Investigating the role of endothelial nitric oxide synthase in liver ischaemia reperfusion injury and direct ischaemic preconditioning using a transgenic double knockout model of endothelial nitric oxide synthase

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I, Gourab Datta 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 he thesis. Where others have contributed to my work, this has been acknowledged.

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Abbreviations

A1AR	A1 Adenosine Receptor
ADP	Adenosine diphosphate
ALT	Alanine Transaminase
ANOVA	Analysis of Variance
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
Bad	Bcl-2 associated death promoter
Bax	Bcl-2 associated X protein
Bcl2	B cell lymphoma 2
CXCL	Chemokine
CXCR	Chemokine Receptor
CO	Carbon Monoxide
ELISA	Enzyme Labelled Immunosobert Assay
EMSA	Electrophoretic Mobility Shift Assay
eNOS	endothelial Nitric Oxide Synthase isoform
$GdCl_3$	Gadolinium chloride
H&E	Haemotoxylin and Eosin
HIF-1α	Hypoxia inducible factor-1α
HO-1	Haem Oxygenase-1
Hmox-1	Haem oxygenase-1 gene
ICAM	Intercellular Adhesion Molecule
IFN	Interferon

IFNR	Interferon Receptor
IH	Immunohistochemistry
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase isoform
IPC	Ischaemic Preconditioning
IR	Ischaemia Reperfusion
IRF-1	Interferon regulatory factor-1
IRI	Ischaemia Reperfusion Injury
iv	Intravenous
IVM	Intravital Microscopy
JNK	c-Jun N-terminal kinase
KO	Knockout
LDF	Laser Doppler Flowmetry
LDH	Lactate Dehydrogenase
L-NAME	N(G)-nitro-L-arginine methyl ester
L-NIL	L-NG-(1-1minoethyl)lysine hydrochloride
MAP	Mean arterial pressure [1/3 x (systolic blood pressure-diastolic blood pressure)]
MAPK	Mitogen-activated protein kinase
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NKT	Natural Killer T cell
NO	Nitric Oxide
NOS	Nitric Oxide Synthase

OLT	Orthotopic Liver Transplantation
PARP	Poly ADP ribose polymerase
PCR	Polymerase Chain Reaction
p-eNOS	Phosphorylated endothelial nitric oxide synthase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
RCT	Randomised controlled trial
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEC	Sinusoidal Endothelial Cell
STAT	Signal Transducer and Activator of Transcription
TCR	T cell receptor
TGF	Transforming growth factor
THI	Total hepatic ischaemia
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UWS	University of Wisconsin Solution
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular endothelial growth factor
WB	Western Blot
WT	Wild Type

THESIS ABSTRACT

Background

Liver ischaemia reperfusion injury (IRI) occurs after prolonged ischaemia followed by reperfusion in the clinical setting, such as liver resection surgery and liver transplantation and is associated with increased morbidity and mortality. Ischaemic preconditioning (IPC) is a therapeutic strategy to lessen liver IR injury. Liver IPC is a mechanical technique whereby a short period of occlusion of the blood supply to the liver confers protection against IR injury. Understanding the molecular mechanisms of IPC offers the possibility of developing techniques and pharmacological agents that will reduce IRI and improve clinical outcomes.

Hypothesis, Aims and Objectives

The molecular mechanisms of liver IPC are not clearly established. Nitric oxide is an important mediator, but the role of the activation and expression of nitric oxide synthase (NOS) and its isoforms endothelial NOS (eNOS) and inducible NOS (iNOS) is unclear. Haem oxygenase-1 (HO-1) protects, but it is unclear if this depends on NOS. There appear to be two phasese of liver IRI and IPC: an early and late phase. There is evidence that NOS has roles in both phases, but that HO-1 has a role only in the late phase. The principal aim was to develop a transgenic eNOS knockout (eNOS-/-) model of liver IRI and IPC to specifically probe the in vivo physiological roles of eNOS and its interactions with HO-1 these processes.

Methods

An in vivo mouse model of partial (70%) warm hepatic ischaemia reperfusion (IR) was used. Normal and transgenic double knockout for eNOS (eNOS-/-) mice were used. A partial warm liver IR model was used where ischaemia was applied to cephalic lobes only followed by reperfusion. Ischemic preconditioning (IPC) consisted of ischaemia applied directly to the cephalic lobes followed by reperfusion then IR to the cephalic lobes. Final reperfusion was either for 2 hours (normal and eNOS-/- mice) representing early phase IRI or for 24 hours (normal mice) representing late phase IRI after which the animals were terminated for tissue and blood samples. The endpoints measured were surface laser Doppler flow to assess liver

microcirculation during the experiment and blood and liver tissue samples at the end of the experiment for serum ALT, liver histological injury scores, Western blotting for eNOS, iNOS, phosphorylated eNOS (p-eNOS), HO-1 protein and RT-PCR for HO-1 mRNA.

Results

1. In this model of early phase partial hepatic IR consisting of 45 minutes ischaemia to the cephalic lobes and 2 hours reperfusion (index IR), there was IRI in normal mice across the three endpoints for IRI of serum ALT, histological injury and microcirculatory dysfunction. IPC consisting of 5 minutes ischaemia and 10 minutes reperfusion (IPC 5/10) preceding index IR reduced IRI across the three endpoints in normal mice. In eNOS-/- knockout mice, there was also IRI across the three endpoints, with greater hepatocellular and histological injury than normal mice, but no difference in the microcirculatory dysfunction compared to normal mice. In eNOS-/- knockouts IPC 5/10 did not reduce IRI across the three endpoints. This indicates that eNOS is a mediator of the protective effects of IPC in early phase liver IRI and baseline eNOS reduces the severity of IRI even without IPC. Based on the differences on the effects on the endpoints between all the experimental groups, it appears that IPC protection is mediated by eNOS in hepatocytes and sinusoidal endothelial cells (SECs) and baseline eNOS protection in liver IR without IPC is mediated by hepatocyte eNOS only.

2. It was demonstrated that in the early phase partial hepatic IR model that both IR and IPC increased eNOS protein expression and eNOS activation by phosphorylation with no additional effect of IPC. This indicates that the protective effect of eNOS with IR alone may at least be partly mediated by increased eNOS protein expression and activation by phosphorylation, but the benefits of IPC in reducing early phase IR injury are mediated by eNOS activation by other mechanisms. Expression of iNOS protein does not play a role in IR injury in our model. Haem oxygenase-1 (HO-1) protein was not expressed in our model, but HO-1 mRNA was expressed in both normal and eNOS-/- animals following liver IR and IPC, indicating that HO-1 expression is not dependent on eNOS. HO-1 may therefore still have a protective effect in the late phase of IR injury acting in a parallel pathway to eNOS.

3. In the model of late phase partial (70%) hepatic IR consisting of 45 minutes ischaemia, recovery from anaesthetic and 24 hours reperfusion (index IR) developed using normal mice only, there was liver IRI across two endpoints of serum ALT and histological injury (microcirculatory dysfunction was not studied in the late phase) with progression of histological injury from mainly sinusoidal congestion to hepatocyte necrosis. IPC consisting of 5 minutes ischaemia and 10 minutes reperfusion (IPC 5/10) preceding index IR reduced IRI across the endpoints. HO-1 protein was detected with late phase IR and IPC, indicating timecourse of early phase HO-1 mRNA expression followed by late phase HO-1 protein expression. This is consistent with the possibility of HO-1 potentially having a protective role in late phase liver IR and IPC.

Conclusions

We have developed a model of early and late phase liver IRI and IPC, which has demonstrated that eNOS is a protective mediator in IRI and IPC in the early phase, HO-1 is activated independently of eNOS and may have a role in late phase IRI and IPC. This model should prove useful in investigating the roles of eNOS and HO-1 in liver IPC and IR injury. This would ultimately be used to identify pathways for development of pharmacological agents that would effectively reduce liver IR injury in the clinical setting and improve patient outcomes related to this.

CHAPTER 1

BACKGROUND:

1.1. Clinical Liver Ischaemia Reperfusion Injury and Therapeutic Interventions

1.1.1. Introduction

Ischaemia reperfusion injury (IRI) is a major cause of hepatic injury following temporary clamping of the hepatoduodenal ligament (Pringle manoeuvre) during liver resection surgery and liver transplantation. This injury is associated with posthepatectomy liver insufficiency and posttransplantation primary nonfunction (PNF) of a graft or initial poor function (IPF) (Huguet et al. 1992; Jaeschke et al. 1996; Rosen et al. 1998; Serracino-Inglott et al. 2001) Even moderate reperfusion injury that does not severely affect the graft can impair recovery of liver function and is a risk factor for patients developing postoperative sepsis and mulitorgan dysfunction or failure (Serracino-Inglott et al. 2001). Steatotic livers are particularly susceptible to IRI with its associated complications (Koneru et al.2002; Selzner et al. 2001). In this context and considering the shortage of suitable organ donors, therapeutic strategies have been explored looking at preconditioning livers to reduce the severity of IRI.

1.1.2. Clinical relevance of Liver Ischaemia Reperfusion Injury

During hepatic resection surgery bleeding from the cut liver surface can be a major problem. It has been well documented that occlusion of the portal vessels by clamping the hepatoduodenal ligament is a safe means of minimising intraoperative blood loss. Although this technique is effective in limiting blood loss, it increases the risk of liver ischaemia with resulting severe liver injury. With prolonged ischaemia, reperfusion can also cause injury which is a distinct entity from the ischaemic injury, called ischaemia reperfusion injury (IRI). Longer periods of continuous warm ischaemia are associated with worse liver IRI which translates into worse postoperative morbidity and mortality (Belghiti et al. 1999; Huguet et al. 1992).

The prolonged ischaemia results in depletion of ATP and accumulation of anaerobic metabolites. On reperfusion, sudden availability of oxygen to ischaemic tissues generates reactive oxygen species which directly injure cells and also activate injurious molecular

signalling pathways. This activates an inflammatory signalling cascade which leads to liver cell and tissue injury. The liver plays an important part in the metabolic stress response, detoxification, immunological function and synthesis of albumin and clotting factors. With severe injury, there is an impaired stress response associated with organ dysfunction, increased risks of sepsis and fluid sequestration and difficulties in dosing drugs metabolised by the liver, such as certain antibiotics like erythromycin and metronidazole. Severe liver injury may be irreversible and can also lead to a systemic inflammatory response and disseminated intravascular coagulation leading to mulitorgan dysfunction then multiorgan failure, with a mortality of 80-100% when three or more organs have failed. Livers with abnormal parenchyma, particularly steatosis and cirrhosis, are more susceptible to IRI with greater risks of liver failure and other postoperative complications, including sepsis and multiorgan dysfunction (Selzner et al. 2001; Belghiti et al. 1999; Huguet et al. 1992; Koneru et al.1992; Nguyen et al. 2009).

In liver transplantation, during procurement of the organ, there is a period of warm ischaemia. The graft is then perfused with and stored in cold preservation solution, typically University of Wisconsin solution. This period of organ ischaemia is cold ischaemia and is followed by a period of warm ischaemia when the graft is being transplanted into the recipient. So, in liver transplantation the IRI that occurs is composed of two separate periods of warm ischaemia and a longer period of cold ischaemia in between before reperfusion of the graft in the recipient. During implantation, when the graft is ambiently exposed to body temperature it is rewarming and the time period while the graft is in the donor body cavity until it is revascularised is the warm ischaemia time. The length of the cold ischaemia time is mainly determined by logistic factors, such as the distance, the facilities available and personnel (Totsuka 2002). The length of the warm ischaemia time is less variable. It depends mainly on the quality and anatomical configuration of the recipient vessels and to a lesser degree the experience of the surgeon. The anhepatic phase is the period of time from clamping of the recipient hepatic vessels to restoration of portal flow, which is closely related to the warm ischaemia time (Figure 1.1.1). Graft reperfusion comprises three phases. Initially the graft is flushed via the portal vein and washed out through the superior vena cava anastomosis which is unclamped. The second phase consists of inferior vena cava and portal vein unclamping. Finally the arterial anastomosis is formed and the native hepatic artery is unclamped to complete graft reperfusion.

Dysfunction of a liver transplant graft varies from a reversible dysfunction (initial poor function, IPF) to liver failure requiring urgent retransplantation (primary nonfunction, PNF). The lack of a clear definition and diagnostic criteria for PNF and IPF has hindered the evaluation of the pathogenesis of these entities and partly explains the wide range of incidences reported in the literature. PNF represents failure of the allograft to function following revascularisation with no discernible cause requiring urgent retransplantation for the patient to survive. Various criteria have been used to define IPF with no consensus, but most definitions use various combinations and cutoffs for deranged serum liver enzymes, coagulopathy and bile output in the early postoperative period typically within the first 7 days (Ploeg 1993; Gonzalez 1994; Heise M 2003). A major risk factor for PNF and IPF of a graft is the cold ischaemia time, especially if it is longer than 13 hours (Burroughs AK 2006). There is evidence that the warm ischaemia time is also a risk factor if it is longer than 45 minutes and the risk is synergistic with a cold ischaemia time of greater than 12 hours for PNF and IPF of a graft (Totsuka 2004). An anhepatic phase of longer than 100 minutes is associated with increased IPF and PNF, which is associated with increased one year mortality (Ijjtsma 2009).

The overall incidence of PNF is 1.7-7.6% with the higher rates seen with donation after cardiac death (Nguyen et al. 2009). Where patients have received an allograft considered to have severe IRI the incidence of PNF is 41% (Rosen et al. 1998). PNF is an indication for super urgent retransplantation, so there is a clear need to understand the mechanisms that underpin liver IRI to guide therapeutic developments. Suboptimal grafts are particularly susceptible to IRI, where grafts with severe steatosis have high rates of PNF (Behrns et al. 1998; Huguet et al. 1994). These complications are associated with increased length of ICU stay, with each extra day in ICU following a liver transplant costing on average £1800 (Foxton 2010). There is some evidence that the severity of IRI is associated with acute rejection and Hepatitis C recurrence (Baron et al. 2000) in an allograft.

The incidence of biliary complications following hepatic resection and transplantation surgery is anywhere between 10-15%, with bile leaks and biliary strictures being the most common (Eghtesad 2005, Mehrabi 2008). Anastomotic leaks occur early in the postoperative course and strictures tend to be late complications. Strictures can be classified as anastomotic and non-anastomotic. Anastomotic strictures are usually related to surgical problems and fibrous healing of the anastomosis. The most common cause of nonanastomotic stricture is ischaemia of the biliary epithelium caused by impaired arterial blood flow, hepatic artery thrombosis and ischaemia reperfusion injury (Sanchez-Urdazpal 1992, Buis 2006).

Liver IRI can occur following prolonged periods of shock where there has been initial inadequate resuscitation or delayed resuscitation. This can occur both in haemorrhagic shock (Raddatz 2006) and in septic shock (Rushing Annals of Surgery 2008). This can lead to multiorgan dysfunction syndrome with an increased mortality. The mechanisms for this are not well understood, but a greater understanding of this phenomenon offers the possibility of pharmacologically modifying the response and improving the outcome. In the context of septic shock, the molecular mechanisms are multifactorial with many parallel pathways so it may be that there will never be a "magic bullet" for sepsis related liver injury and the focus is on first principles of basic resuscitation, early appropriate antibiotics and source control.

1.1.3. Methods of Assessment of Liver IRI

The extent of liver injury in IRI is normally measured by raised levels of serum liver enzymes, most commonly aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and/or serum glutamic-oxaloacetic transaminase (sGOT). There are other conditions that may raise these markers such as intrinsic liver disease, biliary conditions and direct liver trauma and liver sepsis. Other serum markers have been used in research to assess liver IRI, such as serum and liver tissue cytokines TNFα, IFNγ and tissue markers of apoptosis, such as Bcl2 and caspase3 but these are not validated measures clinically and are still very expensive.

Liver IRI is demonstrated histologically. The Suzuki classification, with or without modifications, is the most widely used in liver IRI (Suzuki et al. 1993). In this classification sinusoidal congestion, hepatocyte necrosis and ballooning degeneration are graded 0 to 5. No necrosis, congestion/centrilobular ballooning is given a score of 0 whereas severe congestion/ballooning degeneration, as well as >60 % lobular necrosis is given a score of 5.

Other classification systems have been used based on hepatocyte changes, extent of necrosis, haemorrhage and neutrophil infiltration (Saidi, Chang 2007).

By definition liver IR injury involves a derangement of liver microcirculation. This has been demonstrated physiologically in a number of ways, including intravital fluorescence microscopy, thermodiffusion and surface laser Doppler flowmetry (Klar et al. 1997 and 1999; Koti et al. 2002; Vajdova et al. 2004; Tapuria et al. 2008). Surface Doppler probes placed on the liver surface can be used to assess the liver microcirculation. In the clinical context its use is currently limited to the intraoperative phase, and therefore the very early phase, of liver IRI. One group has used a large animal model of liver transplantation to validate the use dynamic functional MRI (dMRI) to assess liver microcirculation as measurements with dMRI correlated well with laser Doppler flow and thermodillution measurements (Zapletal et al. 2009). This offers the possibility of noninvasively assessing liver microcirculation clinically postoperatively, but its use has yet to be studied clinically.

Overall currently the best way of assessing liver IRI and its severity is by having an overall picture based on a clinical history with evidence, such as patient deterioration, failure to progress and right upper quadrant pain/tenderness especially with evidence of jaundice, deranged liver function tests and histological evidence of liver injury.

1.1.4. Conclusions

Liver IRI is a major clinical problem in hepatic resection surgery, transplantation and shock states. It is associated with increased morbidity and mortality. The diagnosis of liver IRI as a cause of patient deterioration is not straightforward, as there are often multiple factors contributing and depends on a combination of clinical findings and various investigations. With the advances in immunosuppression that have improved long term survival of allografts, acute postoperative complications in liver surgery still pose an important problem with IRI being directly or indirectly the underlying pathophysiology in many of these cases. This type of injury is more severe in marginal grafts. In an era where demand greatly outstrips supply, there is a greater pressure to use more marginal grafts from extended criteria donors. An understanding of the pathophysiology and molecular mechanisms of liver IRI is of great clinical importance, as it will help lead to interventions that reduce this major cause of morbidity and mortality in patients.

1.2. Mechanisms of Ischaemia Reperfusion Injury (IRI)

We have seen that ischemia reperfusion injury is a major obstacle in liver resection and liver transplantation surgery. Prolonged organ ischemia is characterised by lack of tissue oxygenation and depletion of tissue ATP with a transition to activation of anaerobic metabolic pathways, which cannot maintain cellular function for prolonged periods ultimately leading to cell death. Restoration of blood flow is necessary to restore cellular function, but paradoxically reperfusion can initiate a cascade of pathways that cause further cellular injury after prolonged ischaemia. Understanding the mechanisms of liver IRI and developing strategies to counteract this injury will therefore reduce acute complications in hepatic resection and transplantation, as well as expanding the potential pool of usable donor grafts.

The initial liver injury is initiated by reactive oxygen species (ROS) which cause direct cellular injury and also activate a cascade of mediators leading to microvascular changes, increased apoptosis and acute inflammatory changes with increased necrosis. Not all pathways activated are injurous and some adaptive pathways are activated during reperfusion that dampen the reperfusion injury. Classically two phases of liver injury have been described, an early phase of injury (<6 hours), characterised by Kupffer cell and NKT cell activation, release of proinflammatory cytokines and generation of ROS, and a late phase (>12 hours) characterised by neutrophil infiltration and further release of cytokines. In reality, this is a somewhat artificial distinction, as liver injury occurs as a continuum during reperfusion where pathways are activated at various often overlapping time points.

1.2.1 Liver Anatomy and Physiology

The liver is the largest visceral organ in the body. It has a large number of functions. It is important in carbohydrate metabolism and glycogen storage, protein and lipid metabolism. The liver synthesizes a number of proteins, including albumin. It produces coagulation factors II, VII, IX and X and Protein C and S. The liver stores Vitamins A, D and K. The liver breaks down a large number of substances, including hormones such as insulin and drugs such as erythromycin. Red blood cells are broken down in the liver and the biliverdin released is converted to bilirubin, which is excreted by the liver in bile. The liver forms part

of the reticuloendothelial system, containing many immunologically active cells that process and present antigen arriving from the portal circulation.

In relation to IRI, it is important to have an understanding of the basic macroscopic and microscopic circulation of the liver. It receives 75 % of its blood supply from the deoxygenated portal circulation draining gut and other abdominal viscera and the other 25% from the hepatic artery. The oxygen delivery is 50% from the hepatic artery and the other 50% from the portal vein. Occlusion of either portal vein or hepatic artery for a given time results in equivalent liver ischaemia. The blood from the portal vein and hepatic artery enters the liver lobes as left and right hepatic artery and the corresponding portal vein branches and drains the liver lobules through the sinusoids which drain into the central hepatic venules and eventually back to the inferior vena cava via the hepatic veins, which are normally three in number (Fig1.2.1). Sinusoidal endothelial cells (SEC or endothelial cell) line the sinusoids. The Space of Disse which drains bile to bile ductules separates hepatocytes from SECs. Kupffer cells lie within the sinusoids (Fig 1.2.1). The hepatocytes, SECs and Kupffer cells are the three liver cell types that are central to liver IRI.

1.2.2. Liver Ischaemia Reperfusion Models

Liver ischaemia reperfusion injury (IRI) has been demonstrated in different animal models with the various endpoints discussed (See **1.1.3**). The models studied have used either large animals such as pigs or canine or small animals such as rats or mice. Large animal models can be used to replicate surgical procedures and anaesthetic conditions that occur in liver resection surgery and transplantation in humans more closely than small animal models and therefore act as a good indicator of the pathophysiological insults that occur in humans. Experiments on large animals however are very costly, labour and time intensive. Hence, small animal studies have usually been used to develop models of liver IRI and probe and study molecular mechanisms underlying it and accounts for the vast majority of our understanding of liver IRI.

A wide range of transgenic animals has been generated for mice. For reasons that are not fully understood, transgenic large animals are difficult to generate and rarely survive to term. Therefore, few are available. Transgenic mice, particularly genetic knockout models, have become a powerful tool at elucidating mechanisms of liver ischaemia reperfusion injury and are complementary to pharmacological studies and I will focus on mechanistic insights derived from transgenic knockout models of liver ischaemia reperfusion injury. This is because there is very specific targeted disruption of the protein at the genetic level rather than disruption that is not necessarily as specific as maintained when "specific" pharmacological inhibitors or stimulants of the same protein are used. Where there are discrepancies in results with knockout models or particular gene knockouts have not as yet been used to study liver IRI, large animal models, such as pig or canine models, of liver IRI have provided mechanistic insights into what is likely to occur physiologically in human liver IRI.

The animal ischaemia reperfusion (IR) models studied have used global or partial hepatic ischaemia followed by reperfusion with or without liver resection only or liver transplantation. Global hepatic ischaemia results in more severe liver IRI and replicates the conditions during major liver resections and liver transplantation. This has been demonstrated in large animal models with warm ischaemia time (WIT) of up to 2 hours using passive bypass during ischaemia (Uhlmann et al. 2003,), although most studies used a WIT of around 30 minutes without bypass, and cold ischaemia times up to 16 hours (Uhlmann et al. 2006; Brockmann et al. 2005; Qing et al. 2005). Similar results have been shown in a large number of small animal studies using global hepatic ischaemia with warm ischaemia times of up to

60 minutes is tolerated in rats and up to 30 minutes in mice (Shinohara et al. 1990; Minor et al. 1992; Suzuki et al. 1997; Shimamura et al. 2005). Partial hepatic ischaemia prevents splanchnic congestion that leads to gut oedema, therefore allowing decompression of the portal venous flow through perfused liver lobes. This in theory more specifically probes the pathophysiological mechanisms of liver IRI without interactions from effects on the gut influencing results, as well as replicating conditions in partial liver resection surgery. The animals tolerate longer periods of partial hepatic ischaemia and the liver IRI is less severe than for the equivalent global hepatic ischaemia times. This has been demonstrated in large animal models with ischaemia times of 1 to 2 hours and reperfusion of 5 hours to 4 days (Helling et al. 1999; Kannerup et al. 2010). Similar results have been demonstrated by the large number small animal studies of partial liver ischaemia reperfusion with ischaemia times of up to 2 hours in rats and 1 hour in mice (Metzger et al. 1988; Caldwell et al. 2005). The small animals studies of global and partial hepatic IR will be discussed in the subsequent sections with reference to mechanisms of IRI focusing on transgenic mouse models.

1.2.3. Generation of a Genetic Knockout Organism

Evans and Kaufman (Evans et al. 1981) and Martin (Martin et al. 1981) separately described the growth and maintenance of euploid cells in culture obtained from normal mouse embryos that had pluripotent potential, subsequently known as embryonic stem cells (ES cells). These cells were shown to be capable of giving rise to germline chimera after microinjection into blastocysts (Bradley et al. 1984). Thus, normal diploid ES cells could be grown in culture for multiple passages that could still contribute to normal development. The ES cells were also shown to form the germline with high frequency (germline competence). The haploid genomes of these cells were transmitted to the next generation. This set the stage for the introduction of mutations in these cells to produce mice with a targeted alteration in its genome.

In an elegant series of papers using mammalian cell lines (Lin et al. 1985; Smithies et al. 1985; Thomas et al. 1986), based primarily on prior work in Saccharomyces cerrivisae (a species of budding yeast), it became clear that cloned DNA could be precisely altered in vitro, and when introduced into cells via a number of methods (infection, transfection vector) would homologously recombine with the resident gene and introduce the desired mutation at that site in the genome. Homologous recombination in this context is a process of genetic recombination that occurs between similar sequences of DNA during mitosis or repair of

damage to DNA sequences. In higher eukaryotes (Drosiphilia and mouse), unlike in lower eukaryotes (such as yeast), the frequency of homologous recombination occurs at a low rate. This is improved by the use of the combination of positive and negative selection. In positive selection, these rare events of homologous recombination are selected for by introduction of genes conveying resistance to otherwise toxic metabolites, such as neomycin. This method is frequently used to mutate the gene of interest. The frequency of these rare events can further be increased by negative selection, most commonly performed by using a thymidine kinase (TK) gene flanking the targeting vector. ES cell clones which retain the *TK* gene (nonhomologous recombination) do not survive the addition of gancyclovir (or homologues) to the media due to the accumulation of toxic nucleosides, whereas cell clones which have undergone an authentic recombination lose the *TK* gene. This combined strategy is known as *positive-negative* selection (Mansour et al. 1988) and routinely increases the targeting frequency by at least an order of magnitude.

Once these *mutated* ES cells are isolated as a pure clone, they can be introduced into the blastocoele cavity of a normal embryo, where they participate in the development of all tissues and result in the production of chimeras (usually assessed at birth by acquisition of the dominant coat color phenotype Agouti). In matings of these chimeric mice with normal wild type mice, if the ES cells have contributed to formation of germ cells in the chimeras, half the offspring are heterozygotes for the mutated gene. Mating heterozygotes, each carrying a mutated copy of the gene of interest (detected by analysis of the isolated DNA), produces homozygotes in a quarter of the offspring. If these homozygotes have a targeted gene *deletion* they are knockout mice for that gene, and hence the protein it encodes. This whole process is known as gene targeting and is currently the best way of producing knockout organisms.

Understanding the cellular and molecular mechanisms of liver IRI offer the possibility of minimising its debilitating effects in the clinical setting. The attraction of using genetic knockouts to study models of liver IRI is that knockouts can provide robust mechanistic insights, as the disruption is specific and complete at the genetic level rather than relying on pharmacological agents that act on proteins but may not be as specific as advertised. To this effect, genetic knockout models have been used to study liver IRI as will be discussed in sections 1.2.3 to 1.2.17.

1.2.4. Reactive Oxygen Species (ROS)

Depletion of intracellular and extracellular ATP during ischaemia results in increased ATP degradation products, including adenosine, hypoxanthine and xanthine and a shift towards anaerobic metabolism. On reperfusion, initially the increase in oxygen delivery exceeds the rate at which cellular metabolism returns to aerobic pathways, that generates damaging free radicals (ROS). A wide variety of ROS are generated, the most widely implicated being superoxide, hydrogen peroxide and nitric oxide.

There are thought to be three main pathways for the generation of ROS: conversion of xanthine dehydrogenase to xanthine oxidase during ischaemia, NADPH oxidase activation and uncoupling of the mitochondrial electron transport chain (Andrukhiv et al. 2006; Jaeschke et al. 1993). Although hepatocytes can directly produce ROS, physiologically Kupffer cells are thought to be the main source of ROS in the early stages of liver IRI with NKT cells generating ROS later and neutrophils being the main source in the very later stages (Andrukhiv et al. 2006; Jaeschke et al. 1993). The role of these various cells, NADPH oxidase and mitochondrial depolarisation have been supported by knockout animal models (Ozaki et al. 2000; Theruvath et al. 2008). No xanthine oxidase knockout studies have been used on liver IRI. These mice only survive up to 6 weeks and are runted. Intriguingly, one study of XO deficient rats in intestinal IRI showed no role for XO. Another pathway for the generation of ROS is dependent on Fe^{2+}, a byproduct of haem oxygenase-1 which is activated by IR injury, called the Haber-Weiss reaction. The first step of the catalytic cycle involves reduction of ferric ion to ferrous:

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$$

The second step is the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

Net reaction:

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

1.2.5. Microcirculatory dysfunction

Microcirculatory changes play an important part in hepatic IRI. Reduction in sinusoidal diameter and blood flow are among the earliest changes in reperfusion injury. This results from a combination of direct damage to sinusoidal endothelial cells (SECs) with ischaemia

and reperfusion, vasoconstriction mediated by vasoactive substances and expression of adhesion molecules with accumulation of platelets and leucocytes.

Two of the key vasoactive substances that maintain sinusoidal vascular tone are endothelin-1 (ET-1), a vasoconstrictor, and nitric oxide (NO), a vasodilator and inhibitor of platelet aggregation. There appears to be a relative excess of ET-1 in the early stages of liver IRI. Large animal models of orthotopic liver transplantation using pigs have provided evidence that after reperfusion Kupffer cells are activated which leads to increased release of ET-1 which binds to SEC and hepatocyte ET(A) receptor, thereby reducing hepatic micro and macroperfusion after ischaemia and during reperfusion with associated increased liver injury (Frankenberg et al. 2005; Uhlmann et al. 2006). The activation of this pathway is associated with increased expression of TNF α, IL 6 and endothelial NOS (eNOS) (Frankenberg et al. 2005; Uhlmann et al. 2006). Knockout models for ETB receptor or heterozygote knockout for endothelin-1 have not been studied in liver ischaemia reperfusion injury. Double knockouts of endothelin-1 (ET-1), ET-2 and ETA receptor are lethal pre- or perinatally. It has become apparent products of haem oxygenase, namely carbon monoxide (CO) and biliverdin, are likely play a role in reducing the severity of liver IRI in vivo at least partly by directly improving liver microcirculation (Katori et al. 2002 x2; Miyagi, Iwane et al. 2008).

1.2.6. Cell injury and death

Hepatocytes and SECs are the two main cell types that are injured in IRI. Hepatocytes are more sensitive to warm ischaemic injury ($37^{o}C$), while SECs are more sensitive to cold ischaemia ($4^{o}C$) found in cold preservation of donor liver grafts before transplantation. Physiologically, exclusive injury of one cell type is not found and there is evidence that both cell types have been injured **directly** in both cold and warm IRI by ROS. The SECs and Kupffer cells are actively involved in initiating and maintaining IRI, the former mainly through expression of adhesion molecules activating inflammatory pathways and the latter through antigen independent activation of CD4+ T cells.

