Nitric Oxide Metabolism in Ischaemic Preconditioning of the Liver

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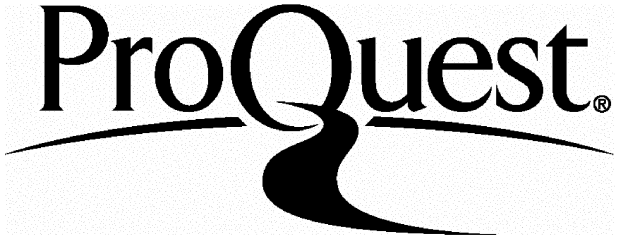

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This thesis is dedicated to my parents

Abstract

Ischaemic preconditioning is a term used for the reduction of ischaemic damage to the liver by a transient period of ischaemia and reperfusion. This thesis evaluates the hypothesis that ischaemic preconditioning may protect against ischaemia reperfusion injury of the liver via nitric oxide formation. Two study models were used. In the first model, lobar ischaemia reperfusion injury in the rat was used to investigate the relationship of nitric oxide metabolism with hepatic oxygenation, microcirculation, and function with ischaemic preconditioning of the liver. As the fatty liver is less tolerant of ischaemic injury the second study model has evaluated ischaemic preconditioning in a steatotic liver model.

In the first study model, Sprague Dawley rats were subjected to 45 mins lobar ischaemia followed by 2 hr reperfusion. Ischaemic preconditioning was performed with 5 min lobar ischaemia and 10 min reperfusion before the sustained ischaemia. L-arginine or N^{ω}-nitro-L-arginine methyl ester (L-NAME) was administered to stimulate or block nitric oxide synthesis. Ischaemic preconditioning resulted in significantly increased hepatic intracellular oxygenation, microcirculation and hepatic tissue ATP, and decreased hepatocellular injury. Preconditioning significantly increased nitric oxide production measured by plasma nitrite/nitrate and cGMP. L-arginine treatment reproduced the protective effect of ischaemic preconditioning. Nitric oxide inhibition with L-NAME antagonized the protective effect of ischaemic preconditioning. Nitric

oxide synthase detected by NADPH diaphorase was induced by ischaemic preconditioning. Immunohistochemistry and Western blotting showed this to be due to overexpression of the constitutive isoform eNOS rather than the inducible isoform iNOS.

The second study involved model of hepatic steatosis in rats induced by a high fat diet. Liver histology in these animals showed moderate grade macrovesicular steatosis. The same protocol for ischaemic preconditioning resulted in increased intracellular oxygenation, microcirculation and ATP, and decreased hepatocellular injury. These results were similar to those observed with preconditioning in the normal livers.

This thesis has confirmed a protective effect of hepatic ischaemic preconditioning. This is associated with increased eNOS derived nitric oxide production. Furthermore, the protective effect of ischaemic preconditioning can be applied to the steatotic liver. These data may have important implications in liver surgery and transplantation and may lead to the development of pharmacological strategies for protecting the liver from ischaemic injury.

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List of abbreviations

ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma gene
Bcl-xl	Bcl-2 related gene
cGMP	Guanosine 3', 5'-cyclic monophosphate
Cyt Ox Cu_A	Cytochrome oxidase Cu_A redox changes
eNOS	endothelial nitric oxide synthase
Hb	Deoxyhaemoglobin
HbO_2	Oxyhaemoglobin
HbT	Total haemoglobin (HbO2 + Hb)
HM	Hepatic microcirculation
HR	Heart rate
iNOS	inducible nitric oxide synthase
IPC	Ischaemic preconditioning
IR	Ischaemia reperfusion
IRI	Ischaemia reperfusion injury
LDF	Laser Doppler flowmeter

L-NAME	N^{ω}-nitro-L-arginine methyl ester
NADPH	Nicotinamide adenosine diphosphate hydrogenase
NIRS	Near infrared spectroscopy
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Plasma nitrate + nitrite
MABP	Mean arterial blood pressure
SaO_2	Oxygen saturation

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Declaration of originality of work for this thesis

The work presented in this thesis is completely original and was carried out by myself. Contributions of other colleagues to this work have been mentioned in the text and the acknowledgements.

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Chapter 1

Introduction

1.1 Overview of Ischaemia reperfusion injury and the clinical problem

Liver injury caused by ischaemia and reperfusion occurs in various surgical interventions including hepatectomy and liver transplantation [1, 2]. Ischaemia reperfusion injury (IRI) is associated with an acute inflammatory response and microvascular dysfunction, which finally lead to irreversible cell injury (figure 1.1). The pathogenesis of hepatic IRI is multifactorial and various mechanisms have been suggested [3, 4]. Recent studies have shown that Kupffer cell activation following reoxygenation of ischaemic tissue generates reactive oxygen species [5, 6], which result in direct tissue damage and initiate a series of complex pathophysiological events [7]. Production and release of inflammatory cytokines such as interleukins and TNF-α cause endothelial cell activation, leading to synthesis of adhesion molecules and secretion of platelet activating factor, which promote leukocyte endothelial cell adhesion [7-9]. Activated endothelium in addition produces more reactive oxygen species [8], and promotes microcirculatory failure [9]. Neutrophils adhere to the activated endothelium where they amplify the inflammatory cascade [10]. Furthermore, inflammatory cytokines activate vascular endothelium in other organ systems leading to systemic inflammatory response syndrome [11]. IRI is also associated with a progressive loss of adenine nucleotides [12]. ATP depletion causes alterations in membrane ion translocation by inhibition of ATP dependant Na^+ /K^+ ATPase, resulting in sodium influx and intracellular sodium accumulation with cell

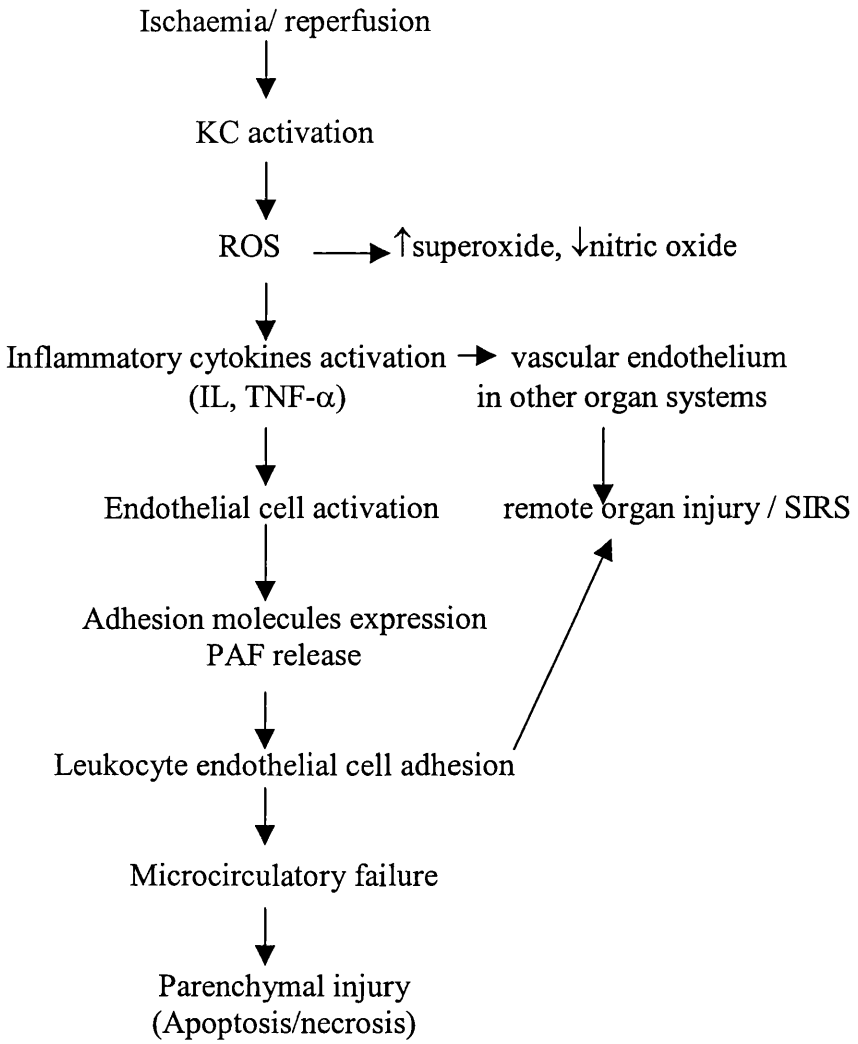

Figure 1.1. Schematic illustration of pathophysiological events following reperfusion of ischaemic liver. Keys: KC, Kupffer cell; ROS, Reactive oxygen species; IL, interleukin; TNF, tumour necrosis factor; PAF, platelet activating factor; SIRS, systemic inflammatory response syndrome; ↑, increased; ↓, decreased.

swelling and death [13]. These factors are not unique to a particular organ as they contribute to IRI to liver, heart and lung, as well as kidney (reviewed by [14]).

Ischaemic and hypoxic liver injury are caused by absolute and relative deficiency of oxygen, respectively [143]. Ischaemic liver injury occurs during storage of livers for transplantation surgery, hepatic artery thrombosis of liver allografts, and interruption of portal vascular flow in liver resections (Pringle maneuver) [15]. Hypoxic liver injury is the consequence of hypotensive emergencies and various shock syndromes [16]. Furthermore, hypoxic liver injury has been suggested as a contributory factor in alcoholic liver disease [17]. Re- oxygenation or reperfusion injuries represent an aggravation of the hypoxic or ischaemic insult caused by the introduction of oxygenated blood [15]. In addition to the two global, mechanistic categories of ischaemic and reperfusion injury, various liver cell types have a different temperature-dependent susceptibility to ischaemic and reperfusion injury, adding more complexity to the clinical manifestations of this type of liver injury [9, 18].

Some degree of IRI is inevitable in harvesting and transplanting the liver. The allograft is subjected to varying periods of warm and cold ischaemia. If hemodynamic compromise is present in the donor, warm ischaemia may occur before the organ is removed. After allograft harvesting, a prolonged period of cold ischaemia occurs during transportation of the organ and preparation of the recipient. During transplantation of the allograft into the recipient, ischaemia may occur due to technical factors and/or

microcirculatory abnormalities. Warm ischaemia is tolerated less well than cold ischaemia due to, at least in part, the higher metabolic rate with the consequent higher demand for energy when ischaemia occurs at body or room temperature. Preservation injury is a major determinant of outcome following orthotopic liver transplantation transplantation and contributes to serious complications such as primary non-function, primary dysfunction and non-anastomotic biliary strictures of transplantated livers [19]. These preservation-related complications are major causes of retransplantation and mortality, and become more important with increasing numbers of orthotopic liver transplantations and the concomitant lack of suitable donor organs. At present only about two thirds of organs offered for transplantation are accepted, and fatty livers, organs of donors with prolonged intensive care or prolonged ischaemia time are rejected [19]. These organs are more vulnerable to ischaemia-reperfusion damage, and graft and patient survival is diminished after use of such organs [19, 20]. Better protection against IRI would decrease the rate of preservation-related complications and, moreover, should increase the number of organs available for liver transplantation.

Another common cause of liver ischaemia is temporary clamping of the portal triad (Pringle manoeuvre) practised to achieve a bloodless field and minimise intraoperative blood loss during parenchymal resection for liver tumours. It has been the practice of most surgeons to reperfuse the liver at regular intervals to minimise the total ischaemic time. As this is both inconvenient, bloody and of no proven benefit [21], studies have focussed on the ischaemic duration that the liver can tolerate. In 1992 Huguet et al

demonstrated that the healthy liver can tolerate normothermic ischaemia for an hour [22]. However with the trauma of surgery and underlying hepatic disease, safe ischaemic times are not known.

1.2 Therapeutic Perspective

It can be assumed that a combination of various interventions will be required for optimal prevention of ischaemia reperfusion injury. The quest for protection from ischaemia reperfusion injury is not new, but in the past several years, this concept has been approached more rigorously by investigators addressing important clinical problems, particularly myocardial infarct, cardiopulmonary bypass, stroke, peripheral vascular embolism, and the preservation of organs for transplantation. Much of the recent work has been focussed on the remarkable observation that relatively short periods of ischaemia sustained just prior to a more prolonged (i.e., clinically relevant) episode, appears to have improved the tolerance to the latter in a number of organs including the heart [23], brain [24], spinal cord [25], skeletal muscle [26], retina [27], kidney , intestine [28], and liver [29].

The term ischaemic preconditioning was introduced in 1986 by Murry and co-workers [23]. In this classical study the authors referred to ischaemic preconditioning as an adaptation of the myocardium to ischaemic stress induced by repetitive short periods of ischaemia and reperfusion. In the liver, brief periods of ischaemia and reperfusion (5-10

min) protected against injury after warm ischaemia [30]or hypothermic preservation in Euro-Collins or UW- solution [31, 32] in animal models. The concept has been applied clinically by Clavien et al [29] who showed reduced apoptosis of sinusoidal endothelial cells by ischaemic preconditioning in patients undergoing hemihepatectomies under inflow occlusion. A broader and more fundamental question about ischaemia preconditioning is, how does it work? Defining the mechanisms of the preconditioning effect may allow for a powerful, targeted intervention against an anticipated period of liver ischaemia. Clearly, agents that induce the preconditioning response may assist the preservation of liver for transplantation and be an important adjunct to liver resection for tumors. Most studies suggest that ischaemic preconditioning modulates critical mechanisms of organ injury, however the underlying molecular mechanisms are yet to be clearly defined. The next chapter (Chapter 2) has reviewed the potential mechanisms of ischaemic preconditioning.

1.3 Aim and objectives of this thesis

This study has investigated the potential role of nitric oxide as a mechanism of ischaemic preconditioning of the liver. The objectives of the study were:

1. To investigate the relationship of nitric oxide metabolism with hepatic oxygenation, microcirculation, and function with ischaemic preconditioning of the liver.
2. To investigate whether the protective effect of ischaemic preconditioning is applicable to the fatty (diseased) liver.

1.4 Hypothesis and thesis description

The review of literature in the following chapter has highlighted that nitric oxide (NO) is a player rather than a spectator in the IPC cascade. However the link between NO and the protective effects of IPC is speculative. NO regulates perfusion of the hepatic microcirculation; the breakdown of microvascular perfusion with subsequent impairment of tissue oxygenation plays a central role in the pathophysiology of IR induced injury of the liver [33]. We have therefore hypothesized that IPC may act through local release of NO and influence hepatoprotection through modulation of hepatic tissue oxygenation and hepatic microcirculation. An approach to study the role of NO in hepatic IRI involves the use of agonists and antagonists; in this study the endogenous substrate for NO synthesis, L-arginine and NO synthase inhibitor, Nω-Nitro-L-arginine methyl ester (L-NAME) have been used.

In Chapter 2, the potential mechanisms of IPC have been critically reviewed.

In Chapter 3, the methodology and experimental study model have been described.

The changes in liver oxygenation and HM with IPC and their relationship with NO metabolism will give an important indication of the effect of IPC on IRI. In chapters 4 and 5 the relationship of NO metabolism with hepatic oxygenation, microcirculation and function in IPC of the liver has been demonstrated. The association of increased nitric oxide production with the preconditioning effect has been demonstrated.
In chapter 6, the distribution and expression of nitric oxide synthase isoenzymes in IPC of the liver has been studied.

In chapter 7, the application of the protective effect of ischaemic preconditioning against ischaemia reperfusion injury in the steatotic liver has been investigated.

In chapter 8, general discussion of the thesis including methodological considerations, results and conclusions drawn from the experiments has been carried out and future directions suggested.

References and, Appendix listing the presentations and publications arising out of work described in this thesis appear at the end of the thesis.

Chapter 2

Ischaemic Preconditioning of the Liver- Mechanisms and Clinical Applications: A Review of Literature

2.1 Introduction

Brief episodes of ischaemia followed by a period of reperfusion called ischaemic preconditioning (IPC) have been shown to protect organs against subsequent sustained ischaemia. IPC was first described by Murry and co-workers in 1986 [23]. In a canine model, they demonstrated that multiple brief ischaemic episodes (coronary vascular occlusions) protected the heart from a subsequent sustained ischaemic insult. Since then myocardial ischaemic preconditioning has been shown to occur in many animal species [34] and in humans [35]. Subsequently, IPC has been demonstrated in other organ systems including skeletal muscle [26], brain [36], spinal cord [37], kidney [38], intestine [28]and liver [30]. Although these studies suggested a preconditioning response in most organ systems the mechanism of the preconditioning effect remains uncertain.

IPC has been described as an endogenous adaptive mechanism for prevention of injury resulting from ischaemia reperfusion [23]. The phenomenon is fascinating, as it is easily reproducible and potentially readily applicable in clinical situations. In the liver the preconditioning effect is a promising strategy in assisting preservation of the liver in clinical situations of anticipated hepatic ischaemia such as transplantation and during resection for tumors using hepatic vascular occlusion. Clearly, identifying the mechanism of preconditioning may allow recognition of a pharmocological agent, to protect the liver from ischaemic injury.

The mechanisms underlying the preconditioning effect have not been defined. In contrast, various potential mediators have been proposed and investigated. Most of the data on mediators of preconditioning in organs including the liver has been extrapolated from information gathered in the heart. This chapter reviews the major developments in characterization of mechanisms of IPC in the liver. In addition, clinical applications of IPC to minimise ischaemic injury to the liver have been discussed.

2.2 Methods of search

All the studies were identified by PubMed, ISIS and CAS searches between years 1966-2002 with the following keywords: ischaemia, ischaemia reperfusion injury, preconditioning, ischaemic preconditioning, hepatoprotection. Other sources include review articles and textbooks.

