Investigating the molecular mechanisms of hypothermia-mediated hepatic protection following intestinal ischaemia-reperfusion injury

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Declaration of originality

I, Emma Jane Parkinson confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 22nd February 2011

Abstract

Introduction:

The pathophysiology of hepatic energy failure following intestinal ischaemia-reperfusion injury (IIR) is not fully understood. In an experimental model of IIR, moderate hypothermia reduces hepatic dysfunction. The aim of this thesis was to investigate the molecular mechanisms of hypothermia-mediated hepatic protection following IIR.

Results:

Although heat shock proteins (HSPs) can be induced by hypothermia, there were no differences in levels of cytosolic HSPs between normothermic and hypothermic IIR groups. Normothermic IIR (NIIR), however, caused a significant increase in activated (phosphorylated) STAT (Signal Transducers and Activators of Transcription) proteins compared with normothermic sham (NS), an action which was completely abolished by moderate hypothermia. Microarray technology revealed increased hepatic gene expression of several acute phase proteins following NIIR which decreased following hypothermia. These effects were investigated further using PCR and measurement of protein amount of targets identified from the gene array. NIIR results in a significant increase in both IL-1β protein and IL-1 receptor antagonist (IL-1Ra) compared with sham operation. In contrast, hypothermic IIR (HIIR) results in a significant decrease in hepatic IL-1β as compared to NIIR but did not significantly change the level of IL-1Ra. In order to investigate whether mitochondrial dysfunction was central to the pathogenesis of hepatic energy failure, biochemical measurements of mitochondrial function and proteomic studies of isolated hepatic mitochondria was performed. Although there were no differences in mitochondrial function,

there were several differences observed in important mitochondrial proteins (e.g. mitochondrial HSPs and thioredoxin-dependent peroxidise reductase) revealed by 2D-DIGE.

Conclusions:

Hypothermic protection does not appear to act via cytosolic HSPs, but activation of STAT pathways may be involved, which may subsequently reduce the hepatic acute phase response. Although primary energetic failure of hepatic mitochondria does not appear to occur following NIIR, there may be other mitochondrial effects mediated at the protein level which ultimately lead to hepatic energy failure.

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Detail of Personal Contribution

The work contributing to this thesis is all my own, except where I have specifically acknowledged the contributions of others appropriately in the text, and detailed as follows:

Chapter 5: Samples were processed and histologically stained by Professor Neil Sebire and staff of the Department of Histopathology, Great Ormond Street Hospital.

Chapter 6: I prepared the samples for microarray analysis, which were then analysed by Dr Nipurna Jina and Dr Mike Hubank at UCL Genomics. EGF ELISAs were performed by the group of Professor Ebba Nexø at Aarhus University, Denmark. Cardiotrophin ELISAs were performed by the group of Professor L Ng, University of Leicester.

Chapter 7: I prepared the samples for 2D-PAGE electrophoresis and performed preliminary 2D-PAGE analyses myself. The definitive 2D-PAGE analyses appearing in the chapter were run by Dr Wendy Heywood (WH) and Dr Kevin Mills, ICH.

List of Abbreviations

ADP	adenosine 5’-diphosphate
ALT	alanine aminotransaminase
AMP	adenosine 5’-monophosphate
AMPK	AMP-activated protein kinase
APS	ammonium persulphate
APP	acute phase protein
APR	acute phase response
AST	aspartate aminotransferase
ATP	adenosine 5’-triphosphate
C/EBP	CCAAT- enhancer binding protein
CO	carbon monoxide
CPT 1	carnitine palmitoyl transferase 1
CRP	C reactive protein
DIC	disseminated intravascular coagulation
DNP	2, 4 -dinitrophenol
DTNB	5, 5’-dithiobis-(2-nitrobenzoic acid)
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EPR	electron paramagnetic resonance
EST	expressed sequence tag
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)

HGF	hepatocyte growth factor
HO-1	heme oxygenase-1 (HSP32)
HSP	heat shock protein
IEF	isoelectric focusing
iNOS	inducible nitric oxide synthase
IκB	inhibitor of NF-κB
ICAM	intercellular adhesion molecule
IKK	IκB kinase
IL-1	interleukin-1 (α/β)
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IFN	interferon
IIR	intestinal ischaemia-reperfusion
IPC	ischaemic preconditioning
LCA	leucocyte common antigen
LPS	lipopolysaccharide (i.e. glycolipids on outer membrane of all gram-negative bacteria)
LBP	lipopolysaccharide binding protein
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MODS	multiorgan dysfunction syndrome
MPO	myeloperoxidase
MPT	mitochondrial permeability transition
MS	mass spectrometry
mtDNA	mitochondrial DNA

NAD	nicotine adenine dinucleotide (oxidised)
NADH	nicotine adenine dinucleotide (reduced)
NEC	necrotising enterocolitis
NF-κB	nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase
PAMP	pathogen associated molecular pattern
PARP	poly (ADP-ribose) polymerase
PDH	pyruvate dehydrogenase complex
P_i	inorganic phosphate
PTP	mitochondrial permeability transition pore
ROS	reactive oxygen species
RCR	respiratory control ratio
SDS	sodium dodecyl sulphate
SEC	sinusoidal endothelial cells
SIRS	systemic inflammatory response syndrome
SOCS	suppressors of cytokine signalling
SOD	superoxide dismutase
STAT	signal transducers and activators of transcription
TEMED	tetramethylethylenediamine
TIMP	tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF-α	tumour necrosis factor α

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Chapter 1

Introduction

Chapter 1 – Introduction

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1.1 General introduction

Intestinal ischaemia-reperfusion (IIR) injury appears to be an important element of the pathology of many surgical emergencies and other more broad pathologies such as overwhelming sepsis. It is a reliable experimental model for reproducible gut injury but the wider, systemic influence of such an injury is not fully understood. This experimental scenario may actually reproduce the clinical situation of many paediatric surgical emergencies more closely than first anticipated as there is inevitably a delay between the initial insult occurring and the institution of supportive intensive therapy and/or laparotomy. During this time the distant influences of intestinal ischaemia-reperfusion are able to develop, promoting what is believed to be a systemic inflammatory response. It is often these distal sequelae which contribute most significantly to the morbidity and mortality seen in these clinical situations, hence my interest in this seemingly simple gut injury and its complex connections with the whole organism. This introductory chapter sets out the background of intestinal ischaemia-reperfusion injury, its known systemic influences, particularly focusing on the hepatobiliary system and the potential protective influence of moderate hypothermia in IIR.

1.2 Ischaemia-reperfusion

Ischaemia is defined as the cessation of blood flow preventing the delivery of nutrients and oxygen to a tissue; reperfusion is the restitution of this blood supply providing essential fuel but also promoting a complex set of reactions that may paradoxically injure the local and distant tissue (Carden and Granger 2002). The intestine is extremely vulnerable to ischaemia-reperfusion (Granger et al.

1986); this local injury produces an intense systemic inflammatory response resulting in progressive distal organ dysfunction.

1.3 Intestinal ischaemia-reperfusion

Intestinal ischaemia-reperfusion (IIR) is recognised as a common element in the pathogenesis of several diseases in neonates, children and adults including necrotizing enterocolitis, midgut volvulus, intussusception, severe sepsis and haemodynamic shock (Schoenberg and Beger 1993). IIR is also a major limiting factor in development of small bowel transplantation. Despite significant improvement in immunosuppressive agents and post-operative management, the small intestine graft sustains injury not only during the ischaemic phase but also during reperfusion (Yamamoto et al. 2001). Multiple Organ Dysfunction Syndrome (MODS), including liver failure, occurs in a significant proportion of these patients (Morecroft et al. 1994, Deitch and Sambol 2002) and it is this rather than the intestinal injury *per se* which is responsible for the high mortality (Neary and Redmond 1999).

Necrotising enterocolitis (NEC) is a devastating condition which, despite advances in neonatal intensive care, continues to be a leading cause of morbidity and mortality. The aetiology of NEC is unclear and as a consequence, despite significant efforts to improve outcome, little progress has been made in the treatment of NEC over the last 35 years. No preventative treatments exist and modern treatment strategies still rely on supportive intensive care and surgical excision for failed medical management, frankly necrotic bowel or perforation. No reliable markers of severity or progression of disease exist and the decision to operate or not continues to be largely subjective. Prematurity and low birth weight

are consistently reported to be inversely related to incidence of NEC and NEC related mortality, indeed as improvements in both obstetric and neonatal care are made the increased survival of very low birth weight infants (VLBW) (<1500g) increases and as a consequence so does the population at risk of NEC. Recent population based studies report an incidence of NEC of 7% in 20,488 VLBW infants between 1996 and 1997 (Sankaran 2004). Mortality rates for NEC range from 15-30%, high rates of mortality are particularly seen in small infants, those with more severe disease (where it is reported as nearing 100%) and in those infants requiring surgery (i.e. 20-40% of infants with NEC) (Sankaran 2004, Luig 2005). Morbidity in survivors is also significant, post-operative complications include recurrent disease, intraabdominal abscesses, wound dehiscence, intestinal strictures and the long-term sequelae of short bowel syndrome. Involvement of other organ systems also causes significant morbidity, such as neurodevelopmental outcomes, recent data has demonstrated that survivors of NEC are at higher risk of poor growth, cerebral palsy, vision and hearing impairment and lower developmental milestones at 18-22 months corrected for gestational age. Those infants who undergo surgery for NEC appear to be at the greatest risk for poor neurodevelopmental outcome (Stoll 2004, Hintz 2005).

The pathophysiology of NEC remains to be fully elucidated however it is widely accepted to be multi-factorial. Prematurity and its related intestinal sequelae appear to be most frequently implicated in NEC: immature intestinal function including intestinal motility and digestion; intestinal circulatory regulation; intestinal barrier function and immune defense all may play a role together with premature institution of enteral feeding and the abnormal bacterial colonisation of an ex-utero infant in an intensive care unit setting (Lin 2008).

NEC is predominantly a disease of premature infants and is rare in the full term, however when this does occur it is usually associated with a circulatory disorder such as congenital heart disease, cardiac-pulmonary by-pass procedures, hypoxic-ischaemic episode, polycythaemia or inutero growth restriction. These risk factors implicate an ischaemia-reperfusion mode of injury especially when coupled with the fact that the ileocaecal region is most frequently involved in NEC, such 'water-shed' areas of the gut are particulary vulnerable to hypoxia-ischaemia (Ng 2001, Lin 2008). Whilst NEC in the premature infant is likely to have a more complex aetiology the role of selective mesenteric ischaemia and reperfusion is widely accepted to be an important factor and the ileocaecal area is also recognised to be most commonly affected in this group (Gellen et al 2003, Ito et al 2007).

Current knowledge regarding the pathogenesis of NEC is largely deduced from epidemiological studies, histological data from surgical biopsy or autopsy (frequently demonstrating coagulation necrosis in resected samples) and a variety of experimental models which attempt to mimic the various identified risk factors. Such experimental models may involve premature animals, exposure to bacterial colonisation, using various feeding regimens, including dam feeding, different feed formulae and various rates of feeding (Okada 2010), some models also involve induced asphyxia for short periods (e.g. by exposing animals to breathing 100% nitrogen gas for 60 seconds) and brief periods of cold stress (e.g. 4°C for 10min) to induce the 'diving reflex' of newborns which protects essential organs (e.g brain and heart) whilst the gut is necessarily exposed to relative levels of hypoxia/ischaemia. One of the most common models is the neonatal rat NEC model as described by Crissinger (Crissinger 1995).

Severe NEC in premature neonates is frequently associated with multiple organ dysfunction (i.e pulmonary, circulatory, renal and hepatic dysfunction) (Morecroft et al. 1994). In addition a number of studies have reported significant pathological changes in hepatic hepatobiliary function in patients with NEC, especially in those who have been treated with parenteral nutrition (Moss et al 1996; Muguruma et al 1997; Andorsky et al 2001). The development of such hepatobiliary dysfunction is multifactorial and the lack of enteral feeding coupled with the risks of long-term parenteral nutrition may be significant enough to explain any liver dysfunction exhibited clinically. However experimental data does suggest a more subtle gut-liver axis which is not fully understood and could act as an 'engine' for promoting further gut inflammation and systemic sequelae seen more frequently in NEC. For example, Halpern et al demonstrated activation of hepatic Kupffer cells in a neonatal rat model of NEC with upregulation of TNF-α production by Kupffer cells that correlated with severity of ileal disease. Interestingly TNF-α levels in the luminal intestinal content of the ileum in animals with NEC is attenuated when Kupffer cell activation is selectively inhibited (by injection of gadolinium chloride), in addition inhibition of Kupffer cells is associated with decreased severity of intestinal damage (Halpern et al 2003). In the same model Khailova et al have demonstrated interesting changes in the composition and structure of hepatic cellular junctions (tight junctions and adherens junctions) which occur during NEC. Significant disturbance in the cellular junctions between the sinusoidal space and the canalicular lumen which normally act to form a paracellular barrier between bile and blood may act as a route for inflammatory cytokines and gut-derived bacterial toxins from the portal

circulation to enter into the hepatobiliary system further exacerbating intestinal injury (Khailova et al 2009).

The experiments in this thesis all relate to an interest in necrotising enterocolitis but are specifically designed to investigate the experimental model of intestinal ischaemia-reperfusion (IIR) in adult male rats. This works stems from original findings in the same reliable model which relate to the influence of IIR, temperature and hepatic bioenergetics (Vejchapipat et al. 2001). This thesis explores these interesting findings further and focuses specifically on the influence of IIR on the liver. IIR is a well-described experimental model; however the pathophysiology of distant organ damage is not fully understood, before turning to the effect of IIR on the liver (Section 1.3.5) the following sections will expand on what is known regarding the mechanisms of IIR induced injury in both the intestine and in other distant organ systems.

IIR is associated with intestinal mucosal epithelial cell damage (Park et al. 1990), loss of mucosal barrier integrity and translocation of bacterial products (e.g. lipopolysaccharide [LPS]) from the intestinal lumen to mesenteric lymph nodes and portal blood (Deitch et al. 1994). Enteric macrophages are activated by these bacterial products and other mediators such as reactive oxygen species (ROS) promoting the production of inflammatory cytokines, such as tumour necrosis factor α (TNFα) which are capable of promoting leukocyte-endothelial cell adhesion, transendothelial leukocyte migration and platelet-leukocyte aggregation (Eppihimer et al. 1997). Several studies have indicated that IIR may prime leukocytes and endothelial cells directly via metabolites (e.g. reactive oxygen and nitrogen species) independently of bacteria and bacterial products (Koike et al. 1994, Tamion et al. 1997). Once this cycle of events commences it

becomes increasingly complex and self sustaining, inducing systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Carden and Granger 2002).

1.3.1 Intestinal ischaemia-reperfusion and the respiratory system

Pulmonary injury following IIR has been extensively investigated (Carden et al. 1993, Simpson et al. 1993). Clinically, the pulmonary damage associated with MODS may result in severe respiratory failure or the acute respiratory distress syndrome (ARDS), a common cause of mortality and complications in such diseases (Carden and Granger 2002). ARDS is characterised by increased microvascular permeability and increased pulmonary vascular resistance both of which contribute to the development of pulmonary oedema. Studies have previously demonstrated that such an increase in microvascular permeability occurs in models of IIR (Iglesias et al. 1998). The mechanism of increased microvascular permeability involves a complex cellular and humoral response resulting in increased adhesion molecule expression and neutrophil infiltration (Ishii et al. 2000, Schmeling et al. 1989). Previous work within our group, using the same experimental model as described in this study, has demonstrated that IIR results in a significant increase in pulmonary neutrophils (Vinardi et al. 2003).

1.3.2 Intestinal ischaemia-reperfusion and the cardiocirculatory system

IIR has been previously demonstrated to result in significant myocardial dysfunction resulting in bradycardia and decreased left ventricular function (Horton and White 1991). This effect may in part be due to distant haemodynamic influences of IIR rather than a direct influence upon the heart itself. However,

further studies by the same group went on to demonstrate preservation of cardiac function during IIR following the introduction of free-radical scavengers and inhibitors of neutrophil adherence (Horton and White 1993a, Horton and White 1993b).

Stefanutti *et al* investigated cardiac energy metabolism using the same model of IIR and demonstrated interestingly that levels of ATP, phosphocreatinine and inorganic phosphate were unchanged as compared to sham animals (Stefanutti et al. 2004). However, the production of free radicals promoted by IIR such as superoxide may react with nitric oxide to produce peroxynitrite which in turn may decrease cardiac contractility by uncoupling ATP production from mechanical work rendering the levels of ATP an inaccurate measure of potential energy substrate.

Fatty acid oxidation provides approximately 75% of cardiac ATP requirements under the majority of conditions (Neely and Morgan 1974). Stefanutti's experiments also demonstrated significant changes in a cardiac enzyme important in the control of fatty acid oxidation, cardiac carnitine palmitoyl transferase (CPT 1). IIR resulted in significant CPT 1 inhibition and in turn decreased myocardial fatty acid oxidation and increased myocardial requirements of other substrates such as glucose, lactate and ketone bodies (Stefanutti et al. 2004). Cardiac CPT 1 had been previously demonstrated to be impaired by peroxynitrite in a model of neonatal sepsis from the same group and has been postulated as a potential mechanism in IIR (Fukumoto et al. 2002).

1.3.3 Intestinal ischaemia-reperfusion and the renal system

Experimental models of IIR have also demonstrated a significant reduction in renal blood flow (by approximately 80%) (LaNoue et al. 1996). It is of note that the maximal reduction in renal perfusion occurs during the first 5 minutes of reperfusion, this finding correlates with other studies examining the effect of IIR on splanchnic blood flow (e.g. intestine) (Turnage et al. 1995) and the liver (Turnage et al. 1996)) where an initial profound reduction in perfusion gradually increases with time but never re-establishes control levels and eventually falls again prior to death. In addition to a reduction in renal blood flow, intestinal reperfusion is associated with a 25% reduction in renal parenchymal ATP levels and early, acute tubular dysfunction as demonstrated by increased sodium loss from the kidneys (LaNoue et al. 1996).

Several pathophysiological mechanisms may be responsible for the reduction in renal blood flow. IIR may influence systemic haemodynamics by directly impairing cardiac performance. Reduced intravascular fluid volume and the increased levels of inflammatory (neutrophils and complement) and vasoactive (catecholamines and renin-angiotensin) mediators will contribute to reduced renal perfusion. In addition local factors produced by the kidney itself act to alter microvascular perfusion e.g. eicosanoids (vasoconstrictors such as thromboxane A_2 and leukotriene C_4 and vasodilators such as prostaglandin E_2 and prostacyclin), NO and platelet activating factor (LaNoue et al. 1996). Several studies have demonstrated that the kidney responds to IIR by producing increased levels of thromboxane A_2 (vasoconstrictor) and reduced levels of vasodilators (e.g. prostaglandin E_2 and prostacyclin) (Rothenbach et al. 1997).

1.3.4 Intestinal ischaemia-reperfusion and systemic oxidative stress

IIR results in reoxygenation (during reperfusion) following a period of relative anoxia (ischaemia), and the increased production of reactive oxygen species (hydrogen peroxide, superoxide, hydroxyl and perhydroxyl radicals) and nitrogen species (NO and peroxynitrite) by activated neutrophils, macrophages, mitochondria and other intracellular sources. The production of such reactive species may exceed the defensive scavenging potential of the endogenous antioxidant system (e.g. glutathione) resulting in a state of oxidative stress, within the intestine and in distant organ systems as part of the SIRS. This, in turn, results in cellular dysfunction as a result of protein modification, lipid membrane disruption and DNA damage (Stefanutti et al. 2006). Recent research demonstrates that this process varies from organ to organ in response to IIR, for example a marker of lipid peroxidation, malondialdehyde (MDA), was markedly raised in plasma, ileum and lung tissue following IIR, whereas there was no significant increase in lipid peroxidation in kidney and liver tissue. In addition, intestinal levels of glutathione were significantly reduced following IIR whereas levels of glutathione within the liver remained unchanged (Stefanutti et al. 2006).

1.3.5 Intestinal ischaemia-reperfusion and the hepatobiliary system

IIR is also associated with hepatic impairment, the mechanisms of which are not fully understood. IIR has been demonstrated to result in hepatic dysfunction characterised by hepatic hypoperfusion, leukocyte accumulation, liver enzyme release and cholestasis (Horie et al. 1996, Poggetti et al. 1992, Turnage et al. 1996).

The blood supply to the liver under normal circumstances is composed of the combined flow of the hepatic artery (25% of hepatic blood flow and 50% of total oxygen) and the portal vein (75% of hepatic blood flow) (Teoh and Farrell 2003). The relative contribution of these routes is profoundly altered when blood supply to the gut is impaired. During intestinal ischaemia, portal blood flow is decreased by 70% compared to sham operated animals, whereas there is a compensatory increase in hepatic arterial flow, so that total hepatic inflow is only decreased by about 25%. Upon reperfusion, there is an immediate restoration of hepatic portal vein flow, but this quickly falls again so that it is reduced by about 50% after one hour of reperfusion. Hepatic arterial flow, which during ischaemia is elevated by about 25% compared with sham animals, falls rapidly on reperfusion so that it is 25% less than sham animals after one hour of reperfusion. These changes result in a brief period immediately following reperfusion when total hepatic inflow is similar to sham animals, followed by a progressive decrease in total hepatic inflow compared with sham animals (Williams et al. 2001). However, these findings may also depend on the length of the period of ischaemia and other variables; Turnage et al found similar effects during the reperfusion phase, but a difference in hepatic arterial flow (decreased rather than increased) during ischaemia (Turnage et al. 1996).

However, hepatic dysfunction following IIR is not fully explained by hypoperfusion, an *ex vivo* study demonstrated that IIR induced liver dysfunction despite sustained oxygen delivery and maintained perfusion pressure to the liver (Poggetti et al. 1992). This suggests that liver dysfunction may not be primarily due to hypoperfusion and compromised oxygen delivery but via circulating mediators (e.g. TNF-α and IL-6 within the portal circulation).

A widely accepted view of the intestine as the 'engine' of multiple organ dysfunction corresponds to a failure in the intestinal barrier, bacterial translocation, portal bacteraemia and endotoxaemia, however a more recently developed concept includes potential mediators within the lymphatic circulation. Such lymphatic mediators may be responsible for IIR related hepatic dysfunction. Approximately 20% of the total lymphatic circulation travels through the liver and 25-50% of the lymph is produced in the liver predominately from the fluid filing the space of Disse (perisinusoidal spaces). Hepatic lymph contains a large number of cells, mainly lymphocytes and macrophages (Wick et al. 2002). Recent interest has developed regarding the influence of mesenteric lymph in promoting the systemic inflammatory response in various disease states including haemorrhagic shock, trauma and IIR (Nakamura et al. 2004). The mechanism of action exerted by factors within mesenteric lymph remains poorly understood. Cavriani et al reported that IIR resulted in intestinal and lung injuries that were partially mediated by TNF-α and were prevented by the ligation of the thoracic duct (Cavriani et al. 2005). There are numerous animal studies demonstrating surgical methods to prevent mesenteric lymph from entering the peripheral circulation subsequently preventing distal organ failure, such as: mesenteric duct ligation, thoracic duct ligation and thoracic duct external drainage. Interestingly temperature change has also been found to influence mesenteric lymph flow, hypothermia decreases flow whereas rewarming has been demonstrated to increase mesenteric lymph flow (Ackerman 1975, Hassoun et al. 2002).

IIR has been described to result in leukocyte plugging of sinusoids, leukocyte adherence in postcapillary venules, a reduction in the number of perfused sinusoids, hepatocellular hypoxia and leakage of enzymes (e.g. alanine

aminotransaminase [ALT] and aspartate aminotransferase [AST]) from hepatocytes (Horie et al. 1996, Horie et al. 1997, Yamamoto et al. 2001). Such hepatic microvascular dysfunction and hepatocellular injury has been demonstrated in some studies to be dependent upon leukocyte sequestration, for example improved sinusoidal perfusion and blunted hepatocellular injury are observed in knock-out mice deficient in one of a variety of adhesion glycoproteins (e.g. P-selectin) (Horie et al. 1996, Horie et al. 1997).

In addition, cholestasis, as defined as an impaired hepatocellular excretion of constituents of bile, occurs in response to a variety of inflammatory stimuli including IIR (Siewert et al. 2004). *In vitro* and *in vivo* studies in rats have demonstrated that systemic inflammation decreases the uptake and secretion of bile constituents (Bolder et al. 1997). In IIR a fall in bile flow rate is initiated soon after intestinal reperfusion and becomes more pronounced throughout reperfusion (Turnage et al. 1994, Turnage et al. 1996). Molecular studies using several models of cholestasis have demonstrated a selective down-regulation of organic anion transporters at the basolateral and canalicular membranes (Green et al. 1996). The cytokine mediated mechanisms underlying transcriptional regulation of these hepatocellular transport systems during the acute-phase response remains unknown. Bile salts have an important protective role in acting to neutralize LPS therefore impaired excretion of bile salts may enable increased quantities of LPS to enter the circulation resulting in endotoxaemia (Koike et al. 1994).

The liver is a complex, heterogenous organ with a central role in coordinating homeostasis and the metabolic response to systemic inflammation. Detailed characterisation of the molecular responses and interrelationships of the many different cell types within the liver remains largely unknown.

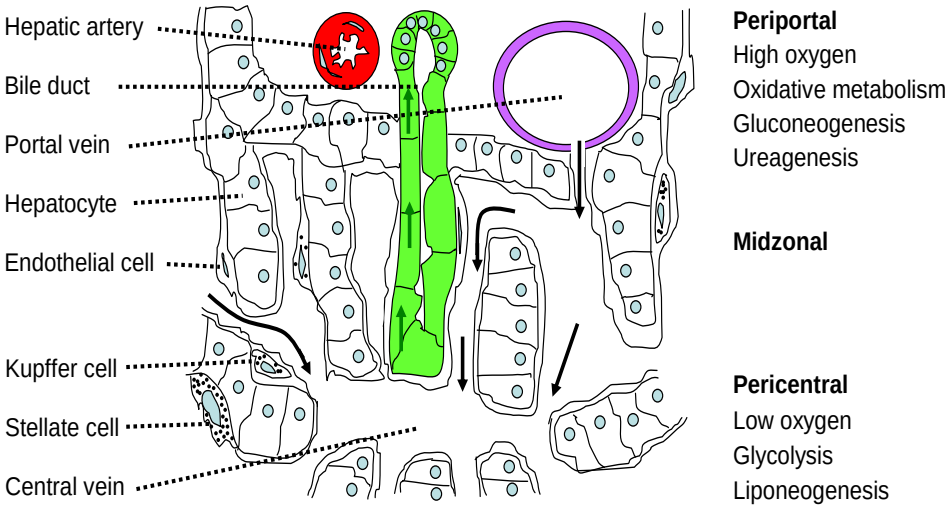

Figure 1.01: Schematic representation of a liver lobule.

As blood flows (black arrows) hepatic tissue extracts oxygen and other blood borne substances (e.g. hormones, bile acids) while adding metabolic waste and synthetic products. Gradients of oxygen tension and metabolites develop between periportal and pericentral regions. In low flow or hypoxaemic states the pericentral hepatocytes are the first to experience hypoxic stress. Gradients of hepatic enzymes also exist, enzymes for oxidative phosphorylation, gluconeogenesis and ureagenesis concentrate in the periportal region, enzymes for glycolysis, lipogenesis and drug metabolism concentrate in the pericentral region. Bile flow is countercurrent to blood flow (green arrows) (Pollard and Earnshaw 2002).

1.4 Gene transcription in mammalian cells

Gene transcription in mammalian cells is controlled by interaction between transcription factors and specific DNA sequences known as ‘promoters’ or ‘enhancer motifs’. Binding of transcription factors in these regions brings them into close proximity with RNA polymerase II and the basic transcription complex which is located at the coding region of the gene. In this way transcription factors may have a positive or negative influence on the transcription of genes. In turn transcription factor activity is controlled at multiple levels, e.g. modulation of expression, post-translational modifications (phosphorylation, acetylation, ubiquitination) and interaction with co-activators and inhibitors.

1.5 Hepatocellular pathophysiology

The liver is a heterogenous organ composed of many different cell types which contribute to the immune and metabolic response to injury. Constituent liver cells may be divided into either parenchymal (hepatocytes) or non-parenchymal cells (comprising approximately 40% sinusoidal endothelial cells [SEC], 20% Kupffer cells and 20% intrahepatic lymphoid cells). The intrahepatic lymphoid cells are composed of a large population of Natural Killer cells (25%), T-cells (65%), dendritic cells (5%), few B cells (<5%) and rare ‘stem-like’ cells (Wick et al. 2002).

Infiltrating neutrophils have also been implicated in the hepatic dysfunction secondary to sepsis. In addition, every T-lymphocyte, Natural Killer cell or dendritic cell that leaves the intestine or spleen via the portal system passes through the liver (Wick et al. 2002). Kupffer cells are tissue-specific macrophages which originate from bone marrow monocytes and comprise 80% of the total

body macrophage population. These cells are classified as a resident cell population of the liver but they are also able to actively move within the lumen of the sinusoids, both with and against the blood flow (Wick et al. 2002). In addition, Kupffer cells are not found evenly distributed throughout the hepatic lobule but have decreasing density from the periportal to central region. Kupffer cells are primarily responsible for bacterial scavenging and inactivation of bacterial products, entering the liver from portal venous blood preventing them from entering the systemic circulation. Activated Kupffer cells produce cytokines (TNF-α, IL-1β, IL-2, IL-6, IL-10 and IL-12) which either regulate hepatocytes and endothelial cells via paracrine interactions or are released into the systemic circulation. In addition, Kupffer cells act as scavengers clearing inflammatory mediators from the systemic and portal circulations thereby limiting the inflammatory cascade (Dhainaut et al. 2001).

Kupffer cell activation occurs as response to lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram negative bacteria and the prototypical endotoxin, which is released in the gastro-intestinal tract and is drained primarily into the liver sinusoids (Monshouwer and Hoebe 2003). LPS then binds to LPS binding protein (LBP) which is produced by neighbouring hepatocytes. The LPS-LBP complex binds to cell surface receptors (e.g. CD14) resulting in the activation of IκB kinase (IKK) which phosphorylates IκB (a naturally occurring nuclear factor κB [NF-κB] inhibitor) which in turn is degraded allowing the translocation of NF-κB to the nucleus. NF-κB is a transcription factor which is composed of homo-or heterodimers of members of the Rel protein family (the p65:p50 heterodimer is the classic NF-κB). NF-κB also acts as an extremely redox-sensitive molecule central to the stress response, most pathways

that activate NF-κB are modulated by ROS or their producers (Ali and Mann 2004). NF-κB, once in the nucleus, binds κB sites in the promoter sequence of inducible genes such as cytokines, adhesion molecules, acute phase proteins and pro-apoptotic genes resulting in an rapid increase in transcription and translation (Su 2002a).

Hepatocytes, like Kupffer cells, are able to respond directly to LPS by activation of NF-κB. In addition to the production of cytokines, hepatocytes may also produce acute phase proteins and iNOS (Monshouwer and Hoebe 2003). Hepatocytes also have many receptors and respond directly to many different chemokines and cytokines (IL-1, IL-4, IL-6, IL-10, IL-11, TNF-α, IFN-αβ, IFN-γ, complement factor C5a) produced by activated Kupffer cells. Hepatocytes also produce many CXC chemokines but only few cytokines (IL-6 and IL-7). Hepatocytes respond to IL-6 by modifying their metabolic pathway to restore homeostasis, this is characterised by an increase in gluconeogenesis, amino acid uptake and the acute phase response (APR) where protein synthesis is directed towards coagulant and complement factors and protease inhibitors (Moshage 1997). TNF-α may have a dual effect on hepatocytes resulting in either apoptosis or regeneration and repair. Expression of membrane-bound and secreted CD14 (classified as an acute phase protein) is upregulated in hepatocytes in response to endotoxaemia (Liu et al. 1998). In addition to controlling the APR, hepatocytes influence the innate and specific immune function of the liver regulating both resident and transient immune cell populations (Wick et al. 2002).

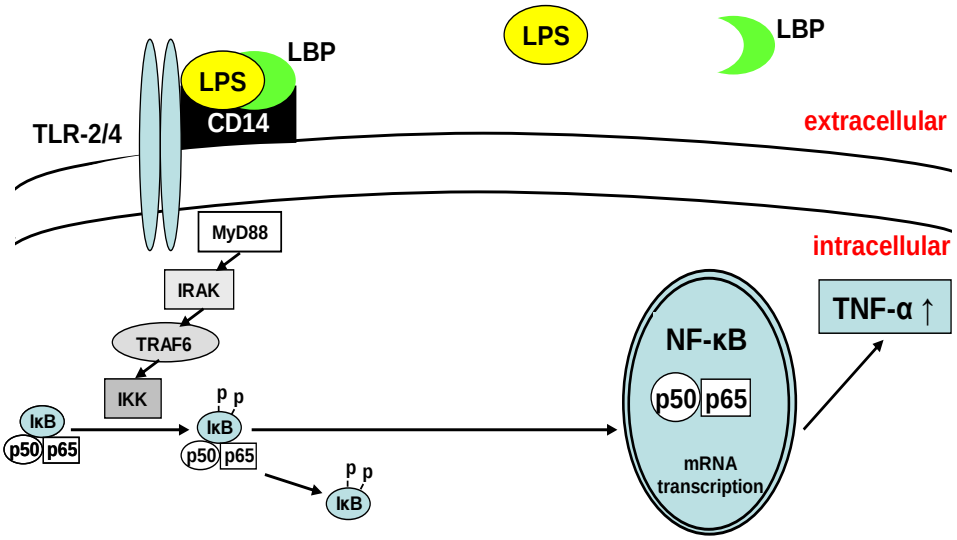

Figure 1.02: Activation of Kupffer cells.

LPS binds to LPS binding protein (LBP), produced by hepatocytes. The LPS-LBP complex binds to CD14 (outer membrane of Kupffer cell) and results in Toll-like receptor activation (TLR). Further activation of the signalling pathway including IL-1 receptor associated kinase (IRAK) and TNF activated factor 6 (TRAF-6) results in the activation of IκB kinase (IKK). IKK phosphorylates IκB which in turn degrades and permits translocation of NF-κB to the nucleus where it promotes transcription of inducible genes such as pro-inflammatory cytokines e.g. TNF-α (Monshouwer and Hoebe 2003).

Direct cell-to-cell interaction between Kupffer cells and hepatocytes appears to be an important determining factor for their subsequent function. Experiments which enable cell-to-cell contact have demonstrated an increased production of pro-inflammatory cytokines and NO. However, the mechanism of such interactions is not fully elucidated (Hoebe et al. 2001).

Sinusoidal endothelial cells (SEC) line the intrahepatic low pressure capillaries, sinusoids; they differ from other endothelial cells as they enable free diffusion of soluble substances into the perisinusoidal space (Figure 1.01). SECs express adhesion molecules (e.g. ICAM-1, ICAM-2 and VCAM-1) and produce cytokines (TNF-α, IL-1, IL-6 and IL-10) and nitric oxide (NO). NO production by endothelial cells promotes antimicrobial activity and limits tissue injury by scavenging superoxide anions and inhibiting leukocyte adhesion to the hepatic endothelial cells (Bautista et al. 1990). Both neutrophils and cytotoxic lymphocytes have also been implicated in both hepatocyte and endothelial cell injury. Activated Kupffer cells produce cytokines (e.g. TNFα, IL-2, IL-12 and leukotriene B4). Neutrophils and lymphocytes are attracted to and activated by Kupffer cells and up-regulate their surface adhesion molecules (CD11b/CD18) and bind to SEC (Jaeschke et al. 1993). Neutrophils then migrate into the parenchyma where they produce oxygen-free radicals and proteases which may damage the hepatocytes (Doi et al. 1993).

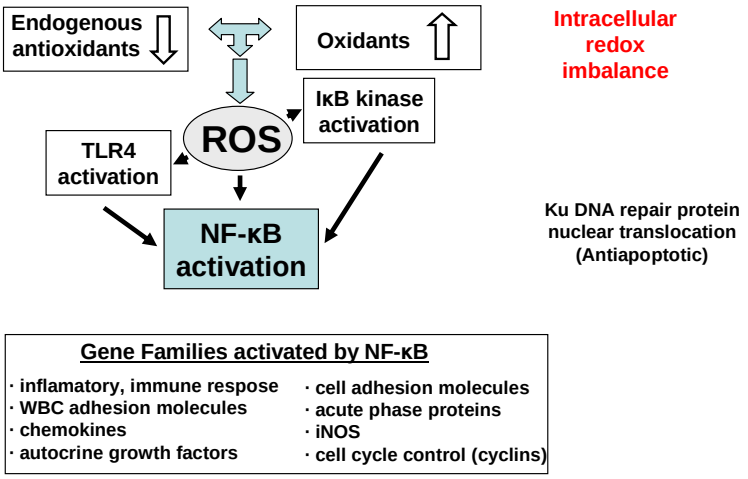

Figure 1.03: The interrelationships between reactive oxygen species (ROS) and the nuclear transcription factor NF-κB.

ROS are generated when the homeostatic balance between constitutively expressed oxidants and antioxidants results in excess oxidants. Important oxidation sensitive molecules are TLR4 and IκB kinase which inactivates the NF-κB suppressor molecule, IκB. Several gene families are activated by NF-κB, many are responsible for modulating the immune response and promote apoptosis, however an important compensatory action of NF-κB is to promote nuclear translocation of Ku DNA repair protein which is anti-apoptotic (Hubbard et al. 2004).

Hepatic stellate cells (HSC) have recently been the subject of much interest. These cells when quiescent generate the extracellular matrix of the perisinusoidal space but on activation can 'transdifferentiate' into wound-healing myofibroblasts with both proinflammatory (e.g. cytokines IL-1α, TNF-α and IFN-γ) and profibrogenic activities (e.g. hepatic growth factor [HGF], collagen expression, proliferation and resistance to apoptosis) (Wick et al. 2002, Mann and Mann 2009). HSC activation may be a critical step in the balance between hepatic inflammation, regeneration and fibrosis and is tightly controlled by changes in the expression of a vast number of genes. For example several studies have shown that HSC activation is associated with elevation of activity and subsequent increase in the expression of NF-κB responsive genes (IL-6 and ICAM-1) (Elsharkawy et al. 1999). Activator protein 1 (AP-1) is a homodimeric or heterodimeric protein (composed of proteins from the Jun and Fos families), which acts as an important transcription factor in the HSC inducing expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) and IL-6 (Smart et al. 2001).

Primary hepatic dysfunction has been previously described as a result of profound liver hypoperfusion and reduced oxygen delivery (Dhainaut et al. 2001). This frequently occurs acutely and results in a significant derangement in normal liver function. Clinically resulting in abnormal liver function tests, disseminated intravascular coagulation (DIC) and bleeding sequelae. Secondary hepatic dysfunction is not well understood and has previously been largely over-looked as it often results in an early, subtle failure to control the level of bacterial, endotoxin and inflammatory mediators passing through the liver into the systemic circulation contributing to MODS (Dhainaut et al. 2001). Recent clinical evidence supports the prognostic influence of early hepatic dysfunction, a large

prospective, multicentre cohort study recorded early hepatic dysfunction (serum bilirubin >2mg/dL within 48 hours of admission) occurring in 11% of critically ill patients. Multiple logistic regression analysis showed an independent mortality risk of hepatic dysfunction which exceeded the impact of all other organ dysfunction (Kramer et al. 2007).

There are methodological difficulties in assessing early, subtle, cellular alterations in function. In the past assessment of liver function has relied on the indirect measurements of plasma proteins and biochemistry; such methods are limited in the accurate assessment of cellular function. Recently with the development of 31-phosphorus magnetic resonance spectroscopy (^{31}P MRS) non-invasive sequential *in vivo* measurements of cellular energy metabolism are now possible leading to new insights into hepatic pathophysiology (Mann et al. 2002).

In an animal model of IIR, our group has previously demonstrated using ^{31}P MRS that intestinal ischaemia alone does not affect liver energy metabolism, whereas reperfusion causes liver energy failure. This is characterised by an increase in hepatic inorganic phosphate (P_i) and a decrease in adenosine triphosphate (ATP) and is followed by 100% mortality within 4 hours. Mortality occurred exclusively during the intestinal reperfusion phase following an increase in the hepatic P_i: ATP ratio (a measure of hepatic energy status) (Vejchapipat et al. 2000, Vejchapipat et al. 2001).

