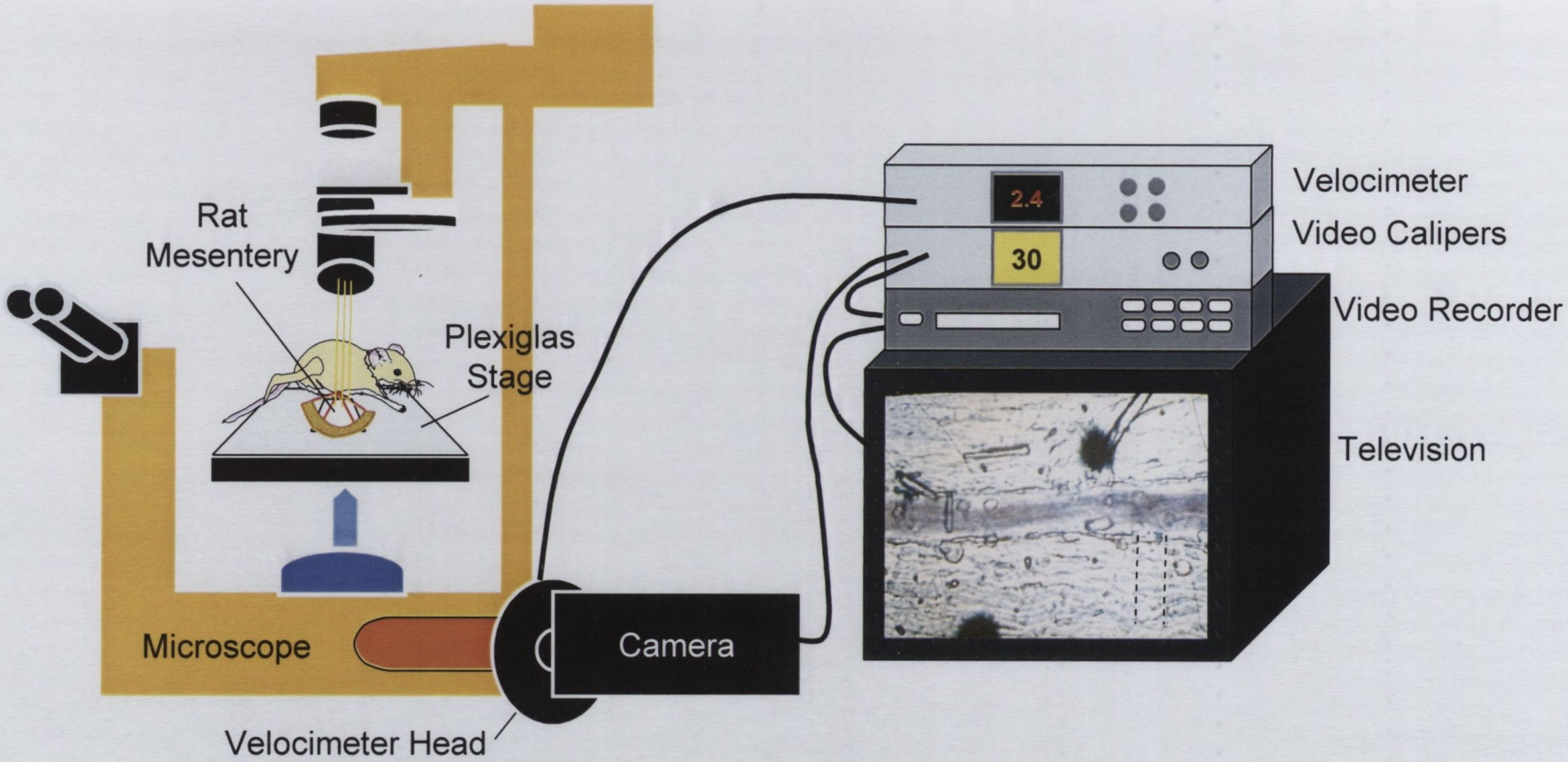


Figure 8.1 Diagramatic representation of the intravital videomicroscopy set-up for the rat mesentery. The black spots on the monitor are from the optical doppler velocimeter and the dashed lines are from the video calipers.

8.2.3 Experimental Protocol

The experimental protocol was performed as demonstrated in Figure 8.2. Asterisks represent 10 min recording periods. A 100 μm length of suitable vessel was selected according to the following baseline criteria: D_v 20-35 μm, WSR≥500s^{-1}, ≤6 adherent leucocytes (per 100 μm), and ≤8 emigrated leucocytes (per field of view). The vessel was allowed to reach equilibrium, at which time the image was recorded for 10 min. This represented baseline measurements. At the end of this period on-line parameters were noted i.e. D_v, V_{rbc}. Infusion of saline (rIL-2 group) or 2 μM adenosine (Aden+rIL-2 group) was commenced @1 ml/h (time=-15 min) via the right internal jugular vein and continued for the duration of the experiment. After 15 min (time=0 min), a bolus of $1x10^6$ U rIL-2 in 0.5 ml saline was administered into the right internal jugular vein. Video recordings of 10 min and measurements of on-line parameters were made at 30, 60, 90 and 120 min following the rIL-2 bolus. The mesentery was kept superfused with warm bicarbonate-buffered saline throughout the experiment.

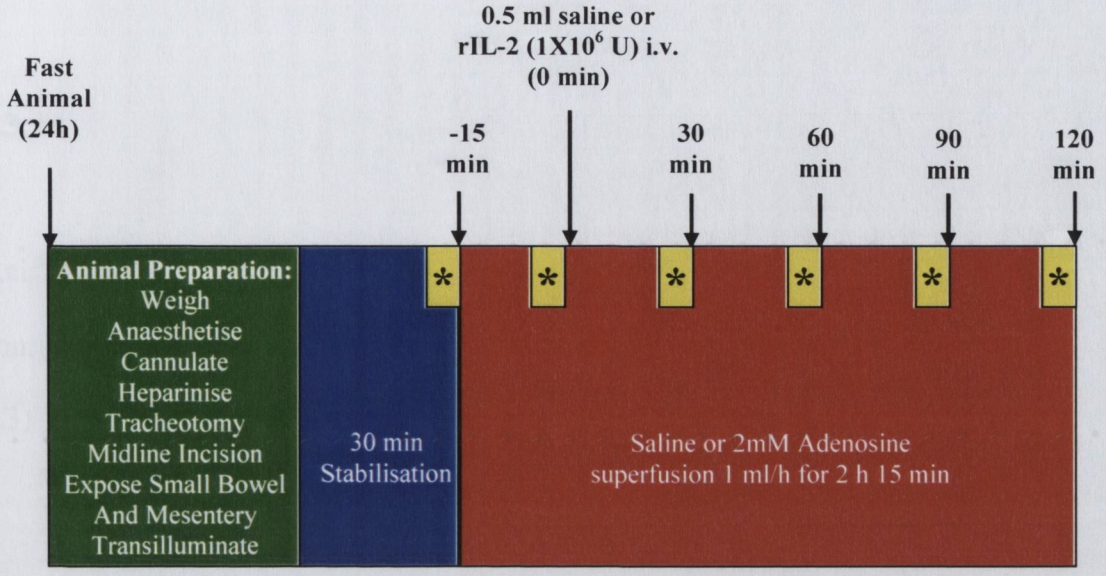


Figure 8.2 Diagrammatic representation of the timeline used to prepare groups for observation using intravital microscopy, in a model of rIL-2-induced inflammation with or without pre-treatment with adenosine.

8.2.4 Adhesion Molecule Expression

A separate group of experiments were performed in which flow cytometry was used to measure the expression of the adhesion molecules L-selectin and CD18 on neutrophils (n=3/group). The left internal jugular vein was cannulated and samples of whole blood were drawn into heparinised tubes (20 IU/ml) at -15, 0, 2, 10, 30, 60, 90 and 120 min as described in section 2.6.

8.2.5 Statistics

All data is expressed as mean±SEM. Unpaired (between groups) and paired (between timepoints within a group) t-tests were performed to compare all parameters.

8.3 RESULTS

8.3.1 The Effect Of Interleukin-2 And Adenosine On Mean Arterial Pressure

Animals in both rIL-2 and Aden+rIL-2 groups exhibited baseline blood pressures comparable to each other. These remained constant throughout the experiment (Figure 8.3).