2.3 Evidence that ischaemic preconditioning occurs in the liver

2.3.1 Studies on warm ischaemia

Over the last decade many investigators have studied the effects of IPC on regional and global ischaemia in the liver, and the evidence is encouraging (see table 2.1). Lloris-Carsi in 1993 first demonstrated in the rat liver that a single episode of preconditioning with 5 minutes portal triad clamping followed by 10 minutes reperfusion showed improved survival and decreased liver enzyme levels after subsequent 90 minutes

Table 1: Current data on Hepatic IPC- published studies.

Key words: I, ischemia; R, reperfusion; *, cold ischemia; ↑, increased; ↓, decreased; LFT's, liver function tests; NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; GPT, glutamate pyruvic transaminase; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; HSP, heat shock protein; HM, hepatic microcirculation; TNF-α, tumour necrosis factor-α; MPO, myeloperoxidase; SEC, sinusoidal endothelial cell; IL-6, interleukin-6; AMPK, adenosine monophosphare activated protein kinase; ICAM, intercellular adhesion molecule; ICG, indocyanine green; OLT, orthotopic liver transplantation; PKC, protein kinase C; IEG's, immediate early genes; LPO, lipid peroxidase; SOD, superoxide dismutase; XOD, xanthine oxidase; 5'-NT, 5'-nucleotidase; MIP-2, macrophage inflammatory protein-2; MDA, malondialdehyde; ROS, reactive oxygen species; cAMP, cyclic adenosine monophosphate; SAPK's, stress activated protein kinases; p38MAPK, p38 mitogen activated protein kinase.

Study Group	Year	Species	IPC time (min)	Ischaemia time (min)	Reperfusion time (min)	Hepatic ischaemia	Pharmacological manipulations	Parameters assessed	Outcome of IPC	Proposed mechanism
Lloris-Carsi (30)	1993	Rat	1x3(5I+10R)	90	3 days	Total	Nil	LFT's & Survival	70% 3 day survival + ↓ liver enzyme levels	Not addressed
Hardy (39)	1996	Rat	5I+10R	0-45	1 to 8 days	partial	Nil	LFT's, Histology & Survival	90% 1 day survival, ↑ prothrombin time	not addressed
Peralta (41)	1996	Rat	10I+10R	90	90	partial	spermine NONOate; L-NAME/Bosentan	LFT's, Tissue endothelin & NOS activity, histology	↓ GPT & endothelin ↑ cNOS.	Nitric oxide
Kume (56)	1996	Rat	15I	30	10 & 40	Total	hyperthermia- 42°C for 15 min	ATP,transaminase,LDH, HSP72 , survival	100% survival, ↑ ATP, ↓ transaminase & LDH	HSP72
Peralta (45)	1997	Rat	10I+10R	90	90	partial	spermine NONOate; Adenosine; L-NAME	LFT's, HM	↓transaminases & LDH	Adenosine & Nitric oxide
Peralta (46)	1998	Rat	2-30I+10R	90	90	partial	Adenosine deaminase/Xanthine; spermine NONOate	LFT's	↓transaminases	Adenosine & Nitric oxide
Yoshizumi (40)	1998	Rat	5I+10R	40	120	partial	Nil	LFT's, Bile flow, Tissue ATP, Histology	↓transaminase, LDH & tissue necrosis, ↑ ATP	not addressed
Yin (31)	1998	Rat	5I, 10I or 20I+10R	*16 to 24 hrs	60 to 5 days	cold storage	L-arginine/adenosine; L-NAME	LFT's, Bile flow, TNF-α, Survival	87.5% 1day & 75% 5 days graft survival	Nitric oxide
Adam (82)	1998	Rat	5 or 10I + 10R	*24 hrs	180	cold storage (UW)	Nil	Bile, transaminases, LDH release, vascular resistance	↑ transaminases, LDH & vascular resistance	not addressed
Peralta (48)	1999	Rat	10I + 10R	90	90	partial	Adenosine; adenosine deaminase; DPCPX; DMPX	Transaminases, hepatic perfusion,nitrite/nitrates	Adenosine A2 receptor antagonist abolished	Adenosine A2 receptors & Nitric oxide
Peralta (47)	1999	Rat	10I + 10R	90	90	partial	Gadolinium chloride, TNF, L-NAME, spermine NONOate	TNF, transaminases, vascular permeability, edema, MPO, histology	↓ TNF & tissue injury	Nitric oxide
Yadav (54)	1999	Mouse	10I + 10R	75-90	60 to 180	partial & total	Nil	LFT's, Hepatocellular apoptosis, Survival	↓ apoptosis of hepatocytes & SEC	modulation of apoptosis cascade
Nakayama (55)	1999	Rat	10I + 10R	45	40 min to 24 hrs	Total	AdoR A1, A2 agonists & antagonists	LFT's, Tissue ATP, histology, Survival	↑ adenosine ↓ tissue damage	Adenosine A2 receptors
Zapletal (67)	1999	Rat	5I+ 10R	70	30	partial	Nil	Intravital microscopy	↑ perfusion parameters, ↓ leucocyte adherence	
Nilsson (52)	2000	Rat	10I + 15	60	60	Total	Dipyridamole	LFT's, HM	↑ peripheral liver blood flow & ↓ transaminase	Adenosine

Howell (53)	2000	Mouse	5I + 10R	30	30 min to 24 hrs	partial	Dipyridamole	LFT's, leukocyte/ endothelial cell adhesion	↓endothelial/leukocyte interaction & transaminase	Adenosine
Clavien (29)	2000	Human	10I + 10R	30	30	Total	Nil	LFT's, Hepatocellular apoptosis,	↓ transaminases & apoptotic sinusoidal lining cells	Modulation of apoptosis cascade
Peralta (49)	2000	Rat	10I + 10R	10-90	90	partial	SQ-22536, forskolin	adenine nucleotides, glycogen, glucose-6-P, fructose-6-P, transaminases	preserved energy metabolism during sustained ischemia	cAMP dependent PKC
Tsumaya (58)	2000	Mice	10/15/20I + 20R	70	1 to 48 hrs	Total	Nil	TNF, IL-6, transaminase, Histology, survival	↑ survival, ↓ transaminase, TNF, IL-6 & liver necrosis	not addressed
Peralta (50)	2001	Rat	10I + 10R	90	0 to 360	partial	AICAR/ 8-bromo-AMP/ araA; ZVAD; spermine NONOate/ L-NAME	AMPK activity, nucleotides, lactate, transaminases, apoptosis, histology	AMPK activation, ↑ATP, ↓ lactate & hepatic injury	AMP via PKC
Peralta (51)	2001	Rat	10I + 10R	90	90	partial	ICAM, P-selectin & TNF blockade; TNF/ gadolinium	MPO & lipid peroxidation, vascular permeability, TNF, transaminases, hepatic perfusion, histology, ICAM & P-selectin expression	remote organ protection by hepatic preconditioning	TNF & P-Selectin
Schulz (83)	2001	Pig	10I + 10R X 3	120-200	480 or 300	Total	Nil	ICG clearance, bile flow, transaminases, ATP, Glycogen & lactate contents, histology	No protection against prolonged ischemia	not addressed
Arai (76)	2001	Rat	5 or 10I + 10R	*30 hrs	15 or 240	cold storage	Nil	SEC injury, superoxide formation in Kupffer cells, graft survival & TNF after OLT	↑ graft survival & protection to contra-lateral liver	not addressed
Ricciardi (77)	2001	Pig	15I + 15R	*120	240	cold storage	PKC inhibitor Chelerythrine	graft function & circulation, LDH, endothelial cell damage, PKC levels	↑ graft function	PKC
Ricciardi (78)	2001	Pig	15I + 15R	*120	240	cold storage	Tyrosine kinase inhibitor Genistein	graft function & circulation, Tyrosine kinase activity	↑ graft function	Tyrosine Kinases
Saito (60)	2001	Rat	10I + 10R	40	6 to 48 hrs	partial	Nil	Transaminases, endothelial cell injury, apoptosis, transcription of IEG's	↓ transaminases, endothelial cell injury, necrosis, apoptosis & IEG's transcription	not addressed
Yamada (59)	2001	Rat	10I + 10R	40-120	upto 7 days	partial	Nil	Transaminases,LDH, necrosis,hepatocyte regeneration	↓ transaminases, LDH & necrosis.	not addressed
Zhang (61)	2001	Mice					Nil	Transaminase, LDH, LPO, SOD	↓ transaminases, LDH, LPO& ↑ SOD	not addressed
Ishii (66)	2001	Rat	10I + 10R	40	6 to 48 hrs		Nil	transaminases, LDH, necrosis, apoptosis, IEG's transcription alterations	↓ transaminases, LDH, necrosis, apoptosis, IEG's transcription alterations	not addressed

Peralta (62)	2002	Rat	10I + 10R	90	90	partial	Xanthine, xanthine oxidase, allopurinol, GSH ester	Xanthine, glutathione, superoxide dismutase, lipid peroxidation, transaminases	↓ xanthine, XOD in liver with ↓ neutrophil accumulation, oxidative stress, & microvascular disorders in lung	Xanthine/XOD pathway for ROS generation
Sindram (75)	2002	Rat	10I + 15R	*30 hrs	60	cold storage (UW)	N-acetyl-cysteine	SEC detachment, apoptosis, peroxide, gelatinolytic & gelatinase activity	↓ SEC detachment & activities of matrix metalloproteinase	Oxygen free radicals
Rudiger (63)	2002	Mice	10I + 15R	75-120	180	partial	Nil	transaminase, apoptosis markers, histology, survival	↓ transaminase, no apoptosis or necrosis, 100% survival for ischemic period upto 75 mins but not 120 mins	modulation of apoptosis csacade
Ajamieh (64)	2002	Rat	10I + 10R	90	90	partial	Ozone	transaminases, 5'-NT, oxidative stress, histology	↓ hepatocellular injury & oxidative stress	not addressed
Peralta (42)	2002	Mice	10I + 15R	90	6 to 24 hrs	partial	Gadolinium chloride, TNF, MIP-2, antibodies against TNF and MIP-2.	Transaminases, TNF, MIP-2, MDA, MPO, P-selectin expression	Preconditioning & Bcl-2 overexpression together abolished liver injury	Via TNF and MIP-2 inhibition
Serafin (73)	2002	Rat	10I+10R, 10I+15R or 5I+10R	60	2 to 24 hrs	partial	NO donors and inhibitors, glutathione ester	Microcirculation, neutrophil activity, lipid peroxidation	↓ hepatic injury in normal and fatty livers	Nitric oxide
Teoh (43)	2002	Mice	2 to 20I+10R	90	24 hrs	partial	Nil	Transaminases, histology,	↓ hepatocellular injury	NF-kappaB and SAPKs
Fernandez (44)	2002	Rat	10I+10R	90	16 hrs	Total	Xanthine, XOD	Transaminases, ROS	↓ liver and lung injury	Via xanthine/XOD blockade
Koti (69)	2002	Rat	5I+10R	45	120	partial	L-arginine, L-NAME	Transaminases, NOx, hepatic oxygenation	↓ hepatocellular injury, ↑ intracellular oxygenation	Nitric oxide
Koti (68)	2002	Rat	5I+10R	45	120	partial	L-arginine, L-NAME	Transaminases, NOx, cGMP, microcirculation	↓ hepatocellular injury, ↑ microcirculation	Nitric oxide
Iwasaki (65)	2002	Rat	10I+ 10R	15 x 3, or 45	Upto 180 min	total	Nil	Transaminases, TNF, histology	↑ protective effect for intermittent than continuous I	Not addressed
Funaki (87)	2002	Mice	15I+ 20R	70	0 to 24 hrs	total	Nil	NF-κB activity	↓ NF-κB activation	NF-κB
Ricciardi (88)	2002	Pig	15I+ 15R			cold storage	Genestein, chelerythrine	TK, PKC, NF-κB	TK, PKC, NF-κB activation	PKC and TK, NF-κB
Arai (32)	1999	Rat	5I + 5R	*30 hrs	15	cold storage (UW)	Nil	SEC killing, Kupffer cell activation	↓ SEC death & KC activation.	not addressed
Arai (74)	2000	Rat	5I + 5R	*30 hrs		cold storage (UW)	AdoR A1, A2 agonists & antagonists	SEC killing, SEC cAMP	↓ SEC death & ↑ cAMP	Adenosine A2 receptors via cAMP
Carini (79)	2000	Rat	10I + 10R	90		Hypoxia	Protein kinase C stimulators & inhibitors	Hepatocyte viability, pH, Na+, ATP	↓ hepatocyte cell death	PKC
Carini (80)	2001	Rat	10I + 10R	90		Hypoxia	AdoR A1, A2 agonists (& antagonists; PKC, MEK inhibitors	cell viability, PKC isoenzymes activity, P38 MAPK activity	hepatocyte killing ↓ reduced by 35%	Adenosine A2 receptors, Gi proteins, Phospholipase C, PKC, P38 MAPK
Compagnon (81)	2002	Rat	10 anoxia+ 10 reoxygenation	30 + *24 – 48 hrs	60	Warm ischaemia + cold storage	Nil	LDH, ATP, oxygen uptake, protein synyhesis	↑ hepatocyte viability, ↑ ATP & protein synthesis	Not addressed

ischaemia [30]. Hardy et al showed improved survival in rats undergoing liver resection during 45 minutes ischaemia after prior 5 minutes ischaemia with 10 minutes reperfusion [39]. Similarly Yoshizumi and co-workers have demonstrated improved survival and increased tissue ATP with preconditioning in a rat liver resection model [40]. Subsequently the ischaemic preconditioning effect in the liver has been reproduced in several *in vivo* rodent models of partial and global liver ischaemia [31, 41-65]. Preconditioning protected from partial and global ischaemia. Since none of these studies have directly compared effects of preconditioning on partial vs global ischaemia, it is not known whether any differences exist between the two effects. These studies have demonstrated that liver ischaemic preconditioning for warm ischaemia resulted in decreased hepatocellular injury [45], increased tissue ATP [40, 50], decreased TNF-α [47,58]and IL-6 [58] release, decreased leukocyte/ endothelial cell interactions [53], decreased endothelial cell injury [66], increased peripheral liver blood flow [52], increased microcirculation [67, 68], decreased hepatocellular apoptosis [54], preserved energy metabolism [49], increased hepatic intracellular oxygenation [69] and remote organ protection [51]. These studies provide considerable evidence that preconditioning ameliorates ischaemia reperfusion induced liver injury in the rodent liver. Encouragingly, recent investigations by Clavien and coworkers have shown that IPC exists in the human liver [29]. In this study, patients undergoing hemihepatectomies under inflow occlusion showed inhibition of sinusoidal endothelial cell apoptosis within 30 minutes of reperfusion in the preconditioned livers. In a murine model of partial

hepatic ischaemia, the same group showed that IPC also inhibits apoptosis of hepatocytes at later stages of reperfusion [54].

The steatotic liver is particularly susceptible to ischaemia reperfusion injury resulting in poor outcome following liver surgery [70]and transplantation [71, 72]. There is therefore, an urgent need for strategies to reduce IRI in steatosis. The recent report by Serafin et al [73] shows IPC increases the tolerance of fatty livers to ischaemia reperfusion injury in rats. In this study obese Zucker rats subjected to 60 minutes of lobar liver ischaemia had 70% survival at 30 days with IPC there were no survivors without IPC.

2.3.2 Studies on cold ischaemia

The protective effect of IPC is not restricted to warm ischaemia and decreased tissue damage in cold preserved livers (cold storage- reperfusion injury) after IPC has been demonstrated in small and large animal models. In rat livers, IPC prior to storage in cold University of Wisconsin (UW) solution for 30 hrs, decreased sinusoidal endothelial cell (SEC) death and Kupffer cell (KC) activation [32, 74]. In another study combining two sets of experiments, IPC prior to preservation of rat livers in cold UW solution for 30 hrs decreased SEC detachment and activities of matrix metalloproteinases, and also decreased SEC apoptosis after 1 hour of reperfusion in an isolated perfused rat liver model [75]. In a rat liver transplant model IPC protected liver grafts from ischaemia reperfusion injury [31]. Furthermore, in a recent study in cold preserved rat livers Arai et al [76] have observed that the benefit of IPC extends not only to the ipsilateral lobe, but

also to the contralateral lobe resulting in an improved graft survival after orthotopic liver transplantation. In this study the authors observe that 'such heterologous preconditioning provides a new means to protect liver tissue against ischaemia reperfusion injury without imposing ischaemia on the target tissue' [76]. The effects of IPC on cold organ preservation has been evaluated in animal models other than rodent, Ricciardi et al [77] demonstrating that IPC reduced IRI in cold ischemic porcine livers[78].

2.3.3 Studies on isolated hepatocytes

The hepatoprotective response of IPC has also been shown in isolated hepatocytes. In, *in vitro* studies on freshly isolated hepatocytes, preconditioned (with brief hypoxia) hepatocytes showed increased resistance to cell death during hypoxic incubation [79, 80]. Normothermic IPC has been shown to improve hepatocyte viability and energy metabolism in isolated rat hepatocytes subjected to hypothermic preservation injury [81].