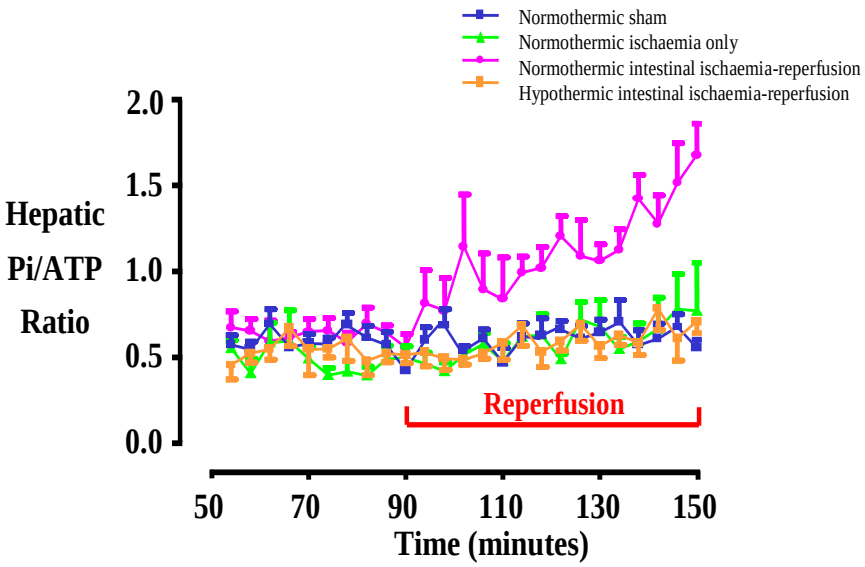

Figure 1.04: Relationship between intestinal ischaemia-reperfusion and hepatic Pi to ATP ratio monitored during time course experiments in all groups.

There is a progressive increase in hepatic Pi to ATP ratio during normothermic IIR as compared to the other experimental groups with a significant increase ($P<0.05$) in the slope of the trend lines during this period. Data are expressed as mean ± SEM (Vejchapipat et al. 2001).

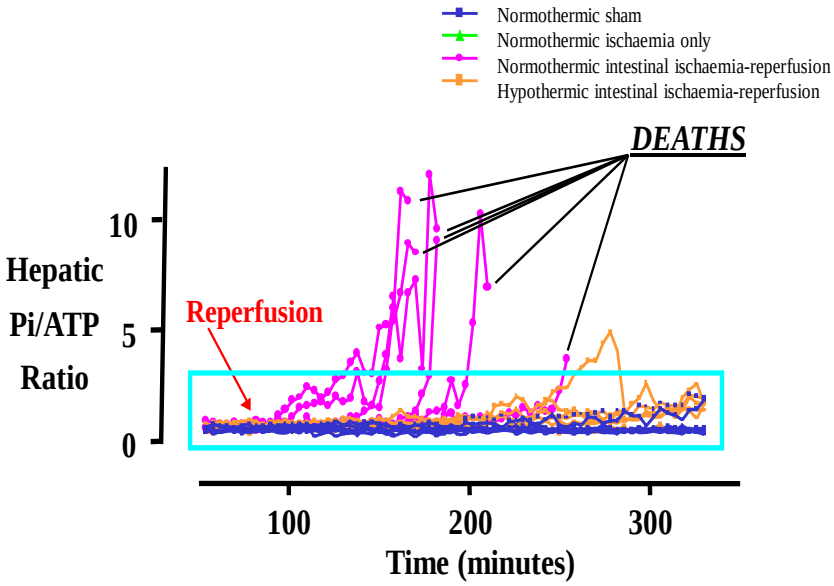

Figure 1.05: Relationship between intestinal ischaemia-reperfusion and mortality during extended time course experiments in all groups.

Data are expressed as the ratio of hepatic Pi to ATP in individual animals. All animals exposed to normothermic intestinal ischaemia-reperfusion died during intestinal reperfusion (Vejchapipat et al. 2001).

1.6 Moderate hypothermia

Whole body moderate hypothermia (30-33°C) has previously been demonstrated to be protective in an animal model of IIR: induced throughout intestinal ischaemia and reperfusion hypothermia significantly reduced intestinal damage (Vejchapipat et al. 2002), diminished portal and systemic pro-inflammatory and anti-inflammatory plasma cytokine response (Williams et al. 2003), decreased lung neutrophil infiltration (Vinardi et al. 2003), preserved intestinal antioxidant potential and significantly reduced levels of lipid peroxidation in plasma, ileum and lung (Stefanutti et al. 2006). Hypothermia completely abolished mortality within the experimental period following 90 minutes of ischaemia and 4 hours of reperfusion (Vejchapipat et al. 2000, Vejchapipat et al. 2001). In addition moderate hypothermia prevented the development of liver energy failure in an animal model of IIR where liver energy failure immediately precedes death (Vejchapipat et al. 2001). Further experiments have also demonstrated that when hypothermia is applied as a 'rescue therapy' (i.e. after ischaemia at the time of reperfusion) its remains protective resulting in reduced intestinal injury, distal organ damage and improved survival (Stefanutti et al. 2008c).

There has been much interest in the protective effects of hypothermia in many different models of disease. Ischaemia-reperfusion of organs such as the heart, kidney and lungs which results in local damage is significantly attenuated by moderate hypothermia (Gordon 2001). Moderate hypothermia has also been shown to be one of the most robust and effective methods of reducing hypoxic-ischaemic cerebral damage in experimental models (Gunn et al. 1997). Accordingly, hypothermia has been proposed as a therapy for cerebral ischaemia-

reperfusion injury following cardiac arrest, stroke and trauma (Eisenberger et al. 2001, Holzer et al. 2005, Kochanek and Safar 2003, Marion et al. 1997, Reith et al. 1996) and is used routinely during prolonged cardiopulmonary bypass (Griepp et al. 1997).

Current evidence has lead to guidelines from the International Liaison Committee on Resuscitation recommending that unconscious adults with spontaneous circulation after out of hospital arrest should be cooled to 32-34ºC for 12-24 hours when the initial rhythm was ventricular fibrillation (Nolan et al. 2003). Induced hypothermia may also be considered for unconscious adult patients with spontaneous circulation despite the original rhythm or whether the arrest occurred in or outside hospital and many intensive care units in the UK will cool comatose patients following cardiac arrest (Soar and Nolan 2007).

There has also been much research into the protective effects of induced hypothermia in asphyxial encephalopathy with experimental evidence that by reducing the body temperature to 3-5ºC below normal results in reduced cerebral injury and improved neurological outcome (Bona et al. 1998, Thoresen et al. 1995). Clinical safety studies were subsequently performed that found no serious adverse effects following moderate hypothermia (Azzopardi 2010, Gunn et al. 1998). More recently several large randomised controlled trials have been conducted investigating outcome after cooling following asphyxial encephalopathy. The CoolCap trial (Gluckman et al. 2005) and National Institute of Child Health and Human Development (NICHD) trial (Shankaran et al. 2005) used different cooling regimens but only the NICHD trial reported a significant reduction in death or disability following hypothermia. However neither study had enough power on systematic review to result in a consensus for

recommending induced hypothermia as a standard therapy (Jacobs et al. 2003). The Total Body Hypothermia for Neonatal Encephalopathy Trial (TOBY) was a multicentre, randomized trial comparing intensive care therapy plus total body hypothermia for 72 hours with intensive care therapy alone (Strohm and Azzopardi 2010). This study found no difference in its primary outcome measure of combined rates of death or severe disability, between the cooled and non-cooled groups, however cooling did result in a significant increase in the rate of survival without neurological abnormalities and improved neurodevelopmental outcomes amongst survivors at 18 months of age (Azzopardi and Strohm 2009). All three of these trials despite their homogeneity of results had discrepancies which may be partially explained by there variability in method of cooling, target temperatures and timing of induction. The influence of hypothermia on less severe neurological impairment resulting from perinatal asphyxial encephalopathy that may not be detected until a later age also requires further investigation (Azzopardi and Strohm 2009).

There has been considerable interest in the potential therapeutic benefits of induced hypothermia since the 1950s however the mechanisms of protection remain unknown. The vast majority of research into possible mechanisms of hypothermic protection has previously been applied in models of cerebral hypoxia-ischaemia. Induced hypothermia during cerebral hypoxia-ischaemia is thought to have a favorable effect on many different pathways: including excitatory amino acids (Thoresen and Wyatt 1997); cerebral blood flow (Baldwin et al. 1991); nitric oxide production (Thoresen and Wyatt 1997); the cerebral energy state and apoptosis (Shankaran et al. 2005). In the brain hypothermia has been demonstrated to prevent the decline in high-energy phosphates that seem to

initiate both apoptotic and necrotic cell death and also to lower the production of local toxic oxygen and nitrogen metabolites (Edwards et al. 1998). Recent studies have also shown that hypothermia alters activation of transcription factors from the AP-1 family (e.g. Fos and Jun) following brain and spinal cord ischaemia (Kamme et al. 1995, Yang et al. 2000). In addition hypothermia has been demonstrated to selectively alter stress-induced heat shock protein expression (e.g. HSP70) in the brain in a model of asphyxial cardiac arrest (Hicks et al. 2000).

The mechanisms of hypothermic protection have also been investigated in other models of ischaemia-reperfusion, for example in the heart both ATP production and expenditure rates are reduced during hypothermia, maintaining tissue levels (Ning et al. 1999). Pro-inflammatory cytokine production (e.g. TNF-α, IL-1ß, MIP-2) was significantly reduced by hypothermia in a model of hepatic ischaemia-reperfusion, where it was also demonstrated that hypothermia resulted in reduced activation of mitogen activated protein, JNK, and transcription factor activator protein, AP-1 (Kato et al. 2002).

Despite the considerable interest in the potential therapeutic role of hypothermia in many different disease processes, including those characterised by ischaemia-reperfusion, the molecular basis of its protective effect remains to be fully elucidated. It is not clear whether the preservation of energy metabolism is the primary mechanism by which hypothermia affects its influence or whether it represents an indicator of cellular protection mediated by other pathways (Edwards et al. 1998).

In IIR hypothermia may act via a global, non-specific, reduction in metabolism, or it may make the liver less responsive to injurious stimuli via

specific molecular pathways (Vejchapipat et al. 2001). For example other groups have demonstrated that moderate hypothermia may decrease the formation of free radical and mediators within the injured intestine (e.g. by inducing heme oxygenase-1 [HO-1] (Attuwaybi et al. 2003) and/ or preventing an increase in NF-κB and inducible nitric oxide synthase (iNOS) (Hassoun et al. 2002). In addition the administration of an HO-1 inhibitor, Sn Protoporphyrin IX (SNP IX) abrogated the protective effects of hypothermia, suggesting that the protective mechanism is partially due to HO-1 induction (Attuwaybi et al. 2003). This evidence suggests that the mechanism of hypothermic protection is not explained simply as a result of reducing global cellular metabolism.

Defining the mechanism of hypothermic protection is not purely an academic exercise, it is important to determine as full a characterisation of the phenomenon as possible to enable the development of induced hypothermia as a potential therapeutic modality. Indeed with increased understanding it may also become possible to develop techniques which reproduce the protective effect of hypothermia without requiring cooling and therefore without the potentially deleterious side-effects of hypothermia (Gadkary et al. 2003, Polderman 2004a, Polderman 2004b, Thoresen and Whitelaw 2000). Even mild hypothermia may result in serious adverse effects, such as cardiovascular instability, respiratory depression, metabolic acidosis, coagulopathy, hyperglycaemia, increased plasma amylase, hypophosphataemia and hypomagnesaemia (Polderman 2004a, Polderman 2004b, Sessler 2001).

1.7 Aims and objectives of this thesis

1. The aim of this thesis is to investigate the mechanism of hypothermic protection during IIR. The hypothesis is that during moderate hypothermia, transcription of specific hepatoprotective genes may be altered and their protein products may be responsible for the preserved hepatic function in IIR.

2. To achieve this aim individual objectives were:

    i) to characterise the influence of IIR on expression of an important family of protective proteins, Heat Shock Proteins (HSPs).

    ii) to investigate the characteristics of hepatic injury following IIR.

    iii) to examine global changes in hepatic gene expression following IIR.

    iv) to explore the influences of IIR on hepatic mitochondria.

Chapter 2

Materials and Methods

Chapter 2 - General Methods and Materials

Contents

2.8 2D-DIGE methods

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2.8.4 First dimension – focusing of strip

2.8.5 Casting gels for second dimension

2.8.6 Second dimension - re-equilibration of strip

2.8.7 Second dimension - PAGE

2.8.8 Gel scanning and fixing

2.1 Animals

The study was approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986. Adult male Sprague-Dawley rats (250-320g) were studied. The rats were kept under standardised conditions for food, water, light and temperature. All animals were fed standard rat chow and water *ad libitum.*

2.2 Surgical procedure

The animals were anaesthetised with an oxygen/nitrous oxide mixture with 1.5-2.0% isoflurane via a nose cone. Animal core temperature was continuously monitored with a rectal probe. Normothermia (36-38°C) was maintained with a heating blanket and a lamp. Moderate hypothermia (30-33°C) was induced by exposing the animals to a room temperature of 22-23°C and using heat sources to prevent the temperature falling below 30°C.

In sham groups (normothermic sham [NS], hypothermic sham [HS]) a midline laparotomy was performed and the origin of the superior mesenteric artery (SMA) was identified and dissected only, the abdomen was then closed and the animal monitored until the end of the experimental period. Intestinal ischaemia was induced (in groups normothermic intestinal ischaemia-reperfusion [NIIR], hypothermic intestinal ischaemia-reperfusion [HIIR]) by occluding the SMA by traction on a silicone loop (Surg-I-Loop; Scanlan, Minnesota, USA.) slung around its origin. After a variable length of time (depending on each experiment and therefore described within individual Chapter's Methods and Materials section) the silicone loop was removed and reperfusion confirmed by visualisation of pulsation in the vascular arcades of the mesentery. Reperfusion

was continued for a set period of time until the experiment terminated (varied according to individual experiments and described within individual Chapter's Methods and Materials section). At the end of the experimental period the whole liver was excised and either snap frozen in liquid nitrogen or placed directly in neutral buffered formaldehyde 10% v/v. Frozen liver samples were then stored at -80°C until RNA/protein extraction.

2.3 Western blotting

2.3.1 Protein extraction for Western blotting

100mg liver was defrosted and diced in 1ml of ice-cold phosphate-buffered saline. 300µl of ice-cold homogenization buffer (10mM Tris pH 7.4/ 0.1% Triton X-100/ 0.1% β-mercaptoethanol) was added to the sample in a tight-fitting glass-on-glass homogeniser. The samples were each homogenised with 5 strokes. Total protein was extracted from 150µl of the homogenate by methanol/chloroform precipitation (Wessel and Flugge 1984). The final pellet was resuspended in 200µl of Laemelli buffer (without bromophenol blue) (Severn Biotech, Worcs, UK) and sonicated twice in a water bath for 2min. Protein concentration was determined using the Pierce BCA method following the manufacturer's instructions using BSA as a standard (Perbio Science Ltd, Cheshire, UK).

2.3.2 Western blotting methods

Western blotting was performed using standard protocols (Packham et al. 1997). An equal amount of protein (100μg) from each sample was denatured at 100°C in protein sample buffer (100mM Tris-HCl (pH6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM dithiothreitol [DTT]) for 3 minutes and loaded onto a (8%, 10% or 12%) SDS-polyacrylamide gel.

2.3.2.1 Preparing resolving gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

Prior to casting the resolving gel, all solutions were de-gassed by sonication for 60s. N,N,N',N'–tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) were added to the polyacrylamide solution with stirring immediately prior to casting of the gels (see Table 9.1). Four gels (1.5 mm thickness) were cast at a time between 2 glass plates (10 cm by 8 cm) by adding the gel solution to within 1.5 cm of the top of the plate. The gels were immediately over-layered with butan-2-ol, covered with aluminium foil and allowed to polymerise for 30 minutes.

2.3.2.2 Preparing stacking gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

A stacking gel (5%, w/v) was used to concentrate the proteins into tight bands prior to entering the resolving gel. Prior to casting the stacking gel (see Table 9.1), the butan-2-ol overlay was removed by inversion of the plates and any residual butan-2-ol removed by washing the surface of the gel with 5 ml of ddH_20. After pouring the stacking gel on to the top of the resolving gel, a 10-well

comb (1.5 mm thick) was placed in the stacking gel polyacrylamide solution. Any bubbles under the comb were removed by gently tapping the side of the plates with a metal spatula. Gels were allowed to polymerise at room temperature overnight.

Table 2.01: Composition of resolving and stacking gels used in Western blotting

Solution	Resolving gel (for 25ml)			Stacking gel (for10ml)
	8 %	**10%**	**12%**	**5%**
Acrylamide (30%, w/v)	**6.7ml**	**8.3 ml**	**10.0 ml**	**1.7 ml**
1.5 M Tris-HCl pH 8.8	**6.3 ml**	**6.3 ml**	**6.3ml**	**-**
SDS (10%, w/v)	**250 µl**	**250 µl**	**250 µl**	**100 µl**
1.0 M Tris-HCl pH 6.8	**-**	**-**	**-**	**1.25 ml**
APS (10%, w/v)	**250 µl**	**250 µl**	**250 µl**	**100 µl**
TEMED	**15 µl**	**10 µl**	**10 µl**	**10 µl**
	Make up to 25 ml with H_2O	**Make up to 25 ml with H_2O**	**Make up to 25 ml with H_2O**	**Make up to 10 ml with H_2O**

2.3.2.3 Transferring and blotting Tris-glycine SDS-Polyacrylamide Gels

Protein samples were electrophoresed on 8-12% Tris-glycine SDS-Polyacrylamide gels using the Mini-PROTEAN electrophoresis system (Bio-Rad, Herts., UK) according to the manufacturers' specifications, using Protein Running Buffer (25mM Tris base, 192mM glycine, 0.1% SDS pH 8.3) at 200 Volts/400mAmps for 45-60 minutes. The proteins were transferred using Transfer Buffer (25% methanol, 10% Protein Running Buffer, 65% ddH_2O) onto

nitrocellulose membranes (GE Healthcare Life Sciences, Bucks, UK) at 100 Volts/400mAmps for 60 minutes. After blocking for 30 minutes in 1x TS buffer (10mM Tris-HCl pH8.0, 150mM NaCl), containing 5% dried skimmed milk powder and 0.05% Tween-20, the membranes were blotted with primary antibodies diluted in TST (1x TS, 0.05% Tween-20), containing 5% dried skimmed milk powder, at 4°C overnight. The membranes were then washed 3 times (for 5 minutes each wash) in TST (1x TS, 0.05% Tween-20) and blotted with the relevant Horseradish Peroxidase labelled secondary antibody (Dako, Denmark) at 4°C for 1 hour and washed 3 times. Blots were visualized using an Enhanced Chemiluminescence (ECL) kit (GE Healthcare Life Sciences, Bucks, UK). Quantitative assessment of band density was performed by densitometry (GS-800 Calibrated Densitometer and Quantity One software, both from Bio-Rad, Herts, UK) and expressed as a ratio of the signal produced by α-tubulin.

Table 2.02: Primary antibodies for Western blotting.

Antibody	Species	Clone	Source
Anti-HSP27	rabbit	polyclonal (SPA-523D)	Stressgen Cambridge Bioscience
Anti-HSP47	mouse	monoclonal (M16.10A1)	Stressgen Cambridge Bioscience
Anti-HSP60	mouse	monoclonal (LK-1)	Stressgen Cambridge Bioscience
Anti-HSPi70	mouse	monoclonal (C92F3A-5)	Stressgen Cambridge Bioscience
Anti-HSC70	mouse	monoclonal (B6)	Santa Cruz Biotechnology
Anti-HSP90	mouse	monoclonal (Ac88)	Mayo Graduate School
Antiphosphorylated STAT-1 (Tyrosine 701)	mouse	monoclonal (ST1P-11A5)	Zymed Cambridge Bioscience
Anti-STAT-1 (p84/p91)	rabbit	polyclonal (sc-98783)	Santa Cruz Biotechnology
Antiphosphorylated STAT-3 (Tyrosine 705)	mouse	monoclonal (3E2)	Santa Cruz Biotechnology
Anti-STAT-3	rabbit	polyclonal (sc-482)	Santa Cruz Biotechnology
Anti-interleukin 1β	rabbit	polyclonal (ab9887)	Abcam Limited
Anti-α-2 macroglobulin	goat	polyclonal (sc-8517)	Santa Cruz Biotechnology
Anti-α tubulin	mouse	monoclonal (DM1A)	Sigma-Aldrich

2.4 Enzyme linked immunosorbent assay (ELISA)

2.4.1 Protein extraction for ELISA

100mg liver was defrosted and diced in 1ml of ice-cold phosphate-buffered saline. 300µl of ice-cold homogenization buffer (phosphate-buffered saline/ 0.05% sodium azide/ 0.5% Triton X-100/ 5mM EDTA / protease inhibitor cocktail (Sigma-Aldrich, Poole, UK) at pH 7.2) was added to the sample in a tight-fitting glass-on-glass homogeniser. The samples were each homogenised with 5 strokes.

Samples were then centrifuged at 13000 rpm at 4°C for 10 minutes. The supernatant was collected and protein concentration was determined using the Pierce BCA method following the manufacturer's instructions (Perbio Science Ltd, Cheshire, UK).

2.4.2 ELISA methods

Rat IL-1Ra ELISA (BioSource International, California, USA) is a commercially available solid-phase sandwich Enzyme Linked Immunosorbent Assay (ELISA). Samples of known standards, control and unknown specimens are added to individual microtiter wells previously coated with polyclonal antibody specific for rat IL-1Ra. Samples are then incubated for 2 hours at 37°C; IL-1Ra antigen binds to this immobilised (capture) antibody (first incubation). After washing a biotinylated polyclonal antibody specific for IL-1Ra for a further hour incubation at room temperature (second incubation), this (secondary) antibody binds to the immobilised IL-1Ra captured during the first incubation. After removal of excess secondary antibody Streptavidin-Peroxidase (enzyme) is

added (third incubation). This enzyme binds to the biotinylated antibody and after washing to remove the unbound enzyme a substrate is added. The bound enzyme acts upon the substrate to produce colour, absorbance is read with an automated plate reader at 450nm. The absorbance of the standard samples is plotted against the standard concentrations to produce a standard curve from which the concentrations of unknown samples may be read. The amount of bound enzyme and therefore intensity of the colour produced is proportional to the concentration of IL-1Ra present in the original sample.

Lipolysaccharide binding protein (LBP) was also measured by a commercially available testing kit (Hycult Biotechnology, Cambridge Bioscience Ltd, Cambridge, UK), a solid-phase ELISA based on the sandwich principle outlined above.

Cardiotrophin (CT-1) ELISA was kindly performed in collaboration with Professor L. Ng, University of Leicester, using a proprietor designed method incorporating human specific anti-CT-1 antibody. No commercial rat CT-1 antibody was available for either Western blotting or ELISA analysis at the time these experiments were performed. There is however, a significant amount of homology with rat and human CT-1 (Pennica et al. 1996). Epidermal growth factor (EGF) ELISA was kindly performed in collaboration with Professor E. Nexø, Aarhaus University, Denmark, with a proprietor designed method (Jorgensen et al. 1996). Lipopolysaccharide binding protein (LBP) was measured using a commercially available solid-phase testing kit (Hycult Biotechnology, Cambridge Bioscience Ltd, Cambridge). Interleukin-1 receptor antagonist (IL-1Ra) was also measured using a commercially available solid-phase testing kit (Biosource International, California, USA).

2.5 Affymetrix Gene Chip methods

2.5.1 Total RNA extraction and quantification

200 mg of frozen liver was ground to a powder using a cold pestle and mortar. Total RNA was isolated from the tissue using the RNA isolator solution TRIZol (Gibco-BRL, Invitrogen Ltd, Paisley, UK). Sample preparation and quality of RNA is a critical aspect of gene chip analysis to minimise detection of false-postive changes in gene expression (Shackel et al. 2002).

RNA was qualitatively and quantitatively assessed by:

1) Electrophoresis on a 1% agarose gel (containing 0.8 μg/ml ethidium bromide): a good quality RNA extraction demonstrates a more intensely illuminating 28S than 18S ribosomal RNA band when viewed under ultraviolet illumination.

2) RNA integrity was assessed by a fluorescent RNA Nano Assay (Bioanalyzer 2100, Agilent Technologies, Berks, UK): intact RNA demonstrates the two ribosomal peaks

3) Spectrophotometry: a good quality RNA extraction (with little contamination of other cellular molecules, particularly proteins) has a ratio of absorbance at 260nm and 280nm close to 2.0 (the concentration of RNA in each sample is measured by the absorbance at 260nm)

The total RNA extracted was then frozen at -80°C and used for Affymetrix Gene Chip analysis and subsequent PCR reactions

2.5.2 First-strand cDNA synthesis

Approximately 5µg of total RNA from each sample was incubated at 37°C for 1 hour with an oligo dT(24) primer containing a 3' T7 RNA polymerase promoter sequence and reverse transcribed using SuperScript II reverse transcriptase (Gibco-BRL, Invitrogen Ltd, Paisley, UK).

2.5.3 Second-strand synthesis

First-strand cDNA was added to a second-strand synthesis reaction containing DNA polymerase I, RNAse H (Promega) and *Escherichia coli* DNA ligase (Gibco-BRL, Invitrogen Ltd, Paisley, UK) and incubated for two hours at 16°C. After this reaction the ends of the double-stranded DNA were blunted using T4 DNA polymerase and then extracted from the reaction mixture using phenol/chloroform and precipitated. The DNA pellet was resuspended in diethylpyrocarbonate-treated water (0.01%).

2.5.4 Preparation of the labelled antisense cRNA target

Double-stranded DNA was *in vitro* transcribed (Bioarray High Yield RNA Transcript Labelling Kit, Affymetrix, Santa Clara, CA, USA) by incubating with T7 RNA polymerase in the presence of biotinylated nucleotides at 37°C for five hours. Biotinylated cRNA was fragmented into segments approximately 100 bases in length by incubating in fragmentation buffer (Affymetrix) at 95°C for 35 minutes.

2.5.5 Target hybridisation

A hybridization cocktail was prepared including the fragmented cRNA target from each of the samples, probe array controls, bovine serum albumin (BSA) and herring sperm DNA. This mixture was hybridised to rat genome RAE230A microarrays (Affymetrix) at 45°C for 16 hours.

2.5.6 Washing and staining

The microarrays were washed several times in nonstringent and stringent wash buffer according to the Affymetrix fluidics station protocol. Phycoerythrin linked to streptavidin was used to label the hybridised target.

2.5.7 Probe array scanning

The stained microarrays were then exposed to laser-induced, excitation fluorescence. Each probe was scanned twice, the Affymetrix software calculated an average of the two images to define the probe cells and provide an intensity value for each cell (a measure of transcript abundance). The double scan improves assay sensitivity and reduces background noise.

2.6 Polymerase Chain Reaction (PCR)

Single-strand cDNA was synthesised: 2µg of total RNA was hybridised with an oligo (dT) primer for 10 min at 70°C then cooled on ice. A master mix was created for each reaction containing: 4µl avian myeloblastosis virus (AMV) reaction buffer, 1µl (10mM stock) dNTPs, 1µl RNasin, 2 µl (18 units) of AMV reverse transcriptase (Promega, Southampton, UK) and diluted with water to give a total volume per sample of 20µl. The mixture was incubated at 37°C for 1 hour

then at 70°C for 3min then frozen at -20°C until use. PCR was performed using 1µl of single-stranded cDNA combined with 5µl magnesium free buffer, 1µl (10mM stock) dNTPs, 2-4µl (25mM stock) $MgCl_2$, 2µl 1:5 dilution of Taq (*Thermos aquaticus*) DNA polymerase (5 units per µl) and 2µl of both forward and reverse primers at a 1:20 dilution.

The following primers (Thermo Electron Corp, Germany) were used to demonstrate semi-quantitative transcript levels of IL-1β and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a control.

Table 2.03: Forward and reverse rat primers for PCR.

Gene	Direction	Sequence	Product Size (base pairs)
α -2 macroglobulin (α-2M)	forward reverse	5'gttacactggaagccgttctg 3' 5' cacttcttattcactgcgtcc 3'	344
Epidermal Growth Factor (EGF)	forward reverse	5' gatctactgtctcgacgttg 3' 5'caacatacatgcacacgccac 3'	257
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	forward reverse	5' ggcattgctctcaatgacaa 3' 5' tgtgagggagatgctcagtg 3'	230
Leptin receptor (LEP-R)	forward reverse	5' cagatcatccacccaatgtg 3' 5' ctggaggattctgatgtcac 3'	230
Lipopolysacharide binding protein (LBP)	forward reverse	5' cttctgaatgtcagtcctgg 3' 5' cagctcatcgttgacatcag 3'	291
Interleukin 1ß (IL-1ß)	forward reverse	5' cattagacagctgcactgcag3' 5' gcttgagaggtgctgatgtac3'	348

2.7 Mitochondrial Methods

2.7.1 Isolation of rat liver mitochondria

Rat liver mitochondria were isolated immediately after sacrifice by homogenisation and differential centrifugation (Causey et al. 1986, Watmough et al. 1989). Immediately after sacrifice fresh liver samples were placed in ice-cold isolation medium (250mM sucrose, 2mM HEPES, 0.1mM EGTA, pH 7.4 at 25˚C). Samples were hand homogenised (10 strokes) in isolation media using a loose fitting Potter-Elvehjem perspex homogeniser and Teflon pestle keeping the samples on ice to prevent local heating. All subsequent operations were performed at 0-4˚C.

The homogenate was then centrifuged (Sorvall Super T-21 ST-750, fixed angle, $7.1x10^{6}rad^{2}.s^{-1}$, 3000rpm for 10 minutes). The resulting nuclear pellet was discarded and the supernatant re-homogenised (10 strokes) and re-centrifuged at 3000rpm for 10 minutes. Again the resulting nuclear pellet was discarded and the supernatant re-centrifuged at 13500rpm for 10 minutes. The resulting pellet consisted of a layer of cellular debris loosely lying over a mitochondrial fraction that was in turn lying over an erythrocyte fraction. The supernatant was discarded with as much of the cellular debris as possible with care to preserve the mitochondrial pellet. The mitochondrial pellets were resuspended in 20ml of isolation medium and re-centrifuged at 13500rpm for 10 minutes. The supernatant was again discarded and the mitochondrial pellet resuspended in a small amount of isolation medium transferred to 2ml microcentrifuge tubes and vortexed gently before being re-centrifuged at 13500rpm for 3 minutes. Following this any remaining debris was removed from the top of the mitochondrial pellet with a

Pasteur pipette and the remaining mitochondrial pellets (from duplicate tubes) combined whilst leaving the remaining erythrocytes behind. The fresh mitochondrial pellet was then resuspended in 500µl of isolation medium and gently vortexed prior to use and placed on ice.

2.7.2 Citrate Synthase assay

Citrate synthase activity was measured spectrophotometrically as described by Shepherd and Garland (Shepherd and Garland 1969) to quantify mitochondria to normalise oxygen consumption values. The method is based on the chemical coupling of CoA, released from acetyl-CoA during the enzymatic synthesis of citrate, to 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The release of the absorbing ion is measured spectrophotometrically at 30˚C.

In a cuvette, 10µl of 10mM DTNB, 10µl of 5mM acetyl-CoA and 965µl of 100mM Tris-HCl pH 8.0 were added then 10µl of fresh mitochondria (1:10 solution diluted in isolation medium). A baseline measurement was taken at this time then 5µl of 50mM oxalacetic acid to obtain a concentration of 0.25mM. 10µl of 10% (v/v) Triton X-100 was then added to disrupt mitochondrial membranes and obtain total citrate synthase activity, which is a measure of the amount of mitochondria. Absorbance was measured at 412nm, and activity calculated in U (i.e. µmol/min), using an extinction coefficient of 13.6mM/cm for the 5-thio-nitrobenzoate ion.

2.7.3 Mitochondrial oxygen consumption

Oxygen consumption was measured polarographically using a Clark type oxygen electrode. Fresh isolated mitochondria were incubated in 0.75ml of

medium containing 110mM KCl, 10mM HEPES, 2.5mM KH_2PO_4, 1mM EDTA, 5mM $MgCl_2$ at pH 7.2. Glutamate 10mM plus 1mM malate or 1.5mM succinate were used as respiratory substrate. State 3 respiration, which represents maximum oxidative flux, was measured after addition of 250nmol ADP. State 4 respiration, which represents leakage of extruded protons, was measured as all of the ADP is converted to ATP. Uncoupling conditions were obtained by addition of 40μM 2,4-dinitrophenol (DNP). The respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration (Figure 7.05, pg 197).

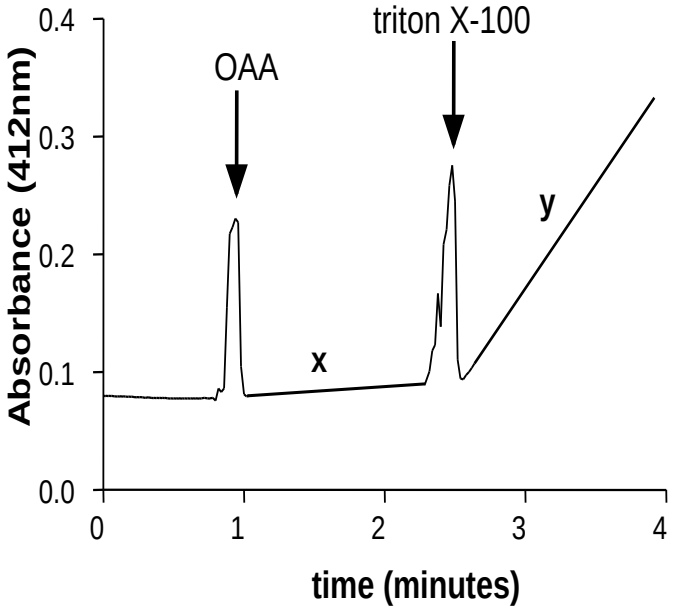

Figure 2.01: Schematic diagram of spectrophotometric citrate synthase assay.

Baseline absorbance is established, 0.25 mM oxalacetic acid (OAA) is added then rate of change (x) is recorded, 10 µl of Triton X-100 is added and rate of change (y) calculated. The value y equates to the total amount of mitochondria. The percentage of disrupted mitochondria is equal to the ratio of x to y multiplied by 100 (> 95% acceptable for liver mitochondria).

2.8 2D-DIGE methods

Two Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) is a method to label proteins with commercially available CyDye Fluors (Minimal dyes) (Amersham, GE Healthcare UK Ltd) and subsequently separate these proteins using 2 dimensional gel electrophoresis. DIGE technology has been developed to simplify the process of 2D protein electrophoresis and increase accuracy in detecting and identifying proteins.

2.8.1 2D-DIGE sample preparation

As per the method of section 2.7.1 the mitochondrial pellet is resuspended by gently vortexing in 500µl of ice-cold isolation medium (250mM sucrose, 2mM HEPES, 0.1mM EGTA, pH 7.4 at 25˚C) and stored at -80ºC. Protein precipitation of the samples is then required to remove contaminants that can affect the isoelectric focusing of gels (IEF). Protein precipitation is performed by taking 1 volume of aqueous sample and combing with 3 volumes of ice-cold acetone, vortexing and storing at -20ºC overnight. The sample is then centrifuged for 5 minutes at 10000g, the supernatant is discarded (containing potential contaminants e.g. salts, lipids, metabolites and DNA). The pellet is the protein fraction which is then freeze-dried.

2.8.2 Protein labelling with CyDye

Three CyDyes are used in the assay; Cy2 is used to label the standard, Cy3 and Cy5. The standard consists of an equal, pooled mix of all 3 experimental samples. This means that every protein from every sample is represented in the

standard that is present on all the gels, this in turn increases the confidence in matching between gels and enables the generation of accurate 'spot statistics' by minimising gel to gel variation.

The CyDyes are initially reconstituted into high quality anhydrous dimethylformamide (DMF) [specification: ≤ 0.005% H_2O, ≥99.8% pure, Sigma). CyDye DIGE Fluors have an NHS-ester reactive group and are designed to covalently bond to the epsilon amino group in proteins via an amide linkage. The quantity of dye added to the sample is limiting in the reaction, therefore this method is termed 'minimal' labelling. This method ensures that dyes label approximately 1-2% of the available lysine, only a single lysine per protein molecule. The pI of the protein does not significantly alter after labelling as the lysine's +1 charge is then replaced with the +1 charge of the CyDye.

The precipitated protein is re-suspended in 20µl of DIGE label buffer (7M Urea, 2M Thiourea, 2% CHAPS, 10mM Tris-HCL pH 8.3), vortexed well and centrifuged briefly. The CyDye in reconstituted in DMF to make a stock solution of (1nmol/µl), a further 1:5 dilution in DMF is made to make a working solution of CyDye (200pmol/µl). 2µl of working CyDye solution is then added to the relevant protein sample (manufacturers recommend concentration of CyDye to protein is 8pmol to 1µg protein), vortexed and centrifuged briefly. This process was repeated for all 3 CyDyes, samples were then left in the dark on ice for 30 minutes, and the reaction was then halted by adding 1µl of 10mM lysine and then kept on ice in the dark for a further 10 minutes.

2.8.3 First dimension - rehydrating of the isoelectric focusing strip

Combined the 3 CyDye labelled samples make a total volume 60µl, this is then made up to total volume of 450µl with rehydration solution B (Urea 8M, Thiourea 2M, CHAPS 4%w/v, DTE 65M, Resolyte^{TM} 50µl in 5mls and trace of bromophenol blue) for a 24cm isoelectric focusing (IEF) strip. This solution is then incubated at 37ºC for 30 minutes. The IEF strip is removed from the freezer, placed face-down in a rehydration cassette containing the sample solution applied evenly, the strip is then covered in mineral oil to prevent oxidization and left overnight.

2.8.4 First dimension – focusing of strip

The IEF strip is removed from the rehydration cassette and placed in the IEF unit manifold, gel side up. 1cm length wicks are moistened with 150µl H_2O and placed at anodic and cathodic ends of the IEF strip after removing excess. The anode and cathode electrodes are then clamped to the wicks and 110ml mineral oil added to the IEF manifold, immersing the strip completely. The strips are then focused at 25ºC as per Table 2.03 below.

Table 2.04: First dimension protocol, focusing of IEF strip.

Time (24hr clock)	Hours	Volts	Accumulative volt hours
16	0	0-300	n/a
17	1	300	300
18	2	300	600
19	3	300	900
20	4	300	1200
21	5	300	1500
22	6	1000	2500
23	7	2000	4500
24	8	4000	8500
1	9	6000	14500
2	10	7000	21500
3	11	8000	29500
4	12	8000	37500
5	13	8000	45500
6	14	8000	53500
7	15	8000	61500
8	16	8000	69500
9	17	8000	77500
10	18	8000	85500
11	19	8000	93500
Total volt hours		**93500**	

2.8.5 Casting gels for second dimension

Gel plates are thoroughly cleaned with detergent, deionised H_2O and HPLC grade methanol. Gel plates are spaced to give a gel width of 1.5mm and 12% acrylamide analytical gels were prepared as Table 2.05 below.

Table 2.05: Constituents of 12% acrylamide analytical gels.

Solution	**Volume / Weight**
Acrylamide solution (30% w/v)	499mls
1.34M Tris-HCL, pH8.8	250mls
Piperazine diacrylamide (PDA)	1.92g
Sodium thiosulphate (5mM)	4.9ml
TEMED	110µl
Ammonium persulphate	Dissolve 0.5g in 5ml of H_2O and add slowly

All components were then mixed thoroughly and made up to a total volume of 1L with H_2O. The casting chamber was filled with acrylamide to within 1.5cm of the top of the plate and then overlaid with 2ml of H_2O saturated with butan-2-ol. After casting overnight the surface of the gels is washed with a 1:4 dilution of 1.34M Tris-HCl, pH8.8.

2.8.6 Second dimension - re-equilibration of strip

A stock solution of 50mM Tris-HCl, pH 6.8, 6M Urea and 30% glycerol is required and from this 2 working solutions are prepared: Solution A is composed of 5g SDS and 1g DTE and made up to 50ml with the stock solution. Solution A acts to re-solubilise the proteins, re-breaks any disulphide bonds which may have reformed during focusing and corrects the pH ready for SDS-PAGE. Solution B is composed of 5g SDS, 1.25g of iodoacetamide and a trace of bromophenol blue made up to a total volume of 50 ml with stock solution. Solution B also acts to re-solubilise proteins, it carboamidomethylates all cysteine groups which gives higher resolution spots on analytical gels and also makes peptides generated after in-gel tryptic digestion more amenable to mass spectrometry analysis. Solution B also acts to correct the pH for SDS-PAGE.