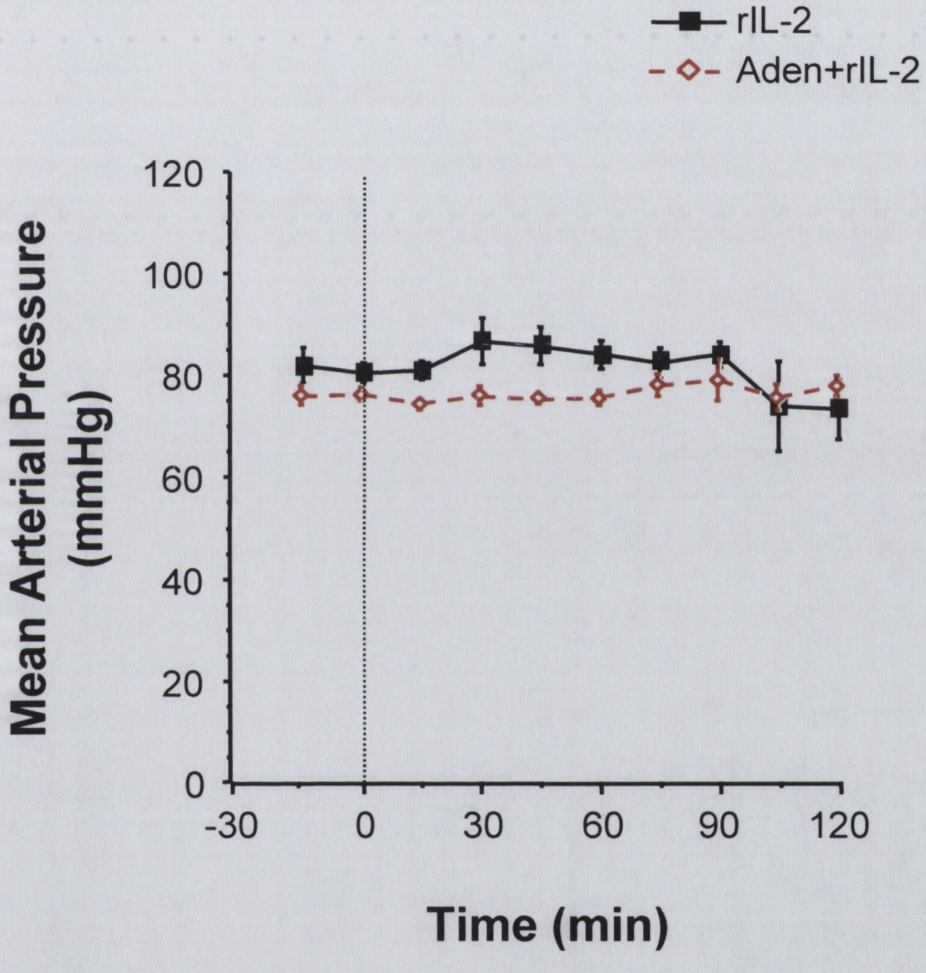


Figure 8.3 Mean arterial pressure. Saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion was began 15 min before animals received a bolus of recombinant interleukin-2. Mean arterial blood pressure was monitored every 15 min for 2 h. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=5/group.

8.3.2 The Effect Of Interleukin-2 On Leucocyte Rolling

The V_{wbc} was decreased from 40.6±3.04 μm/s under baseline conditions to 33.3±1.65 μm/s at 30 min post-rIL-2 (Figure 8.4). This parameter rose slightly towards baseline values at 60 min and 90 min following rIL-2 before decreasing to 31.9±3.39 μm/s by 120 min. However, these values never reached significance compared with baseline levels. The number of rolling leucocytes fell rapidly from a baseline value of 2.9±0.53 cells/100 μm to 1.8±0.37 cells/100 μm by 30 min following rIL-2 administration (Figure 8.5). The number of leucocytes rolling levelled off thereafter. These values were not significantly different from baseline levels at any time.

8.3.3 The Influence Of Adenosine On Interleukin-2-Induced Alterations In Leucocyte Rolling

The V_{wbc} was increased above baseline levels in the Aden+rIL-2 group within 30 min following rIL-2 administration, staying above baseline for the entire 120 min observation period. The V_{wbc} was significantly higher in the Aden+rIL-2 group (54.3±8.80 μm/sec) compared with the rIL-2 group (33.3±1.65 μm/sec) at the 30 min time point ($P<0.05$) (Figure 8.4), remaining elevated above the saline-treated rIL-2 group thereafter. Animals treated with adenosine demonstrated a sharp drop in the number of rolling leucocytes between baseline and 30 min after rIL-2 administration (Figure 8.5). This value remained below baseline for the duration of the experiment. In fact the Aden+rIL-2 group exhibited a significantly lower number of leucocytes rolling along the endothelium 30 min after injection of rIL-2 (0.5±0.07 cells/100 μm) compared with the untreated rIL-2 group (1.8±0.35 cells/100 μm) ($p<0.05$).

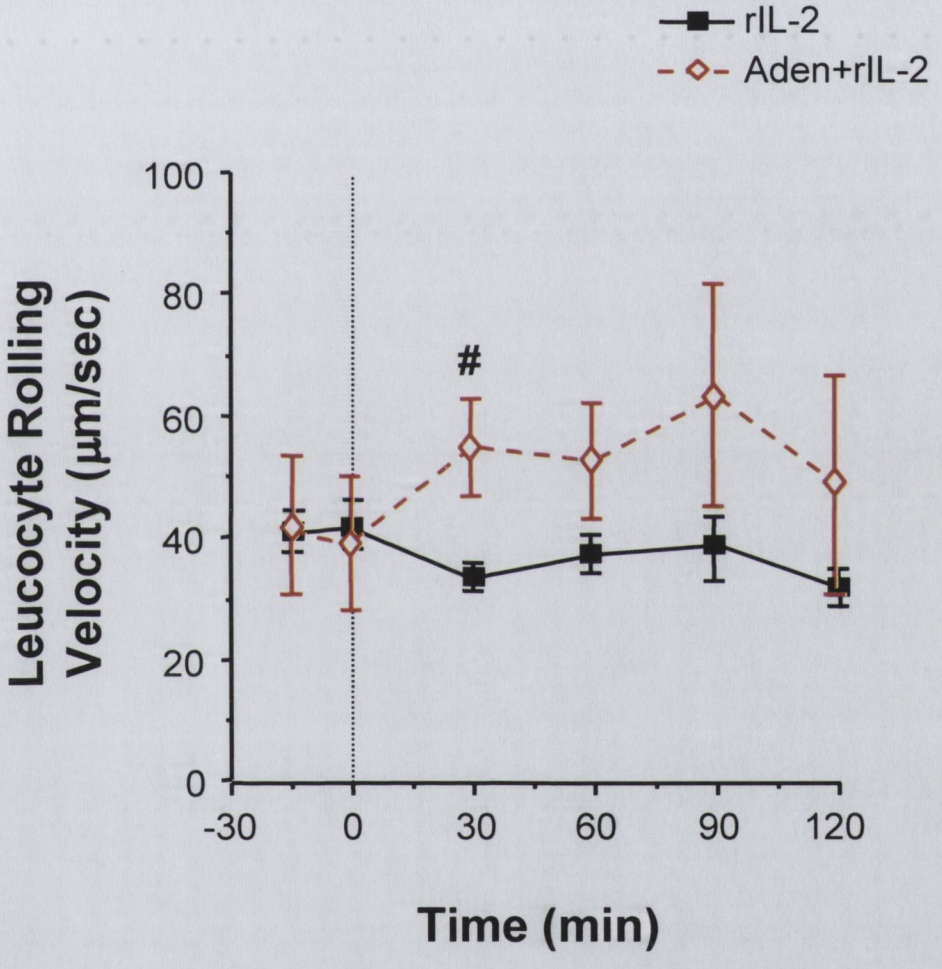


Figure 8.4 Baseline levels of leucocyte rolling velocity were recorded before and 15 min after (T=0 min) saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion began. All animals received a bolus of recombinant interleukin-2 @ 0 min, and leucocyte rolling velocity was measured every 30 min for 2 h. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=5/group. # $P<0.05$ vs. rIL-2 group.