There has been debate about what the primary mode of cell death is in liver IRI: apoptosis or necrosis. Apoptosis is an energy dependent process, so in theory when there is greater depletion of ATP, necrosis should dominate. Also, necrosis takes longer to become apparent, normally more than 3 hours. This is challenging to show experimentally in vivo, as tissue ATP before and after reperfusion would need to be measured as well as the change in

metabolic state of the cell. Therefore, varying degrees of necrosis and apoptosis have been shown in the literature for given ischaemia reperfusion protocols.

Different assays have been used to implicate apoptosis, including activation of various genes such as caspase-3 which is thought to be a specific indicator of apoptosis, and Bax. One isolated ex vivo perfused liver model using knockout of Bax showed reduced liver IRI (Table 2), apoptosis and caspase-3 activation in the knockouts compared to the normal wild type livers (Ben-Ari et al. 2007). The TUNEL assay has been used to indicate apoptosis, but it now appears that it does not specifically distinguish between apoptosis and necrosis. Many of the same initiators and pathways are involved for both types of cell death, so there is much overlap. Some authors refer to the process as necro-apoptosis.

1.2.7. Adhesion Molecules

The adhesion to the hepatic sinusoidal endothelial cells and transmigration into liver tissue require sequential steps in which many molecules are involved. The selectin family (P- E- and L-selectin) of adhesion molecules is expressed by SECs early in reperfusion. They mediate loose or rolling adhesion of platelets and leucocytes. Knockout models indicate that there is an initial peak of P-selectin expression 20 to 30 minutes after reperfusion which is required for early IRI (Sawaya et al. 1999; Singh et al. 1999). Functionally, some groups have found that E-selectin expression, and not P-selectin, is required for IRI to occur (Young et al. 2001). This is followed by firmer adhesion of leucocytes on SECs by upregulation of integrins, such as Mac-1 and LFA-1, and intercellular adhesion molecules (VCAM-1 and ICAM-1), respectively (Table 1.2.1).

1.2.8. Platelets

Platelets and leucocytes begin to adhere to SECs within 5 minutes of reperfusion (Table 1.2.1). Khandoga et al. used an ICAM-1 knockout model of liver IRI to show that the very early phase of IRI characterised by increased lipid peroxidation, apoptosis and reduced sinusoidal perfusion, depends on platelet rather than leucocyte adhesion, mediated by fibrinogen deposited on adhesion molecules: E-selectin, VCAM-1 and ICAM-1 on SECs (Khandoga et al. 2002).

1.2.9. Neutrophils

Neutrophils are important cellular mediators of liver IRI after 6 hours of reperfusion (Hines et al. 2002; Kawachi et al. 2000). The neutrophil oxidative burst is the main source of reactive oxygen species in the later stages of IRI and contributes directly to necrosis and apoptosis. This has been supported by immunologically deficient knockout models of liver IRI (Shimamura et al. 2005; Ozaki et al. 2000). Neutrophil recruitment is mediated, at least in part, by MIP-2 binding to the chemokine receptor CXCR2 on neutrophils, supported by a study using a CXCR2 knockout model (Kuboki et al. 2009). Neutrophil transmigration within liver sinusoids occurs over fibronectin (Hamada et al. 2009). A complex mixture of substances including cytokines, chemokines and adhesion molecules produced by other leucocytes and various liver cell types coordinates these responses. These will be discussed further.

1.2.10. CD4+ T cells: NKT cells and αβ T cell receptor (TCR) T cell

CD4+ T cells, but not B cells or CD8+ T cells (Caldwell et al. 2005) or NK cells (Kuboki et al. 2009; Shimamura et al. 2005) are activated and recruited into liver sinusoids in liver IRI (Figure 1.2.2). They have a dual role either contributing to injury or reducing the extent of injury depending on the CD4+ subtype and mechanism of cellular activation (Table 3). The majority of CD4+ T cells can be subdivided into αβ TCR (the most common subtype) expressing cells, γδ TCR expressing cells, NKT cells and regulatory T cells (Treg).

NKT cells contribute to liver injury in the early stages from 1 hour of reperfusion onwards. This has been supported by immunologically deficient knockout models, such as nu/nu and CD1d knockout mice (Shimamura et al. 2005; Kuboki et al. 2009; Lappas et al. 2006). NKT cells are also thought to contribute to neutrophil activation mediated by cytokines they release, such as interferon gamma (Lappas et al. 2006). A study using T cell subtype specific knockouts showed Treg cells are not involved in IRI and γδ TCR T cells recruit neutrophils but this does not affect the severity of IRI (Kuboki et al. 2009).

There is a large body of evidence that CD4+ T cell activated in IRI is by antigen independent pathways (Huang et al. 2007). This is supported by knockout models, in which activation of the Toll-like receptor 4 (TLR 4) on Kupffer cells is most consistently implicated (Shen et al. 2007). One knockout model showed that CD4+ T cell related liver IRI depended on costimulatory activation of the CD4 with the CD154 receptor on activated T cells (Shen et al. 2002). There is emerging evidence that there are also antigen dependent pathways activated in liver IRI. One model using a knockout for a TCR specific for ovalbumin self antigen

showed reduced IRI in the knockout, indicating that a small subset of T cells sensitised to self antigen contribute directly to liver IRI at least up to 8 hours into reperfusion (Kuboki et al. 2009).

CD4+ T cells of the αβ TCR variety are recruited into liver sinusoids within 1 hour of reperfusion. CD4+ T cell knockout models of liver IRI with adoptive transfer of functional CD4+ T cells into the knockout mice indicate that these cells are involved in neutrophil recruitment via cytokines such as interleukin 17 (IL 17) and MIP-2, but these T cells inhibit the neutrophil oxidative burst. Overall they reduce the extent of liver IRI both indirectly via cytokines they release affecting other leucocytes and directly acting on hepatocytes (Caldwell et al. 2005).

Therefore, in summary NKT cells contribute directly to early liver IRI and indirectly to late IRI by recruiting neutrophils. αβ TCR CD4+ T cells are activated by Kupffer cells by an antigen independent mechanism, which recruits neutrophils into liver sinusoids, but these CD4+ T cells inhibit the neutrophil oxidative burst and overall reduce the severity of IRI. A small subset of CD4+ T cells activated by self antigen contribute to IRI.

Figure 1.2.2: Schematic diagram of cellular mechanisms of liver IRI within a liver sinusoid and the surrounding area containing hepatocytes. Initial sinusoidal perfusion failure from platelet plugging, then Kupffer cells activate CD4+T cells that activate NKT cell which cause SEC and hepatocyte injury, followed by neutrophil activation, adhesion and transmigration causing more cell injury.

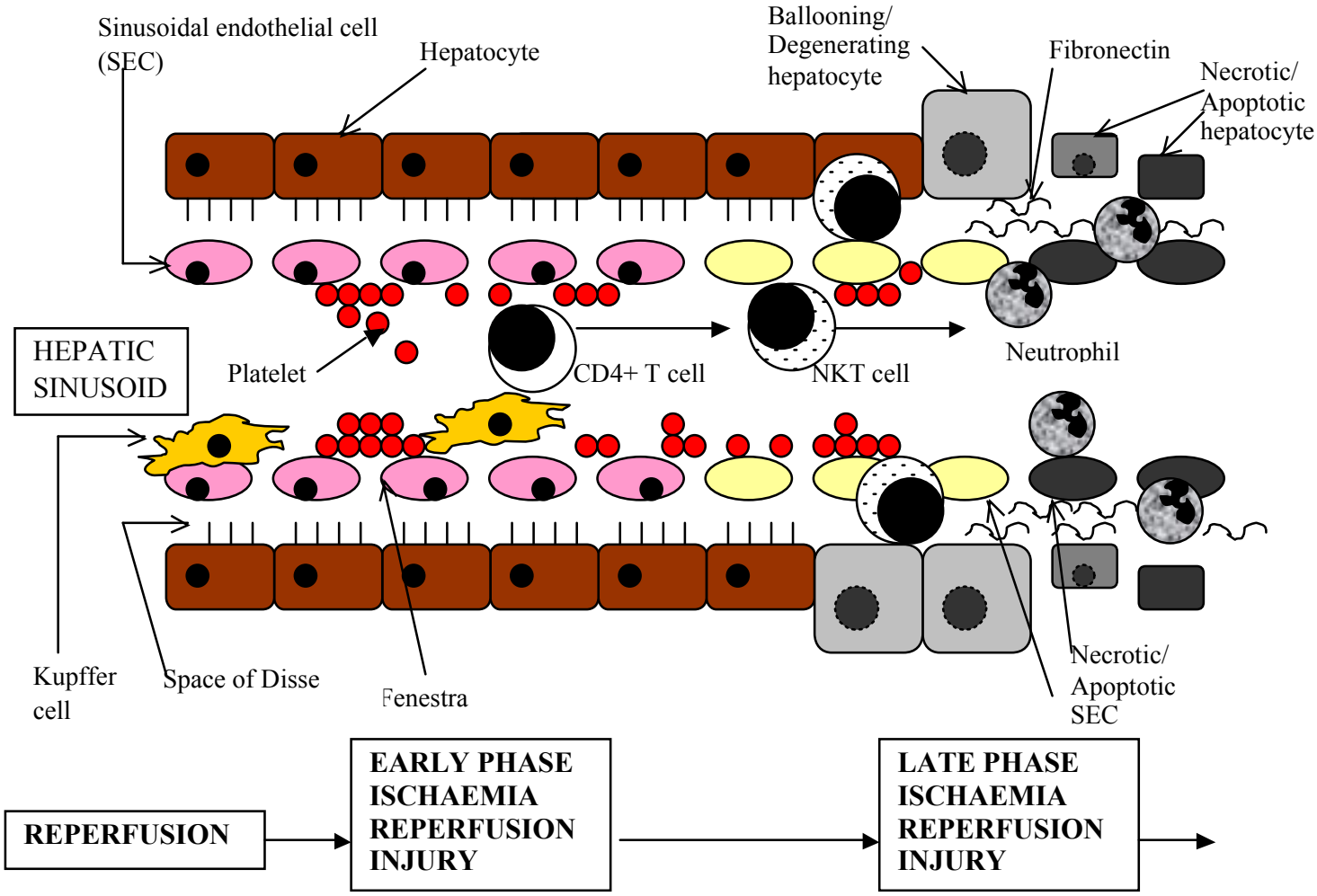


TABLE 1.2.1: Summary of knockout models of liver ischemia reperfusion injury pertaining to reactive oxygen species, adenosine and cells involved in the injurious mechanisms

Author/Yr	Knockout model	IR Protocol	Outcome Measure	Agent	Adaptive Responses	Injurous Responses
Kuboki et al., 2009	OTII; TCRδ deficient	70% I 90 min/R 4,8h	Histology; serum ALT; MPO	antiCD1d Ab; anti NK1.1 Ab; anti CD25+ Ab		Antigen dependent CD4+ T cell activation via TCR and NKT cell activation increase IRI; γδ T cell recruit PMN but not affect IRI
Evans et al., 2008	ob/ob or double knockout of leptin and UCP2	Total hepatic iscahemia 15 min/R 1, 24h	Histology; serum ALT; WB; liver ATP assay; lipid peroxidation			In steatotic livers of ob/ob mice only, UCP-2 depletes liver ATP which increases IRI 1h onwards
Hanschen et al., 2008	IL6(-/-); CD4(-/-); TNFR1(-/-)	Left lobe I 90 min/R 30 min, 2,3,4h	Kupffer cell activity (fluorescent latex beads and IVM); IH; serum AST and ALT	GdCl3 or glutathione to wild types (WT) only		Kupffer cells activation, ROS, IL6 and TNFα increase SEC VAP-1 expression and CD4+ Tcell sinusoidal recruitment which increase IRI. CD4+ T cells inhibit Kupffer cell phagocytic activity
Kim et al., 2008	Adenosine A1 receptor (A1AR)(-/-)	70% I 1h/R 24h	Histology; ALT; IH; PCR; WB;	CCPA (AIAR agonist); DPCPX (A1AR antagonist)	Endogenous adenosine via A1AR reduces IRI	Exogenous adenosine increase IRI not via A1AR
Lappas et al., 2006	Rag1(-/-), i.e.lack mature lymphocytes A2AR(-/-); IFNγ(-/-)	70% I 72 min/R 2,24h	Histology; serum ALT; intracellular IFNγ	i.p. ATL146 (A2AR agonist); PK136 (NK1.1 depletion); CD1d Ab (inhibit NKT cell); NKT cell adoptive transfer from WT, A2AR and IFNγ KO to Rag1 KO	Exogenous and endogenous adenosine acts through A2AR to reduce NKT cell recruitment	NKT cell recruitment increases IRI through release of IFNγ from at least 2 h reperfusion onwards and increased neutrophil recruitment from at least 24h after reperfusion
Caldwell et al., 2005	CD4 (-/-); B cell (-/-)	70% I 90 min/R 1,2,4,8h	Histology; serum ALT; MPO	Adoptive transfer CD4+T cell to CD4(-/-); Anti-IL17 Ab	CD4+ T cell only 1-4 h after reperfusion secrete IL17 increasing PMN infiltration, but inhibiting their oxidative burst	

Khandoga et al., 2002	ICAM(-/-)	Left lobe I 90 min/R 20 min	Serum AST and ALT; IH; IVM caspase-3 assay;	Anti-fibronectin Ab		Platelets bind fibronectin deposited on ICAM-1 on SECs
Shen et al., 2002	nu/nu; CD154(-/-)	70% I 90 min/R 4h	Serum ALT; histology; MPO; WB	Anti-CD154 Ab to WT; adoptive transfer T cell to KO or Ab group	IRI induces HO-1 protein	CD4-CD154 T cell costimulation in IRI
Wyllie et al., 2002	Nramp(-/-)	70%I 45 min/R 30,60 min	Plasma GOT and TNFα; histology; WB; IH; EMSA		HO-1 expressed in this model is protective in IRI	Macrophage activation after reperfusion increases TNFα release and NFκβ activity
Young et al., 2001	P-selectin/ ICAM-1 double KO	70% I 90 min/R 1.5,3,6h	Serum ALT; histology			P-selectin and ICAM-1 do affect the severity of IRI upto 6 h reperfusion, midzonal PMN infiltration
Ozaki et al., 2000	gp91 phox component of phagocyte NADPH oxidase(-/-)	70% I 60 min/R 5,8,24h+/- i.v. injection 3 days preop of adenovirus	Serum ALT; histology (H&E; ELISA for DNA histone fragments); TUNEL; IH; WB; assays for lipid peroxidation, hydrogen peroxide and superoxide; EMSA (NFκβ)	Replication deficient adenovirus encoding Rac1 (control: Adβgal)	Rac1 is activated in IRI and is protective	Liver tissue releases ROS within 5 min of reperfusion. PMN recruited 8h onwards, associated with increased lipid peroxidaion, necro-apoptosis. NFκβ DNA binding is associated with increased IRI. NADPH oxidase regulated by Rac1 small GTP binding protein is a source of ROS in IRI
Sawaya et al., 1999	P-selectin(-/-)	Left lobe I 30 min/R 15, 30, 60 120 min	Serum AST, ALT, LDH; histology; IVM	Radiolabelled Anti P-selectin Ab		P selectin on SECs increases rolling/ adherent leucocytes (peak 30 min R)

KO: transgenic knockout; I: ischemia; R: reperfusion; IR: ischemia reperfusion; IRI: ischemia reperfusion injury; ROS: reactive oxygen species; ATP: adenosine triphosphate; IH: Immunohistochemistry; H&E: haemotoxylin and eosin; WB: Western blot; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; ELISA: Enzyme labelled immunosorbent assay; EMSA: Electrophoretic mobility shift assay; AST: Aspartate transaminase; ALT: Alanine transaminase; LDH; lactate dehydrogenase; GOT: glutamic oxaloacetic transaminase; NADPH; nicotinamide adenine dinucleotide phosphate; IR: ischemia reperfusion; IVM: intravital microscopy; THV: terminal hepatic venule; A2AR: adenosine (subtype 2A) receptor; Nramp: natural resistance associated macrophage protein; PMN: polymorphonuclear cell; NKT: natural killer T cell; NK: natural killer cell; IFN: interferon; Ab: antibody; TNF: tumour necrosis factor; TNFR1: tumour necrosis factor receptor (subtype 1); TCR: T cell receptor; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: interleukin; ICAM: intercellular adhesion molecule; VAP: vascular adhesion protein

1.2.11. Cytokines and Chemokines

The interplay between cytokines and chemokines in liver IRI is not fully understood. The most extensively studied cytokines are TNFα, interferon (IFN) β, IFN γ and IL6.

TNFα has a directly injurous effect on ischaemic but not normal liver tissue (Teoh et al. 2004). Release of TNFα is stimulated by a cytokine cascade involving activation of interferon regulatory factor (Tsung et al. 2006). Antigen independent macrophage/Kupffer cell TLR 4 activation stimulates TNFα release (Shen et al. 2005, 2007). The effects of TNFα are mediated by binding to its receptor TNFR1 leading to increased apoptosis (Rudiger et al. 2002; Tian et al. 2006) and increased CD4+ T cell sinusoidal recruitment (Hanschen et al. 2008). One knockout model of mouse liver transplantation showed the deleterious effects of TNFα are mediated by TNFR outside the liver, most likely infiltrating leucocytes, but TNFR on liver cells appear to reduce IRI in this model (Table 1.2.2). The possible downstream pathways mediating this dual role will be discussed (Figure 1.2.3).

Interferon beta (IFN β) has emerged as a cytokine that appears to be involved throughout the reperfusion period in liver IRI, substantiated by work using knockout mice (Fig 1.2.2 Zhai et al. 2004). The damaging effects of IFN β are mediated by binding to the interferon receptor subtype IFN AR (Type 1) (Zhai et al. 2008) which upregulates interferon regulatory element 1 (IRE-1) (Figure 1.2.3) (Tsung et al. 2006). Knockout models support other studies in that **IFN γ** produced by NKT cells contribute to liver IRI from early on in reperfusion onwards (Shen et al. 2007; Tsung et al. 2006; Hamada et al. 2008; Tsuchihashi et al. 2006). Activation of innate immune pathways via TLR 4 stimulate release of IFN β and IFNγ in liver IRI, confirmed by TLR 4 knockout models (Shen et al. 2007).

Some cytokines released during liver IRI appear to reduce the severity of injury. The best evidence for this from knockout studies is for **IL6** (Camargo et al. 1997). There was worse IRI in livers of IL6 knockout mice than wild type mice, which was reversed by administration of recombinant IL6 to the knockout mice before ischaemia.

Chemokines (CXCL) are very small molecules, which are highly localised and form concentration gradients that guide leucocyte chemotaxis. One knockout model of CXCL10 found showed that this chemokine contributed to liver IRI (Figure 1.2.3 and Table 1.2.2) from 1 hour reperfusion onwards with associated activation of neutrophils, Kupffer cells and increased TNF α and IL 1β release (Zhai et al. 2008). A study using knockouts of chemokine receptor 2 (CXR 2) showed CXCR 2 activation contributes to liver IRI and neutrophil recruitment (Kuboki et al. 2008).

TABLE 1.2.2: Cytokine, chemokine and toll like receptor knockout models of liver IRI

Author/Yr	Knockout model	IR Protocol	Outcome Measure	Adaptive Responses	Injurous Responses
Kuboki et al., 2008	CXCR2	70% I 90 min/R 12,24,48, 96h	Histology; MPO; serum ALT, TNFα; WB: EMSA;	CXCR2 activates STAT3 hepatocyte proproliferative pathway	CXCR2 increases neutrophil recruitment and IRI. NFκβ activity reduced in IRI
Zhai et al., 2008	CXCL10(-/-)	70% I 90 min/R 2,4,8h	Histology; serum ALT; IH; quantitative RCR; WB		CXCL10 increases TNFα, IL6, IL1β, iNOS, PMN and Kupffer cell activation
Zhai et al., 2008	IFNAR type1 (-/-); IFNAR type 2 (-/-)	70% I 90 min/R 6h	Histology; quantitative PCR		IFNβ (not IFNγ) mediates IRI by binding to IFNAR type 1
Tsung et al., 2006	Interferon regulatory factor-1(IRF-1)(-/-) +/-Adenovirus IRF-1 vector	70% I 60 min/R 1,3,6,12h	Histology;serum ALT; WB;PCR		IFNγ, IFNβ, TNFα, IL1β all activate IRF-1 which increase Jnk (not p38 MAPK) and TNFα and iNOS expression in IRI
Shen et al., 2005	TLR4(-/-); TLR2(-/-)+/-SnPP (inhibit HO-1)	70% I 90 min/R 6h	Histology; serum ALT; MPO; WB; PCR	HO-1 is expressed which inhibits TLR4	TLR4 activation increases TNFα expression associated with increased IRI
Teoh et al., 2004	TNFα(-/-) +/- low or high dose TNFα i.p.	70%I 90 min/R 2,4,24h	Serum ALT and TNFα; IH; WB EMSA(NFκβ)		TNFα injurous to ischaemic liver, increased NFκβ activity
Kato et al., 2002	IL1R(-/-)	70%I 90 min/R 1,2,4,8,16,24h	Serum ALT, IL1β,TNFα and MIP-2; Histology (PMN score); MPO; EMSA		IL1R not involved in IRI
Rudiger and Clavien, 2002	TNFR(-/-); Fas(-/-); FasL(-/-) +/- Pentoxyphylline	70% I 75 min/R 3h	Serum AST; TUNEL; caspase-3 assay;ELISA; WB		TNFα binds to TNFR1 which increases apoptosis in IRI. Fas and FasL not involved in this model
Camargo et al., 1997	IL6(-/-)+/- recombinant IL6	Median lobe (45%) I 90 min/R 30,60,90, 120 min	Serum AST and ALT; histology; PCR	IL6 released in IRI is protective	TNFα expression during reperfusion is associated with worse IRI

IH: Immunohistochemistry; WB: Western blot; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; ELISA: Enzyme labelled immunosobert assay; EMSA: Electrophoretic mobility shift assay; IH: immunohistochemistry; ALT: Alanine transaminase; I: ischemia; R: reperfusion; IR: ischemia reperfusion; IRI: ischemia reperfusion injury; IFN: interferon; TNF: tumour necrosis factor; TNFR1: tumour necrosis factor receptor (subtype 1); TLR: Toll-like receptor (subtype); TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); IL: interleukin; CXCR: chemokine receptor; CXCL: chemokine ligand (subtype) NF: nuclear factor; i.p.: intraperitoneal

1.2.12. Matrix metalloproteinase-9 (MMP-9)

MMP-9 is a zinc dependent secreted gelatinase which catalyses degradation of type IV collagen and gelatin. MMP-9 knockout models of liver IRI have shown increased expression of MMP-9 on macrophages and neutrophils occurs during reperfusion (Table 1.2.3) which increases neutrophil transmigration over fibronectin in liver sinusoids and increases TNF α and interferon γ secretion and CD4+ T cell activation by mechanisms that remain to be elucidated. This leads to increased liver cell apoptosis and necrosis (Hamada et al. 2009; Hanschen et al. 2008).

1.2.13. Nitric Oxide Synthase

Nitric oxide synthase (NOS) catalyses formation of nitric oxide (NO) from L-arginine. NO is a versatile molecule which is vasoactive, is involved in activating molecular signalling pathways in cell survival, has immunological effects as well as directly injurous effects in high levels as a free radical itself. There are three isoforms of NOS: constitutive calcium (Ca2+) dependent forms which are endothelial NOS (eNOS) and neuronal NOS (nNOS), and an inducible calcium independent form, namely inducible NOS (iNOS). Only eNOS and iNOS are expressed in liver. Double knockouts of eNOS (eNOS-/-) and iNOS (iNOS-/-) have been generated. They have complete genetic knockout of eNOS or iNOS respectively, so the animal with that gene knocked out does not express the protein in any cell. The animals are viable and have been used in validated liver IR models to study IRI in vivo. The IR protocols in most studies involve using partial hepatic ischaemia of between 45 minutes and 1 hour and reperfusion of between 1 to 6 hours (Hines et al 2002; Lee et al. 2001).

Most studies agree that eNOS is upregulated in liver IRI and this reduces the severity of IRI. This is confirmed by eNOS knockout models of IRI (Table 1.2.3), where eNOS expression is related to reduced liver necrosis, apoptosis, leucocyte infiltration and increasing liver sinusoidal diameter and blood flow compared to control normal mice (Hines et al. 2002; Kawachi et al. 2000; Lee et al. 2001; Theruvath et al. 2006).

The role of iNOS is more controversial (Table 1.2.3) with different studies showing either no effect, iNOS contributing to IRI or iNOS reducing IRI. Some knockout models of iNOS show no role for iNOS in liver IRI, although apparently similar IR protocols and liver IRI models were used in the different studies (Kawachi et al. 2000; Hines et al. 2001; Khandoga et al. 2002). One knockout study of iNOS showed in the liver IRI model used that iNOS was protective which was at least partly mediated by activation of iNOS by eNOS (Hines et al.

2002). Yet another set of knockout studies conclude iNOS contributes to IRI. Hamada et al. used separate iNOS and matrix metalloproteinase-9 (MMP-9) knockout mice to show in their model of liver IRI that iNOS is upregulated in macrophages which increases IFN γ release and NO which increases MMP-9 expression on the macrophages and neutrophils. This signalling cascade contributes to increased liver IRI. Hines et al. (2001) used iNOS knockouts in a partial (70%) liver IR model to show increased IRI in iNOS-/- mice compared to normal mice, but no iNOS mRNA was detectable in normal mice nor any effect of an iNOS inhibitor. This suggests that genetic compensation occurs.
Large animal models of warm liver ischaemia reperfusion and an orthotopic liver transplantation using pigs have found that there is increased iNOS expression, highest in Kupffer cells and neutrophils in the centrilobular region, with associated higher levels of serum nitrite/nitrate, reduced capillary perfusion with more thrombi and ultimately increased liver injury and increased mortality (Kimura et al. 2003; Meguro et al. 2002,2003).
These discrepancies in the role of iNOS in liver IRI are likely to reflect differences in the experimental protocol and some genetic compensation in iNOS knockout mice. The overall impression from the literature is that eNOS and iNOS are both induced during liver IRI from 1hour reperfusion onwards (for mRNA and 2 hours reperfusion onwards for protein) and eNOS reduces injury. Low levels of iNOS induction are probably protective while high expression of iNOS contributes to increased injury and the overall effect of iNOS physiologically most likely depends on how ischaemia and reperfusion is produced.

1.2.14. Haem Oxygenase-1

Haem oxygenase-1 (HO-1 or heat shock protein 32, hsp 32) is the inducible isoform of haem oxygenase, the constitutive isoform being HO-2. This enzyme catalyses the formation of carbon monoxide (CO), biliverdin and Fe^{2+} from haem degradation. HO-1 has been implicated as having a protective role in IRI through CO and biliverdin associated with microcirculatory beneficial effects and reduced apoptosis and necrosis (Katori et al. 2002). HO-1 is typically expressed three or more hours after liver reperfusion (Shen et al. 2005; Su e al. 2006). The protective effects of HO-1 are supported by knockout models of IRI (Table 5) (Shen et al. 2005; Tsuchihashi et al. 2006). One knockout model demonstrated that targeted deletion of the HO-1 gene (Hmox-1-/-) resulted in aberrant Kupffer cell differentiation and increased susceptibility to ischaemia reperfusion insults (Devey et al. 2009).

TABLE 1.2.3: NOS, hsp/Hmox-1, MMP knockout models of liver IRI.

Author/Yr	Knockout model	IR Protocol	Outcome Measure	Adaptive Responses	Injurous Responses
Hamada et al., 2009	iNOS(-/-); MMP-9(-/-) +/- ONO-1714 (iNOS inhibitor) or NO donor (DETA NONOate)	70% I 90 min/R 3,6,24h	Histology; serum ALT, NO2-/NO3- MPO activity; IH; PCR, Western Blot (WB); MMP-9 assays; PMN migration assay		Increased macrophage iNOS producing NO increases PMN MMP-9 and PMN transmigration over fibronectin
Hamada et al., 2008	MMP-9 (-/-)	70% I 90 min/R 6,24h +/-Anti MMP-2/9 inhibitor	Histology; serum and GOT; MPO; IH; PCR		IRI: MMP-9 (not MMP-2) increase TNFα, IFNγ, IL2, IL6 and increase PMN and CD4+ T cell recruitment
Theruvath et al., 2006	eNOS(-/-)	Donor(WT/KO) to WT recipient; organ stored 18h, 4oC,UWS	Histology; serum ALT; IVM; TUNEL; IH (macrophage infiltration)	eNOS activation reduces necro-apoptosis, inhibits macrophage infiltration, increased sinusoidal diameter and flow	
Tsuchiashi et al., 2006	Hmox-1(+/-); Hmox-1(-/-);CoPP (induces HO-1) in WT	70% I 90 min/R 6h	Histology; serum GOT; MPO; TUNEL; PCR; WB	HO-1 upregulated which inhibits expression of cytokines TNFα and IFNγ	TNFα and IFNγ expression increased overall in IRI associated with increased apoptosis and necrosis
Hines et al., 2002	eNOS (-/-); iNOS (-/-)	70% I 45 min/R 1,3h	Serum ALT; histology; PCR	IRI reduced by eNOS (inhibits TNFα); and iNOS	Liver IRI in eNOS (-/-) and iNOS (-/-)
Lee et al., 2001	eNOS(-/-); iNOS(-/-)	70% I 1h/R 1,3,6h	Serum ALT, AST;	eNOS activated during IRI is protective	Increased iNOS mRNA from 3 h with increased IRI
Hines et al., 2001	iNOS(-/-)	70% I 45 min/R 1,3,6h+/- L-NIL (iNOS inhibitor)	Serum ALT; histology; MPO		Increased IRI in iNOS(-/-); no iNOS mRNA or L-NIL effect in WT; genetic compensation effect
Kawachi et al., 2000	eNOS(-/-); iNOS(-/-)	70% I 45 min/R 5h	Serum ALT; histology; MPO	eNOS is activated in IRI and is protective	No PMN infiltration and iNOS is not activated
Devey et al. 2009	Hmox(-/-); Hmox(+/-)	Left lobe I 40 or 50 min/R 24 h	Serum ALT; histology; WB; IH	IRI activates Kupffer cells. HO-1 is protective	

IH: Immunohistochemistry; WB: Western blot; MPO: Myeloperoxidase; PCR: polymerase chain reaction; ELISA: Enzyme labelled immunosobert assay; EMSA: Electrophoretic mobility shift assay; AST: Aspartate transaminase; ALT:Alanine transaminase; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling (assay for cell death); GOT: glutamic oxaloacetic transaminase; I: ischemia; R: reperfusion; IR: ischemia reperfusion; IRI: ischemia reperfusion injury; TNF: tumour necrosis factor MMP: matrix metalloproteinase; Hmox: heme oxygenase gene; eNOS:endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; HO-1: heme oxygenase (subtype 1); PMN: polymorphonuclear cell (neutrophil); WT: wild type animal

1.2.15. Downstream Pathways

A wide range of downstream pathways have been studied in liver IRI. The majority of systems which are activated in ischaemia reperfusion are effective through these pathways. Some of the key mediators activating these downstream pathways in liver IRI are TNFα, IFNβ, IFNγ and CXCL10 (Figure 1.2.3.). In particular, the roles of the transcription factors nuclear factor kappa beta (NFκβ) (Luedde et al. 2006), the survival kinases (JNK, MAPK's, PKC, PI3K/Akt), signal transducer and activator of transcription (STAT's) (Stephanou et al. 2004), Poly ADP ribose polymerase (PARP), Peroxisome proliferator-activated receptor (PPAR) from pharmacological studies have been supported by knockout models of liver IRI.