The IEF strips are placed in a re-equilibration tube (gel side up), solution A is added to completely immerse the gel strip which is then rotated on a mixer for 15 minutes. Solution A is then poured off and solution B is added in the same way and also rotated on a mixer for 15 minutes. Following this SDS-PAGE second dimension is immediately performed.

2.8.7 Second dimension – PAGE

After the IEF strips have been re-equilibrated the top of the resolving gel is filled with running buffer 2X Tris-glycine-SDS [25mM-198mM-0.1%w/v]. The IEF strip is then carefully added to the surface of the resolving gel. The upper reservoir of the gel tank is then filled with 2X Tris-glycine-SDS [25mM-198mM-0.1%w/v] and the current is immediately applied at 45mAmp per gel, 60 Volts.

The gels are then left running overnight a 2 Watts per gel with a tank buffer temperature of 15ºC.

2.8.9 Gel scanning and fixing

To perform 2D-DIGE the electrophoresis is stopped, the gel removed from the tank is replaced by a blank and 1 Watt is continued to prevent spot dissolution. The gel plate is rinsed and wiped clean using double-distilled H_2O. The gel is then scanned using a Typoon scanner (GE Healthcare, Bucks, UK). The gels are initially scanned at low resolution to optimize settings then fluorescence settings are selected for CyDye 2, 3 and 5 with an initial voltage setting of 550. Image analysis was then performed using Progenesis SameSpots software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK).

Chapter 3

The influence of IIR on hepatic Heat Shock Protein expression

Chapter 3 – The influence of IIR on hepatic Heat Shock Protein expression

Contents

3.1 Introduction

There is considerable interest in the potential therapeutic role of induced hypothermia in many different disease processes; however the molecular basis of its protective effect remains unknown. The following experiments set out to investigate the hepatic response of a family of important proteins known as 'heat shock proteins' (HSPs) to IIR during both normothermia and hypothermia.

HSPs are constitutively expressed and/or induced within the cell following thermal (both hyper- and hypothermic) (Ning et al. 1999) and non-thermal stressful stimuli. HSPs are a natural cytoprotective response against such stressful stimuli (Kim et al. 2002, Latchman 1999), an adaptive response which is highly conserved across species. Recent experiments have demonstrated that induced hypothermia selectively alters stress-induced HSP expression (e.g. HSP70 expression was significantly reduced as compared to HSP40 in rat brains following asphyxial cardiac arrest) (Hicks et al. 2000). Conversely induction of profound hypothermia in a model of lethal haemorrhage in swine was reported to significantly increase levels of HSP70 and attenuate IL-6 production in serum as compared to normothermic cases (Chen et al. 2005). Recently reported proteomic analysis of post–ischaemic induced hypothermia in rat brain has demonstrated a sustained increase in HSP70, whereas expression of other HSP proteins were unaffected by hypothermia. Interestingly, hypothermia did not increase, but decreased, the upregulation of HSP70 mRNA expression by ischemia, suggesting that HSP70 abundance is controlled by an unknown posttranscriptional regulation (Terao et al. 2009)

HSPs act as a 'chaperone' within the cytoplasm or mitochondrion by binding to misfolded and damaged proteins, preventing their proteolysis and

aiding their refolding back to the native state. Therefore HSPs can protect cells from apoptosis and are involved in many other essential cellular functions, such as metabolism, growth and differentiation. Experimental over-expression of specific HSPs induced by unrelated stress (Javadpour et al. 1998), via viral vectors (Brar et al. 1999,Wagstaff et al. 1999) or transgenic mice (Radford et al. 1996, Rajdev et al. 2000) have demonstrated protection against ischaemia-reperfusion injury.

HSP are termed according to their molecular mass (e.g. HSP70 has a molecular mass of 70kDa). HSP70 is one of the most closely studied heat shock proteins and has a remarkably simple gene, a single exon with no introns, which enables rapid transcription and translation (Feder and Hofmann 1999, Pilon and Schekman 1999). Within the 70kDa HSP 'family' there are subdivisions, the 72-kD inducible protein HSP70 (also known as HSP72) is expressed during periods of cellular stress, the 73-kD constitutive heat shock cognate protein (HSC70) is also known as HSP73 and expressed within the cytosol at most times (Kregel 2002). HSP70 in addition to being an intracellular chaperone for damaged proteins within the cell also decreases the production of specific cytokines (e.g. TNF, IL-1, and IL-6), regulates NF-κB expression, attenuates NO production and decreases cellular apoptosis. In addition there is evidence from experimental models that increased levels of HSP70 have correlated with increased survival from inflammatory shock (Qing et al. 2009, Van Molle et al. 2002).

The potential role of HSPs in IIR injury has received little attention. Previously in a rat model of IIR, extracted ileal levels of heme oxygenase-1, (HO-1), otherwise known as HSP 32, were significantly increased following hypothermic injury (Attuwaybi et al. 2003, Hassoun et al. 2002). In addition the

administration of an HO-1 inhibitor, Sn Protoporphyrin IX, acted by reducing the protective effects of hypothermia in IIR. HO-1 as with many other HSPs has been demonstrated to be cytoprotective in many different models of oxidant stress (Otterbein and Choi 2000, Otterbein et al. 2010). However HO-1 appears to differ from other HSPs as in addition to its role as a classical 'heat shock protein', HO-1 also has as catalytic role: HO-1 acts by catalyzing the first and rate-limiting step in the degradation of heme, thereby yielding biliverdin, carbon monoxide (CO) and free iron. These products are rapidly converted into bilirubin and ferritin, together with CO they constitute three major anti-oxidants (Otani et al. 2000), (Figure 3.01).

HO as with other HSPs are highly conserved evolutionarily across species, HO for example is present in prokaryotic bacteria, plants and fungi. Such conservation suggests a significant role in modulation of critical cellular pathways to maintain homeostasis (Otterbein and Choi 2000). The protective effects of induced hypothermia in IIR may be mediated via such a family of important cellular modulators; the following experiments describe the testing of this hypothesis.

3.2 Materials and Methods

Animals were anaesthetised and underwent either sham or IIR at either normothermia or moderate hypothermia according to the methods in Chapter 2 (pg 47-48). The reperfusion time of 60 minutes was based on previous experiments where normothermia IIR resulted in significant liver energy failure which was prevented in moderately hypothermic IIR (Vejchapipat et al. 2000, Vejchapipat et al. 2001).

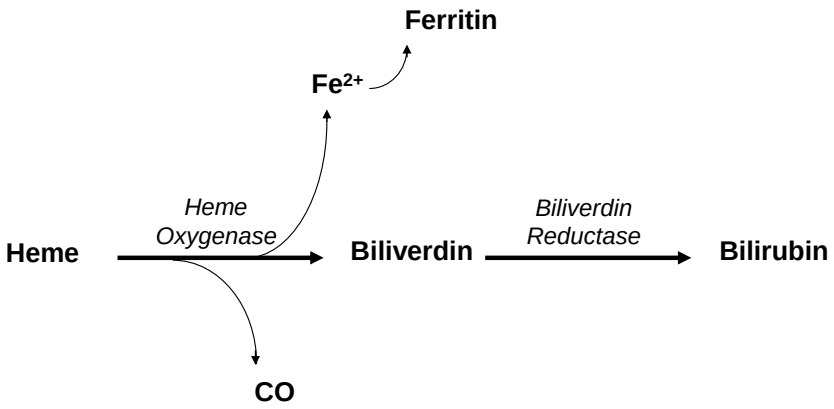

Figure 3.01: Enzymatic reaction of heme oxygenase.

The animals were divided into four groups:

NS = sham operation for 120 minutes at normothermia

(n=6)

NIIR = 60 minutes ischaemia and 60 minutes reperfusion at normothermia

(n=6)

HS = sham operation 120 minutes at moderate hypothermia

(n=6)

HIIR = 60 minutes and 60 minutes reperfusion at moderate hypothermia

(n=6)

3.2.1 Western blotting

Western blotting was performed using standard protocols outlined in Chapter 2 with equal amounts (100µg) of protein loaded for each sample.

Table 3.01: Primary HSP antibodies for Western blotting.

	Antibody	**Species**	**Clone**	**Source**
1.	Anti-HSP27	rabbit	polyclonal (SPA-523D)	Stressgen Cambridge Bioscience
2.	Anti-HSP47	mouse	monoclonal (M16.10A1)	Stressgen Cambridge Bioscience
3.	Anti-HSP60	mouse	monoclonal (LK-1)	Stressgen Cambridge Bioscience
4.	Anti-HSPi70	mouse	monoclonal (C92F3A-5)	Stressgen Cambridge Bioscience
5.	Anti-HSC70	mouse	monoclonal (B6)	Santa Cruz Biotechnology
6.	Anti-HSP90	mouse	monoclonal (Ac88)	Mayo Graduate School
7.	Anti-α Tubulin	mouse	monoclonal (DM1A)	Sigma-Aldrich

3.2.2 *Statistical analysis*

Data are expressed as mean ± SEM. Statistical analysis was performed using repeated-measures ANOVA, grouping samples run on the same gel, p values of less than 0.05 were considered significant. Statistical evaluation of densitometric data across several Western blots is problematic as the absolute densitometric values are very dependent upon between-gel variation (e.g. background density, washing strategies, radiographic exposure etc). For this reason I chose to use repeated-measures ANOVA for statistical evaluation. Thus, a single gel is considered as an 'individual' on which repeated measures (corresponding to the experimental groups NS, NIIR, HS, HIIR) are made.

3.3 Results

Surprisingly, given that we suspected that the liver had been exposed to a significant stress there were no significant differences between the experimental groups (normothermic sham [NS], normothermic intestinal ischaemia-reperfusion [NIIR], hypothermic sham [HS] or hypothermic intestinal ischaemia-reperfusion [HIIR]) in expression of major constitutive HSPs such as HSC70 (Figure 3.02A), in important inducible HSPs such as HSP27 (Figure 3.02B) and HSP70 (Figure 3.02C) or other relevant HSPs measured (HSP47, HSP60, and HSP90 [Figure 3.03]).

This is clearly illustrated in representative Western blots of samples taken from two sets of experimental animals probed for HSP27, HSP70, HSC70, and α-tubulin (loading control) (Figure 3.04A) and HSP47, HSP60 and HSP90 and α-tubulin (loading control) (Figure 3.04B). These findings clearly demonstrate that

increased hepatic expression of HSPs is not the mechanism responsible for hepatic protection at this time point.

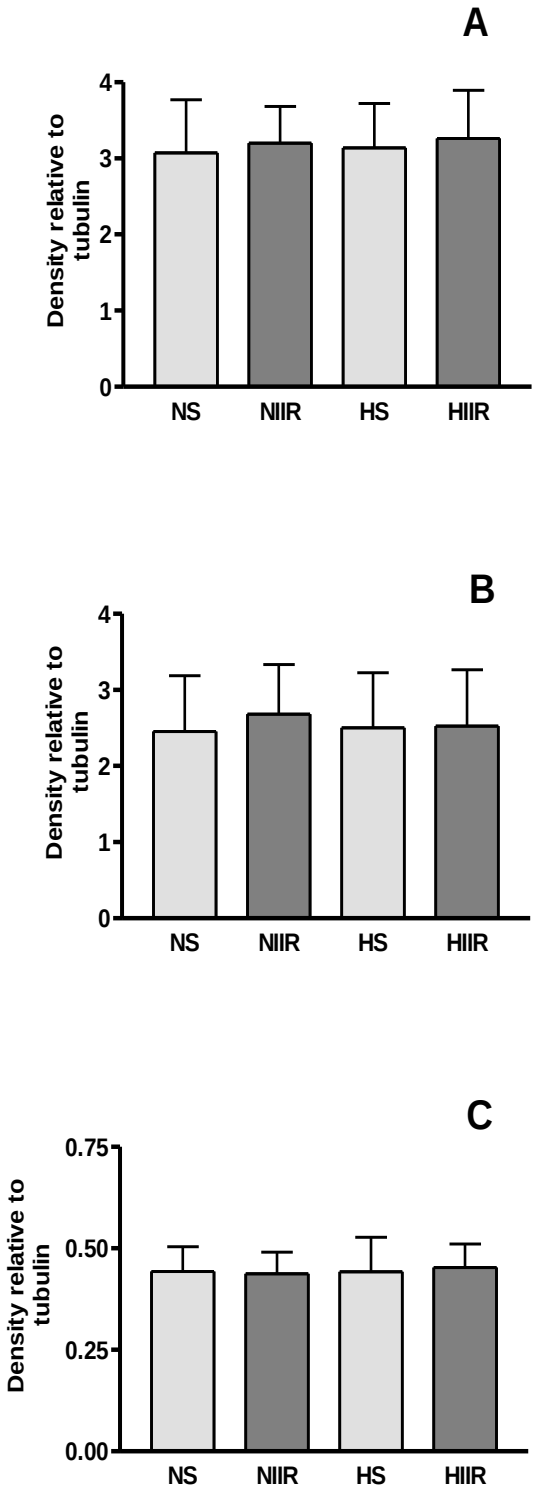

Figure 3.02: Protein levels of [A] HSC70, [B] HSP27 and [C] HSP70.

Samples were analysed by Western blotting as indicated in the Materials and Methods. Density of sample bands was normalised to α-tubulin, and results were compared by repeated-measures ANOVA, grouping samples run on a single gel; there were no significant differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS; hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

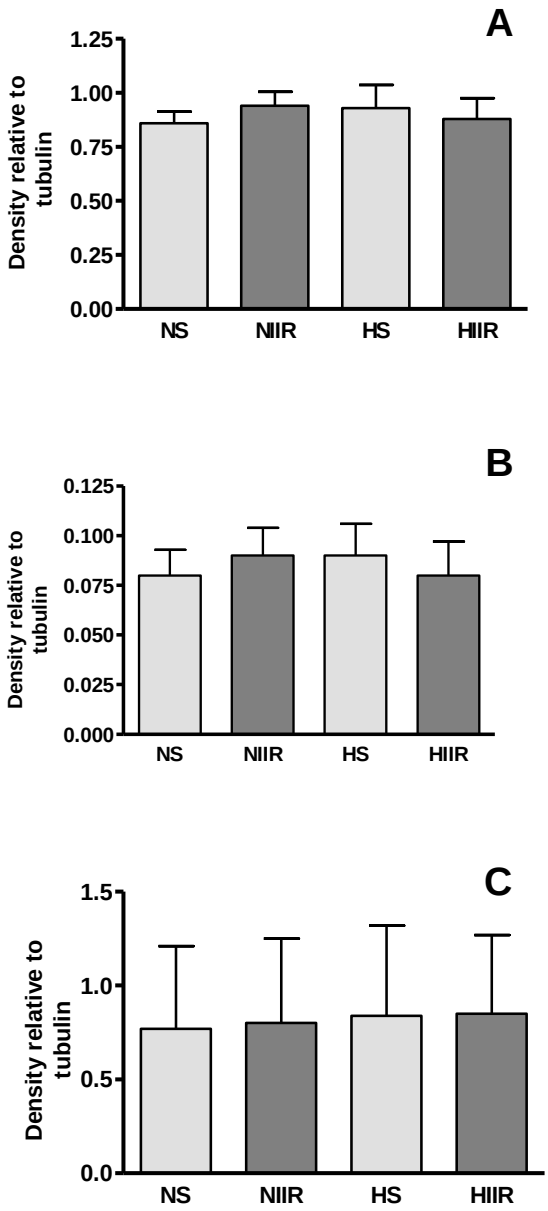

Figure 3.03: Protein levels of [A] HSP47, [B] HSP60 and [C] HSP90.

Samples were analysed by Western blotting as indicated in the Materials and Methods. Density of sample bands was normalised to α-tubulin, and results were compared by repeated-measures ANOVA, grouping samples run on a single gel; there were no significant differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS; hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

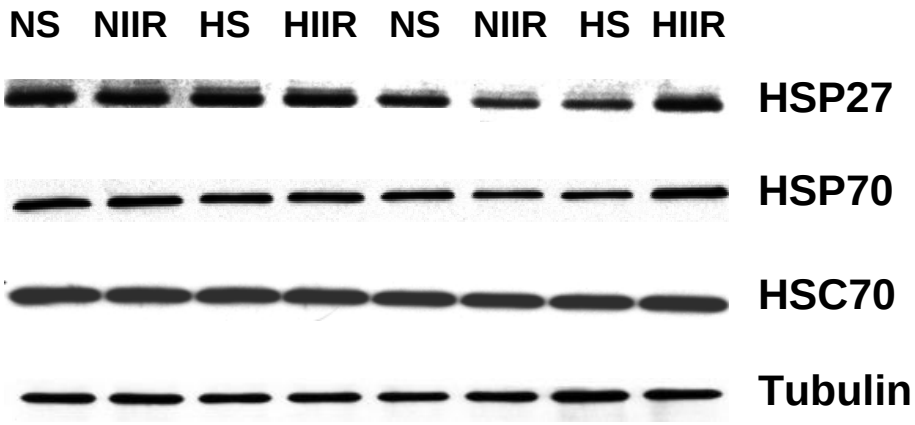

Figure 3.04A: Representative Western Blot of HSP27, HSP70, HSC70 and α-tubulin (loading control).

NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

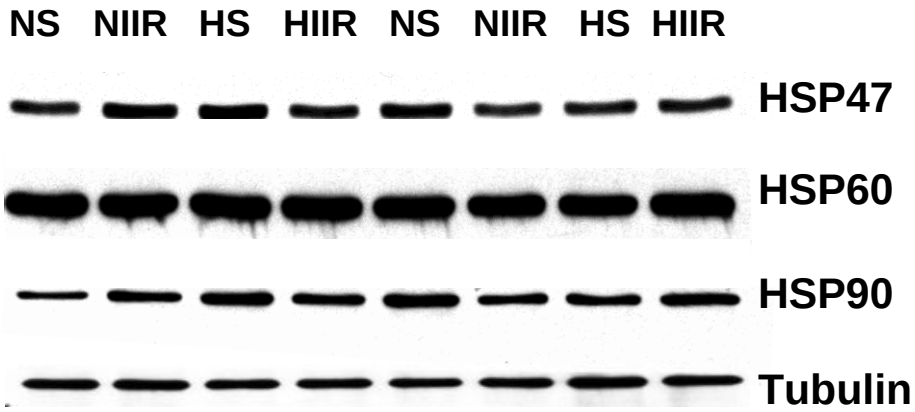

Figure 3.04B: Representative Western Blot of HSP47, HSP60, HSP90 and α-tubulin (loading control).

NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

3.4 Discussion

Previous studies have demonstrated significant protection of the liver from the deleterious effects of IIR occurring at 60 minutes of reperfusion during moderate hypothermia (Vejchapipat et al. 2001). In the present study, this data demonstrates that the protective effect of moderate hypothermia does not appear to be mediated by changes in hepatic HSP expression, and that HSP expression in the liver is not altered by IIR.

These experiments also confirm that hepatic expression of important inducible HSPs (e.g. HSP27, HSP70) does not appear to alter in response to normothermia IIR injury at 60 minutes of reperfusion. Previous studies have demonstrated that at this time point there is significant increase in liver energy failure (Vejchapipat et al. 2001). A change in expression of these important protective proteins may have been expected but was clearly not demonstrated at this particular time-point. However, these findings do not exclude the possibility that HSPs are induced and/or protective at a later time point in this experimental model. Of note, previous studies have reported increased levels of HSP 32 (HO-1) in ileal tissue following hypothermic IIR but most significantly at 24 hours of reperfusion (Attuwaybi et al. 2003, Hassoun et al. 2002) However, it is clear that even if HSPs are demonstrated to be protective at a later time they do not provide the mechanism of protection afforded by moderate hypothermia at this experimental time point.

It is also of note that at the time of the above experiments the appropriate HO-1 (HSP32) antibody was not commercially available and unfortunately has not subsequently been investigated in hepatic tissue following IIR in our model. However, in view of the addition role of HO-1 as a catalyst, any influence of IIR

or temperature change on HO-1 levels could potentially influence its role as a catalyst and/or as a 'heat shock protein'. A HO-1 inhibitor, Sn Protoporphyrin IX, has been previously demonstrated to reduce the protective effects of hypothermia in IIR by acting as an inhibitor of catalytic function rather than an inhibitor of heat shock 'chaperone' function (Attuwaybi et al. 2003). Therefore influence of our model on HO-1, although as yet unexamined, is unlikely to influence our final conclusion that at this particular time-point HSPs are not responsible for the metabolic changes in the liver following IIR injury and/or moderate temperature change.

The following chapter sets out to investigate the influence of moderate hypothermia on a more rapid mechanism of modulating protein function, post-translational modification. Post-translation features (e.g. phosphorylation) provide the cell with a mechanism for activating quiescent constitutive proteins that are then able to contribute to rapid signalling (e.g. by altering transcription factor activity) without the time and machinery required for protein synthesis. Such rapid changes in cell signalling provides may explain the prompt, protective effect of moderate hypothermia in IIR and thus the subsequent experiments examine the influence of IIR, induced hypothermia and activation of an important family of cell signalling molecules, Signal Transducers and Activators of Transcription (STATs).

Janus Kinase-Signal Transducers and Activators of Transcription (JAK-STAT) pathway is a highly conserved signalling network that is involved in a wide range of distinct cellular processes including, inflammation, cell-cycle control and development. The JAK-STAT pathway has been demonstrated to be an important modulator of apoptosis: STAT-3 is an anti-apoptotic transcription

factor (target genes Bcl-2, Bcl-X_L, inhibits p53 and Caspase expression) whereas STAT-1 has been demonstrated to be pro-apoptotic (target genes Fas, Fas ligand, Caspase-1 and p53 STAT-3 has been demonstrated to be protective in experimental models of ischaemia-reperfusion in the heart (Barry et al. 2007, Hilfiker-Kleiner 2004, Oshima 2005). The influence of STATs in intestinal ischaemia-reperfusion is not known.

Chapter 4

The influence of IIR on a family of transcription factors (STATs) in the liver

Chapter 4 -

The influence of IIR on a family of transcription factors (STATs) in the liver

Contents

4.1 Introduction

Subsequent to the results demonstrating unchanged HSP expression in the four experimental groups a further hypothesis was developed to address whether an earlier 'stress' response was elicited in the liver following IIR. The level of post-translational activation (phosphorylation) of an important family of constitutively expressed transcription factors, STAT (Signal Transducers and Activators of Transcription) proteins was selected. This important family of signalling molecules have previously been demonstrated to be involved in protective pathways in experimental models of cardiac injury (Barry et al. 2007). In addition any difference detected in transcription factor activation may indicate altered transcriptional activity of specific genes within the liver down-stream during the different experimental conditions, thereby enabling the identification of potential protective pathways.

STATs comprise of a family of latent cytoplasmic proteins activated by a large number of extracellular signalling molecules including reactive oxygen species and specific cytokines (e.g. IL-6, TNFα, and IFNγ). At the time of these experiments seven mammalian STAT genes have been identified in three chromosomal clusters (STAT-1, STAT-2, STAT-3, STAT-4, STAT-5A, STAT-5B and STAT-6) (Copeland 1995).

STAT activation occurs in the cytoplasm following phosphorylation of a conserved tyrosine residue (e.g. STAT-1 residue 701, STAT-3 residue 705). Ligand-activated receptors that catalyse this reaction include receptors with intrinsic tyrosine kinase activity (e.g. epidermal growth factor receptor [EGFR]) and receptors which lack intrinsic tyrosine kinase activity but are non-covalently associated with Janus kinases (JAKs) (e.g. cytokine receptors). Following tyrosine

phosphorylation STAT molecules form homo- or heterodimers which are then able to translocate into the nucleus and bind to DNA. STATs that form homodimers are STATs 1, 3, 4, 5 and 6; DNA-binding heterodimers are STAT 1:2 and STAT 1:3 (Battle and Frank 2002, Darnell 1997). In addition STAT-1 and STAT-3 may also be phosphorylated on a single serine residue (residue 727) which is required for maximal gene activation by these members of the STAT family. Tyrosine phosphorylation is obligatory for transcription activation whereas serine phosphorylation is supplementary (Darnell 1997).

STAT binding to specific promoters of target genes results in modulation of expression of mediators of both inflammatory (e.g. haptoglobin, complement C3, C/EBP and CRP) and apoptotic pathways (e.g. Fas/FasL, p53, Bcl-2) (Horvath 2000, Hosui and Hennighausen 2008). As changes in protein expression are preceded by changes in activation of specific transcription factors this chapter examines the phosphorylation (activation) of two related transcriptional factors, STAT-1 and STAT-3, in the same experimental groups examined previously in Chapter 3.

4.2 Materials and Methods

The same samples of extracted liver protein were used in the experiments outlined below as were used for Heat Shock Protein Western Blots in Chapter 3 (pg 79-80).

The four experimental groups were:

NS = sham operation for 120 minutes at normothermia

(n=6)

NIIR = 60 minutes ischaemia and 60 minutes reperfusion at normothermia

(n=6)

HS = sham operation for 120 minutes at moderate hypothermia

(n=6)

HIIR =60 minutes and 60 minutes reperfusion at moderate hypothermia

(n=6)

4.2.1 Western blotting

Western blotting was performed using the standard protocols outlined in Chapter 2 (pg 53-57).

Table 4.01: Primary Antibodies for Western blotting.

	Antibody	**Species**	**Clone**	**Source**
1.	Antiphosphorylated STAT-1 (Tyrosine 701)	mouse	monoclonal (ST1P-11A5)	Zymed Cambridge Bioscience
2.	Anti-STAT-1 (p84/p91)	rabbit	polyclonal (sc-98783)	Santa Cruz Biotechnology
3.	Antiphosphorylated STAT-3 (Tyrosine 705)	mouse	monoclonal (3E2)	Santa Cruz Biotechnology
4.	Anti-STAT-3	rabbit	polyclonal (sc-482)	Santa Cruz Biotechnology
5.	Anti-interleukin 1β	rabbit	polyclonal (ab98871)	Abcam Limited
6.	Anti-α-2 macroglobulin	goat	polyclonal (sc-8517)	Santa Cruz Biotechnology
7.	Anti-α tubulin	mouse	monoclonal (DM1A)	Sigma-Aldrich

4.2.2 Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using repeated measures ANOVA, grouping samples run on the same gel, p values of less than 0.05 were considered significant. Statistical evaluation of densitrometric data across several Western blots is problematic as the absolute densitrometric values are very dependent upon between-gel variation (e.g.

background density, washing strategies, radiographic exposure etc). For this reason I chose to use repeated-measures ANOVA for statistical evaluation. Thus, a single gel is considered as an 'individual' on which repeated measures (corresponding to the experimental groups NS, NIIR, HS, and HIIR) are made.

4.3 Results

IIR at normothermia caused significant increase in phosphorylated STAT-1 (p-STAT-1), reflecting activation of this transcription factor ($p<0.05$; Figures 4.01A, 4.02A). In addition, there was a dramatic increase in phosphorylated STAT-3 (p-STAT-3), again reflecting transcriptional activation ($p<0.001$; Figures 4.01B, 4.02B). Moderate hypothermia completely abolished this activation of both p-STAT-1 (HIIR vs. NIIR $p<0.05$, Figures 4.01A, 4.02A) and p-STAT-3 (HIIR vs. NIIR $p<0.001$, Figures 4.01B, 4.02B) such that levels of phosphorylation of these important active transcription factors were reduced to the same levels demonstrated in sham groups.

In addition to activation by phosphorylation, STATs may also be synthesised *de novo* in response to other transcriptional regulators, leading to increased levels of total STATs. There were no differences between the study groups in total STAT-1 (p = not significant, Figures 4.01C, 4.02A). However, interestingly, there was a significant increase in total STAT-3 following normothermic injury (NIIR vs. sham $p<0.05$, Figures 4.01D, 4.02B) but this increased synthesis was abolished following moderate hypothermia (HIIR vs. NIIR $p<0.01$, Figures 4.01D, 4.02B) such that total STAT-3 levels were similar to normothermic sham animals. Despite this increase in total STAT-3 protein amount, there was a large relative increase in the proportion of phosphorylated

STAT-3 in the normothermic injury group, reflected by the ratios of p-STAT-3 *vs.* total STAT-3 (0.68±0.06 in the normothermic sham group, but significantly increased in the normothermic ischaemia-reperfusion group NIIR 0.94±0.08; $p<0.01$, Figure 4.03).

These results demonstrate that at this particular time point normothermic IIR does not cause an increase in hepatic HSP expression but phosphorylates (activates) STAT-1 and STAT-3. Interestingly, moderately hypothermic IIR does not change hepatic HSP expression but prevents STAT-1 and STAT-3 activation.

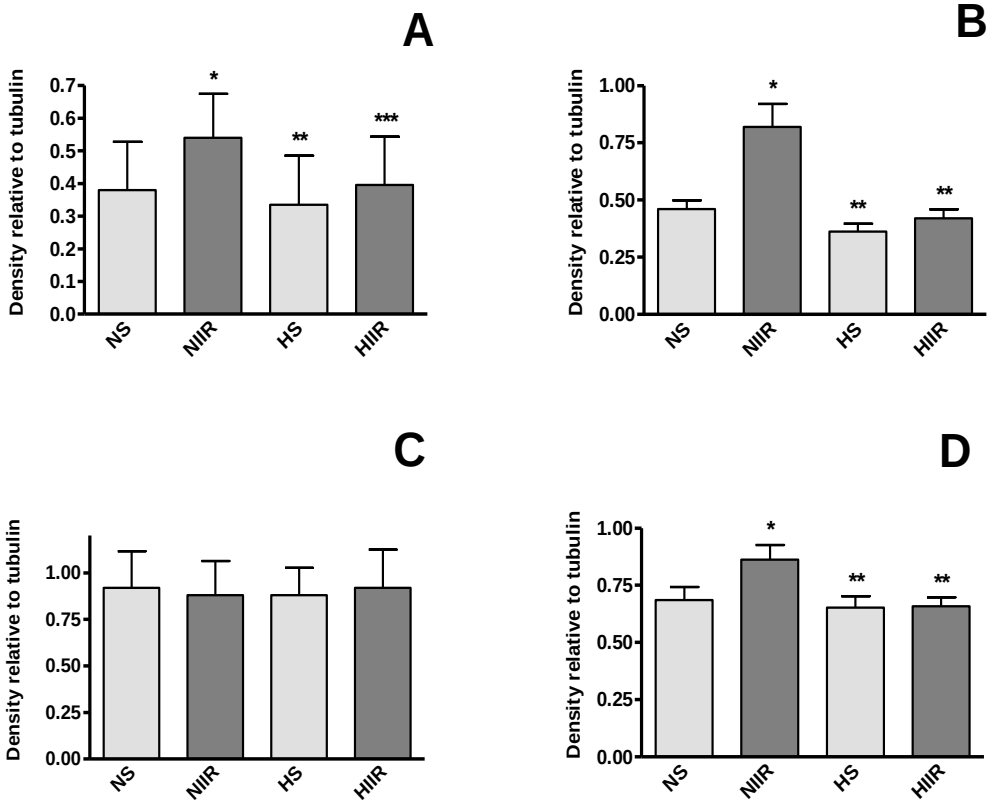

Figure 4.01: Protein levels of [A] p-STAT-1, [B] p-STAT-3, [C] Total STAT-1 and [D] Total STAT-3.

Samples were analysed by Western Blotting as indicated in the Materials and Methods section. Density of sample bands was normalised to α-tubulin, and results were compared by repeated-measures ANOVA, grouping samples run on a single gel: [A] * $p<0.05$ *vs.* NS, ** $p<0.01$ *vs.* NIIR, *** $p<0.05$ *vs.* NIIR; [B] *$p<0.001$ *vs.* NS, ** $p<0.001$ *vs.* NIIR; [C] no significant differences between any of the experimental groups; [D] * $p<0.05$ *vs.* NS, ** $p<0.01$ *vs.* NIIR.
NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS; hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

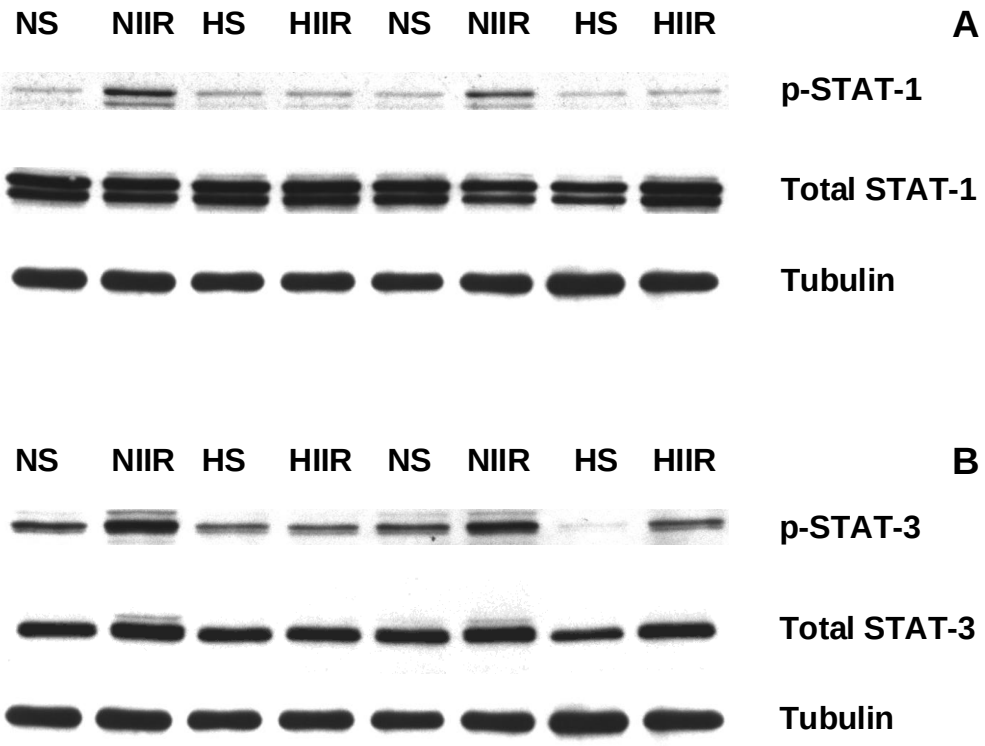

Figure 4.02: Representative Western blots of [A] phosphorylated STAT-1, total STAT-1 and α-tubulin (loading control) and [B] phosphorylated STAT-3, total STAT-3 and α-tubulin (loading control).

NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS; hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

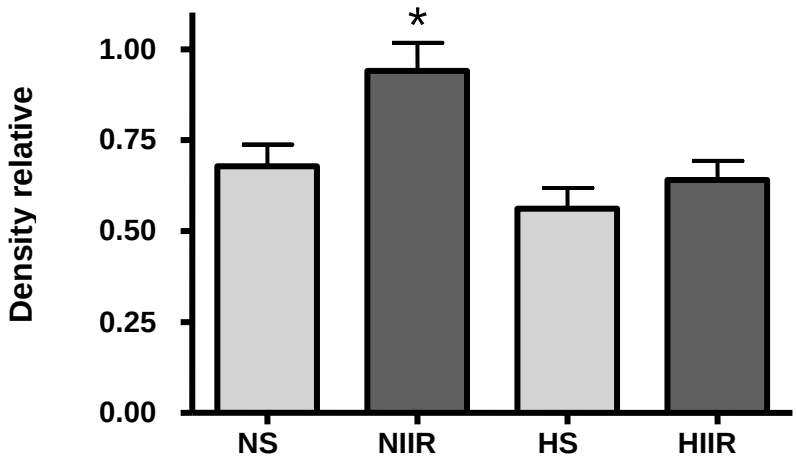

Figure 4.03: Protein levels of p-STAT-3 *vs.* Total STAT-3.

Samples were analysed by Western blotting as indicated in the Materials and Methods section. Density of sample bands was normalised to Total STAT-3, and results were compared by ANOVA with Tukey post-test. * $p<0.01$.
NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HS hypothermic sham, HIIR; hypothermic intestinal ischaemia-reperfusion.

4.4 Discussion

Excitingly these experiments have revealed a novel finding: IIR results in the hepatic activation of two important signalling molecules which are known to play roles in both apoptosis and inflammation. In addition induction of moderate hypothermia completely abrogates this effect. These results could explain, in part, the mechanism of the deleterious hepatic influence of IIR and the protective effect of hypothermia against liver dysfunction described in previous studies (Vejchapipat et al. 2001).

This study has described experiments designed to elucidate the mechanism of hepatic protection, Chapter 3 demonstrated that levels of several important HSPs were found to have remained unchanged in the liver at 60 minutes of intestinal reperfusion time. However, this chapter has demonstrated that IIR increases the phosphorylation and activation of transcription factors STAT-1 and STAT-3 in the liver at this same time point, and that induction of moderate hypothermia during IIR prevents such activation.

Early events in the inflammatory response are mediated by factors that induce changes in transcription. STATs are key transcription factors in this cascade, coupling acute mediators (e.g. STAT-1 activation is linked with interferons [IFN-α and IFN-γ], EGF and IL-6, STAT-3 activation with the IL-6, IL-10, Leptin and G-CSF family) to the complex cellular regulatory response to these stimuli (Schindler and Plumlee 2008, Takagi et al. 2002). In IIR such acute mediators may be released early in the reperfusion phase and STATs act as an important element in the subsequent cascade of inflammatory events. STAT-1 activation has been previously associated with a proinflammatory response but STAT-1 target genes have also been associated with promoting suppression of

cellular proliferation, this contrasts directly with the anti-inflammatory and pro-proliferative activities linked to STAT3 (Schindler and Plumlee 2008). The ability of several cytokines of the same families (e.g. IL-6) to activate both STAT-1 and STAT-3 with their seemingly contradictory activities contributes to the belief that such a dual activation leads to a more balanced and complex response.

STATs are also known to play a role in modulating apoptosis (Figure 4.04). STAT-1 is part of the pro-apoptotic signalling cascade that has been shown to be pivotal in triggering the apoptotic response in several experimental models. Studies in the heart have demonstrated that activation of STAT-1 is a critical step in the propagation of apoptotic cell death in ischaemia-reperfusion both by activation of pro-apoptotic pathways and by switching off anti-apoptotic genes *Bcl-2 and Bcl-x* (Stephanou et al. 2001). STAT-3 activation can have either a positive or negative effect on cell survival depending on the stimulus and cell type (Battle and Frank 2002). Interaction of these STAT proteins within the complex apoptosis/inflammation signalling cascade appears to be an important determinant of cell fate.

The specificity and strength of this transcriptional response is unknown. It is likely to depend on a number of factors, including oligomerisation with other STATs, potentiation of transcription of downstream targets by additional serine phosphorylation, and association with other transcription factors both before and during DNA binding (Horvath and Darnell 1997). For example, the *c-Fos* promoter contains neighbouring binding sites for multiple transcription factors including STAT-3. Experiments in transgenic mice have revealed that this promoter requires the simultaneous activation of at least four of these sites, interaction of such a cluster of proteins has been subsequently described as an

‘enhancesome’ (Thanos and Maniatis 1995). Another characteristic feature of JAK-STAT signalling is its rapid onset and decay of activation, again this is not determined by these experiments in our model but is likely to involve further classes of regulators beyond tyrosine phosphorylation e.g. nuclear import/export machinery and members of the SOCS (Suppressors of Cytokine Signaling) family (Schindler and Plumlee 2008).

These results demonstrate that normothermic IIR promotes hepatic activation of both STAT-1 and STAT-3. The downstream effect of this activation is unknown and may be postulated to be pro/anti-inflammatory and/or pro/anti-apoptotic. Thus activation of STAT proteins may be involved in an intricate mechanism of antagonistic responses to IIR within the liver, this may ultimately contribute to explaining the hepatic dysfunction observed during IIR (Vejchapipat et al. 2001). Excitingly, moderate hypothermia during IIR abolishes hepatic STAT-1 and STAT-3 activation, suggesting that the protective role of hypothermia in the liver could be exerted through mediation of these extremely complex signalling cascades.

Subsequent experiments described in Chapters 5, 6 and 7 are designed to investigate the downstream effects of modulation of such signalling molecules including the effects on markers of apoptosis and gene transcription.