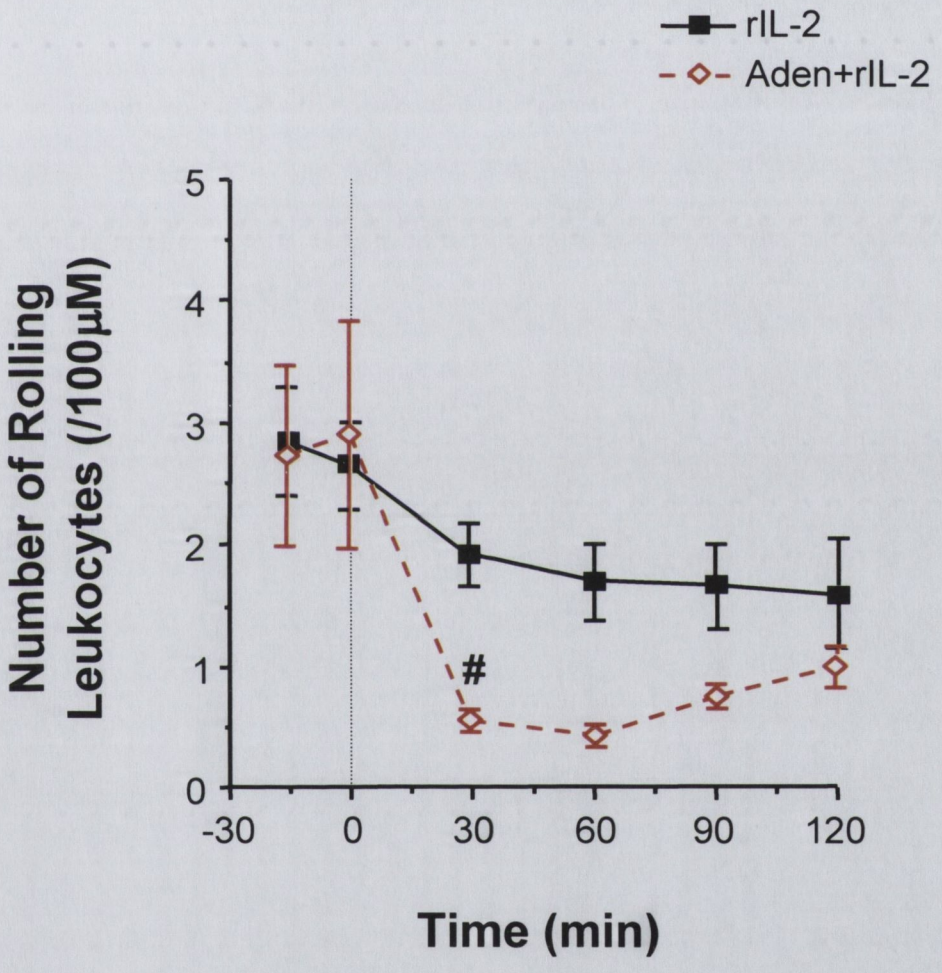


Figure 8.5 The number of rolling leucocytes were recorded under baseline conditions and 15 min after saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion began (T=0 min). All animals received a bolus of recombinant interleukin-2 @ 0 min, and leucocyte rolling was counted every 30 min for 2 h. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=5/group. # $P<0.05$ vs. rIL-2 group.

8.3.4 The Effect Of Interleukin-2 On Leucocyte Adhesion

The baseline level of leucocyte adhesion in the rIL-2 group was 2.8±0.65/100 μm. Administration of rIL-2 caused a significant increase in the number of leucocytes adherent to the vascular endothelium within 30 min ($P<0.01$ vs. baseline) (Figure 8.6). This continued to rise throughout the experiment peaking at 43.5±6.67 cells/100 μm at 90 min; $p<0.0001$ vs. baseline).

8.3.5 The Influence Of Adenosine On Interleukin-2-Induced Leucocyte Adhesion

Leucocyte adhesion in the adenosine-treated group did not change significantly from baseline values following bolus administration of rIL-2 (Figure 8.6). However, when compared with the rIL-2 group, animals in the Aden+rIL-2 group exhibited lower levels of leucocyte adhesion at all times following administration of rIL-2, which reached significance from 30 min onwards ($P<0.05$). By 120 min, the number of adherent leucocytes was 3.6±2.60 cells/100 μm. ($P<0.005$ vs. rIL-2 group).

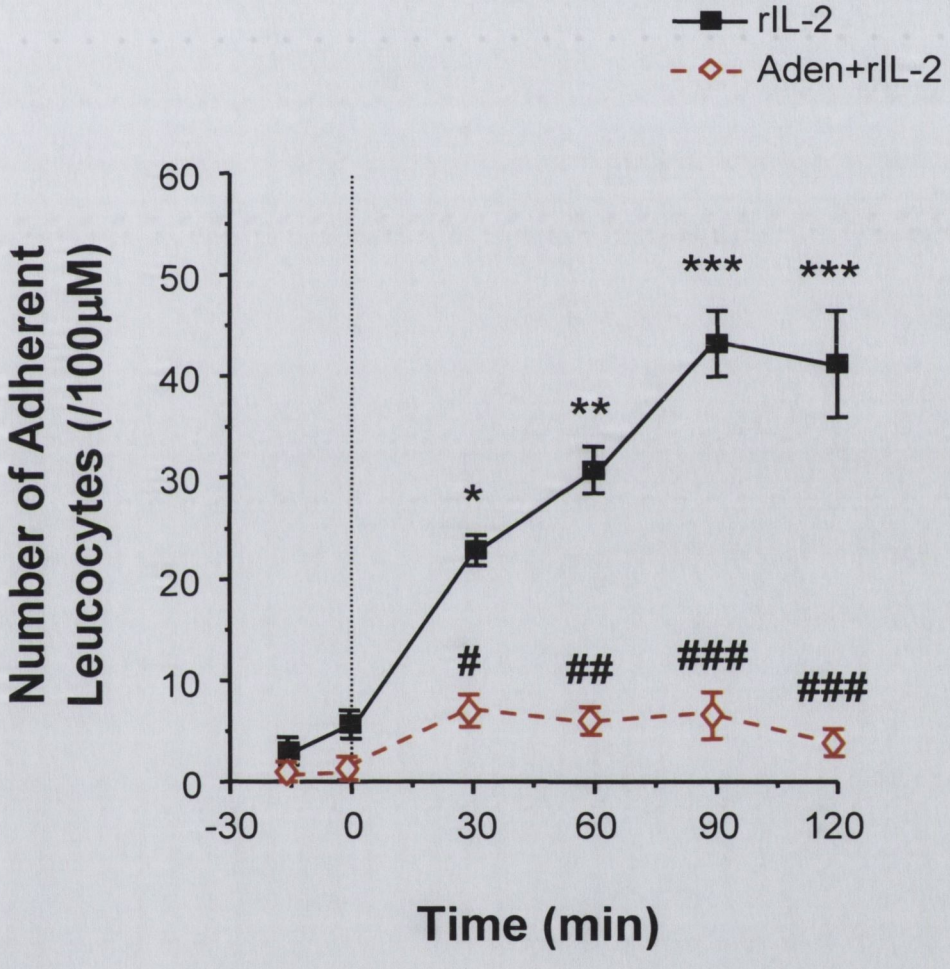


Figure 8.6 The number of adherent leucocytes was assessed under baseline conditions and 15 min after saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion began (T=0 min). All animals received a bolus of recombinant interleukin-2 @ 0 min, after which leucocyte adhesion was measured every 30 min for 2 h. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=5/group. * $P<0.01$ vs. rIL-2 baseline; ** $P<0.005$ vs. rIL-2 baseline; *** $P<0.0001$ vs. rIL-2 baseline; # $P<0.05$ vs. rIL-2 group; ## $P<0.01$ vs. rIL-2 group; ### $P<0.005$ vs. rIL-2 group.

8.3.6 The Effect Of Interleukin-2 On Leucocyte Emigration

The number of leucocytes transmigrating through the endothelial barrier into the interstitium was significantly enhanced from a baseline value of 1.3±0.62 cells/field to 29.0±11.76 cells/field at 90 min ($p<0.05$) and 56.0±14.50/field at 120 min ($p<0.005$) in the rIL-2 group (Figure 8.7).