NFκβ DNA binding increases and contributes to liver IRI (Wyllie et al. 2002; Kato et al. 2000; Kuboki et al. 2007). TNF α increases NFκβ activity (Kato et al. 2000; Teoh et al. 2004). One knockout study, in contrast, showed a fall in NFκβ activity following liver reperfusion (Kuboki et al. 2008), although a longer period of ischaemia and reperfusion was used than other studies. Another group used a conditional NFκβ knockout to study liver IRI and showed NFκβ activity was protective, reducing necrosis, apoptosis, JNK expression and TNF α expression (Table 1.2.4). Unlike the other studies, the caudate lobe was resected in their protocol, which would activate inflammatory pathways following this surgical resection and cause additional circulatory and microcirculatory dysfunction. It is therefore unclear if the role of NFκβ in IRI is protective, injurous or depends on the IR protocol.
A knockout model has provided evidence that interferon regulatory factor-1 (IRF-1) is released by IR, which in turn activates jnk-2, but not p38 MAPK, which contributes to liver IRI after reperfusion (Table 1.2.2) (Tsung et al. 2006). A jnk-2 knockout model of left lobe ischaemia using inhibitors of HO-1 and Kupffer cell depletion found a mechanism for IRI where IR activates jnk-2 which inhibits HO-1 mainly within Kupffer cells which contributes to IRI (Devey et al. 2009).

Knockout models of PI3K and Protein kinase C (PKC) have been used in the heart, but not in the liver (Table 1.2.4) (Ban et al. 2008; Xuan et al. 2005). One group showed in a large animal pig model of orthotopic liver transplantation (OLT) using chelerythrine (a PKC inhibitor) and/or ischaemic preconditioning (IPC) of the donor liver before cold storage to show that PKC activity was not affected by IRI alone of OLT, although PKC was strongly

activated by IPC reducing the severity of IRI (Cursio et al. 2002; Ricciardi et al. 2001; Yanagida et al. 2004).

Activation of the STAT family of transcription factors is mediated by extracellular signalling molecules such as cytokines which bind to membrane receptors which activate intracellular Janus kinases on the cytoplasmic face of the plasma membrane, which in turn activate a STAT protein which is then transported to the nucleus where they bind DNA to affect gene expression (Jak/STAT signalling pathway). STAT 6 activation does not appear to be involved in liver IRI based on results from knockout models (Kato et al. 2000; Shen et al. 2003). Kato et al. found in their model of IRI using STAT 4 knockouts that STAT 4 did not affect the extent of liver injury after 8 hours reperfusion (Kato et al. 2000). In contrast, Shen et al. showed STAT 4 expression was related to IRI after 6 hours of reperfusion, although it was specifically its expression within CD4+ T cells that mediated the liver injury (Table 1.2.4) (Shen et al. 2003).

A knockout of PARP has been used to show that PARP activation contributes to early liver IRI (Table 1.2.4) and activates signalling pathways increasing expression of adhesion molecules on SECs (Khandoga et al. 2002). A liver IRI model using a PPAR knockout demonstrated background PPAR activity reduces the severity of liver reperfusion injury acting via signalling pathways that remain to be elucidated but appear not to involve NO or TNF α, both of which act independently of PPAR in this model (Okaya et al. 2004).

TABLE 1.2.4: Knockouts of downstream mediators and models of liver IRI

Author/Yr	Knockout model	IR Protocol	Outcome Measure	Agent	Adaptive Responses	Injurous Responses
Devey et al. 2009	jnk-2(-/-)	Left lobe I 40 min/R 24h	Serum ALT; histology; WB; IH	ZnPP (HO-1 inhibitor); liposomal clodronate (Kupffer cell depletion)	HO-1 reduces IRI	jnk-2 activation inhibits HO-1 in Kupffer cells contributing to IRI
Shen et al., 2003	STAT4(-/-); STAT6(-/-); nu/nu	70%I 90 min/R 6h	Serum ALT; histology; MPO; WB; PCR	Adoptive transfer of CD4+ T cells	HO-1 expressed at very low levels after 6h in this model, but protective	CD4+T cell activation involving T cell STAT 4 activation, but not STAT 6 increased IRI
Kato et al., 2002	P50 NFκβ(-/-)	70% I 90 min/R 1,8h	Serum ALT; histology; WB;EMSA			No effect of p50 subunit deletion
Kato et al., 2002	STAT 4(-/-)	70% I 90 min/R 30 min, 1,2,4,8h	Serum ALT; histology; MPO; WB	Anti IL12 Ab		IL12 associated with IRI. STAT 4 not activated

KO: transgenic knockout; WT: wild type (normal animals); IH: Immunohistochemistry; WB: Western blot; MPO: Myeloperoxidase assay; PCR: Polymerase chain reaction; EMSA: Electrophoretic mobility shift assay; ALT: Alanine transaminase; I: ischemia; R: reperfusion; IR: ischemia reperfusion; IRI: ischemia reperfusion injury; IFN: interferon; Ab: antibody; TNF: tumour necrosis factor; IL: interleukin; NF: nuclear factor; STAT: Signal Transducer and Activator of Transcription; Jnk: a survival kinase;

1.2.16. Therapeutic Interventions

A greater understanding of liver IRI from in vivo and in vitro pharmacological studies, knockout models and large animal studies have provided insights into possible therapeutic interventions at reducing IRI and improving clinical outcome. This has led to some initial clinical trials, using pharmacological preconditioning or surgical preconditioning (i.e. ischaemic preconditioning), the results of which are conflicting (Table 1.2.5).
N-acetylcysteine (NAC) given to patients before liver transplantation showed no benefit, although the sample size was small, the recipients were at the healthier end of the spectrum and marginal donor livers were not used (Khan et al. 2005). One clinical trial in which patients inhaled NO preoperatively before liver transplantation showed improved liver function tests postoperatively (Lang et al. 2007). Another clinical trial involved patients being given methylprednisolone intravenously preoperatively before liver transplantation showed reduced IRI (Aldrighetti et al. 2006). A preoperative dose of the protease inhibitor gabexate mesilate given to patients before major hepatic resection reduced the severity of IRI in one clinical trial (Kim et al. 2002). A multicentre prospective randomised control trial using a pancaspase inhibitor (IDN-6556) during cold storage/flush and intravenously after liver transplantation showed reduced liver cell apoptosis in recipients (Baskin-Bey et al. 2007).
Ischaemic preconditioning (IPC) where one or more short cycles of ischaemia and reperfusion preceding the main iscahemia reperfusion insult of the target organ, including the liver, has been shown to reduce IRI in most laboratory studies. In hepatic resection surgery, there is evidence that intermittent occlusion of the portal triad, although resulting in greater blood loss, is associated with lower rates of postoperative liver failure (9.6 vs. 0%) and lower mortality (4.6 vs. 0%) than continuous occlusion. Overall, however, the results have been conflicting, ranging from showing reduced IRI (Cescon et al. 2006; Clavien et al. 2003; Li et al. 2004), delayed graft function but reduced injury (Azoulay et al. 2005), no effect (Koneru et al. 2007) or even increased IRI (Koneru et al. 2007) with the IPC protocols used.
A number of Cochrane reviews have been published assessing the benefits of protective strategies against liver IRI. Several Cochrane reviews of IPC found not enough evidence to support or refute the use of IPC, but few trials were included and some were of high bias (Gurusamy et al. 2009). A Cochrane review assessing the effects of various pharmacological interventions concluded that trimetazidine, methylprednisolone and/or dextrose may protect

against ischaemia reperfusion injury in elective liver resections performed under vascular occlusions (Abu-Amara et al. 2009). There are several criticisms of this review. Only fifteen trials were used, they were high bias, different pharmacological interventions were pooled together for analysis and there were no statistically significant differences between groups in any of the clinically relevant outcomes of morbidity and mortality.

1.2.17. Conclusions

Liver IRI is a clinically relevant phenomenon in a wide range of settings including trauma surgery, hepatic resection and transplantation, affecting clinical outcome. Laboratory work using knockout models and large animal studies have provided insights into the mechanisms of liver IRI. Liver IRI occurs as a continuum beginning from the moment of reperfusion onwards for up to a week.

Ischaemic preconditioning is a surgical method of reducing the severity of liver IRI. Understanding its molecular mechanisms and how this affects the pathways of liver IRI is a useful way of identifying putative protective pharmacological agents. In the context of large scale multicentre clinical trials that are more robust than previous trials offer the possibility of providing convincing evidence of techniques that can greatly reduce complications resulting from IRI in liver surgery as well as increasing the pool of usable allografts.

TABLE 1.2.5: Clinical Preconditioning Trials in Liver Surgery.

Author/Year (Trial Type)	Patient Group and Preconditioning Protocol	Endpoints	Results
Azoulay et al./2005 (RCT)	OLT cadaveric transplant (n=45); Portal triad IPC (10I/10R) (n=46)	Serum AST, ALT, INR, bilirubin (POD 1, 3)	No effect of IPC
Cescon et al. /2006 (RCT)	OLT (n=24); IPC (10I/15R) (n=23)	iNOS, neutrophil infiltration, apoptosis, serum AST,ALT	No clinical effect of IPC, but reduced apoptosis
Khan et al./2005 (RCT)	OLT (n=9); NAC (i.v. portal flush) at donor operation (n=9)	Peak AST, 1 hr post reperfusion biopsy	No benefit of IPC
Lang et al./2007 (RCT)	OLT (n=10); Inhaled NO (iNO) at 80 ppm during recipient operation (n=10)	Serum AST, ALT, APTT, hepatocyte apoptosis, length of hospital stay	iNO improved rate of improvement of liver function and reduced length of hospital stay
Aldrighetti et al./2006 (RCT)	Liver resection (n=38); Preoperative methylprednisolone (n=38)	Serum AST, ALT, bilirubin, INR, IL6, TNFα (POD 1, 2)	Lower complication rate and serum markers in steroid group
Kim et al./2002 (RCT)	Liver resection (n=25); Gabexate mesilate (GM) i.v. 24 hr preop until POD 3 (n=25) or GM beginning of surgery only (n=16)	Serum ALT, TNFα, IL1β, IL6	Lower complication rate, including mortality and serum markers in GM group
Baskin-Bey et al./2007 (Multicentre Phase II RCT)	OLT(n=23); Pancaspase inhibitor IDN-6556 to organ storage/flush with (n=27) or without (n=23) 0.5 mg/kg IDN-6556 to recipient	Serum AST, ALT, apoptosis (TUNEL assay), caspase 3/7 assay, histology	Reduced apoptosis and serum markers when IDN-6556 added, but no difference in complication rate
Petrowsky, Clavien et al./2006 (RCT)	Major liver resection (>bisegmentectomy) inflow occlusion > 30 min (n=50); IPC (10I/10R) (n=50)	Serum AST, ALT, Liver ATP	No clinical benefit of IPC, but improved markers in younger and steatotic livers
Li et al./2004 (RCT)	Hepatic resection in HCC and cirrhosis under hepatic inflow occlusion (n=15); IPC (5I/5R) (n=14)	Serum AST, ALT, bilirubin (POD 1, 3, 7), SEC apoptosis	Improved serum markers, reduced apoptosis, length of stay and liver failure in the IPC group
Koneru et al./2007 (RCT)	IPC (10I/10R) (n=50)	Serum AST, ALT, bilirubin, INR (POD 1, 2, 7), TNFα, IL6, IL10, Histology	IPC associated with worse serum liver function tests but no significant clinical effect

RCT=randomised controlled trial; OLT=orthotopic liver transplantation; IPC=ischaemic preconditioning; I=ischaemia time in minutes; R=reperfusion time in minutes; POD=postoperative day AST=aspartate transaminase; ALT=alanine transaminase; IL=interleukin; TNF=tumour necrosis factor; NAC=N-acetylcysteine

1.3. LIVER ISCHAEMIC PRECONDITIONING

1.3.1. INTRODUCTION

Liver preconditioning is a pretreatment of the organ to reduce the severity of liver IRI. Ischaemic preconditioning (IPC) is a mechanical technique whereby a short period or several short periods of occlusion of the blood supply to an organ confers protection to the organ against IRI. This effect was first demonstrated in the canine heart by Murry et al (1986). This type of IPC is called direct IPC. Another form has been identified more recently where IPC of an organ or tissue different to the organ or tissue undergoing IRI reduces that injury, known as remote IPC (Kanoria et al. 2007). This chapter focuses on direct IPC to the liver. Current evidence supports the concept of two phases of protection of the liver against IRI by direct IPC. An early phase that protects within minutes of reperfusion lasting up to 3 hours, and a delayed phase of protection starting from 12 to 24 hours of reperfusion lasting several days (Bolli et al. 2000; Cohen et al. 2000; Yellon et al. 2000). Understanding the molecular signalling pathways for liver IPC offers the possibility of identifying agents that can pharmacologically precondition livers and eliminate the troublesome complications resulting from liver IRI in the clinical setting.

1.3.2. INFLAMMATORY RESPONSE

IPC decreases endothelial P-selectin expression in warm IRI of the rat liver (Sawaya et al. 1999) related to TNFα downregulation (Peralta et al. 2001). The resultant decrease in neutrophil adhesion, transmigration and activation protects the parenchyma. This decreases serum transaminase levels in hepatic IRI (Howell et al. 2000).

Rat pretreatment with low dose TNFα (1-5 μg/kg body wt) before hepatic IR substantially reduces liver injury and TNFα release, while high dose TNFα exacerbates injury (Teoh et al. 2003, 2004). This protective effect is associated with increased hepatocyte proliferation, increased IL-6 levels and increased STAT3 DNA binding (Teoh et al. 2006). It appears that IL-6 rather than TNFα itself seems to be the mediator of the hepatoprotective and proproliferative effects of IPC (Teoh et al. 2006). This is supported by using an IL-6 knockout model of total hepatic ischaemia (THI), where IPC protected livers of wild type mice and was associated with increased STAT3 phosphorylation, whereas IPC has no effect in the knockout mice (Matsumoto et al. 2006).

1.3.3. MICROCIRCULATION

A decrease in ROS production and neutrophil mediated injury by IPC leads to preservation of the microcirculatory architecture. This improvement in microcirculatory perfusion can be demonstrated by intravital microscopy showing improved sinusoidal perfusion (Vajdova et al. 2004; Zapletal et al. 1999). This microcirculatory improvement is further substantiated by laser Doppler studies of liver microcirculation (Koti et al. 2002). This improvement in microcirculation is associated with a fall in endothelin-1 (ET-1) (Kadono et al. 2006)and an increase in hepatic nitric oxide (NO) (Koti et al. 2002).

1.3.4. ADENOSINE

Peralta et al. showed adenosine acts as a trigger and mediator in liver IPC (Peralta et al. 1998). This adenosine is produced by endothelial cells (Peralta et al. 1997, 1998)**.** Nakayama et al. (1999) showed an A2 receptor agonist but not an A1 receptor agonist enhanced protection against THI. So, although liver cells express both A1 and A2a adenosine receptors, liver IPC acts through A2a receptors only (Dixon et al. 1996).

In isolated hepatocytes, preconditioning is abolished by inhibiting heterotrimeric G_i protein or phospholipase C (PLC) with pertussus toxin and U73122, respectively (Carini et al. 2001). Direct stimulation of protein kinase C (PKC) with a DAG analogue prevents hypoxic liver cell injury, whereas PKC inhibition by chelerythrine prevents preconditioning protection in isolated hepatocytes or perfused livers (Carini et al. 2000; Ricciardi et al. 2001).This evidence supports that Gi proteins, PLC and PKC mediate the protective effects of liver ischaemic preconditioning by adenosine acting through A2a receptors.

Stimulation of the adenosine A2a receptor is associated with a 2 to 3 fold increase in the activity of protein kinase B (PKB/Akt) (Carini et al. 2004). Experiments in isolated hepatocytes show activation of PKC leads to p38 MAPK phosphorylation (Carini et al. 2001)**.** The addition of activators of p38 MAPK protect while selective inhibition of p38 MAPK during hepatocyte preconditioning abolishes the protection (Carini et al. 2001).

1.3.5. NITRIC OXIDE (NO)

NO plays an important role in mediating hepatic preconditioning. An increase in NO production is associated with the onset of liver tolerance to reperfusion injury, which is abolished by inhibiting NO release with L-NAME (Koti et al. 2002; Peralta et al. 1997, 2001; Arai et al. 2001). NO donors promote preconditioning even in the presence of NO synthase

inhibitors (Peralta et al. 2001). NO appears to act as a trigger in early IPC (Lochner et al. 2002) and both a trigger and mediator in late IPC (Rakhit et al. 1999)

The source of the NO is not established. The two main enzymes responsible for endogenous NO production are endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). eNOS is constitutively present in liver endothelial cells and iNOS is expressed in virtually all cell types in response to stressful stimuli. There is evidence that eNOS is the source of NO in early IPC (Koti et al. 2005; Serracino-Inglott et al. 2005) and iNOS is the source of NO in late IPC (Shah et al. 2003).

Isolated rat hepatocytes cultured under hypoxic conditions have improved cell survival when preconditioned by an NO donor (NOC-9), this protection being mediated by activation of soluble guanylate cyclase and cGMP dependent kinase (cGK), but not PKC. This cytoprotection is associated with increased p38 MAPK phosphoryation, which is abolished by inhibiting either guanylate cyclase or cGK (Carini et al. 2003).

1.3.6. HAEM OXYGENASE-1 (HO-1)

HO-1 is the inducible isoform of haem oxygenase and catalyses the formation of carbon monoxide (CO), biliverdin (which is converted to bilirubin by biliverdin reductase) and Fe^{2+} from haem degradation. Liver IPC is associated with increased HO-1 mRNA, followed by HO-1 protein expression and increased activity (Patel et al. 2004). In a rat model of warm liver IRI, the protective effects of liver IPC are associated with increased expression and activity of HO-1 (Coito et al. 2002; Lai et al. 2004; Massip-Salcedo et al. 2006). Cobalt protoporphyrrin (CoPP), an inducer of HO-1 reduces warm IRI, which is most dramatically evident after 24 hours reperfusion following the index ischaemia (Massip-Salcedo et al. 2006). Hemin, another inducer of HO-1, reduces IRI in a rat ex vivo perfused liver model following storage of the liver for 16 hours at 4 ^{o}C in University of Wisconsin solution (Kato et al. 2003)**.** The protection of IPC is abolished by inhibiting HO-1 with zinc protoporphyrrin (ZnPP) (Lai et al. 2004; Massip-Salcedo et al. 2006; Kato et al. 2003).

HO-1 exerts its protective effects through its end products. CO reduced liver IRI in an ex vivo rat perfused liver model, where after 24 hours storage in cold preservation solution, ex vivo livers perfused with CO enriched blood showed less injury and this protective effect was even maintained in livers treated with ZnPP to inhibit HO-1 (Amesi et al. 2002). The CO mediated protection in this model was p38 MAPK dependent. In rat orthotopic liver transplantation (OLT) models, studies have shown that both CO being given to recipients or

CO enriched cold preservation solution reduced IRI (Ikeda et al. 2009; Kaizu et al. 2008). This protection relates microscopically to reduced sinusoidal endothelial cell (SEC) damage and reduced neutrophil extravasion (Ikeda et al. 2009). In contrast to the ex vivo model of Amersi et al., Kaizu et al found in their in vivo OLT model that p38 MAPK and JNK MAPK pathways were unaffected by CO (Kaizu et al. 2008).

In one ex vivo rat perfused liver model, there was less liver IRI following 16 hours storage of livers in cold preservation solution when livers were perfused with bilirubin or biliverdin supplemented perfusion solution (Kato et al. 2003; Amersi et al. 2002; Ikeda et al. 2009; Kaizu et al. 2008; Fondevila et al. 2004). Similarly in a rat in vivo OLT model following 24 hours storage of livers at 4 ^{o}C in University of Wisconsin solution, recipients and/or donors given biliverdin intravenously had less graft IRI (Fondevila et al. 2004). The other product of HO-1, Fe^{2+}, in contrast, contributes to IRI. Fe^{2+} is thought to participate in the generation of free radicals through the Fenton reaction. Increased expression of the Fe^{2+} sequestering protein ferritin with IPC limits the severity of liver IRI (Berberat et al. 2003). Bilirubin is a powerful antioxidant, its efficacy being enhanced under the hypoxic conditions of ischaemia and at micromolar concentrations scavenges ROS, protecting cells from peroxide radicals. Its antioxidant capacity may arise from a cycle in which biliverdin reductase undertakes NADPH-dependent reduction of biliverdin to bilirubin. The bilirubin is then oxidised by free radicals back to biliverdin and so this cycle continues (Baranano et al. Proc Natl Acad Sci USA 2002; Stocker et al. 1987).

In summary, HO-1 is a protective mediator of liver IPC. This protection appears to be most evident in the late phase of IPC. This protection appears to be mediated through two of the the byproducts of HO-1: CO and biliverdin (Fig 1.3.1).

1.3.7. HO-1 and NOS: IS THERE A CONNECTION?

We have seen that NOS and HO-1 are important mediators of liver IPC. There is little data exploring the relationship between HO-1 and the NOS isoforms in liver IRI and the conclusions from the studies that have been done are conflicting.

Acquaviva et al. (2008) used a warm THI rat model (30 minutes ischaemia and 3 hours reperfusion) to study the effects of coadministration of the antioxidants L-arginine and L-rutin (Acquaviva et al. 2009). The antioxidants reduced liver IRI and increased eNOS and HO-1 protein expression and reduced iNOS expression. Since L-arginine is a substrate for NOS, it was concluded that eNOS expression increases HO-1 expression, which in turn

inhibits iNOS expression. In a rat OLT model, where donor rats were treated with adenovirus expressing HO-1 (or a control Ad-β Gal virus), it was shown that that HO-1 was induced in the Ad-HO-1 treatment groups with there being less liver IRI and reduced expression of iNOS in liver macrophages (Coito et al. 2002).

In contrast, an in vivo mouse model of partial warm hepatic ischaemia (45 minutes ischaemia and 5 hours reperfusion) using wild type mice and transgenic overexpressors of eNOS with or without various HO-1 inducers and inhibitors showed that both eNOS and HO-1 activity reduced IRI, but they were independent of each other (Duranski et al. 2006).

It is likely that there is a common activator for HO-1 and NOS induction in IPC. A good candidate for this is the oxygen sensitive transcription factor, hypoxia inducible factor, HIF-1. HIF-1 is a transcription factor expressed ubiquitously in cells in response to hypoxia. HIF regulates the expression of nearly 200 genes that can affect the cellular adaptive responses to hypoxia and/or ischaemia. HIF-1 activates transcription of genes whose protein products either increase oxygen delivery to tissues (erythropoietin, EPO, via erythropoiesis and vascular endothelial growth factor, VEGF, via angiogenesis) and provide metabolic adaptation under conditions of reduced oxygen availability by affecting a wide range of genes including those encoding glycolytic enzymes and glucose transporters (Hill et al. 2008; Loor et al. 2008; Semenza et al. 2000). In a study of cultured rat hepatocytes exposed to the effects of late phase hypoxic preconditioning, A2a receptor agonists and PKC and PI3K antagonists and assays of HIF-1 activation and hepatocyte death, it was shown that A2a receptor activation, PI3K and PKC activation increase HIF-1 activation which reduces hepatocyte death (Alchera et al. 2008).

HIF is also thought to affect cell survival in IRI by regulating genes for HO-1, iNOS and cyclooxygenase-2 (COX-2), all associated with increased resistance to IRI. Double knockouts of HIF usually die in utero. Heterozygote knockouts for a single allele are viable (hif+/-), but have not been studied in liver IRI. Hill et al. used hif1a+/- and hif2a+/- mice in a model of unilateral renal ischaemia reperfusion injury (30 minutes ischaemia/72 hours reperfusion) to show that wild type mice had less renal reperfusion injury. In this model, HIF-1 activation occurs during renal IRI and reduces the extent of renal IRI.

1.3.8. CONCLUSIONS

IPC has been shown to be protective in a number of animal models and some small clinical trials. Adenosine, NO and HO-1 are important mediators of liver IPC. It appears that

adenosine acts as a trigger and mediator in the early phase of IPC. NO is a key mediator in all phases of liver IPC. It is less clear what the source of the NO is. Current evidence supports that in early IPC, NO is released by eNOS and in late IPC it is released by iNOS. HO-1 appears to be protective in the late phase of IPC acting through its products CO and biliverdin with elimination of the toxic product Fe^{2+}. The interrelationship between the upstream mediators is poorly understood.

There are few transgenic knockout or overexpressor models to provide *robust* mechanistic information about IPC and substantiate findings of other studies. The role of nitric oxide synthase in the protective effects of liver IPC is not well understood and its relationship to HO-1 expression and the in vivo timecourse of expression of these enzymes need to be clarified. This will provide important information that can be used in the consideration of identification and designs for pharmacological studies aimed at reducing liver IRI clinically.

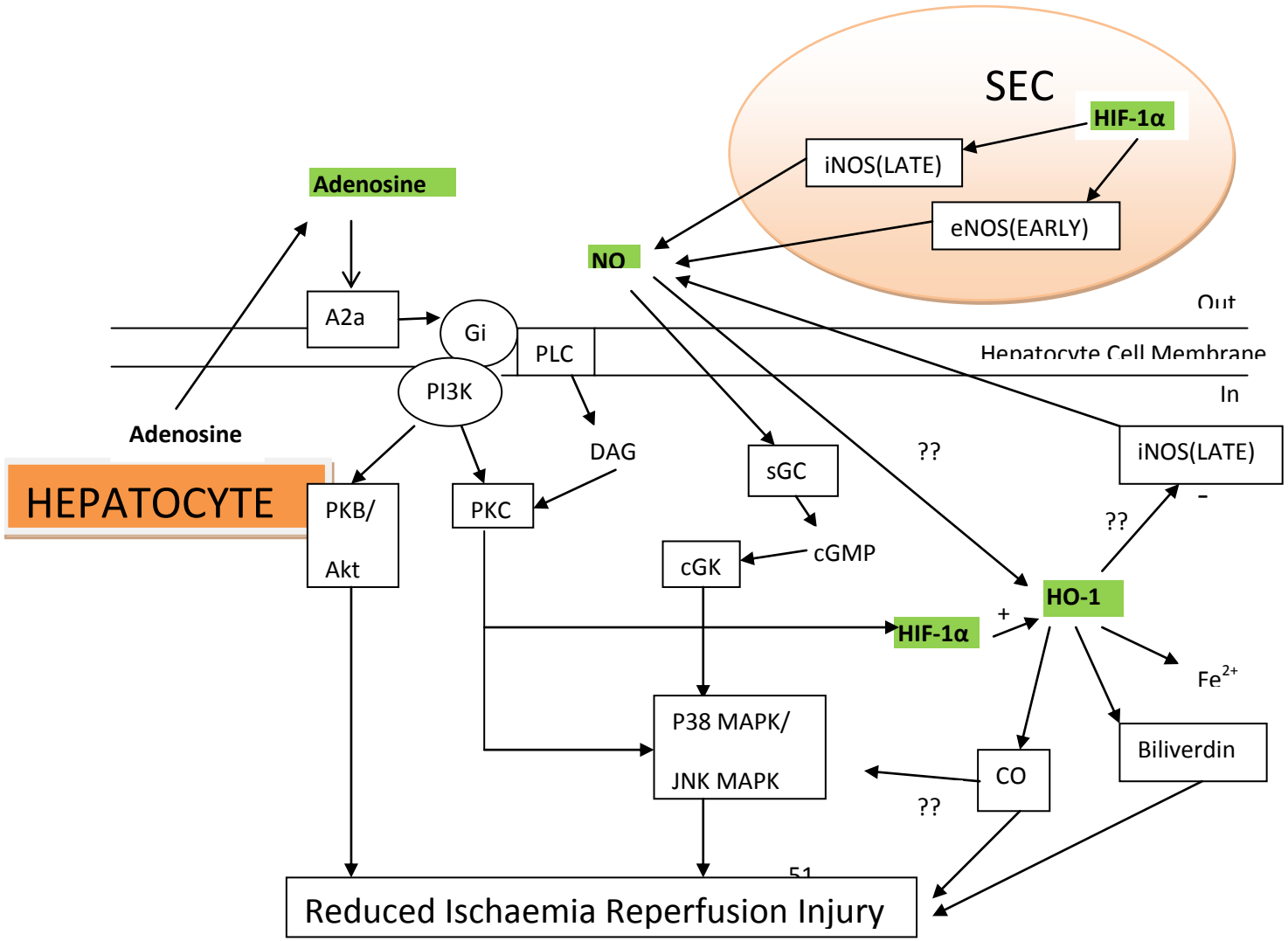


Figure 1.3.1: Summary of current understanding of molecular mechanisms of Liver Ischaemic Preconditioning. sGC=soluble guanylate cyclase; cGK=cGMP dependent kinase; ??=inconclusive evidence for this pathway. Some key triggers and mediators are highlighted in green. Following ischaemic preconditioning, adenosine released activates A2a receptors which acts through the PI3/Akt, PKC and PLC/DAG pathways to reduce the severity of cellular IRI. The downstream mediators for this protection are the survival kinases, specifically p38 and JNK MAPK's. Nitric oxide (NO) released early from eNOS and late from iNOS is stimulated by adenosine and HIF-1. Acting through sGC (soluble guanylate cyclise), cGMP, cGK (cytosolic guanylate kinase) and p38/JNK MAPK in sequence, cell injury is reduced. HO-1 is activated possibly by NO or HIF-1, which protects through its products CO and biliverdin and by inhibiting excessive activation of iNOS, as high levels of NO can exacerbate cell injury. The protection of CO may be mediated by p38/JNK MAPK's.

CHAPTER 2

Methods

An in vivo mouse model of partial warm hepatic ischaemia reperfusion (IR) was used. Normal and transgenic double knockout for eNOS (eNOS-/-) mice were used. A partial warm liver IR model was used where ischaemia was applied to cephalic lobes only followed by reperfusion. Ischemic preconditioning (IPC) consisted of ischaemia applied directly to the cephalic lobes followed by reperfusion then IR to the cephalic lobes. The endpoints measured were surface Laser Doppler flow to assess liver microcirculation during the experiment and at the end of the experiment serum ALT, liver histological injury scores, Western blotting for eNOS, iNOS, phosphorylated eNOS (p-eNOS), HO-1 protein and RT-PCR for HO-1 mRNA.

2.1 Animals

All procedures and animal care were in accordance with the U. K. Home Office licensing regulations. C57 BL6 animals with targeted deletion of the *enos* gene by neomycin resistance gene insertion were supplied by the Welcome Institute (Biological Research, UCL) and bred by homozygote to homozygote mating in accordance with local regulations. *Wild-type* strain specific controls were obtained from commercial sources (Harlan UK Ltd, Bicester, Oxford). All animals were male, 8 to 12 weeks old.

The eNOS-/- mice were viable at 8 to 12 weeks, appeared as healthy as wild type mice, there were no anatomical differences intraabdominally on laparotomy and were as stable as wild type mice under general anaesthesia.

2.2. Operative Procedure

Animals had free access to food and water. All procedures were in accordance with the U. K. Home Office regulations and conformed with the NIH guidelines for the

care and use of laboratory animals. Animals were not fasted before the procedure, as this is not required for mice as they do not cannot vomit.

Animals were anaesthetised with 2% isoflurane inhaled within an induction chamber. Anaesthesia was maintained by 1-5-2% isoflurane via a concentric oronasal mask connected to an anaesthetic circuit. Respiratory rate and depth were monitored visually and core temperature was monitored by a rectal probe and maintained using a heating pad. The abdomen of animals was shaved and was cleaned with 2% chlorhexidine solution. The abdomen was draped with a single sterile drape with rectangular cut out to expose the operative site. Normal saline was administered subcutaneously to animals at the end of ischaemia 10 ml/kg to cover the duration of the experiments. For recovery experiments, analgesia in the form of buprenorphine 0.01 mg/kg subcutaneously was administered at the end of the experiment before recovering the animals.