The above studies, mostly in rodent livers, have shown liver protection by IPC to warm and cold ischaemia. However there are few published studies which have suggested that hepatic IPC may be of limited or no benefit. A study by Adam et al [82] in fact suggested that preconditioning had a deleterious effect on hepatic tolerance to cold ischaemia. This study used a model of isolated perfused livers from Wistar rats [82]. Preconditioning protocols of 5 or 10 min ischaemia followed by 10 min reperfusion before liver harvesting, prior to extended cold ischaemia of 24 hrs resulted in extensive reperfusion injury, increased vascular resistance and increased transaminases and LDH release. In a

larger animal model using pigs, a preconditioning protocol of repeated 10 min ischaemia followed by 10 min reperfusion, prior to 120 min or 200 min sustained ischaemia was tested[83]. In the 120 min ischaemia group IPC increased bile flow and ATP, but the degree of hepatic necrosis and apoptosis was not different from control groups. With 200 min ischaemia IPC resulted in no significant differences in bile flow, ATP and liver enzymes from control groups, and the degree of necrosis and apoptosis was in fact greater in preconditioned livers. It should be emphasized that 200 min of global hepatic ischaemia is likely to cause irreversible injury and this time period is considerably longer than would be considered for human liver surgery. This study suggested that IPC conferred some functional protection against reversible ischaemia but no protection from prolonged ischaemia in pigs [83]. The major difference between this study and those showing benefits with IPC is the use of three cycles of preconditioning in comparison with a single episode. In a more recent study Rudiger et al [63] noted that in mice IPC resulted in 100% animal survival with no morphological parenchymal injury after 75 minutes sustained ischaemia as against 14% survival with significant parenchymal injury after 120 minutes ischaemia.

Thus, a large body of evidence favours liver protection by IPC from injury in both warm and cold ischaemia. The existence of IPC in the liver has been demonstrated in rodents, pig and humans. Although most of the data on hepatic IPC has been gathered in rodents and it is recognised that information on preconditioning in rats may not always be extrapolated to larger species and humans, the recent report by Clavien and co-workers

of the first human study [29] is a thoughtful example of potential clinical application of the preconditioning effect.

2.4 Possible mechanisms of preconditioning

The precise mechanism of the IPC response is unknown. From studies on preconditioned myocardium, it is widely accepted that IPC is mediated via a receptor targeting mechanism [84, 85]. Molecules released during ischaemia attach to cellular receptors and contribute to the preconditioning response. The candidate compounds implicated in liver IPC include adenosine [32, 45, 48, 53, 74], protein kinase C [78-80], nitric oxide [31, 41, 45, 48, 68, 69], heat shock proteins [56, 86], tyrosine kinases [77], mitogen activated protein kinases [80], oxidative stress [62, 75], nuclear factor κB [87, 88] and modulation of apoptosis cascade [42]. However the characterizations of these candidate compounds into different processes in the preconditioning cascade such as initiating trigger, signalling pathway and end-effector are not defined and the inter-relationship between these processes is unknown. In the liver, the most investigated molecules are nitric oxide (NO) [41], adenosine [74], protein kinase C [79] and heat shock proteins [56]. This chapter reviews the major developments in characterization of these proposed mechanisms of preconditioning (Fig.2.1).

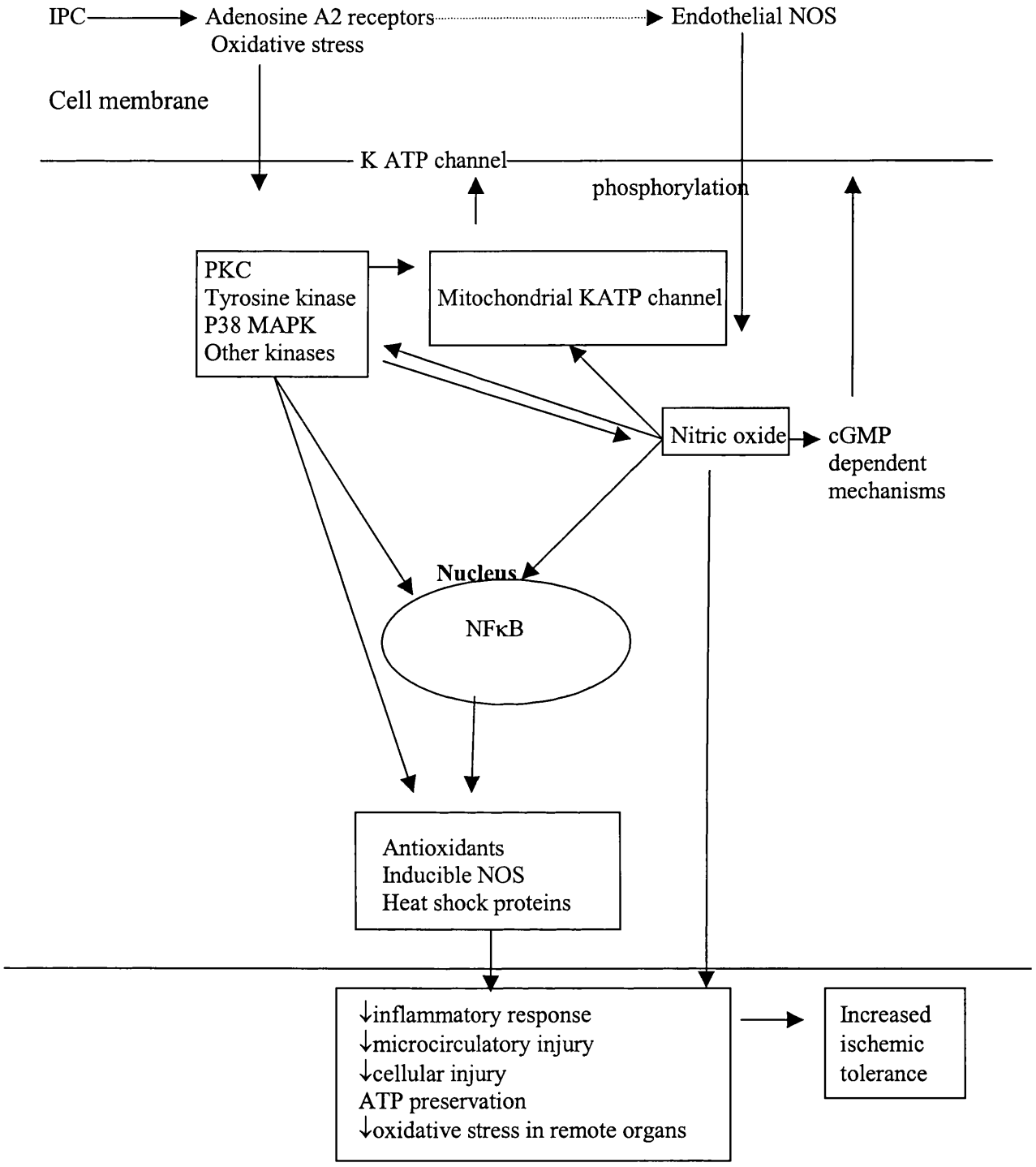

Figure 2.1. Schematic illustration of possible mechanisms involved in ischaemic preconditioning. Keys: NOS, nitric oxide synthase; KATP, potassium dependent ATP channel; PKC, protein kinase C; cGMP, cyclic guanosine monophosphate; NFκB, nuclear factor κB.

2.5 The role of adenosine

Adenosine is an extracellular molecule proposed both as "trigger" and "mediator" of IPC [34]. During ischaemia, adenosine triphosphate is degraded to adenosine. The extracellular adenosine released in large quantities during ischaemia is believed to play a role in the protective effect of IPC during reperfusion of the ischaemic tissue. Ischaemia reperfusion injury is associated with neutrophil and leukocyte activation and primary microvascular failure. Adenosine inhibits leukocyte adhesion, decreases expression of vascular adhesion molecules and inhibits neutrophil and platelet function[89, 90]. Adenosine also inhibits free radical production,[91, 92] important mediators of cellular damage in the early phase of ischaemia reperfusion injury, and is a potent vasodilator[93]. The above would suggest adenosine may be protective against ischaemia reperfusion injury and the effects of adenosine in IPC likely to be multifactorial. Most of the data on the role of adenosine in IPC has been gathered in the cardiac muscle[94-96] and extrapolated to skeletal muscle[26] and kidneys [38].

Over the recent years a few studies have gathered evidence of the involvement of adenosine in liver IPC. Whereas A1 receptors have been implicated in the myocardium [97], A2 receptors have been proposed to be the adenosine receptor subtype likely to be expressed in the liver [74]. The existence of adenosine A2 receptors on hepatic SEC is supported indirectly by demonstrating dose dependent increase in cAMP by adenosine and selective A2 receptor agonist CGS-21680 [74]. In this study by Arai et al, adenosine

A2 receptors blockade prevented the protective effect of IPC in rat livers preserved in cold UW solution [74]. IPC and administration of adenosine A2 receptor agonist, in this study, decreased SEC death and increased cAMP levels [74]. The authors have proposed that SEC protection by IPC is mediated by activation of adenosine A2 receptors producing an increase in cAMP levels in sinusoidal endothelial cells, but the mechanism downstream to increased cAMP, by which adenosine decreases SEC injury is not explained. The same authors have previously shown that IPC suppressed KC activation and have stipulated the involvement of adenosine A2 receptors in this response [32]. IPC induced protection of SEC's could have profound implications for preservation of livers for transplantation, since SEC's are susceptible to cold preservation injury[9, 98] whereas hepatocytes are vulnerable to warm ischaemia reperfusion injury [18]. SEC injury rather than hepatocellular injury has been shown to be responsible for graft failure from cold ischaemia reperfusion injury [9, 98, 99]. Peralta et al have postulated activation of adenosine A2 receptors with subsequent formation of NO to play a role in mediating IPC against warm ischaemia reperfusion injury [45]. In this study adenosine administration in the presence of a NO donor reproduced the protective effect of IPC on hepatic parenchymal cells. In another study, a three-fold increase in adenosine after IPC was associated with decreased parenchymal tissue damage[48]. Both IPC and increasing endogenous adenosine concentrations with the non-specific adenosine uptake inhibitor dipyridamole, decreased hepatic leukocyte/endothelial cell interactions after ischaemia reperfusion injury [53]. All of the above studies have been carried out in rats and although the evidence is limited, suggest that adenosine modulates IPC induced

protection of non parenchymal and parenchymal cells against cold and warm hepatic ischaemia reperfusion injury in the rat liver. There are no studies challenging the involvement of adenosine in the rat liver.

The data on adenosine from IPC studies in rat livers contradicts the information gathered in the rat heart. The role of adenosine in myocardial preconditioning is supported indirectly by studies in rabbits [100], pigs [101], dogs [102], and humans [103] demonstrating abolition of preconditioning by adenosine receptor blockade. However in rats, it is evident that adenosine has no role in IPC of the myocardium [104]. IPC is effective in the absence of extracellular accumulation of adenosine in the rat heart [105]. Thus, adenosine does not appear to be endogenous trigger or obligatory mediator of preconditioning in rat hearts. Thus, in the rat species the adenosine concept does not seem to apply consistently to different tissues. It therefore seems likely that adenosine may only be a mediator to IPC of the liver but not a sole mechanism.

2.6 The role of protein kinase C

The PKC mediated signalling pathway of myocardial preconditioning was proposed by Downey and colleagues [85, 106]. The hypothesis proposes that during preconditioning ischaemia G-protein activation following G-protein coupling with adenosine receptors leads to PKC activation and subsequent translocation from the cytosol to the membrane where it phosphorylates substrate proteins to induce tolerance to subsequent ischaemia

[85]. However conflicting results in some species, particularly large animals where the concept does not apply consistently, would suggest that PKC activation is an epiphenomenon or secondary effect and not a primary mediator of the cardioprotective effects of preconditioning [107, 108]. Most of the evidence surrounding the PKC hypothesis is indirect and based on pharmacological approaches using PKC activators and inhibitors. Many of the inhibitors are not specific to PKC and are also isoform nonspecific. The above reviewers [107, 108] highlighted the limitations of pharmacological methods and also the fact that studies using isoform specific antibodies may not indicate activity of these specific PKC isoforms. Further, information on events downstream of PKC activation and the end effector of preconditioning is lacking at present.

In recent years, a few studies have evaluated the evidence for involvement of PKC in preconditioning of the liver. This evidence is indirect and based on a pharmacological approach. Carini et al used an in vitro model of isolated rat hepatocytes and proposed that hypoxic preconditioning was mediated via PKC mediated activation of vacuolar proton ATPase (V-ATPase) [79]. In this study the increased tolerance of preconditioned hepatocytes to hypoxia was abolished by inhibition of PKC with chelerythrine or blocking V-ATPase with bafilomycin A1 and mimicked by stimulators of PKC, 4β-phorbol-12-myristate-13-acetate (PMA) and 1,2 dioctanoyl-glycerol (1,2 DOG). The authors observed that the prevention of intracellular acidosis and of cytosolic Na+ increase during hypoxia was associated with decreased hypoxic injury in preconditioned hepatocytes [79]. In another study, the same authors observed that preconditioning for

isolated hepatocytes was abolished by adenosine A2a receptor antagonist and have proposed a signalling pathway involving adenosine A2a receptors, PKC and kinases downstream of PKC (p38 mitogen activated protein kinase) to be involved in hypoxic preconditioning of isolated rat hepatocytes [80]. However downstream of this point, the mechanisms by which liver injury is decreased have not been explained. In the heart, it has been suggested that the kinase cascade activated during preconditioning leads to the opening of mitochondrial K_{ATP} channels [109] but there is no evidence that these are the end effectors. There is data to suggest that mitochondrial K_{ATP} channels may simply act as another signal transduction step [110]. The kinase cascade can also stimulate phosphorylation of heat shock proteins [111], activation of the transcription factor nuclear factor κB [112] and upregulation of inducible nitric oxide synthase [113] but the link with end effects of preconditioning has not been established. Ricciardi et al have extended support for involvement of PKC and tyrosine kinase in liver IPC in larger animals [77, 78]. In one study, tolerance of ischaemically preconditioned pig livers to cold ischaemia was abolished by pretreatment with PKC inhibitor chelerythrine [78]. In another study by the same authors pretreatment with tyrosine kinase inhibitor genistein abolished the preconditioning effect in cold preserved pig livers [77]. While these data support a role for PKC in ischaemic preconditioning, they still do not prove that PKC is responsible for preconditioning.

2.7 The role of heat shock proteins

HSP's are intracellular stress proteins that have been shown to accumulate after hyperthermia and ischaemia[114]. The concept of sublethal whole animal hyperthermia conferring tolerance to other stresses such as ischaemia and lethal endotoxin exposure, is referred to as hyperthermic preconditioning and has been associated with HSP accumulation[115, 116]. In the rat liver, tolerance to ischaemic injury has been associated with production of various inducible HSP's; HSP72 [57, 117], HSP73 [57] and, HSP70 and HO-1/HSP32 [118, 119]. Ishikawa et al have proposed that in heat shock preconditioned rat livers HSP's maintain mitochondrial membrane integrity during the ischaemic episode, to produce energy rich phosphates during reperfusion and thus contribute to ischaemic tolerance [86]. In an in vivo study in rats by Kume et al the reduced postischaemic hepatocellular injury and improved survival was associated with overexpression of HSP72 in ischaemically preconditioned livers as well as in the livers preconditioned with heat shock [56]. In this study HSP72 was detected within 6 to 72 hrs after heat exposure and the authors have proposed that HSP72 production is associated with delayed protective effect of IPC. The link between HSP72 and delayed effect of IPC has not been explained. It is also not clear whether HSP production and accumulation is the reason for resistance to ischaemia or merely a marker of tolerance [120].

While these studies demonstrate that HSP's are detected after preconditioning, the molecular mechanism of protection associated with HSP accumulation is not explained

and these studies do not prove that HSP's are responsible for preconditioning.

2.8 The role of nitric oxide

Nitric oxide (NO) is a colourless, odourless, free radical gas which has been identified as an important signaling molecule in almost every tissue in the body. NO is produced from L-arginine by the enzyme nitric oxide synthase. In the liver, as in many other organs NO has many actions and cellular sources. Several observations suggest that NO is a major player in modulating ischaemia reperfusion induced liver injury. Recent evidence supports the role of NO in regulating perfusion of the hepatic microcirculation (HM) [121]. The breakdown of microvascular perfusion with subsequent impairment of tissue oxygenation plays a central role in the pathophysiology of IR induced injury of the liver [33]. Treatment of rats with nonspecific NOS inhibitors resulted in failure of hepatic microvascular perfusion and development of patchy necrosis [122, 123]. Inhibition of nitric oxide synthase (NOS) in rats also resulted in aggravated hepatic injury following the oxidative stress of endotoxaemia [123]. Augmentation of NO synthesis with NO donors has been shown to attenuate hepatic IRI and improve post transplant survival [124]. NO may modulate microvascular perfusion through it's vasodilatory effect [6] and through it's anti-inflammatory actions including inhibitory effects on stellate cell activation [125], neutrophil adhesion [126] and platelet aggregation [123]. The other vasoactive substances which modulate blood flow in the HM are endothelins (ET). ET evokes sinusoidal constriction by contraction of Ito cells [127]. NO produces relaxation of

hepatic stellate cells and opposes the vasoconstrictive effects of stellate cell activation [125] and as a result may limit microcirculatory disturbances. Blocking ET receptors or providing a NO donor, protected HM and reduced hepatic IRI in an experimental model.[128]. These data suggest that NO may influence liver injury either directly or through effects on blood flow. Furthermore, NO is a scavenger of superoxide. The early reperfusion injury is characterized by a profound increase in production of superoxide and a decrease in endogenous NO [8]. Current data suggests that IRI induces an acute inflammatory response and microvascular dysfunction by altering the balance between superoxide and NO in endothelial cells [8]. Furthermore, the decreased availability of NO following IRI may contribute to the pathogenesis of SIRS by facilitating neutrophil endothelial cell interactions and inducing an oxidative stress in remote organs [124]; Augmentation of NO synthesis with NO donors has been shown to attenuate IRI and improve post transplant survival [124]. From the above observations it may seem likely therefore, that the adaptational phenomenon of preconditioning following transient ischaemia may exert its protective effect through local production of NO [31, 45, 49, 68, 69, 73, 129]

It has been proposed that NO plays a key role in both initiating and mediating ischaemic preconditioning. While functional evidence in the heart indicates that NO modulates both acute (< 24 hrs) and delayed (> 24 hrs) preconditioning, downstream of this point in the biochemical pathway hypotheses are less well established [130, 131]. A recent study by Lochner et al has proposed that NO through generation of cGMP acts as a trigger of

acute preconditioning in rat hearts [132]. Parratt [133] has suggested that endocardial endothelium derived NO may mediate stimulate cGMP resulting in the induction of the cGMP-sensitive cAMP phosphodiesterase enzyme. The resulting increase in cAMP may mediate IPC. It appears that whereas the acute phase of preconditioning is protein synthesis independent, the late phase requires new protein synthesis. It has been proposed that eNOS derived NO leads to activation of PKC and other kinases which in turn through nuclear factor-κB (NF-κB) and other transcription factors leads to increase in transcription of iNOS [131]. The end effector of IPC in the supposed NO pathway is speculative and cGMP dependent mechanisms and ATP sensitive potassium channel have been proposed [131].