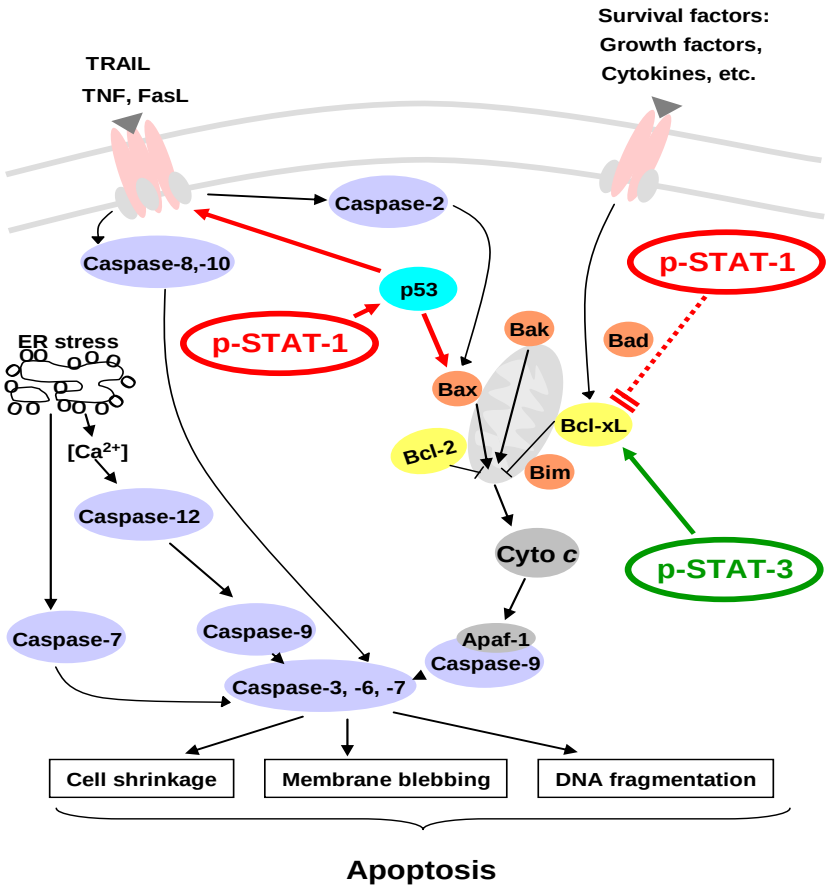

Figure 4.04: Simplified overview of the apoptotic cascade.

Sites of action of phosphorylated transcription factors STAT-1 (p-STAT-1) and STAT-3 (p-STAT-3) are indicated, arrows (activates), blunt end (inhibits). Mitochondrial cyclins (pro-apoptotic) labelled orange, mitochondrial pro-survival factors are yellow. Common pathway leads to the effector phase of apoptosis.

Chapter 5

The influence of IIR on hepatic cell death

Chapter 5 – The influence of IIR on hepatic cell death

Contents

5.1 Introduction

It has been previously demonstrated that hepatic transcription factors, STATs, are significantly influenced by IIR and temperature. STATs are known to be involved in the signalling of both inflammatory and apoptotic cascades. This chapter sets out to explore the influence of STAT initiators and STAT responsive elements of the apoptotic cascade in the liver.

Classification of modes of mammalian cell death usually refers to two main subtypes, apoptosis and necrosis. Apoptosis, previously defined as 'programmed cell death', is characterised by a specific set of morphological changes: cell (and nucleus) shrinkage, DNA fragmentation and rapid phagocytosis by neighbouring cells. Apoptosis is thought to be distinct from another form of cell death, necrosis, which results in a local inflammatory response and swelling of cells and organelles. Autophagy has been proposed as a third mode of cell death, autophagy describes a process whereby cells digest their own macromolecules and generate energy to aid survival when starved of nutrients. Autophagy-associated cell death occurs when all available substrate has been consumed preventing the survival of the cell (Hotchkiss et al. 2009).

The term 'programmed cell death' refers to genetic control, apoptosis is tightly controlled and associated with normal tissue processes of growth and repair and is integral to development and differentiation of cells (Sookhai et al. 2002). Autophagy is genetically 'programmed' and there is evidence that necrosis may also be modulated by control mechanisms (Hotchkiss et al. 2009).

There are three distinct steps of apoptosis (Figure 5.01) described as the: *(1) initiation phase*, pro-apoptotic stimuli (i.e. death receptor 'extrinsic' pathway

stimulation [e.g. Fas is a death receptor triggered by Fas-ligand, Tumour necrosis factor receptor (TNFR) a death receptor triggered by TNF-α] ; *(2) effector phase*, executioner mechanisms (e.g. caspase 3 (a member of the family of cysteine-dependent aspartate specific proteases) and *(3)* the *degenerative phase* where the characteristic morphological features of apoptosis become apparent (e.g. DNA fragmentation and plasma membrane blebbing).

In addition, apoptosis may be triggered in death receptor-independent mechanisms which directly influence mitochondrial permeability and promotes the release of pro-apoptotic molecules such as cytochrome *c* from the mitochondrial intermembrane space. Examples of death-receptor independent initiators include increased intracellular reactive oxygen species, DNA damage, the unfolded protein response and the deprivation of growth factors. This 'intrinsic' mitochondrial pathway of apoptosis is regulated by the Bcl-2 family of proteins (the pro-apoptotic e.g. Bax and Bak and anti-apoptotic proteins e.g. Bcl-2 and Bcl-xL, which inhibit cytochrome *c* release) and caspase 9. There is also significant interaction between the two (extrinsic and intrinsic) pathways as pro-apoptotic proteins e.g. Bax and Bak translocate to the mitochondria from the cytosol to promote the release of cytochrome *c* in response to death receptor signaling (e.g. Fas/Fas-Ligand) (Sookhai et al. 2002), (Figure 5.01).

Activated Caspase 8 (extrinsic/death-receptor pathway) and caspase 9 (intrinsic/mitochondrial pathway) then activate 'executioner' caspases 3, 6 and 7 leading to the degenerative phase of apoptosis.

When apoptosis becomes deregulated it can become a significant cause of disease including cancer and autoimmune disease. There is growing evidence that neuronal apoptosis occurs both in neonatal hypoxic brain injury and in stroke

patients and apoptosis occurs in addition to necrosis in the heart following myocardial infarction. Recent evidence has also suggested that significant insults such as Gram-negative sepsis results in widespread cellular deregulation (induction or inhibition) of apoptosis contributing to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Sookhai et al. 2002).

Review of published evidence has lead to a hypothesis of a significant role of apoptosis in the pathogenesis of hepatic dysfunction following IIR. For example, in an animal model of IIR, Horie *et al* demonstrated features of hepatic apoptosis histologically using TUNEL staining (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling), features of necrotic cell death were not demonstrated suggesting that hepatic dysfunction (as measured by plasma alanine aminotransferase activity) is secondary to increased hepatic apoptosis rather than necrosis in the liver (Horie et al. 2003). Similarly normothermic IIR has been demonstrated to result in a significantly increased level of plasma TNF-α which is then attenuated during hypothermic injury (Williams et al. 2003). TNF-α, an important pro-inflammatory cytokine which also acts as a major pro-apoptotic mediator capable of inducing apoptosis in a variety of tissues including hepatocytes (Leist et al. 1995). In addition, as previously described in Chapter 4, normothermic IIR results in a significant increase in activation of hepatic STAT-1 and STAT-3, important transcription factors both known to influence apoptotic signalling (Battle and Frank 2002, Stephanou et al. 2001).

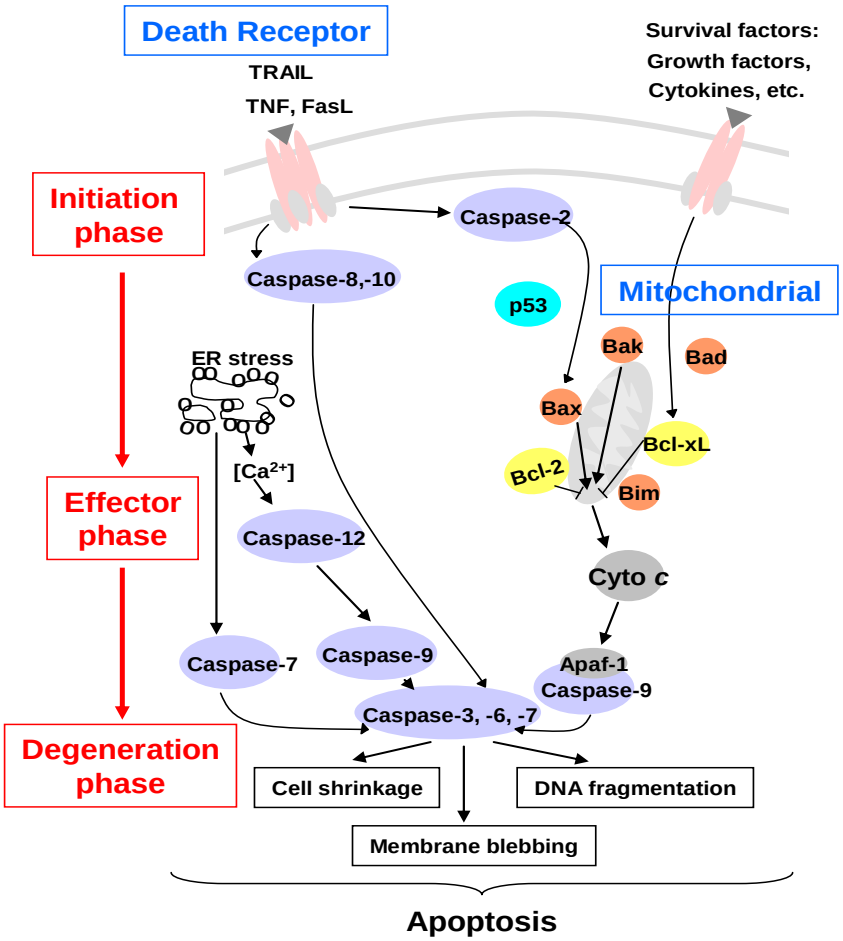

Figure 5.01: Overview of the apoptotic cascade.

Overview of the apoptotic cascade highlighting the initiation phase (e.g. death receptor pathway), the effector phase (executioner mechanisms e.g. caspase 3), and the degenerative phase (morphological features of apoptosis e.g. DNA fragmentation, cell shrinkage and membrane blebbing).

The aim of this study was to identify possible down-stream effectors (and up-stream initiators) of STAT-1 and STAT-3 activation in hepatic tissue exposed to IIR. Hepatic STAT-1 and STAT-3 activation has been previously demonstrated to occur under the same experimental conditions which resulted in hepatic energy failure, namely normothermic IIR. Conversely, markedly attenuated hepatic energy failure occurred during induced moderate hypothermia which correlates significantly with reduced STAT-1 and STAT-3 activation seen under the same experimental conditions. In order to further elucidate the pathogenesis of IIR the effects of such important signalling modulators are explored, this chapter describes several methods employed to attempt to characterise the influence of the apoptotic cascade in the liver following normothermic and moderately hypothermic IIR: 1) levels of up-stream (initiators) and down-stream (effectors) of STATs within the apoptotic cascade are investigated; 2) morphological changes of apoptosis were also investigated including the presence of DNA fragmentation and the gross histological appearance of liver sections which were exposed to haematoxylin and eosin (H&E) and immunohistochemical staining.

5.2 Materials and Methods – assessment of hepatic cell death

5.2.1 Western blotting

Experimental groups were as per the table overleaf. In all cases the same protocol for IIR and sham operation as described in Chapter 2 was performed, however all of these samples were taken from animals maintained at normothermia. In addition in the IIR group the ischaemic period was fixed at 60 minutes however the range of reperfusion times was extended for these experiments (from 30 minutes to 180 minutes, n=2 in each group). These conditions were applied to attempt to examine samples beyond a single time-point, across a spectrum from sham, mild to the most severe injury.

Table 5.01: Experimental groups for Section 5.2.2 and 5.2.3.

Group	Ischaemia (minutes)	Reperfusion (minutes)	Sham operation (minutes)	Total time (minutes)
NS 120			120	120
NIIR 120	60	60		120
HS 120			120	120
HIIR 120	60	60		120

Western blotting was performed using the standard protocols outlined in Chapter 2 (pg 48-52). The antibodies used in these experiments are described in the table overleaf.

Table 5.02: Primary antibodies for Western blotting

Antibody	Species	Clone	Source
Anti-Fas	rabbit	polyclonal (sc-1023)	Santa Cruz Biotechnology
Anti-Fas ligand	rabbit	polyclonal (sc-956)	Santa Cruz Biotechnology
Anti-Bcl-xL	mouse	monoclonal (H-5)	Santa Cruz Biotechnology
Anti-phosphorylated p38-MAPK	mouse	monoclonal (D-8)	Santa Cruz Biotechnology
Anti-total p38 MAPK	rabbit	polyclonal (sc-7149)	Santa Cruz Biotechnology
Anti-caspase 3	rabbit	polyclonal (sc-7148)	Santa Cruz Biotechnology
Anti-caspase 7	rabbit	polyclonal (sc-33773)	Santa Cruz Biotechnology
Anti-caspase 8	rabbit	polyclonal (sc-7890)	Santa Cruz Biotechnology
Anti-caspase 9	rabbit	polyclonal (sc-8355)	Santa Cruz Biotechnology
Anti-phosphorylated JAK-2	rabbit	polyclonal (sc-16566-R)	Cell Signalling Technology
Anti-total JAK-2	rabbit	polyclonal (sc-278)	Cell Signalling Technology
Anti-PARP	mouse	monoclonal (C-2-10)	Abcam Limited

5.2.2 DNA Fragmentation

200µg of frozen liver was homogenised in 600 µl of TE (10mM Tris-HCl pH 7.4, 1mM EDTA,) with 5 strokes of a glass-on-glass homogeniser then centrifuged at 47700 *g* for 30 minutes. Genomic DNA was precipitated from the supernatant by addition of 0.5M sodium chloride and cold absolute ethanol at -80°C for 20 minutes and extracted by phenol-chloroform-isoamyl alcohol (25:24:1) and then precipitated in the presence of 0.3M sodium acetate and cold absolute ethanol at -80°C for 30 minutes and then resuspended in TE.

Apoptosis results in endonuclease cleavage of double stranded DNA at the internucleosomal linker region which generates mono- and oligonucleosomes of multiples of 180 base pairs. A semiquantitative determination of DNA fragmentation may be demonstrated by a so called 'DNA-ladder' i.e. the pattern of multiples of 180 base pairs following electrophoresis on a 1% agarose gel stained with ethidium bromide.

5.2.3 ELISA Cell Death Detection

Using the same centrifugation sample as that prepared for Section 5.2.2 above, the supernatant was then diluted 200 fold and used directly to determine internucleosomal degradation using a commercially available ELISA Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany). This is a quantitative sandwich enzyme immunoassay using mouse monoclonal antibodies against DNA and histones (small positively charged proteins associated with DNA that maintain and regulate chromosome structure), designed to quantify cytosolic oligonucleosome-bound DNA and therefore apoptosis.

5.2.4 Histology

A total of 10 samples were analysed by a Consultant Histopathologist (Prof. Neil Sebire, Histopathology Department, Great Ormond Street Hospital, London, UK) who was blinded to the experimental groups. Sham operation was as described in Chapter 2; however samples were collected from an additional 'sacrifice' group (S) for this experiment which enabled analysis of tissue harvested immediately following induction of adequate anaesthesia. This additional group was examined in an attempt to eliminate the influence of prolonged anaesthesia as a potential insult in its own right.

Table 5.03: Experimental groups for histological analysis.

Group	Ischaemia (minutes)	Reperfusion (minutes)	Sham operation (minutes)	Total time (minutes)	N
S			sacrifice		1
NS 120			120	120	2
NIIR 120	60	120		120	2
NS 180			180	180	1
NIIR 180	60	120		180	1
HS 120			120	120	1
HIIR 120	60	120		180	2

Samples were immediately fixed in formaldehyde solution following harvesting. Subsequent processing of samples was kindly performed by technical staff of the histopathology department at Great Ormond Street Hospital.

Formaldehyde fixed liver samples were initially sectioned in paraffin wax. Specimens were then treated in xylene and graded alcohols, then stained with Mayer's haematoxylin and counterstained in 1% eosin and rinsed in water. Specimens were then dehydrated through ascending grades of alcohol and mounted.

5.2.5 Immunohistochemistry

Immunohistochemistry specimens were dewaxed as previously described. Endogenous peroxidase was blocked with 10% hydrogen peroxide in phosphate buffered saline. The slides were then rinsed and incubated in the appropriate dilution of primary antibody for 1 hour: TUNEL (Apoptag Plus Peroxidase In Situ Kit, Millipore, Watford, UK); mouse monoclonal anti-annexin (Santa Cruz, Insight Biotechnology, Middlesex, UK.); mouse monoclonal anti-Caspase 3 (Novocastra, Milton Keynes, UK); rabbit polyclonal anti-myeloperoxidase and mouse monoclonal anti-leukocyte common antigen (Dako, Denmark).

5.3 Results

Both methods of assessing DNA fragmentation and the ELISA Cell Death Detection kit failed to produce reliable results. The quality of genomic DNA extracted for assessment of DNA fragmentation was poor; despite repeated attempts the DNA extracted remained degraded. Figure 5.02 illustrates a representative gel taken from this experiment demonstrating degraded DNA and an example of a positive result that may have been expected given the initial hypothesis.

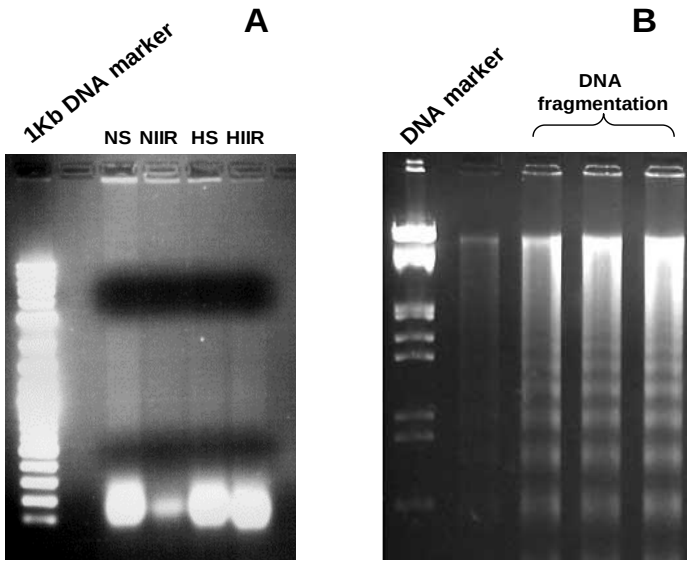

Figure 5.02: DNA fragmentation.

A) A representative example of degraded genomic DNA samples. NS, normothermic sham (120min); NIIR, normothermic intestinal ischaemia-reperfusion (60min ischaemia, 60min reperfusion); HS, hypothermic sham (120min); HIIR, hypothermic intestinal ischaemia-reperfusion (60min ischaemia, 60min reperfusion); B) An example of a positive result demonstrating DNA fragmentation (Zhang et al. 2003).

The ELISA Cell Death Detection kit (which used the same poor quality genomic DNA) also failed to produce reliable results (Figure 5.03). Despite using volumes of homogenate within the range recommended by the manufacturer, all samples appeared to be saturated, i.e. there was no volume dependency.

These results, despite being assumed to saturate the assay samples, gave very different absorbance values, suggesting that something was present in the samples which either interfered with binding in the ELISA, or interfered with the absorbance readings. Even in the unsaturated range (i.e. <5µl), 3 out of 5 samples, including one normothermic control, in which apoptosis was assumed to be minimal, yielded absorbance values in excess of the positive control. It was therefore decided to abandon the ELISA Cell Death Detection method for detection of apoptosis and use well-validated histological and immunohistochemical methods to unambiguously document the presence or absence of apoptosis in these samples.

Western blotting was performed using the protein samples extracted in Section 5.2.1 (Table 5.01) and the antibodies (listed above in Table 5.02) of up-stream initiators and down-stream mediators of STAT-responsive elements of the apoptotic cascade (Figure 5.04). After an extensive search it was concluded that no significant differences could be demonstrated between any of the experimental groups and that there was no evidence that apoptosis (e.g. cleavage of caspase 3 and PARP) was occurring in any of the samples tested (Figure 5.06).

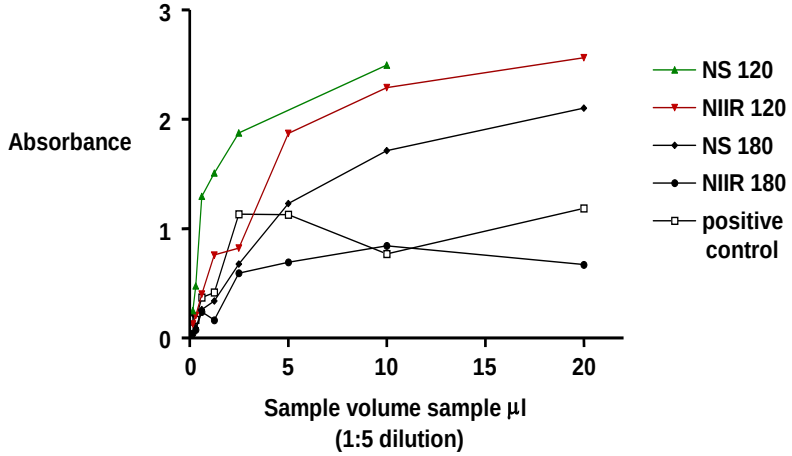

Figure 5.03: Representative results of Cell Death detection ELISA.

The experimental groups are labelled NS 120 (normothermic sham, 120 min), NIIR 120 (normothermic IIR, 60 min ischaemia, 60min reperfusion), NS 180 (normothermic sham, 180mins), NIIR 180 (normothermic IIR, 60min ischaemia, 120min reperfusion), and positive control. Technical problems with the ELISA failed to produce reliable results. It is of note, that even in the unsaturated range (e.g. less than 5µl) 3 out of the 5 experimental readings were higher than the positive control.

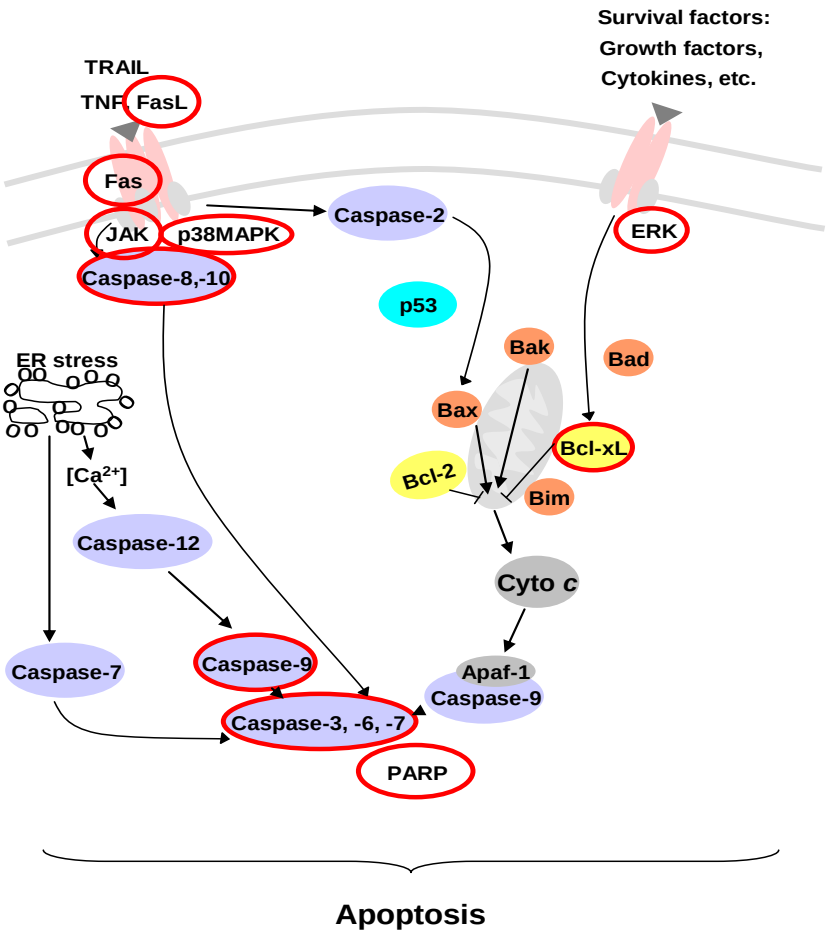

Figure 5.04: Apoptotic mediators investigated by Western blotting.

Mediators investigated by Western Blotting have been highlighted in red.

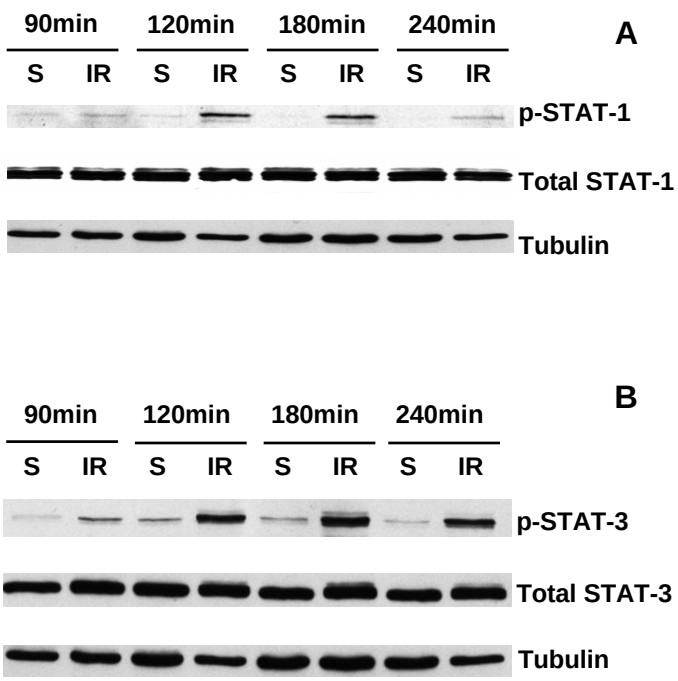

Figure 5.05: Representative Western Blots of [A] phosphorylated STAT-1, total STAT-1 and α-tubulin, [B] phosphorylated STAT-3, total STAT-3 and α-tubulin (loading control).

S; normothermic sham, IR; normothermic intestinal ischaemia-reperfusion. Reperfusion times vary 90min (60min ischaemia, 30min reperfusion), 120min (60min ischaemia, 60min reperfusion), 180min (60min ischaemia, 120min reperfusion), 240min (60min ischaemia, 180min reperfusion).

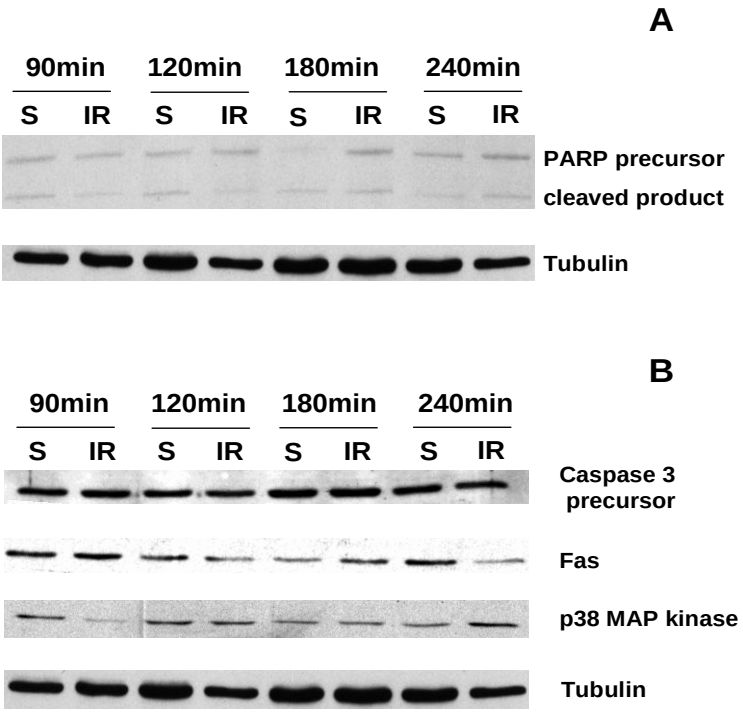

Figure 5.06: Representative Western Blot of [A] PARP precursor, cleaved PARP product and α-tubulin, [B] Caspase 3 precursor, Fas, p38 Map kinase (total) and α-tubulin (loading control).

S; normothermic sham, IR; normothermic intestinal ischaemia-reperfusion. Reperfusion times vary 90min (60min ischaemia, 30min reperfusion), 120min (60min ischaemia, 60min reperfusion), 180min (60min ischaemia, 120min reperfusion), 240min (60min ischaemia, 180min reperfusion).

Histopathological analysis was performed on formaldehyde fixed tissue. Conventional light microscopy of specimens stained with haematoxylin and eosin (H&E) is one of the least sensitive methods to detect apoptosis, but it is highly specific if the characteristic morphological changes are observed. A Consultant Histopathologist (Prof. Neil Sebire, Great Ormond Street Hospital, London) reviewed blinded H&E stained sections from each of the experimental groups; this revealed no morphological differences between any of the specimens. There were specifically no features characteristic of necrosis or apoptosis; in addition there were no demonstrated inflammatory changes such as infiltration of leukocytes around the portal triad (Figures 5.07, 5.08).

The histological sections examined essentially appeared identical, whether they were from a sacrifice sample, sham operation or IIR. Immunohistochemistry confirmed these findings. TUNEL (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labelling technique) (Figure 5.09), annexin and caspase 3 stains revealed only low level apoptosis occurring equally in all groups, interestingly including the sacrifice sample. In addition myeloperoxidase and leukocyte common antigen staining revealed no significant differences in inflammatory cell infiltrate (Figure 5.10).

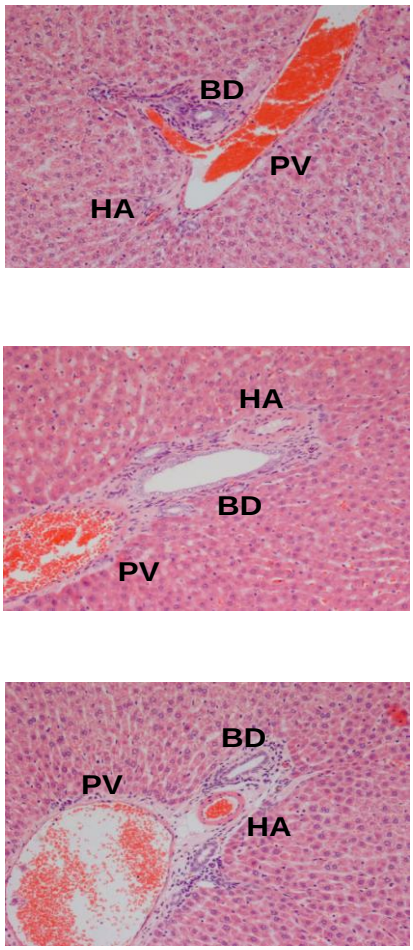

Figure 5.07: Representative hepatic histological sections demonstrating A) sacrifice, B) normothermic sham (180 min) and C) normothermic intestinal ischaemia-reperfusion (60min ischaemia, 120min reperfusion) animals.

Each histological section demonstrates a low power original magnification x40 image of the portal triad region of the liver lobule. Cross-sections of the constituentss of the portal triad are indicated: HA (hepatic artery), PV (portal vein) and BD (bile duct). Of note there are no characteristic morphological features suggestive of necrosis or apoptosis, there are no demonstrated inflammatory changes such as infiltration of leucocytes around the portal triad.

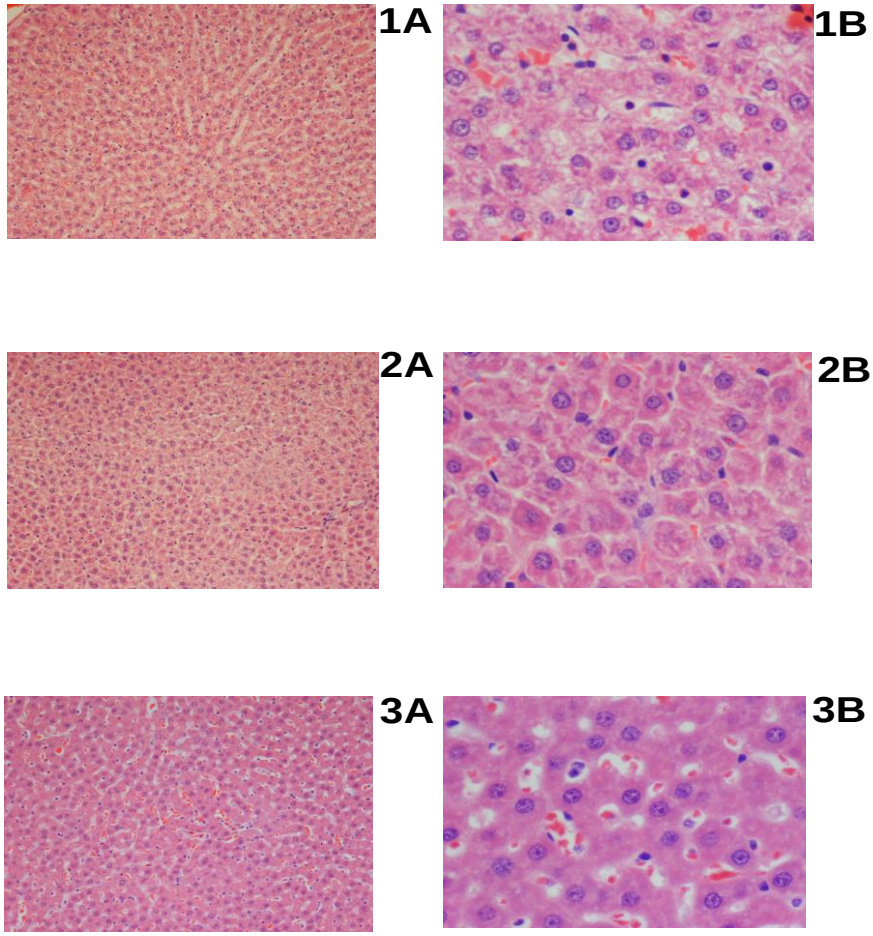

Figure 5.08: Representative hepatic histological sections (haematoxylin and eosin) demonstrating 1) sacrifice, 2) normothermic sham (180 min) and 3) normothermic intestinal ischaemia-reperfusion (60min ischaemia, 120min reperfusion) animals.

Each section demonstrates A) low power original magnification x40 and B) high power original magnification x200 images. Of note, there are no characteristic morphological features suggestive of necrosis or apoptosis and there are no demonstrated inflammatory changes such as infiltration of leucocytes around the portal triad.

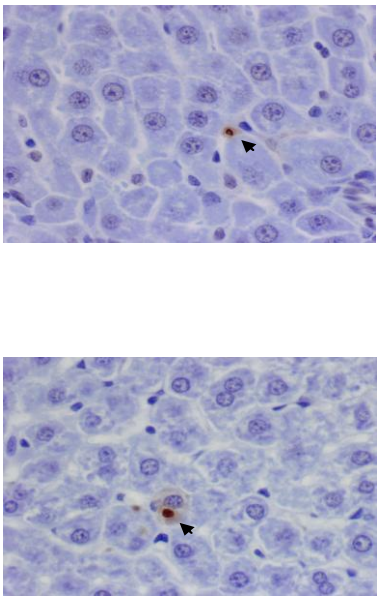

Figure 5.09: Representative hepatic histological sections demonstrating terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end (TUNEL) labelling technique.

Liver samples taken from A) normothermic intestinal ischaemia-reperfusion (60min ischaemia, 120min reperfusion), B) hypothermic intestinal ischaemia-reperfusion (60min ischaemia, 120min reperfusion). Very low levels of apoptotic hepatocytes were demonstrated equally by this technique in each of the experimental groups including the above examples and in sham and sacrifice samples (not shown).

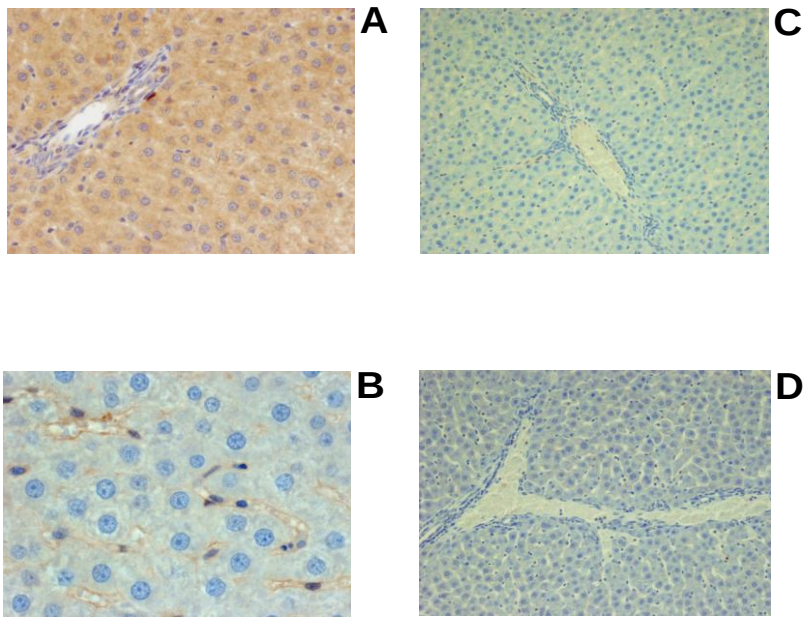

Figure 5.10: Representative hepatic histological sections demonstrating results of immunohistological staining techniques.

Methods of A) annexin and B) caspase 3 staining revealed very low levels of apoptosis equally in each of the experimental groups. No significant differences in inflammatory cell infiltrate were demonstrated by C) leucocyte common antigen (LCA) and D) myeloperoxidase (MPO) in each of the experimental groups.

5.4 Discussion

The histopathological findings above confirm that there is neither hepatic necrosis nor apoptosis occurring in any of the experimental groups including, sacrifice, sham operation and normothermic IIR. This establishes that even at extended time-points neither of these modes of cell death is responsible for the hepatic dysfunction demonstrated with this model. These results also confirm that apoptosis is not the primary effect of STAT-1 and STAT-3 activation in the liver at this time. Levels of many of the usual activator molecules of STATs (e.g. JAK-2, p38-MAPK) and the downstream effectors of the apoptotic cascade (e.g. caspase 3 and PARP cleavage) have been measured as part of this study and no differences have been demonstrated between the experimental groups.

Interestingly, the fact that no significant inflammatory cell infiltrate is noted is in marked contrast with findings of another group with a similar model who reported significant changes in leukocyte accumulation in the sinusoids of the midzonal and pericentral regions of the liver lobule (Horie et al. 2003). It does, however confirm previous biochemical findings with this model of a lack of significant neutrophil infiltration as measured by myeloperoxidase activity in liver tissue (Vinardi S. personal communication) as compared to lung and gut tissue where normothermic IIR causes a significant increase in myeloperoxidase activity indicating neutrophil infiltration (Stefanutti et al. 2008, Vinardi et al. 2003).

Despite the significant changes in hepatic STAT protein phosphorylation and synthesis that have been described in Chapter 4 normal histological findings in the same experimental groups suggest that IIR does not result in direct liver injury. However previous evidence that IIR results in reduced hepatic ATP levels does suggest that liver bioenergetic failure occurs as a consequence of IIR

(Vejchapipat et al. 2001). Apparently contradictory evidence of markedly deranged organ function coupled with normal histological appearance is known to occur in other disease states, for example the majority of organ dysfunction in sepsis occurs despite normal histology and a paradoxical rise in oxygen tension suggests that oxygen is freely available but is being underutilised. This evidence has provided a new perspective in explaining MODS and a new direction for potential therapeutic modalities (Hotchkiss et al. 1999).

STAT activation also influences modulation of the intra-hepatic acute phase response and may contribute via this mode to the coupling of 'gut injury' to multiple organ dysfunction. STATs may act principally as transcription factors and therefore subsequent investigations were designed to examine the influence of each of the experimental groups on as many gene products as possible by employing gene microarray technology.

Chapter 6

Hepatic Gene Expression Profiling

Chapter 6 - Hepatic Gene Expression Profiling

Contents

6.1 Introduction

Previous experiments revealed significant activation of two important hepatic transcription factors (STAT-1 and STAT-3) following normothermic intestinal ischaemia-reperfusion (IIR). In turn STAT activation was highly attenuated following moderate hypothermic IIR. Subsequent experiments however failed to provide evidence of 'upstream' or 'downstream' activation of typical STAT responsive genes (e.g. Fas, p38-MAPK, JAK-2, Bcl-xL) (see Chapter 5). In addition there were no clearly demonstrated histopathological sequelae indicative of hepatic apoptosis or inflammation within the experimental groups.

To broaden our analysis further, this chapter describes experiments applying gene microarray technology to whole liver tissue extract following IIR at normothermia, moderate hypothermia and sham operation. This is, as far as I am aware, the first reported study using this methodology to attempt to define previously unrecognised molecules and signalling pathways within the context of hepatic function following IIR injury. Analysis of the subsequent microarray data was applied to identify specific genes of interest and to investigate the levels of corresponding protein products.