A midline laparotomy was performed. The cephalic end of the wound was retracted avoiding contact with the viscera to obtain good exposure of the liver. The falciform ligament was cut in all groups. Viscera were handled gently with the ends of cotton buds wetted in sterile normal saline only. The small bowel was extruded and wrapped in swabs soaked in normal saline to optimise exposure of the liver. When undertaking ischaemia and reperfusion with or without preceding ischaemic preconditioning, a partial (70 %) hepatic ischaemia model was used where portal vessels to the lateral and median lobes were occluded (the so called cephalic lobes). This prevents mesenteric congestion by allowing portal vein decompression through the right and caudate lobes, bypassing the cephalic lobes.

The ligament between the caudate and lateral lobes was cut up to the portal vessels under a dissecting microscope (Zeiss). The plane under the portal vessels was developed by gentle blunt dissection using curved microsurgical forceps. Haemostasis was ensured and bleeding was minimal, soaking the end of one wetted cotton bud at most. An atraumatic clip was placed across the portal triad to the cephalic lobes under direct vision. Correct placement was confirmed by a colour change of the cephalic lobes to an ischaemc dark ruddy purple, the right and caudate lobes turned a suffused

red colour and the small bowel did not change colour. On removing the clip at the end of the ischaemia time, all lobes became a suffused purple/red colour before returning to a normal pink colour within 5 minutes. During the index ischaemia period the laparotomy was closed with clips to ensure good core body temperature control. The clips were removed at the end of the ischaemia time.

For nonrecovery experiments, the laparotomy was reclosed with clips once reperfusion had been established until the end of the designated reperfusion period, when blood samples were obtained by cardiac puncture through an anterolateral thoracotomy. The animal was terminated by cervical dislocation. Tissue samples were stored in liquid nitrogen for protein and mRNA analysis and at 4oC in 10% formalin for histology. For recovery experiments, the laparotomy was closed in two layers, with mass closure with 4.0 Vicryl as a continuous layer and 4.0 Vicryl interrupted, buried layer to skin. 0.05 mL of buprenorphine was given subcutaneously for postoperative analgesia and the animals were terminated 24 hours later as with the nonrecovery groups.

2.3. Histopathology

After euthanasia, representative pieces of ischemic, I/R, or bypass lobes are quickly removed and fixed in ice-cold 10% phosphate-buffered formalin for 24 h at 4°C. The tissue is then partially dehydrated with ethanol and embedded in plastic mounting medium using standard histological methods. Five-micrometer sections are cut and stained with hematoxylin and eosin. Several sections from each animal and given an overall score by an experienced liver pathologist blinded to the experimental group and conditions. The scoring system used was the Suzuki classification for liver ischaemia reperfusion injury. This is summarised in Table 2.1 below. The maximum score is 12, minimum score is 0. The mean score was taken for each group +/- sem.

Numerical Assessment	Sinusoidal Congestion	Vacuolisation/Ballooning	Necrosis
0	None	None	None
1	Minimal	Minimal	Single Cell
2	Mild	Mile	<30%
3	Moderate	Moderate	30-60%
4	Severe	Severe	>60%

Table 2.1: Suzuki classification of histological injury following ischaemia reperfusion

2.4. Measurement of Hepatic Microcirculation (HM)

HM was measured by a surface LDF (DRT4, Moor Instruments Limited, Axminster, **UK)** in flux units. The Doppler signal varies linearly with the product of the total number of moving red blood cells in the measured volume of a few cubic mm multiplied by the mean velocity of these red blood cells. The numeric product is termed perfusion units or blood cell flux units. The LDF probe was placed on a fixed site on the surface of the left lateral lobe of the liver and was held in place by a probe holder. LDF dam were collected continuously at sampling rate of 2 Hz. LDF measurements at the relevant time points were collected as a mean of 1-minute data.

2.5. Measurement of Hepatocellular Injury

A sample 0.2-0.5 mL of blood was collected from each animal by cardiac puncture via an anterolateral thoracotomy followed immediately by euthanasia by cervical dislocation. Serum alanine aminotransferase (ALT) was measured on a Hitachi 747 auto-analyzer using commercially available enzymatic kit tests. The tests were determined using reagents supplied by Boehringer Mannheim Ltd (Lewes, Sussex, UK).

2.6. Western Blot

Western blots were performed on liver samples from the animal groups to assess protein expression of eNOS, phosphorylated eNOS (p-eNOS), iNOS and HO-1.

Preparation of Protein Extracts

Samples of liver tissue (100–200 mg) were crushed in liquid nitrogen using a sterilised pestle and mortar. The crushed tissue was immediately scooped into fresh sterilised tubes and weighed. The cell pellet was suspended in 300 microlitres of Ripa Buffer with protease inhibitor (Thermo Scientific EDTA-free protease inhibitor cocktail 10 microlitres per I mL cell extract) and vortexed for 15 minutes with breaks

in between. The samples were centrifuged at maximum speed for 5 minutes at 4 ^{o}C. Aliquots of 50 microlitres of the supernatant were taken.

Lowry Technique of Protein Quantification

Standard solutions of albumin solution were prepared with Phosphate Buffered Saline (PBS) according to manufacturers instructions (xxx) and 10x and 100x dilutions of samples were prepared. Modified Lowry Protein assay Reagent (1 mL) was pipetted into a second set of labelled tubes followed by 200 microlitres of diluted standards and sample into the individually labelled tubes. The tubes were vortexed and left to incubate for 10 minutes at room temperature. A preprepared dilution of 1x Folin-Ciocalteu reagent (100 microlitres) was then added to all the standard tubes and samples, the tubes were vortexed and incubated for 30 minutes at room temperature. The standards were run in the spectrophotometer (read at 750 nm) programmed to calibrate absorbance against a standard Lowry protein concentration graph. The diluted samples were then run in the spectrophotometer. The sample concentration read outs were used to calculate the original sample protein concentrations in the sample aliquots. This was used to standardise protein concentration across wells during Western Blotting.

SDS gel electrophoresis and Western blotting

In order to load enough total proteins to each well, protein samples were diluted to 10 micrograms/microlitre made up to a volume of 30 microlitres with Ripa Buffer in new sterilised tubes. Laemlli Sample buffer 2x (30 microlitres) was pipetted into the new tubes. The samples were vortexed, heated in a water bath set at 95^{o}C. This cycle of vortexing and heating was repeated another two times. The samples were then centrifuged at 7400 rev/minute for a few seconds.

The samples were then loaded onto precast NuPAGE 4–12% gradient gels. Solubilized proteins were subjected to NuPAGE system (Invitrogen Life Technologies, Paisley,UK) on the precast gels using NuPAGE MOPS running buffer and electrophoretically transferred onto PVDF membranes using NuPAGE Western

blot Transfer buffer. After blotting the membranes, the transfer of proteins was checked using Ponceau S stain. All subsequent steps were performed under gentle agitation. The membranes were then blocked using PBS containing 5% Marvel, 1% and 0.05% Tween 20 for 30 minutes. Next, the membranes were incubated with appropriate primary antibody (polyclonal rabbit anti-eNOS, anti-phosphorylated eNOS, anti-iNOS, anti-HO-1 and 1:200 dilution, (Santa Cruz Biotechnology) overnight at 4°C. Next the membranes were washed three times for 5 min and one time for 15 min with PBS Tween 0.05%. Then the membranes were incubated for 2 h at room temperature with donkey anti- rabbit IgG HRP secondary antibody (1:200 dilution). The washing steps were then repeated, and to detect any proteins that bound the antibody, the membranes were incubated with West Dura reagents (Perbio, Cheshire,UK) for 5 min according to the manufacturer's instructions.

The membrane was exposed to a digital camera as part of an electronic imaging system to visualize the proteins bound to the antibody. Blots were quantified by densitometric analysis of each protein band compared with a background reading from each membrane using an image analysis system (Molecular Analyst/PC, Windows Software for Bio-Rad's (Hercules, CA) Image Analysis Systems Version 1.5, and the data are expressed in relative optical density (OD) units.

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR):

RT-PCR of liver samples was performed in all animals to assess expression of HO-1 mRNA.

Purification of Total RNA from Liver Tissue

Weighed frozen tissue (20-30 mg) was immediately placed in liquid nitrogen and ground thoroughly with sterilised pestle and mortar. Tissue powder was decanted into RNase-free 2 mL microcentrifuge tube. 600 microlitres of Buffer RLT with β-Mercaptoethanol (made up to 1:100 in Buffer RLT) was added and vortexed for 15 minutes. The lysate was centrifuged for 3 minutes at full speed and the supernatant was removed and transferred into a new microcentrifuge tube. Only the supernatant

was used in subsequent steps. One volume of 70 % ethanol in RNase-free H20 was added to the cleared lysate. Up to 700 microlitres of the sample supernatant was transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. 700 micrlitres of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. This washed the spin column and the flow-through was discarded. 500 microlitres of Buffer RPE with 4 volumes of 96-100% ethanol was added to the RNeasy spin column. The spin column was centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. The last step was repeated centrifuging the spin column for 2 minutes rather than 15 seconds to dry the spin column and preventing ethanol is not carried over in the next steps. The RNeasy spin column was placed in a new 2 mL collection tube and centrifuged at full speed for 1 minute. The RNeasy spin column is placed in a new 1.5 mL collection tube and 50 microlitres of RNase-free H20 is added directly to the spin column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA. The concentration of the RNA was measured by Gene Speck Machine.

Reverse Transcription Reaction

A volume of 10.5 microlitres of total RNA is mixed with 1 microlitre of 100 micromol/litre of the REVERSE primer in a RNase free sterilised tube incubated for 5 minutes at 95 oC to denature the linear RNA. The tube is spin centrifuged for a few seconds at maximum speed. While the tube is on ice, 2 microlitres of RT Buffer (10x), 5 microlitres of 4 dNTP, 0.5 microlitres of RNase Inhibitor and 0.5 microlitres RNase-free H20 were added to the tube. The mixture was vortexed and span down for 1 minute at maximum speed. 1 microlitre of reverse transcriptase enzyme 9RT) is added to the mixture, vortexed and span down. The mixture is incubated for 2 hours at 37oC to start the RT reaction of transformation from mRNA into cDNA. After the incubation is completed, the tube is transferred into a water bath at 70oC for 10 minutes to deactivate the RT enzymes.

Polymerase Chain Reaction (PCR)

All reactions used a RNase free tube. The PCR mix was made up over ice by adding 25 microlitres of TopTaq Master Mix 25 microlitres, 1 microlitre of 1:4 of 100 micomol/litre of FORWARD and of REVERSE primer, 5 microlitres of 10x CoralLoad, 16 microlitres of RNase-free H20 and 2 microlitres cDNA. This gives a total PCR mix volume of 50 microlitres. A control mix should be made up substituting the 2 microlites of cDNA with RNase-free H20. The PCR mix tube was placed on a PCR Express Machine on the following cycles: 95 oC for 15 minutes for 1 cycle followed by 94 oC for 1 minute then 56 oC for 1 minute and then 72 oC for 1 minute repeated for 30 cycles. The thermal cycling programme is ended with 1 cycle of 72 ^{o}C for 10 minutes. The samples are loaded into wells in a 2% E Gel for 15 minutes and viewed using the image analyser system.

Table 2.2. Haem Oxygenase -1 (HO-1) Primers

Forward Primer	5'-CACAGGGTGACAGAAGAGGCTAA-3'
Reverse Primer	5'-CTGGTCTTTGTGTTCCTCTGTCAG-3'

Statistical Analysis

Data was entered into and analysed using SPSS 14.0. The values were expressed as means+/- standard error. Means were compared using one-way ANOVA with posthoc Tukey analysis unless otherwise stated. $P<0.05$ was considered significant.

CHAPTER 3

OVERVIEW OF EXPERIMENTS

3.1. Partial Liver IR Model

During hepatic resection and liver transplantation surgery there are distinct periods of warm hepatic ischaemia followed by reperfusion. When the period of ischaemia is prolonged in this clinical setting, reperfusion results in liver IR injury, which is associated with organ dysfunction, acute liver failure, multiorgan failure and death. Developing in vivo models that reflect human IR injury help us better understand the mechanisms of liver injury and will help us to develop interventions that protect against IR injury clinically.

In models of warm hepatic ischaemia and reperfusion, hepatic ischaemia induced by occluding the portal vessels either with a tie or clamp most closely approximates the intraoperative situation that occurs during liver surgery.

Partial hepatic ischaemia prevents splanchnic congestion allowing decompression of the portal venous flow through perfused liver lobes. Total hepatic ischaemia causes portal venous congestion resulting in gut oedema causing increased gut wall permeability leading to translocation of bacteria forming the normal microflora into the portal system leading to portal endotoaxeamia and a systemic inflammatory response and sepsis. Thus partial lobar hepatic ischaemia in theory more specifically probes the pathophysiological mechanisms of liver IR injury without interactions from effects on the gut influencing results, in addition to replicating conditions in partial liver resection surgery. Small animals tolerate partial hepatic ischaemia better and the liver IR injury is less severe than for the equivalent global hepatic ischaemia times as discussed in Chapter 1.2.2. In mice, partial (70%) ischaemia of 45 to 60 minutes with reperfusion from 1 hours onwards consistently show evidence of IR injury with minimal animal mortality (Hines et al. 2002, Kawachi et al. 2000, Lee et al. 2001, Tsung et al. 2006, Wyllie et al. 2002). Therefore in the present study, the use

of a mouse warm partial (70%) hepatic ischaemia reperfusion injury model with 45 minutes ischaemia and 2 hours reperfusion was felt to be justified to study liver IRI.

3.2. Endpoints of Liver Injury

The three endpoints of serum ALT, surface Laser Doppler flowmetry assessment of liver microcirculatory dysfunction and histological injury scoring were used as endpoints of liver IR injury. A prolonged period of liver ischaemia followed by reperfusion results in liver IR injury. The resulting hepatocellular injury releases liver transaminases into the circulation which are detected within 1 hour of reperfusion (Wyllie et al. 2002; Hines et al. 2001). Therefore, with liver IR injury serum liver transaminases, such as ALT, GGT and AST are raised. Liver serum transaminases reach peak levels in IR between 6 and 12 hours of reperfusion (Shimamura et al. 2005; Kuboki et al. 2008). Longer periods of liver ischaemia result in greater IR injury and higher levels of serum transaminases for a given period of reperfusion (Yoshizumi et al. 1998; Yadav et al. 1999).

Liver IR injury can be demonstrated histologically initially by progressive sinusoidal congestion, hepatocyte vacuolisation and ballooning occurring within an hour of reperfusion, followed by progressive hepatocellular necrosis and apoptosis and neutrophil infiltration from 6 hours reperfusion onwards (Kato et al. 2002; Hines et al. 2002; Kawachi et al. 2000). Longer periods of ischaemia result in more severe histological liver injury with enhanced hepatocellular necrosis evident as more extensive pyknotic nuclei, cytoplasmic blanching and loss distinct hepatocellular borders (Hines, Hoffman et al. 2003). These are best observed under high power microscope of stained sections of liver and with electron scanning microscope. The most widely used histological scoring system in liver IR injury research is the Suzuki classification (Table 3.1), which is a validated scoring system for liver IR injury as discussed in Chapter 1.1.3.

By definition liver IR injury involves a derangement of liver microcirculation. This has been demonstrated physiologically in a number of ways, including intravital fluorescence microscopy (IVFM) showing reduced red blood cell velocity and surface laser Doppler flowmetry signal (LDF). Surface LDF readings of the liver during IR have been validated as a technique of assessing the microcirculatory dysfunction that

occurs associated with liver IR injury. A previous study demonstrated in a rat model a linear correlation between red blood cell velocity as measured by IVFM and LDF signal across sham laparotomy, liver IR with hepatic artery ligation (HA) and nonarterialised liver transplantation (NOLT) groups. They showed greater microcirculatory dysfunction with the more severe IR injury that occurred in the NOLT group of animals compared to the HA group (Tawadrous et al. 2001). Longer periods of liver ischaemia causing more severe IR injury and microcirculatory dysfunction are associated with lower LDF signals (Kazuo et al. 1998). Therefore LDF assessment of liver microcirculatory is a validated assessment technique and endpoint of liver IR injury.

The more endpoints that are used, the more robust the validation is of a model. In human liver transplantation increased warm and cold ischaemia time is associated with increased serum ALT, histological liver injury and impaired liver blood flow which are associated with reduced graft survival and increased mortality (Kelly DM et al. 2011; Friedman et al. 2012; Tzimas et al. 2004).As serum ALT, histological injury scoring and Laser Doppler flow assessment of liver microcirculation are validated endpoints for demonstrating liver IR injury, in the current study, we used all three endpoints to validate our model of liver IR injury. We found that anaesthesia for 3 hours with sham laparotomy did not result in liver IR injury, as there was no rise in serum ALT, no histological injury, or any derangement in liver microcirculation.

3.3. Selection of IPC Protocol to Study Protection versus Liver IR Injury

Ischaemic preconditioning (IPC) is a therapeutic strategy to reduce liver IR injury. The protective effect is dependent on the timing of ischaemia and reperfusion in IPC that precedes index IR that results in IR injury. The mechanism of IPC remains controversial and hence no individual measurement can determine the adequacy of the preconditioning stimulus. As a result it is the effect on liver IR that is being measured via the validated endpoints of IR that were discussed above. Systematic studies of different IPC protocols on murine models of partial hepatic IR showed that IPC of 10 minutes ischaemia (but not 5 or 15 minutes) and 10 or 15 minutes reperfusion protected against 75 and 90 minutes ischaemia followed by reperfusion (Yadav et al. 1999; Teoh et al. 2002; Vajdova, Heinrich and Clavien 2004). These previous

systematic studies showed somewhat variable results between them for timeframes of ischaemia and reperfusion of IPC that were protective against IR injury with small differences leading to a loss of protection by IPC. Also, in our mouse partial (70%) warm hepatic IR model of liver IR injury, the ischaemia period of 45 minutes we used is shorter than the above systematic studies of IPC protocols. It was therefore appropriate to establish an IPC protocol that reduces the liver IR injury in our model with a preliminary systematic study of different IPC protocols and their effects on liver IR injury.

3.4. EXPERIMENTAL GROUPS

Normal (i.e. wild type) C57Bl6 mice were randomly allocated to three groups, 5 animals per group:

1. Sham laparotomy.
2. IR only: index ischaemia reperfusion (45 min ischaemia/120 min reperfusion).
3. Ischaemic preconditioning (IPC) followed immediately by index ischaemia reperfusion.

In the Results Chapter 4, preliminary experiments done to establish best protective protocol of IPC. The IPC protocols investigated in the current study consisted of ischaemia of 3 minutes and reperfusion of 10 minutes (IPC 3/10), IPC 5/10, IPC 10/10 and IPC 10/15, which were felt to be reasonable IPC protocols based on previous systematic IPC studies discussed above.

In Chapter 4, we found that only IPC 5/10 was protective against liver IR injury in our model of partial liver IR. Therefore, in subsequent chapters we used this IPC protocol to study the effects of IPC on liver IR injury.

Nitric oxide is an important mediator of liver IPC and protection against liver IR injury, but the role of endothelial nitric oxide synthase (eNOS) is unclear. The main advantage of using mice for IR research is that many transgenic strains are available. The use of transgenic strains offers a robust method of studying the functions of various genes and their products, thereby providing insights into the molecular mechanisms underlying liver IR injury and IPC. Therefore the role of eNOS in IR injury and IPC was studied using eNOS transgenic knockout (eNOS-/-) mice, which

do not express eNOS. The same partial hepatic IR model was used as for wild type mice and the effect of IPC of 5 minutes ischaemia and 10 minutes reperfusion was evaluated. Three groups of eNOS-/- mice were studied:

1. Sham laparotomy.
2. IR only: index ischaemia reperfusion (45 min ischaemia/120 min reperfusion).
3. Ischaemic preconditioning (IPC) followed immediately by index ischaemia reperfusion.

In **Results Chapter 5** the effects of sham laparotomy and IR only in eNOS-/- animals was compared to wild type (WT) animals on the three endpoints of serum ALT, liver histological injury and liver microcirculation (as measured by LDF). **In Results Chapter 6** the effects of IPC on liver IR injury on the three endpoints was compared between eNOS-/- and WT animals. In **Results Chapter 7** the effects of sham laparotomy, IR only and of IPC on IR injury on liver protein expression (eNOS, phosphorylated eNOS, HO-1) and HO-1 mRNA was compared in wild type animals and eNOS -/- animals. Protein expression for eNOS and phosphorylated eNOS was also studied, but only in wild type animals, as eNOS-/- mice do not express eNOS.

In Chapter 7 we found that HO-1 protein was not detected in ay of the experimental groups, but HO-1 mRNA was detected in all experimental groups. Therefore, in **Results Chapter 8** we developed a model of late phase IR with recovery from anaesthesia ("recovery experiments") to assess if HO-1 protein expression followed a delayed timecourse. This consisted of 3 groups of wild type mice:

1. Sham laparotomy, recovery, euthanased 24hr later.
2. IR only: index ischaemia reperfusion (45 min ischaemia/ 24 hr reperfusion) only, recovery, euthanased 24 hr later.
3. IPC (5 minutes ischaemia and 10 minutes reperfusion) followed immediately by index ischaemia reperfusion (45 min ischaemia/ 24 hr reperfusion), recovery, euthanased 24 hr later.

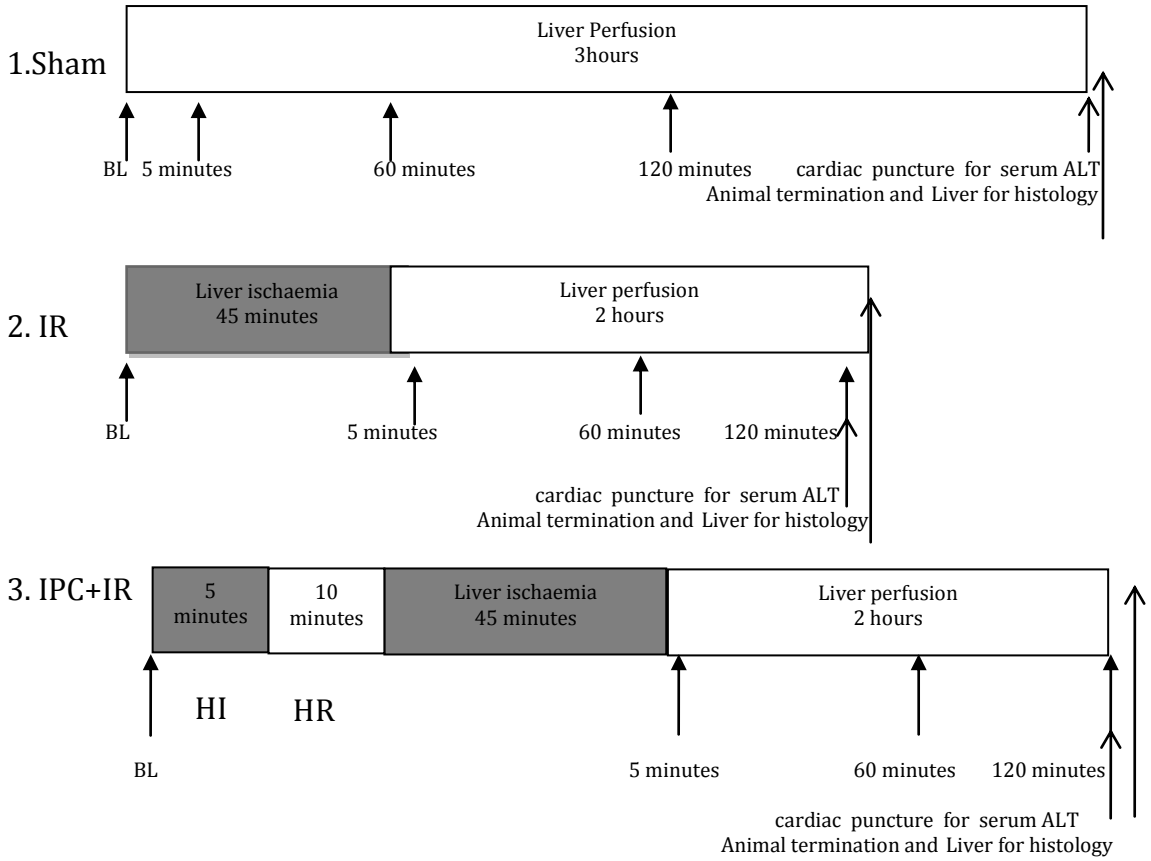


Figure 3.1. Experimental groups for nonrecovery experiments.

Thin arrows as labelled; BL. Baseline; HI. Ischaemia period during IPC; HR. Reperfusion period during IPC. For the longer term recovery experiments, ischaemic interventions were identical to those shown in 3. IPC + IR except that the recovery period was extended from 2h to 24h

CHAPTER 4:

SYSTEMATIC STUDY OF IPC PROTOCOLS IN A MOUSE MODEL OF LIVER IRI

4.1. INTRODUCTION

Liver ischaemia reperfusion injury (IRI) occurs after prolonged ischaemia followed by reperfusion in the clinical setting, such as liver resection surgery and liver transplantation, as discussed in Chapter 1. This is corroborated by animal models of liver IR injury. As discussed in Chapter 1.2, the pathophysiology of liver IR injury is initiated by reactive oxygen species (ROS) during reperfusion which cause direct cellular injury and also activate a cascade of mediators leading to microvascular changes and activation of acute inflammatory pathways with leading to liver injury.

Ischaemic preconditioning (IPC) is a potential therapeutic strategy to lessen liver IR injury. Liver IPC is a mechanical technique whereby a short period of occlusion of the blood supply to the liver confers protection against IR injury. This has been demonstrated in many large and small animal models of liver IR (Giovarnardi et al. 2009, Yoshizumi et al.1998, Koti et al.2002, Funaki et al. 2002, Berasain 2006, Izuisii et al. 2006, Teoh et al. 2004, 2006). As discussed in Chapter 3.3., systematic studies of different IPC protocols on murine models of partial hepatic IR showed somewhat variable results between them for timeframes of ischaemia and reperfusion of IPC that were protective against IR injury with small differences leading to a loss of protection by IPC.

In this chapter, the first aim was to confirm that early phase liver IR injury occurred in a mouse partial (70%) warm hepatic IR model with an IR protocol of 45 minutes partial (70%) ischaemia and 2 hours reperfusion (index IR). The second aim was to different IPC protocols were protective against liver IR injury (Methods Chapter 2 and Overview of Experiments Chapter 3), and therefore would be useful in studying the underlying protective mechanisms.

4.2. RESULTS

During the operative procedure all animals remained stable and tolerated anaesthesia, IR and IPC. There was no intraoperative mortality in any of the animals. All the endpoints were achieved.

Anaesthesia and Sham Laparotomy

There was no evidence of liver injury in the animals with 3 hours of anaesthesia and sham laparotomy (control group), reflected by normal serum ALT and absence of histological liver injury. (Figure 3.1 and Table 3.1).
Liver microcirculation did decrease progressively from baseline to 120 minutes, but this was only a relatively small decrease to 90% of baseline (Figure 3.2).

Effect of Liver IR without IPC

All the animals tolerated partial (70%) ischaemia of the cephalic lobes for 45 minutes followed by 2 hours reperfusion. IR injury was demonstrated by raised serum ALT (1805+/-454 IU vs sham 63+/- 33IU, $p<0.05$) and histological liver injury (score 3.67+/-0.47 vs sham no injury with score zero all samples) in the form of hepatocyte necrosis, cellular ballooning and sinusoidal congestion at the end of 2 hours reperfusion (Figure 3.1, Figure 3.3 and Table 3.1). Liver microcirculation was impaired relative to baseline for the duration of reperfusion . The liver blood flow progressively decreased from 5 minutes after reperfusion to 2 hours of reperfusion down to 65% of baseline blood flow(Figure 3.2, $P<0.01$ 1-way ANOVA).

The effect of different IPC protocols

In the previous sections it was established that sham laparotomy with anaesthesia was not associated with liver IR injury and that the IR protocol of 45 minutes partial (70%) hepatic ischaemia and 2 hours reperfusion (index IR) did result in liver IR injury. Following this, different IPC protocols were tested consisting of a single cycle of a short period of ischaemia followed by a short period of reperfusion, as described

in the Methods section (3.2), to assess if any of these IPC protocols had a protective effect in reducing the IR injury resulting from the index IR. The endpoints used to assess the effects of different IPC protocols were serum ALT and LDF measurements of liver microcirculation as described in section 3.2.

There was no significant difference in the serum ALT compared to index IR with an IPC protocol of IPC 3/10 (IR serum ALT 1805 IU+/- 454 versus IPC 3/10 serum ALT 2050 IU+/-378 P=0.38) and IPC 10/15 (IR serum ALT 1805 IU+/- 454 vs IPC 10/15 serum ALT 1580 IU+/-393 P=0.26) (Figure 3.1). There was no improvement in the microcirculatory dysfunction measured by LDF resulting from index IR alone when IPC 3/10 or IPC 10/15 preceded IR (P=0.32 and P=0.21, respectively Figure 3.2).

Animals undergoing IPC 10/10 preceding index IR had significantly higher serum ALT than animals in the IR only group (serum ALT 2910 IU+/- 219 versus 1805 IU+/-454 respectively, P<0.05, Figure 3.1). The liver microcirculatory dysfunction found in the IR only group was not improved by IPC 10/10 and actually appeared on visual inspection to be more severe in the IPC 10/10 group than the IR only group, but this did not reach statistical significance (P=0.10, Figure 3.2).

IPC of 5 minutes ischaemia and 10 minutes reperfusion (IPC 5/10) was the only IPC protocol used that reduced the severity of IR injury. This was reflected by a reduction in serum ALT compared to the IR only group of animals (560+/-218 IU versus 1805+/-454 IU respectively, P<0.05, Figure 3.1). The impairment in liver microcirculation observed throughout the 2 hour reperfusion period in the IR only group was also abrogated with IPC 5/10 (P<0.05 ANOVA, Figure 3.2). As this was the only IPC protocol that reduced IR injury based on the two endpoints, liver histopathology was also examined at the end of index IR, as described in the Methods section 3.2, as a third endpoint to validate the protective effects of IPC 5/10 more robustly. There was reduced liver histological injury with less hepatocyte necrosis and ballooning at the end of index IR in the IPC 5/10 group of animals compared to the IR only group (IPC 5/10 group histological score 1.67+/-0.47 versus IR only group score 3.67+/-0.47, P<0.05, Figure 3.3).

4.3. DISCUSSION

In this study a mouse model of partial (70%) hepatic IR was used to investigate liver IR injury and the effects of different IPC protocols preceding index IR on IR injury. The model was found to be robust and reliable. In this model sham laparotomy did not result in IR injury. Index IR consisting of partial (70%) ischaemia of cephalic lobes and 2 hours reperfusion resulted in liver IR injury. An IPC protocol consisting of 5 minutes partial (70%) ischaemia to cephalic lobes and 10 minutes reperfusion (IPC 5/10) followed immediately by index IR reduced the hepatocellular injury, histological changes and microcirculatory dysfunction of IR injury. IPC 3/10, IPC 10/10 and IPC 10/15 did not reduce IR injury. So, in this model, of the IPC protocols studied, only IPC 5/10 was protective against liver IR injury.

The animals tolerated partial (70%) ischaemia of the cephalic lobes for 45 minutes followed by 2 hours reperfusion. There was liver IR injury in this group. Liver IR injury was demonstrated in this mouse model of warm IR as elevated transaminases, impaired parenchymal perfusion and increased histological injury score. The demonstration of IR injury in this mouse model of liver IR and no liver injury with sham laparotomy is as noted in previous studies (Hines et al. 2001, 2002).

There was liver IR injury in all the IPC groups. Liver IR injury was demonstrated with all the IPC protocols in this mouse model by elevated transaminases and impaired parenchymal perfusion. In the IPC 3/10 and IPC 10/15 groups there was no statistically significant difference or obvious visual difference from the graphs of serum ALT and LDF in the severity of the IR injury compared to the IR group without IPC (Figure 3.1 and 3.2). This is consistent with reports from previous studies using a similar IR model and IR protocol, although there are far fewer mouse models of IPC with IR than larger animal models and many of these mouse models use longer ischaemia times for index IR than our model (Ishii et al. Teoh et al. 2003). We did not use an index IR ischaemia time of longer than 45 minutes, as this was associated with high intraoperative animal mortality, which was felt not to add greatly to relevant data collection.