Interaction of NO with other mediators of IPC

In the liver it has been suggested that depending on the rate of its production, NO may also play a mediating role in preconditioning [134]. NO has been implicated in IPC associated decreased tissue damage in both warm ischaemia [41] and cold ischaemic storage [31] of the rat liver. However the link between protective effects of IPC and NO is speculative. Peralta et al suggested that liver IPC in rats is mediated by the inhibitory action of NO on endothelin [41]. In other studies in rats, the same authors have demonstrated that inhibition of adenosine and simultaneous administration of NO donor offered similar results to ischaemic preconditioning [45] and have proposed that activation of adenosine A2 receptors with subsequent NO formation mediates IPC in the

rat liver [48]. Yin et al have postulated that IPC increased resistance to cold ischaemic liver injury in rats through stimulation of endogenous NO [31]. In this study pharmacological NO stimulation mimicked and NO inhibition antagonized IPC associated protection of liver grafts from preservation reperfusion injury in a rat liver transplantation model but the mechanism has not been explained. A recent report by Serafin et al [73] has implicated NO in the preconditioning response for ischaemia reperfusion injury in fatty livers. In this thesis the relationship of hepatic oxygenation [69] and hepatic microcirculation [68] with NO metabolism in IPC of the liver have been evaluated.

NO and apoptosis

NO suppresses apoptosis in endothelial cells. In recent years it has been suggested that apoptosis is the dominant mechanism for cell turnover in the human liver. Apoptosis is a rapid process terminating in nuclear pyknosis and cell death. Apoptosis of sinusoidal endothelial cells (SEC) and hepatocytes are a feature of ischaemia reperfusion injury in warm [135] and cold [136] ischaemia of the liver. The signalling pathways leading to nuclear apoptosis in response to extracellular stimuli, involve activation of cysteine proteases known as caspases and release of cytochrome c from the mitichondria [137]. Subsequent activation of downstream caspases such as caspase 3 ultimately executes nuclear apoptosis [138]. Antiapoptotic molecules such as Bcl-2 and caspase inhibitors have been shown to prevent release of mitochondrial cytochrome c [139]. In an

experimental model of partial hepatic ischaemia, IPC inhibited apoptosis of SEC and hepatocytes and was associated with inhibition of caspase 3 activity [54]. In the study IPC was not associated with higher Bcl-2 or Bcl-xl expression. The link between IPC and inhibition of caspase activity is speculative. NO has been shown to inhibit caspase activity in vitro [140]. Apoptosis in hepatocytes exposed to TNF-a and actinomycin-D was prevented by NO. In this study NO produced by an NO donor or through iNOS gene expression, inhibited caspase family proteases by S-nitrosylation and prevented cytochrome c release [137]. Other mechanisms for antiapoptotic effect of NO are increase in cGMP [140] and upregulation of Bcl-2 [141] and HSP's [142]. Thus, potentially liver IPC may be mediated through NO modulation of the apoptosis cascade.

2.9 Role of IPC in hepatic surgery

Ischaemia reperfusion injury is a major cause of morbidity and mortality following liver surgery and transplantation. Ischaemia reperfusion injury after prolonged ischaemia has been shown to occur in virtually all organ systems. Ischaemic (and reperfusion) injury to the liver occurs during liver resections performed under temporary inflow occlusion (Pringle manoeuvre) or inflow and outflow occlusion commonly used to reduce intraoperative blood loss, and during storage and implantation of livers for transplantation. The liver tolerates prolonged ischaemia poorly and safe ischaemic times particularly for the diseased liver are not known. Both warm and cold ischaemias result

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in significant liver injury [143], and ischaemia reperfusion injury of the liver can result in multiple system organ failure and systemic inflammatory response syndrome.

In the setting of liver resections, the effects of intermittent inflow occlusion, continuous inflow occlusion and total vascular exclusion during liver resections have been studied in clinical trials [144-146]. Whereas total vascular exclusion was effective in reducing blood loss, it led to unpredictable hemodynamic intolerance, increased morbidity and longer hospital stay [144]. This is not surprising since the state of total vascular exclusion is akin to the anhepatic phase of liver transplantation and hemodynamic consequences on reperfusion would be anticipated. In a prospective evaluation of intermittent inflow occlusion versus no inflow occlusion in patients undergoing liver resections, the former resulted in less blood loss and better preservation of liver function in the early postoperative period [145]. When intermittent versus continuous inflow occlusion were studied in patients undergoing liver resections [146], the group subjected to intermittent inflow occlusion was associated with decreased hepatocellular injury indicated by lower postoperative liver enzymes and serum bilirubin levels. However the intraoperative blood loss during liver transection was significantly higher in this group and this is most likely related to bleeding from the transected surface during successive reperfusion episodes [146]. Thus, the increased blood loss and likely increased duration of surgery due to successive reperfusion episodes may outweigh the benefit of intermittent occlusion on parenchymal tolerance to ischaemia. Although some liver resections can be performed without vascular inflow occlusion, prolonged ischaemia may be

unavoidable to achieve radical tumor resection. An ideal protective strategy for human liver surgery would allow a bloodless parenchymal transection and an increased parencymal tolerance to ischaemia. In theory, ischaemic preconditioning may obviate the need for intermittent releases of hepatic vascular occlusions and extend safe periods of ischaemia by increasing hepatic tolerance to ischaemia during hepatic surgery. The potential for clinical application of ischaemic preconditioning for hemihepatectomies under inflow occlusion has been demonstrated by Clavien et al [29]. In this study ischaemic preconditioning protected against 30 minutes of continuous inflow occlusion with patients showing a twofold decrease in serum transaminases compared to patients subjected to continuous ischaemia only, but no significant differences in duration of surgery, need for intensive care or mortality. This study provides evidence that ischaemic preconditioning occurs in the human liver.

In the setting of liver transplantation, ischaemia time of the donor liver is a major determinant of graft outcome and patient survival after liver transplantation(19). Liver transplantation requires mandatory organ ischaemia. Warm ischaemia to the graft may occur at organ harvest in an unstable donor and cold ischaemia occurs during preservation of the liver for transplantation. During implantation of the graft in the recipient, the liver is subjected to further warm ischaemia until the vascular anastomoses are completed. Finally reperfusion injury is inevitable following revascularization. Prolonged ischaemia results in primary non function or dysfunction of the transplanted liver graft and is associated with biliary and vascular

complications[19, 147] often resulting in retransplantation. This adversely affects patient outcome and survival. Therefore ischaemic preconditioning is an attractive strategy to assist liver preservation and protect the liver from ischaemia reperfusion injury during transplantation by increasing ischaemic tissue tolerance of the liver. As yet there are no reported studies demonstrating clinical benefits of ischaemic preconditioning in patients undergoing liver transplantation. Most animal studies have shown that ischaemic preconditioning offers a degree of protection against cold ischaemia in experimental liver transplantation. This data in animal models is encouraging and clinical studies are required to clarify the potential application of ischaemic preconditioning in human liver transplantation.

2.10 Conclusions

The past decade has provided interesting new data establishing the existence of IPC in the liver. IPC is a powerful endogenous means to protect the liver from ischaemia. To date one study has demonstrated human clinical benefits of liver ischaemic preconditioning. Further clinical studies are required to prove unequivocally that ischaemic preconditioning is possible in the human liver. However the central mechanism of ischaemic preconditioning remains undefined. Current research has demonstrated that IPC is an endogenous adaptive phenomenon that can be reproduced easily in different models of warm and cold ischaemia, and in animals as well as humans. However the causal relationship between the initiating event, biochemical

pathways and end effector molecules remains mechanistically undefined and controversial. As the field advances with mechanistically descriptive studies, these controversies in interrelationships in the preconditioning cascade are likely to be resolved and will hopefully lead to pharmacological strategies for protecting the liver from ischaemic injury.

Chapter 3

Methods

In this chapter, the various investigative techniques and the experimental model used have been described in detail. To avoid repetition of methods description, in future chapters reference is made to the relevant sections in this chapter.

3.1 Assessment of hepatic tissue oxygenation by near infrared spectroscopy

3.1.1 Principle of near infrared spectroscopy

The immediate effect of ischaemia reperfusion on hepatic tissue oxygenation was measured using near infrared spectroscopy (NIRS). Light at visible wavelength (450-700 nm) is strongly attenuated in tissue and as a result can only penetrate a maximum distance of a few millimetres [148]. However, the absorption of light by the tissue chromophores is significantly lower at near infrared wavelengths (700-1000 nm), allowing photons at this wavelength to penetrate deeply [148]. NIRS relies upon this relative transparency of biological tissue to light in the NIR region (700 to 1000 nm wavelength range) to measure tissue properties noninvasively. There are three main tissue chromophores in liver whose near infrared absorption characteristics vary with their oxygenation status, namely oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb) and the mitochondrial enzyme that consumes oxygen, cytochrome oxidase. NIRS directly measures changes in haemoglobin oxygenation and concentration (HbO_2 and Hb) which reflects extracellular tissue oxygenation [149, 150]. NIRS also directly measures the reduction oxidation (redox) changes of the copper centre (Cu_A) of cytochrome oxidase.

The Cu_A dimer rapidly accepts and donates electrons, and therefore changes in the redox state of Cu_A in cytochrome oxidase in principle can reflect changes in intracellular oxygenation and mitochondrial function (35,40). The change in the concentration of these chromophores can be quantified using a modified Beer Lambert Law [151]. NIRS measurements of liver parenchymal oxygenation correlate with liver blood flow and arterial oxygenation [150] and with hepatic vein oxygen partial pressure [152]. Furthermore, a significant correlation between changes in redox state of cytochrome oxidase measured by NIRS and tissue ATP has been demonstrated [149]. NIRS also measures HbT (total haemoglobin, Hb + HbO2) which reflects the blood volume in liver tissue. The use of NIRS to measure liver oxygenaton has been extensively investigated and it has recently been applied to severity grading of IRI [161].

3.1.2 Near infrared spectrophotometer

The NIR spectrometer used in this study is the NIRO 500 (Hamamatsu Photonics K.K., Hamamatsu, Japan) (Figure 3.1). This spectrometer is the commercial version of an instrument developed by colleagues in the Department of Medical Physics and Bioengineering, University College London [151]. In the NIRO 500, the light source is monochromatic light generated from semiconductor laser diodes (LD). The light is produced at four wavelengths (774, 826, 849, and 906 nm). The choice of the wavelengths is based on 765 nm, the absorption maximum for Hb; 810 nm, the isobestic wavelength at which the extinction coefficients of HbO_2 and Hb are equal which can be

used to calculate haemoglobin concentration independent of oxygen saturation; 845 nm, the absorption maximum for oxidised Cyt Ox; and 900 nm, a reference wavelength [153-155].

The light is produced by laser diodes and carried to the liver via a bundle of optical fibres in sequential pulses. The optical fibres are covered by a light proof protective sheath and its distal end terminated in a very small glass prism which reflects the light through 90° to direct it into the tissue [156]. Photons emerging from the liver are collected by the second bundle of optical fibres and detected by a photomultiplier tube (PMT) light detector [156]. The incident and transmitted light intensities are recorded and from these the changes in the concentration of tissue chromophores (μmole/L) are calculated using an algorithm incorporating the known chromophores absorption coefficients and an experimentally measured optical pathlength [157-159].

3.1.3 Recording of hepatic tissue oxygenation in the rat

The NIRS instrument (NIRO-500, Hamamatsu Photonics K.K., Hamamatsu, Japan) used in this study produces near infrared light at four wavelengths which is transmitted in sequential pulses via a bundle of optical fibres to the liver. Photons emerging from the liver are collected by a second bundle of optical fibres and detected by a photomultiplier tube. The difference between transmitted and received light intensity at each wavelength was used to determine the optical density changes at each wavelength.

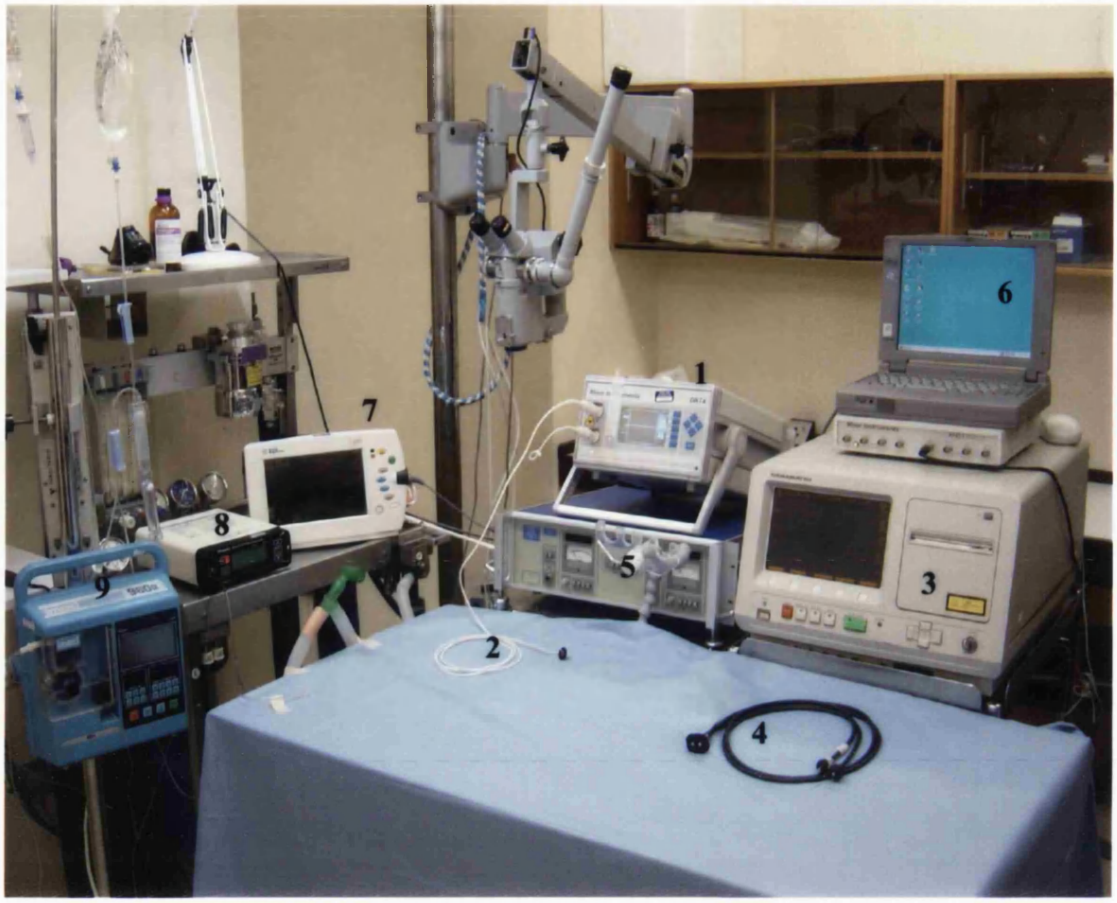

Figure 3.1 Experimental operating room equipped with 1) Dual channel surface laser Doppler flowmeter and 2) its probes (DRT4, Moor instruments Ltd, Devon, UK); 3) Near infrared spectrometer and 4) its probes (NIRO 500, Hamamatsu Photonics, Hamamatsu, Japan); 5) Probes holder; 6) Computer; 7) Blood pressure monitor; 8) Pulse oximeter; 9) Infusion pump

The optical fibre bundles (NIRO probes) (figure 3.1) were mounted inside a probe holder and placed on the surface of the median lobe of the liver with a 10 mm separation. A flexible probe holder (figure 3.1) was used to ensure a satisfactory contact with the liver surface and a fixed interprobe spacing [149, 160].This probe holder ensured that the sites of light entry and exit are maintained at a constant and known spacing distance which minimises the possibility of artefact due to changes in the distance between the probe ends. Also, it allows a satisfactory contact between the liver surface and the probe ends.

The NIRO includes the facility to set the attenuation and therefore chromophore concentration changes to zero with the NIRO initial setting. Since all the measurements are changes from an arbitrary initial zero, this function is important to ensure that artefacts such as system drift, optode movement, and excessive light have a minimal effect on the data. For collection of NIRS data, a sampling time of 1 HZ was used. The NIRS data were continuously collected in a laptop computer connected to the NIRO. These data are the changes in light attenuation (optical densities: OD) at four wavelengths due to absorption by the tissue chromophores. A NIRS algorithm developed specifically to convert these data changes into concentration changes of hepatic HbO_2, Hb and redox state of Cu_A in cytochrome oxidase (Cyt Ox Cu_A redox state) in μmole/L [161] was used[149, 150, 152, 160]. In addition the sum of HbO_2 + Hb (HbT) was computed continuously and reflects the liver blood volume [161]. To determine absolute changes in chromophore concentration, the optical path length in the tissue

must be known as a function of wavelength. The differential path length factor has been determined specifically for the liver by measuring the absorption coefficient as a function of wavelength [149, 161]. The differential path length factor of the liver is 2.7, and this value was used to adjust the NIRS algorithm for calculating the changes in the chromophore concentration [149, 161]. This was then transferred to excel® data sheets (Microsoft Company, Seattle, USA) for analysis. The data at the relevant time points were collected as the mean of 1-minute data and calculated in regard to the baseline value at the start of the experiment. In all the experimental groups the preischaemic baseline was taken as a baseline against which changes were recorded.