Gene microarray technology has developed a huge amount of interest in recent years; as opposed to a traditional approach of investigating one gene at a time this methodology enables researchers to apply a genome wide search. Gene microarrays have successfully been used in clinical studies to classify cancers e.g. breast carcinoma (Weigelt and Reis-Filho 2009), identify single nucleotide polymorphisms (Gomase et al. 2009) and genotype viruses (Miller and Tang 2009). A large number of gene microarray studies have been performed to

investigate malignancy whereas relatively few have been conducted into pathological conditions characterized by inflammation and sepsis. In view of the negative results in recent years of many large, prospective, randomized clinical trials conducted into sepsis a demand, has developed, for a fresh, new approach to investigate this complex pathology. In the past more traditional approaches have focused predominantly on the 'magic bullet theory' of single gene/anti-mediator treatment strategies for sepsis-induced MODS. The era of gene microarray technology has facilitated broad analysis tools which are capable of profiling tens of thousands of genes in a single experiment, in this way the sepsis 'transcriptome' may be characterised and exploited to develop new therapeutic strategies (Cobb et al. 2002).

Several studies have attempted to identify gene expression profiles in animal models of sepsis (Chinnaiyan et al. 2001, Cobb et al. 2002, Fang et al. 2004). Most recently microarray technology has been applied to large clinical studies of paediatric septic shock, the genomic signature of this complex pathology has been identified and is characterised by large-scale repression of genes related to zinc homeostasis and lymphocyte function (Cvijanovich et al. 2008). Interpreting the massive amount of microarray data generated by such a study is fraught with potential error, as there are as yet no standard statistical approaches to analysing such data. The various statistical and filtering methods available can lead to markedly different results from a single experiment. Validation of microarray data may be desirable if particular genes are to be studied in detail, this may be achieved by real-time polymerase chain reaction for confirmation of differentially expressed gene probes. However to confirm that these gene changes are translated in the 'effector' protein molecules further

experiments are required including Western blotting and ELISA, more recently proteomic technology has also be used directly to further validate such data (Cvijanovich et al. 2008).

Gene lists may be further analysed by applying pathway-based analytical tools such as the Ingenuity Pathways analysis application (Ingenuity Systems, Redwood City, USA), this tool facilitates the discovery of signalling pathways and gene networks. This method has been applied to large human studies such as that recently conducted in paediatric septic shock, the gene networks found to be most significant are those involved with innate immunity and inflammation, a finding which is consistent with the current paradigm of sepsis research (Shanley et al. 2007). The most interesting, and novel finding of the same analysis was the down-regulation of gene networks involved with T-cell immunity and the MHC antigen presentation system, suggesting an element of immune suppression associated with septic shock (Shanley et al. 2007).

By employing such exciting technology the hypothesis for this study is that IIR results in differential gene expression in the liver as compared to sham operation and that this response is in turn modulated by temperature. Such changes in gene expression may be coordinated with our previous findings of STAT activation and the influence of IIR on hepatic bioenergetic failure.

6.1.2 Gene microarray

Gene expression varies with cell type and is highly dynamic; the complement of messenger RNA (mRNA) changes over time in response to signals and stresses. Because the mRNA complement, or ‘transcriptome’, encodes proteins that in turn determine the phenotype of the cell, transcriptional analysis

provides an opportunity to investigate the molecular changes that underlie cell behaviour and disease (Hubank 2004).

The principle of gene array experiments relies on the binding of the test sample (target, e.g. labeled antisense RNA) to a complementary, immobilized, detecting reagent (probe, synthetic oligonucleotide array e.g. Affymetrix Gene Chips (Santa Clara, CA, USA), (Figure 6.01).

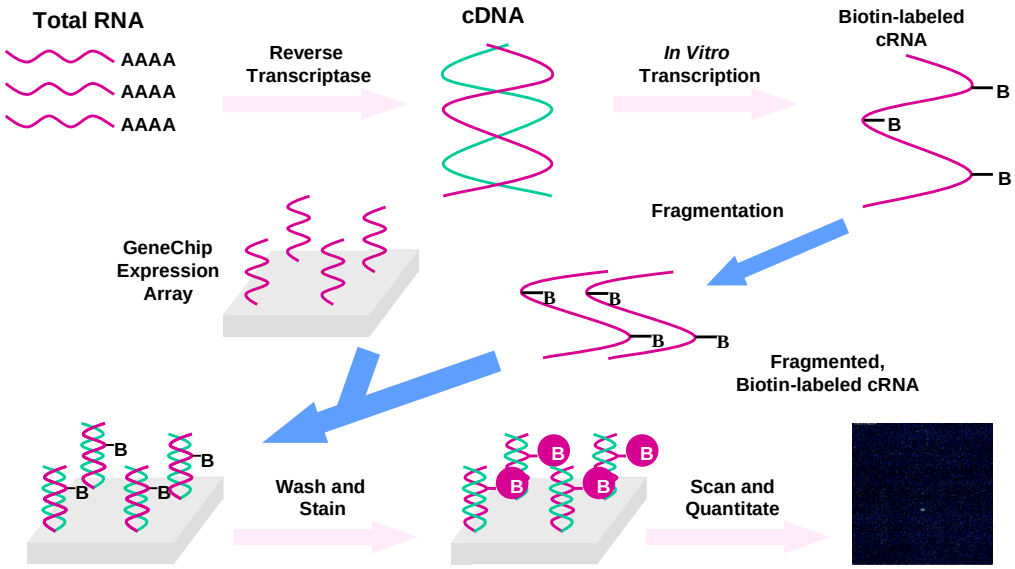

Figure 6.01: Standard eukaryotic gene expression assay.

Labelled cRNA targets derived from the mRNA of the experimental sample are hybridised to nucleic acid probes attached to the solid support. The Gene Chip is then laser scanned to generate raw data **(**from Affymetrix Gene Chip Expression Analysis Technical manual).

Affymetrix rat RAE230A oligonucleotide microarrays consist of approximately 15000 transcripts. Each transcript corresponds to a known gene or an as yet uncharacterised transcribed sequence (an expressed sequence tag; EST). Each of these transcripts is represented by 11 oligonucleotide probes (of 25 bases in length), with each of these probes having a partner oligonucleotide that has a single base mismatch to determine the degree of non-specific binding (Shackel et al. 2002).

Biotin-labelled cRNA fragments (target) are hybridised to the probe array. The hybridised probe array is stained with a streptavidin phycoerythrin conjugate and scanned by the Affymetrix Gene Array Laser Scanner at the excitation wavelength of 488nm. The amount of light emitted at 570nm is proportional to the bound target at each location on the probe array. This raw data is then processed to generate a list of relative gene expression.

6.2 Materials and Methods

6.2.1 Surgical procedure

Animals were anaesthetised and underwent either sham operation or IIR, at normothermia or moderate hypothermia, according to the methods in Chapter 2. In this experiment the reperfusion time was extended from 60 to 120 minutes. This time point was based on the previous time-course experiment (see Chapter 5 pg113 and 119) where significant STAT activation occurred at 120 minutes of reperfusion, a time when significant changes in gene expression could be expected to occur.

The animals were divided into three groups:

NS = sham operation for 180 minutes at normothermia (n=3)

NIIR = 60 minutes ischaemia and 120 minutes reperfusion at normothermia (n=3)

HIIR = 60 minutes ischaemia and 120 minutes reperfusion at moderate hypothermia (n=3)

The methods of Affymetrix microarray target preparation and probe hybridisation are detailed in Chapter 2 (pg 55-57).

6.2.2 Data collection and normalization

Expression values were obtained for all nine hybridisations using Affymetrix Microarray Suite (MAS) 5.0 software. Data quality was assessed using MAS 5.0 report files and GeneSpring 6.0 (Silicon Genetics, San Carlos, CA, USA). Arrays were included in the analysis if they met minimum requirements for establishing data quality: 3' to 5' ratio (less than 3), background (less than 100), noise and the total number of positively identified transcripts (percentage call, approximately 50%).

6.2.3 Data filtering and analysis

Analysis was performed with GeneSpring 6.0 software; values were normalised to the median signal value for each array. A list of expressed genes was created which passed a series of accepted exclusion criteria:

1) ***Normalisation***: arrays were normalised for chip wide variability in signal; values were normalised to the median *per chip* and to the median value (of chip medians) *per gene* across the experiment.
2) ***Filtering***: this applied further restrictions to the gene list by excluding those genes whose expression did not reach a threshold value for reliable detection (*filtering by data file restriction* p>0.1*).* Finally, genes whose level of expression did not vary in response to ischaemia-reperfusion or temperature change, by more than 1.5-fold were also excluded (*filtering on expression*). This is a negative filter, i.e. genes whose expression failed to exceed this level are less likely to be different between experimental groups.

The remaining genes were considered informative and log-transformed replicate data sets were subjected to a Welch's approximate t-test (where variance is not assumed to be equal between 2 samples) between 2 conditions to determine the most significant changes.

6.3 Validation of differential expression of candidate genes

Validation of differential expression of candidate genes was performed by semi-quantitative Polymerase Chain Reaction (PCR). In addition the observed change in gene expression was investigated further by quantifying specific protein products using Western blotting and ELISA techniques.

6.3.1 PCR

Polymerase chain reaction (PCR) experiments were performed as per methods described in Section 2.6, Chapter 2. PCR products were resolved on a 1% agarose gel, with ethidium bromide 0.8 µg/ml.

6.3.2 Western Blotting

Protein analysis was performed by Western blotting according to the protocols described previously in Section 2.3, Chapter 2. Protein was extracted from the same liver samples (n=3 per group) used for the gene array analysis and an additional 3 liver samples per group (total n=6 per group).

Western blotting was performed using antibodies commercially available for those genes of interest (IL-1β and α-2 macroglobulin) as compared to a control, α-tubulin. Detection of phosphorylated STAT-1 and STAT-3 was also performed to confirm previous findings and to demonstrate that samples were comparable to those used in previous experiments. Data are expressed as mean ± SEM. Statistical analysis was performed using repeated measures ANOVA, grouping samples run on the same gel, a p value of less than 0.05 was considered significant.

6.3.3 ELISA

Sandwich type enzyme-linked immunosorbent assays (ELISA) were used to measure tissue concentration of specific proteins where appropriate antibodies were not available for Western blotting analysis; methods are described in Section 2.4, Chapter 2.

ELISA experiments were performed on n=6 samples for each of the experimental groups (except EGF and IL-1Ra where n=3 samples). That is the original n=3 samples used for the microarray analysis plus extra n=3 for each experimental group. Each of the liver samples were tested in triplicate and a mean value taken, one-way ANOVA was then performed.

6.4 Results

6.4.1 Affymetrix Gene Microarray

Nine Affymetrix RAE230A oligonucleotide microarrays were used to analyse gene expression in NS, NIIR and HIIR (n=3) per experimental group. After normalising and filtering the data (see Methods above) expression of 1232 transcripts were found to change between the three experimental conditions. By comparing gene expression following normothermic sham and normothermic IIR, Welch's t-test identified 222 transcripts whose expression differed significantly (range 0.0004 - 0.0997; $p< 0.1$). 140 transcripts were ESTs and 82 represent Genbank database identified genes. Of these known genes, 59 had increased expression following IIR and 23 had decreased expression.

Expression following normothermic and hypothermic IIR were also compared, Welch's t-test identified 155 transcripts whose expression differed significantly (range 0.0002 – 0.0999; $p<0.1$), 105 transcripts were ESTs and 50 represent Genbank database identified genes. Of these known genes, 22 had increased expression following hypothermic IIR and 28 had decreased expression.

As there is still controversy regarding the optimum method to analyse such data, I used a consensus strategy to identify groups or 'clusters' of genes that

were similarly expressed. A non-hierarchical method of clustering, K-means with Pearson correlation, was used on the pooled data of 1232 transcripts to demonstrate grouping of samples with similar patterns of expression. Using this method 10 data sets were produced (Figure 6.02).

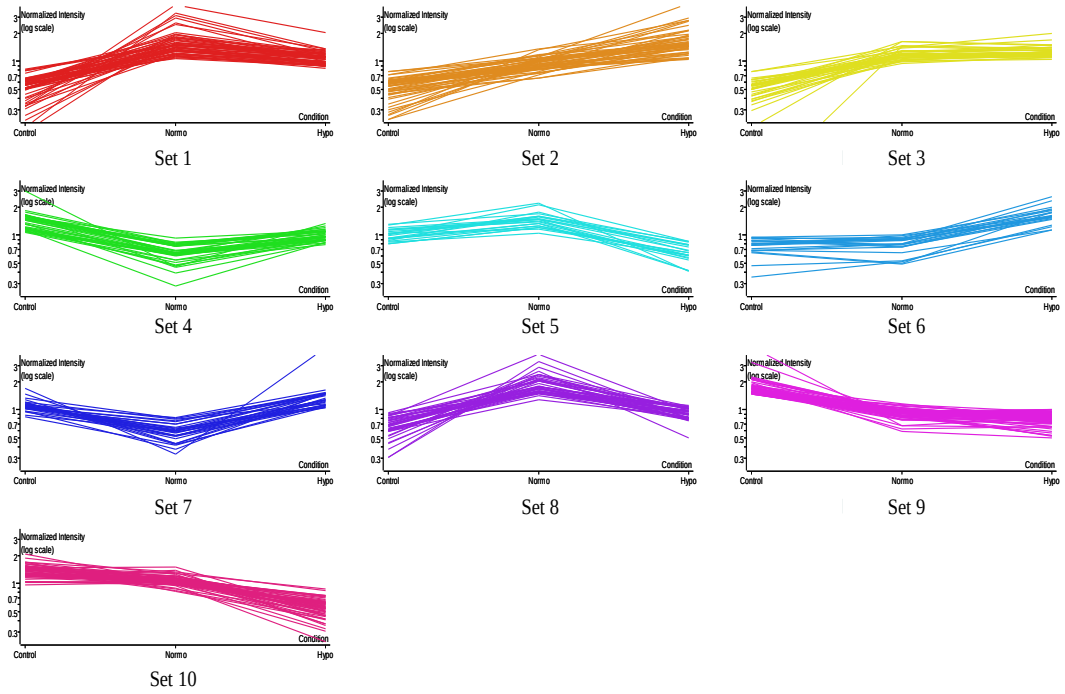

Figure 6.02: A non-hierarchical method of clustering, K-means with Pearson correlation, is used to group samples with similar patterns of expression.

The 10 cluster sets illustrated result from applying K-means clustering with Pearson correlation to the pooled data of 1232 transcripts. The three experimental conditions are labelled, Control; normothermic sham, Normo; normothermic intestinal ischaemia-reperfusion, Hypo; hypothermic intestinal ischaemia-reperfusion and plotted against normalised intensity of signal (continuous log of ratio).

The most interesting patterns of expression between the experimental groups, NS (normothermic sham), NIIR (normothermic intestinal ischaemia-reperfusion) and HIIR (hypothermic intestinal ischaemia-reperfusion) can be grouped as:

1) 'up-down' expression profile, combining sets 5 and 8 (Figures 6.03A, 6.04 / Table 6.01.), this includes those genes whose pattern of expression may be anticipated to increase in normothermic IIR relative to normothermic sham and subsequently decrease following moderately hypothermic IIR.

Combining sets 5 and 8 (Fig 6.04), which both follow a similar expression profile, results in a total of 61 transcripts, 32 ESTs and 29 known genes (listed in Table 6.01). By examining this group in detail specific genes may be discovered by which hypothermic injury affords protection: by either reducing a toxic product or by attenuating signalling in a damage pathway.

Prominent in this group in terms of biological function were acute phase protein (APP) encoding genes, such as α–2-macroglobulin (α-2M) and lipopolysaccharide binding protein (LPS-BP). Genes encoding the proinflammatory cytokine interleukin-1ß (IL-1ß) and the leptin receptor (LEP-R), a member of the cytokine receptor family, were also of interest.

Within this group genes were also identified with a role in the regulation of transcription (e.g. hepatic nuclear factor-3 [HNF-3], nuclear factor kappa B subunit, DNA binding (centaurin–α2), superoxide dismutase 2, and eicosanoid synthesis (arachidonate 12-lipoxygenase).

Figure 6.03: Schematic representation of gene expression profiles.

Experimental groups are labelled S (normothermic sham), N (normothermic IIR), H (hypothermic IIR). A) 'up-down' pattern (combined sets 5 and 8), B) 'down-up' pattern (combined sets 4 and 7).

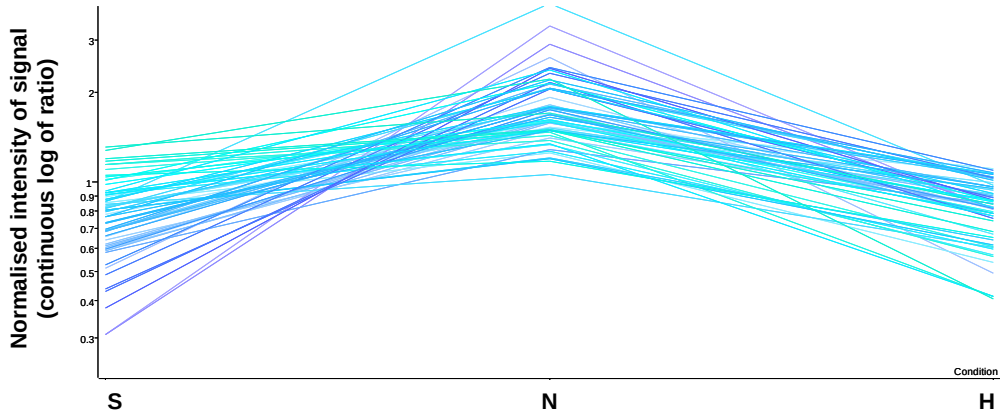

Figure 6.04: K-means cluster sets 5 and 8 are combined, resulting in a group of 61 transcripts with a similar 'up-down' expression pattern.

A non-hierarchical method of clustering, K-means with Pearson correlation, is used to group samples with similar patterns of expression. By combining sets 5 and 8 a group of 61 transcripts with a similar expression profile, 'up-down' pattern, are selected, within this group are 29 known genes. S; normothermic sham, N; normothermic intestinal ischaemia-reperfusion, H; hypothermic intestinal ischaemia-reperfusion.

Table 6.01: Gene ontology of sets 5 and 8 ('up-down' expression pattern).

	Affymetrix Probe set ID	Gene title	Biological Process	Molecular function	Cellular component
Set 5	1367794_at	Alpha-2 macroglobulin	·Acute phase response ·Regulation of Ca-mediated signalling	Endopeptidase inhibitor	Extra cellular
Set 8	1368139_s_at	Alkaline phosphatase	Metabolism	Alkaline phosphatase activity	Extra cellular
	1368645_at	Protein tyrosine phosphatase	Amino acid dephosphorylation	Tyrosine phosphatase activity	Plasma membrane
	1368748_at	Testis-specific kinase 2	Amino acid phosphorylation	Protein kinase activity	·Nucleus ·Cytoplasm
	1369420_at	Neuraminidase 3	Carbohydrate metabolism	·Exo-alpha-sialidase activity ·Hydrolase activity	Plasma membrane
	1368550_at	Hepatocyte nuclear factor-3	·Regulation of transcription ·DNA dependent	Transcription factor	Nucleus
	1369725_at	Centaurin-alpha2 protein	·Regulation of GTPase activity ·Inisitol lipid-mediated signalling	GTPase activator activity	·Plasma membrane ·Cytosol
	1370173_at	Superoxide dismutase 2		Superoxide dismutase activity	Mitochondrion

Set 8 cont	Affymetrix Probe set ID	Gene title	Biological Process	Molecular function	Cellular component
	1370968_at	Nuclear factor of kappa light chain gene enhancer in B cells	·Regulation of transcription ·DNA dependent apoptosis	DNA binding transcription factor activity	·Nucleus ·Cytoplasm
	1386869_at	Actin	Cytoskeleton	Structural molecule	Cytoskeleton
	1387796_at	Arachidonate 12-lipoxygenase	Carbohydrate metabolism	Arachidonate 12-lipoxygenase activity	·Cytosol ·Sarcolemma
	1387868_at	Lipopolysaccharide binding protein	Defence response to bacteria	Lipopolysaccharide binding activity	·Extracellular space ·Plasma membrane
	1398256_at	Interleukin-1 beta	Inflammatory response	Signal transducer activity	·Extracellular space

The second pattern of expression of interest:

2) ‘down-up’ expression pattern, combining sets 4 and 7 (Figures 6.03B, 6.05, Table 6.02). This pattern of expression demonstrates decreased levels of expression resulting from normothermic IIR and an increase towards sham levels in hypothermic IIR. This group may demonstrate genes which are protective when expression is increased or indicate a low level of signalling within injury pathways.

Combining sets 4 and 7, (Figure 6.05), which both follow a pattern of decreased expression in normothermic IIR relative to sham then increased expression in moderately hypothermic IIR, provides a total of 66 transcripts. This total of 66 transcripts represented 45 ESTs and 21 known genes (Table 6.02).

Genes of particular interest in this group are cardiotrophin-1 (CT-1), a cytokine of the IL-6 family, and epidermal growth factor (EGF), a growth factor that binds to a specific gp130 receptor (EGF-R). This group also included several transcription factors and genes encoding transporter proteins.

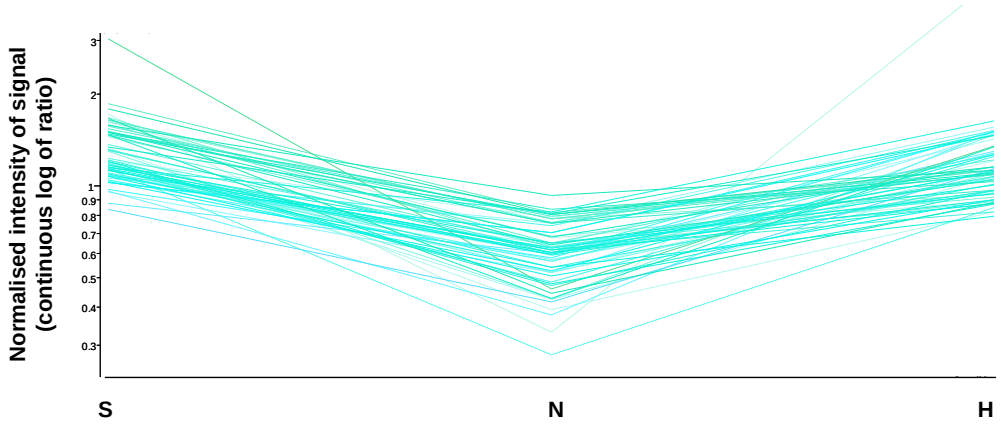

Figure 6.05: K-means cluster sets 4 and 7 are combined, resulting in a group of 66 transcripts with a similar 'down-up' expression pattern.

A non-hierarchical method of clustering, K-means with Pearson correlation, is used to group samples with similar patterns of expression. By combining sets 4 and 7 a group of 66 transcripts with a similar expression profile, 'down-up' pattern, are selected, within this group are 21 known genes. S; normothermic sham, N; normothermic intestinal ischaemia-reperfusion, H; hypothermic intestinal ischaemia-reperfusion.

Table 6.02: Gene ontology of sets 4 and 7 ('down-up' expression pattern).

	Affymetrix Probe set ID	Gene title	Biological Process	Molecular function	Cellular component
Set 4	1367741_at	Homocysteine-inducible ER stress-inducible ubiquitin-like domain member1	·Response to stress ·Response to unfolded protein		Endoplasmic reticulum
	1367911_at	Isocitrate dehygrogenase 3 NAD^+ alpha	TCA cycle metabolism	·Isocitrate dehydrogenase (NAD^+) activity ·Oxidoreductase activity	Mitochondrion
	1367937_at	Aldehyde reductase	Cellular osmoregulation	Aldehyde reductase activity	·Cytoplasm ·Inclusion body
	1367941_at	Mitochondrial transcription factor A	Mitochondrial DNA replication	DNA binding transcription factor activity	Mitochondrion
	1368057_at	ATP-binding cassette, subfamily D, member 3	Peroxisome transport	·Nucleotide binding ·ATP binding ·ATPase activity	·Extracellular ·Mitochondrion ·Peroxisome membrane
	1368325_at	Epidermal growth factor	Protein kinase cascade	Epidermal growth factor receptor binding	·Extracellular ·Plasma membrane
Set 7	1368020_at	Mevalonate (diphospho) decarboxylase	·Cholesterol biosynthesis ·Isoprenoid biosynthesis	Diphosphomevalon-ate decarboxylase activity	·Peroxisomal matrix ·Cytosol
	1370023_at	Gap junction membrane channel protein alpha 4	·Blood vessel development ·Cell to cell signalling	Gap-junction forming channel activity	Plasma membrane
	1387083_at	Cardiotrophin 1	· Stress induced cytokine (IL-6 family)	· Cytokine activity · Hepatocyte survival factor	

6.4.2 Polymerase Chain Reaction

Gene microarray data demonstrating changes in RNA levels was examined further to attempt to confirm transcriptional changes for specific genes of interest. Genes were identified and selected for confirmatory experiments on the basis of their expression pattern (i.e. 'up-down', 'down-up'), their relationship with STAT transcription factors and their potential biological role. Semi-quantitative reverse transcription PCR was performed on single representative samples from each of the experimental groups and compared to a GAPDH control. Microarray findings were confirmed in 5 genes: Interleukin-1ß (IL-1ß), Lipopolysaccharide binding protein (LBP), α-2 macroglobulin (α-2M), leptin receptor (LEP-R) and epidermal growth factor (EGF). Several other selected genes were investigated [e.g. Hepatocyte nuclear factor (HNF-3), Cardiotrophin-1(CT-1)] but PCR results failed to generate a reliable product despite several attempts using differently designed primers and altering cycle variables, these experiments were therefore abandoned.

The PCR transcript was greatly increased for IL-1ß, LBP, α-2M and LEP-R following normothermic IIR, but this increased expression was abolished following hypothermic IIR, confirming their microarray expression pattern as 'up-down' (Figure 6.06). EGF demonstrated a reverse pattern, again confirming the microarray pattern of 'down-up' expression with decreased expression following normothermic IIR and an increase with hypothermic IIR (Figure 6.07).

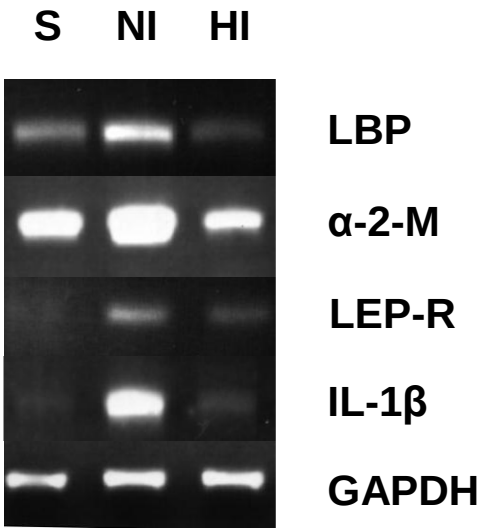

Figure 6.06: PCR products resolved on a 1% agarose gel: Lipopolysaccharide binding protein (LBP); α-2 macroglobulin (α-2-M); leptin receptor (LEP-R); interleukin-1β (IL-1β); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.

The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

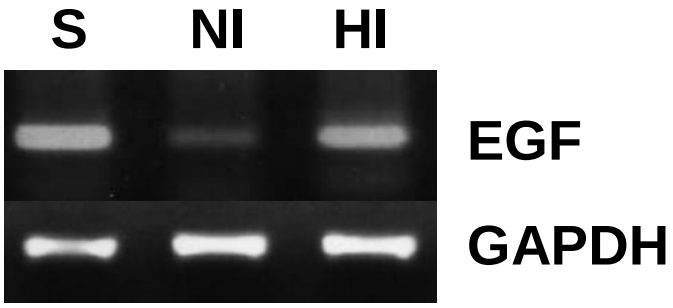

Figure 6.07: PCR products resolved on a 1% agarose gel: epidermal growth factor (EGF); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.

The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

6.4.3 Western blotting

In order to determine whether altered levels of mRNA (demonstrated by both microarray and PCR data) resulted in corresponding changes in levels of hepatic protein both Western blotting and ELISA techniques were performed.

Western blotting was performed on the original 9 liver samples (n=3 per experimental group) used in the initial microarray experiments. Firstly protein levels of phosphorylated STAT-1 (p-STAT-1) and STAT-3 (p-STAT-3) were determined compared to control protein α-tubulin. These experiments were performed in order to confirm changes in phosphorylated STATs seen in previous experiments (Chapter 4). Normothermic IIR caused significant increase in p-STAT-1, reflecting activation of this transcription factor ($p<0.05$; Figure 6.08). In addition, there was a dramatic increase in phosphorylated STAT-3 (p-STAT-3), again reflecting transcriptional activation ($p<0.05$; Figure 6.08). Phosphorylation of STAT-1 and STAT-3 in these experimental groups corresponded to previous findings.

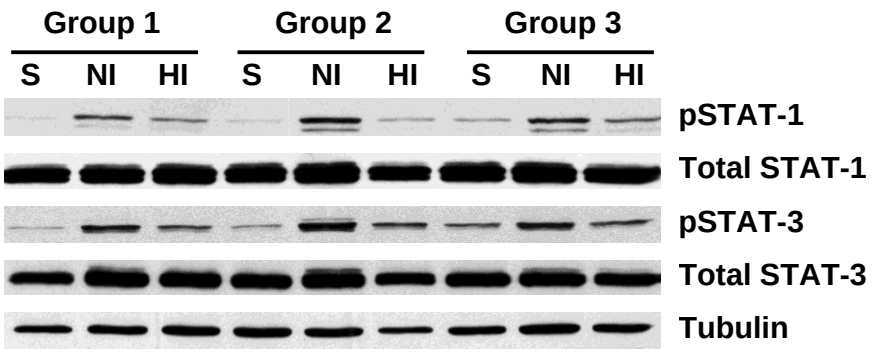

Figure 6.08: Representative Western Blot of [A] phosphorylated STAT-1, total STAT-1, phosphorylated STAT-3, total STAT-3 and α-tubulin, [B] interleukin-1ß and α-tubulin (loading control).

The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

Western blotting was performed on these same samples (n=3) to determine protein expression for α-2 macroglobulin as compared to control protein α-tubulin. There was no significant difference in levels of α-2 macroglobulin across the experimental groups (Figure 6.09A).

Western blotting was also used to determine levels of IL-1ß protein (n=6) in each of the experimental groups, 18 samples in total. Normothermic IIR resulted in a significant increase in IL-1ß protein ($p<0.05$), whereas moderate hypothermia prevented this increase reducing the levels of intra-hepatic IL-1ß to that of sham groups ($p<0.01$) (Figures 6.09B and 6.10).

The action of IL-1ß is usually opposed by its specific, physiological receptor antagonist, IL-1Ra. I therefore decided to investigate the expression of IL-1Ra in the same experimental groups which had demonstrated a significant difference in IL-1ß (see Section 6.4.4).

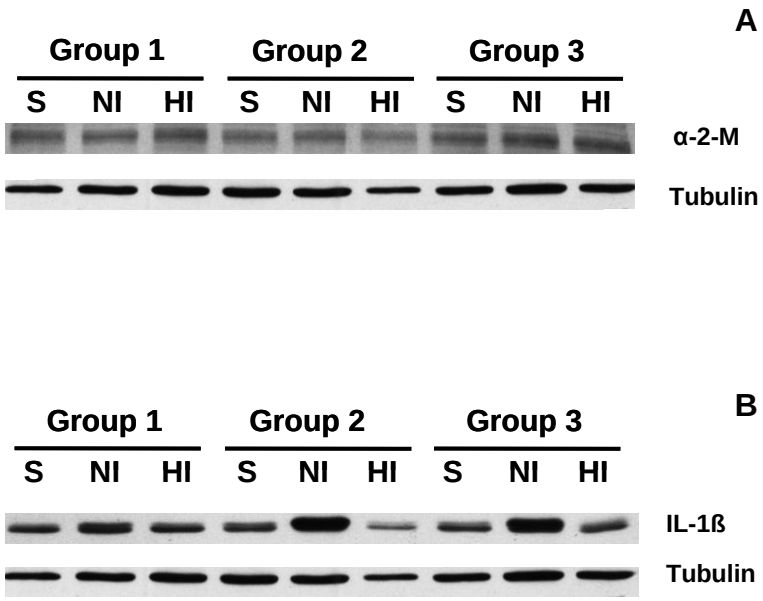

Figure 6.09: Representative Western Blot of [A] α-2-macroglobulin (α-2-M) and α-tubulin (loading control), [B] interleukin-1ß (IL-1β) and α-tubulin (loading control).

The three experimental conditions are S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.
The three groups represent the original nine samples used in the initial microarray experiments.

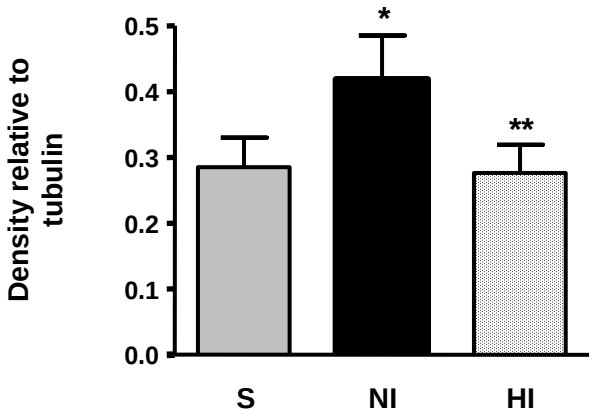

Figure 6.10: Protein levels of interleukin 1 β (IL-1β).

Samples (n=6) were analysed by Western blotting as indicated in the Materials and Methods section. Density of sample bands were normalised to tubulin and results were compared by repeated-measures ANOVA: * $p<0.05$ vs. S, ** $p<0.01$ vs. NI. The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

6.4.4 ELISA

ELISA experiments were undertaken to determine the protein levels of cardiotrophin (CT-1), lipopolysaccharide binding protein (LBP), epidermal growth factor (EGF) and hepatic interleukin-1 receptor antagonist (IL-1Ra).

The levels of CT-1 (Figure 6.11) and EGF (Figure 6.12) detected did not change significantly across the experimental groups. LBP apparently exhibited the 'up-down' pattern of expression as detected by the microarray and confirmed by PCR. However, the amount of LBP protein detected did not change significantly across the experimental groups (Figure 6.13).

The levels of IL-1Ra were significantly increased in normothermic IIR as compared to normothermic sham ($p<0.01$). However, there was no significant difference between normothermic IIR and hypothermic IIR (Figure 6.14).

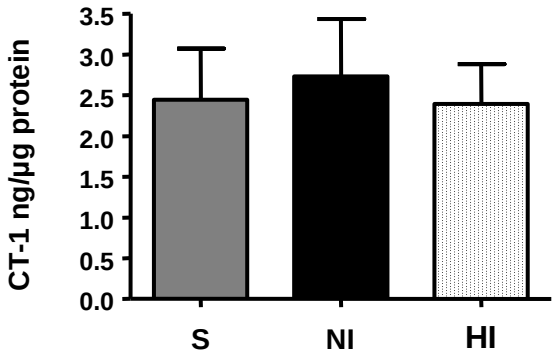

Figure 6.11: Protein levels of cardiotrophin-1 (CT-1).

Samples (n=6) were analysed by ELISA as indicated in the Materials and Methods section 2. Levels of CT-1 were normalised to total protein and results were compared by one-way ANOVA. The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

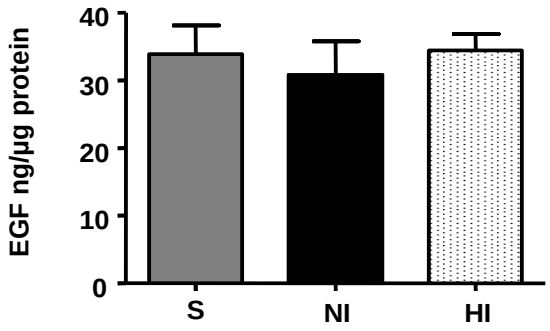

Figure 6.12: Protein levels of epidermal growth factor (EGF).

Samples (n=3) were analysed by ELISA as indicated in the Materials and Methods section. Levels of EGF were normalised to total protein and results were compared by one-way ANOVA. The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia- reperfusion.

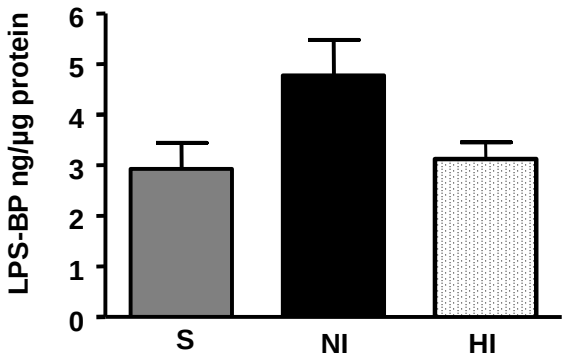

Figure 6.13: Protein levels of lipopolysaccharide binding protein (LBP).

Samples (n=6) were analysed by ELISA as indicated in the Materials and Methods section. Levels of LBP were normalised to total protein and results were compared by one-way ANOVA. The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

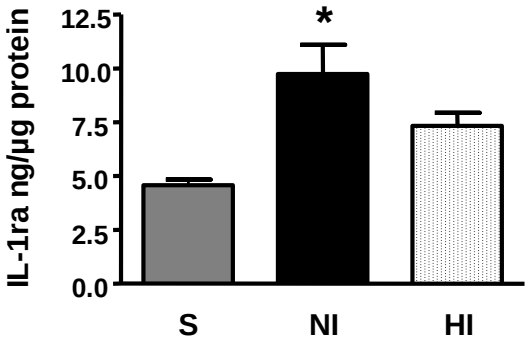

Figure 6.14: Protein levels of interleukin 1 receptor antagonist (IL-1Ra).

Samples (n=3) were analysed by ELISA as indicated in the Materials and Methods section. Levels of IL-1Ra were normalised to total protein and results were compared by one-way ANOVA with post-test Tukey's multiple comparison test: * $p<0.01$ vs. S. The three groups represent S; normothermic sham, NI; normothermic intestinal ischaemia-reperfusion, HI; hypothermic intestinal ischaemia-reperfusion.

6.5 Discussion

This study indicates that hepatic gene and protein expression changes following IIR injury and that altering temperature of the injury can modulate this expression.

There are significant advantages to using microarray technology despite its limitations; it remains a powerful method for assessing transcriptional changes of a very large number of known genes. Such a tool is highly relevant to studying models of disease, such as in this study, comprise of complex systems and pathways such as Multiple Organ Dysfunction Syndrome (MODS). These complex systems are not adequately explained by current 'linear' models investigating few molecules and pathways at a time, microarray technology can facilitate a relatively unbiased approach compared to that of purely single pathway investigations.

In this study, we were primarily interested in identifying genes whose hepatic transcription increased following normothermic IIR but then decreased following hypothermic injury and which therefore could be involved in the protective effect of hypothermia on the liver and distal organ damage. The influence of normothermic and hypothermic IIR on hepatic (STAT) transcription factor activation exhibits this pattern and was confirmed in the same samples examined for gene expression.

The number of microarrays (n=3) within each experimental group is limited, due to financial constraints, consequently the accuracy of this investigation is also limited. In analysing the data the Welch's t-test provided a list of genes which changed between two experimental groups with $p<0.1$ acting as a crude but inclusive filter. However, K-means clustering provided an

important tool in further analysing this data grouping changes in expression according to the direction of behaviour. The most obvious patterns of interest are those highlighted above.

Ontologies of genes with expression changes of most interest have been constructed (see Tables 6.01 and 6.02). The candidate genes selected for validation of gene expression and protein levels were selected from these ontologies, following a detailed review of the literature enriched for potential association with hepatic and systemic injury. Time constraints have limited the investigation of seemingly unrelated genes and those of unknown association (ESTs).

The main aim of this study was to identify genes that are differentially expressed in the liver following normothermic and moderately hypothermic IIR. These results provide an important insight into the relationship between IIR and its influence on hepatic gene expression both during normothermic and hypothermic injury.

6.5.1 Acute Phase Proteins

Interleukin-1β (IL-1β), lipopolysaccharide binding protein (LBP) and α-2 macroglobulin (α-2M) gene expression showed increased expression following normothermic IIR that decreased following hypothermic injury. All of these genes represent members of the acute phase response (APR). Protein levels of IL-1β also demonstrated this same pattern, increasing following normothermic IIR and then decreasing following hypothermic IIR. However, both hepatic LBP and α-2 macroglobulin protein levels did not change significantly across the experimental groups at this time-point.