8.3.7 The Influence of Adenosine on Interleukin-2-Induced Leucocyte Emigration

Animals treated with adenosine prior to receiving rIL-2 demonstrated levels of leucocyte emigration that were comparable to baseline throughout the 120 min period of observation (Figure 8.7). In fact only 1.7±0.88 cells/field had emigrated by 120 min in this group. The administration of adenosine in the Aden+rIL-2 group prevented rIL-2-induced leucocyte emigration at 90 min ($P<0.05$) and 120 min ($P<0.05$). Figure 8.8 shows representative images of post-capillary venules from rIL-2 group under baseline conditions (a), and every 30 min after rIL-2 administration up to and including 120 min (b-e). It can clearly be seen that under baseline conditions, very few leucocytes are adherent or emigrated (a). However, leucocyte adhesion and emigration increase progressively following rIL-2 administration. Conversely in the Aden+rIL-2 group at 120 min post-rIL-2 bolus, there are very few leucocytes inside the vessel or emigrated into the tissue (f).

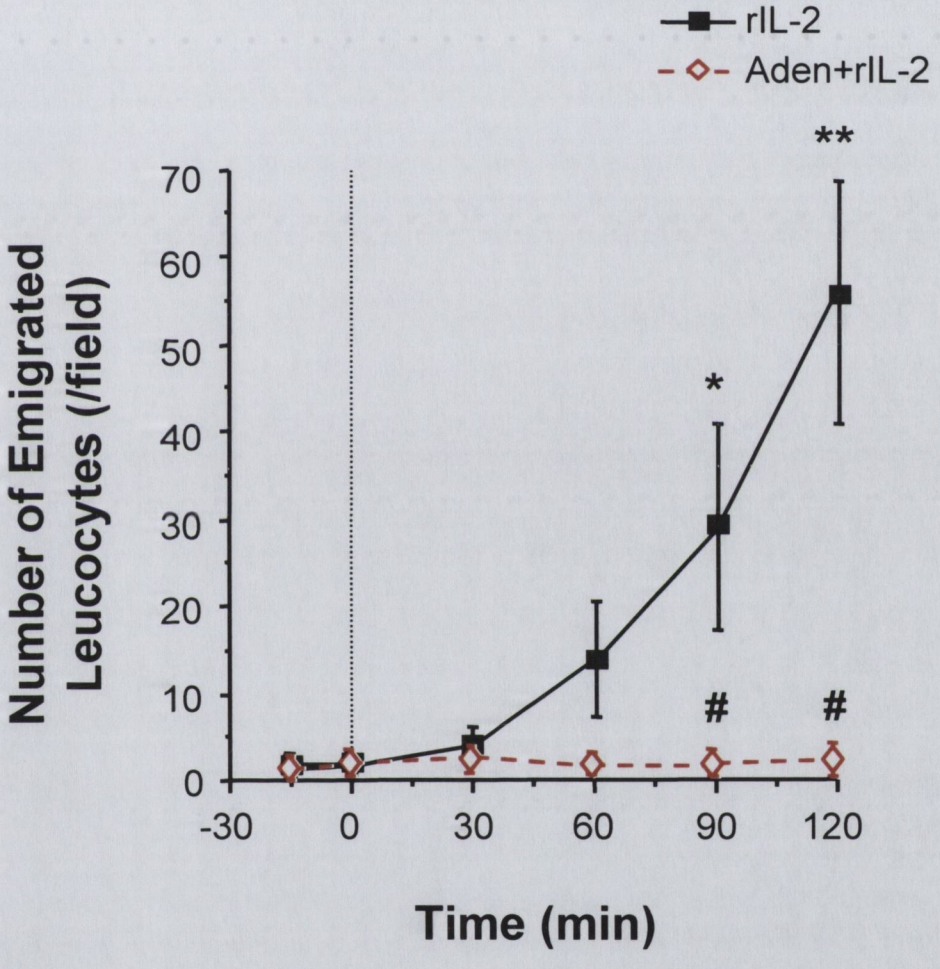


Figure 8.7 The number of emigrated leucocytes was assessed under baseline conditions (T=-15 min) and 15 min after saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion began (T=0 min). All animals received a bolus of recombinant interleukin-2 @ 0 min, after which leucocyte emigration was measured every 30 min for 2 h. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=5/group. * $P<0.05$ vs. rIL-2 baseline; ** $P<0.005$ vs. rIL-2 baseline; # $P<0.05$ vs. rIL-2 group.

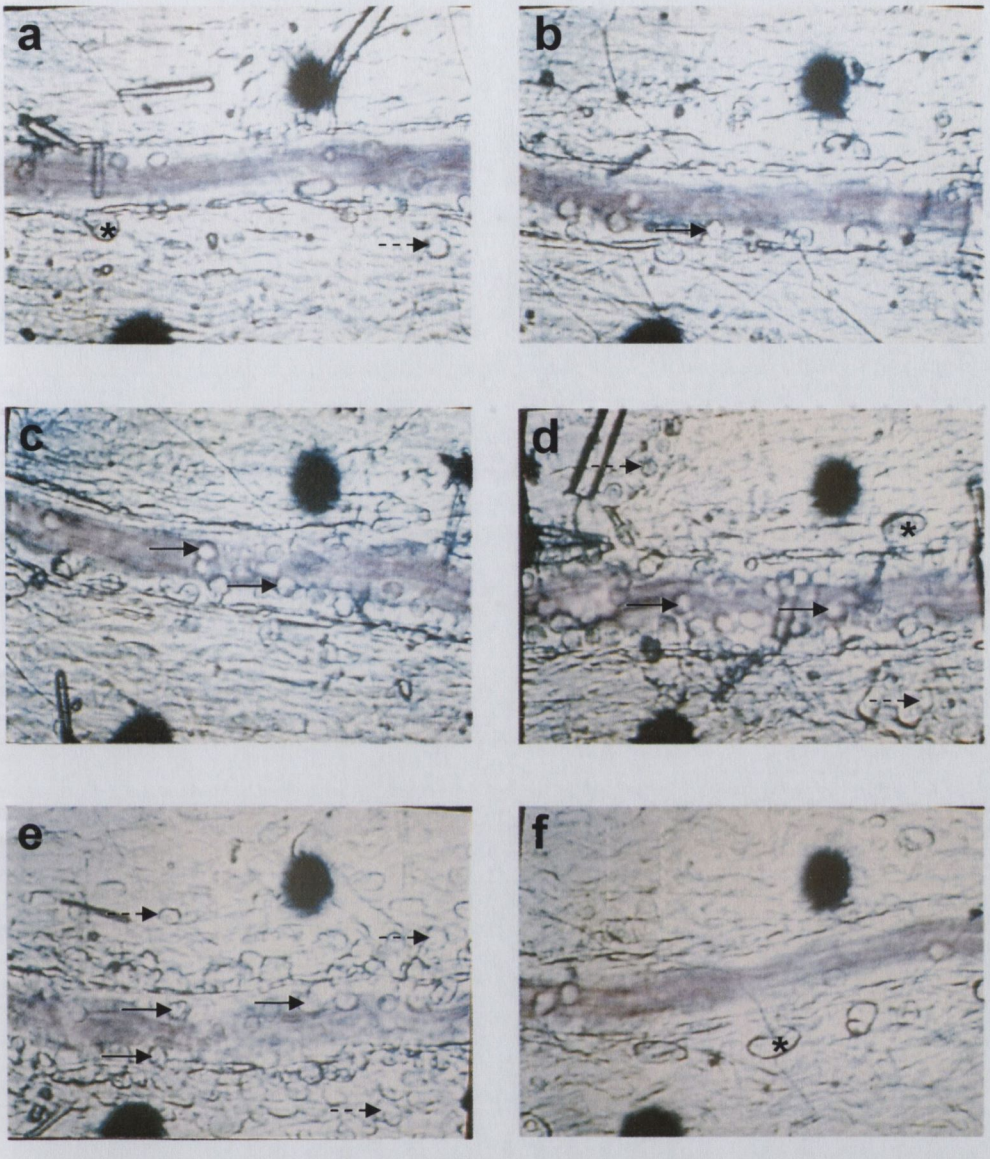


Figure 8.8 Images of postcapillary venules during intravital microscopy captured during the recording of post-capillary venules in animals treated with saline and rIL-2 (a-e) or adenosine and rIL-2 (f). Images a-e represent a post-capillary venules under (a) baseline conditions, and (b) 30 min, (c) 60 min, (d) 90 min and (e) 120 min after administration of 10^6 U rIL-2 i.v. The black dots are on the head of the optical doppler velocimeter, used to measure red blood cell velocity. Note the steady increase in the numbers of leucocytes both inside (rolling/adherent) and outside the vessel (emigrated) in images a-e. However, in image f, there are very few leucocytes present either inside or outside the vessel. This is a post-capillary venule from the adenosine-treated group 120 min after a bolus of rIL-2. Solid arrows point out adherent leucocytes; broken arrows mark emigrated leucocytes. The asterisk denotes a mast cell.