3.2 Assessment of hepatic microcirculation by laser Doppler flowmetry

3.2.1 Principle of laser Doppler flowmetry

Laser Doppler flowmetry is an optical technique for assessing tissue microcirculation. Measurement is easy to perform and provides a continuous assessment of microcirculation without interference with tissue blood flow [162-166]. The theory of operation of this technique has been described in detail in many studies [162-166].

Briefly, a monochromatic laser light from a 2mV-helium neon laser operating at 632 nm is guided to the tissue via optical fibres. The back-scattered light from the tissue is transmitted through optical fibres to photodetectors. Only the photons which are

scattered by moving red blood cells will have a Doppler frequency shift, whereas those from the static tissue matrix will not be Doppler shifted. Mixing of these components at the photodetector surface produces an electrical signal containing all of the Doppler frequency shift information. Further processing of the signal produces an output voltage that varies linearly with the product of total number of moving red blood cells in the measured volume of a few cubic millimetres multiplied by the mean velocity of these red blood cells. The numeric product is termed perfusion units or blood cell flux units[167, 168].

Linearity of the laser Doppler flowmeter (LDF) signal from the liver with total organ perfusion has been demonstrated and the technique has been shown to be sensitive to rapid changes in organ blood flow [162-166]. The LDF measurements are expressed in flux units. Due to the problems associated with variation in signal across the surface of the liver, it is not possible to apply a conversion factor so that the LDF signal can be expressed in absolute flow units [163, 164, 165, 166].

The application and reproducibility of LDF measurement for assessment of liver microcirculation has been validated in both experimental animals [164, 166, 169] and human liver transplantation[170].

3.2.2 Laser Doppler flowmeter

The hepatic microcirculation in this study was measured using a commercially available dual channel surface laser Doppler flowmeter (DRT4, Moor Instruments Ltd., Devon, UK) (Figure 3.1). The LDF was calibrated before each study against a standard reference (Brownian motion of polystyrene microspheres in water) provided by the manufacturer.

3.2.3 Recording of the hepatic microcirculation in the rat

To minimise any disturbance to blood flow by the LDF probe pressure on the tissue, the probe was mounted on a probe holder so that the actual probe was just in contact with the surface of the left lateral lobe of the liver without any pressure by the probe weight. LDF data were collected continuously at sampling rate of 1 Hz. LDF measurements at the relevant time points were collected as a mean of one-minute data. Data from the continuous measurement by LDF was collected via the NIRS program that can accept the input of 4 different clinical monitors. After conversion of the NIRS data to excel sheets, the LDF data at the relevant points in each experiment was calculated as a mean of 1-minute data.

3.3 Assessment of hepatocellular injury (liver transaminases)

Liver transaminases alanine aminotransferase (ALT) aspartate transferase (AST) was measured in plasma. The blood samples were heparinised and then centrifuged at 2,000 g for 10 minutes at room temperature to sediment the erythrocytes. The plasma supernatant was removed and analysed on a Hitachi 747 auto-analyzer using commercially available enzymatic kit tests. The tests were determined using reagents supplied by Boehringer Mannheim Ltd UK.

3.4 Assessment of nitric oxide production

The transient and volatile nature of nitric oxide (NO) makes it unsuitable for most convenient detection methods. However, since most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions have been used as a quantitative measure of NO production.

Plasma nitrite + nitrate (NOx) was measured using a 280 Nitric Oxide Analyser (Sievers Instruments) by chemiluminescence method [171].

NO was determined as its decay products, nitrite (NO_2^-) and nitrate (NO_3^-):

$$3\ NO + 2O_2 \rightarrow NO_3^- + 2\ NO_2^-$$

The method is based on the gas phase chemiluminescence reaction between NO and ozone (O_3), viz:

$$NO + O_3 \rightarrow NO_2^* + O_2 \text{ and } NO_2^* \rightarrow NO_2 + h\nu$$

Briefly, the plasma sample (0.5 ml) was diluted one in ten and centrifuged at 1,000 g for 60 minutes at room temperature to remove protein. NO_3^- was then reduced by nitrate reductase prior to chemiluminescence which involved incubating the sample with 40 μM NADPH, 1 μM FAD and 10 mU nitrate reductase in 20 mM Tris buffer pH 7.4 at 37 °C for 60 minutes. Standard solutions of NO_2^- or NO_3^- reduced sample were then added to the purging (reaction) vessel which contained a reducing solution of 1.5 ml KI (50 mg/ml) and 200 μl anti-foaming agent in 6 ml concentrated acetic acid to generate NO. NO then reacted with O_3 produced from O_2. The Nitric Oxide Analyser was attached to a PC computer and with a NOA-Excel linked program, which allowed real time NO_2^- and NO_3^- measurements to be recorded. Results were compared with a standard NO_2^- calibration graph.

3.5 Assessment of hepatic bioenergetics (adenosine triphosphate)

At termination, samples of ischaemic and non-ischaemic lobes of liver were freeze clamped in liquid nitrogen for adenosine triphosphate (ATP) determination. ATP levels in ischaemic and non-ischaemic liver tissue were assayed spectrophotometrically.

ATP levels in ischaemic or non-ischaemic liver tissue were assayed from liver extract using a Unicam UV1 spectrophotometer. Liver extract was prepared as follows: approximately 1 g frozen liver was ground up in liquid N_2 with a mortar and pestle and then added to 5 ml perchloric acid. This homogenate was spun at 3667 r.p.m. for 20

minutes at 4°C and the supernatent neutralized with KOH. The supernatent was centrifuged again at 3667 r.p.m. for 20 minutes at 4°C to remove a $KClO_4$ precipitate.

ATP levels were measured as a reduction of NAD^+ to NADH followed spectrophotometrically at 340 nm. The reaction mechanism was as follows: *ATP + glucose → ADP + glucose-6-phosphate → NADH + 6-phosphoglucolactose.* In brief, 150 µl liver extract was added to 1 ml of assay solution (containing 0.4 mM glucose; 5 mM $MgSO_4$; 0.2 mM NAD^+; 50 mM Tris, pH 8.0) and 0.2 U hexokinase (Sigma H5500) at 4°C. The absorbance was read at 340 nm and this provided blank readings. 0.2 U glucose-6-phosphate dehydrogenase (Sigma G5760) was then added and the sample left in the dark for 60 minutes before the absorbance was again read at 340 nm.

3.6 Guanosine 3',5'-cyclic monophosphate (cyclic GMP, cGMP)

cGMP has been shown to be present in most tissues and is formed by the action of the enzyme guanylate cyclase on GTP. It is involved in a number of important biological reactions. Nitric oxide (NO), a stimulator of guanylate cyclase, also stimulates cGMP levels. The interaction of NO with guanylate cyclase allows cGMP to act as a third messenger in some cells.

cGMP accumulation in liver tissue was determined by using a commercial ELISA kit

(Cayman chemical, USA) [172]. Briefly, immediately at termination of experiment liver tissue was frozen in buffer, homogenised, centrifuged and supernatant collected for assay. Each sample supernatant was then individually acetylated by addition of 100 μL of 4 M KOH and 25 μL Acetic Anhydride, vortex for 15 seconds, and finally 25 μL of 4 M KOH and vortex. Samples (50 μL) or standard solutions of cGMP (3 pmol/ 50 μL to 0.0234 pmol/ 50 μL), were incubated together with rabbit anti- cGMP (50 μL), and cGMP linked to acetylcholinesterase (50 μL), in pre-coated plates at room temperature for 18 hrs. After plates were washed, colour development was initiated by the addition of Ellman's Reagent (200 μL) for 60 mins. Absorbance was measured at 412 nm on a plate reader.

3.7 NADPH Diaphorase histochemical stain

Nitric oxide (NO) is synthesised from L-arginine by the enzyme NO synthase (NOS). 3 isoforms of this enzyme has been identified: neuronal NOS (nNOS/ NOS 1), inducible NOS (iNOS/ NOS 2) and endothelial NOS (eNOS/ NOS 3) [173]. NO is extremely labile with a short half-life of less than 10 seconds and it is not possible to identify NO on tissue sections. However, NOS distribution can be studied using histochemical techniques and *in vitro* autoradiography. These techniques provide important *in situ* evidence to supplement biochemical data. NOS expression can be studied using Northern blotting and Western blotting on tissue homogenates allowing quantitative analyses.

NADPH Diaphorase histochemical stain is a rapid technique that localizes NOS on tissue sections. Diaphorases are a group of redox enzymes, which are able to reduce various chromagen in the presence of a reduced co-factor. A diaphorase with absolute dependence on NADPH previously used to stain neurons in the brain was found to be related to NOS [174]. All isoforms of NOS have since been found to have NADPH diaphorase activity and no other enzyme unrelated to NOS have shown the same activity. NADPH diaphorase is therefore recognised as a simple, convenient and reliable marker for detecting NOS.

Fresh tissue was frozen and stored at -70°C. Five μm transverse sections were cut with a cryostat at approximately –25°C and thaw-mounted onto polylysine-coated microscope slides.For staining, sections were allowed to equilibrate at room temperature (approx 22°C) for 30 min and post-fixed for 30 min in 3% paraformaldehyde in 0.01M PBS buffer at 4°C. After rinsing in PBS and drying in cold air, sections were incubated for 1 hour at 37°C with 1 mg/ml ß-NADPH (cofactor) and 0.2 mg/ml nitroblue tetrazolium (chromagen) dissolved in PBS buffer (pH 7.6), containing 0.2% Triton X-100. The reaction was stopped by removing the incubation solution, blotting the sections and rinsing for 5 min in running tap water. Sections were then stained for 3 min with 1% eosin, and prepared for microscopic examination. Controls were incubated with nitroblue tetrazolium and no NADPH. NADPH diaphorase-positive staining gives blue stain.

3.8 Immunohistochemistry

NADPH diaphorase staining does not differentiate between the 3 isoforms of NOS. Polyclonal antibodies against the specific NOS isoforms are commercially available (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and can identify the isoforms on tissues sections.

Cryostat-cut sections thaw-mounted onto polylysine-coated slides were used. Sections were allowed to equilibrate at room temperature for 30 min and fixed in acetone for 20 min at -20°C and rinsed in 0.01M PBS. Endogenous enzyme activity was inhibited by pre-incubating sections in 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were incubated in 5% normal goat serum in PBS at room temperature for 20 min to block background staining. Tissue was then incubated in the appropriate primary antibody (rabbit anti-e NOS or anti-I NOS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature. After incubation sections were rinsed with PBS and incubated with biotinylated secondary goat antibody for 30 minutes. Following further rinses, sections were incubated with streptavin/biotinylated horseradish peroxidase solution for 30 min (StreptABC Complex/HRP duet, Mouse/Rabbit kit, DAKO). DAB was used as the chromogenic substrate solution and sections were counterstained with Mayer's haematoxylin and prepared for microscopic examination. For negative controls, sections were processed without primary antibody.

3.9 Western blot analysis

eNOS and iNOS proteins were identified using Western blotting.

3.9.1 Preparation of protein extracts

Samples of liver tisssue (100- 200 mg) were homogenised in ice-cold lysis buffer (20 mM HEPES [pH 7.2], 1 mM EDTA, 0.2 M Sucrose, 20 μg/ml Soybean trypsin inhibitor, 20 μg/ml leupeptin, 5 μg/ml peptastin, 5 mg/ml DTT, 5 μg/ml E-64, 5 μg/ml bestatin, 5 μg/ml aprotinin, 5 μg/ml antipain, 0.1 mM PMSF). After centrifugation at 10,000g at 4°C for 30 min, the supernatants were stored at -80°C. Protein determination of samples was performed using the Bicinchoninic acid protein assay (Pearce).

3.9.2 SDS gel electrophoresis and Western blotting

Solubilized proteins were subjected to Nu-polyacrylamide gel electrophoresis (NuPAGE) system (Invitrogen life technologies Ltd, Paisley, UK) on precast NuPAGE 4- 12% gradient gels using NuPAGE MOPS running buffer and electrophoretically transferred onto nitrocellulose membranes using NuPAGE western blot buffer. After blotting the membranes the transfer of proteins was checked using Ponceau S stain. The membranes were then blocked using PBS containing 0.5% bovine serum albumin, 1% polyvinylpyrrolidone 10, 1% polyethylene glycol, 0.2% Tween 20 and 10 mM NaF.

Next, the membranes were incubated with appropriate primary antibody (polyclonal rabbit anti e-NOS, anti i-NOS, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Next the membranes were washed for 3 x 5 min and 1 x 15 min with PBS Tween 0.05%. After this the membranes were incubated for 1 hr at room temperature with anti rabbit IgG horseradish peroxidase secondary antibody (1:200 dilution). Next the washing steps were repeated. To detect any proteins, which have bound the antibody, the membranes were incubated with West Dura reagents (Perbio, Cheshire, UK) for 5 min according to manufacturers instructions. After this the membrane was exposed to a digital camera as part of an electronic imaging system to visualise the proteins bound to the antibody.

3.10 Histology

Liver biopsies were taken from the ischaemic lobes at the end of the experiment in the fatty liver model. The tissue sections were fixed in neutral buffered formalin (10%), embedded in paraffin and were stained with haematoxylin and eosin (H&E). Sections were examined under light microscope to determine the presence and extent of inflammation and necrosis. For fatty livers the grade of steatosis was analysed in a semi-quantitative manner: mild (<30%), moderate (30- 60%), and severe (>60%) using a clinically applied grading system [72].

3.11 Animal preparation and surgical procedure

The study was conducted under a project license granted by the home office in accordance with the Animals (Scientific Procedures) Act 1986. Male Sprague- Dawley rats, each weighing 250- 300g were used for the experiments. All animals were kept in temperature controlled environment with 12 hours light- dark cycle and allowed tap water and standard rat chow pellets ad libitum. Animal research protocols were approved by the hospital ethics committee

Animals were anaesthetised using Urethane 1mg/kg body weight intraperitoneally and prepared for aseptic surgery. Animals were allowed to breathe spontaneously via concentric mask connected to an oxygen regulator during the procedure. The animal's body temperature was maintained at 37-39 oC using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with rectal temperature probe. The arterial oxygen saturation and heart rate were continuously monitored with pulse oximeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda Louisville Co., USA). Polyethylene catheters (PE-50, 0.38-mm inner diameter, Portex, Kent, UK) were inserted into the right femoral artery and connected to a pressure transducer for monitoring of mean arterial blood pressure (MABP), and in the right femoral vein for administering normal saline (1 ml/100g body weight/hr) to compensate for intraoperative blood loss.

Laparotomy was carried out through a midline incision. The ligamentous attachments

from the liver to the diaphragm were severed and the liver was exposed. Ischaemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and hepatic artery branches using an atraumatic microvascular clamp. The other hepatic lobes were not handled during the procedure. This method produces ischaemia to the left and median lobes of the liver (about 70% of the liver) while leaving the blood supply to the right and caudate lobes uninterrupted [33]. At the end of the ischaemia period the vascular clamp was removed and reperfusion was allowed. Hepatic tissue oxygenation and hepatic microcirculation were measured on the liver surface, in separate but identical experiments, to avoid interference with each other. Hepatic tissue oxygenation was continuously measured via optodes placed on the surface of the median and left lateral lobes during ischaemia and reperfusion periods. Hepatic microcirculation was continuously measured via a probe placed on a fixed site on the left lobe of the liver and held in place by a retort holder during ischaemia and reperfusion period. The animal's abdomen was covered with a plastic wrap to prevent fluid evaporation. At the end of the experiment the animals were killed by exsanguination.

3.12 Experimental protocols

The rats were randomly allocated to one of 5 study groups.

Group 1. Control (sham) group (n=6): The liver was exposed for 3 hours. There was no liver ischaemia.

Group 2. Ischaemia-reperfusion (IR) (n=6): Ischaemia was induced in the median and left lateral hepatic lobes for 45 minutes, followed by a 2 hour period of reperfusion.

Group 3. Ischaemic preconditioning (IPC) + IR (n=6): The median and left lateral lobes were preconditioned with 5 minutes ischaemia followed by 10 minutes of reperfusion. This was followed by IR (group 2 procedure).

Group 4. L-arginine + IR (n=6): Animals were treated with L-arginine (100 mg/kg body weight, intravenously) 10 minutes prior to IR.

Group 5. L-NAME + IPC + IR: Animals were treated with Nω-Nitro-L-arginine methyl ester (L-NAME) (30 mg/kg body weight, intravenously) 10 minutes prior to group 3 procedure.

3.13 Blood collection

Blood samples were collected in separate but identical experiments so as not to interfere with systemic hemodynamic stability, NIRS and LDF measurements. Samples (1 ml each) for plasma ALT, AST and NOx measurements were collected from the inferior vena cava at baseline and subsequently at the end of, 5 min ischaemia, 10 min reperfusion (preconditioning protocol), 45 min ischaemia and 2 hrs reperfusion. Samples were heparinised and centrifuged at 2,000 g for 10 minutes at room temperature to sediment the erythrocytes. The plasma supernatent was removed and stored at –20 °C until required for assay.

3.14 Tissue preparation

At termination of reperfusion phase, sections of ischaemic and non-ischaemic lobes of liver were (1) freeze clamped in liquid nitrogen for adenosine phosphate determination and (2) collected on dry ice and immediately stored at –80 C for immunohistochemistry, cGMP determination and western blotting.