The liver is a major target for proinflammatory cytokines (e.g. TNF-α, IL-1α, IL-1ß and IL-6) which promote the APR by modulating the synthesis of acute phase proteins (APP). The APR is an important role of the liver, particularly Kupffer cells, as part of the innate immune response to inflammation or infection. In acute infection, binding of pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) on Gram-negative bacteria and lipotechnoic acids on Gram-positive bacteria, to the Toll-like family of receptors (TLR) on inflammatory cells e.g. (Kupffer cells) results in the activation of the APR.

Acute phase proteins have been divided into several different groups according to different classifications: 1) positive (up-graded, e.g. α-2 macroglobulin) or negative (down-graded, e.g. albumin); 2) Type-1 (regulated primarily by IL-1 type cytokines, e.g. α_1-acid glycoprotein, haptoglobin) or Type-2 (regulated primarily by IL-6 type cytokines, e.g. α-2 macroglobulin). Most attention has focused previously on the function of secreted plasma APPs: 1) transport proteins (e.g. albumin, haptoglobin), 2) protease inhibitors (e.g.) and 3) coagulation factors (e.g. fibrinogen) (Olivier et al. 1999).

Although the secreted acute phase plasma proteins are a well characterised response of the liver to systemic inflammation there are also resident intracellular and membrane proteins of the liver whose synthesis also modified (acute phase related intracellular proteins, APRIP). APRIPs include transcription factors (e.g. CAAT/enhancer binding protein [C/EBPß], STAT-3 and Hepatocyte Nuclear Factor-3 [HNF-3]), intracellular enzymes (e.g. glutathione *S*-transferase) and surface receptors (e.g. the polymeric immunoglobulin receptor) (Olivier et al. 1999). Most liver transcriptions factors are prone to APR associated transcriptional regulation themselves in addition to post-transcriptional

modifications (e.g. STAT-1 and STAT-3 are phosphorylated in response to IL-6 activation proteins).

α-2 macroglobulin is an important positive APP in rats but not in humans. α-2 macroglobulin acts not only by inhibiting a wide range of proteases (e.g. neutrophil elastase and matrix metalloproteinases) (Milland et al. 1990) but also as a specific cytokine carrier that binds and inactivates cytokines (e.g. IL-1ß and TNF-α), superoxide and hydroxyl radicals (Gourine et al. 2002). α-2-macroglobulin gene transcription is promoted primarily by IL-6 (Type-2) type cytokines through STAT-3 activation (Lerner et al. 2003). In this study however, despite the increased α-2-macroglobulin gene transcription demonstrated in the microarray and PCR data there is no difference between the experimental groups in hepatic α-2 macroglobulin protein. This may represent a functionally significant increase in transcription in the normothermic injury if there is a delay in protein synthesis or if secreted (plasma) levels of the protein are increased, or conversely, a functionally insignificant increase in transcription which is not translated into protein synthesis. Additional experiments would be required to determine this relationship, however there is evidence that α-2 macroglobulin protein synthesis may be delayed for up to 12 hours after onset of the APR (Ruminy et al. 2001a).

Lipopolysaccharide binding protein (LBP) is another positive APP produced predominately by hepatocytes, LBP gene is also transcriptionally activated by STAT-3 (Schumann et al. 1996). Within the liver Kupffer cells are consequently exposed to high concentrations of LBP and it may also be secreted directly into the plasma to act distally, LBP is also produced locally in the lung and skin (Su 2002). Lipopolysaccharides (LPS) are glycolipids found on the

surface of gram-negative bacteria, on their release they are capable of resulting in a significant inflammatory response. Endogenous, gut-derived, LPS is predominately cleared from the portal circulation by the liver but the liver also has an ability to react strongly to LPS (e.g. Kupffer cells) by producing cytokines (e.g. TNF-α, IL-1ß) and reactive oxygen species.

Within the liver LBP binds to LPS with a high degree of specificity and affinity and facilitates its transfer to membrane CD14 on Kupffer cells, signalling is then mediated via Toll-like receptor 4 resulting in the activation of Kupffer cells and the release of proinflammatory mediators (Su 2002). More recent research has demonstrated that LBP may act differently with varying concentrations of LPS, for example at low concentration of LPS transfer to isolated Kupffer cells ex vivo is facilitated by LBP resulting in an accelerated production of TNF-α (Su et al. 2000). LBP also acts to transfer LPS to lipoproteins such as high-density lipoprotein (HDL) which results in its neutralization. LBP is known to have dose dependent effects on monocyte and neutrophil activation (Su 2002b). In addition in a LPS-galactosamine model of liver injury in vivo administration of recombinant murine LBP diminishes LPS response, i.e. TNF-α levels, ALT levels and mortality (Lamping et al. 1998). The exact mechanism whereby LBP can exert these opposing effects is not known, but it appears from LBP knock-out studies that the primary role of LBP in vivo is not to potentiate the effects of LPS but to protect organisms from such bacterial infections (Wurfel et al. 1997).

In this study LBP gene expression is increased following normothermic IIR and reduced at moderate hypothermia. The initial microarray findings were confirmed by PCR and hepatic protein levels of LBP followed the same pattern as

the original microarray findings but no statistically significant differences were established. With further experiments and higher numbers of samples this difference may have reached statistical significance, however no such conclusions can be drawn from the data with a current sample size n=6.

Significant results have been established with regard to the expression of hepatic IL-1ß following IIR. Transcription of IL-1ß is up-regulated within 3 hours of superior mesenteric artery occlusion, but significant levels of *de novo* synthesised protein is also present within a very short time (Green et al. 1996). Interleukin-1 is an important multifunctional cytokine which exists in two highly agonist forms (IL-1α and IL-1ß). IL-1β is not usually expressed in healthy individuals but transcription and synthesis of IL-1ß is increased rapidly following a variety of different microbial and non-microbial stimuli. The initial translational product is an inactive precursor activated by action of caspase-1 (interleukin cleavage enzyme) (Dinarello 2004). In the liver, IL-1ß is predominately synthesised by the resident macrophages, Kupffer cells (Kato et al. 2002, Koo et al. 1999). Hepatocytes are the major target for IL-1ß within the liver, where IL-1β acts locally on specific IL-1 receptors present on the plasma membrane (Zhu and Liu 2003). IL-1ß in turn up-regulates the expression of other important inflammatory mediators, via the activation of transcription factors STAT3 and NF-κB (Yoshida et al. 2004), including cytokines both pro-inflammatory (e.g. IL-6 and TNF-α) and anti-inflammatory (e.g. IL-10), colony-stimulating factors and several growth factors, including hepatocyte growth factor (HGF). IL-1ß also stimulates the production of other acute phase proteins in the liver, such as C-reactive protein (CRP), α_1-proteinase inhibitor, haptoglobin, fibrinogen, and complement protein C3, whereas in contrast, the expression of housekeeping

genes such as albumin, transferrin and cytochrome p450 are suppressed (Peters 1996). Increased hepatic IL-1ß may also have systemic effects due to distant activation of IL-1receptor resulting in fever, leucocytosis, thrombocytosis, growth of fibroblasts and induced expression of adhesion molecules and receptors on endothelial and inflammatory cells (Dinarello 1996). IL-1ß, as well as affecting the acute phase response, could also influence hepatic metabolism and therefore be involved in the pathogenesis of hepatic energy failure following IIR. IL-1ß inhibits ATP synthesis (Berthiaume et al. 2003) and gluconeogenesis (Ceppi et al. 1996) in hepatocytes *in vitro* and as a potent inducer of hepatocyte nitric oxide synthase (Ceppi et al. 1996), IL-1β could potentially influence several other metabolic pathways (see Chapter 8).

Therefore, increased levels of IL-1ß in the liver may represent an increased acute phase response to inflammatory mediators produced in normothermic IIR and may act to promote pro-inflammatory changes both locally and distally. Hypothermic IIR significantly reduces IL-1ß gene transcription and protein levels in the liver as compared to normothermic injury. This reduction may represent a reduced acute phase response with a reduction of the pro-inflammatory potential of IIR and hypothermia may act in part by attenuating this highly potent, proximal element of the cytokine cascade.

IL-1 signalling incorporates a naturally occurring antagonist, IL-1 receptor antagonist (IL-1Ra). IL-1 initially binds to the type 1 receptor (IL-1R1) and this complex then recruits the IL-1R accessory protein, formation of this three-part complex then leads to signal transduction. IL-1Ra acts by binding to the same receptor as the agonists but does not elicit an agonist response as this two-part complex fails to recruit the accessory protein (Irikura et al. 2002). IL-1Ra

therefore represents a naturally occurring mechanism whereby the pro-inflammatory actions of IL-1β may be modulated (Carl et al. 2004).

The term IL-1Ra actually refers to three closely related isoforms: secretory (sIL-1Ra); intracellular (icIL-1Ra) and alternative translation initiation site (icIL-1RaII). The relative roles of the different forms of IL-1ra in the regulation of the inflammatory and immune response are not known. However all functions of IL-1Ra depend upon the presence of a functioning IL-1 receptor as this is its only target (Irikura et al. 2002). Recently it has been demonstrated that human hepatocytes in culture produce sIL-1Ra and icIL-1Ra following stimulation with IL-1β and IL-6 (Gabay et al. 1997). Hepatocytes have been shown to be the major *in vivo* source of sIL-1Ra contributing significantly to circulating levels of IL-1Ra (Gabay et al. 2001). The presence of a functional STAT-3 binding site within the proximal sIL-1Ra promoter has also been demonstrated. IL-10 plays a significant role in inducing sIL-1Ra gene expression via the activation of STAT-3. This response is consistent with the anti-inflammatory role of both IL-10 and IL-1Ra (Carl et al. 2004).

The liver is known to be an important target for IL-1ß which promotes the acute phase response, a central element of the innate immune system. The pro-inflammatory actions of IL-1ß are carefully controlled by the corresponding local levels of IL-1Ra, which is itself produced by the liver as an acute phase protein (Gabay et al. 1997). The relationship between IL-1ß and IL-1Ra has been extensively studied in a variety of different models of many diseases, and in each the local overproduction of IL-1 and/or the underproduction of IL-1Ra has predisposed to the disease (Arend 2002).

In this study the influence of normothermic and hypothermic IIR on hepatic IL-1Ra was also examined. Normothermic IIR results in a significant increase in both IL-1β and IL-1Ra as compared to sham operation. In contrast, hypothermic IIR results in a significant decrease in hepatic IL-1β as compared to normothermic IIR but did not significantly change the level of IL-1Ra. This response may indicate a protective action of hypothermic injury resulting in reduced production of the pro-inflammatory cytokine IL-1β and a relative increase in its naturally occurring antagonist IL-1Ra (i.e. increase in IL-1Ra: IL-1β ratio). The ratio between a cytokine and its antagonist is known to be a major determinant for its biological efficacy and therefore its role in physiological or pathological situations (Maedler et al. 2004). The elevated ratio of IL-1Ra to IL-1β in patients with fulminant hepatic failure who survived compared with those who died suggests a potential protective role in liver disease (Sekiyama et al. 1994).

By altering the levels of IL-1Ra in animal experiments the course of disease states has been influenced. Mice lacking the gene for IL-1Ra show abnormal development and homeostasis as well as an altered response to inflammatory stimuli. IL-1Ra knock-out mice also develop two distinct diseases spontaneously; these are similar to rheumatoid arthritis and arteritis in humans. (These knock-out mice also produce antibodies against double-stranded DNA, antibodies to type II collagen and excessive amounts of immunoglobulins. Levels of IL-1 mRNA in the joints of these mice were 10 times as high as the levels in control mice). Another group demonstrated lethal arterial inflammation in IL-1Ra knock-out mice, characterised by transmural infiltration of neutrophils, macrophages and T lymphocytes. The inflammatory appearance is consistent with

the biological effect of IL-1 on endothelial and smooth muscle cells (Arend 2002, Nicklin et al. 2000).

A reduction in the level of IL-1 signalling, that is deletion of the IL-1 receptor (IL-1R) or by increased expression of IL-1Ra, does not effect development or homeostasis but does alter the immune response (Irikura et al. 2002). Over-expression of IL-1Ra in a mouse model of arthritis results in a significant reduction in incidence and severity. Pre-treatment with IL-1Ra has been demonstrated to improve the survival of animals following hepatic ischaemia-reperfusion injury (Shito et al. 1997). In several models of animal inflammatory bowel disease neutralisation of IL-1Ra exacerbates disease and treatment with IL-1Ra attenuates the disease (Ferreti et al. 1994, McCall et al. 1994).

IL-1Ra blood level has been demonstrated in human studies to be a valuable predictor for mortality in sepsis by logistic regression analysis (Arnalich et al. 2000). Arend *et al* have also demonstrated that it is important to maintain the balance of the IL-1Ra: IL1β ratio for host defence in critical illness (Arend 2002). An elevated ratio of IL-1Ra: IL-1β in patients with fulminant hepatic failure who survived compared to those who died, also suggests a protective role for IL-1Ra in liver failure (Molnar et al. 2004).

There is evidence to suggest that IL-1Ra controls the activity of IL-1 and that the administration of exogenous IL-1Ra should ameliorate inflammatory diseases (Dinarello 2000). Pre-treatment with exogenous Il-1Ra has been demonstrated to improve the survival of animals following hepatic ischaemia-reperfusion injury (Shito et al. 1997). In addition human trials of IL-1Ra have been successful in the treatment of rheumatoid arthritis. The recombinant form of

IL-1Ra is commercially available and called Anakinra (Kineret^{TM}). The advantage of IL-1Ra is that even in massive doses Anakinra does not have an agonist role. However there are disadvantages, IL-1 receptors are present on all cells except red blood cells so the challenge for occupying receptor sites is huge, in addition Anakinra is rapidly excreted by the kidney and blood levels are low after 24 hours. IL-1 receptors are regenerated everyday therefore at least daily injections of Anakinra are required (Dinarello 2004).

However there is conflicting evidence from studies that have demonstrated treatment with exogenous IL-1Ra enhances the severity of local and systemic injury following IIR and increases lethality, in addition administration of IL-1β or blockade of IL-1Ra has conversely been demonstrated to protect from reperfusion injury (Souza et al. 2003). These results may demonstrate the effect of excess IL-1Ra, increasing the susceptibility to systemic inflammation by impairing the innate immune response. *In vivo* IL-1 and IL-1Ra are co-regulated. Parallel levels of IL-1 and IL-1Ra may reflect an accumulation of proteins in the serum due to competition for receptor binding that would reduce the level of free cytokine rather than an alteration in de novo protein/mRNA synthesis. In this way maintaining the balance between IL-1 and IL-1Ra may be essential for an appropriate immune response *in vivo*.

It is also of note that the function of the APR is not fully established, previously it has been assumed to be beneficial as many APPs have anti-inflammatory actions (e.g. haptoglobin) or act as antiproteases (α_1-antichymotrypsin). However, several APPs initiate or sustain inflammation such as classic complement components. The complex patterns of the APR may be determined in part by the initial causative inflammatory condition. For example,

molecular studies using endotoxin-induced systemic inflammation in rats have shown a selective down-regulation of both mRNA and protein of organic anion transporters at the basolateral and canalicular membrane of hepatocytes (Green et al. 1996, Utili et al. 1976). Additional pathophysiological studies confirm these findings with an endotoxin-induced reduction in bile flow and hepatic bile salt excretion (Utili et al. 1976). The pathophysiological characteristics of the APR in IIR are unknown.

Regulation of the APR is also complex and not fully delineated. STAT-3, itself produced as an acute phase protein, mediates transcription of both pro-inflammatory and anti-inflammatory genes and appears to have an important role controlling the APR. Experiments in cell-specific knock-out mice suggest that it is the anti-inflammatory effects of STAT-3 which are important for terminating the APR. Knock-out mice deficient in STAT-3 in macrophages and neutrophils had increased mortality in LPS-induced shock associated with increased production of pro-inflammatory cytokines (Takeda et al. 1999). STAT-3 deficient hepatocytes stimulated by LPS do not produce IL-6 responsive acute phase proteins (Alonzi et al. 2001, Carrithers et al. 2005).

Inflammation is known to be a highly complex process, involving many different cell types and molecules many of which are multi-functional, each contributing to and abating injurious processes in turn (Gabay and Kushner 1999). The APR is not a uniformly beneficial phenomenon and may indeed contribute to the morbidity associated with the SIRS and MODS (Bone 1996).

Epidermal growth factor (EGF) exerts its effects by binding to its receptor a transmembrane protein tyrosine kinase which activates the JAK/STAT signalling pathway. EGF has been demonstrated to be protective in various

pathologic conditions including IIR protecting enterocytes from necrosis and apoptosis via decreased production of nitrogen and oxygen free radicals. In addition EGF promotes the recovery of damaged enterocytes, preserves intestinal barrier function and decreases bacterial translocation (Xia et al. 2003). The role of EGF in the liver following IIR is unknown.

The linking of expression data with gene function is particularly difficult in solid organs such as the liver because of its heterogeneous cell population. The situation in diseased liver is even less well characterised as transcriptomes can double or triple in complexity during disease (Shackel et al. 2002). In these experiments, as whole liver was studied, hepatocytes and Kupffer cells will be the predominant cell types contributing to the microarray data, whereas sinusoidal endothelial cells and stellate cells, which constitute a relatively small percentage of the total number of cells, may have an important role in determining progression of disease. Previous results suggest that there is very little hepatic inflammatory cellular infiltrate following IIR (see Chapter 5) thus establishing that gene expression changes do not appear to be a result of an influx of an additional cell population but transcriptional changes of the same resident cell types in all three experimental groups.

One of the most challenging aspects of gene expression analysis remains the selection, from a vast amount of data, of those genes which have a real causal role. A significant change in the transcriptional level of a gene does not necessarily correlate with a causal role of that gene. A small change in a key gene may produce a large biological effect. In addition changes in gene expression are not invariably associated with changes in protein expression and provide no information regarding post-translation modifications (Napoli et al. 2003). This

corresponds with previous work which has demonstrated huge variation in mRNA and protein expression (Coulouarn et al. 2004)

This study illustrates many of the limitations of current microarray technology. Measuring relative levels of RNA and extrapolating to suggest changes in gene transcription is subject to potential error. Despite several measures to minimise the significance of false hybridization, including normalisation and filtering of the data, false positive gene expression changes are detected. False positives arise as the size of genomic data generated is vast, enormously complex and highly dimensional, and as such conventional statistical methods fail to handle such data adequately. For example, a traditional statistical testing criterion of a significance of 5% applied to a 10K microarray study would be likely to produce 500 false positive genes, in this case any truly differentially expressed genes will be mixed with these genes without any discriminatory information to discern them. By tightening the significance level e.g. 1% or lower will simply result in a high false-negative error rate with failure to identify many important biological targets. In addition conventional statistical techniques perform optimally when applied to a sample size, 'n' (i.e. the number of independent observations) that is much larger that the number of candidate targets, 'p', this situation is reversed in many genomic data analyses (Lee et al. 2008).

An initial approach to identify differentially expressed genes is known as the fold-change estimation, this detects the average log-ratio between two groups and categorises all genes that exceed an arbitary cut-off as being differentially expressed. However, fold-change measurements lack the rigor of statistical methods and lead to a high rate of false positive gene expression changes.

Welch's t-test has been a popular statistical method for gene array analyses but it has also been critisised for its use with small experimental sample sizes, which through the constraints of limited resources and high cost is frequently the case in gene array technology (Jeanmougin et al. 2010). Clustering analysis is also widely applied to mine genomic data, the advantage of these techniques are that they can effectively reduce the high-dimensional gene expression data generated into two-dimensional diagrams that are organised by each gene's expression patterns. Clustering analysis is defined as a measure of similarity or dissimilarity which is then applied to hierarchical or partitioning allocation algorithms [e.g. K means clustering is a partioning algorithm that divides the data into a pre-specified number of subsets (Storey 2003)]. Despite their popularity clustering analyses are also subject to criticism as interpreting their results can be difficult and the risk of false-positive gene expression remains an issue.

It is inevitable that gene microarray experiments incorporate an element of pragmatism as no single method of experimental design or statistical analysis removes the risk of fale-postive detection. It is recognised that the p value cut-off is arbitrary and that data generated at the 'gene list' level is generally treated with great caution, but still, within this list there will be genuine gene expression changes of interest. Other methods, as in this study, can be applied to probe these 'lists' further in the form of down-steam confirmatory experiments to determine accurate changes in gene expression and associated protein levels.

In whole tissue, as in this study, there are difficulties in whether apparent gene expression changes are due to true changes in gene expression or due to alterations in numbers of different cells in the liver. For example, hepatic neutrophil infiltration could cause an apparent change in gene expression when

whole tissue gene arrays are examined. In whole liver over 80% of the tissue mass is composed of hepatocytes therefore the majority of gene expression changes reflect that of hepatocytes. Microarray studies investigating gene expression in particular cell lines avoid this confounding variable of cellular heterogeneity (Cobb et al. 2002), although this approach would not be easily applicable to investigating the distant effects of IIR on organs such as the liver.

Analysis of microarray data may also lead to the prediction of false negative gene expression. Genes which may have been anticipated to demonstrate significantly altered expression such as transcription factors (e.g. NF-κB and STATs) and plasma membrane receptors (e.g. CD14) may be viewed as false negatives. These suspected false negatives may 'true' negatives if the selected time-point in these experiments is too late to detect changes in the corresponding levels of mRNA but demonstrates significant increase in the *de novo* synthesis of the specific protein (e.g. STAT3 in normothermic IIR, Chapter 4).

Other limitations of this study include the decision to not attempt to explore the nature of expression changes in expressed sequence tags (ESTs). These, as yet, unclassified genes, or fragments of genes, have not been investigated further secondary to time and financial constraints.

In addition in our process of detecting patterns of change in gene expression between the experimental groups the 'down-regulation' of genes in the most injurious group (NIIR) appears to be less likely in our experiments, this has been reported by others in different pathological conditions e.g. in a model of sepsis (Cobb et al. 2002). It is not clear whether is a technical limitation of the methods used or an accurate reflection of biology. This is a phenomenon which would perhaps warrant further investigation in the future.

Understanding of the modulations that control the net activity of the whole array of hepatic transcription factors is still in its infancy and so is our understanding of the mechanisms that control the transcription of a given acute phase protein gene (Ruminy et al. 2001). Progress in these fields will lead to improved understanding of the hepatic acute phase response to insults such as IIR. Modulation of the acute phase response may in turn lead to the development of potential therapeutic techniques for the treatment of conditions characterised by intestinal ischaemia-reperfusion. In addition this study also supports the potential therapeutic role of moderate hypothermia, which acts in part by attenuating an important hepatic pro-inflammatory cytokine, IL-1β.

In summary, this study has established that moderately hypothermic IIR influences hepatic (STAT) transcription factor activation and the expression of several genes. Some groups of genes are down-regulated (e.g. IL-1ß, α-2M, LBP) in response to hypothermia others are up-regulated (e.g. EGF). Both gene expression and protein levels of an important hepatic pro-inflammatory cytokine, IL-1ß, are significantly attenuated in response to moderately hypothermic IIR injury. This cytokine may be pivotal in terms of controlling the subsequent inflammatory changes in IIR but also represents only the ‘tip of the iceberg’ in analyzing the potential influence of IIR and temperature change on gene expression and protein levels.

Chapter 7

Hepatic bioenergetics and Mitochondrial protein expression

Chapter 7 –

Hepatic bioenergetics and Mitochondrial protein expression

Contents

7.1 Introduction

All experiments previously performed in this thesis used whole liver tissue to explore the influence of IIR injury on the liver; this chapter enables a specific sub-cellular fraction of liver tissue to be examined more closely, mitochondria. Previous experiments using whole liver tissue have demonstrated changes in hepatic gene expression, protein phosphorylation and protein synthesis following IIR (Chapters 4 and 6) however normal histological features of liver tissue exposed to the same conditions (Chapter 5) suggest that IIR does not result in 'direct liver injury'. In addition, evidence published previously in the same experimental model (Vejchapipat et al. 2001) demonstrates that IIR results in reduced hepatic ATP levels suggesting that liver bioenergetic failure does occur as a consequence of IIR. Recently other work has focused attention on a bioenergetic theory of MODS with evidence that suggests that mitochondria may play an important role (Brealey et al. 2002, Carre and Singer 2008). Together, evidence that the majority of organ dysfunction occurs in sepsis despite normal histology and a paradoxical rise in oxygen tension suggests that oxygen is freely available but is being underutilised, this has provided a new perspective in explaining MODS and a new direction for potential therapeutic modalities (Hotchkiss et al. 1999, Vejchapipat et al. 2001). This chapter explores the possibility that IIR may directly influence hepatic mitochondrial function.

Mitochondria are thought to have derived originally from bacteria; they exist within eukaryotic cells in an endosymbiotic relationship retaining many of their original bacterial features: 1) mitochondria have their own genome (in most cases DNA exists as a circular chromosome with no associated histones);

mitochondria also synthesize their own proteins; and 3) mitochondrial proteins have a formyl methionine at their N terminus (Hubbard et al. 2004).

Under normal aerobic conditions ATP is produced by mitochondrial oxidative phosphorylation. Electrons derived from the oxidation of glucose or fatty acids are transferred through the respiratory chain complexes I-IV. Complexes I, III and IV pump protons out of the mitochondrial matrix into the intermembrane space which results in the generation of an electrochemical proton gradient used by a fifth enzyme complex (ATP synthase) to drive ATP synthesis (Figure 7.01).

Cellular dysfunction following hypoxia (and/or the action of circulating mediators, e.g. NO and peroxynitrite, termed ‘cytopathic hypoxia’) results in restricted ATP production through aerobic pathways and anaerobic glycolysis predominates. Eventually ATP production fails with an associated development of lactic acidosis (Vanhorebeek et al. 2005).

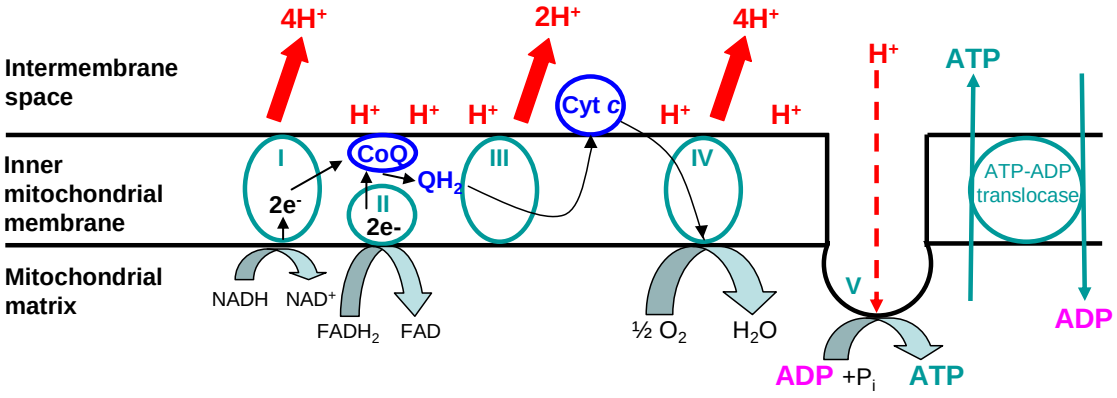

Figure 7.01: The mitochondrial respiratory chain.

Energy obtained through the transfer of electrons (e-, black arrows) is used to pump protons (H+, red arrows) from the mitochondrial matrix into the intermembrane space. This creates an electrochemical proton gradient across the inner mitochondrial membrane which allows Complex V; ATP synthase to use the flow of protons back into the matrix to generate ATP. Complex I; NADH dehydrogenase, accepts electrons from NADH and passes them to CoQ; Coenzyme Q (ubiquinone) which aslo receives electrons from Complex II; Succinate Dehydrogenase. QH2; Reduced Coenzyme Q then passes electrons to Complex III; Cytochrome *c* reductase, which in turn passes them to Cyt *c*; Cytochrome *c*. Cytochrome *c* passes electrons to Complec IV; Cytochrome *c* oxidase which uses the electrons and hydrogen ions to reduce molecular oxygen to water.

Mitochondria act as the ‘power house’ of eukaryotic cells providing over 95% of ATP production. Mitochondria act by constantly monitoring intracellular protons, calcium ions, reactive oxygen species (ROS), redox state, matrix pH and nitric oxide (NO) to regulate electron transfer activity. As a consequence mitochondria have a central role within the cell acting as a control between homeostasis and cellular dysfunction. This is an essential role as insults upon the cell may produce high metabolic demands requiring increased ATP production. However, if the insult is overwhelmingly severe, mitochondria may respond by dysfunction of oxygen metabolism, accelerated oxidant production and fission which in turn may lead to apoptosis and necrosis (Crouser 2004a). However less severe insults may result in potentially reversible mitochondrial dysfunction which does not lead to inevitable cell death.

Previous animal experimental work has demonstrated that the severity of MODS and eventual rates of survival were associated with nitric oxide overproduction and mitochondrial dysfunction (respiratory chain complex I inhibition and decreased ATP concentration) (Brealey et al. 2004). These findings were demonstrated in vital (e.g. liver) and non-vital (e.g. skeletal muscle) organs. In addition skeletal muscle biopsies taken from critically ill patients with septic shock revealed a significant association between disease severity, outcome and mitochondrial dysfunction (30% reduction in respiratory chain complex I performance and decreased ATP concentration). More severe abnormalities (lower tissue ATP concentration and significant reduction in activity of mitochondrial complexes I and IV) were associated with a higher risk of organ failure and adverse outcome of septic shock in these patients (Brealey et al. 2002). Most recently it has been observed that the early decrease in mitochondrial

functional capacity seen in critically ill patients is associated with a decrease in mitochondrial respiratory protein subunits [Complex I and Complex IV (e.g. mitochondrial encoded COX1 and COX2 and nuclear-encoded COX4)] and transcripts of mediators involved with mitochondrial respiration (e.g. mitochondrial respiratory complex subunit OXPHOS) (Carre et al. 2010). Notably in this study, patients that eventually recovery from critical illness demonstrated an early activation of 'mitochondrial biogenesis', that is the expression, import and assembly of mitochondrial proteins from both nuclear and mitochondrial genomes [e.g. increased mRNA content of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α] and the regulation of mitochondrial morphology. PGC-1α is a particularly interesting mediator as it is known to have an important role in global regulation of oxidative metabolism and is itself closely regulated by multiple different mediator pathways that are suspected to have pivotal roles in multiorgan dysfunction [e.g. AMP-activated protein kinase (AMPK), p38 MAPK and NO synthase/guanylate cyclise] (Carre et al. 2010). The majority of these important clinical studies linking multiorgan dysfunction syndrome with mitochondrial dysfunction have necessarily been restricted to investigating non-vital tissue such as skeletal muscle. However there has been extensive investigation of morphological differences in livers of septic mice and rats and a post-mortem study of liver sections from patients with sepsis demonstrated that hepatocyte autophagic vacuolisation increased during sepsis (Watanabe et al. 2009). Vanhorebeek *et al.*, investigated mitochondrial morphological features and functional data in post-mortem liver tissue from patients admitted to a surgical intensive care unit that were treated randomly with either conventional (when hyperglycaemic) or intensive (aggressively maintained

normoglycaemia) insulin therapy. Their results demonstrated that mitochondrial morphological abnormalities were reduced significantly if patients were in the normoglycaemic group and this preservation of normal ultrastructure was associated with higher respiratory-chain enzyme activity of Complex I and Complex IV. The authors report that by aggressively maintaining normoglycaemia normal hepatic mitochondrial function and cellular energetics is either maintained or restored and thereby suggests that this mechanism is responsible for the improved morbidity and mortality in critically ill patients treated with intensive insulin therapy (Vanhorebeek et al. 2005).

Other exciting developments have occurred in the science of non-invasive, real-time measurement of bioenergetic parameters that have provided a novel perspective on organ metabolism. For example, in animal models of sepsis and in patients, by using nuclear magnetic resonance (Taylor et al. 2004) and near infra-red spectroscopy (Rhee et al. 1997), a fall in ATP in both vital (e.g. liver) and non-vital (e.g. skeletal muscle) organs has been demonstrated (Hubbard et al. 2004).

It is also known that depressed ATP production may continue for some time after shock despite adequate supply of oxygen and substrate with modern intensive care therapy. This dysregulation of cellular energy metabolism has been termed ‘cytopathic hypoxia’ (Fink 2001). There are a number of possible mechanisms which have been postulated for impaired cellular oxygen utilization e.g. depletion of substrate for oxidative phosphorylation (i.e. inhibition of the pyruvate dehydrogenase complex [PDH]), consumption of NADH by PARP, poly (ADP-ribose) polymerase (a nuclear repair enzyme capable of consuming large quantities of NADH, the main source of reduction in oxidative phosphorylation),

inhibition of electron transport via mitochondrial complexes, abnormal permeability of outer mitochondrial membranes to cytochrome *c* and dissolution of the electrochemical gradient via opening of the mitochondrial permeability transition pore (PTP) (Crouser et al. 2004).

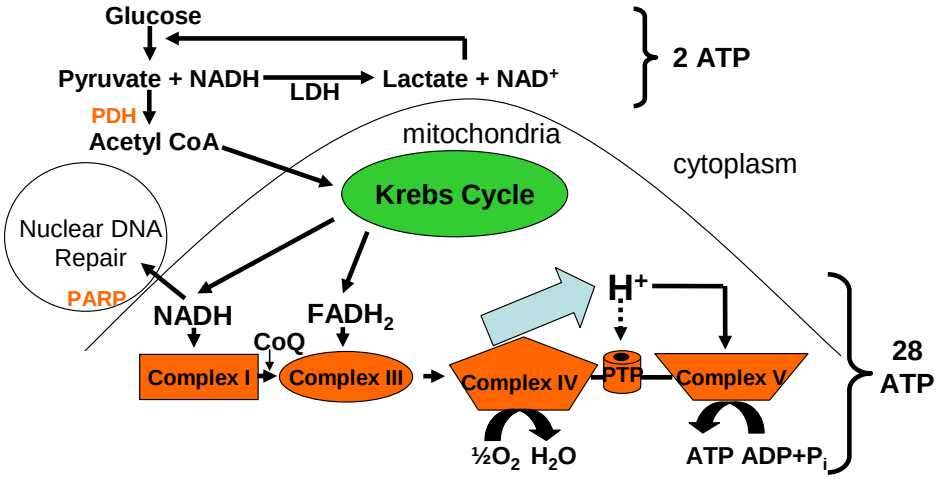

Figure 7.02: 'Cytopathic hypoxia' or dysregulation of cellular energy metabolism during sepsis.

Under normal conditions oxidative phosphorylation is dependent upon the development of an electrochemical gradient across the inner mitochondrial membrane, this is primarily related to the efflux of hydrogen ions (H+ represented by large grey arrow). In sepsis, critical components of this pathway are compromised (orange labels), including inhibition of pyruvate dehydrogenase complex (PDH), consumption of NADH by poly-(ADP-Ribosyl)-Polymerase (PARP), inhibition of electron transport via mitochondrial complexes, abnormal permeability of outer mitochondrial membrane to cytochrome *c* and opening of mitochondrial permeability transition pore (PTP). (CoQ = coenzyme Q or Complex II) (Crouser 2004).

Despite the efficiency of normal functioning mitochondria to respire and use oxygen highly effectively (98%) to produce ATP, mitochondria remain the largest producers of reactive oxygen species (ROS) within the cell. In mitochondrial dysfunction the efficiency of oxidative phosphorylation is impaired by the formation of the PTP, mitochondrial membrane damage and failed cytochrome complex function. As a consequence electrons are diverted away from the electron transport chain and generate high levels of superoxide. Effective anti-oxidant mechanisms exist within the cell to prevent damage to membranes, proteins and DNA which would lead to the destruction of the cell. For example, superoxide reacts immediately with NO to form peroxynitrite, a biologically active species capable of inhibiting enzyme and membrane functions, or is reduced by superoxide dismutase (SOD) to form hydrogen peroxide. In turn, hydrogen peroxide may be reduced further by catalase or by glutathione to produce water (Crouser 2004).

Nitric oxide is a ROS that has a number of roles in several different biological processes and has been implicated in the regulation of mitochondrial function. Animal models of sepsis have demonstrated an over expression of inducible NOS resulting in a dramatic increase in NO and modification of proteins by nitration (Crouser 2004). Mitochondrial proteins such as the complexes of the respiratory chain are specifically susceptible to nitration, inhibiting their function and promoting the mitochondrial PTP. In addition recent research using non-invasive electron paramagnetic resonance (EPR) techniques confirms that NO actively inhibits mitochondrial respiration in the liver in a model of sepsis (Davies et al. 2003). It has also been postulated that NO and its reactive metabolites while potentially toxic could also have a protective role by

modulating the rate of oxidative phosphorylation when mitochondrial function is impaired thereby limiting further oxidative stress (Crouser 2004).

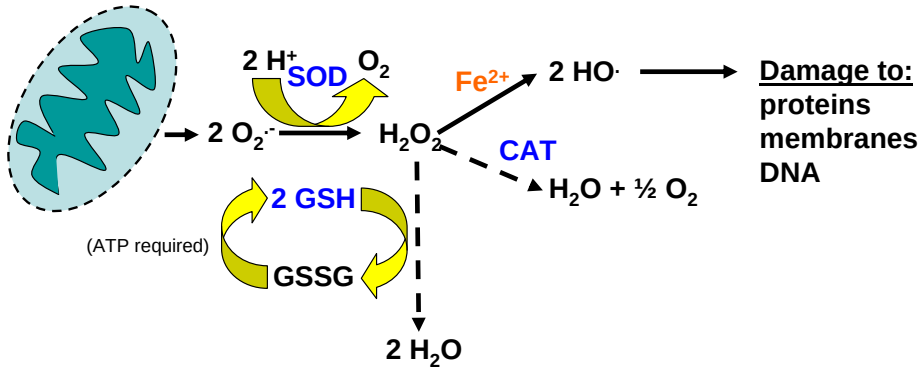

Figure 7.03: Mitochondrial-dependent oxidative stress during sepsis.

Sepsis-induced damage to mitochondria increases superoxide production ($O_2^{\cdot -}$) in conjunction with an acquired imbalance of the activity of intracellular antioxidants e.g. superoxide dismutase (SOD) activity is preserved whereas catalase (CAT) and glutathione (GSH) are depleted leading to increased production of hydrogen peroxide (H_2O_2). In sepsis H_2O_2 then reacts with iron (Fe^{2+}) to form highly toxic hydroxyl radicals ($HO^{\cdot}$) which are damaging to proteins, lipid membranes and DNA (Crouser 2004).

Mitochondrial DNA (mtDNA) is particularly sensitive to oxidative stress as it lacks the histones and repair enzymes that are associated with nuclear DNA. In addition expression of the whole of mitochondrial DNA is essential to preserve functional integrity. Therefore mitochondria are vulnerable to oxidative stress which may result in irreversibly reduced respiratory activity (Crouser 2004). Cells are able to replace dysfunctional mitochondria but this resynthesis of respiratory chain components requires several days of undisturbed protein synthesis in the absence of catabolism and will cause a necessary delay to fully functional recovery (Tavakoli and Mela 1982).

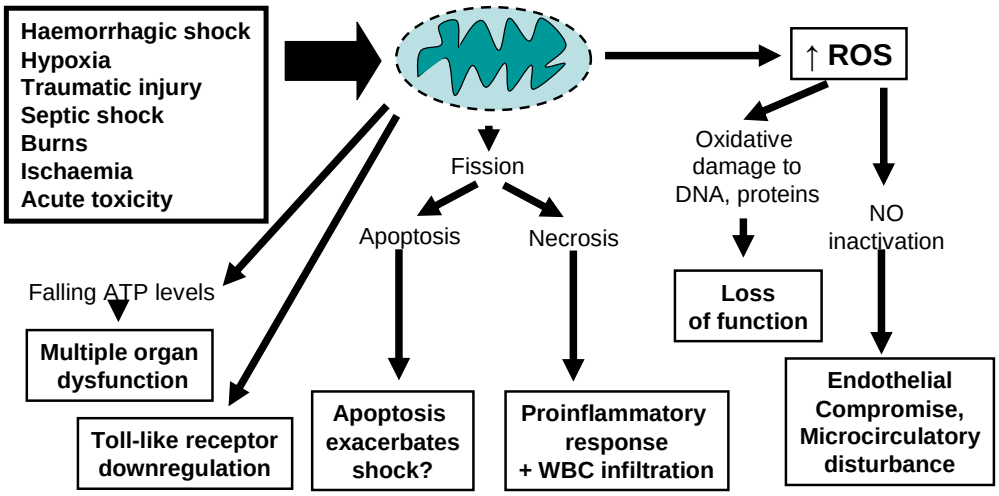

Figure 7.04: A schematic diagram of the multiple pathways affected by the mitochondrion as a consequence of trauma and shock (Hubbard et al. 2004).