IPC 10/10 increased serum ALT compared to index IR without IPC and there was a visual trend in the graph of LDF towards increased microcirculatory dysfunction compared to index IR without IPC, although the LDF trend did not reach statistical significance. This would suggest that IPC of 10 minutes ischaemia followed by 10 minutes reperfusion (IPC 10/10) before index IR of 45 minutes ischaemia and 2 hours reperfusion increases IR injury compared to index IR only in our mouse model. There is no data in the literature relating to these specific timings of IPC and IR for mouse partial (70%) hepatic IR models, however for equivalent rat models, studies have shown IPC 10/10 to reduce IR injury (Yoshizumi et al.1998; Koti et al.2002; Funaki et al. 2002; Berasain 2006; Izuisii et al. 2006 (BalB); Teoh et al. 2004, 2006). There may be a narrower time window of protection of IPC in mice than larger animals when index ischaemia times are 45 minutes or shorter in partial (70%) warm IR models, which would explain this difference between mice and larger animals. IPC 10/10 causes more severe IR injury than IPC 10/15 in our model. This may be because in our mouse model 10 minutes of reperfusion is too short a period to activate any protective pathways following 10 minutes ischaemia before the injurious 45 minutes index ischaemia is initiated.

IPC of 5 minutes ischaemia followed by 10 minutes reperfusion (IPC 5/10) before index IR of 45 minutes ischaemia and 2 hours reperfusion reduced the severity of IR injury. This was demonstrated by three endpoints as a reduction in serum ALT, lower histological injury scores and improved liver microcirculation. One of the aims of this chapter was to establish an IPC protocol that was protective against liver IR injury. The groups of animals that underwent the other IPC protocols did not exhibit reduced liver IR injury based on serum ALT and LDF assessments of liver microcirculation. As there was no protection against IR injury with IPC 3/10, IPC 10/10 and IPC 10/15 based on two endpoints, it was felt unnecessary to look at liver histology also as a third endpoint in these IPC groups. Therefore, IPC 5/10 was the only IPC protocol of those used in the present study that reduced liver IR injury. This IPC protocol of 5 minutes ischaemia is long enough to generate adequate substrates that initiate a protective pathway, but not so long as to be injurious, on reperfusion, while 10 minutes reperfusion is long enough to set off a cascade of protective molecular signalling pathways. Previous studies using a mouse model of partial (70%) hepatic

IR with index ischaemia of 45 minutes or less have also found that IPC 5/10 is protective against liver IR injury (Sawaya et al. 1999; Howell JG et al. 2000).

During index ischaemia livers were friable and the LDF probe occasionally caused visible trauma during liver ischaemia readings invalidating further LDF readings. As the experiments in this chapter were preliminary experiments for assessing liver IR injury and establishing a protective IPC protocol, having established LDF is severely impaired with IR with or without any of the IPC protocols, it was not felt justified to use a larger number of animals for each group without altering the conclusion of which, if any, IPC protocol was protective. Once this had been established, we intended to measure LDF during ischaemia, so in subsequent chapters LDF data during the ischaemia period will be included mainly for presentation reasons and to show similarly impaired microcirculation during ischaemia across the experimental groups.

In conclusion, we described and validated a mouse model of liver IR that results in IR injury and established a direct IPC protocol that significantly reduces this liver IR injury. This model should prove useful in investigating liver IR injury and the protective mechanisms of direct IPC and opens up the possibility of using transgenic knockout animals to study the underlying mechanisms of liver IPC and IR injury.

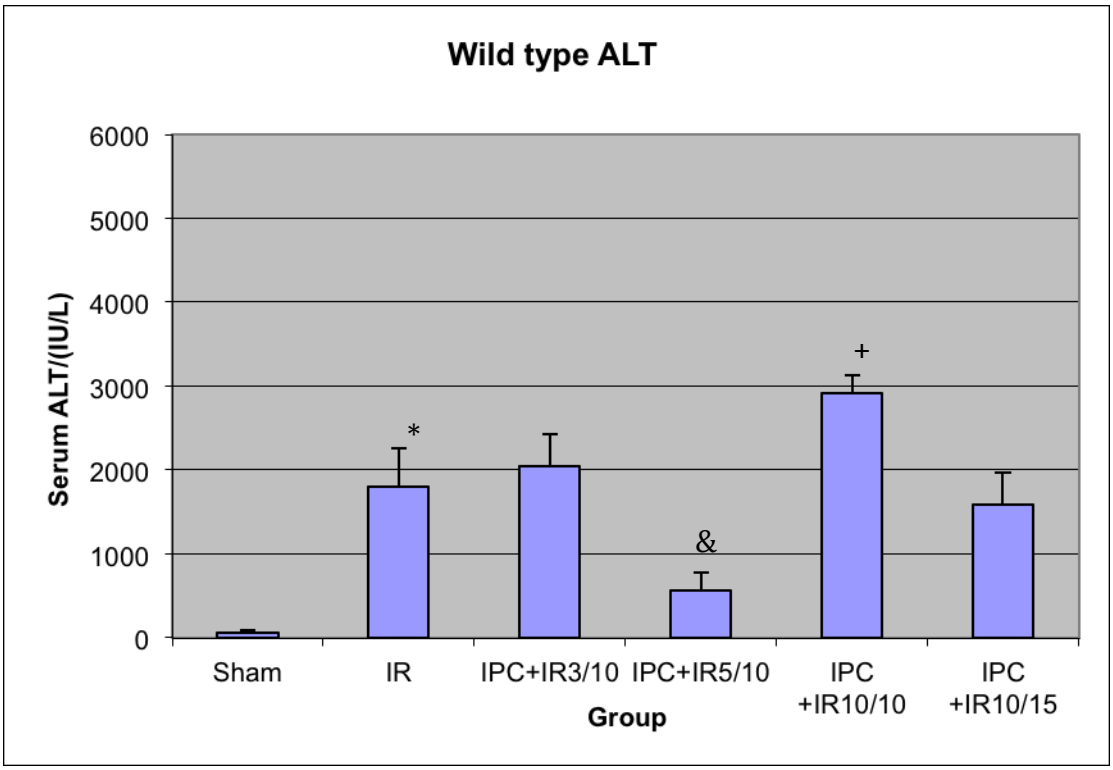


Figure 4.1: Serum ALT in sham, IR and various IPC protocol groups. Values are means +/- s.d. of 5 animals in each group (*P<0.05 vs. sham and IPC 5/10; $^{+}$P<0.05 vs. sham, IR, IPC 3/10, IPC 5/10, IPC 10/15; &P<0.05 vs. sham). IR=ischaemia reperfusion; IPC+IR X/Y= ischaemic preconditioning (X minutes ischaemia, Y minutes reperfusion) preceding ischaemia reperfusion.

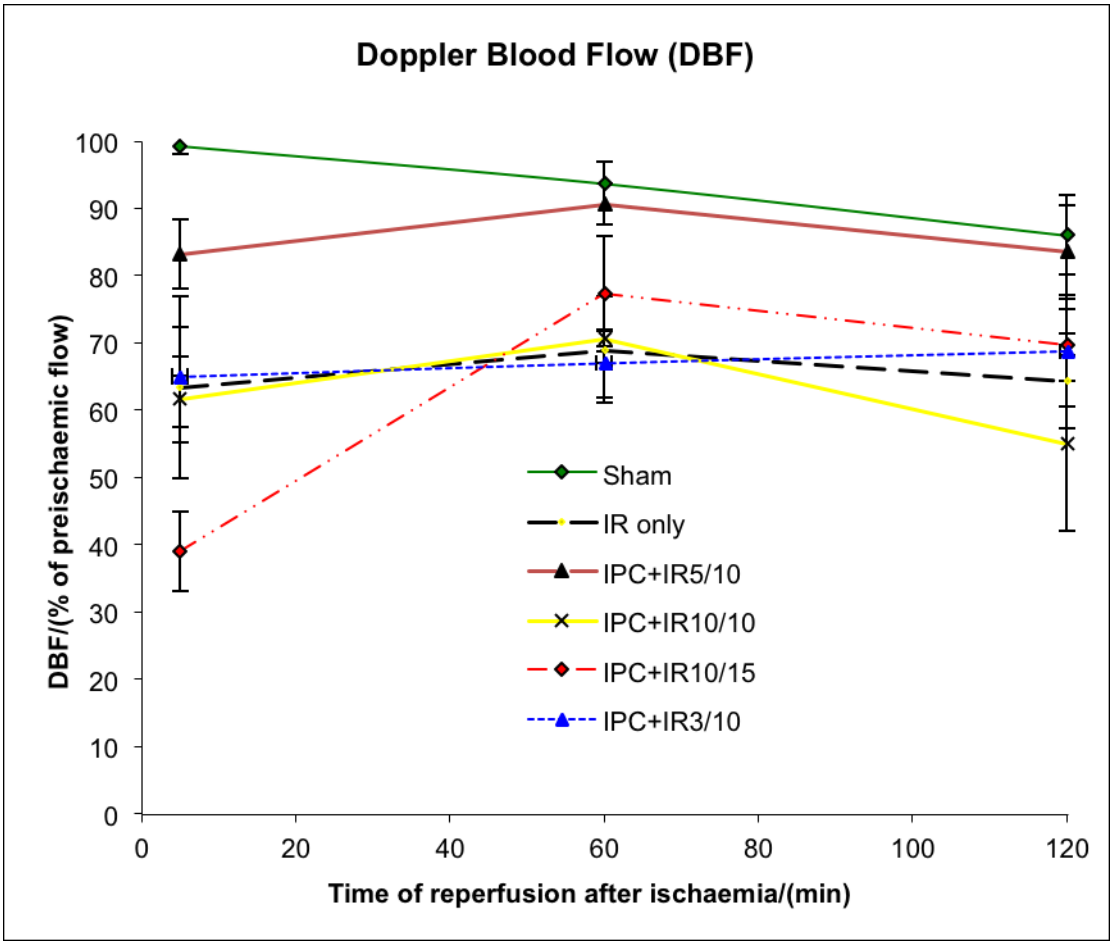
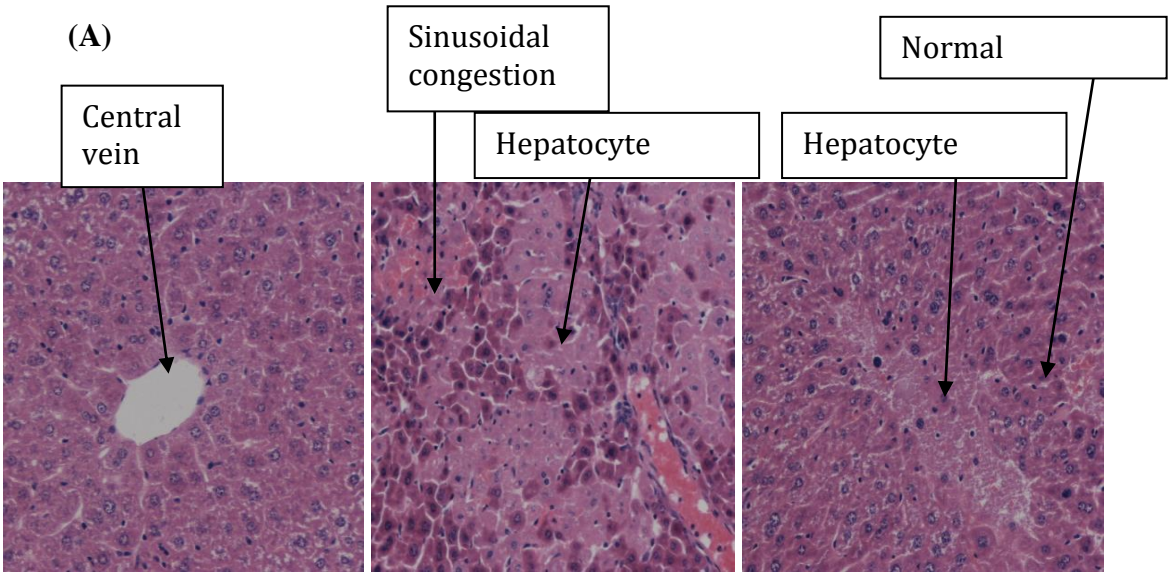


Figure 4.2: Liver microcirculation with sham, IR and different IPC protocols. Laser Doppler Flow (LDF) as percentage of preischaemia measurement at various timepoints during reperfusion after index ischaemia of 45 minutes for IR only and various IPC protocol groups and sham (no ischaemia). LDF was measured over the left lateral lobe of the liver for each group preischaemia (DBF 100% baseline value), following index ischaemia at 5 minutes, 60 minutes and 120 minutes reperfusion. Values are means+/-s.d. of 5 animals in each group. IR=ischaemia reperfusion; IPC+IR X/Y= ischaemic preconditioning (X minutes ischaemia and Y minutes reperfusion) preceding ischaemia reperfusion.



(B)

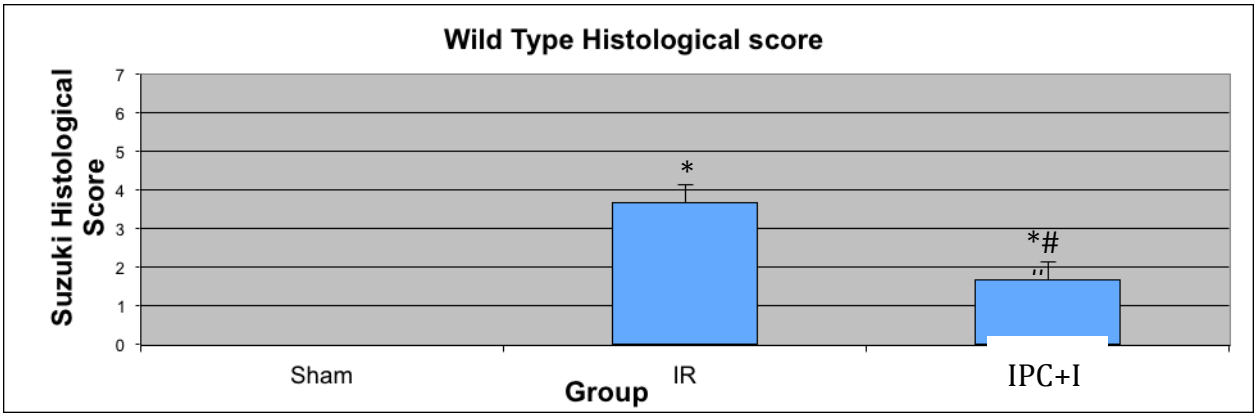


Figure 4.3: Liver histology for sham, liver IR only and IPC+IR groups. (A) Sections from the experimental groups (400X). (B) Bar graph summarising histological scores. Values are mean +/- s.d. of 5 animals in each group (*P<0.05 vs. sham, #P<0.05 vs. IR). IR=ischaemia reperfusion only; IPC+IR= ischaemic preconditioning of 5 minutes ischaemia and 10 minutes reperfusion preceding ischaemia reperfusion.

CHAPTER 5

COMPARISON OF ISCHAEMIA REPERFUSION INJURY (IRI) in eNOS-/- and WILD TYPE ANIMALS

5.1. INTRODUCTION

Our current understanding of the mechanisms of liver IR injury was discussed in detail in Chapter 1.2. Nitric oxide synthase (NOS) is an enzyme expressed in the liver that has drawn much attention in the field of IR injury. NOS is activated by liver IR and although it appears to modulate IR injury, its exact role is not well understood.

Nitric oxide (NO) is endogenously produced by NOS. Nitric oxide synthase (NOS) is an enzyme that catalyses the formation of NO and L-citrulline from L-arginine. The transient and volatile nature of NO makes it difficult to measure directly. However, because most of the NO is oxidised to NO2- and NO3-, the concentrations of these anions (NOx) are often used as a quantitative measure of NO production (Zeballos et al. 1995). Koti et al. (2002) found that NOx levels were reduced with liver IR injury in a rat partial (70%) hepatic IR model consisting of 45 minutes ischaemia and 2 hours reperfusion. L-arginine treatment (100 mg/kg intravenously) 10 minutes before liver IR significantly increased NOx levels, which resulted in reduced hepatocellular injury compared to IR only. An inhibitor of NOS, L-NAME (30 mg/kg iv) given 10 minutes before liver IR reduced NOx levels and increased hepatocellular injury compared to IR only. These findings are suggestive of NO having a protective role in liver IR injury with NO levels falling with prolonged ischaemia followed by reperfusion. This is corroborated by other studies on models of partial hepatic ischaemia reperfusion (IR) that have shown reduced ischaemia reperfusion injury (IRI) in the form of reduced serum transaminases and improved liver microcirculation when NO donors are used (Uhlmann et al. 1998, 2000). In contrast, very high levels of NO become injurious, as found by a group using a total heaptic ischaemia model consisting of 40 minutes ischaemia and 90 minutes reperfusion. This produces more severe liver IRI than the partial (70%) IR model. L-arginine (3 mg/kg iv) as an NO

donor given 20 minutes before liver IR resulted in increased liver IR injury and conversely L-NAME (5 mg/kg iv) given 20 minutely before IR reduced hepatocellular injury (Lin, Wang 2004).

NOS has two isoforms in the liver: a constitutive endothelial isoform (eNOS) and an inducible isoform (iNOS). Their respective contribution to liver IR injury is not well understood. Highly specific pharmacological inhibitors or activators of eNOS and iNOS are not currently available. Although L-NAME and L-NIL for instance are often advertised as inhibitors of eNOS and iNOS ,respectively, the specificity of their action on a particular isoform is in fact limited. The use of transgenic models of IR offers a robust method of studying the functions of various genes and their products, including eNOS and iNOS, thereby providing insights into the molecular mechanisms underlying liver IR injury, as discussed in the previous sections. Transgenic double knockouts of eNOS (eNOS-/-) and iNOS (iNOS-/-) are available and have been studied in models of liver IR injury.

Transgenic eNOS -/- animals have been used in a partial (70%) hepatic IR model to study IR injury. These studies have shown that liver IR injury is more severe in eNOS-/- animals than normal mice. In the eNOS-/- mice there is greater hepatocyte apoptosis and necrosis, increased leucocyte infiltration within liver lobules and higher serum transaminases compared with normal wild type animals (Kawachi et al. 2000; Lee et al. 2001; Hines et al. 2002). Consistent with this, mice with transgenic overexpression of eNOS sustain less severe liver IR injury than wild-type mice (Duranski et al. 2006). Overall, these findings support a protective role of liver eNOS in IR injury. Although there is liver IR injury in normal mice, more severe IR injury in mice lacking eNOS suggests that eNOS in normal mice blunts the severity of injury resulting from prolonged ischaemia followed by reperfusion. These findings have also been corroborated with experiments studying the effects of global combined cold and warm liver ischaemia followed by reperfusion in a mouse liver transplantation model using either donor wild type or eNOS-/- liver to a recipient wild type mouse. This study showed there was greater hepatocellular and histological injury, increased hepatocyte apoptosis and increased microcirculatory dysfunction as measured by intravital fluorescence microscopy with eNOS-/- donor livers than wild type donors (Theruvath et al. 2006). This would indicate that the protective effect of eNOS in liver

IRI, at least in a liver transplantation model, requires eNOS expression within the liver.

The current evidence suggests that NO derived from eNOS has a protective effect in liver IR injury. The previous studies of liver IR using transgenic eNOS mice have used two different endpoints of liver IR injury. In this chapter we describe the development of a transgenic eNOS-/- mouse model of partial (70%) hepatic IR for the study of the role of eNOS in liver warm IR injury using the three endpoints of liver IR injury used in the previous chapter (Chapters 2 and 3). The aim of the experiments in this chapter is to study the effect of IR in wild type and eNOS-/- to determine if NOS has a central role in liver IR and IPC.

5.2. RESULTS

During the operative procedure all animals remained stable and tolerated anaesthesia, IR and IPC. There was no intraoperative mortality in any of the wild type and eNOS-/- knockout animals. All the endpoints were achieved. The eNOS-/- mice were viable at 8 to 12 weeks, appeared as healthy as wild type mice, there were no anatomical differences intraabdominally on laparotomy and they were as stable as wild type mice under general anaesthesia.

Anaesthesia and Sham Laparotomy in eNOS-/- versus Wild Type Animals

Although in wild type animals there was no evidence of liver injury in the sham group, in eNOS-/- animals there was evidence of liver injury with anaesthesia and sham laparotomy. Serum ALT was raised (120+/- 59 IU vs 63+/- 33 IU in wild type mice)) and there was histological liver injury, mainly hepatocyte necrosis (Figure 4.1 and 4.3). There was no statistically significant change in the liver microcirculation in the sham group for the duration of the experiment although there was a trend towards reduced microcirculation over the duration of anaesthesia (Figure 4.2). The LDF did not differ between eNOS-/- sham and wild type normal sham groups.

Effect of IR on eNOS-/- versus Wild Type Animals

The eNOS-/- animals tolerated the period of liver ischaemia and reperfusion. There was acute hepatocellular injury (ALT 3655+/- 1607 vs sham120+/-59IU, $p<0.05$) and histological injury (score 4.75+/-1.3 vs shams 1.7+/-1.3, $p<0.05$). The IR injury was more severe in the eNOS-/- than wild type animals ($P<0.05$) (Figure 4.1 and 4.3).

Liver microcirculation was impaired in the eNOS-/- animals undergoing IR throughout the 2 hours of reperfusion compared with the sham eNOS-/- group ($P<0.01$ 1-way ANOVA, Figure 4.3). There was a greater percentage reduction in microcirculation with IR in wild type animals at all the time points of measurement during reperfusion compared with eNOS-/- mice (wild type 30-40% vs eNOS-/- 10-20%), but this did not reach statistical significance ($P=0.18$).

5.3. DISCUSSION

The eNOS-/- knockout model of partial hepatic IR to investigate the role of eNOS in IR injury was found to be robust and reliable. In this model sham laparotomy was not associated with liver injury in wild type animals, but there was mild liver injury with eNOS-/- animals. IR injury was more severe in the eNOS-/- knockout animals than the normal wild type animals.

Unlike wild type animals, in eNOS-/- animals anaesthesia with sham laparotomy was associated with a rise in serum ALT and histological injury at the end of 3 hours anaesthesia, although there was no effect on liver microcirculation. This indicates that anaesthesia alone with sham laparotomy activates physiological and molecular mechanisms that would result in liver histological injury, but baseline expression of eNOS protects against this. The stress response initiated by laparotomy activates inflammatory cascades that result in hepatocellular injury. NO released from baseline expression of eNOS in liver dampens this inflammatory response (Okajima et al. 2004, neutrophil elastase). Without an anaesthesia only without sham laparotomy group for eNOS-/- knockouts it cannot be ruled out that isoflurane anaesthetic sensitivity contributed to liver injury. Some reports have shown that liver injury can

result with isoflurane anaesthetic (Nishiyama et al. 1998) which may be related to increase intracellular Ca2+ in hepatocytes (Iaizzo et al. 1990) and reduced liver blood flow (Goldfarb, Debaene et al. 1990). In our study, the anaesthetic regimen was not associated with microcirculatory dysfunction in the sham laparotomy group of eNOS-/- animals. This is consistent with previous reports where after sham laparotomy and anaesthesia, eNOS-/- livers have the same sinusoidal with and flow velocity (as measured by intravital flow microscopy) as wild type livers, indicating no significant difference in baseline liver microcirculation with the absence of eNOS (Theruvath et al. 2006). Systemically, eNOS-/- mice are hypertensive at baseline, under anaesthesia and postoperatively by 20-30% compared to wild type animals with greater MAP variability (Huang et al. 1995, 1999; Strauss et al. 1999). These effects are mediated through reduced systemic baseline vasodilatation. This may act to increase susceptibility to anaesthetic injury by increasing the bioavailability of anaesthetic.

In the eNOS-/-animals there was increased liver IR injury compared to wild type animals demonstrated by increased hepatocellular injury and increased histological IR severity score. The more severe histological injury resulting from liver IR injury in the eNOS-/- animals than wild types was related to increased hepatocyte necrosis evident as more extensive pyknotic nuclei, cytoplasmic blanching and loss of distinct hepatocellular borders. The demonstration of IR injury in wild type and eNOS-/- animals with more severe IR injury in eNOS-/- in this IR model is as noted in previous studies using eNOS-/- models of lobar hepatic ischaemia (Kawachi et al. 2000; Hines et al. 2001).

The 2 hours reperfusion time following the prolonged 45 minutes ischaemia is too early in the phase of IR injury for neutrophils to accumulate within the liver sinusoids as seen with longer reperfusion periods in the IR injury process (Kawachi et al.2000). This indicates that baseline eNOS partially protects against hepatocellular injury occurring with early phase IR injury. It does this by inhibiting hepatocyte necrosis, but not by affecting the liver microcirculation in vivo. NO from eNOS acts as a scavenger of reactive oxygen species (ROS) and there is evidence that it activates survival kinase pathways (Kim JS, Ohshima S et al. 2004) through guanylate cyclase (Duranski et al. 2006). This would explain a partial protection mediated by baseline

hepatocyte eNOS against hepatocellular injury in the absence of a cellular inflammatory response. Previous reports have, however, also shown that NO maintains perfusion of the hepatic microcirculation (Shibayama et al. 2002) and modulates liver injury through its vasodilatory effects (Jaeschke et al. 2006), but this has involved the use of exogenous agents, such as inhaled NO, in models of hepatic IR rather than in vivo studies of endogenous NO in liver IR.

In conclusion, we described a mouse model of IR that results in IR injury and that in eNOS-/- mice results in more severe liver IR injury than wild type normal mice. This shows that eNOS is protective in early phase warm liver IR injury. eNOS exerts its protective effects through the reduction of hepatocellular injury, but not by preserving liver microcirculation. This would suggest that eNOS within hepatocytes rather than sinusoidal endothelial cells blunts the severity of IR injury following liver IR without preconditioning. This model should prove useful in investigating the protective mechanisms of eNOS in liver IR injury. Having evaluated the effect of anaesthesia and IR in the eNOS animal model in this chapter, in the next chapter we wished to analyse the mechanism of IPC using the model.

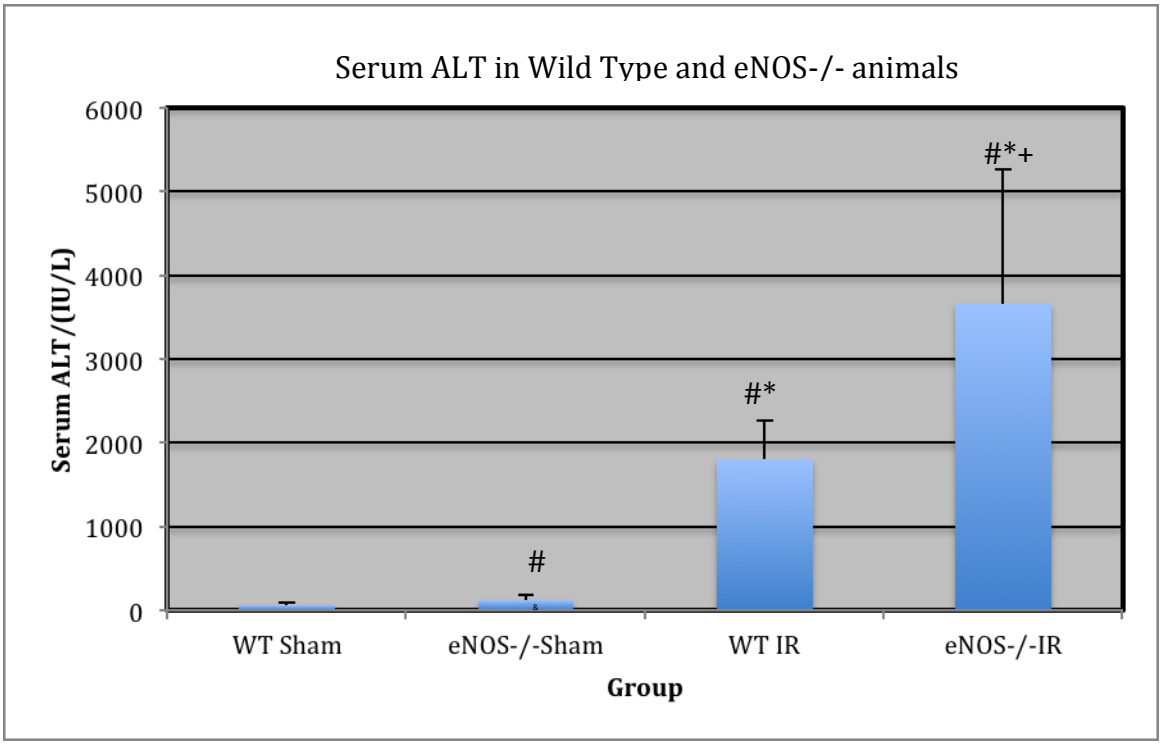


Figure 5.1: Serum ALT in Wild Type and eNOS(-/-) groups. Values are means+/-s.d. of 5 animals in each group (*P<0.05 vs. eNOS-/- sham, #P<0.05 vs. WT sham, +P<0.05 vs. WT IR). IR=ischaemia reperfusion only; IPC=ischaemic preconditioning preceding ischaemia reperfusion.