3.15 Data collection and statistical analysis

Data from the NIRS, LDF and the pulse oximeter (SaO2, heart rate, mean arterial blood pressure) were collected continuously on a laptop computer. A NIRS algorithm specifically developed for liver NIRS [149] was used. The data were calculated as one-minute averages at baseline, at the end of 45 mins of ischaemia and at the end of 30, 180 mins of reperfusion. The values are expressed as mean ± SD of 6 animals in each group. One way analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated where unpaired Student's t test was used for statistical analysis between groups. $P<0.05$ was considered statistically significant. The relationships between hepatic oxygenation changes, transaminases, ATP and NOx, and between hepatic microcirculation changes, transaminases, ATP and NOx were tested using Spearmans correlation coefficient.

Chapter 4

The relationship of hepatic tissue oxygenation with nitric oxide metabolism in ischaemic preconditioning of the liver

4.1 Introduction

In this chapter the effects of IPC and NO stimulation and inhibition on hepatic tissue perfusion and oxygenation have been studied. The major determinants of ischaemia-reperfusion induced injury of the liver are capillary perfusion failure and impairment of tissue oxygenation after reperfusion [33, 175]. Hepatic tissue oxygenation correlates with microcirculatory impairment and liver dysfunction and is an indicator of the severity of ischaemia reperfusion injury (IRI) [176]. Hepatic tissue oxygenation has also been correlated with early graft function and survival in liver transplantation in experimental animals [177] and humans [178]. Studying the changes in liver oxygenation following IPC may provide insight into the mechanism by which IPC modulates liver IRI.

The use of near infrared spectroscopy (NIRS) to measure liver oxygenation has been described in section 3.1. It has recently been applied to severity grading of IRI [161]. In the present study, plasma alanine aminotransferase was used as an indicator of hepatocellular injury and plasma nitrite + nitrate (NOx) an indicator of NO production.

4.2 Material and Methods

The methods used have been described in details in chapter 3 and only a brief description is given below.

4.2.1 Animal preparation, surgical procedure and experimental protocols

Briefly, the animals were anaesthetised and laparotomy was carried out through a midline incision as described in section 3.11. The ligamentous attachments from the liver to the diaphragm were severed and the liver was exposed. Ischaemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and hepatic artery branches using an atraumatic microvascular clamp. The other hepatic lobes were not handled during the procedure. At the end of the ischaemia period the vascular clamp was removed and reperfusion was allowed. Hepatic tissue oxygenation was continuously measured via optodes placed on the surface of the median and left lateral lobes during ischaemia and reperfusion periods as described in section 3.1. The experimental protocols were as described in section 3.12, and the study groups of 6 animals each were 1) Sham, 2) ischaemia reperfusion (IR), 3) Ischaemic preconditioning (IPC) + IR, 4) L-arginine + IR and, 5) L-NAME + IPC + IR). At the end of the experiment the animals were killed by exsanguination.

4.2.2 Measurements of hepatocellular injury and nitric oxide production

Blood samples were collected in separate but identical experiments so as not to interfere with systemic hemodynamic stability and NIRS measurements. Samples (1 ml each) for measurements of liver specific enzyme alanine aminotransferase (ALT) in plasma and plasma nitrite + nitrate (NOx) were collected from the inferior vena cava at baseline and subsequently at the end of, 5 min ischaemia, 10 min reperfusion (preconditioning

protocol), 45 min ischaemia and 2 hrs reperfusion. ALT was a marker for hepatocellular injury and was measured in plasma (see section 3.3). NOx was a marker for NO production and was measured using a 280 Nitric oxide analyser by chemiluminescence method [171] (See section 3.4).

4.2.3 Data collection and statistical analysis

As described in section 3.15, the data were collected continuously on a laptop computer and a NIRS algorithm specifically developed for liver NIRS (149) was used. One way analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated where unpaired Student's t test was used for statistical analysis between groups. The relationship of hepatic oxygenation with plasma ALT and NOx was tested using Spearmans correlation coefficient.

4.3 Results

In all animals in the experimental groups the heart rate and arterial oxygen saturation did not change significantly relative to baseline or between the groups, throughout the experiment ($p>0.05$). In the L-arginine treated group, a transient fall in blood pressure was observed immediately after L-arginine administration, but this was statistically not significant. In the other groups blood pressure did not change significantly throughout the experiment.

4.3.1 Hepatic tissue oxygenation

Figure 4.1 shows the pattern of changes in tissue oxygenation with lobar ischaemia and reperfusion.

Tissue oxygenation did not alter significantly during the course of the experiment in the sham laparotomy group (non- ischaemic control group)

In IR group (group 2), with ischaemia there was a significant decrease in HbO2 and Cyt Ox Cu_A redox state and an increase in Hb. These parameters did not change significantly until the end of ischaemia (Fig.4.1 and Table 4.1). On reperfusion, there was a significant further increase from ischaemic levels in Hb and further decline in Cyt Ox Cu_A redox state (Fig.4.1 and Table 4.2). HbO2 levels rose on reperfusion but were not significantly higher than ischaemic levels (Fig.4.1 and Table 4.2).

Following IPC (group 3), HbO2 and Hb levels during ischaemia were not significantly different from IR group (Fig.4.1 and Table 4.1). Preconditioning was associated with an increased Cyt Ox Cu_A redox state during 45 minutes of ischaemia in comparison to the IR group with levels similar to those of controls (Fig.4.1 and Table 4.1). On reperfusion there was significant rise in Cyt Ox Cu_A redox state which persisted throughout the reperfusion period (Fig.4.1 and Table 4.2). The levels of HbO2 increased on reperfusion in the IPC group but this was statistically not significant when compared with IR alone group at the end of 2 hrs of reperfusion (Fig.4.1 and Table 4.2). The levels of Hb on

reperfusion showed an increase from ischaemic levels which was not significantly different from IR alone group (Fig.4.1 and Table 4.2). There was a significant increase in HbT on reperfusion indicating an increased liver blood volume. There were significant differences in Cyt Ox Cu_A redox state at the end of 2 hrs of reperfusion between this group and other groups ($p<0.0001$).

In L- arginine treated group (group 4), the pattern of changes in HbO2, Hb and Cyt Ox Cu_A redox state during ischaemia and reperfusion were similar to those observed in IR group (Fig.4.1). The differences in tissue oxygenation between theses two groups during ischaemic and reperfusion periods were not significant (Tables 4.1, 4.2).

In the L- NAME + IPC group (group 5), the changes in HbO2, Hb and Cyt Ox Cu_A redox state during ischaemia were not significantly different from IR group (Fig.4.1 and Table 4.1). On reperfusion, however, levels of Hb increased and Cyt Ox Cu_A redox state further declined from ischaemic levels and both changes were significant when compared with IR group at the end of 2 hrs of reperfusion (Fig.4.1 and Table 4.2). The levels of HbO2 on reperfusion were not significantly different from IR group (Fig.4.1 and Table 4.2). There were significant differences in Hb and Cyt Ox Cu_A redox state at the end of 2 hrs of reperfusion between this group and other groups ($p<0.0001$).

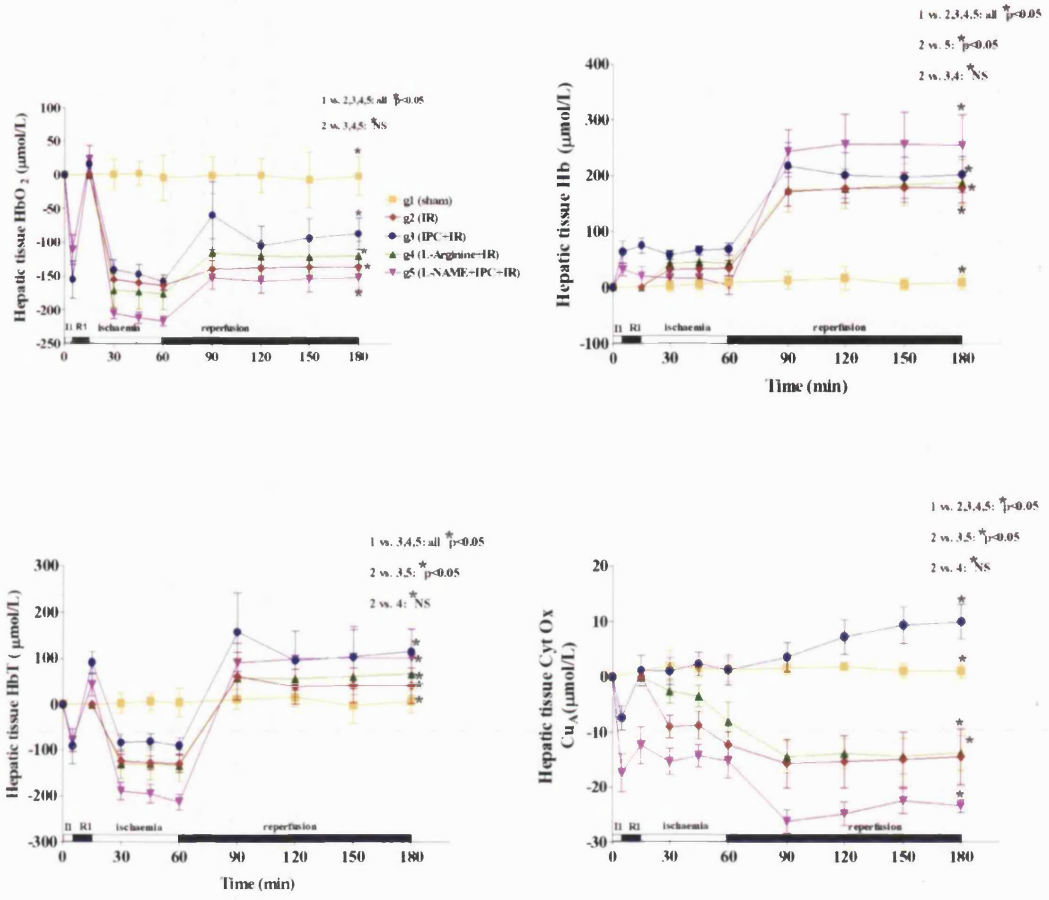

Figure. 4.1 Hepatic tissue oxygenation during 45 min of ischaemia and 2 hrs of reperfusion, measured by NIRS. Values are mean ± SD of 6 animals in each group. I1= 5 mins ischaemia, R1= 10 mins reperfusion. Keys: HbO2: Oxyhaemoglobin, Hb: Deoxyhaemoglobin, HbT: Total haemoglobin (HbO2 + Hb),;Cyt Ox CuA: Cytochrome oxidase CuA redox changes , NS: not significant

Table 4.1. Changes in hepatic tissue oxygenation (μmole/L) vs. baseline at end of 45 min of warm ischaemia (pre-reperfusion). Values are mean ± SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC)	Group 4 (L-arginine + IR)	Group 5 (L-NAME + IPC + IR)
HbO_2	-4.0 ± 2.1	-163.9 ± 16.8*	-158.4 ± 10.8 ^{NS}	-176.2 ± 58.2^{NS}	-198.2 ± 19.0 ^{NS}
Hb	8.6 ± 2.3	34.3 ± 2.5*	68.4 ± 4.1 ^{NS}	42.9 ± 7.0 ^{NS}	33.7 ± 9.7 ^{NS}
HbT	4.6 ± 3.8	-129.6 ± 21.2*	-90.0 ± 28.2**	-133.2 ± 82.4^{NS}	-154.5 ± 45.3**
CytOx Cu_A	1.3 ± 0.1	-12.4 ± 5.8*	1.2 ± 0.6**	-8.7 ± 0.5 ^{NS}	-15.3 ± 7.4^{NS}

*$p<0.01$ vs. Group 1; **$p<0.05$ vs. Group 2; ^{NS}Not significant ($p>0.05$ vs. group 2) using unpaired t test.

Keys: HbO_2: Oxyhaemoglobin, Hb: Deoxyhaemoglobin, HbT: Total haemoglobin, Cyt Ox Cu_A: Cytochrome oxidase Cu_A redox state.

Table 4.2. Hepatic tissue oxygenation in μmole/L at end of two hours of reperfusion. Values are mean ± SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC + IR)	Group 4 (L-arginine + IR)	Group 5 (L-NAME + IPC+ IR)
HbO_2	-2.1 ± 1.1	-136.8 ± 27.3*	-87.1 ± 25.1 ^{NS}	-120.2 ± 53.9^{NS}	-152.6 ± 44.6^{NS}
Hb	8.6 ± 3.3	167.8 ± 69.3*	202.2 ± 79.5^{NS}	186.9 ±103.1^{NS}	253.9 ± 132.7**
HbT	6.5 ± 5.0	31.0 ± 26.7 ^{NS}	115.1 ± 64.4**	66.7 ± 22.9^{NS}	101.3 ± 31.1**
CytOx Cu_A	1.0 ± 0.2	-14.5 ± 9.9*	9.9 ± 7.6**	-13.8 ± 7.7^{NS}	-23.4 ± 2.8**

*$p<0.01$ vs. Group 1; **$p<0.05$ vs. Group 2 ; ^{NS}Not significant ($p>0.05$ vs. group 2) using unpaired t test.

Keys: HbO_2: Oxyhaemoglobin, Hb: Deoxyhaemoglobin, HbT: Total haemoglobin, Cyt Ox Cu_A: Cytochrome oxidase Cu_A redox state.

4.3.2 Hepatocellular injury

IR (group 2) resulted in a significant increase in Plasma ALT measured at the end of both the ischaemic and reperfusion phases. Both IPC and L-arginine treatment reduced ALT levels measured at the end of reperfusion phase. ALT levels were not significantly different at the end of preconditioning period. The addition of L-NAME to IPC (group 5) significantly increased ALT levels (Fig.4.2).

4.3.3 Nitric oxide activity

IR (group 2) resulted in significant reduction in NOx measured at the end of ischaemic and reperfusion phases. Both IPC and L-arginine treatment increased NOx. The levels of NOx were significantly increased at the end of preconditioning period. In the L-NAME + IPC group (group 5) NOx levels were significantly reduced. (Fig.4.2)

4.3.4 Correlation of hepatic tissue oxygenation with plasma ALT and NOx

Changes in plasma ALT correlated significantly with changes in HbO2, Hb and Cyt Ox Cu_A redox changes. Changes in NOx also correlated significantly with changes in HbO2, and Cyt Ox Cu_A redox changes, however no significant correlation was found with Hb. (Table 4.3)

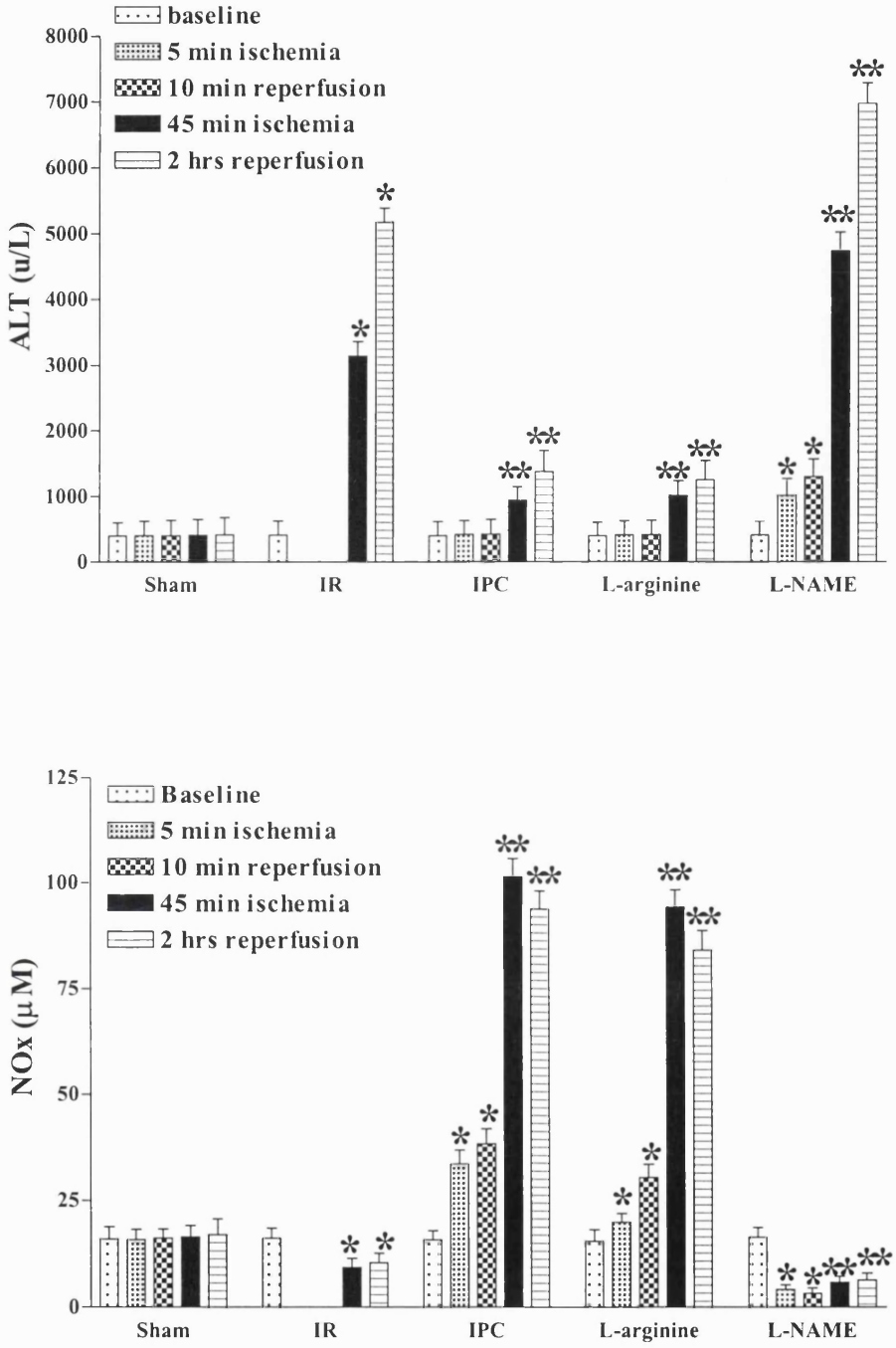

Fig. 4.2. Plasma Alanine aminotransferase (ALT) levels (u/L) and Plasma nitrite + nitrate (NOx) levels (μM). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR.