8.3.8 Adhesion Receptor Expression

Administration of rIL-2 caused a shedding of L-selectin by circulating neutrophils (identified by forward scatter versus side scatter characteristics using flow cytometry), as indicated by the sustained decrease in expression of this adhesion molecule which reached significance by 60 min ($P<0.01$ vs. baseline) (Figure 8.9). The Aden+rIL-2 group exhibited a small reduction in L-selectin expression, although this was not significant. In fact, the administration of adenosine significantly attenuated the rIL-2-induced shedding of L-selectin by approximately 50% ($P<0.05$ at 60 and 120 min; $P<0.01$ at 90 min) (Fig. 8.9). The expression of CD18 on peripheral venous blood neutrophils remained unchanged versus baseline throughout the experiment in both the rIL-2 and the Aden+rIL-2 groups (Figure 8.10).

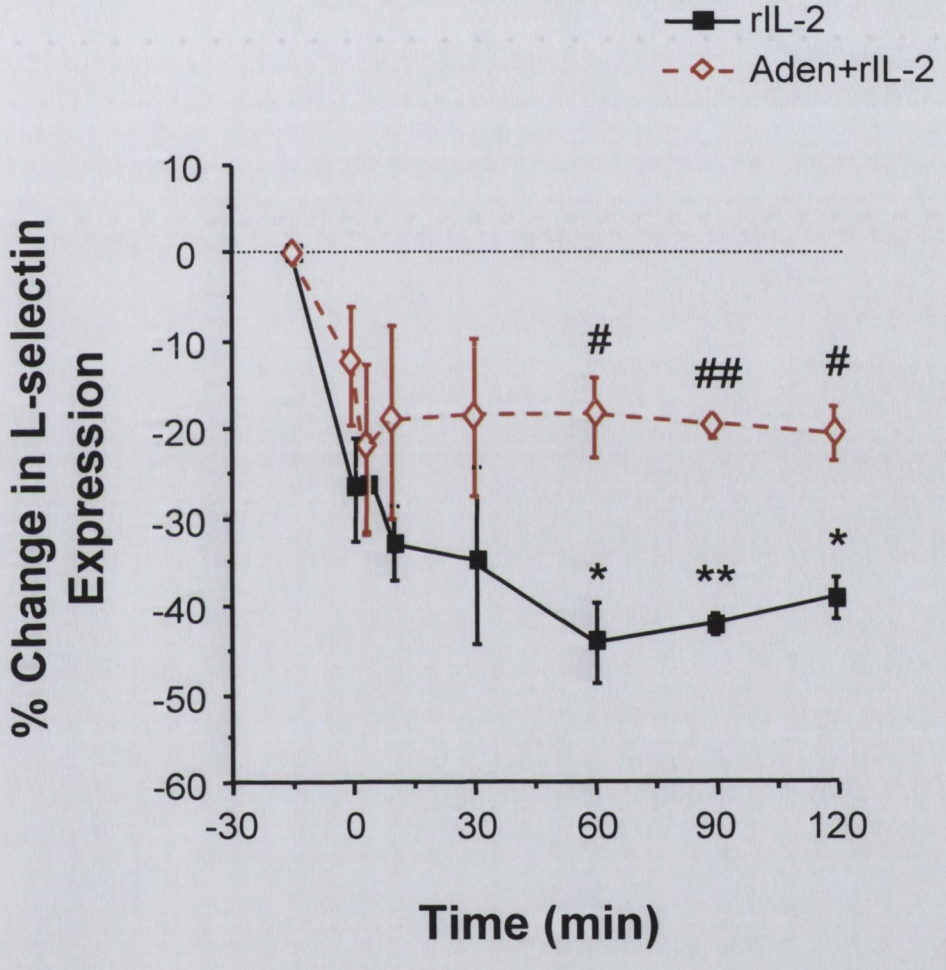


Figure 8.9 % Change in L-selectin Expression on circulating neutrophils as assessed using flow cytometry. The change in expression was calculated as a % of baseline values. Once blood was drawn for measurement of baseline levels (T=-15 min), saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion began. A blood sample was drawn at 0 min, immediately prior to the administration of recombinant interleukin-2 to all animals. Thereafter, blood samples were taken at various intervals over 2 h and L-selectin expression was measured. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=3/group; * $P<0.01$ vs. baseline; ** $P<0.005$; # $P<0.05$ vs. rIL-2 group; ## $P<0.01$ vs. rIL-2 group.

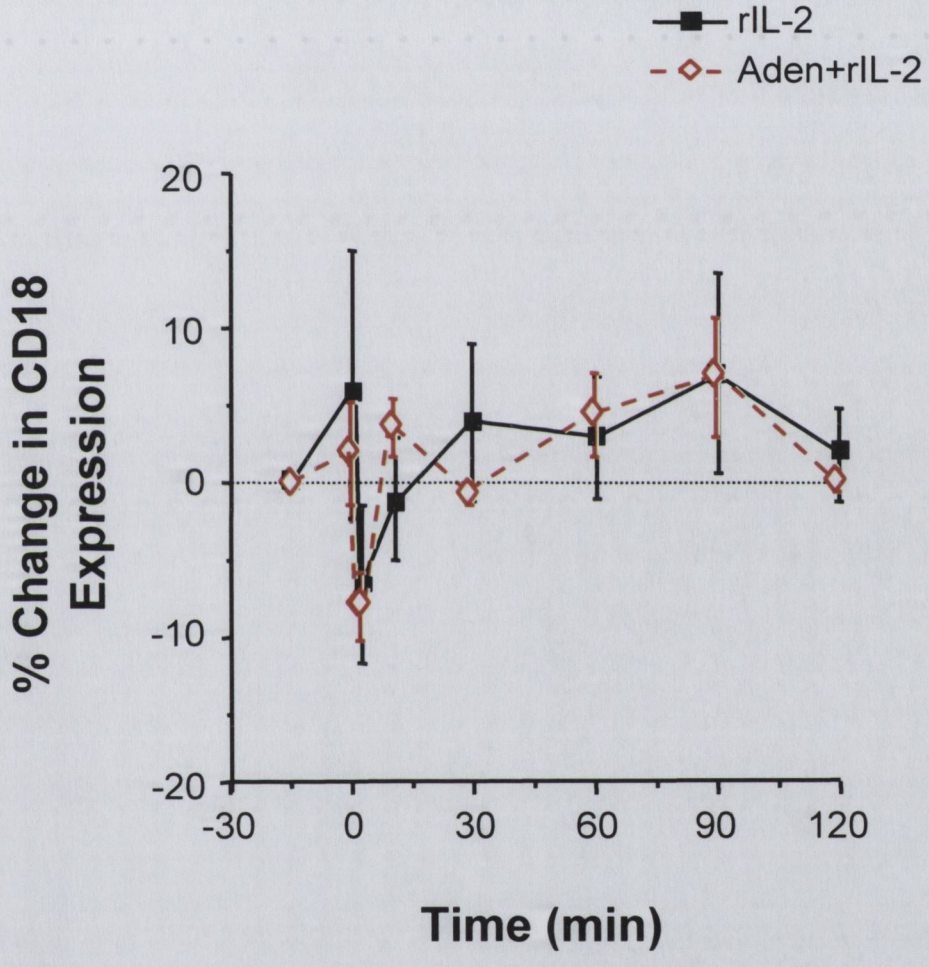


Figure 8.10 % Change in CD18 expression on circulating neutrophils as assessed using flow cytometry. The change in expression was calculated as a % of baseline values. Once blood was drawn for measurement of baseline levels (T=-15 min), saline (rIL-2 group) or adenosine (rIL-2+Aden) infusion was started. A blood sample was drawn at 0 min, immediately prior to the administration of recombinant interleukin-2 to all animals. Thereafter, blood samples were taken at various intervals over 2 h and CD18 expression was measured. Mean±SEM; unpaired (between groups) and paired (between timepoints within a group) t-tests; n=3/group.