Therapeutic strategies have been postulated that would augment ATP production until time of recovery e.g. administration of exogenous ATP-$MgCl_2$ (Mahmoud et al. 1997) or other methods of pharmacologically boosting ATP production e.g. glutamine (Hubbard et al. 2004). ATP-$MgCl_2$ administration after shock and ischaemia preserves many normal physiological functions such as macro- and microcirculatory blood flow, specific and non-specific immunological function, hormone balance and prevent apoptosis (Hubbard et al. 2004). Other treatments have been shown to protect ATP levels in animal models e.g. glutamine, prostaglandin E1, intensive insulin therapy (Vanhorebeek et al. 2005), allupurinol and adenosine (Hubbard et al. 2004).

Together this evidence has generated interest in a potential pathophysiological mechanism describing the hepatic bioenergetic failure noted in IIR. In addition by reversing the reduction in hepatic ATP and improving survival in experimental animals' moderate hypothermia may act by restoring normal mitochondrial function and thereby preventing or reversing cytopathic hypoxia.

Hypotheses

1. That normothermic IIR results in liver mitochondrial dysfunction and that hypothermic IIR may reverse or lessen this trend.
2. That the liver mitochondrial proteome is likely to demonstrate altered protein expression in normothermic IIR.
3. That the liver mitochondrial proteome may also be subject to altered protein expression in hypothermic IIR.

Subsequently preliminary experiments investigating the role of mitochondria in this IIR model have been performed. Mitochondrial oxygen consumption was chosen as a well established technique for assessing mitochondrial function. In addition the mitochondria proteins were further examined by employing an established proteomic technique, two dimensional fluorescence difference gel electrophoresis (2D-DIGE).

7.2 Materials and Methods

7.2.1 Surgical procedure

Animals were anaesthetised and underwent either sham operation or IIR, at normothermia or moderate hypothermia, according to the methods in Chapter 2 (pg 47-48).

The animals were divided into three groups:

NS = sham operation for 180 minutes at normothermia (n=3)

NIIR = 60 minutes ischaemia and 120 minutes reperfusion at normothermia (n=3)

HIIR= 60 minutes ischaemia and 120 minutes reperfusion at moderate hypothermia (n=3)

7.2.2 Mitochondrial oxygen consumption

Established techniques were used to isolate sub-cellular fractions of fresh liver samples to produce mitochondrial isolates (Chapter 2 pg 59-60). Fresh mitochondrial isolates (n=3) from each of the three experimental groups (NS, NIIR and HIIR) were prepared and quantified for citrate synthase activity (pg 60-61). Oxygen consumption was measured polarographically according to the methods described in Chapter 2 (pg 61-63) and State 3 oxygen consumption, State 4 oxygen consumption, respiratory control ratio (RCR) and 2, 4–dinitrophenol (DNP) stimulated oxygen consumption rate were calculated.

Rates of oxygen consumption are measured following addition of quantified mitochondrial sample and substrate (i.e. either glutamate and malate or succinate in these experiments). State 3 oxygen consumption is defined as ADP

dependent respiration; it is measured following addition of substrate in the presence of ADP and represents maximum mitochondrial oxidative flux coupled to ATP production. State 4 oxygen consumption is resting respiration, measured after all of the ADP has been converted to ATP representing oxygen consumption that is uncoupled, wasted (proton leak) and not used for ATP production. The respiratory control ratio (RCR) is calculated as the ratio of State 3 to State 4 and reflects the overall efficiency of oxidative metabolism to produce ATP. DNP stimulated oxygen consumption rate acts as a measure of maximum possible oxygen consumption by uncoupling oxidative phosphorylation.

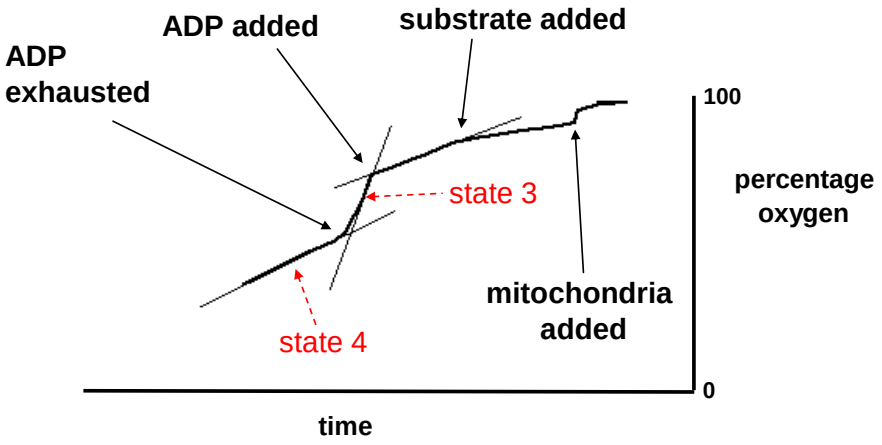

Figure 7.05 Schematic diagram of oxygen electrode recording trace.

Oxygen electrode trace is calibrated to initially record 100% oxygen absorbance. Fresh isolated mitochondria are added to the media and then the respiratory substrate glutamate, malate or succinate. State 3 respiration (maximum oxidative flux) is measured after addition of ADP substrate. State 4 respiration (leakage of extruded protons) is measured as all ADP is converted to ATP. Respiratory control ratio (RCR) is calculated as ratio of State 3 to State 4.

7.2.3 2D-DIGE

Two Dimensional Fluorescence Difference in Gel Electrophoresis (2D-DIGE) is a commercial method available to label and separate proteins using 2-dimensional gel electrophoresis enabling increased reproducibility and accuracy in detecting and identifying proteins (Amersham, GE Healthcare UK Limited).

A total of eight mitochondrial samples were prepared and labelled as per Chapter 2 (pg 59-60):

NS = sham operation for 180 minutes at normothermia (n=2)

NIIR = 60 minutes ischaemia and 120 minutes reperfusion at normothermia (n=3)

HIIR = 60 minutes ischaemia and 120 minutes reperfusion at moderate hypothermia (n=3)

These are samples of the same experimental animals used in the mitochondrial oxygen consumption experiments (Section 7.2.1), however due to financial constraints only 8 labelled samples were permitted and therefore NS samples were limited to 2. First and second dimension separation of the different constituent proteins were then performed as per Chapter 2 (pg 64-70).

The standard consists of an equal, pooled mix of all 8 samples and is labelled with red, Cy2 (CyDye Fluor, Amersham, GE Healthcare UK Limited). For the purposes of illustrating the results a single NIIR sample labelled with blue, Cy3 and a single HIIR sample labelled with green, Cy5 have been selected.

7.2.4 Statistical analyses

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption statistical analyses were performed by one-way ANOVA using mean values ± standard error of mean. Results were then compared by Tukey multiple comparison post-test analyses.

2D-DIGE

2D PAGE gel image analysis was performed by Progenesis SameSpots software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). Limiting criteria were used to identify the most significant changes in protein expression between the three experimental groups NS, NIIR and HIIR, spots that did not change by more than 1.5-fold were excluded. Pixel intensity values are normalised using the internal standard and ANOVA analysis performed (Table 7.01).

7.3 Results

7.3.1 Mitochondrial oxygen consumption

State 3 oxygen consumption, representing maximum mitochondrial oxidative flux remained unchanged between the sham group and that of normothermic IIR. In addition, despite an upward trend, there remained no significant difference between the State 3 oxygen consumption of normothermic and hypothermic IIR (Figure 7.06). State 4 oxygen consumption, representing wasted oxygen consumption (proton leak), also appeared to increase between the sham, normothermic IIR and hypothermic IIR groups however, again there was no significant difference demonstrated (Figure 7.07). Therefore the respiratory control ratio (RCR), the ratio of State 3 to State 4, was unchanged between the 3 experimental groups (Figure 7.08). The DNP rate was also calculated as a measure of the maximum possible rate of oxygen consumption by uncoupling oxidative phosphorylation, but again no significant difference was calculated between the 3 experimental groups (Figure 7.09).

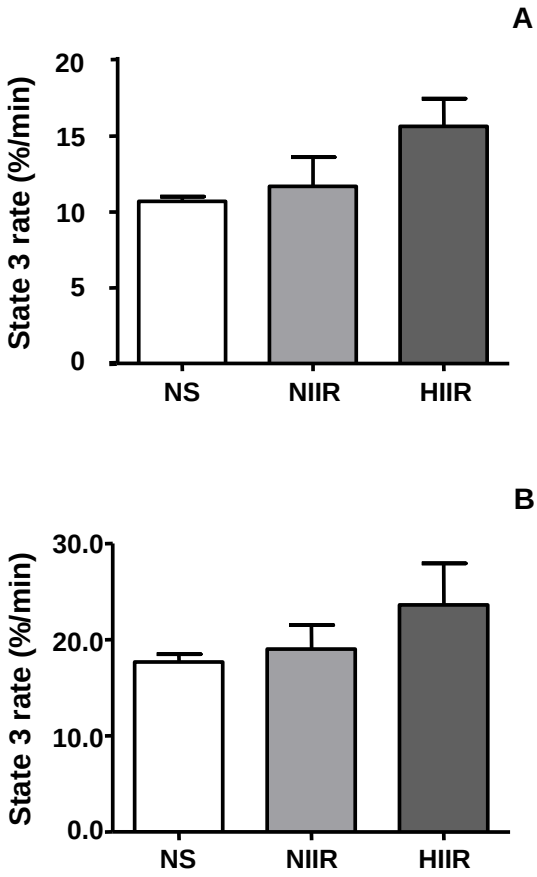

Figure 7.06: Rate of change of State 3 oxygen consumption (% per minute).

The initial substrates were A) glutamate and malate, B) succinate. Results were compared by one-way ANOVA; there were no statistical differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HIIR; hypothermic intestinal ischaemia-reperfusion.

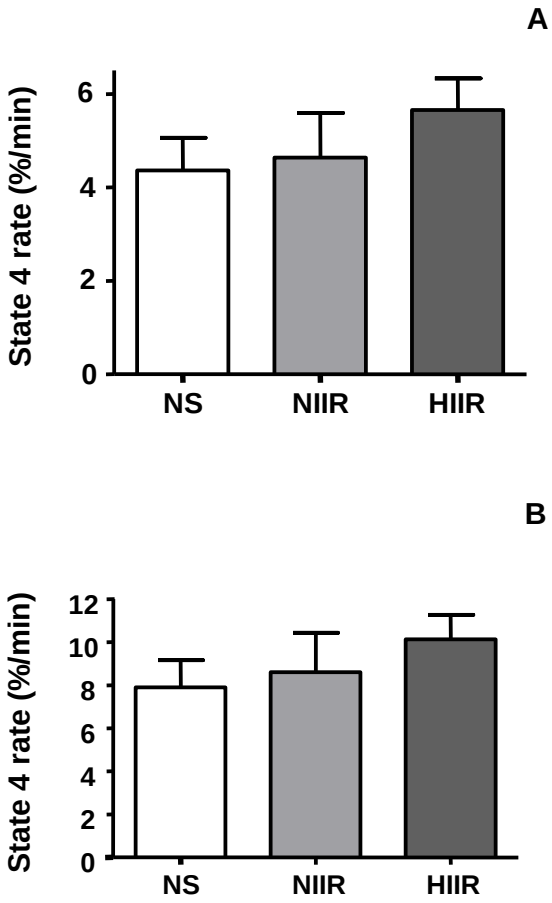

Figure 7.07: Rate of change of State 4 oxygen consumption (% per minute).

The initial substrates were A) glutamate and malate, B) succinate. Results were compared by one-way ANOVA; there were no statistical differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HIIR; hypothermic intestinal ischaemia-reperfusion.

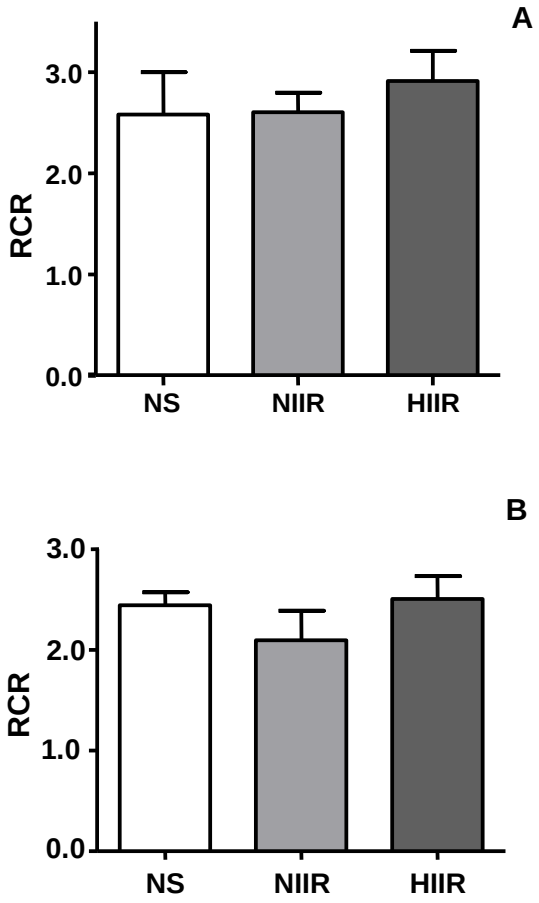

Figure 7.08: Respiratory control ratio (RCR) a ratio of mitochondrial State 3 to State 4 oxygen consumption.

The initial substrates were A) glutamate and malate, B) succinate. Results were compared by one-way ANOVA; there were no statistical differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HIIR; hypothermic intestinal ischaemia-reperfusion.

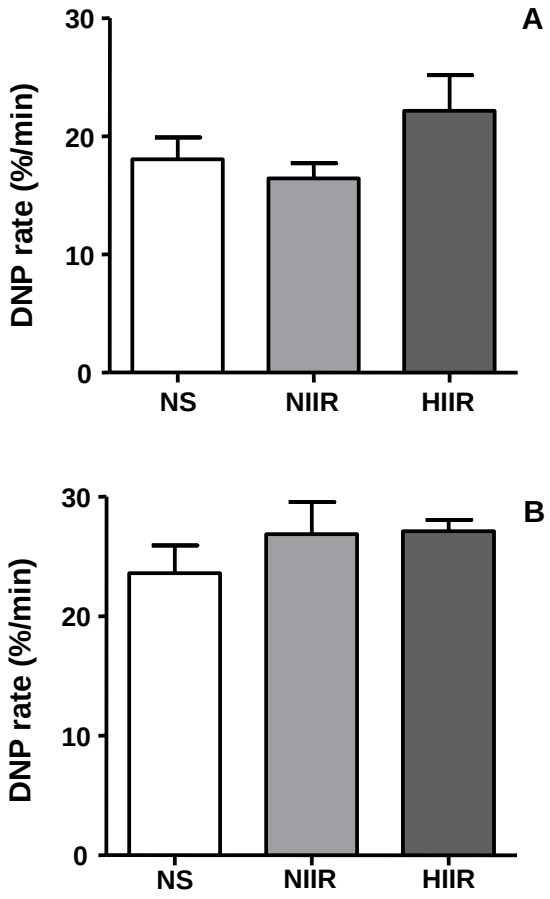

Figure 7.09: DNP rate of change (% per minute).

The initial substrates were A) glutamate and malate, B) succinate. Results were compared by one-way ANOVA; there were no statistical differences between any of the experimental groups. NS; normothermic sham, NIIR; normothermic intestinal ischaemia-reperfusion, HIIR; hypothermic intestinal ischaemia-reperfusion.

7.3.2 2D-DIGE

The resulting 2D-PAGE gels: Figure 7.10 demonstrates the collective standard of all eight experimental samples as described in Section 7.2.3. Figure 7.11 demonstrates a representative 2D-PAGE gel of a single experimental sample of NIIR liver mitochondria (Figure 7.11B representing blue CyDye, Cy 3), Figure 7.12 a representative 2D-PAGE gel of a single experimental sample of HIIR liver mitochondria (Figure 7.12B representing green CyDye, Cy 5). Figure 7.13 demonstrates the 3 overlaid 2D-PAGE gels of Cy dyed mitochondrial samples: combined standard, Cy2, NIIR, Cy3 and HIIR Cy5.

Statistical analysis identified eight 'spots' (areas corresponding to unique proteins) as differing significantly between the three experimental groups NS, NIIR and HIIR (Table 7.01).

Table 7.01 2D-DIGE results

Spot label	**Fold Change**	**P value**
5	1.9	0.008
628	1.8	0.016
7	2.4	0.032
226	1.6	0.033
1	2.6	0.051
779	1.8	0.210
691	1.5	0.241
3	2.1	0.549

Spots were ‘flagged’ as a potential significant change by Progenesis SameSpots software if a fold change between two of the experimental groups exceeded 1.5 fold, ANOVA reveals p values that are recorded as a wide range across the 8 spots selected (0.008-0.549) (Table7.01), all spots ‘flagged by the software program then had their imaging individually reviewed and confirmed by two researchers (myself and WH).

Significantly increased expression occurred following moderately hypothermic conditions (HIIR) in three distinct proteins labelled 1, 226 and 779. Significantly increased expression occurred following normothermic IIR in 3 proteins labelled 5, 628 and 691. Reduced protein expression significantly occurred following moderately hypothermic conditions as compared to the other 2 groups in 2 proteins labelled 3 and 7. By comparison with an online 2D PAGE rat ‘mitochondrial map’, that is a corresponding gel whose ‘spots’ have been previously identified from a 2D-PAGE gel using mass spectrometry (http://bioinfo.nist.gov/hmpd/mito2d2.html courtesy of Dr M. Foutoulakis and Dr F. Hoffman at La Roche Ltd, Basel, Switzerland). The resultant ‘mitochondrial map’ was carried out under similar 2D PAGE conditions and therefore is very similar in appearance to the 2D PAGE gels resulting from the experiments described above. This information then facilitates provisional protein identification of the areas on 2D PAGE gel which have been previously selected by statistical analysis as changing significantly between the experimental groups (Table 7.02, Figure 7.14).

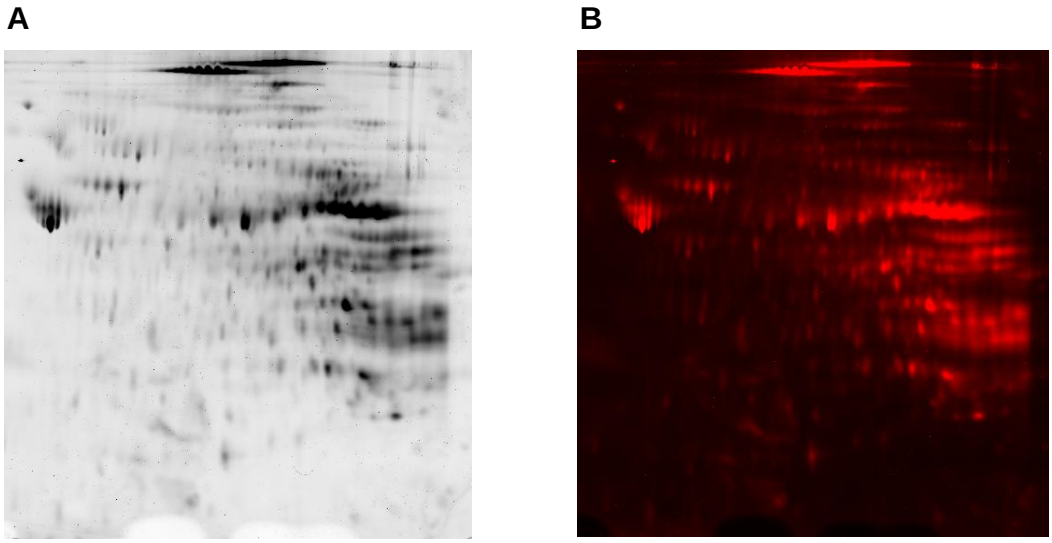

Figure 7.10: Two dimensional PAGE gel collective standard of all 8 sample groups.

The collective standard consisting of all eight sample groups as described in Section 8.2.3, A) conventional black and white photographic representation B) Colour labelled as per red CyDye, Cy 2.

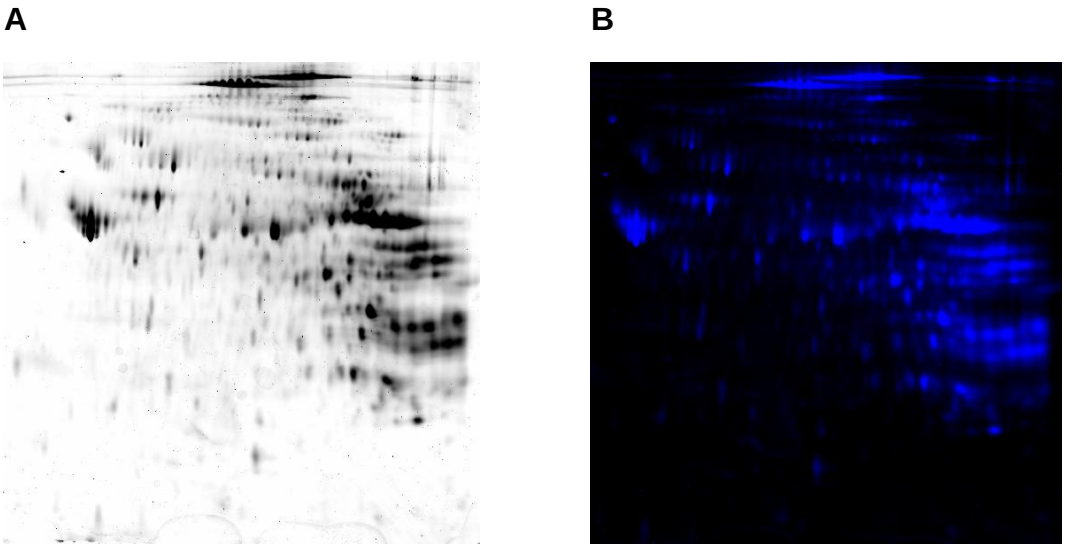

Figure 7.11: Two dimensional PAGE gel of a single sample of normothermic intestinal ischaemia-reperfusion (NIIR) liver mitochondria.

A) Conventional black and white photographic representation B) Colour labelled as per blue CyDye, Cy 3

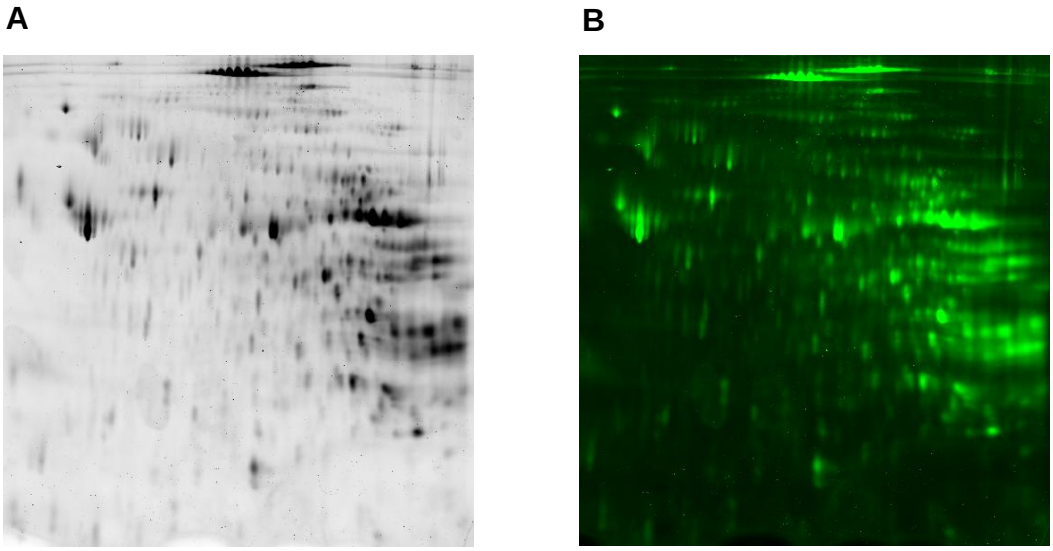

Figure 7.12: Two dimensional PAGE gel of a single sample of hypothermic intestinal ischaemia-reperfusion (HIIR) liver mitochondria.

A) Conventional black and white photographic representation B) Colour labelled as per green CyDye, Cy 5.

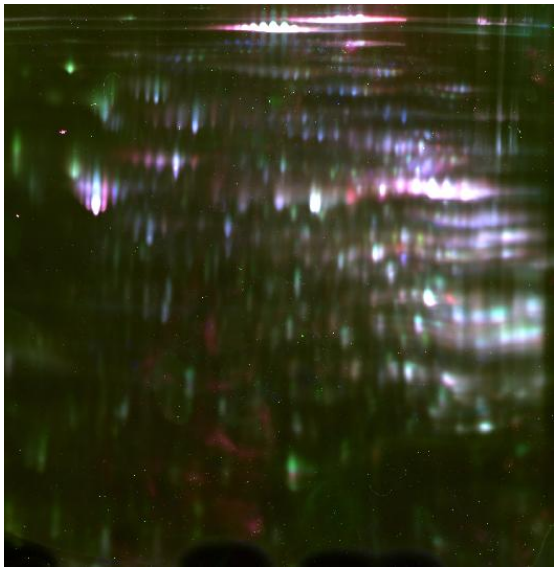

Figure 7.13: Three overlaid two dimensional PAGE gels of Cy dyed mitochondrial samples.

Mitochondria are labelled with Cy dyes: combined standard Cy2, normothermic intestinal ischaemia-reperfusion (NIIR) Cy 3, hypothermic intestinal ischaemia-reperfusion (HIIR) Cy5.

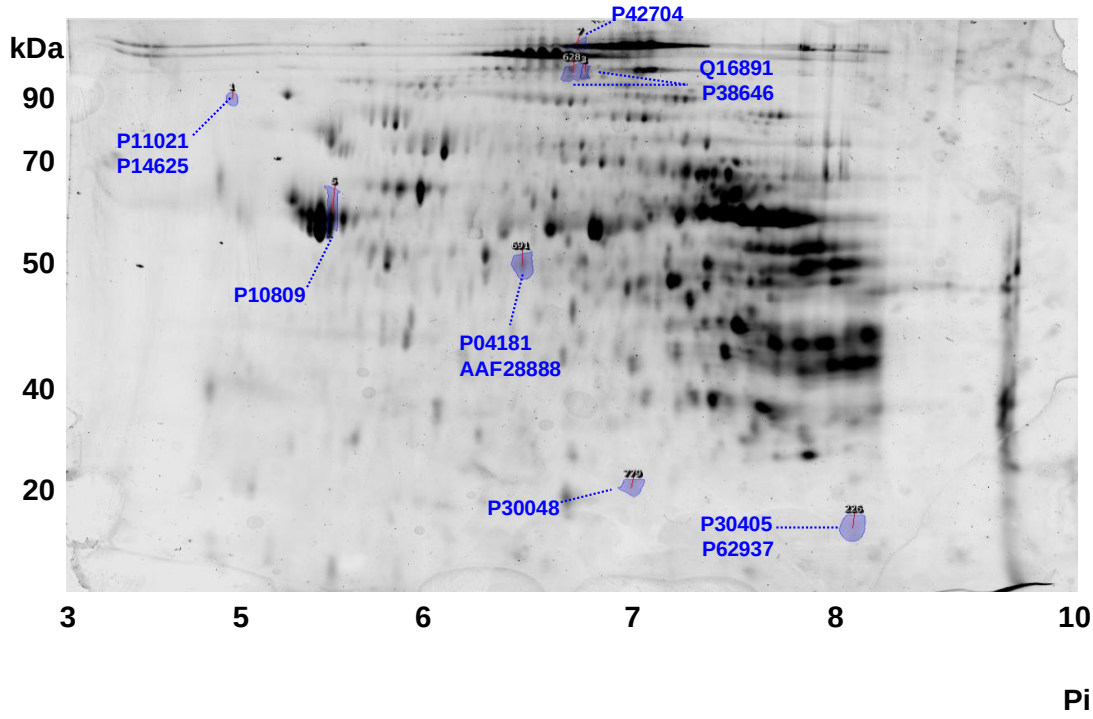

Figure 7.14: Experimental mitochondrial map.

Scanned image of 2D Page gel demonstrates Pi range and molecular weight (kDa). The eight mitochondrial proteins found to differ significantly between the experimental groups and their associated Swiss-Prot identifier number(s) are labelled in blue (see Table 7.01)

Table 7.02 Ontology of 8 mitochondrial proteins following 2D-DIGE

Label	Direction of change in expression	Swiss-Prot identifier (alternative numbers if spot position in dispute)	Recommended protein name	Alternative protein names	Protein molecular function
1	Increased in HIIR	P11021 P14625	· 78kDa glucose-regulated protein · Endoplasmin	· Heat shock 70kDa protein 5 · Heat shock 90kDa protein beta member 1 · 94kDa glucose-regualted protein	· Cellular chaperone · Cellular chaperone · ATPase activity
226	Increased in HIIR	P30405 P05092 (aka P62937)	· Peptidyl-prolyl cis-trans isomerase · Peptidyl-prolyl cis-trans isomerase A	· Cyclophililn F · Cyclophilin A · Cyclosporin A-binding protein	· Accelerates folding of proteins · Accelerates folding of proteins
779	Increased in HIIR	P30048	· Thioredoxin-dependent peroxide reductase	· Peroxiredoxin-3 · Antioxidant protein 1	· Redox regulation of the cell
3*	Reduced in HIIR	Q15092 (aka Q16891) P38646	· Mitochondrial inner membrane protein ·Stress-70 protein	·Mitofilin · 75kDa glucose-regulated protein ·Heat shock 70kDa protein 9	· Mitochondrial inner membrane protein · Control of cell proliferation · Cellular chaperone

Label	Direction of change in expression	Swiss-Prot identifier (alternative numbers if spot position in dispute)	Recommended protein name	Alternative protein names	Protein molecular function
7	Reduced in HIIR	P42704	Leucine-rich PPR motif-containing protein		· Role in RNA metabolism · Translation/ stability of cytochrome c subunits · Transcription regulation
628*	Increased in NIIR	Q15092 (aka Q16891) P38646	· Mitochondrial inner membrane protein ·Stress-70 protein	·Mitofilin · 75kDa glucose-regulated protein ·Heat shock 70kDa protein 9	· Mitochondrial inner membrane protein · Control of cell proliferation · Cellular chaperone
5	Increased in NIIR	P10809	· 60kDa heat shock protein	· 60kDa chaperonin	· Mitochondrial protein import · Cellular chaperone
691	Increased in NIIR	P04181 AAF28888	· Ornithine aminotransferase · Calcium binding mitochondrial carrier protein	· Ornithine-oxo-acid aminotransferase	· Amino-acid biosynthesis · Carrier protein

* denotes experimental 2D-PAGE spot labels with closely related position on the mitochondrial map, both correspond potentially to the same Swiss Prot identifier numbers and therefore the same proteins. Interestingly the protein labelled 3 is significantly increased in hypothermic intestinal ischaemia-reperfusion whereas the protein labelled 628 is significantly increased in normothermic intestinal ischaemia-reperfusion.

7.4 Discussion

Previous experiments have demonstrated that there is a significant increase in the hepatic ratio of inorganic phosphate to ATP following normothermic IIR and that this effect is prevented in experiments performed under moderately hypothermic conditions (Vejchapipat et al. 2001). By extracting and examining mitochondria from animals exposed to each of the experimental conditions, the working hypotheses for this chapter were that IIR influenced hepatic mitochondrial function and altered the mitochondrial proteomic profile.

It is of note that these experiments required the extraction of good quality hepatic mitochondrial samples, the oxygen consumption experiments required fresh, functional mitochondria which was reliably performed following IIR. The 2D-DIGE experiments used in this study had undergone previous freeze-thaws. However, no lower molecular weight protein bias was observed in the subsequent 2D-DIGE analyses which are often indicators of sample degradation. In addition, the experimental gels obtained in this study were very similar in terms of pI/Mr to the proteins observed in the online rat mitochondrial gel. The similarity between the experimental and 'clickable' online database-inked gel, provided significant confidence in the identification of the proteins detected in our experimental gels without the need for subsequent mass spectrometry identification.

Hepatic mitochondrial function was examined in terms of oxygen consumption in the three experimental groups NS, NIIR and HIIR. At this particular time-point of either a sham equivalent of 180 minutes or of 60 minutes intestinal ischaemia and 120 minutes of reperfusion there was no significant difference between the experimental groups of any of the functional measures

used, e.g. State 3 oxygen consumption, State 4 oxygen consumption, respiratory control ratio and DNP stimulated oxygen consumption rate. This finding is surprising given that Vejchapipat *et al.,* found that hepatic ATP levels fell dramatically over the same experimental time period (Vejchapipat et al. 2001).

Oxygen consumption rates are used as a reliable method of assessing mitochondrial function (Exline and Crouser 2008) and it must be concluded therefore that hepatic mitochondrial respiratory function is not influenced significantly by normothermic IIR as compared to sham animals. Moderately hypothermic IIR resulted in an increased level of both State 3 and State 4 oxygen consumption as compared to both sham and NIIR animal but again this was not significant and particularly because of the small numbers of samples in each of the experimental groups no additional conclusions may be drawn from this trend.

There has been previous robust evidence in the same experimental model demonstrating hepatic energy failure in normothermic IIR which has been prevented by induced moderate hypothermia (HIIR). Vejchapipat demonstrated an increased hepatic ratio of inorganic phosphate to ATP following NIIR using real time, *in vivo,* MR techniques, hepatic ATP levels were then demonstrated to be preserved following HIIR as previously described in Chapter 1 (Vejchapipat et al. 2001). Stefanutti also demonstrated significant changes in hepatic ATP, inorganic phosphate and energy charge which confirm these initial findings; these experiments used high performance liquid chromatography (HPLC) techniques (Stefanutti et al 2008). Stefanutti's experiments were conducted using the same experimental model as Vechapipat and those described in this study; however these experiements were designed to investigate hypothermia as a potential 'rescue' therapy so hypothermia was induced later, at the time of reperfusion, and

continued for the rest of the experiment. In these experiements sham operation was for 180 minutes, intestinal ischaemia 60 minutes and reperfusion 120 minutes (Figure 7.15). Despite these important supportive data the results described in this chapter using the same experimental model at an equivalent time-point have failed to demonstrate an influence on hepatic mitochondrial oxygen consumption, there are in turn no histological features of hepatic apoptosis and/or features of hepatic inflammation and no molecular evidence of apoptosis within the liver as described in Chapter 5. These seemingly contradictory results may be explained by a feature of measuring absolute levels of ATP using one technique and subsequently making assumptions of expected balance of synthesis, dysfunction and modes of dysfunction as a consequence. Simply, a measurement of low levels of hepatic ATP does not equate with mitochondrial dysfunction as these experiments have demonstrated. In addition other experiments described in Chapter 5 have also confirmed that low levels of hepatic ATP does not equate with measures of gross cellular-dysfunction and damage. There is similar absence of a histological evidence of cell death and necrosis in post mortem studies from patients with severe MODS (Hotchkiss et al. 1999, Rossi et al. 2007) this fits with clinical findings that organ recovery is the norm in surviving patients without pre-existing organ disease.

Another possible explanation is the influence of AMP-activated protein kinase (AMPK). AMPK acts as a downstream component of a molecular stress response pathway, which senses cellular energy charge, being activated by a rising AMP and a falling ATP. AMPK then acts to preserve cellular viability by reducing anabolic reactions and increasing catabolic actions which increase ATP production. Specifically AMPK stimulates ATP production from glycolysis, fatty

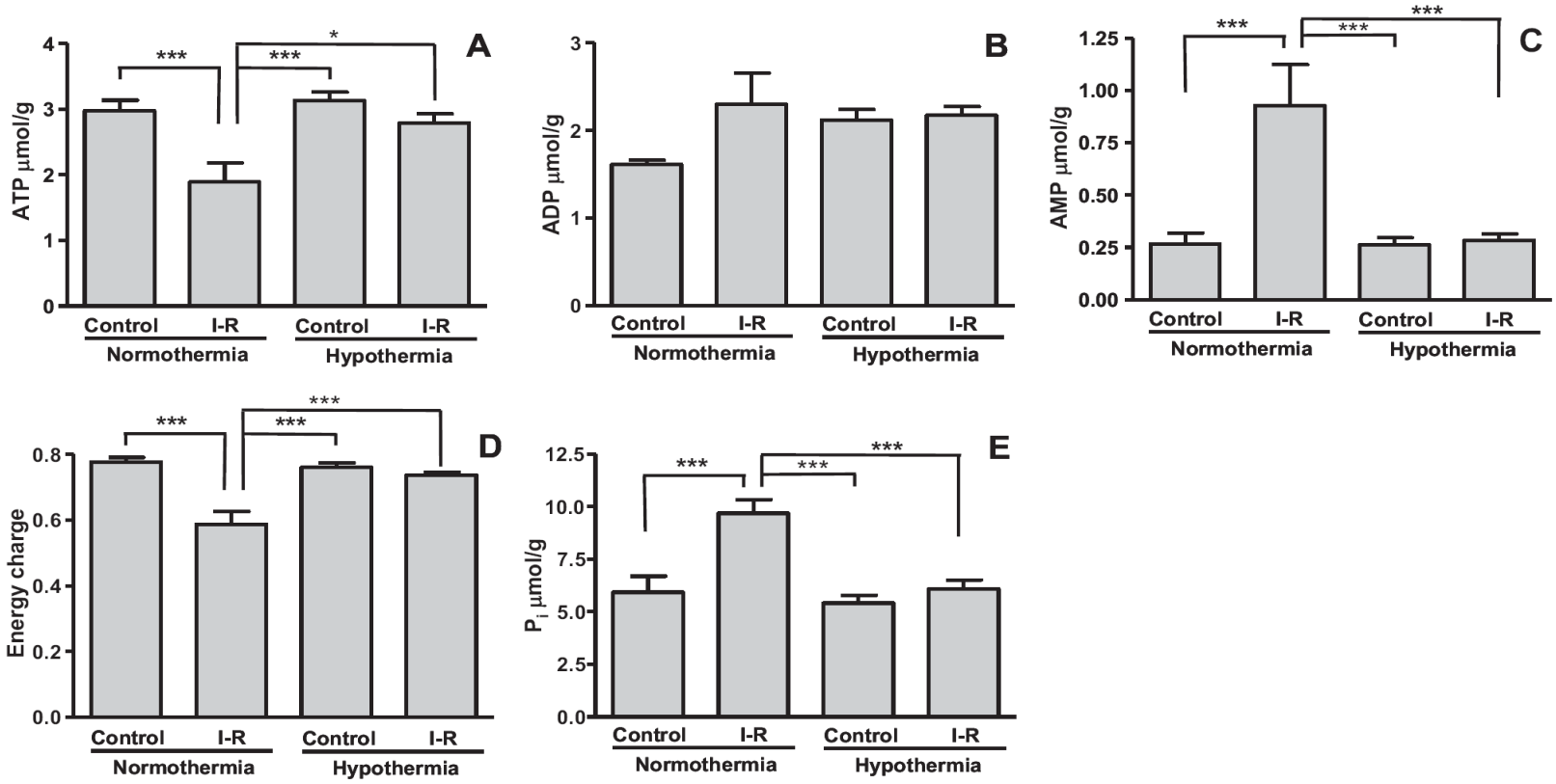

Figure 7.15 Effect of rescue hypothermia on hepatic phosphoenergetics.

Concentration of hepatic (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP), (C) adenosine monophosphate (AMP), (D) energy charge (ATP + [1/2]ADP)/(ATP + ADP + AMP), and (E) inorganic phosphate (P_i). Following sham operation [Control] (180mins) or intestinal ischaemia (60mins) and reperfusion (120mins) [I-R] at either normothermia or moderate hypothermia (n=8 per group). One-way analysis of variance with Tukey post-test was used for group comparison. *p<0.05; ***p<0.001 **(Stefanutti et al 2008)**

acid oxidation and mitochondrial biogenesis and inhibits anabolic pathways such as gluconeogenesis, glycogen, fatty acid and protein synthesis (Lim et al. 2010). There is also evidence that AMPK acts to reduce mitochondrial dysfunction directly during cardiac ischaemia-reperfusion (Young 2008). AMPK is a cytosolic enzyme which, as it is regulated at the post-translation level by reversible phosphorylation, would not have been detected in the previous experiments described. Such post-translational regulatory changes are evidently not detectable by gene array technology (as previously applied to whole liver tissue samples) and a cytosolic enzyme such as this would have been necessarily excluded in the mitochondrial sub-cellular fraction prepared for the proteomic experiments. To examine the influence AMPK between the experimental groups it would be necessary to specifically measure AMPK activity and degree of phosphorylation, this theory is discussed further in Section 8.4 pg 239.