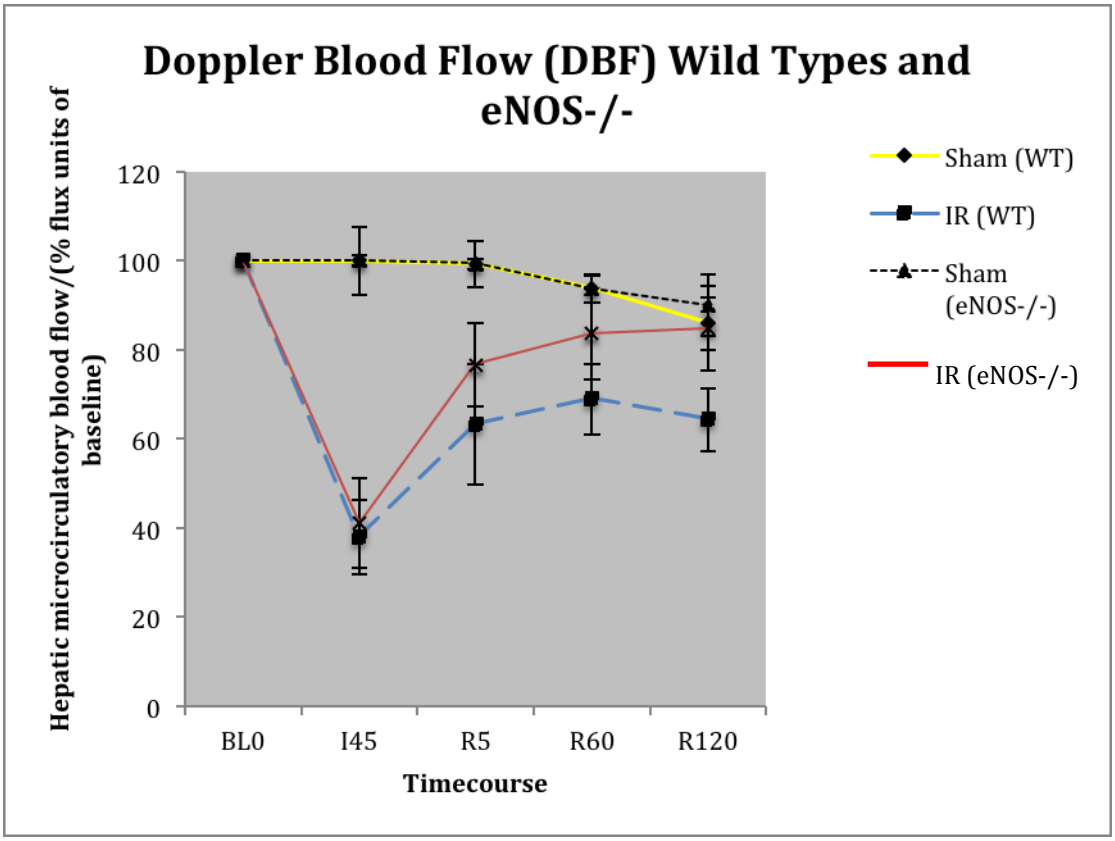
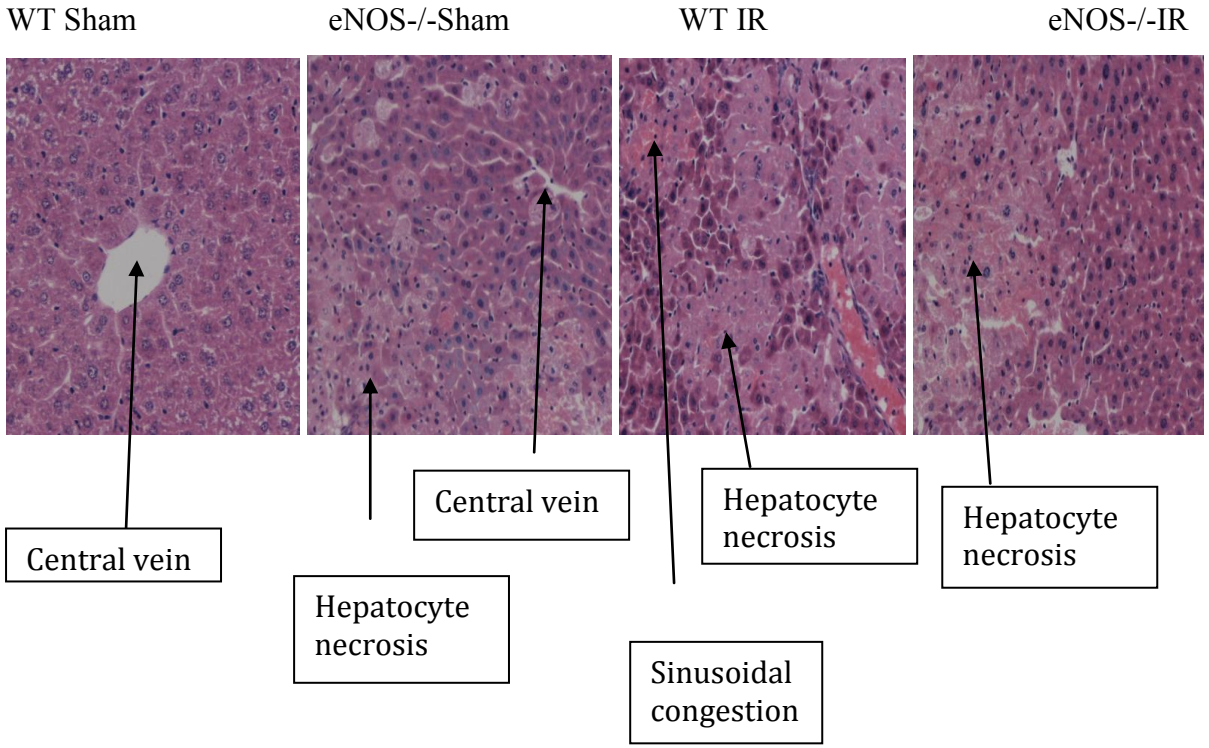


Figure 5.2: Hepatic Microcirculation during reperfusion after Index ischaemia of 45 minutes in eNOS Knockouts and wild type animals. BL= baseline microcirculation at sham laparotomy (time 0); I45= 45 minutes partial (70 %) hepatic ischaemia; R5= 5 minutes reperfusion after ischaemia; R60= 60 minutes reperfusion after ischaemia; R120= 120 minutes reperfusion after ischaemia IR=ischaemia reperfusion only; WT=wild type animals; eNOS-/-=eNOS knockout animals. Data points are mean+/- sem.


WT Sham
eNOS-/-Sham
WT IR
eNOS-/-IR
Central vein
Hepatocyte necrosis
Central vein
Sinusoidal congestion
Hepatocyte necrosis
Hepatocyte necrosis

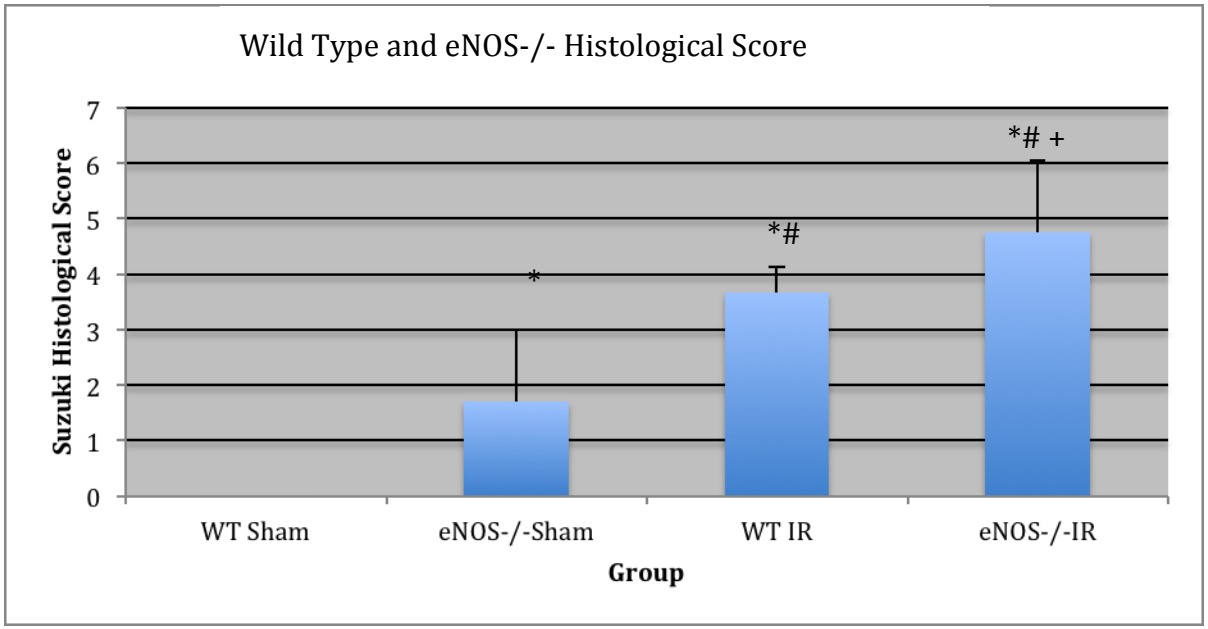


Figure 5.3: Liver histology eNOS-/- and WT animals. (A) Sections from the experimental groups (400X). (B) Bar graph summarising histological scores. Values are mean +/- s.d. of 5 animals in each group (*P<0.05 vs. sham WT, #P<0.05 vs. eNOS sham, +P<0.05 vs. WT IR). WT=wild type animals; IR=ischaemia reperfusion only; IPC= ischaemia preconditioning preceding ischaemia reperfusion.

CHAPTER 6:

COMPARISON OF EFFECT OF ISCHAEMIC PRECONDITIONING (IPC) on eNOS-/- and WILD TYPE ANIMALS

6.1. INTRODUCTION

The role of eNOS in liver IPC is not well understood. A rise in blood and periportal nitric oxide (NO) levels is associated with the development of liver tolerance to reperfusion injury, which is abolished by adding inhibitors of NO release such as L-NAME. NO donors promote liver preconditioning, even in the presence of NO synthase inhibitors (Koti et al. 2002, Peralta et al. 1997, Arai M et al. 2001,Peralta et al. 2001). NO appears to act as a trigger in early IPC (Rakhit et al. 1999). Understanding the mechanisms by which NO release is activated and how it reduces liver IR injury in IPC offers the possibility of developing specific pharmacological agents for clinical use to protect against liver IR injury and its sequelae.

Endogenous NO production in liver IR injury is by the two isoforms eNOS and iNOS, but it is unclear what the dominant source in vivo is in IPC. Koti et al. (2005) showed in a rat model of partial (70%) hepatic ischaemia that early IPC is associated with increased hepatic vein nitrate and nitrite (NO_x) and with increased eNOS protein expression localised to hepatocytes and sinusoidal vascular endothelium associated. Addition of a nonspecific NOS inhibitor, L-NAME, abolished the increased expression of eNOS and rise in hepatic vein NO_x. iNOS expression was absent in all groups. This suggests that eNOS is the source of NO in early IPC. This study relied on pharmacological agents to arrive at conclusions about the role of eNOS in early IPC.

An eNOS -/- knockout has not been used to study liver IPC and would give robust mechanistic insights into the role of eNOS in liver IPC, as the disruption is highly specific and complete at the genetic level. In this chapter we studied the effect of IPC on liver IR injury in vivo in eNOS-/- and wild type animals (Chapters 2 and 3).

6.2. RESULTS

During the operative procedure all animals remained stable and tolerated anaesthesia, IR and IPC. There was no intraoperative mortality in any of the wild type and eNOS-/- animals. All the endpoints were achieved.

The effect of IPC followed by IR on eNOS-/- animals

The eNOS-/- animals underwent direct IPC of 5 minutes ischaemia followed by 10 minutes reperfusion to the cephalic liver lobes followed by 45 minutes ischaemia of the cephalic lobes and then 2 hours reperfusion. Although IPC preceding IR was still associated with liver IR injury, the mean serum ALT was reduced by 50% in the IPC+IR group compared to the IR group in the eNOS-/- animals, but with 5 animals per group this difference did not reach statistical significance (P=0.20). IPC did not reduce the histological liver injury score in eNOS-/- groups (P=0.29; Figure 5.4), unlike wild type animals. IPC failed to reverse the reduction in the liver microcirculation found in the in eNOS-/- group (P=0.34, Figure 5.3). Taken together there is no protection against IR injury with IPC preceding IR in eNOS-/- animals in this model of liver IR. This is in contrast to the effects of IPC in wild type animals in this model.

6.3. DISCUSSION

In this chapter we have described a new model for the investigation of the mechanisms of ischaemic preconditioning using an eNOS-/- knockout animal. The model was found to be robust and reliable. Ischaemic preconditioning (IPC) reduced the hepatocellular injury of IR in the eNOS -/- knockouts, although this did not reach statistical significance, but IPC did not reduce histological changes or the reduced microcirculation found during the period of reperfusion. This would suggest that eNOS is a mediator of the beneficial effect of IPC.

Throughout our study we have used a model of partial (70%) hepatic IR in which liver IR injury was demonstrated consistently for wild type and eNOS-/- animals and where IPC of 5 minutes partial ischaemia and 10 minutes reperfusion reduced IR injury in wild type animals.

It was therefore felt reasonable to use the same IPC protocol on eNOS-/- animals to assess the role of eNOS in the protective effects of IPC against liver IR injury.

Nitric oxide (NO) has previously been shown to be a key mediator of liver IPC (Peralta et al. 1999; Koti et al. 2002). IPC is associated with increased nitric oxide metabolites and increased expression of nitric oxide synthase in hepatocytes and vascular endothelium in the centrilobular zone (Koti et al. 2002). In the present study it was shown IPC of 5 minutes ischaemia followed by 10 minutes reperfusion reduced IR injury in normal wild type animals, but the same IPC protocol failed to show significant protection against liver IR injury in eNOS-/- animals as demonstrated by no improvement in liver microcirculation, no decrease in histological injury and highly elevated serum ALT. This indicates that eNOS is a protective mediator of IPC and reduces IR injury by not only reducing hepatocellular injury but also by improving liver microcirculation. This would suggest that IPC activates eNOS in both hepatocytes and sinusoidal endothelial cells (SECs). This is consistent with previous reports, which have shown that IPC improves liver microcirculation and SEC wall integrity in hepatic IR models (Vajdova, Heinrich et al 2004; Glanemann et al. 2003). In a rat liver transplantation model IPC was shown to reduce SEC injury, which is a predominant feature of liver donor cold storage and reperfusion injury, by more than 50 %, improving graft survival (Arai M et al. 2001). IPC activates eNOS which releases NO. NO activates soluble guanylate cyclase (sGC) which catalyses formation of cGMP and GTP which causes relaxation of vascular smooth muscle by a number of mechanisms, including inhibiting release of the vasoconstrictor endothelin-1 (Rensing et al. 2002; Suematsu et al. 1996; Peralta et al. 1996). The cGMP also acts as a second messenger activating downstream survival MAP kinase pathways (Carini et al. 2003).

In the present study the model of partial hepatic ischaemia with an index IR of 45 minutes ischaemia and 2 hours reperfusion causes greater liver IR injury in eNOS-/- than wild type animals. Therefore, a limitation of the present study is that an optimized IPC protocol was not used for eNOS-/- animals, as although IPC of 5 minutes ischaemia followed by 10 minutes reperfusion before index IR has only very limited protective effect in eNOS-/- knockouts, it is possible other IPC protocols may be protective for the more severe injury found in knockout animals which involve eNOS independent mechanisms. There is some evidence of this in our model, as IPC did reduce serum ALT by 50% compared to the IR only group, but this did not

reach statistical significance because of the relatively large standard deviation of the mean serum ALT in the knockout animal IR and IPC+IR groups. There is therefore the possibility of a type II error for the serum ALT results in the eNOS-/- animals with five animals per group, precluding a solid conclusion about whether IPC reduced serum ALT in eNOS-/- knockouts. This indicates that this part of the study was underpowered. This could be overcome by using at least 7 animals per group based on retrospective power and sample size calculation (α=0.05 and power 80 %).

Another limitation is that by studying a knockout animal we only obtain information about the effect of the presence or absence of a protein, but not about the **graded effects** of levels or timecourse of that protein (in this case eNOS) or the signalling cascades it is involved in.

In conclusion, we described a new mouse model of direct IPC that in wild type mice significantly reduces liver IR injury and in eNOS-/- mice has no significant hepatoprotective effect against liver IR injury. This shows that eNOS is a protective mediator of direct IPC in early phase warm liver IR injury. eNOS exerts its protective effects through the preservation of liver microcirculation and by directly reducing hepatocellular injury, most likely by activating downstream cell survival signaling pathways. This suggests that IPC activates eNOS in both hepatocytes and SECs. This model should prove useful in investigating the protective mechanisms of eNOS in direct IPC and liver IR injury.

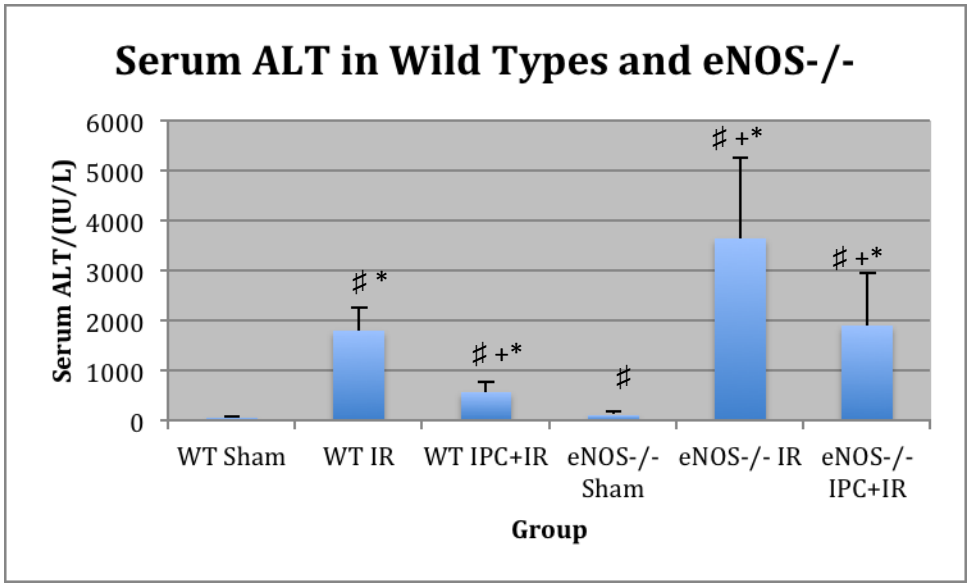
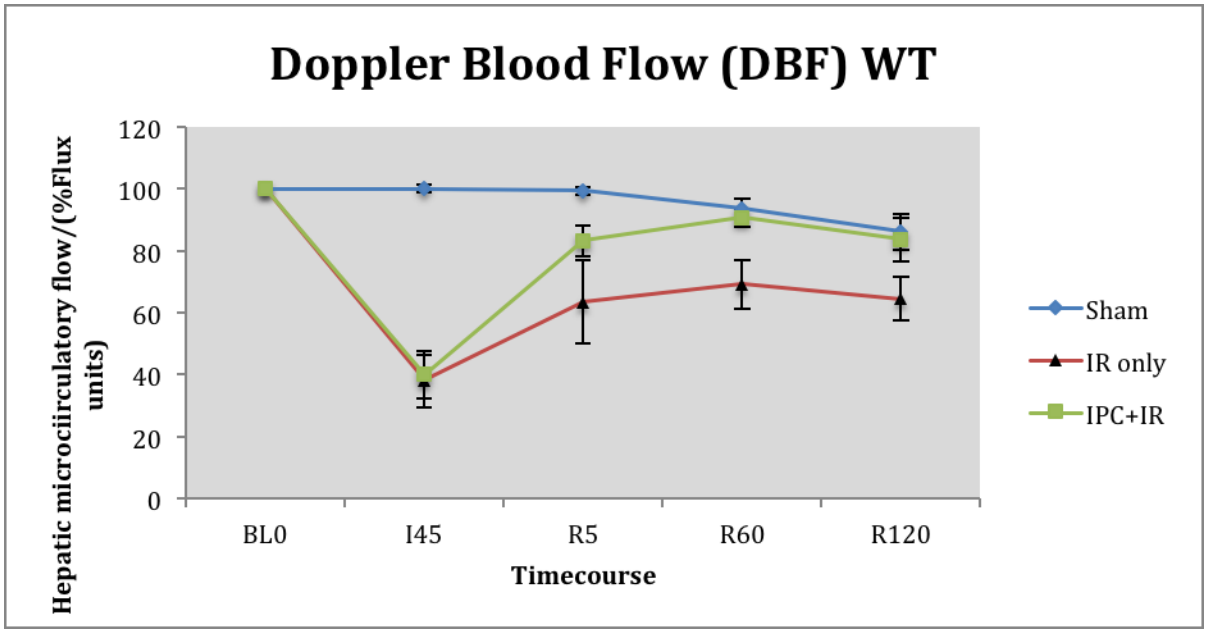


Figure 6.1: Serum ALT in Wild Type and eNOS(-/-) groups. Bar graph summarising serum ALT. Values are mean +/- s.d. of 5 animals in each group (# P<0.05 vs. WT sham, +P<0.05 vs. WT IR, *P<0.05 vs. eNOS -/- sham. IR=ischaemia reperfusion only; IPC+IR= ischaemia preconditioning preceding ischaemia reperfusion; WT=wild type animals; eNOS= endothelial nitric oxide synthase; eNOS-/- =eNOS transgenic double knockout.

(A)



(B)

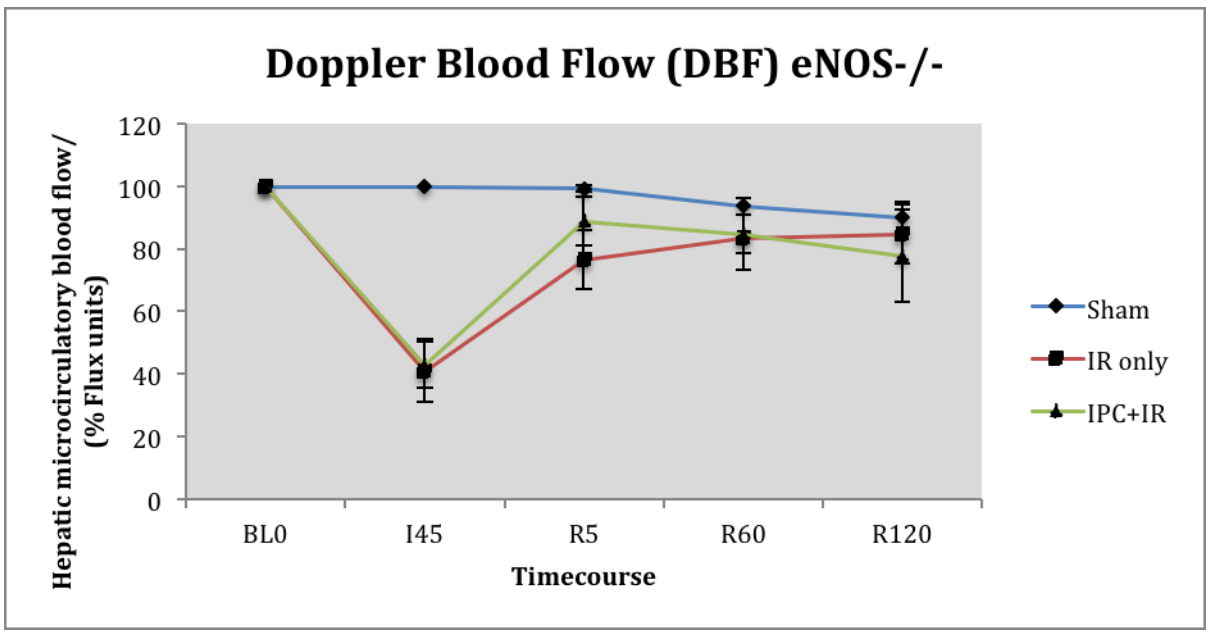
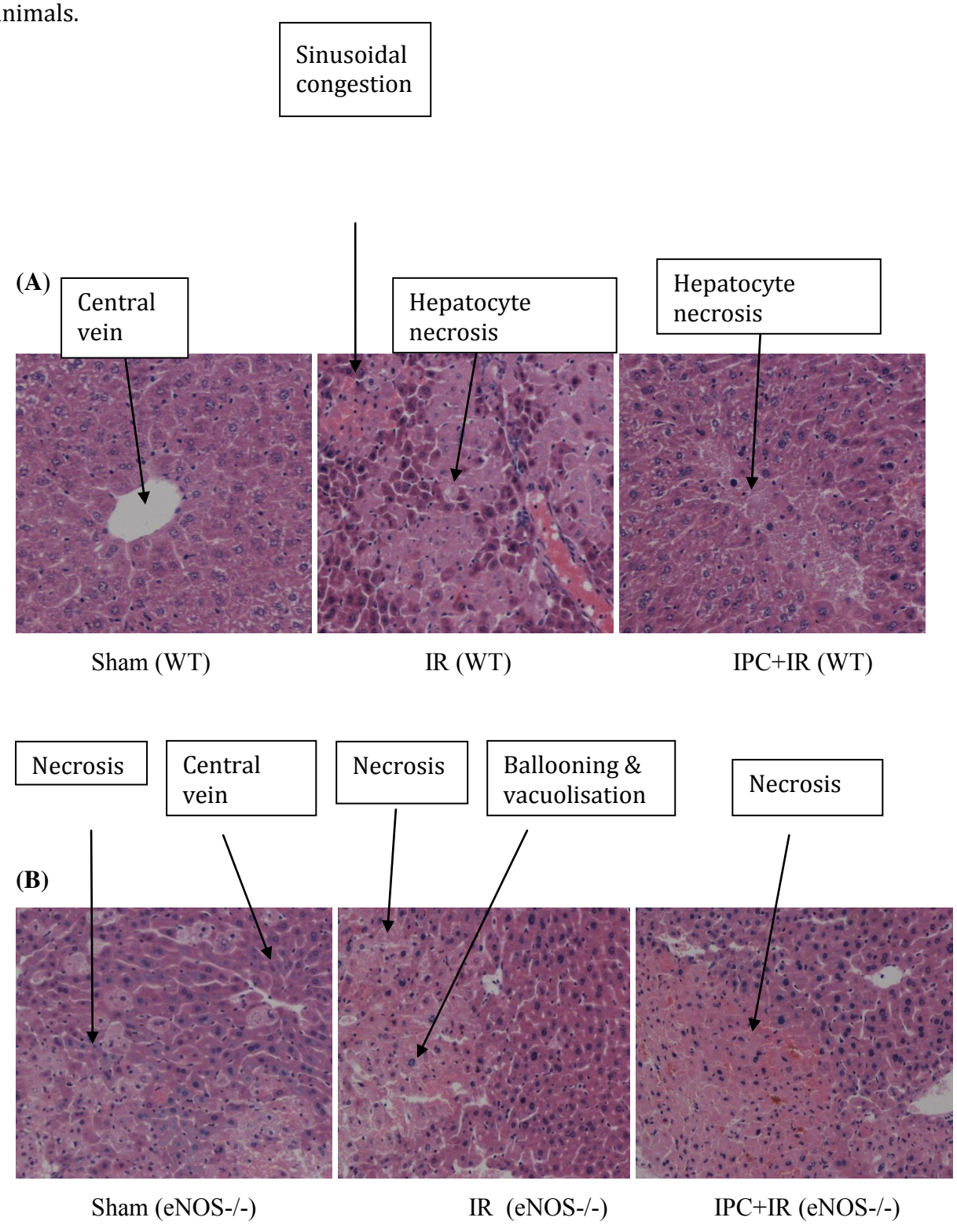
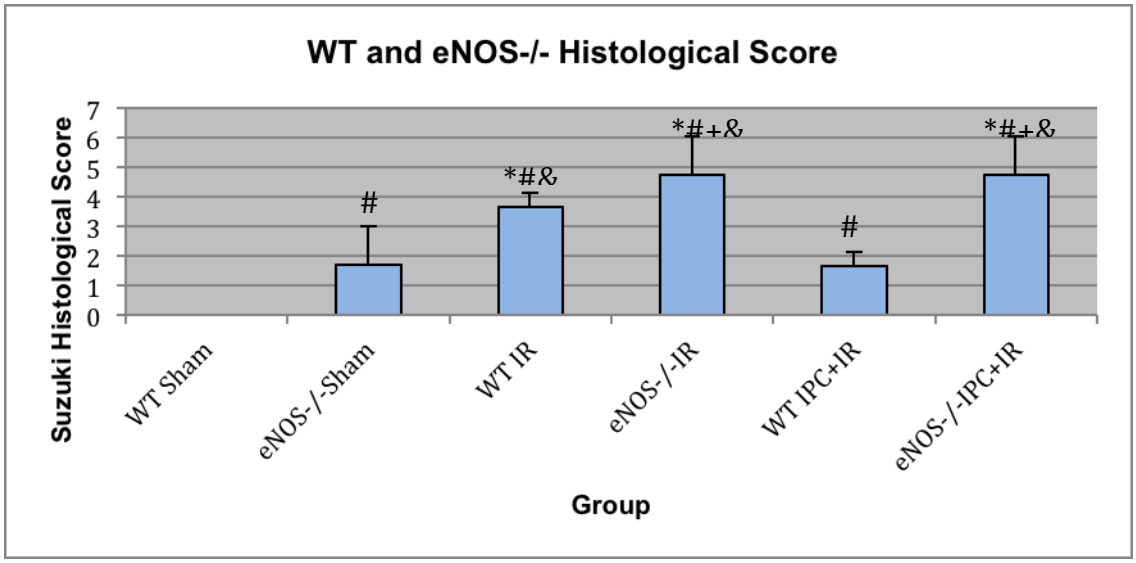


Figure 6.2: Liver microcirculation in WT and eNOS-/- animals. (A) Hepatic Microcirculation during reperfusion after Index ischaemia of 45 minutes in Wild Types. (B) Hepatic Microcirculation during reperfusion after Index ischaemia of 45 minutes in eNOS Knockouts. BL= baseline microcirculation at sham laparotomy (time 0); I45= 45 minutes partial (70 %) hepatic ischaemia; R5= 5 minutes reperfusion after ischaemia; R60= 60 minutes reperfusion after ischaemia; R120= 120 minutes reperfusion after ischaemia IR=ischaemia reperfusion only; IPC+IR= ischaemia preconditioning preceding ischaemia reperfusion; WT=wild type mice. Data points are mean+/-sem.

Figure 6.3: Liver histology for sham, liver IR only and IPC in WT and eNOS-/- groups. (A) Sections from the eNOS-/- groups (400X). (B) Sections from WT groups (400X). (C) Bar graph summarising histological scores. Values are mean +/- s.d. of 5 animals in each group (# P<0.05 vs. WT sham, *P<0.05 vs. eNOS -/- sham, +P<0.05 vs. WT IR, &P<0.05 vs. WT IPC). IR=ischaemia reperfusion only; IPC= ischaemia preconditioning preceding ischaemia reperfusion; WT=wild type animals.



(C)


WT and eNOS-/- Histological Score
Suzuki Histological Score
0
1
2
3
4
5
6
7
#
*#&
*#+&
#
*#+&
WT Sham
eNOS-/-Sham
WT IR
eNOS-/-IR
WT IPC+IR
eNOS-/-IPC+IR
Group

CHAPTER 7:

COMPARISON OF NOS AND HO-1 Protein EXPRESSION in eNOS-/- and WILD TYPE ANIMALS IN EARLY PHASE LIVER IR INJURY

7.1. INTRODUCTION

Endothelial nitric oxide synthase (eNOS) is a key mediator of the protective effects of liver ischaemic preconditioning (IPC) in liver IR injury. Studies have shown increased expression of hepatocyte eNOS in early IPC and the protection of IPC was abolished with inhibitors of NOS (Koti et al. 2005, Serracino-Inglott 2002).

The protective effects of eNOS in early IPC may relate to increased activation and/ or expression of eNOS protein. eNOS is activated in a number of ways: by Ca^{2+}-calmodulin , phosphorylation by protein kinases (such as PI3-Akt) and association with a number of molecules such as hsp-90 (Fleming et al. 1999). It is unclear which of these mechanisms dominate in liver IPC.

The role of iNOS in liver IRI and IPC has not been established. Genetic knockouts of inducible nitric oxide synthase (iNOS -/-) have been used to study liver IR injury, but the various models of liver IR injury have reached some conflicting conclusions. This ranges from some studies showing no role for inducible nitric oxide synthase (iNOS) in liver IR injury (Kawachi et al. 2000;Khandoga et al. 2002), to iNOS being protective (Lee, Baust 2001) to others citing iNOS as contributing to the liver injury (Hamada et al. 2009).

Haem oxygenase-1 (HO-1 or heat shock protein 32, hsp 32) has attracted much research interest, because of its potential cytoprotective roles in diverse disease and injury states, including liver IRI, and its surprising similarities to NOS (Chapter 1.2.14 and 1.3.6). HO-1 is an enzyme that catalyses formation of carbon monoxide (CO), biliverdin and Fe^{2+} from degradation of haem. HO-1 is the inducible isoform of haem oxygenase. It is ubiquitously expressed in organs, including liver, and circulating nucleated cells. The specific mechanism(s) by which HO-1 can mediate cytoprotective functions is not clear, but

byproducts generated during the haem catabolism have been suggested as potential protective mediators (Fujita et al. 2001; Neto et al. 2004; Nakao et al. 2004; Baranano et al. 2002).

Inducers of HO-1 (hsp-32) reduce liver IR injury (Shen XD et al. 2005; Kato Y et al. 2003) while inhibitors of HO-1 exacerbate liver IR injury (Kato Y et al. 2003). HO-1 has been implicated as having a protective role in IR injury through its by-products CO and biliverdin which improve liver microcirculation and reduce hepatocellular apoptosis and necrosis (Katori et al. 2002). Models of early phase liver IPC have shown increased HO-1 mRNA, followed by HO-1 protein expression and increased activity (Patel A et al. 2004; Coito et al. 2002; Lai IR et al. 2004; Massip-Salcedo M et al. 2006).