8.4 DISCUSSION

One of the primary mediators of allograft rejection is the T-lymphocyte-derived cytokine IL-2. In fact immunosuppression protocols target the synthesis and actions of this cytokine, which is responsible for proliferation of alloreactive T-cells following transplantation. Levels of IL-2 are also increased after an IR insult, and coincide with T-cell infiltration of the post-ischaemic tissue and MHC II expression. Furthermore, IL-2 promotes leucocyte-endothelial interactions via the release of other inflammatory mediators. Hyperthermic preconditioning has been shown to reduce both IR-induced and IL-2-induced leucocyte adhesion and emigration in the microvasculature. This suggests that preconditioning could target both inflammatory responses that occur immediately upon reperfusion (CAM upregulation) and those that occur several days later (T-cell-derived cytokine responses). Adenosine exerts a protective effect against renal IR injury and has been implicated as a mediator of preconditioning in several organs. This study demonstrated that adenosine could also minimize leucocyte-endothelial interactions caused by administration of rIL-2 and that this was associated with a reduction in L-selectin shedding from the surface of circulating neutrophils, indicating that activation of these cells was reduced. These data suggest that adenosine might be a useful therapy in the prevention of IR- and IL-2-mediated inflammatory responses during transplantation.

Leucocytes interact with the vascular endothelium via three main stages, rolling, firm adhesion and emigration. Rolling is mediated by members of the selectin family, L-selectin on leucocytes and P- and E-selectin on the endothelium and their ligands which include sialyl Lewis X and PSGL-1. Adhesion and emigration are mediated by integrins such as members of the CD18 family on the leucocytes and Igs including ICAM-1 on the

vascular endothelium, although the nature of the bonds between these molecules are likely to be different for the two stages of interactions. In this study, IL-2 caused a significant increase in leucocyte adhesion and emigration, supporting findings by several other groups (Edwards *et al.*, 1992). Although leucocyte rolling has been shown to be important for subsequent adhesion of leucocytes to the vascular endothelium during inflammation, the finding that increased rolling did not occur is not completely unexpected, as it has been previously demonstrated that baseline levels of rolling are adequate to allow for firm adhesion when these leucocytes are activated. In fact, L-selectin is constitutively expressed allowing leucocytes to roll on activated endothelium. However this molecule is shed upon activation of the leucocytes. Many of these leucocytes will then adhere to the vessel wall via CD18, although some will re-enter the circulation. The decreased expression of L-selectin on circulating neutrophils in the rIL-2 group most likely reflects shedding of the receptor upon activation of these cells. Conversely, the lack of CD18 upregulation, despite the more profound effect of IL-2 on leucocyte-endothelial interactions being on the latter two stages of leucocyte extravasation, that is adherence and emigration which require CD18, appears to be contradictory. However, several studies have failed to find evidence of CD18 upregulation during inflammatory conditions in which CD18 does participate. This may be explained several ways. Firstly, the activated leucocytes that exhibit elevated CD18 expression may be the leucocytes that are adhering firmly to the endothelium and do not re-enter the circulation, hence analysis of circulating neutrophils would not detect upregulation. Alternatively, CD18-mediated adhesion may not require increased expression of this adhesion molecule as it is constitutively expressed and conformational changes rather than elevated expression may occur upon leucocyte activation, which would also not be detected by flow cytometry.

IL-2 synthesis and actions have been targeted since the invention of immunosuppressive protocols for transplantation. It has recently been shown that CsA possesses IL-2-independent anti-inflammatory properties which may contribute to its effectiveness in improving allograft outcome. Of particular interest is the ability of CsA to attenuate ICAM-1 induction on renal tubular epithelium by TNF-α (Frishberg *et al.*, 1996). However, the long-term use of CsA during transplantation is limited by a dose-dependent toxicity. Recently developed agents include chimeric antibodies directed against the IL-2R subunit, CD25, which decreased acute rejection by as much as 30-40% (Wiseman and Faulds, 1999). Levels of IL-2 are elevated in the kidney during renal IR injury and following transplantation into an allogeneic recipient (Takada *et al.*, 1997a; Nagano *et al.*, 1997). This occurs in parallel with infiltration of the kidneys with $CD4^+$ T-cells and the induction of MHC II. In fact CsA can reduce IR injury alongside an associated decrease in infiltrating $CD4^+$ T-cells in syngeneic grafts (Kouwenhoven *et al.*, 1999). Not only does IL-2 exert profound stimulatory actions on T-cells, but this cytokine induces the production of several inflammatory substances e.g. IL-1, PAF and complement, which may mediate the leucocyte-endothelial interactions caused by IL-2 as it does not directly effect neutrophils (Lentsch *et al.,* 1999). These responses in turn promote the development of organ oedema (Edwards *et al.*, 1992). In fact several of these mediators have been implicated in the pathogenesis of renal IR injury, e.g. PAF (Torras *et al.*, 1999).

Adenosine, on the other hand, protected against IL-2-induced leucocyte adhesion. The preservation of L-selectin on neutrophils in the adenosine-treated animals indicates that this compound reduced neutrophil activation. It is not however clear whether this is a mechanism of action of adenosine or if it is merely a marker of decreased cellular activation due to some other anti-inflammatory effect of adenosine. Similar to findings in

this chapter with IL-2, previous studies on IR have also demonstrated that adenosine prevents can prevent migration without any detectable alteration in expression of CD18 by circulating neutrophils (Grisham *et al.,* 1989). Both activated neutrophils and endothelial cells release adenosine, which exerts several anti-inflammatory properties on both cell types. These anti-inflammatory effects of adenosine appear to be mediated by A2a receptor activation. This leads to reduced oxidant release e.g., O_2^- and H_2O_2 from neutrophils, and decreased neutrophil adhesion to endothelium (Cronstein *et al.,* 1986; Cronstein *et al.,* 1992). The direct action of adenosine on endothelial cells leads to activation of adenylate cyclase causing cAMP-mediated endothelial cell relaxation (thereby reducing the intercellular gap) and reduced adhesion receptor expression. In fact, Okusa *et al.* (2000) found that activation of A2a receptors attenuated renal IR-induced organ dysfunction which was associated with reduced P-selectin and ICAM-1 upregulation, and lower levels of neutrophil infiltration of the kidney. Whether the decrease in adhesion molecule expression was mediated directly via actions on the vascular endothelium or indirectly by binding to and stabilizing resident tissue inflammatory cells is unknown. Interestingly, activation of A1 receptors was also shown to protect against post-ischaemic renal dysfunction (Lee and Emala, 2000). In fact, activation of A1 or A2a receptors attenuated oxidant injury in human proximal tubule cells (Lee and Emala, 2002), which may have important implications in renal IR where there is a robust generation of oxidants. Thus adenosine is not only capable of blocking release of oxidants from neutrophils but may also prevent damage by released oxidants, although this protection required new gene transcription and protein synthesis. Exactly how adenosine acted to reduce IL-2-mediated inflammation remains to be determined, although it may be via the modulation of adhesion molecule expression which would directly impact leucocyte adhesion and emigration.

In addition to its anti-inflammatory effects mentioned above, adenosine itself has been shown to precondition myocardium against post-operative dysfunction following bypass surgery (Zarro *et al.*, 1998). Furthermore, this purine nucleotide has been implicated in IPrec models (Liu *et al.*, 1992), although this does not appear to be the case in the kidney (Lee and Emala, 2001; Sugino *et al.*, 2001). However, adenosine can itself protect against post-ischaemic renal dysfunction via a signal transduction pathway that involves PKC, similar to IPrec in the kidney (Lee and Emala, 2001; Chapter 4). In addition, adenosine was implicated in a model where HSP70 transfection conferred protection against IR injury (Sakaguchi *et al.*, 2000). The attenuation of myocardial IR injury by transfection of rat hearts with HSP70 was associated with increased activity of ecto-5'-nucleotidase. Ecto-nucleotidases rapidly convert adenine nucleotides that are released by a variety of cells including endothelium, platelets and leucocytes when injured to adenosine, thereby providing a source of adenosine during inflammation (Cronstein *et al.*, 1986; Linden *et al.*, 2001). Inhibition of ecto-5'-nucleotidase in turn blocked the protection granted by HSP70 transfection against post-ischaemic myocardial injury, suggesting that adenosine may be important in the reduction of injury by HSP70 (Sakaguchi *et al.*, 2000). The exact role of adenosine in HSP70-mediated protection during preconditioning has yet to be elucidated.