Table 4.3. Correlation between Hepatic tissue oxygenation (x) and, ALT and NOx (y)

.

	Regression analysis	Spearman's Correlation coefficient	P value
HbO_2 vs. ALT	y = -34.47x + 677.33	r = 0.8	p< 0.001
Hb vs. ALT	y = 25.85x –575.84	r = 0.9	p< 0.01
CytOx Cu_A vs. ALT	y = -186.17x + 227.14	r = 0.9	p< 0.001
HbO_2 vs. NOx	y = 0.56x + 95.42	r = 0.9	p< 0.001
Hb vs. NOx	y = -0.34x + 106.8	r = 0.4	p> 0.05
CytOx Cu_A vs. NOx	y = 3.11x + 71.5	r = 0.9	p< 0.001

Keys: HbO_2: Oxyhaemoglobin, Hb: Deoxyhaemoglobin, Cyt Ox Cu_A: Cytochrome oxidase Cu_A redox state.

4.4 Discussion

This study has investigated the association between tissue oxygenation and NO metabolism with IPC of the liver. A rat model of partial hepatic ischaemia with temporary interruption of blood flow to the left lateral and median lobes while maintaining normal blood flow to the right and caudate lobes was employed. This maintains splanchnic blood flow and, prevents the systemic hemodynamic instability associated with mesenteric congestion and portal bacteraemia from total inflow occlusion [33]. Rats with total hepatic warm ischaemia develop splanchnic congestion and produce TNFα [179], which may contribute to ischaemia reperfusion injury. Total inflow occlusion in humans leads to increase in mean arterial pressure and systemic vascular resistance and decrease of the cardiac index, which may affect hepatic blood flow and hepatic energy metabolism [180]. In this study, the systemic hemodynamic parameters did not change significantly during the experiments avoiding an additional factor which could produce liver injury. Hemodynamic compensation due to splanchnic vasoconstriction was also excluded, as there was no hypovolemia during the experiments.

The model of 45 minutes partial hepatic ischaemia with 2 hours reperfusion was reliable and simple with no procedure related mortality. 45 minutes of partial hepatic ischaemia is nonlethal but induces substantial liver injury as indicated by the liver enzyme rises in the present study and found by others [181]. Experimental data suggest that IR induced

liver injury occurs in a biphasic manner; an early phase of injury that develops over the course of first 2 hrs of reperfusion and, a later progressive phase that develops at 6 to 24 hrs after reperfusion and, liver injury in the early phase modulates the development of the later phase [10]. In this study, therefore a period of 2 hrs of reperfusion following ischaemia was chosen to assess changes in the early phase of reperfusion injury. Preconditioning times of 5 minutes ischaemia with 10 minutes reperfusion have been shown to protect against liver injury in various experimental models of liver IRI [30] including the present[40]. The protective role of endogenous NO in liver IRI is supported indirectly by studies demonstrating exacerbation of liver injury associated with failure of microcirculation in rats treated with nonselective NOS inhibitors [122, 123]. In this study, L-arginine or L-NAME was administered prior to IR to produce the effect of NO stimulation or inhibition.

IRI resulted in substantial liver injury as assessed by elevation in plasma ALT. Cellular enzymes are released into the circulation following rupture of plasma membrane due to cell injury and, the plasma enzyme levels correlate with the degree of cell injury [182]. IPC and NO stimulation with L-arginine reduced the IR induced liver injury, as both were associated with reduced ALT levels. In contrast, NO inhibition with L-NAME not only prevented the IPC associated reduction in liver injury but actually exacerbated the IR associated liver injury. Wang and colleagues have reported an aggravation of liver injury by 90%, characterized by severe vascular oxidant stress, lipid peroxidation , neutrophil infiltration and reduction in microvascular blood flow after inhibition of NO

synthesis with nonselective NOS inhibitor in an experimental model of liver ischaemia and endotoxemia [183].

The transient and volatile nature of nitric oxide (NO) makes it difficult to measure directly. However, since most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions (NOx) are often used as a quantitative measure of NO production [171]. In this study, increased NO production with L-arginine treatment and reduced NO production with L-NAME treatment affirms the response to pharmacological intervention. NO maintains perfusion of the hepatic microcirculation [122] and modulates liver injury through it's vasodilatory [6, 125] and anti-inflammatory [123, 126] effects. In this study, IRI resulted in reduced NO production. Whereas, IPC and L-arginine treatment significantly increased NO production, L-NAME addition to IPC not only prevented the IPC associated increase in NO production but also further reduced NOx levels. These changes in NO production in the experimental groups, suggest an association of NO with liver injury in relation to IRI and IPC. The significant increase in NOx levels at the end of preconditioning period would further support that NO is important in the mechanism of the protective effect of IPC. When this result is considered together with the significant increase in NOx at the end of 45 mins ischaemia, the findings would suggest that NOS induction occurs with preconditioning ischaemia and further NO production is induced throughout the subsequent sustained ischaemia.

One of the major determinants of IR induced injury of the liver is the breakdown of microvascular perfusion with subsequent impairment of tissue oxygenation [33]. Hepatic tissue oxygenation reflects the adequacy of microvascular perfusion and is the ultimate determinant of hepatocyte viability. It is vital for liver graft function and survival after liver transplantation [176, 184]. Measurements of oxygen consumption in liver grafts after transplantation showed that an increase in oxygen consumption was associated with good graft function [185]. Measurement of hepatic venous oxygen saturation showed that low oxygen saturation correlates with poor function of the graft and high liver enzymes [186]. Direct measurement of hepatic tissue oxygenation by NIRS has demonstrated that tissue oxygenation is significantly correlated with the microcirculatory impairment and the liver dysfunction induced by IRI [176, 184]. In addition, liver graft tissue oxygenation has been shown to provide valuable information on early graft function and survival in both experimental animals [177] and human liver transplantation [178]. In this study, the effect of IRI on hepatic tissue oxygenation was measured directly and the effect of IPC on the magnitude and time course of hepatic tissue oxygenation changes was analyzed continuously by NIRS.

NIRS uses an algorithm (developed for liver NIRS) which computes absolute changes in HbO2, Hb and the redox state of Cytochrome oxidase Cu_A [150, 160, 161]. NIRS assesses changes in tissue oxygenation at the level of capillaries and intracellular uptake of oxygen [187]. Oxygen saturation of haemoglobin in liver tissue depends on the difference between oxygen supply and demand. Since the oxygen demand in the liver differs with

the pathophysiological state, it is essential to measure the tissue oxygenation as well as the blood volume in the liver. NIRS measures HbT (total haemoglobin, Hb + HbO2) which reflects the blood volume in liver tissue [150]. The NIRS algorithm may be sensitive to light scattering changes since there were variations in HbT between the groups in this study. There are no studies investigating the relationship between changes in light scattering and changes in blood volume in the rat liver in the context of liver ischaemia reperfusion. However in the brain large changes in the cerebral energy state and cerebral blood flow do not cause large changes in light scattering in hypoxic-ischaemic piglet brains [190]. In the present model, an assumption made is that the effect of light scattering changes is negligible and all observed optical density changes are caused by changes in chromophore absorbance. Cytochrome oxidase is the terminal electron carrier of the mitochondrial respiratory chain that catalyses the reduction of oxygen to H2O in a four electron reaction with the concomitant synthesis of adenosine triphosphate (ATP) through the oxidative phosphorylation process [188]. In the hepatocytes approximately 90% of the oxygen taken up is consumed by cytochrome oxidase in the mitochondria [189]. Cytochrome oxidase has 3 redox active metal sites which exhibit different absorption characteristics depending on their redox state. The copper centres are optically active in the NIR light in contrast with the haem centres that absorb visible light [153, 190]. However absorption of the NIR light by cytochrome oxidase occurs primarily at the Cu_A centre within cytochrome oxidase. The oxidised Cu_A centre has a characteristic shape spectrum with a broad peak centred around 845 nm which is absent with the reduced enzyme [153, 190]. The contribution of the haem

centres to absorption of NIR is less than 10% of the total signal in the reduced-oxidised spectrum [190]. Thus the signal measured by NIRS is almost entirely due to the Cu_A centre. The signal intensity decreases on reduction of this centre. The redox state of cytochrome oxidase Cu_A is dependant on cellular oxygen availability and a linear correlation exists between the two [191]. In the presence of oxygen, electron transfer takes place and the enzyme becomes oxidised, whereas lack of oxygen results in a decreased flow of electrons and cytochrome oxidase becomes reduced [190]. Thus, assessment of the redox state of cytochrome oxidase indicates oxygen availability and liver tissue cytochrome oxidase redox state may reflect the viability of hepatocytes [192]. In this study hepatic tissue oxygenation was reduced during ischaemia and following reperfusion in all groups when compared with sham operated control values. NIRS measurement of hepatic tissue oxygenation correlates with hepatic venous blood oxygenation which reflects parenchymal tissue oxygenation [152]. With 45 mins ischaemia there was decrease in HbO2 and increase in Hb in all groups, reflecting the dissociation of oxygen from haemoglobin as oxygen is extracted by the hepatic tissue [193]. These changes reflect reduced blood and oxygen supply to the tissue. The reduction in cytochrome oxidase reflects severe cellular hypoxia. On reperfusion, in the IR group the failure of recovery of HbO2, Hb and HbT, decline in CytOx Cu_A redox state indicate persistent tissue and cellular hypoxia due to low liver blood volume and an inability to fulfill the oxygen demand with the reperfusion injury events. Other mechanisms responsible could be an increase in hepatic arterial resistance due to

vasoconstriction, hypocapnia and alkalosis, oxygen transfer failure, decreased ATP and energy dependant pathways all of which accompany tissue hypoxia.

Ischaemic preconditioning significantly raised Cyt Ox Cu_A redox state during 45 minutes of sustained ischaemia. This implies preservation of intracellular oxygenation and is likely due to reduced energy consumption during sustained ischaemia in the preconditioned livers. It is unlikely that there would be any increased collateral flow to the ischaemic lobes due to the peculiar anatomy of the rat liver, which is multilobed with individual blood inflow to each lobe. Furthermore, on reperfusion in preconditioned livers there was significant rise in Cyt Ox Cu_A redox state and which persisted throughout the 2-hr reperfusion period. There was no significant change in extracellular oxygenation despite recovery of HbT indicating recovery of blood volume during the reperfusion period in the preconditioned livers. Taken together these findings indicate a limitation or delay of hepatocellular injury as well as reduced cellular metabolism in the preconditioned livers. Studies on the myocardium have demonstrated that the features contributing to the anti infarct effect of preconditioned myocardium are slower rate of energy metabolism with decreased utilization of high-energy phosphates and delay of cellular ultrastructural damage [23, 194].

In this study, in the ischaemically preconditioned group a raised Cyt Ox Cu_A redox state was observed in the presence of NO. Recently several studies have demonstrated that NO controls mitochondrial respiration through cytochrome oxidase (reviewed by [195]).

The primary effect of exogenous NO in these studies on mitochondrial activity *in vitro* was a reversible and competitive inhibition of cytochrome oxidase activity in the presence of physiological levels of oxygen. However, as it is not easy to measure NO levels and cytochrome oxidase activity *in vivo* accurately, direct evidence for NO inhibition of the oxidase in whole tissues is difficult to obtain [195]. Furthermore NO is also a substrate for cytochrome oxidase and the NO oxidase activity of the mammalian enzyme stoichiometrically converts NO to nitrite[195]. As nitrite is a non toxic product, this activity might play a protective role in processes in which NO production is implicated (e.g., organ preconditioning in this study). Endogenous NO has a rapid but transient effect on the respiratory chain based on the cytochrome oxidase mediated catabolism, which allows a slow but continous flow of electrons through the chain even when a complete suppression of the O_2 consumption has been accomplished [196].

In this study the NIRS changes in redox state of cytochrome oxidase may indicate that cytochrome oxidase or mitochondrial electron transport chain is becoming reduced due to inhibition of oxygen delivery. However, cytochrome oxidase does not become fully reduced, because the binding of NO to haem a_3 of cytochrome oxidase is only transient and reversible (excluding significant interactions to other components of respiratory chain- cytochromes c or b) and the NO is reduced to nitrite by cytochrome oxidase. [196]. Presumably the end effect of NO inhibition on haem a_3 (in NO metabolism of haem a_3) would be apparent in all groups. The effect does not therefore alter the differences between the groups in terms of tissue oxygenation and hepatocellular injury.

NO regulates sinusoidal perfusion [122] and may thus modulate liver injury. In this study, using the amino acid substrate for NO biosynthesis L-arginine before IR, hepatic oxygenation values did not show statistically significant differences when compared with IR group. Treatment with L-arginine did however increase NOx and significantly reduce plasma ALT, comparable to IPC group. Increased NO production may have reduced hepatocellular injury by a direct cytoprotective effect. Use of NO synthesis inhibitor L- NAME with IPC, however, significantly impaired hepatic oxygenation and enhanced ALT levels, showing statistically significant differences when compared with IR group. Overall, the impairment of tissue oxygenation was greater with NO inhibition than that observed with IR and would suggest it may have increased cellular sensitivity to ischaemia. Overall, the data from this study suggests that NO is a major factor influencing tissue damage with IRI. However inhibition of NO synthesis blocks IPC and aggravates IRI. Therefore, endogenous NO may influence or be responsible for the preconditioning effect.

Significant correlation was found between hepatic tissue oxygenation parameters measured by NIRS and both, plasma ALT and NOx in the experimental groups. This data further emphasize that hepatic tissue oxygenation monitored by NIRS correlates with hepatocellular injury and that NO production influences liver oxygenation and also liver cell injury. The former observation may have important implications in clinical application of NIRS to monitor hepatic ischaemia.

In conclusion, the data in this study suggests that IPC has an important role in limiting or downregulating IR induced liver injury and may thereby, increase ischaemic tolerance of the liver. Furthermore, NO production is associated with the hepatic preconditioning effect.

This study has demonstrated that IPC may mediate a hepato protective effect through improved hepatic tissue oxygenation via NO production. Since tissue oxygenation correlates with microcirculatory impairment and hepatic microcirculatory failure is a major determinant of hepatic IRI, the next chapter has evaluated the relationship of hepatic microcirculation with NO metabolism in IPC of the liver.

Chapter 5

Changes in Hepatic Microcirculation with Ischaemic Preconditioning of the Liver and their Relationship to Nitric Oxide Metabolism

5.1 Introduction

In this chapter the effects of IPC and, NO stimulation and inhibition on hepatic microcirculation and liver function have been studied. Key events in the pathophysiology of IRI include an amplified inflammatory response [197] and failure of hepatic microcirculation (HM) [175]. The postischaemic hepatic microcirculatory failure correlates with the degree of hepatocellular damage [198]. NO regulates perfusion of the HM. Because failure of the HM is a key factor of IRI, changes in HM with IPC may give an important indication of the effect of IPC on IRI.

Flow in the HM was continuously measured using laser Doppler flowmeter (LDF) [170, 199]. The use of LDF to measure the HM has been described in section 3.2.

5.2 Material and Methods

The methods used have been described in details in chapter 3 and only a brief description is given below.

5.2.1 Animal preparation, surgical procedure and experimental protocols

Briefly, the animals were anaesthetised and laparotomy was carried out through a midline incision as described in section 3.11. The ligamentous attachments from the liver to the diaphragm were severed and the liver was exposed. Ischaemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular

pedicle containing the portal vein and hepatic artery branches using an atraumatic microvascular clamp. The other hepatic lobes were not handled during the procedure. At the end of the ischaemia period the vascular clamp was removed and reperfusion was allowed. HM was measured by LDF probe placed on a fixed site on the surface of the left lateral lobe of the liver and held in place by a probe holder. The method is described in section 3.2. The experimental protocols were as described in section 3.12, and the study groups of 6 animals each were 1) Sham, 2) ischaemia reperfusion (IR), 3) Ischaemic preconditioning (IPC) + IR, 4) L-arginine + IR and, 5) L-NAME + IPC + IR). At the end of the experiment the animals were killed by exsanguination.

5.2.2 Measurements of hepatocellular injury and nitric oxide production

At the end of 2 hrs of reperfusion, 2ml blood sample each were taken from the inferior vena cava for measurements of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma. (See section 3.3) and, for plasma nitrite + nitrate (NOx) measurements. (See section 3.4).

5.2.3 Hepatic tissue ATP and cGMP measurements and, NADPH diaphorase histochemical staining

At the end of 2 hrs of reperfusion biopsies of the ischaemic lobes of liver were freeze clamped in liquid nitrogen for adenosine triphosphate (ATP) determination. (See section

3.5). Hepatic guanosine 3'5' cyclic monophosphate (cGMP) accumulation in liver tissue was determined [172] by using a commercial ELISA kit (Cayman chemical, USA). (See 3.6). A separate liver biopsy was frozen and stored at –70° C for NADPH diaphorase histochemical stain which was carried out on five μm transverse sections of liver tissue as described in section 3.7.

5.2.4 Data collection and statistical analysis

As described in section 3.15, the data were collected continuously on a laptop computer. One way analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated where unpaired Student's t test was used for statistical analysis between groups. The relationships of hepatocellular injury and NO production with HM were tested using Spearmans correlation coefficient.