Despite the lack of significant differences between the experimental groups in mitochondrial respiratory function, the molecular component of mitochondria remains an area of great interest because of their central role in energy generation, free radical generation and the regulation of apoptosis. This consequently has a pivotal role in the intricate business of cellular homeostasis.

Previous data has demonstrated that insults such as sepsis do result in changes in both mitochondrial structure and function. For example, electron microscopic images detailing mitochondrial ultrastructure in animal models of sepsis reveal high amplitude swelling of mitochondria (associated with MPT-induced changes) which result in the collapse of the electrochemical gradient and impaired ATP production (Crouser et al. 2004). Crouser et al demonstrated that liver mitochondria are early targets of injury during endotoxaemia (LPS induces

damage to both the inner and outer mitochondrial membrane within 4 hours of injury) (Crouser et al. 2002). In addition, the mechanism of membrane injury appears to differ: the inner membrane is mediated through mitochondrial permeability transition (MPT); the outer membrane appears to occur independently and involve Bax (a proapoptotic member of the Bcl-2 family) (Crouser et al. 2004) In addition, in a rat model of endotoxin mediated sepsis mitochondrial gene expression and levels of expressed protein are altered, for example down-regulation of genes encoding elements of the electron transport chain and glycolysis have been demonstrated (Croner et al. 2010, Young 2008).

Interestingly, the provisional 2D-DIGE data described above demonstrates that some protein groups do appear to change significantly between the experimental groups, the statistics generated by Progenesis SameSpots software aid the speed and accuracy with which several gels may be compared with each other, however these statistical methods also produce p values that would not usually pass conventional tests of significance (e.g. $p > 0.05$). I have included these proteins in my initial analysis of the 2D-DIGE data as the numbers of individual observations are necessarily small and the numbers of significant spot changes are also small. In these experiments the resulting data is therefore treated with circumspection and the results remain provisional. Given these caveats interesting results are revealed as some protein groups of particular contextual interest do appear to change significantly (e.g. hepatic mitochondrial heat shock proteins and a protein important in redox regulation of the cell [thioredoxin-dependent peroxidise reductase]) (Table 7.02). Due to time constraints of this study it has not been possible for confirmation of these suspected findings, this would entail identifying each of the eight ‘spots’ which changed significantly

between the three experimental groups using mass spectrometry/proteomic techniques. However by comparing 'spot' position with a known rat 'mitochondrial map' it is possible to draw initial conclusions regarding the identity of the unique protein position. Table 7.02 lists the eight areas of interest, their respective direction of change of expression between the experimental groups and possible Swiss Prot identifier numbers, in the cases where two possible identifiers are suspected both have been listed and will be discussed further below.

For the purposes of this study, possibly the most striking initial finding is the potential significant differences detected in hepatic mitochondrial heat shock protein expression between the experimental groups. HSP60, HSP70 and HSP90 have been detected to change significantly but the pattern of direction of change of expression of each of these HSPs is extremely complex, for example spot 1 corresponds to either HSP70 or HSP90 and demonstrates increased expression in HIIR, spot 3 may correspond to HSP70 or a structural inner membrane protein but expression is reduced in HIIR and as part of the same cluster of spots position 628 exhibits increased expression in NIIR. Spot 5 corresponds to HSP60 and demonstrates increased expression in NIIR. This complex pattern exhibits the limitations of these results and detailed mass spectrometry analysis would define the exact protein involved, however this data does suggest a variable expression pattern of mitochondrial HSPs between the experimental groups. This is an intriguing possibility as HSPs were initially proposed as a family of possible mediators of protection in hypothermic IIR in the first hypothesis of this thesis (see Chapter 3), experiments described there using Western blotting techniques did not find any significant changes in HSPs between the experimental groups

using whole liver tissue extract. The proteomic studies described have suggested that HSPs active specifically within the mitochondria may still be highly relevant in explaining the mechanism of hepatic dysfunction following normothermic IIR and the protective effect of hypothermic IIR.

Mitochondrial HSPs have several important roles, for example, HSPs facilitate import and sorting of proteins into the various mitochondrial compartments (e.g. the outer membrane, intermembrane space, inner membrane and matrix). Most mitochondrial proteins are encoded by nuclear genes (only 1% of mitochondrial proteins are encoded by the mitochondrial genome in the matrix), synthesised as precursors on cytosolic ribosomes and then imported into the mitochondria by a post-translation mechanism described below (Endo and Yamano 2009, Wiedemann et al. 2004). The translocase of the outer mitochondrial membrane (TOM complex) is the principle entry gate for the majority of nuclear-encoded mitochondrial proteins. After the TOM complex, precursor proteins may follow one of three pathways i) transfer to the presequence translocase of the inner membrane (TIM23) which forms a channel across the inner membrane and cooperates with the matrix HSP70 (mtHSP70); ii) hydrophobic carrier proteins (e.g. the ADP/ATP carrier, the phosphate carrier) are transferred to the protein insertion machinery of the inner membrane (carrier translocase, TIM22 complex); precursors of the outer membrane proteins (e.g. porin) are integrated into the outer membrane by the sorting and assembly machinery (SAM complex). Cytosolic chaperones (e.g. HSP70 and HSP90) bind hydrophobic precursors preventing their aggregation in the cytosol, and then specifically interact with TOM. The matrix HSP (mtHSP70) also acts as a core molecule of the presequence translocase-associated motor (PAM) which drives

completed protein transport into the matrix. HSPs also act directly as chaperones within mitochondria, limit the damage caused by cellular stress and may facilitate cellular recovery and inhibit cell death pathways but also as frequently seen in complex integrative pathways in living organisms act conversely, promoting cell death pathways at multiple levels (Arya et al. 2010). For example, Mitochondrial HSP60 over-expression protects cardiac myocytes from ischaemia-reperfusion injury, through maintaining mitochondrial integrity and function, whereas reduced expression of mitochondrial HSP60 by an antisense oligonucleotide precipitates apoptosis, via release of mitochondrial cytochrome *c* and caspase activation (He and Lemasters 2010, Zong et al. 2002). HSP90 is of particular interest in the mitochondria as it is an ATP-dependent molecular chaperone whose mechanism is not yet fully understood (Leskovar et al. 2010).

Another protein of interest that has been revealed by these experiments, corresponding with spot position 779, is mitochondrial thioredoxin-dependent peroxide reductase which expression is increased in HIIR as compared to sham and NIIR. Thioredoxins are a family of small (approximately 12kDa) redox proteins that undergo NADPH-dependent reduction by thioredoxin reductase and then in turn oxidise cysteine groups on proteins (Powis and Montfort 2001). In mammals there are at least two thioredoxin systems, the cytosolic system (Trx 1, thioredoxin reductase 1 and NADPH) and the mitochondrial system (mtTrx, thioredoxin reductase 2 and NADPH). There is evidence that the redox status of mitochondrial thioredoxin (mtTtx) appears to control the sensitivity to oxidative stress, in addition to the significant ROS production in mitochondria the increased sensitivity of mitochondrial proteins to oxidative injury may explain why mitochondria are particularly susceptible to oxidative injury (Chen et al. 2006). It

may be provisionally concluded that HIIR alters the redox status of hepatic mitochondria by increasing availability of an important component of the redox regulatory mechanism. This is a provisional, but nonetheless, exciting finding and would benefit from further analysis.

Mitochondria consist of 1000-2000 different proteins, most of these are synthesised in the cytosol and imported into the mitochondria. The imported proteins are then further sorted into the four mitochondrial components: the outer membrane, intermembrane space, inner membrane and matrix (Endo and Yamano 2009). Potential limitations of 2D-DIGE results, may be initially explained by the methods used to prepare the mitochondrial samples, it is a concern that each of the 4 mitochondrial components listed above are represented in accurate amounts given the extraction techniques and subsequent storage (samples were kept at -80ºC). This may of course contribute to any bias in the results in terms of the type of proteins detected to change most significantly (i.e. these may not be the biologically most significant but just those that survive in sufficient number to be detected). Additionally often these experiments require further refinement and methodological development to 1) optimise the size of protein captured on the gel, proteins at either end of the molecular weight spectrum may 'lost' from the gel and become false negative results 2) the pH range of focusing strip used may also require further refinement as differing pH ranges enable high resolution isoelectric focusing and clarification of areas of interest. Unfortunately, due to time constraints such further methodological development has not been possible in these experiments. Further experiments to examine this issue specifically would include performing detailed mass

spectroscopy analysis of each detected protein to build up an accurate 'experimental mitochondrial map'.

Such an example of the limitations of such provisional results is illustrated by two spots labelled 3 and 628 (Table 7.02) which can also be seen on the 2D-PAGE gel, Figure 7.14, both spots are so closely related that they may both represent either of two same Swiss Prot identified proteins from their respective positions on the 'mitochondrial map'. These two proteins represent mitofilin (a mitochondrial structural inner membrane protein) or HSP70; however this provisional analysis reveals that spot 3 demonstrates reduced expression in HIIR, whereas spot 628 demonstrates increased expression in NIIR. Further experiments including refining methodology in terms of pH range and focusing criteria in addition to fully defining the protein sequence of each spot would aid more meaningful analysis of these initially contradictory findings.

In summary, fresh hepatic mitochondria extracted from animals exposed to NS, NIIR and HIIR demonstrate no significant difference in oxygen consumption. This leads to the conclusion that hepatic mitochondrial function *per se* is unaffected by NIIR as compared to sham operation and that in turn HIIR does not influence mitochondrial function. Despite this important negative functional data, novel, though highly provisional, findings have demonstrated significant differences in the molecular components of hepatic mitochondria exposed to the same experimental conditions. It does remain possible that IIR induces subtle changes in the molecular components of mitochondrial regulatory systems that may influence ATP synthesis but not influence gross cellular appearance or influence global oxygen consumption. Alternatively, hepatic

mitochondrial ATP generation may be impaired due to the action of extra-mitochondrial factors such as cytosolic AMP-activated protein kinase activity.

Chapter 8

General Discussion

Chapter 8 – General Discussion

Contents

8.1 General discussion

This thesis has examined the influence of moderate hypothermia upon several different parameters measured within the liver in an experimental model of intestinal ischaemia-reperfusion. Focus on the liver as the primary organ of interest stemmed from original research by Vejchapipat et al which identified that moderately hypothermic animals exposed to IIR significantly preserved ratio levels of hepatic inorganic phosphate to ATP as compared to those animals maintained at normothermia. In these experiments normothermic animals exhibited a significant increase in the ratio of hepatic inorganic phosphate to ATP which then immediately preceded death (Vejchapipat et al. 2001). These results lead to a hypothesis that at moderate hypothermia hepatic energy homeostasis is preserved following IIR and that this effect results in prolonged survival.

The initial hypothesis of this thesis was that the action of hypothermia was mediated through differential expression of an important family of protective proteins, heat shock proteins. Initial experiments performed as part of this study, have demonstrated that HSP expression does not significantly change in the liver as a response to IIR and that the protective effects of moderate hypothermia do not appear to be mediated by HSPs. It is of note, however, that these conclusions were drawn from results of experiments restricted to a specific time point of 60 minutes of reperfusion and were all conducted in whole liver tissue. To fully delineate the role of HSPs in this injury and the influence of temperature many more experiments would be required: expanding possible time points; examining specific subcellular fractions of liver tissue (e.g. mitochondria); exploring the actions of perhaps the most relevant HSP to the experimental injury, HSP32 (HO-

1) which was unfortunately not examined as no antibody was commercially available at the time of these experiments but subsequent research has shown it to be highly relevant to this experimental model. Several groups have described induced HO-1 expression in animal models of IIR (e.g. via intraperitoneal cobalt protophorphyrin [CoPP] administration) which results in significantly less intestinal tissue injury as compared to control animals (Wasserberg et al. 2007). Mashregi et al went on to perform gene microarray analysis of murine liver following induction of HO-1 and demonstrated that STAT-3 phosphorylation rapidly occurred together with SOCS3 and other STAT-3 responsive genes (Bcl and ICAM-1) (Mashregi et al. 2008).

A retrospective examination of the original microarray data was performed looking specifically for heat shock protein gene expression revealed a significant change in HO-1 expression, that is NIIR expression of HO-1 was significantly higher than that of NS and HIIR ($p<0.05$, unpaired t-test, volcano plot analysis). There were no significant changes in gene expression of HSP 27, HSPi70, HSC70 across the three experimental groups, this finding corresponds directly with the Western Blotting analysis of the HSPs described in Chapter 3. HO-1 would certainly warrant further investigation in this model and given the siginificant changes in gene expression it could be a highly relevant molecule in terms of explaining the action of hypothermic protection in the liver following IIR. This novel finding also neatly exposes the limitations of gene microarray analysis: despite the carefully conducted experiments and the wealth of data generated the handling and mining of this data remains a delicate process. The Gene Spring software has developed since the original analysis (GeneSpring GX, Silicon Genetics, San Carlos, CA, USA) and this may explain why this finding

was not revealed on the previous K-means clustering analysis and it may be also be the case that previous statistical filters applied were too stringent and may have excluded this biologically highly relevant molecule.

There remains no data available regarding heat shock proteins or STATs in human tissue following NEC. However, recent proteomic data from intestinal tissue in a preterm pig model of NEC has demonstrated an increase in HSP60 after just 8 hours of commencing formula feeding. The authors suggest that stress proteins (such as heat shock proteins) released in response to formula feeding and bacterial colonisation may initiate an inflammatory cascade through the actions of Toll-like receptors (Jiang et al 2011). Interestingly the authors introduce a theory that the differences in these inflammatory signaling pathways between preterm and term infants may explain the differences in tolerating bacterial colonization and enteral feeding in these two groups, this may also in time explain the differences between NEC in premature infants and full-term babies (Siggers et al 2011). It would be interesting to investigate levels of intestinal heat shock proteins across the experimental groups previously described in this study to determine whether IIR or temperature change resulted in differential gut expression despite the fact that no differences were detected in hepatic expression. It would also be interesting to examine STAT activation in gut samples taken at the same time-points.

Returning to the initial findings of a significant increase in liver energy failure in IIR and the significant protective influence of moderate hypothermia reported by Vejchapipat were both reported as significant at 60 minutes of reperfusion. The initial time point of 60 minutes of reperfusion in the experiments in this study correlates with this finding. Therefore, this particular time point was

retained as investigations widened to investigate the presence of a 'stress' response in the liver as a consequence of IIR. Signal Transducers and Activators of Transcription, STAT-1 and STAT-3, were found to be activated (phosphorylated) in the liver following IIR at 60 minutes of reperfusion. In addition moderate hypothermia prevented such activation and leads to the further hypothesis that downstream signalling of these important molecules may explain the deleterious effects of IIR and the protective influence of hypothermia on the same injury. Interestingly, *de novo* synthesis of STAT-3 protein was also significantly increased. These results clearly demonstrate two distinct points 1) the liver responds rapidly to IIR and that a family of important signalling molecules are influenced without requiring the time and energy consuming process of protein synthesis; 2) increased hepatic de novo STAT-3 protein synthesis was also demonstrated following IIR indicating that is some circumstances this may remain an important initial response to such an injury.

The discovery of STAT activation lead to the investigation of two of their most important signalling pathways, apoptosis and the influence of IIR on inflammatory changes within the liver. Histopathological studies failed to demonstrate direct hepatic cellular damage following IIR even at the extended time point of 120 minutes reperfusion. Of particular note there was no increased hepatic inflammatory cell infiltrate following IIR, no evidence of necrosis and immunohistochemical markers of apoptosis failed to demonstrate any increase in programmed cell death. In addition several mediators of apoptosis known to be downstream mediators of STAT activation failed to show any differences between the experimental groups. It may therefore be concluded that increased apoptosis does not occur in the liver following IIR and that this mode of cellular death is not

the principle effect of STAT activation in this injury. This evidence does however correlate strongly with previous studies of both animal models of sepsis and post-mortem samples from patients who died of MODS with the presence of severe biochemical dysfunction (Brealey et al. 2004, Hotchkiss et al. 1999). These studies also revealed a significant disparity between the level of physiological dysfunction and relatively unchanged histological findings as compared to normal controls. This is a largely under-recognised phenomenon that suggests that organ failure is primarily a functional rather than structural event and therefore may potentially be reversible suggesting opportunity for therapeutic exploitation.

The lack of inflammatory cell infiltrate did correlate with previous findings of an indirect measure of neutrophil infiltration which failed to demonstrate a significant difference in liver tissue as compared to lung and gut tissue following the same model of IIR (Vinardi S. personal communication). These findings do contradict previously reported findings in a similar animal model of IIR (Horie et al. 2003). Horie et al reported significant changes in leukocyte accumulation in the sinusoids of the liver lobule. In addition results from this study clearly demonstrate that liver dysfunction (i.e. energy failure) following IIR is not the result of an increased inflammatory cell infiltrate as previously postulated by other groups.

STATs act as transcription factors, therefore to further delineate the downstream effect of their activation a broad analysis of hepatic gene expression profiling was performed by applying gene microarray technology. Confirmatory experiments demonstrated that IL-1ß, LBP and α-2M exhibited increased gene expression following normothermic IIR which decreased in hypothermic animals. Hypothermic animals also demonstrated upregulation of a particular gene, EGF.

Proteins encoded by such genes were also investigated but it proved more difficult to demonstrate significant changes in these effector molecules. However, hepatic IL-1ß protein levels were demonstrated to change significantly between the experimental groups, increased levels of hepatic IL-1ß in the liver following normothermic IIR may represent an increased acute phase response and in turn promote both pro-inflammatory and/or anti-inflammatory changes locally and distally. Hypothermic injury significantly reduces hepatic IL-1ß; this may in part explain the protective action of hypothermia as it attenuates levels of this highly potent and proximal element of the cytokine cascade. However cytokine signalling is a highly complex system with a great deal of inherent redundancy, so termed 'pro-inflammatory' mediators often stimulate a seemingly opposite 'anti-inflammatory' action, it is therefore difficult to draw simple conclusions regarding action and effect.

Interestingly, in addition to these complex interactions with the acute phase response IL-1ß may also have a role in hepatic metabolism and therefore potentially in the pathogenesis of hepatic energy failure following IIR. IL-1ß inhibits ATP synthesis (Berthiaume et al. 2003) and gluconeogenesis (Ceppi et al. 1996) in hepatocytes *in vitro* and as a potent inducer of hepatocyte nitric oxide synthase (Ceppi et al. 1996), IL-1β could potentially influence several other metabolic pathways.

These significant changes in IL-1ß protein levels lead to investigation of the physiological antagonist, IL-1Ra. Normothermic IIR resulted in a significant increase in both IL-1ß and IL-1Ra as compared to sham operation. Hypothermic injury significantly reduced IL-1ß, as compared to normothermic injury, but did not significantly change the levels of IL-1Ra. This may be explained by the

protective influence of hypothermia resulting in a relative increase in the IL-1Ra: IL-1ß ratio, ratios between cytokines and their antagonists are known to be a major determinant for the resultant biological effect in various physiological and pathological scenarios (Maedler et al. 2004). There is evidence that IL-1Ra controls the activity of IL-1ß and improves the survival of animals in disease models such as hepatic ischaemia-reperfusion injury (Shito et al. 1997).

It is however worth noting that an enormous amount of work in other disease models, such as sepsis has clearly demonstrated that highly significant correlations with specific cytokines do not prove causation. For example, IL-6 in septic shock highly correlates with mortality but IL-6 does not cause hypotension or any other pathological changes in sepsis (Dinarello 2000). The influence of IIR on hepatic IL-1Ra: IL-1ß ratio raises more questions rather than providing a simple solution.

By investigating hepatic mitochondria directly it was hoped that the limitations of using whole liver extract would be eliminated and that questions regarding the basis of liver energy failure as a consequence of IIR may be addressed. Fresh mitochondria were used to measure the influence of the three experimental groups (NS, NIIR and HIIR) on mitochondrial oxygen consumption. There was no significant difference between the experimental groups of any of the functional measures used including State 3 and State 4 oxygen consumption, respiratory control ratio and DNP rate. It can therefore be concluded that hepatic mitochondrial respiratory function is not directly influenced by IIR and that a further explanation of the significant changes in hepatic inorganic phosphate to ATP ratio must be sought.

Despite the lack of significant differences in mitochondrial respiratory function the molecular components of mitochondria are of interest as they play a pivotal role in cellular homeostasis. 2D-DIGE experiments revealed provisional data that brings the search for the molecular influences of IIR and temperature back to the beginning of our study, namely those mitochondrial HSPs (HSP60, HSP70 and HSP90) appear to change significantly between the experimental groups. In addition thioredoxin-dependent peroxide reductase, an important redox regulatory protein, is found to have increased levels in HIIR as compared to sham and NIIR. This data is necessarily provisional but it may be postulated that moderately hypothermic IIR is protective because it alters the redox status of hepatic mitochondria by increasing availability of an important component of the redox regulatory mechanism.

8.2 Limitations of this study

There are several limitations to interpreting the data from this study. Firstly, by extrapolating the initial results of experiments in small animals to that of potential therapeutic application humans is highly speculative, however there is further animal (Stefanutti et al. 2008) and clinical data (Hall et al. 2010) that supports induction of therapeutic hypothermia in conditions such as necrotising enterocolitis (NEC). A preliminary clinical study of induced mild hypothermia in preterm infants with advanced NEC has been performed within the Paediatric Surgery Unit at Great Ormond's Street Hospital for Sick Children. Mild hypothermia of 35.5ºC, 34.5ºC and 33.5ºC was successfully induced for 48 hours with subsequent, gradual rewarming. This study demonstrates that induction of

mild hypothermia is feasible and safe in the clinical setting as there was no increase in mortality, bleeding, infection or requirement for inotropic support as compared to non-cooled infants (Hall et al. 2010). Further large, randomised trials are required to further investigate the efficacy of therapeutic hypothermia in infants with NEC together with close monitoring of its safety.

Secondly, all interpretations of the possible mechanisms of hypothermic protection from this study have been as the result of experiments where hypothermia is induced prior to intestinal injury and sustained throughout the experimental period of reperfusion. This does not accurately reflect the majority of clinical scenarios where induced hypothermia may have a potential therapeutic role, such clinical scenarios are likely to involve an element of diagnostic delay both pre- and post presentation to such a specialist unit. There is additional evidence from Stefanutti et al demonstrating that delayed hypothermia, induced at reperfusion following normothermic ischaemia, results in improved survival and prevents cardiovascular failure (Stefanutti et al. 2008). It does however remain a huge transition between translating a pre-injury effect of an experimental manoeuvre into a practical post-injury treatment. In addition to the timing of institution of therapeutic hypothermia, the depth and duration remain important unknown variables. It may be postulated however that the benefit of induced hypothermia will be to institute it as rapidly after acute mesenteric event as possible and that the optimum depth of hypothermia will be established once the balance between risk and benefit is established following large, randomised and controlled clinical trials.

In addition there are significant concerns that by normalising the temperature following a period of mild hypothermia (i.e. rewarming),

inflammation maybe exacerbated as demonstrated by an experimental model of haemodynamic shock (Vaagenes et al. 2003). Stefanutti et al have further developed the same animal model of IIR to incorporate induction of moderate hypothermia following normothermic intestinal ischaemia. Their results demonstrate that hypothermia applied as a 'rescue therapy' abolishes mortality as compared to normothermic animals (Stefanutti et al. 2008). Stefanutti also examined the phenomenon of rewarming, 60 minutes of normothermic intestinal ischaemia was followed by 300 minutes of hypothermic reperfusion and then 180 minutes of rewarming with normothermia gradually being achieved after 60 minutes. Hypothermic animals that were rewarmed all survived to the end of the experimental period. Heart rate and MAP were lower than baseline during the hypothermic phase (i.e. equivalent to hypothermic sham animals, MAP>75mmHg) but returned to baseline levels when rewarmed to normothermia and remained stable until the end of the experiment. In the clinical safety study conducted by Hall et al concentration of plasma cytokines IL-1ß, IL-6 and IL-10 were measured; IL-1ß and IL-10 were significantly reduced following 24 hours of hypothermia, on rewarming IL-10 significantly reduced further, whereas there was no change in levels of IL-1ß. IL-6 did not change significantly either with induced hypothermia or rewarming. This lead the authors to conclude that there was no evidence of a rebound inflammatory response on rewarming following a period of 48 hours of mild hypothermia (Hall et al. 2010).

Thirdly the initial premise of this study is based on the findings of Vejchapipat et al (Vejchapipat et al. 2001), in the same animal model of IIR raised levels of the ratio of hepatic inorganic phosphate to ATP were detected by in vivo phosphorus (^{31}P) magnetic resonance spectroscopy and interpreted as a

measure of hepatic energy failure. This forms the basis of the original hypothesis of this thesis namely that normothermic IIR results in hepatic energy failure, an effect that is totally abrogated in moderately hypothermic animals resulting in prolonged survival. However, on closer inspection is this extrapolation of one measure of ATP synthesis sufficient to draw such a significant claim? In 2008 Stefanutti at al subsequently confirmed and expanded on these findings by investigating phosphoenergetics using high-performance liquid chromatography (HPLC). In normothermic animals hepatic ATP decreased significantly with a corresponding significant increase in AMP and inorganic phosphate. Energy failure was further confirmed in NIIR by a significant decrease in a measure of liver energy charge (i.e. (ATP + [1/2]ADP)/(ATP + ADP + AMP), whereas HIIR completely prevented hepatic energy failure with no change in ATP, AMP, energy charge or inorganic phosphate as compared to control animals. In summary hepatic energy failure is a feature of this experimental model of intestinal ischaemia-reperfusion and this effect is abrogated in hypothermic animals but on the direct study of mitochondrial respiratory function no changes in oxygen consumption can be detected between the experimental groups. Hence there is an apparent paradox – mitochondrial respiratory chain function and oxygen consumption appears to be normal, whereas ATP depletion and energy failure certainly occurs. It is possible that signals and/or the necessary metabolic substrates are not supplied to the mitochondria to increase ATP production. The cytosolic enzyme AMP-dependent protein kinase (AMPK), which is a crucial regulator of cellular energy status, could potentially be damaged by ischaemia-reperfusion injury. Under normal conditions, AMPK, which acts as part of a molecular stress response pathway, is activated by increases in the intracellular

concentration of AMP (Young 2008) (Figure 8.1). In conditions of cellular stress as ATP utilisation fails to keep up with production, ADP therefore increases and 2 molecules of ADP are then able to be converted to ATP plus AMP via the action of adenylate kinase. Raised AMP is therefore a very sensitive and early indicator of compromised energy availability (Hardie et al. 2006). AMP acts as a molecular signal directly regulating metabolic pathways that increase cellular energy generation (e.g. AMP activates glycogen phosphorylase that releases glucose from glycogen and similarly activating phosphofructokinase-1 which regulates the entry of glucose into glycolysis), however by activation of the AMP-activated protein kinase (AMPK) pathway the scope of molecular signalling by AMP is dramatically increased (Young 2008). Activation of AMPK leads to activation of catabolic pathways such as glucose oxidation and fatty acid oxidation, and thus to ATP generation.

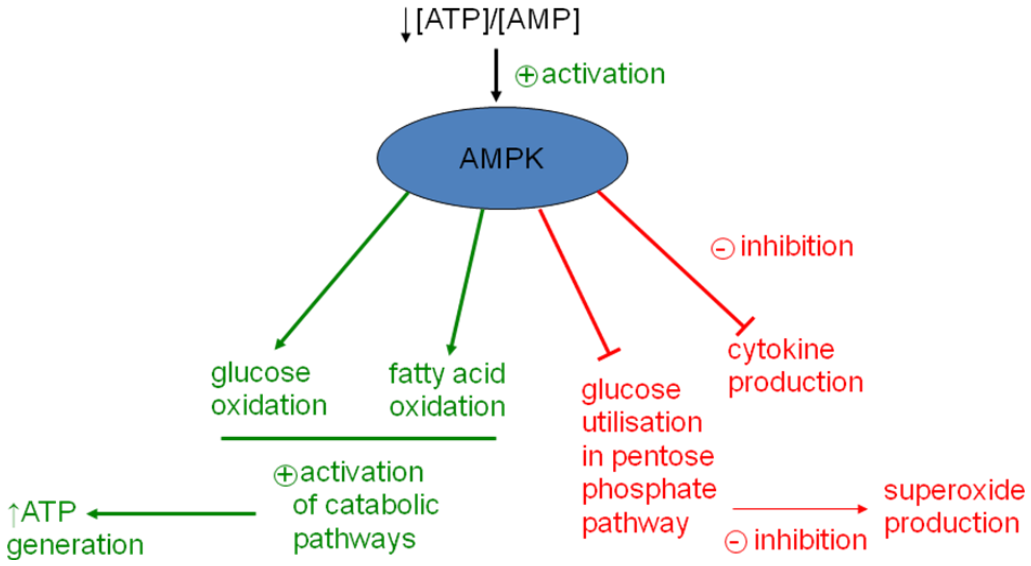

Figure 8.01: Pathways influenced by AMP-activated protein kinase.

Reduction in the ATP: AMP ratio results in AMP- activated protein kinase activation (AMPK). Catabolic pathways such as glucose oxidation and fatty acid oxidation are stimulated resulting in increased ATP generation. Conversely cytokine production and glucose utilisation in the pentose phosphate pathway are inhibited, resulting in increased superoxide production.

In turn, activation of AMPK attenuates anabolic processes such as synthesis of proteins, fatty acids and cholesterol. The known molecular targets of activated AMPK include key enzymes of glucose and lipid metabolism (Carling and Hardie 1989), ion channel activity and the function of specialised organelles (e.g. mitochondrial enzymes and transcriptional coactivators controlling mitochondrial biogenesis) (Winder et al. 2000, Zong et al. 2002). In addition, AMPK is thought to reduce mitochondrial dysfunction during cardiac ischaemia-reperfusion (Young 2008).

In relation to IIR if AMP-activated protein kinase (AMPK) were inhibited by ischaemia-reperfusion injury, then cellular energy failure would be likely to occur, despite mitochondrial function apparently remaining normal, seemingly explaining the paradox of normal mitochondrial activity coupled with energy failure previously described (Lim et al. 2010). Further experiments to address this theory are described in Section 8.4.

The gene microarray results have limitations in terms of their interpretation, the liver as the organ of interest adds to this complexity as in diseased liver the transcriptome may double or triple in size. The cell types that predominate in extracts from whole tissue may overwhelm the small number of biologically highly significant cells (e.g. sinusoidal endothelial cells, stellate cells) and in turn their smaller but highly significant transcripted message. The selection of genes that appeared to warrant particular interest in terms of downstream experiments is a largely subjective exercise and leaves large scope to omit small changes in biologically highly significant genes. There is a profound limitation of microarray technology which recognises that even correctly detected changes in gene expression do not invariably correlate with changes of the corresponding protein. This is why downstream experiments were undertaken to prove changes in the 'effector' protein but with this crude approach the yield of significant results is very poor. In addition, the majority of proteins may be subject to acute post-translational modifications which may profoundly alter activity and/or function independent of gene expression.

The provisional proteomic work is limited in scope and correspondingly the conclusions that may be drawn from such data are also necessarily limited.

However, there is evidence that further experiments are warranted and outlined below.

8.3 Potential mechanisms of therapeutic hypothermia

It may be postulated that prolonged systemic inflammation, with its overproduction of cytokines, reactive oxygen and nitrogen species and associated hypoperfusion-hypoxia, promotes the organism to switch off many of its non-essential energy consuming physiological processes (Brealey et al. 2004). This induced 'hibernation' may be viewed as an attempt to prevent irreversible individual organ damage and protect the organism as a whole (Singer et al. 2004). This phenomenon has been recognised in the heart and it is suggested that this 'dormant phase' is induced via specific cytokine and hormones as part of the acute phase response (Hearse 1997). It may be postulated that induced hypothermia artificially accelerates this naturally protective phenomenon.

It has been previously suggested that the protective effect of induced hypothermia represents a global effect of reducing overall cellular metabolism and is therefore unlikely to be explained by specific cellular actions such as altered gene expression. However, this hypothesis is very difficult to test, as it is difficult to lower metabolic rate without either lowering body temperature or directly affecting individual metabolic processes (and thereby causing alterations in gene expression. In addition using the same experimental model of IIR there is no reduction in the basal rate of production of nitrosative stress products observed between normothermic and hypothermic controls (Stefanutti et al. 2006). Indeed, there is evidence that hypothermia may actively promote protective metabolic

processes e.g. by up-regulating the rate controlling step of production of an endogenous antioxidant (glutathione) within ileum and the liver (Stefanutti et al. 2006).

Experiments in this study have demonstrated a profile of altered gene expression following both normothermic and hypothermic IIR, of note specific gene transcription (e.g. EGF) has been shown to increase following hypothermic injury (see Chapter 6).

There is also associated evidence from recent developments in investigating the mechanisms of another adaptive response to injury common to many species and organs, ischaemic preconditioning (IPC). IPC was initially described as a paradoxical protective effect of the heart; following brief periods of ischaemia the myocardium developed an increased tolerance to subsequent sustained episodes of ischaemia (Murry et al. 1986). IPC has subsequently proved to be a powerful phenomenon, reducing experimental myocardial infarct size being by up to 80-90% (Cohen et al. 2000). Two distinct phases of IPC have been described in the heart: an early phase which lasts from minutes to hours; and a late phase which typically develops after approximately 12 hours and lasts for 3 to 4 days (Millar et al. 1996). The early phase results from rapid post-translational modulation of pre-existing proteins. The late phase is related to upregulation of transcription of cardioprotective genes (e.g. inducible NO synthase, cyclooxygenase-2, aldose reductase and Mn superoxide dismutase) which promote *de novo* synthesis of the relevant proteins (Bolli 2000, Cohen et al. 2000).

The molecular basis of IPC remains incompletely understood. However recent experiments have demonstrated that the JAK-STAT pathway has an

important role in early and late phase of myocardial IPC. Exposure of the heart to brief ischaemia-reperfusion results in rapid tyrosine phosphorylation of JAK1 and JAK2, translocation and activation of STAT-1 and STAT-3 (Hattori et al. 2001, Xuan et al. 2001). Clearly Hattori's work in early IPC has implications for this study's attempt to clarify molecular mechanisms of hypothermic protection in IIR. JAK-STAT signalling has previously been implicated in the relative slow response of translocation of STATs from the cytoplasm to nucleus and subsequent transcriptional activation of genes and protein translation. However early IPC develops in minutes, does this demonstrate an additional protective role of JAK-STAT that may be applicable to IIR injury? (Bolli et al. 2001).

In addition the production of one or more proteins may be implicated in the mechanism of IPC they appear to be involved in modulating the inflammatory response and oxidative stress, e.g. NOS and various HSPs including HO-1 (Tamion et al. 1997). Intestinal IPC has developed significant interest as a potential therapeutic modality in the clinical context of gut transplantation but may also shed light on the mechanisms of intestinal hypoperfusion and the subsequent hepatic acute phase response and development of MODS in other modes of IIR including sepsis and haemodynamic shock.

8.4 Future work

The seemingly contradictory finding in normothermic animals exposed to IIR of no significant differences in hepatic mitochondrial oxygen consumption despite the previous evidence of liver energy failure may be explained by the influence of AMP-activated protein kinase (AMPK). Post-translational

modifications resulting in altered AMPK activity would not have been detected in the microarray experiments conducted in this study. In order to determine whether impaired AMPK-activation could be responsible for the hepatic energy failure, it would be necessary to freeze-clamp liver following IIR and then measure the activity and phosphorylation status of AMPK (Lim et al. 2010).

The proteomic experiments described in Chapter 7 reveal only very preliminary data. These experiments would benefit from further refinement in terms of 2D-DIGE methodology, this would enable the size of the protein captured on the gel to be optimised and increase clarification of the experimental 'mitochondrial map' (see Figure 7.14). In particular, 'spots' of interest may be extracted from the 2D-DIGE gel and exposed to highly sophisticated mass spectrometry techniques enabling accurate identification of each of the specific proteins, this in turn would enable an accurate proteomic profile to be established of the molecular constituents of hepatic mitochondria exposed to the different experimental conditions.

Preliminary data from the mitochondrial 2D-DIGE experiments has revealed that expression of an important redox regulatory protein, thioredoxin-dependent peroxide reductase, is significantly increased in hypothermic IIR as opposed to normothermic IIR or sham operation animals. The thioredoxin system (thioredoxin, thioredoxin reductases, NADPH) act as the major electron donors for enzymes such as thioredoxin-dependent peroxide reductase, by subsequently catalysing the reduction of peroxide this enzyme protects against oxidative stress and apoptosis. Thioredoxins are a highly conserved family of proteins present in most organisms (from Archea to humans), with a wide range of functions including: protecting protein function from redox inactivation and preserving

signalling via redox regulating transcription factors (e.g. NF-κB) (Arner and Holmgren 2000). In addition there is evidence that suggests that thioredoxin reductase itself may be transiently (or permanently) inactivated by raised levels of peroxide, this action of a cellular 'redox sensor' (Sun et al. 1999) may be an important role for the thioredoxin system within the experimental model of IIR and temperature modulation that warrants further analysis.

Functional MRI may be useful to provide a temporal relationship between the development of intestinal ischaemia then reperfusion and the influence over time of temperature and ATP concentration, reduction in ATP turnover and clinical status (Singer et al. 2004)

Preliminary work has been undertaken to develop the clinical application of induced hypothermia in conditions associated with intestinal ischaemia-reperfusion. At Great Ormond Street Hospital a feasibility and safety study was conducted in neonates with a diagnosis of severe necrotising enterocolitis, core temperatures as low as 33.5ºC were demonstrated to be safe and further randomised trials to investigate the role of therapeutic hypothermia in NEC are planned (Hall et al. 2010).

8.5 Conclusion

Normothermic intestinal ischaemia-reperfusion (NIIR) results in significant hepatic energy failure (Stefanutti et al. 2008, Vejchapipat et al. 2001), a phenomenon which is associated with specific molecular events in the liver: activation of an important family of signalling molecules (STAT-1 and STAT-3) and increased protein levels of the cytokine IL-1ß. Hypothermic intestinal

ischaemia-reperfusion (HIIR) attenuates hepatic energy failure and improves survival (Vejchapipat et al. 2001), this important effect is associated with significantly reduced activation of STAT-1 and STAT-3 and an increased IL-1Ra: IL-1ß ratio.

Interestingly the hepatic bioenergetic failure associated with NIIR has been demonstrated to not be associated with evidence of gross hepatocellular dysfunction, i.e. there is no significant difference between NIIR and sham operation in terms of apoptosis, necrosis and inflammatory infiltration. In addition there is no evidence that such bioenergetic failure is associated with impaired hepatic mitochondrial respiratory function. There is preliminary evidence however that mitochondrial protein expression differs between the experimental groups and these results may point towards new avenues of research to define the influence of IIR and temperature modulation on the liver.

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Appendices

Appendices

Contents

9.1 Published papers

Parkinson E, Townsend P, Stephanou A, Latchman D, Eaton S, Pierro A.
The protective effect of moderate hypothermia during intestinal ischaemia-reperfusion is associated with modification of hepatic transcription factor activation.

Journal of Pediatric Surgery 39:696-701, 2004

9.2 Prizes

The *Maria Di Lorenzo* Prize for best basic science research paper
Canadian Association of Paediatric Surgeons 35th Annual Meeting
September 18th-21st 2003, Niagara-on-the-Lake, Canada.

9.3 Presentations

1. Parkinson E, Townsend P, Stephanou A, Eaton S, Latchman D, Pierro A.
Moderate hypothermia attenuates hepatic apoptotic signalling following intestinal ischaemia-reperfusion
35th Annual International Meeting of the Canadian Association of Paediatric Surgeons, Niagara-on-the-Lake, Canada, September 18th-21st 2003, Abstracts book Paper No. 24.

2. Parkinson E, Lawrence K, Stephanou A, Latchman D, Eaton S, Pierro A.
Moderate hypothermia alters hepatic gene expression following intestinal ischaemia-reperfusion.
51st Annual International Congress of the British Association of Paediatric Surgeons, Oxford, UK, 27th-30th July 2004, Abstracts book Paper No. 67.

3. Parkinson E, Lawrence K, Stephanou A, Latchman D, Eaton S, Pierro A.
Protective moderate hypothermia influences hepatic gene expression following intestinal ischaemia-reperfusion injury.
36th Annual International Meeting of the Canadian Association of Paediatric Surgeons, Winnipeg, Canada, 30th September- 3rd October 2004, Abstracts book Paper No. 23.

4. Parkinson E, Lawrence K, Stephanou A, Latchman D, Eaton S, Pierro A.
Hepatic gene expression following intestinal ischaemia-reperfusion is altered by moderate hypothermia.
International Meeting of the American Academy of Paediatrics, San Francisco, USA, 8th-11th October 2004, Abstract book page 52.