Currently, adenosine is incorporated into organ preservation solutions during transplantation to aid with regeneration of ATP levels upon reperfusion. However, with its wide array of actions, adenosine may have further potential as a therapeutic agent during transplantation. While it has been used clinically, caution must be taken as adenosine is also a potent vasodilator, which can induce significant systemic hypotension, although protective properties of adenosine and its receptor agonists have been demonstrated at doses which do not causes these side-effects (Lee and Emala, 2000). Prior to clinical

evaluation this will have to be fully evaluated. One way to overcome this problem is to use a purine precursor, such as acadesine, which is a local adenosine-regulating agent with minimal effects on blood pressure (Fabian *et al.,* 1996). With respect to HSP70-associated preconditioning, it will remain to be seen whether gene transfer of HSP70 into kidneys for transplanting is associated with increased adenosine release. Nonetheless, treatment of organs to induce HSP70 and/or to increase adenosine levels may prove protective not only against IR but also against alloimmune responses, possibly by reducing leucocyte infiltration via decreased adhesion molecule expression, thereby attenuating the release of cytokines and other harmful mediators which could promote allograft rejection.

Chapter 9

Discussion

The stopping (ischaemia) and restoration (reperfusion) of blood flow in a grafted organ is an unavoidable step during transplantation. However IR may negatively influence graft outcome by itself predisposing the tissue to DGF, acute allograft rejection and ultimately chronic rejection. The effect of IR can be appreciated both structurally and functionally. However an inflammatory process characterized by adhesion molecule up-regulation, leucocyte recruitment and increased immunogenicity appears to underlie these changes. Several approaches have been taken to minimize the pro-inflammatory influence of IR, including the reduction of ischaemic time, decreasing ischaemic temperature and the use of protective storage solutions such as University of Wisconsin solution. However, while short-term graft survival has improved enormously, many kidneys are still lost to chronic rejection, a process that may begin with IR injury and be promulgated by episodes of acute rejection. This thesis addresses the premise that rather than targeting individual aspects of the inflammatory cascade triggered by IR, pre-treatment of the organ to initiate its inherent defence mechanisms before it is exposed to IR may prove a more effective therapeutic approach to reducing the influence of IR on allograft outcome.

Preconditioning is a phenomenon whereby exposure of a cell or tissue to a sub-lethal stress paradoxically protects against a subsequent otherwise lethal stress. Many mediators have been shown to be involved in this, including HSPs. These proteins are ubiquitously expressed under normal conditions but are up-regulated by a variety of stresses that include ischaemia, hyperthermia and chemical inducers. The objectives of this thesis were to 1) establish a model of early renal IR injury in the rat; 2) employ this model to study the potential of several methods of preconditioning to reduce acute IR injury; 3) assess whether the mode of preconditioning most effective in attenuating acute IR injury may be acting via an alteration in ICAM-1 expression and organ immunogenicity; 4) investigate

whether this latter means of preconditioning could also reduce long-term organ injury following IR; and finally 5) set up an intravital microscopy facility to begin preliminary assessment of *in vivo* leucocyte-endothelial interactions using a simple non-invasive model of injury.

Initially, a time-course of renal reperfusion injury in a rat uninephrectomy model was established, using an ischaemia time of 45 minutes based on several other studies. Significant renal dysfunction, leucocyte infiltration and tubular deterioration were observed within 2 h reperfusion, one of the earliest reperfusion times examined to date in this model. While renal function continued to deteriorate for at least 24 h reperfusion in accordance with findings by others, 2 h reperfusion was chosen as the endpoint at which to first assess the efficacy of preconditioning treatments, as the injury indices were reproducible and addressed several aspects of injury. Furthermore, this approach allowed preliminary investigation into which early events of the inflammatory cascade initiated by IR may be altered by preconditioning.

Ischaemic preconditioning is where the tissue or organ is exposed to short episodes of non-lethal ischaemia prior to the application of the main injury. This method has been most extensively characterized in the heart, although other organs also respond to this pre-treatment, such that preliminary studies have actually been performed in humans. While initial findings have been mixed there is evidence that IPrec does exist in humans. The first controlled study demonstrated that in humans undergoing routine coronary artery bypass grafting, two 3 min periods of ischaemia separated by 3 min reperfusion before the 10-15 min ischaemia required to perform a coronary vascular anastomosis led to reduced levels of post-bypass serum troponin T. While evidence is lacking for IPrec in human

transplantation, it has been shown that livers from human donors that have undergone a brief period of cardiopulmonary arrest prior to organ procurement exhibit reduced aspartate transaminase and alanine transaminase levels following transplantation despite incurring organ damage during the arrest. Graft and patient survival was not adversely affected by the donor cardiopulmonary arrest, suggesting that preconditioning may have occurred (Raeburn *et al.,* 2001). Importantly, ischaemic preconditioning is protective when applied immediately before IR in many organs. Thus this was chosen as the first method of preconditioning to assess in this thesis, although a limited number of previous studies had revealed conflicting results in the kidney. Short bouts of ischaemia were applied to the kidney before it was exposed to the main IR insult. However, in the absence of a recovery or development period between preconditioning and the IR injury, no protection was observed. This is surprising in light of more recent studies revealing the existence of immediate protection in the kidney following IPrec. As discussed in Chapter 4, this discrepancy is unlikely to be fully explained by differences in protocol and species. Interestingly, after a two hour recovery period between preconditioning and clamping of the renal pedicle for 45 min, protection against the main IR insult was becoming evident. Extending this development period to four hours between preconditioning and IR completely attenuated the post-ischaemic organ dysfunction. This was associated with a reduced leucocyte infiltration, suggesting that blocking early leucocyte-endothelial interactions may a mechanism through which this protection was manifested.

It was discovered that the window of recovery required was associated with the induction of the highly conserved protein HSP70 by the time IR was applied. This stress protein has been implicated as a mediator of preconditioning using numerous methods of preconditioning in many organs. Furthermore, in an attempt to elucidate the mechanism

through which the HSP70 was induced by IPrec, a blocking study revealed that PKC was important in the induction of this stress protein as well as the associated functional protection against IR. Although this kinase has been widely implicated in the immediate protection observed following IPrec in the heart (a HSP70-independent process) only a few studies have suggested that PKC may be important for the induction of HSP70 and the stress response. Exploring IPrec further would have been of great interest had there been immediate protection, which would have allowed the findings to be extended not only to transplantation but to vascular surgery where renal ischaemia may be necessary. However, required development period coupled with the invasiveness of the procedure meant that this was not a clinically appealing method of preconditioning. Rather, since in reality it is counterintuitive for a surgeon to initiate longer ischaemia than is required by the surgery in question, most of the research in this field is actually focused on developing a pharmaceutical strategy that may easier (practically and ethically) to investigate in humans. Thus it was decided to pursue the HSP pathway further by using a pharmaceutical means of inducing stress proteins.

To this end, SA was employed for several reasons, not least of which was that this substance can induce many HSPs, including HSP70, HSP60, HSP27 and HSP90, in both animal and human kidney cells at non-toxic levels, and is readily available. However, despite basing the dose and route of administration on several other studies, no HSP70 expression was found in the kidneys of our animals. This was not due to the SA failing to enter the circulation as HSP70 was induced in the lungs. Furthermore, based on results from a subsequent study in the kidney, it was unlikely to be due to the time at which the kidneys were examined. There was a concomitant lack of improvement in post-ischaemic renal function in SA-treated animals, supporting the proposal that the induction of HSP70

expression is important in conferring protection against renal IR injury by preconditioning. This led to the use of a more established and better characterized inducer of HSPs, namely heat itself, or hyperthermia.

A more detailed approach to establish a time-course of HSP induction was taken in this chapter. Rats were exposed to hyperthermia and allowed to recover for various periods up to 24 h, at each of which HSP27, HSP60, HSP70 and HSP90 expression were assessed. It was determined that HSP60 and HSP70 only were induced. After consulting other studies where the time-course of protection was directly compared with the levels of HSP70 present in the cells/tissue it was decided that the IR injury should be applied after peak HSP70 expression, before the levels of this stress protein reached control levels again. This method of preconditioning not only attenuated renal dysfunction, tubular deterioration and leucocyte infiltration at 2 h reperfusion, but these signs of injury and inflammation remained suppressed at 18 h reperfusion. Because leucocyte interaction with the vascular endothelium is one of the first steps in the chain of inflammatory events culminating in organ injury following IR, and hyperthermic preconditioning attenuated leucocyte accumulation, an adhesion molecule known to be involved in leucocyte-endothelial adhesion in the post-ischaemic kidney was investigated. The expression of ICAM-1 was up-regulated after just 2 h reperfusion in the non-treated ischaemic animals. This was prevented by preconditioning with whole-body hyperthermia, thus suggesting that one mechanism through which hyperthermia was working was by reducing adhesion molecule expression and thereby leucocyte infiltration of the post-ischaemic organs. This would inhibit the release of many inflammatory substances and stop the inflammatory process during the initial phase, providing a potential therapeutic approach to reducing transplantation-associated IR injury.