HO-1 and NOS have surprising similarities in their regulation. HO-1 and NOS are both up-regulated by numerous common stimuli, such as reactive oxygen species, cytokines, and endotoxin. It is well established that NO donors can activate HO-1 gene expression and activity in a variety of tissues (Foresti et al. 1997; Yee EL et al. 1996). Conversely HO-1 activity can modulate NOS activity (White KA et al. 1992). As there is increasing evidence of the protective effects of NOS, particularly the eNOS isoform, and of HO-1 in liver IR injury and mounting evidence of crosstalk between NOS and HO-1, it is possible that this crosstalk may be occurring in liver IR injury and IPC and form part of the mechanisms of protection against IR injury.

The relationship between HO-1 and eNOS in liver IPC and IR injury in vivo is currently unclear. Based on the current knowledge of eNOS, iNOS and HO-1 in liver IPC and IR injury and the results from the previous sections, the following experiments were conducted on wild type animals measuring eNOS and phosphorylated eNOS to clarify whether eNOS protein expression or activation by phosphorylation were affected in our model of partial liver IR and IPC (Chapter 2 and 3). Protein expression of iNOS was measured to assess whether this was related to liver injury with IPC and IR in wild type animals and eNOS -/- animals. We used eNOS-/- knockouts, as well as wild type animals, in the same model to study the in vivo levels and timecourse of HO-1 and eNOS.

7.2. RESULTS

7.2.1. eNOS and phosphorylated eNOS (p-eNOS) Western Blot in Wild Type Animals

Expression of eNOS protein was detected in all the experimental groups of wild type normal animals. Expression of eNOS was higher in the IR group and the IPC+IR group than the control sham laparotomy group reflected by increased band signal intensity normalised to βactin ($P<0.05$) (Figure 6.1). eNOS expression was higher in the IR only group than the IPC+ IR group, but this difference did not reach statistical significance ($P=0.18$) (Figure 6.1). Phosphorylated eNOS levels were higher in IR and IPC groups than sham ($P<0.05$), but there was no difference between IPC+IR and IR only ($P=0.32$) (Figure 6.2). Liver harvested from eNOS -/- animals were used as a negative control, which showed no expression of eNOS or p-eNOS.

7.2.2. iNOS Western Blot in Wild Type and eNOS -/- Animals

Inducible nitric oxide synthase (iNOS) protein was expressed in all the groups of wild type mice. There was no difference in iNOS expression in the groups ($P=0.22$) (Figure 6.3).

Expression of iNOS was detected at low levels in all experimental groups of eNOS -/- animals. As with the wild type animal groups, there was no significant difference between the levels of expression of iNOS between sham laparotomy (control), IR only and IPC preceding IR groups in eNOS-/- animals ($P=0.11$).

7.2.3. HO-1 Western Blot Results in Wild Type and eNOS-/-Animals

A strong band was detected in the lane containing the positive control (mouse spleen), but no bands were detected from any of the liver samples from the experimental groups of wild type animals (Figure 6.5). In eNOS -/- animals there were also no HO-1 bands in the experimental groups (results not shown).

7.2.4. HO-1 mRNA RT-PCR Results in Wild Type and eNOS -/- Animals

RT-PCR detected HO-1 mRNA in all groups of normal wild type animals. This was confirmed by repeating PCR runs on different liver tissue samples from the same animal and different animals for liver samples obtained at the end of 2 hours reperfusion for the IR group and the IPC followed by IR group and at the end of 3 hours anaesthesia for the control sham laparotomy group (n=2). There is a suggestion on these runs that HO-1 mRNA levels are higher in the IR only and IPC groups than the sham group based on the higher intensity of bands on the gels (Figure 6.6).

In eNOS -/- animals liver samples were obtained at the end of 2 hours reperfusion for the IR group and the IPC followed by IR group and at the end of 3 hours anaesthesia for the control sham laparotomy group, as with normal wild type animal liver samples, for RT-PCR testing. RT-PCR detected HO-1 mRNA in all the eNOS-/- knockout experimental groups. Like the previous experiments on wild types, there is evidence in eNOS -/- knockouts that HO-1 mRNA expression is increased by IR with or without IPC compared to sham laparotomy (Figure 6.7).

7.3. DISCUSSION

In this chapter it was demonstrated in our partial hepatic IR model that both IR and IPC increased eNOS protein expression and eNOS activation by phosphorylation. Haem oxygenase-1 (HO-1) protein was not expressed, but HO-1 mRNA was expressed in both wild type animals and eNOS-/- animals following liver IR and IPC, indicating that HO-1 expression is not dependent on expression of eNOS.

It is unclear whether early phase liver IPC protection associated with eNOS is from increased eNOS protein synthesis or increased activity of eNOS and how this activation occurs. In our model, liver IR resulted in detectable increases in eNOS protein expression and phosphorylated eNOS levels (p-eNOS) in the affected lobes compared to sham laparotomy groups, although no additional effect of direct ischaemic preconditioning on eNOS expression or p-eNOS levels was demonstrated in this time period.

Our results would indicate that the protective effects of eNOS against liver IR injury in our model of partial hepatic IR without IPC are mediated by an increase in eNOS activation by phosphorylation and eNOS protein expression. It would appear then that the additional beneficial effects of IPC in reducing IR injury in our model are not by increased expression of eNOS protein or activation of eNOS by phosphorylation, but by activation of eNOS by some other mechanism, as there was no additional effect of IPC on expression of eNOS protein and p-eNOS levels compared to the IR only group. A possible mode of eNOS activation with IPC is by a Ca^{2+} dependent mechanism.

Our study is the first to investigate and report patterns of phosphorylated eNOS levels in both liver IR and IPC. Our findings on levels of eNOS protein expression contrast with previous reports using similar partial hepatic IR models of early phase IR injury with index ischaemia of 45 minutes and reperfusion of 2 to 3 hours. These reports show either no difference in eNOS protein expression with IR or IPC compared to controls (Abu-Amara et al. 2011) or reduced eNOS protein with IR and increased eNOS with IPC (Koti et al. 2005). Western Blotting is a semiquantitative technique, but without normalisation of band intensities against a control protein such as B actin for a given Western blot as used in our study, it is not valid

to make any quantitative assertions about levels of protein expression even from a given Westen Blot as discussed in the Results section of this chapter.

The role of iNOS in liver IR injury and IPC is more controversial, as some studies indicate that it has no role (Kawachi 2000), contributes to increased injury (Lee 2001, Acquaviva 2008) or even may be protective (Taylor BS 1998, Hsu 2002). In this chapter iNOS protein expression was studied in wild type and eNOS-/- animal experimental groups to elucidate this. There was detectable iNOS protein expression in all the experimental groups of wild type normal animals and eNOS-/- animals, but there was no significant difference between the groups. These findings indicate that iNOS protein expression does not play a role in early phase IR injury in our model of partial hepatic IR and IPC.

The conflicting results of the role of iNOS in early phase liver IR injury in different reports in the literature may reflect contrasting roles of iNOS and the regulation of its function in liver IR injury depending on the duration of liver ischaemia. Partial hepatic IR models with prolonged ischaemia of 60 minutes or longer have found that iNOS does have a role in liver IR injury (Hamada et al. 2009; Lee, Baust 2001), while models using shorter ischaemia times of 45 minutes, as used in our model, have shown no role of iNOS (Kawachi et al. 2000). Some of these previous reports have used iNOS-/- knockout animal models of hepatic IR. There is evidence, however, that iNOS-/- knockouts show genetic compensation, as discussed in the Introduction of Chapter 4 (Hines et al. 2001), so conclusions based on models of liver IR using iNOS-/- animals need to be interpreted with caution and bring into question the validity of using iNOS-/- animals in liver IR research.

Haem oxygenase-1 (HO-1) has many molecular similarities and interrelationships with NOS. There is some evidence that it is protective in liver IR injury and IPC. The relationship of HO-1 to NOS in liver IR injury and IPC in vivo is poorly understood. In our model, HO-1 protein was not expressed in any of the experimental groups. RT-PCR detected HO-1 mRNA in all experimental groups of wild type and eNOS-/- animals over the same timeframe as liver samples probed for HO-1 protein, although it was not possible to draw any quantitative conclusions about differences in HO-1 mRNA between groups, as RT-PCR is not a quantitative technique.

Our findings imply that there is increased transcriptional activation of the haem oxygenage-1 gene (hmox-1 gene) resulting in increased HO-1 mRNA but the timecourse is too short for this to be reflected by a detectable increase in HO-1 protein expression. This is consistent with previous reports of partial hepatic IR models, where HO-1 protein is typically expressed after six or more hours of liver reperfusion (Shen XD et al. 2005; Su H et al 2006) and is preceded by an increase in HO-1 mRNA in postischaemic livers reaching peak levels typically after around 3 hours reperfusion (Su H et al. 2006).

In our model, HO-1 expression occurs independently of eNOS, as HO-1 mRNA is detected both in wild type normal animals and animals having a double knockout at the genetic level of enos in all their cells. There have been conflicting conclusions drawn from previous studies (Acquaviva et al. 2008; Duranski et al. 2006). It is possible that in these studies the use of the pharmacological agents L-arginine and HO-1 inhibitors and activators may have influenced other molecular pathways making it impossible to draw firm conclusions of the true in vivo interactions of eNOS and HO-1 in liver IR and IPC.

In conclusion, we have demonstrated in a partial hepatic IR model that both IR and IPC increased eNOS protein expression and eNOS activation by phosphorylation with no additional effect of IPC. This indicates that the protective effect of eNOS with IR alone may at least be partly mediated by increased eNOS protein expression and activation by phosphorylation. The additional benefits of IPC are mediated by an increase in eNOS activation, but not by phosphorylation. Expression of iNOS protein does not play a role in IR injury in our model. Haem oxygenase-1 (HO-1) protein was not expressed in the early phase of liver IR injury with or without IPC, but HO-1 mRNA was expressed in both wild type and eNOS-/- animals. This indicates that HO-1 expression is not dependent on eNOS activation in our model. HO-1 may still have a protective effect in the late phase of IR injury.

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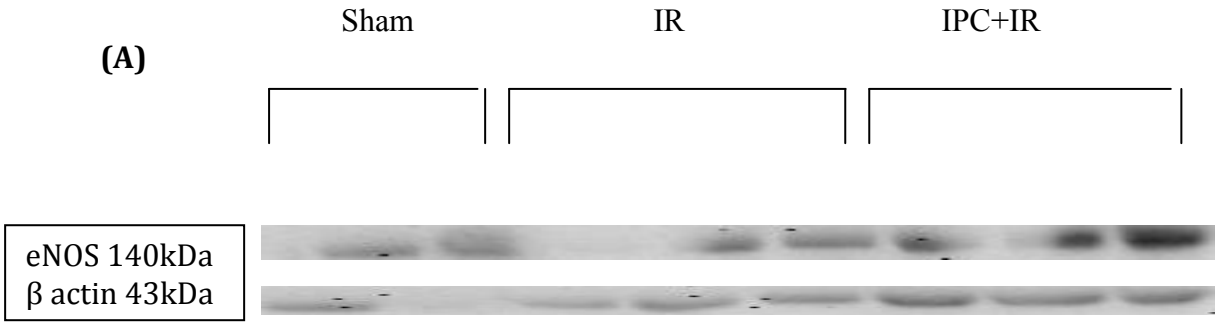

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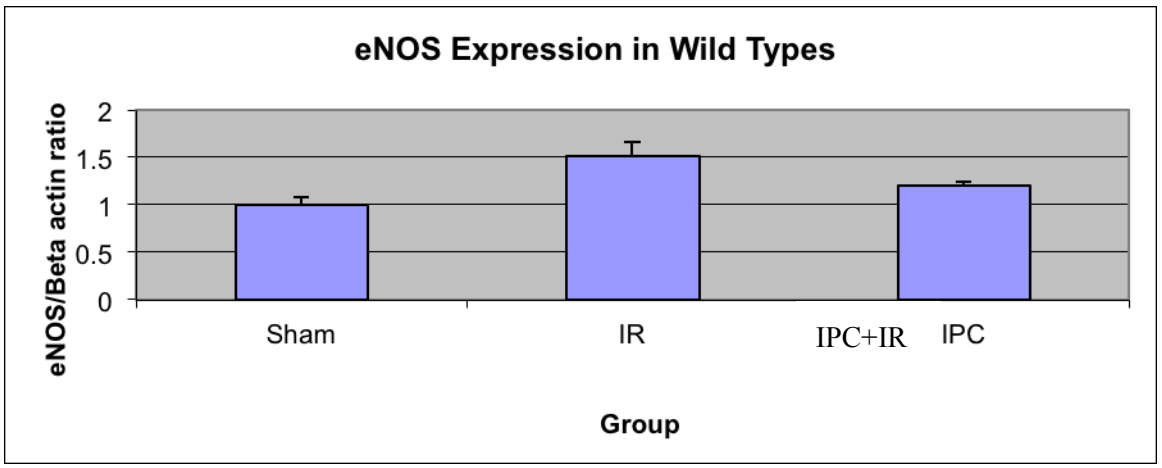
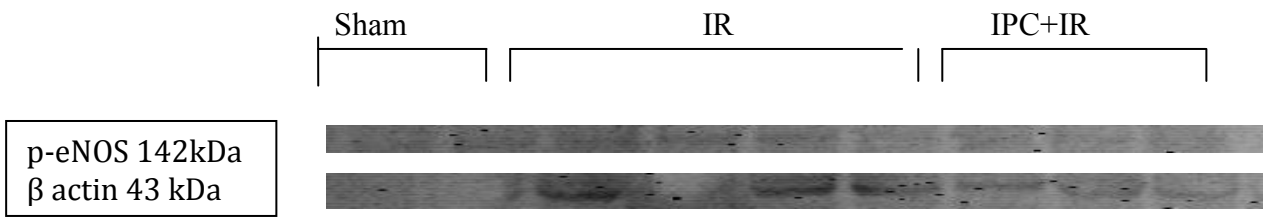


Figure 7.1: Western Blot of eNOS in Wild Type groups. (A) Bands from Western Blot (B) Summary histogram of band density. Densitometric results are expressed as arbitrary units corresponding to signal intensity normalised to βactin. Values are means+/-s.d. of 5 animals in each group. IPC+IR=ischaemic preconditioning (5 minutes ischaemia and 10 minutes reperfusion) preceding index ischaemia reperfusion (45 minutes ischaemia and 2 hours reperfusion); IR only=index ischaemia reperfusion.

(A)



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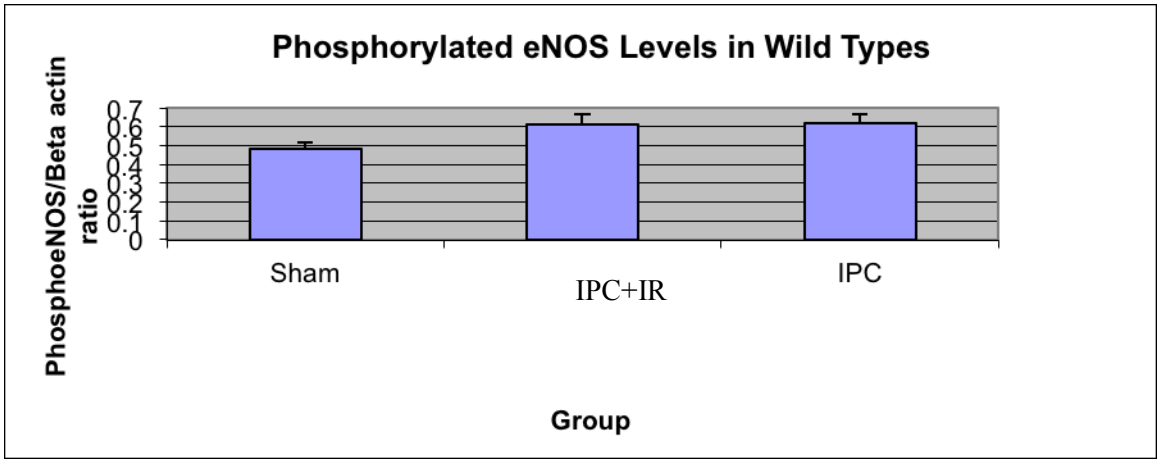
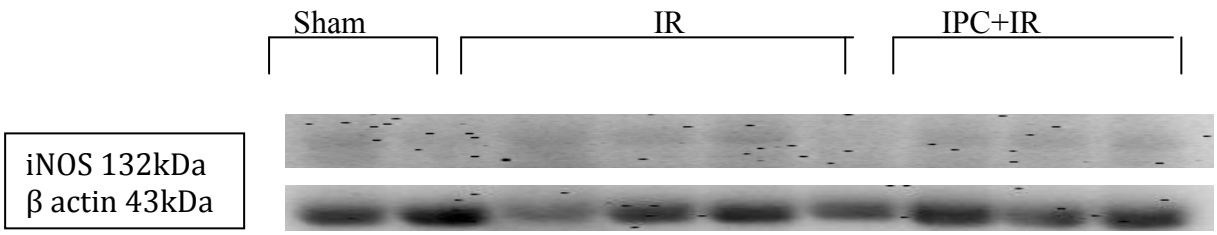


Figure 7.2: Western Blot phosphorylated-eNOS in Wild Type groups. (A) Bands from Western Blot (B) Summary histogram of band density. Densitometric results are expressed as arbitrary units corresponding to signal intensity normalised to βactin. Values are means+/-s.d. of 5 animals in each group. IPC+IR=ischaemic preconditioning (5 minutes ischaemia and 10 minutes reperfusion) preceding index ischaemia reperfusion (45 minutes ischaemia and 2 hours reperfusion); IR only=index ischaemia reperfusion.

(A)



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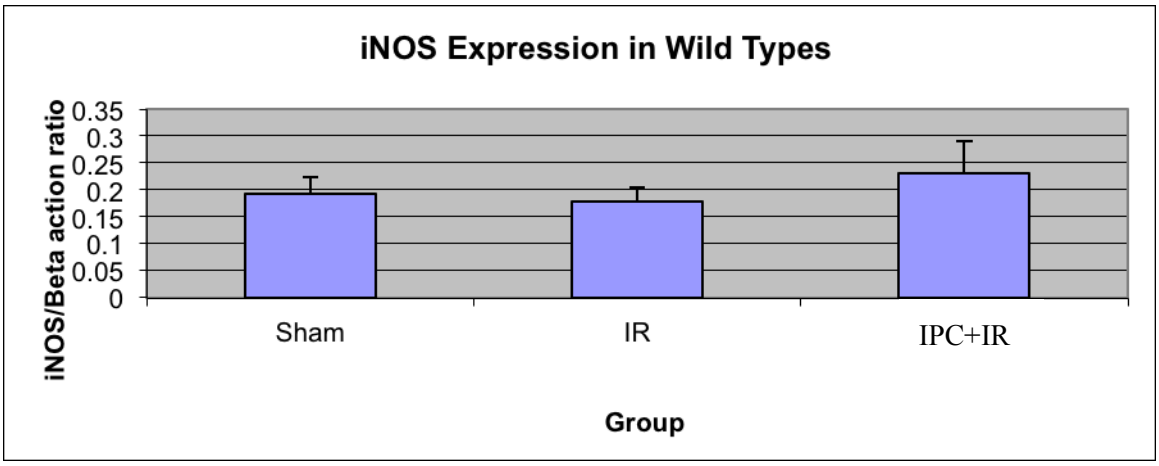
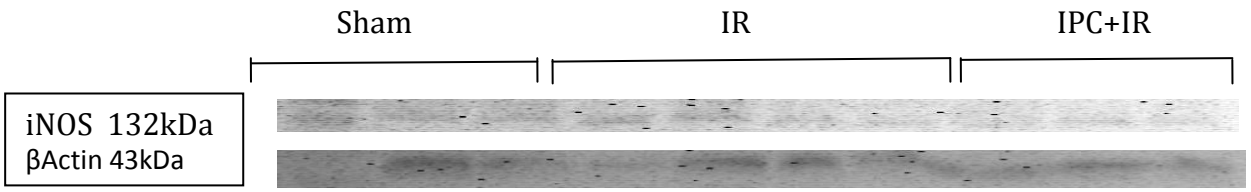


Figure 7.3: Western Blot iNOS in Wild Type groups. (A) Bands from Western Blot (B) Summary histogram of band density. Densitometric results are expressed as arbitrary units corresponding to signal intensity normalised to βactin. Values are means+/-s.d. of 5 animals in each group. IPC+IR=ischaemic preconditioning (5 minutes ischaemia and 10 minutes reperfusion) preceding index ischaemia reperfusion (45 minutes ischaemia and 2 hours reperfusion); IR only=index ischaemia reperfusion.

(A)



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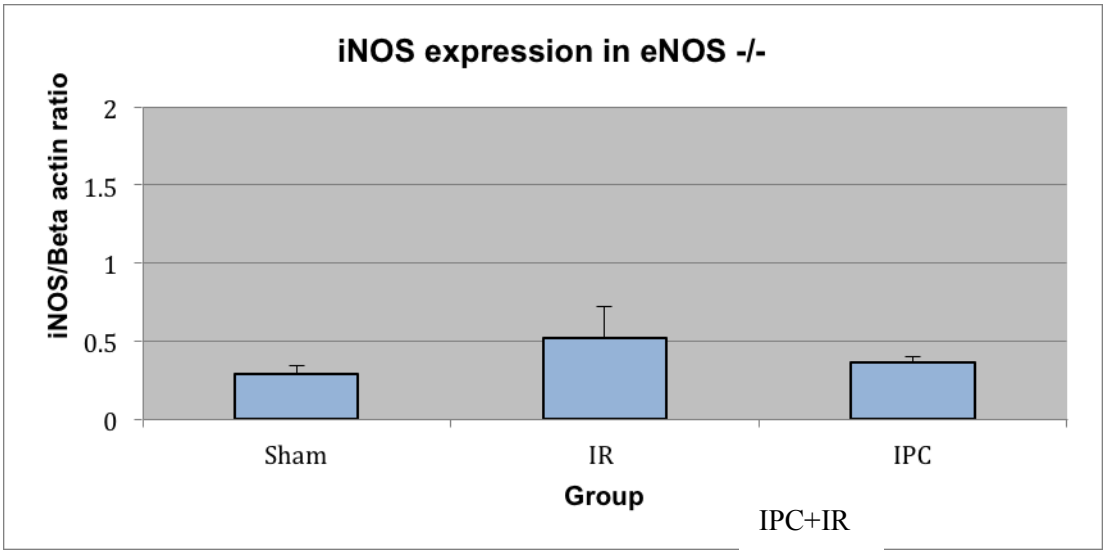


Figure 7.4: Western Blot iNOS in eNOS -/-knockout animals. (A) Bands from Western Blot (B) Summary histogram of band density. Densitometric results are expressed as arbitrary units corresponding to signal intensity normalised to βactin. Values are means+/-s.d. of 5 animals in each group. IPC+IR=ischaemic preconditioning (5 minutes ischaemia and 10 minutes reperfusion) preceding index ischaemia reperfusion (45 minutes ischaemia and 2 hours reperfusion); IR only=index ischaemia reperfusion.

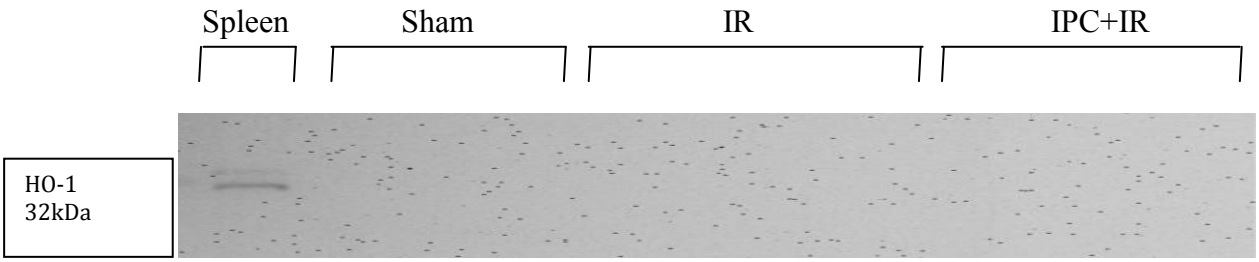


Figure 7.5: Western Blot of HO-1 Wild Type groups. Strong band for HO-1 detected for the spleen sample, but no bands were shown in any liver samples from the experimental groups.

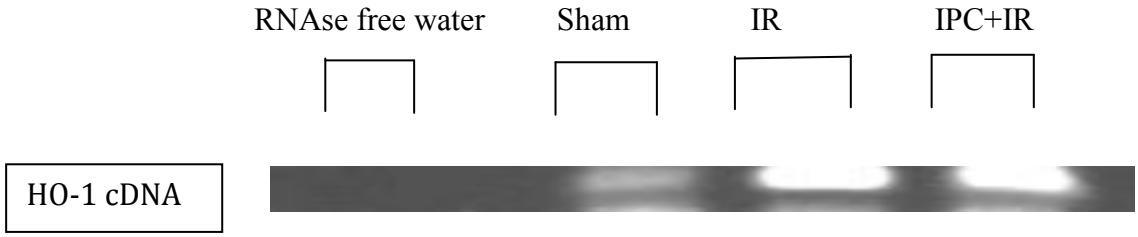


Figure 7.6: RT-PCR for HO-1 mRNA in Wild Type groups. IR=ischaemia reperfusion only; IPC= ischaemic preconditioning preceding ischaemia reperfusion.

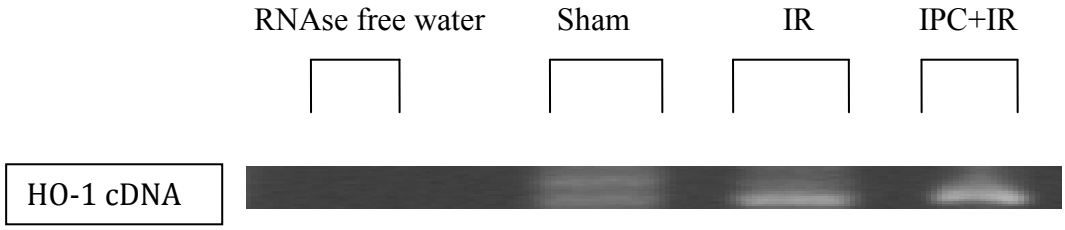


Figure 7.7: RT-PCR for HO-1 mRNA in eNOS(-/-) groups. IR only=ischaemia reperfusion only; IPC= ischaemic preconditioning preceding ischaemia reperfusion.

CHAPTER 8:

STUDIES ON THE LATE PHASE OF LIVER IR INJURY AND IPC

8.1. INTRODUCTION

There is evidence that there are two phases of liver ischaemia reperfusion injury (Yokoyama et al. 2000): an early phase (starting from 30 minutes after reperfusion to 6 hours afterwards) and a late phase (starting 12 hours after reperfusion to 72 hours afterwards). There is some evidence that ischaemic preconditioning (IPC) also has two phases (Centurion et al. 2007; Caban et al. 2006): an early phase (starting within minutes of reperfusion and lasting up to 6 hours) and a late phase (starting 24 hours after reperfusion and lasting several days). This has been shown in large animal models of warm hepatic ischaemia reperfusion, as well as orthotopic liver transplantation (Compagnon et al. 2005). There may be different mechanisms for late phase processes, which has relevance to application in clinical practice meaning that additional molecular pathways would need to be targeted for comprehensive protection against IR injury and its effects (Monbaliu et al. 2009).

In chapter 7 it was shown in the mouse model of early phase partial hepatic IR and IPC that HO-1 mRNA expression was increased, but the timeframe of reperfusion was too short for HO-1 protein to be detectable, and this did not require eNOS activity, as the same patterns of HO-1 expression were observed in wild type normal and eNOS-/- knockout animals. This indicates that HO-1 expression occurs independently of eNOS activity under in vivo physiological conditions of liver IR injury and IPC. The timeframe of HO-1 expression suggests that HO-1 may have a role in late phase IR injury and IPC.

In late phase mouse models of partial hepatic IR, HO-1 protein expression is detectable in postischaemic mouse livers after 24 hours reperfusion (Devey et al. 2009). In a mouse model of partial hepatic IR using 60 minutes ischaemia with reperfusion from 3 to 48 hours, a spatiotemporal relationship of HO-1 expression was established where HO-1 expression was induced and HO-1 mRNA levels in livers peaked after 3 hours reperfusion followed by HO-1

protein expression which was detectable after 6 hours reperfusion peaking at 9 hours reperfusion (Su H et al. 2006).

In this chapter, we sought to develop the mouse model of partial hepatic IR and IPC established for early phase IR injury to evaluate a model of lobar IR injury and IPC with a late phase evaluation of HO-1 expression (Chapter 2 and 3). This model was used to assess whether HO-1 protein expression was detected in this model with IR and IPC consistent with a delayed timecourse of expression of HO-1 and a possible role in late phase liver IR injury and IPC. This model could be used in future studies to develop other mouse models using transgenic animals to investigate mechanisms of delayed phase liver IR and IPC.

8.2. RESULTS

Anaesthesia and Sham Laparotomy in Recovery versus Nonrecovery Animals

There was no evidence of liver injury with 3 hours of anaesthesia and sham laparotomy in animals terminated at the end of the 3 hours anaesthesia (nonrecovery group) reflected by normal serum ALT and absence of histological liver injury. (Figure 2 and 3). There was no evidence of hepatocellular injury with 3 hours of anaesthesia and sham laparotomy with recovery from anaesthesia and termination after 24 hours (recovery group), reflected by normal serum ALT. In the sham recovery group, however, there was evidence of very low grade histological injury in the form of occasional sinusoidal congestion (score 1.0+/-0.8).

Effect of IR in Recovery (Late Phase IR) versus Nonrecovery (Early Phase IR) Animals

In the recovery animal IR group, liver IR injury occurred as demonstrated by hepatocellular injury (ALT 290+/-97 IU/L vs sham 43 +/-7 IU/L, $p<0.05$) as well as significant histological injury (score 4.2+/-0.8 vs shams 1.0+/-0.8, $p<0.05$). The hepatocellular injury was more severe in the nonrecovery than the recovery animals reflected by significantly higher serum ALT in the former ($P<0.05$) (Figure 2 and 3).

The histological injury scores were not significantly different in the recovery and nonrecovery groups ($P=0.09$). In the IR only animals with 2 hours reperfusion (i.e.

nonrecovery group), liver sinusoidal congestion and hepatocyte ballooning were dominant features, but necrosis was less pronounced, while IR with 24 hours reperfusion resulted in extensive centrilobular hepatocyte necrosis with little or no sinusoidal congestion or hepatocyte ballooning. The Suzuki classification scores histological injury on extent of sinusoidal congestion, hepaocyte ballooning and necrosis (Table 1, Chapter 2 Methods).

The effect of IPC in Late Phase IR versus Early Phase IR

All animals underwent direct IPC of 5 minutes ischaemia followed by 10 minutes reperfusion to the cephalic liver lobes preceding ischaemia of the cephalic lobes for 45 minutes followed by 2 hours nonrecovery animals or 24 hours reperfusion for nonrecovery and recovery animals, respectively.

IPC reduced the severity of IR injury in the recovery group, demonstrated by a reduction of serum ALT (IPC+IR 110+/-37 IU/L versus IR only 290+/-97 IU/L, $P<0.05$, Figure 2) and by less marked liver necrosis in the IPC+IR group compared to the IR group at the end of 24 hours of reperfusion (IPC score 2.2+/-0.8 vs IR score 4.2+/-0.8, $P<0.05$, $P<0.05$, Figure 3). Therefore, direct IPC of 5 minutes ischaemia followed by 10 minutes reperfusion to the cephalic liver lobes is protective against both early phase and late phase liver IR injury produced by this model of partial hepatic IR.