The blocking of ICAM-1 may have another benefit during transplantation, when there is another aspect to consider, that is, the alloimmune response. It is important to consider that ICAM-1 is not only used by leucocytes to infiltrate organs, but is also an accessory molecule in T-cell receptor/MHC-mediated interactions between T-cells and antigen-presenting cells. In fact ICAM-1 may serve to strengthen these interactions. Thus, targeting the up-regulation of ICAM-1 may exert a dual effect. Furthermore, blocking the adhesion of leucocytes to the vascular endothelium by preventing ICAM-1 may actually prevent the inflammatory pathway leading to the expression of cytokines such as IFN-γ that would up-regulate MHC in the first place, further reducing the likelihood of an alloimmune response. This idea is supported by studies that have blocked ICAM-1 and found that MHC II induction is also prevented in both post-ischaemic and allografted kidneys. Therefore, the possibility that hyperthermic preconditioning may also inhibit MHC induction was investigated here. It was found that this pre-treatment did indeed prevent the induction of MHC II expression on post-ischaemic tubular epithelium at 5 days reperfusion. This could have important implications for transplantation. However, it must be kept in mind that pre-treatment with antisense oligonucleotides or antibodies against ICAM-1 has been equally effective in attenuating ICAM-1 and MHC II expression, as well as improving renal function in several animal models, but the first studies using blocking antibodies to ICAM-1 in humans have been disappointing. With the generation of chimeric antibodies with higher efficacy, or new modifications for oral or i.v. formulations of antisense oligonucleotides, this approach may still prove to be a useful therapy. It should be remembered here that preconditioning is employing the cells own natural defence system i.e. HSPs. The induction of these proteins is likely to be exerting a more widespread protective influence on the kidney than the use of antibodies. Other

potential pathways through which preconditioning may be acting include aiding in the recovery of damaged cells through chaperone actions of the HSPs and the induction of antioxidants. In fact, several groups have shown that transfection of cells/organs with HSP70 confers protection against a subsequent insult suggesting this protein is not an innocent bystander to the protection exerted by preconditioning.

Another finding of interest in the animals allowed to reperfuse for five days was the fact that the renal tubular damage, while recovering back towards normal at this time-point in the IR group, was nevertheless improved by preconditioning, suggesting that tissue integrity and function may be maintained beyond the immediate post-ischaemic period by preconditioning. Since renal IR injury has been shown to be a biphasic response, with an early inflammatory phenotype which resolves within approximately one week and begins to appear again after 12 weeks, this begged the question whether or not the prevention of this early post-ischaemic injury and leucocyte infiltration by hyperthermic preconditioning would influence the chronic changes associated with IR injury in the kidney. This was addressed in chapter 7.

The assessment of renal damage in long-term models of IR has shown that tubular deterioration is associated with fibrosis. Furthermore, the decline in function has predominantly been addressed using urine protein levels. Based on studies by others, a model of chronic IR injury was set up where 45 min ischaemia was followed by 16 wk of reperfusion, a time-point at which renal function should be decreasing. However, while renal dysfunction was not observed, tubular epithelium did show deterioration, and fibrosis was beginning to appear. This would suggest that renal dysfunction was not far behind. However preconditioning with hyperthermia before ischaemia actually prevented

not only the acute post-ischaemic changes but also these chronic morphological responses. Interestingly, the elevated leucocyte accumulation in the chronic kidney model was also reduced by preconditioning, this possibly being one of the mechanisms whereby morphological damage was attenuated by hyperthermic preconditioning, as appeared to be the case for the acute injury.

These observations that HSP70 induction is associated with reduced leucocyte accumulation further stimulated a long-standing goal in our laboratory to be able to quantify leucocyte-endothelial interactions in real-time *in vivo*. To this end, an intravital videomicroscopy unit was set up. A preliminary study using rIL-2-mediated injury of the rat mesentery was chosen for several reasons. Firstly, unlike the kidney, which requires the development of hydronephropathy to observe leucocyte recruitment (essentially destroying the normal structure of the organ), the mesentery is one of the most widely used tissues studied with intravital microscopy and requires minimal manipulation. Secondly, rather than beginning with an invasive injury model such as IR, a generous donation of rIL-2 prompted this use of this cytokine which has been widely implicated in the pathogenesis of allograft rejection and is in fact targeted by several immunosuppressants. Adenosine was used to pre-treat the animal. It is a nucleotide that has been implicated in many models of preconditioning and is capable of inhibiting neutrophil O_2^- synthesis and adherence to vascular endothelium. Furthermore, adenosine attenuates renal IR injury and is currently used in organ preservation solutions during transplantation. Elevated leucocyte-endothelial interactions were observed following IL-2 administration. This was associated with a shedding of L-selectin, suggesting that circulating neutrophils were activated. Adenosine pre-treatment prevented the inflammatory alterations in the post-capillary venules and was associated with reduced

shedding of L-selectin. Our group has gone on to demonstrate that hyperthermic preconditioning also induces HSP70 expression in the mesentery and that this is associated with reduced microvascular inflammatory responses to both IR and IL-2. This technique has the potential to offer invaluable supporting data to functional studies in many models of injury.

The novel findings in this thesis that protection conferred by preconditioning against subsequent IR injury is associated with the reduction of adhesion molecule and antigen expression, and is not only evident in the immediate post-reperfusion period but influences chronic alterations to IR provide important insight into the possibilities for preconditioning in the transplantation arena as well as other surgeries, such as coronary revascularisation, vascular surgeries where the kidney and intestine undergo ischaemia and skin and muscle flap surgery, where an ischaemic period is unavoidable. The manner in which preconditioning was studied in this thesis allowed enquiry into the potential mechanisms of protection, a common theme among investigators in this area, where the development of pharmaceutical means of preconditioning prevails. For example, with the advent of transfection techniques, delivery of HSP70 to an organ immediately before grafting may become possible in the near future. In humans, support for a protective role for HSP70 in a clinical situation comes from a study by Demidov *et al.* (1999) where patients with high initial myocardial levels of HSP70 exhibited superior cardioprotection during cardiac surgery. Not only could preconditioning improve the survival of organs currently used for transplantation, but successful studies on preconditioning of fatty livers before transplantation (Mokino *et al.,* 2004) have given hope that preconditioning could salvage currently rejected donor organs for use, thereby expanding the organ pool for transplantation.

Before the advent of newer therapies the actual methods of preconditioning employed here while on the surface not appearing practical for use in humans, may be a realistic goal for transplantation, where local heating of an organ may be an easily achievable and clinically sound method of reducing organ procurement-associated IR injury (Van der Zee, 2002). Furthermore, the use of many currently available drugs, such as the adenosine precursor, acadesine, or the K_{ATP} channel opener, nicorandil, that target various mediators involved in preconditioning are receiving great attention for use as preconditioning agents in surgeries that require scheduled ischaemic periods. In conclusion, results herein indicate that HSP70 may be a useful tool to combat renal allograft rejection by not only protecting the organ from the IR injury incurred during transplantation, but also from the immune response elicited by the allogeneic recipient against the graft by reducing organ immunogenicity (MHC expression). This approach is likely to be most relevant for cadaveric transplantation, where the organs are exposed to longer periods of both warm and cold ischaemia. This contributes to their inferior prognosis when compared with organs from living donors, thus minimizing the IR injury aspect of the procedure may improve the long-term survival of these grafts. Furthermore since HSP70 is inducible in many if not all tissues, these results may extend beyond the kidney to multiple other organs systems.

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APPENDIX

Publications arising from this thesis:

Stokes, K.Y., Abdih, H.K., Kelly, C.J., Redmond, H.P., and Bouchier-Hayes, D.J. (1996) Thermotolerance attenuates ischemia-reperfusion induced renal injury and increased expression of ICAM-1. **Transplantation** 62, 1143-1149.

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