HO-1 Western Blot in Late Phase IR Model

After 24 hours reperfusion, a strong HO-1 band was detected in the spleen control lane. In contrast to the nonrecovery groups which showed no HO-1 bands, faint HO-1 bands were detected in the IPC+IR and IR only groups of recovery animals (but not the sham group).

Intraoperative and Postoperative Complications and Animal Survival

During the operative procedure all animals remained stable and tolerated anaesthesia, IR and IPC. There was no intraoperative or postoperative mortality in any of the animals. The animals in the recovery experiments did not show behaviour indicating distress or pain requiring additional analgesia after recovery. All the endpoints were achieved.

8.3. DISCUSSION

In this chapter we studied a mouse partial hepatic IR model of late phase liver IR injury and IPC. We showed that the hepatocellular injury occured in the late phase of IR injury with lower levels of serum ALT in the late phase after 24 hours, but there was greater hepatocte necrosis when compared to the early phase of 2 hours reperfusion of this model. Direct liver IPC was protective against both early and late phase IR injury in this model. There is a delayed expression of HO-1 protein after 24 hours in this model of liver IR.

In our study there was no rise in serum ALT after 3 hours anaesthesia (nonrecovery) or in the 24 hour sham control group (recovery). This was reflected by no histological injury in the nonrecovery sham group, but there was mild sinusoidal congestion in the recovery sham group. The stress response initiated by laparotomy activates inflammatory cascades that result in hepatocellular injury. Without an anaesthesia only group that does not undergo laparotomy, it cannot be ruled out that isoflurane anaesthetic sensitivity contributed to liver injury.

In this mouse model of partial hepatic IR there was IR injury in both the early and late phase of IR as demonstrated by increased serum ALT and histological injury scores. The hepaotcellular injury is more severe in the early phase as reflected by higher serum ALT after 2 hours reperfusion than 24 hours reperfusion. This does not necessarily mean the liver injury is less in the late phase, as serum ALT is cleared by the animal. ALT may decrease with severe injury even on the background of liver failure and histological changes may require a significant time period before the degree of injury is demonstrated histologically.

The histological injury of IR injury progresses from mainly sinusoidal congestion and hepatocyte ballooning in the early phase after 2 hours reperfusion to mainly centrilobular hepatocyte necrosis in the late phase after 24 hours reperfusion. These findings are in agreement with those noted from previous reports of early and late phase liver IR injury using mouse partial hepatic IR models. These studies have shown that after 24 hours reperfusion there is liver IR injury demonstrated by increased serum ALT and histological injury and this injury is less than in early phase IR injury (Uchida et al. 2009; Hamada et al. 2009). In these models of liver IR injury, partial hepatic IR with 90 minutes ischaemia of cephalic lobes was

used and resulted in sinusoidal congestion in early phase IR injury and necrosis and progressive neutrophil recruitment in late phase IR injury (Lappas et al. 2006; Hamada et al 2009; Kato et al. 2002).

In chapter 3, IPC consisting of 5 minutes ischaemia and 10 minutes reperfusion was shown to be protective against early phase IR injury in the mouse model of partial hepatic IR. In this chapter, the same IPC protocol was used to investigate if it was protective against late phase IR injury with the aim of developing a **complete model** of liver IPC with early and late phase IR injury. IPC reduced early and late phase IR injury in our partial hepatic IR model. This was reflected by a reduction in serum ALT and histological injury after 2 hours reperfusion and 24 hours reperfusion.

This is consistent with previous reports using rat models of hepatic IR, supporting that liver IPC reduces hepatocellular and histological injury and its protective actions have two phases (Centurion et al. 2007; Cutrin et al. 2002): an early phase (starting within minutes of reperfusion and lasting up to 6 hours) and a late phase (starting 24 hours after reperfusion and lasting several days). These two phases of IPC have also been found in large animal models of warm hepatic ischaemia reperfusion, as well as orthotopic liver transplantation (Compagnon et al. 2005).

In chapter 6 it was shown in our model of early phase IR and IPC that HO-1 mRNA expression was increased, but no HO-1 protein was detectable. In this chapter, HO-1 protein was detected after 24 hours in IPC+IR and IR only groups of animals in our late phase IR model. This indicates that in our model of liver early and late phase IR, there is transcriptional activation of the HO-1 gene, with HO-1 mRNA detectable after 2 hours reperfusion, followed by HO-1 protein expression detectable after 24 hours reperfusion. This is as noted in previous reports of models of partial hepatic IR, where HO-1 protein expression is detectable in postischaemic livers after 24 hours reperfusion, peaking at 9 hours reperfusion, and preceded by HO-1 mRNA expression peaking after 3 hours reperfusion (Devey et al. 2009; Su H et al. 2006).

There have been no mouse models studying the role of HO-1 in the protective effects of IPC in the late phase liver IR injury. There have, however, been these studies in larger animal

models, where liver IPC is associated with increased HO-1 mRNA expression followed by HO-1 protein expression, consistent with our results. HO-1 protein expression is not detected before 6 hours reperfusion, but HO-1 protein is found in liver 24 hours after reperfusion when the levels are higher than with IR alone without IPC (Massip-Salcedo et al. 2006). The protection conferred by late phase liver preconditioning is abolished by inhibitors of HO-1 such as zinc protoporphyrrin (ZnPP) in partial liver ischaemia models (Shen XD et al. 2005), ex vivo liver and in vivo orthotopic liver transplantation models (Kato Y et al. 2003).

The mechanistic significance of these patterns of HO-1 expression in late phase liver IR injury and IPC is unclear and would need to be explored with further recovery experiments assessing effects on endpoints of liver IR injury using pharmacological inhibitors of HO-1, transgenic HO-1 animal models (hmox+/-, as double knockouts of HO-1, hmox-/- are not viable ex utero) and exploring interactions between HO-1 and eNOS by using eNOS -/- animals. A limitation of this model is that two endpoints of IRI were used rather than the three endpoints used in earlier chapters, as LDF assessment of liver microcirculation was not undertaken.

In conclusion, in this chapter we studied a mouse partial hepatic IR model of late phase liver IR injury and IPC, demonstrating that the hepatocellular injury was less severe in the late phase of IR injury after 24 hours, but there was greater hepatocte necrosis when compared to the early phase of 2 hours reperfusion. Direct liver IPC was protective against both early and late phase IR injury in this model. HO-1 mRNA expression precedes HO-1 protein expression in our model, consistent with previous reports suggesting that HO-1 may have a protective effect in the late phase of IR injury and IPC. This model should prove useful in investigating the role of HO-1 in the late phase of direct IPC and liver IR injury. This would aid in the development of effective pharmacological agents that target various key signalling pathways to reduce liver IR injury in the clinical setting and improve patient outcomes.

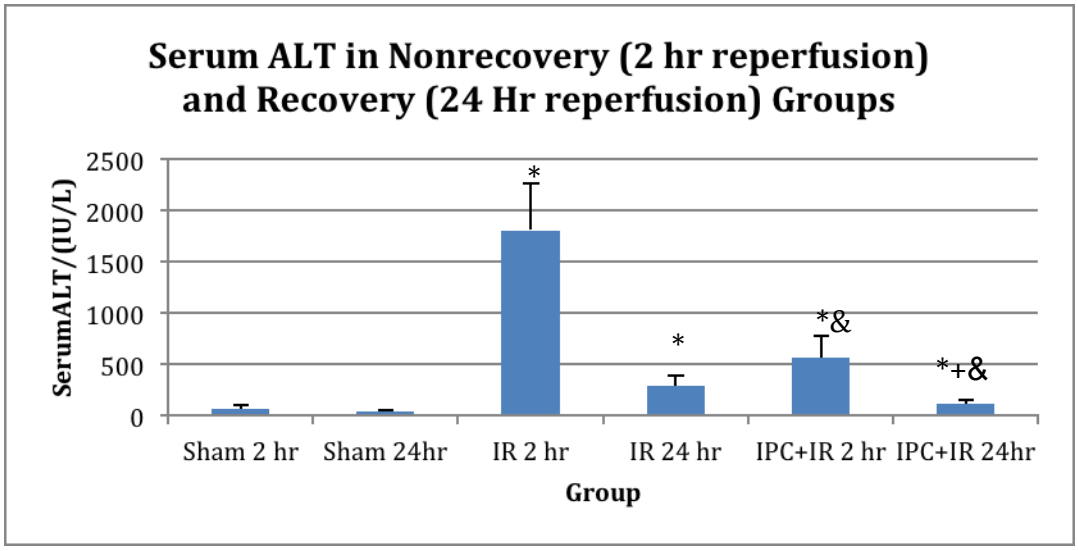
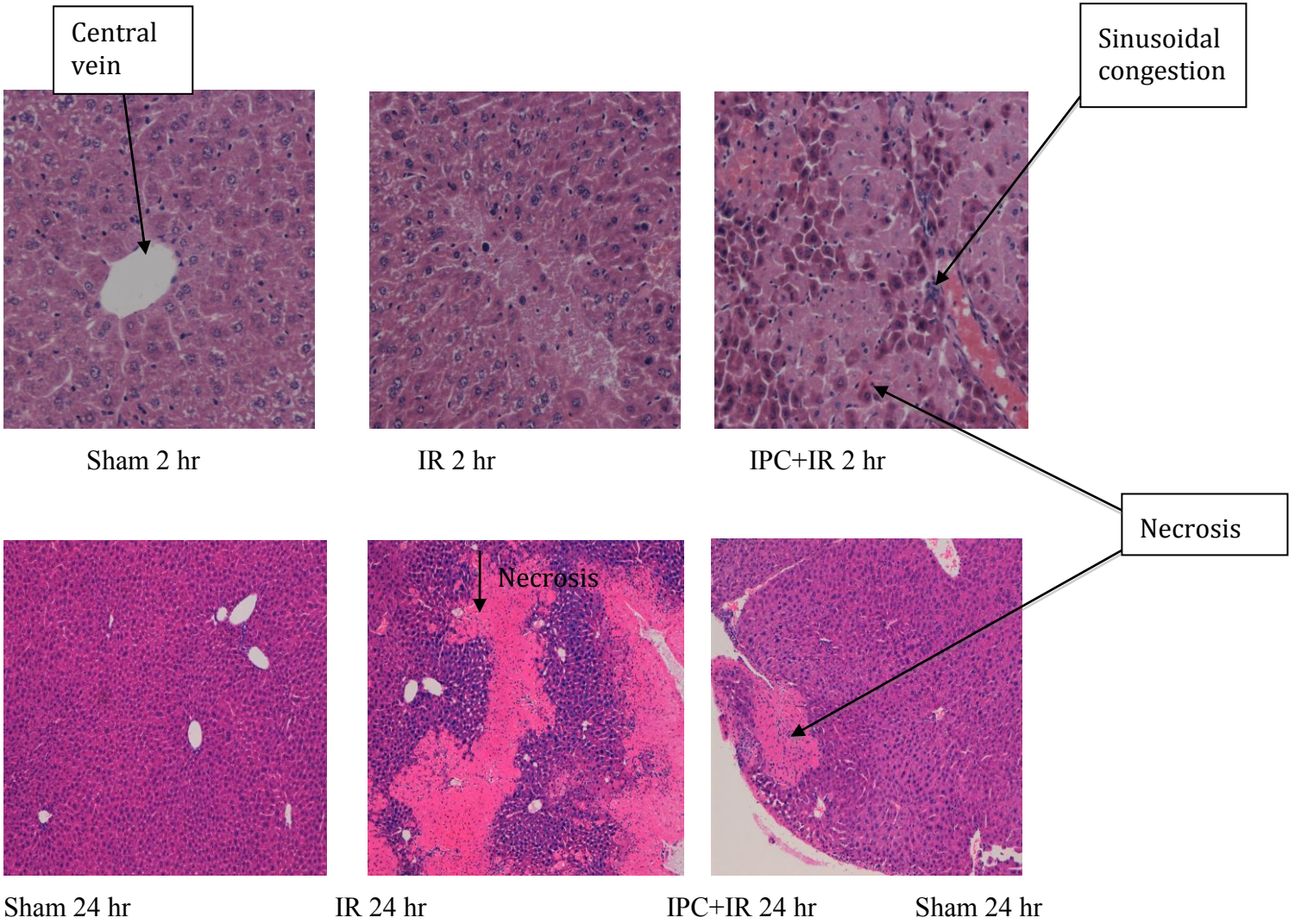


Figure 8.1: Serum ALT for Nonrecovery and Recovery Experiments. Groups with 2 hours reperfusion are nonrecovery and groups with 24 hours reperfusion are recovery. Values are means+/-s.d. of 5 animals in each group (*P<0.05 vs. sham, & P<0.05 vs. IR 2 hr, +P<0.05 vs. IR 24 hr). IR=ischaemia reperfusion only. IPC+IR=ischaemic preconditioning preceding ischaemia reperfusion.

(A)


Central vein
Sinusoidal congestion
Sham 2 hr
IR 2 hr
IPC+IR 2 hr
Necrosis
Necrosis
Sham 24 hr
IR 24 hr
IPC+IR 24 hr
Sham 24 hr

(B)

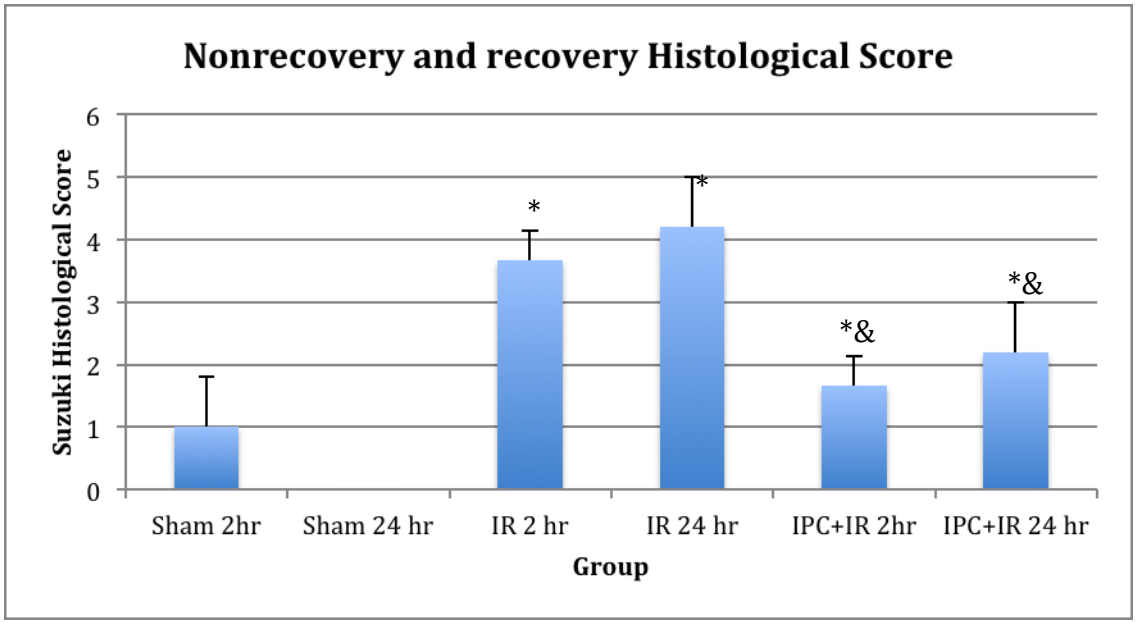


Figure 8.2: Histology and Scores for Nonrecovery and Recovery Groups. Groups with 2 hours reperfusion are nonrecovery and groups with 24 hours reperfusion are recovery. (A) Sections from the nonrecovery and recovery experimental groups (400X). (B) Bar graph summarising histological scores. Values are mean +/- s.d. of 5 animals in each group (*P<0.05 vs. sham, &P<0.05 vs. IR 2hr and IR 24 hr,). IR=ischaemia reperfusion only; IPC+IR= ischaemia preconditioning preceding ischaemia reperfusion.

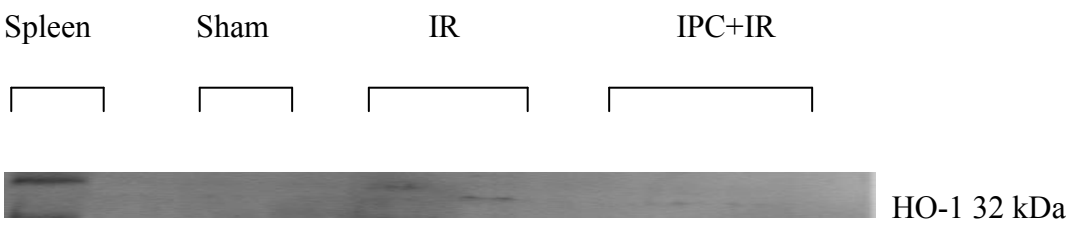


Figure 8.3: Western Blot for HO-1 from 24 hour Recovery Experiments with a spleen control (single band). Two lanes were run on the Western blot for each of the sham, IR and IPC+IR recovery groups and a single lane was run for the spleen control, hence one dark band. Weak HO-1 bands were detected in the IR and IPC groups as well as a strong band in the spleen control for HO-1. Sham=sham laparotomy; IR=ischaemia reperfusion only; IPC=ischaemic preconditioning preceding ischaemia reperfusion

CHAPTER 9:

DISCUSSION

9.1. A MOUSE MODEL OF LIVER IR INJURY AND IPC

In Chapter 4 we sought to verify liver IR injury occurred in our model and an IPC protocol that reduced IR injury.

There was liver IR injury across the three endpoints of serum ALT, histological injury and microcirculation (LDF). Various IPC protocols were used, but only IPC consisting of 5 minutes ischaemia and 10 minutes reperfusion reperfusion was protective against IR injury from the index ischaemia reperfusion (IR) of 45 minutes ischaemia and 2 hours. Systematic studies of different IPC protocols on models of partial liver IR showed that IPC of 10 minutes ischaemia (but not 5 or 15 minutes) and 10 or 15 minutes reperfusion reduced IR injury, although longer index ischaemia times were used of 75 to 90 minutes ischaemia (Yadav et al. 1999; Teoh et al. 2002).

It would appear that the combination of ischaemia and reperfusion times of IPC that are protective vary depending on the severity of the index IR and its context. In our IR model, 10 minutes ischaemia is either not protective or requires a longer reperfusion time than 10 minutes to initiate IPC protective mechanisms. It appears that 3 minutes ischaemia is too short to activate protective mechanisms.

In conclusion, we described and validated a mouse model of liver IR that results in IR injury and established a direct IPC protocol that significantly reduces this IR injury.

9.2. COMPARISON OF LIVER IR INJURY AND THE EFFECT IPC in eNOS-/- and WILD TYPE ANIMALS

Nitric oxide (NO) is a protective mediator in liver IR injury (Shibayama et al. 2002). It is produced endogenously by NOS, but the in vivo role of eNOS in IR injury and

IPC is not established. To specifically study the role of eNOS, we used double knockout eNOS (eNOS-/-) mice in the same model of liver IR injury from Chapter 4.

Anaesthesia with sham laparotomy in eNOS-/- animals was associated with a small rise in serum ALT and liver sinusoidal congestion. The stress response to laparotomy activates inflammatory cascades. NO released from liver eNOS dampens this response and protects against tissue injury (Okajima et al. 2004). Studies have shown that IR injury can also result with isoflurane anaesthesia (Nishiyama et al. 1998), but it is unknown if eNOS-/- animals are more sensitive to this.

In our IR model there was increased liver IR injury in the eNOS-/-animals compared to wild type animals, demonstrated by increased hepatocellular injury and increased histological IR severity score, but the microcirculatory dysfunction between groups was similar. This is consistent with previous studies using eNOS-/- models of lobar hepatic ischaemia (Kawachi et al. 2000; Hines et al. 2001).

This indicates that baseline eNOS partially protects against hepatocellular injury in early phase IR injury. It does this by inhibiting hepatocyte necrosis, but not by affecting the liver microcirculation in vivo. NO from eNOS acts as a scavenger of reactive oxygen species (ROS) and there is evidence that it activates survival kinase pathways (Kim JS, Ohshima S et al. 2004). This would explain a partial protection mediated by baseline **hepatocyte** eNOS in the absence of an inflammatory response.

NO has been shown to be a key mediator of liver IPC (Peralta et al. 1999; Koti et al. 2002). IPC is associated with increased expression of eNOS in hepatocytes and vascular endothelium in the centrilobular zone (Koti et al. 2002). We found that IPC failed to show significant protection in eNOS-/- animals with no improvement in liver microcirculation, no decrease in histological injury or serum ALT. This indicates that eNOS is a protective mediator of IPC by reducing hepatocellular injury and by improving liver microcirculation. This would suggest that IPC activates eNOS in **both hepatocytes and sinusoidal endothelial cells (SECs)**. This is consistent with previous reports which have shown that IPC improves liver microcirculation and SEC wall integrity (Vajdova, Heinrich et al 2004; Glanemann et al. 2003).

9.3. COMPARISON OF NOS AND HO-1 PROTEIN EXPRESSION IN eNOS-/- and WILD TYPE ANIMALS IN EARLY PHASE LIVER IR INJURY

Previous studies have supported the role of eNOS as the source of NO based on localisation of NOS isoforms and Western Blots for isoforms of NOS proteins in liver IR injury and IPC models (Hines 2002, Peralata C 2001, Koti 2005, Serracino-Inglott 2002). It is unclear whether during early phase liver IR that the increased NO from eNOS is related to protein expression and eNOS activation.

In our model, we detected increases in eNOS protein expression and phosphorylated eNOS levels (p-eNOS) in affected liver lobes, although no additional effect of IPC was demonstrated. This would indicate the protective effects of eNOS in liver IR are mediated by an increase in eNOS activation by phosphorylation **and** eNOS protein expression. IPC would appear to activate eNOS by another mechanism. eNOS may be activated by a Ca^{2+} dependent mechanism (Dudzinski et al. 2006;2007).

Our findings on levels of eNOS protein expression contrast with previous reports using similar partial hepatic IR models of early phase IR injury (Abu-Amara et al. 2011, Koti et al. 2005). Western Blotting is a semiquantitative technique, but without normalisation of band intensities against a control protein such as B actin as used in our study, it is not valid to make any quantitative comparisons of protein levels.

Haem oxygenase-1 (HO-1) has molecular similarities and interrelationships with NOS. There is some evidence that it is protective in liver IR injury and IPC (Kaizu 2005; Patel A 2004; Coito 2002; Lai 2004, Massip-Salcedo 2006). We studied the expression of HO-1 protein and mRNA. HO-1 protein was not expressed in any of our experimental groups. RT-PCR detected HO-1 mRNA in all experimental groups.

This is consistent with previous reports, where HO-1 protein is expressed after six or more hours of reperfusion (Shen XD et al. 2005; Su H et al 2006) and is preceded by an increase in HO-1 mRNA reaching peak levels typically after around 3 hours reperfusion (Su H et al. 2006). In our model, the HO-1 mRNA expression without detectable HO-1 protein in eNOS-/- and wild type groups indicate that liver IR is

associated with increased HO-1 mRNA expression **independent of eNOS**. This is consistent with *microarray* studie*s* indicating multiple parallel pathways of protection (Knudsen et al. 2012).

The relationship of HO-1 to NOS in IR injury and IPC is poorly understood. One study showed administration of the NOS substrate L-arginine increased eNOS and HO-1 expression in an in vivo liver IR model and concluded that eNOS causes increased HO-1 expression (Acquaviva et al. 2008). In contrast, another study using eNOS transgenic overexpressors with various HO-1 inducers and inhibitors found that both eNOS and HO-1 activity reduced IR injury independently of each other (Duranski et al. 2006). The limitation of using inhibitors/activators of NOS and HO-1 is that they are relatively nonspecific. Therefore, results may not reflect the physiological interactions of eNOS and HO-1 as evaluated in our study.

Haem oxygenase-1 (HO-1) protein was not expressed in our model, but HO-1 mRNA was expressed in both normal and eNOS-/- animals following liver IR and IPC, indicating that HO-1 expression is not dependent on eNOS. HO-1 may therefore have a protective effect in the late phase of IR injury acting in a parallel pathway to eNOS.

9.4. THE LATE PHASE OF LIVER IR, IPC AND HO-1 EXPRESSION

We developed our IR model for early phase IR injury in the previous chapters to evaluate a mouse model of late phase lobar IR injury and IPC with reperfusion of 24 hours with a late phase evaluation of HO-1 expression.

We found that there was IR injury in the late phase (24 hurs reperfusion) of IR. Histological changes require a significant time period before the degree of injury is demonstrated. The histological injury progressed from sinusoidal congestion and hepatocyte ballooning in the early phase to mainly centrilobular hepatocyte necrosis in the late phase. These findings are in agreement with previous reports of early and late phase liver IR injury (Uchida et al. 2009; Hamada et al. 2009).

In our model, direct IPC reduced late phase IR injury. This is consistent with previous animal models of late phase IR injury where IPC reduced liver IRI. Its protective actions had two phases: an early phase (within minutes of reperfusion and lasting up to 6 hours) and a late phase (starting 24 hours after reperfusion and lasting several days) (Centurion et al. 2007; Cutrin et al. 2002;Teoh et al. 2003; Matsumoto et al).

In Chapter 7 HO-1 mRNA expression, but not HO-1 protein was detected in early phase liver IR and IPC. In our late phase model, HO-1 protein was detected after 24 hours in all groups of animals. Overall, this suggests that in this model of liver IR, there is activation of transcription of the HO-1 gene, with HO-1 mRNA detectable after 2 hours reperfusion, followed by activation of HO-1 protein expression detectable after 24 hours reperfusion. Previous studies have found that late phase IPC protection was abolished by the HO-1 inhibitor zinc protoporphyrrin (ZnPP) in partial liver IR (Shen XD et al. 2005), ex vivo liver and OLT models (Kato Y et al. 2003).

9.5. LIMITATIONS OF THE CURRENT STUDY

- A limitation of our model is the **small size of the mouse**. This limits amounts of tissue and blood that can be used for testing. Unfortunately, larger transgenic eNOS-/- animals are not readily available as discussed in Chapter 1.2.2.

- Our intraoperative monitoring was limited to core body temperature and respiratory rate/depth. **Haemodynamic monitoring** with blood pressure and oxygen saturation provides detailed monitoring on intraoperative animal stability. For instance, portal triad clamping causes an initial drop in blood pressure.

- A limitation of using an eNOS-/- knockout to investigate mechanisms of liver IR injury is that once a role of eNOS is established, it is unclear whether this effect is from the absence of the gene's mRNA, protein or functional products.

- There was evidence in our study that there was mild hepatocellular injury in the sham group of eNOS-/- animals. It was unclear if this was related to sham laparotomy or the isoflurane anaesthetic. This is best addressed by studying

eNOS-/- animals that undergo anaesthesia but no surgery and a "sham" group with neither surgery nor anaesthetic. This would separate the effects of sham laparotomy and isoflurane anaesthetic in the eNOS-/- animals.

- In our model there was greater liver IR injury in eNOS-/- than wild type animals. Therefore, a limitation is that an **optimised IPC protocol** was not used for eNOS-/- animals. It is possible other IPC protocols may be protective for the more severe injury found in eNOS-/- through eNOS independent mechanisms.

- In eNOS-/- animals, there was a trend towards lower serum ALT in the IPC+IR group compared to the IR only group, but this did not reach statistical significance. For a power of 80% and α=0.05, a **sample size estimate of n=10** reduces the chance of a Type II error.

- There was progressive liver histological injury from 2 hours to 24 hours reperfusion. Serum ALT does not reflect this progressive injury, as it is rapidly excreted. **Serum bilirubin** remains elevated with sinusoidal congestion and disruption of bile canaliculi. This is routinely used clinically as a marker of ongoing liver injury. Future late phase IR models should measure serum bilirubin.

- The **LDF probe** used was designed for small animal studies and mounted onto a portable stand. During ischaemia, mouse liver is very friable and is easily damaged by any pressure more than the minimal contact required to obtain reliable LDF readings. In future, this could be avoided by using soft tip probes.

- It was not possible to quantitatively comment on whether eNOS was predominantly in the phosphorylated form following IR or IPC+IR, as **Western blotting** is only a semiquantitative technique and different antibodies were used for the assay for eNOS and p-eNOS.

- **RT-PCR** is a semiquantitative technique at best. Therefore, trends in different levels of HO-1 mRNA expression between groups would need to be assessed by

using quantitative real time PCR, rather than semi-quantitative RT-PCR, and using larger number of animals per group.

9.6. DIRECTIONS FOR FUTURE STUDIES

We justified the use of a partial liver IR model in Chapter 3. The most common clinical scenarios where liver IR injury occurs are in liver resection surgery and OLT. Future studies would use a model of liver resection in mice to more closely replicate the clinical scenario (Beraza et al. 2007). OLT models in mice are technically highly challenging and the animals do not tolerate this procedure well.

Our study findings support that eNOS is the protective mediator of IPC in liver IR injury. These findings could be consolidated using a NO donor, such as L-arginine, in eNOS-/- animals. If the loss of protection in eNOS-/- animals against IR injury was reversed with a NO donor, this would indicate that eNOS derived NO was necessary and sufficient for protection against early phase liver IR injury.

Our recovery experiments were exploratory to study late phase liver IR and IPC and the timecourse of HO-1 expression. Future recovery studies would assess expression of other proteins (e.g. eNOS, phosphorylated eNOS and iNOS). Measurements of liver microcirculation in the late phase would provide information on the timecourse of microcirculatory dysfunction and its recovery. The use of a NO donor and eNOS-/- animals in this late phase IR model will provide a more complete in vivo picture of the timecourse and inter-relationships of molecular events in liver IR and IPC.

The mechanistic significance of the patterns of HO-1 expression we observed in our model of late phase liver IR injury and IPC is unclear. This would need would further exploration with experiments using HO-1 inhibitors/activators, transgenic HO-1 animals (hmox+/-, as double knockouts of HO-1, hmox-/- are not viable ex utero) and exploring interactions between HO-1 and eNOS using eNOS -/- animals.

The benefit of animal models of liver IR and IPC would be translation into clinical practice with clinically beneficial reductions in liver IR injury.

PUBLICATIONS AND PRESENTATIONS

Publications

1. Gourab Datta, Tu Vinh Luong, Barry J Fuller, Brian R Davidson. Endothelial nitric oxide synthase and heme oxygenase-1 act independently in liver ischemic preconditioning. Journal of Surgical Research 2014 Jan; 186 (1):417-28.

2. Gourab Datta, Barry J Fuller, Brian R Davidson. Molecular mechanisms of liver ischemia reperfusion injury: Insights from transgenic knockout models. World Journal of Gastroenterology 2013 Mar 21; 19(11): 1683-98.

Presentations

Patey Prize Session Presentation SARS 2010. Liver Ischaemia Reperfusion Injury and Ischaemic Preconditioning: Insights from an eNOS Knockout Model. Annual Surgical Academic Research Society (SARS) Meeting February 15th 2010.

Gold Medal Winner AsiT Conference 2010. G.Datta, B.Ramesh, B.J.Fuller, B.R. Davidson. Liver Ischaemic Preconditioning reduces liver ischaemia reperfusion injury acting through nitric oxide synthase independent of haem oxygenase-1. Association of Surgeons in Training Meeting (ASiT) March 15th 2010.

Moynihan Prize Session Presentation ASGBI 2011. G.Datta, B.Ramesh, B.J.Fuller, B.R. Davidson. Liver Ischaemic Preconditioning protects against liver ischaemia reperfusion acting through endothelial nitric oxide synthase. Association of Surgeons of Great Britain and Ireland Annual Meeting (ASGBI) March 10th 2011.

Poster Presentation BTS. G. Datta, B.Ramesh, B.J.Fuller, B.R.Davidson. Endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and haem oxygenase-1 (HO-1) in liver ischaemic preconditioning and IRI. British Transplantation Society (BTS) Annual Meeting November 20th 2009.

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