5.3 Results

Hemodynamic data are shown in Table 5.1. In all animals in the experimental groups the heart rate and arterial oxygen saturation did not change significantly throughout the experiment. In the L-arginine treated group, a transient fall in blood pressure was observed immediately after L-arginine administration, but this was statistically not significant. In the other groups blood pressure did not change significantly throughout the experiment.

5.3.1 Hepatic microcirculation

Figure 5.1 illustrates the changes in mean percentage (standard deviation) of HM with respect to the preischaemic baseline level (100%). Table 5.1 lists the changes in HM in absolute values.

There was no significant change in the hepatic microcirculation at one, two or three hours of recording in the sham operated control group when compared with baseline ($p= 0.5$). There were significant differences between this groups and groups 2,3,4 and 5 ($p<0.001$) when HM changes at the end of 45 mins ischaemia and at the end of 30, 180 mins of reperfusion were compared (Fig. 5.1 and Table 5.1).

In IR group, at the end of 45 minutes of ischaemia the mean HM decreased to 20.7% of the preischaemic level. After removal of the microsurgical clamp the mean HM recovered to 31.1% during first 30 minutes of reperfusion period, thereafter slowly declined to reach a value of 22.5% ($p=0.00$ vs. baseline) at the end of 2 hours of reperfusion (Fig. 5.1).

In the IPC group, during the preconditioning period, the mean HM recovered rapidly to 97.3% on declamping after 5 minutes of ischaemia, and remained steady during 10 minutes of reperfusion. After subsequent 45 minutes of sustained ischaemia the mean HM decreased to 18.1% and on declamping showed a rapid recovery to 58.5% during

initial 30 minutes of reperfusion and thereafter showed a slight decline without any peaks to reach a final value of 49% ($p= 0.005$ vs.baseline) during final 15 minutes of recording (Fig. 5.1 and Table 5.1). following 2 hrs of reperfusion flow in the HM was significantly higher in the IPC group than those undergoing IR alone ($p<0.05$) (Table 5.1).

In the L-arginine treated group the mean HM decreased to 81.2 % ($p= 0.01$ vs. baseline) immediately after L- arginine injection. Subsequently after 45 minutes ischaemia the mean HM had decreased to 24.6 % ($p<0.05$ vs. baseline). On declamping the mean HM improved to 46.8 % ($p<0.001$ vs. baseline, $p< 0.05$ vs. IR)) and then gradually declined to 31.6 % ($p<0.05$ vs. baseline) during final 15 minutes of recording (Fig. 5.1). However the differences were not significant when compared with IR group at the end of 2 hrs of reperfusion ($p>0.05$) (Table 5.1).

In the L-NAME treated group, at the end of the preconditioning period, the mean HM recovered to 89.3% ($p<0.05$ vs. baseline). After subsequent 45 mins of sustained ischaemia the mean HM had decreased to 15.8% ($p<0.05$ vs. baseline). On declamping the mean HM recovered to 26.3% and then decreased progressively to around 10.2% during the final 15 minutes of reperfusion (Fig. 5.1). There were significant differences between this group and groups 2 and 3 at the end of 2 hrs of reperfusion ($p<0.001$) (Table 5.1).

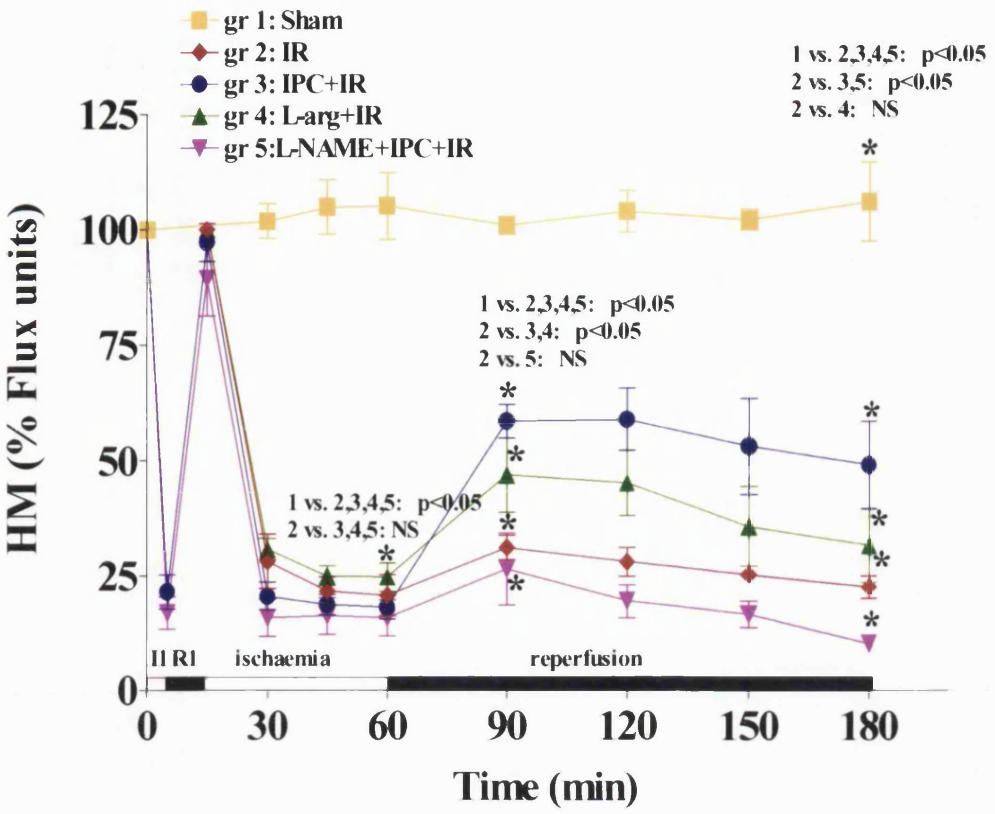

Figure. 5.1. Hepatic microcirculation in Flux units (%) during 45 min of ischaemia and 2 hrs of reperfusion, measured by laser Doppler flowmeter (LDF). Values are mean ± SD of 6 animals in each group. I1= 5 min ischaemia; R1= 10 min reperfusion.

Table 5.1. Hepatic microcirculation (HM) (Flux units, absolute values) at end of 45 min of warm ischaemia (HM-1), at the end of initial 15 minutes of reperfusion (HM-2), and at end of two hours of reperfusion (HM-3), heart rate (HR) (beats/ min), arterial oxygen saturation (SaO2) (%), mean arterial blood pressure (MABP) (mmHg). Values are mean ± SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC)	Group 4 (L-arginine + IR)	Group 5 (L-NAME + IPC)
HM-1	154.3 ± 15.7	32.3 ± 13.9*	30.0 ± 12.9^{NS2}	38.5 ± 13.9^{NS2}	26.3 ± 12.4^{NS2}
HM-2	150.2 ± 21.4	42.4 ± 16.9*	95.3 ± 16.5**	71.3 ± 19.8^{NS2}	43.3 ± 16.0**
HM-3	155.0 ± 14.9	32.2 ± 17.0*	78.7 ± 17.8**	47.1 ± 20.8^{NS2}	16.8 ± 3.6**
HR	235.0 ± 11.9	229.6 ± 7.7^{NS1}	230.0 ± 6.2^{NS2}	233.9 ± 7.7^{NS2}	230.0 ± 9.7^{NS2}
SaO2	98.4 ± 1.1	97.7 ± 0.6^{NS1}	97.2 ± 0.9^{NS2}	96.9 ± 1.4^{NS2}	97.4 ± 1.6^{NS2}
MABP	55.0 ± 2.2	51.0 ± 1.4^{NS1}	53.0 ± 1.6^{NS2}	53.0 ± 2.1^{NS2}	50.0 ± 2.4^{NS2}

*$p<0.01$ vs. Group 1; **$p<0.05$ vs. Group 2; ^{NS1}Not significant ($p>0.05$ vs. group 1);

^{NS2}Not significant ($p>0.05$ vs. group 2) using unpaired t test.

5.3.2 Hepatocellular injury

IR (group 2) resulted in significant increase in plasma ALT and AST. Both IPC and L-arginine treatment reduced ALT and AST levels. Whereas, in L-NAME + IPC group (group 5) ALT and AST levels were significantly increased. (Fig. 5.2)

5.3.3 Hepatic tissue ATP

IR (group 2) resulted in significant decrease in hepatic tissue ATP as compared to Sham values. Both IPC and L-arginine treatment increased ATP levels as compared to IR. Whereas, in L-NAME + IPC group (group 5) ATP levels were significantly reduced as compared to IR. (Fig. 5.3)

5.3.4 Nitric oxide production and hepatic cGMP

IR (group 2) resulted in significant decrease in NOx measured at the end of reperfusion phase. Both IPC and L-arginine treatment increased NOx and cGMP. Whereas, in the L-NAME + IPC group (group 5) NOx and cGMP levels were significantly reduced. (Figs. 5.4 and 5.5)

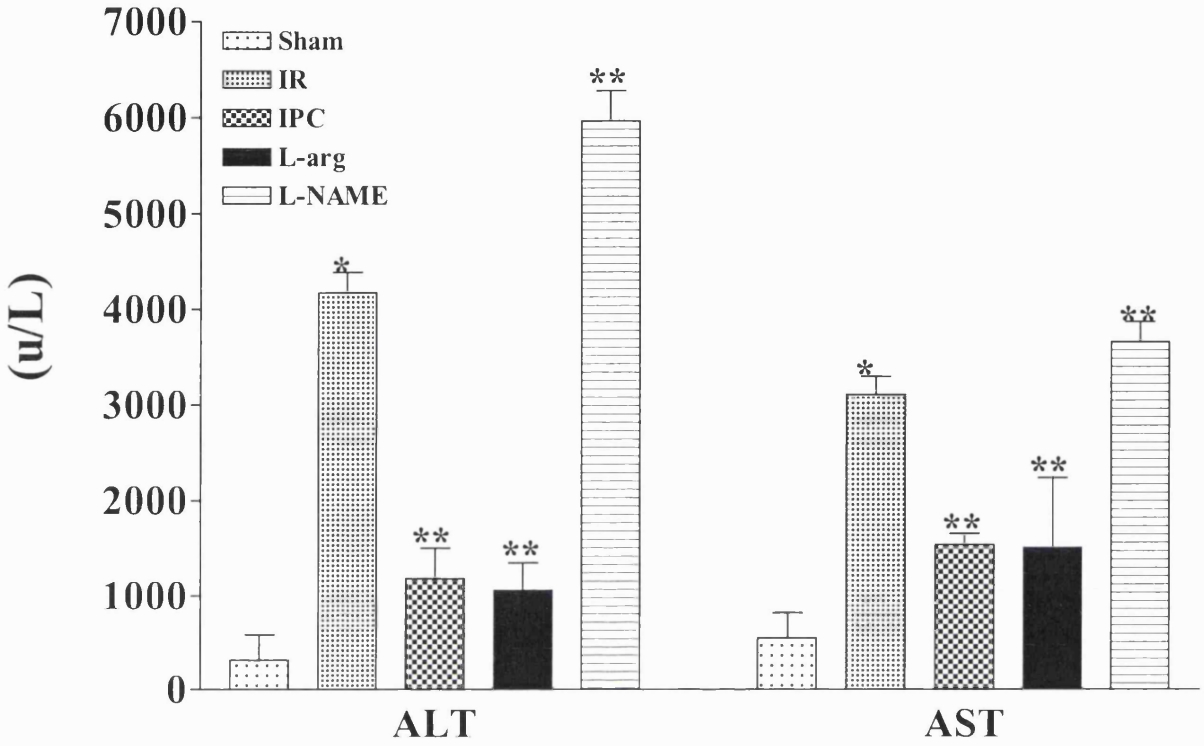

Fig. 5.2. Plasma Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels (U/L) at the end of 2 hrs reperfusion phase. Values are mean ± SD of 6 animals in each group. *$p<0.05$ vs. sham, **$p<0.05$ vs. IR, Students t test.

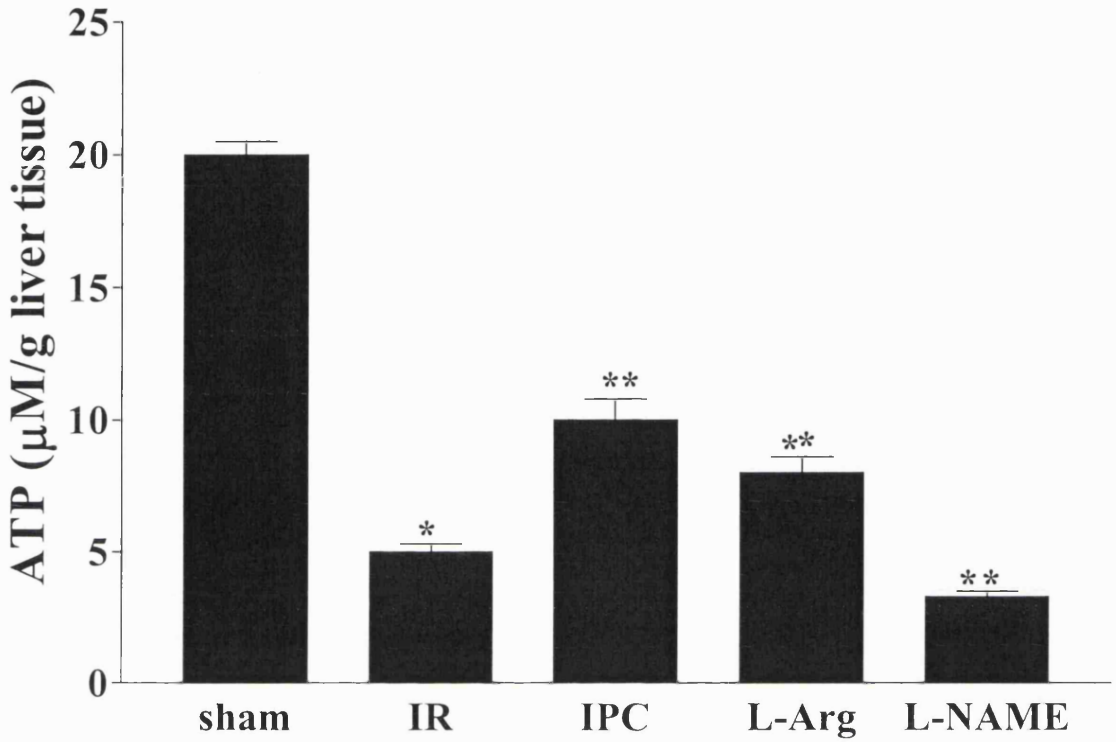

Fig. 5.3. Hepatic tissue ATP levels (μmol/g wet liver tissue) at the end of 2 hrs reperfusion phase. Values are mean ± SD of 6 animals in each group. *$p<0.05$ vs. sham, **$p<0.05$ vs. IR, Students t test.

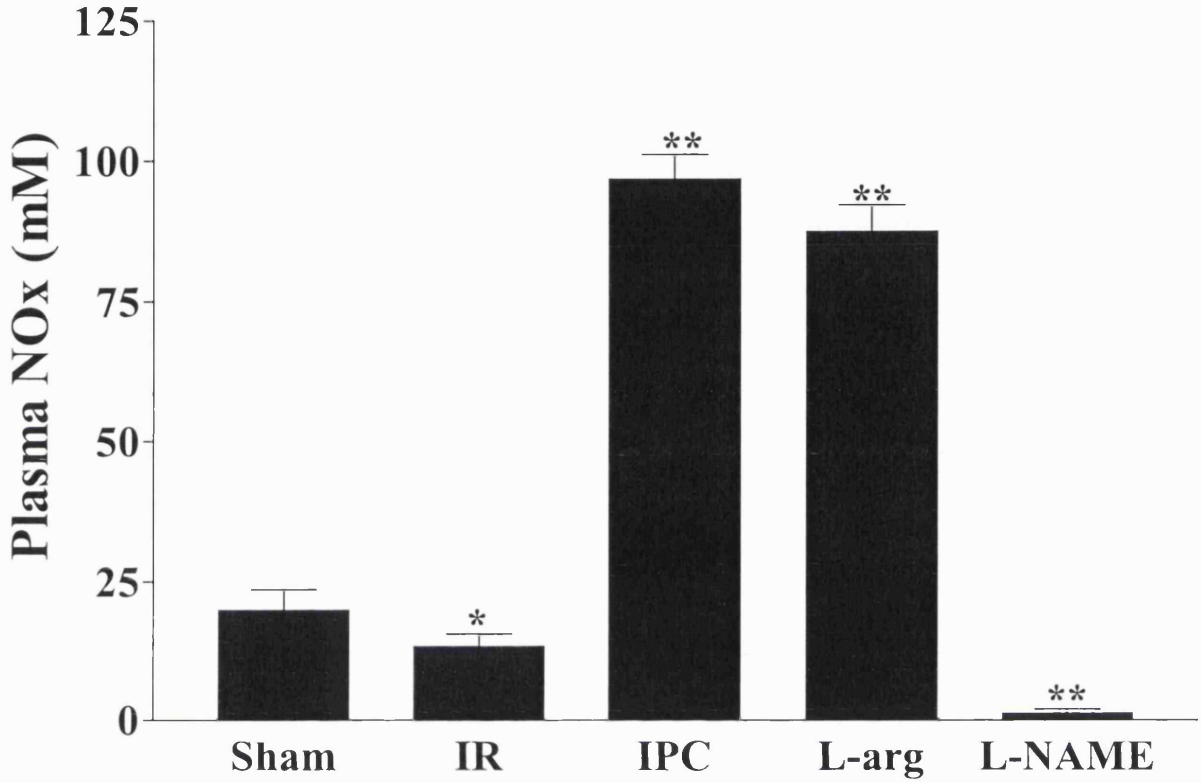

Fig. 5.4. Changes in Plasma nitrite + nitrate (NOx) levels (μM) at the end of 2 hrs reperfusion phase. Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR, Students t test.

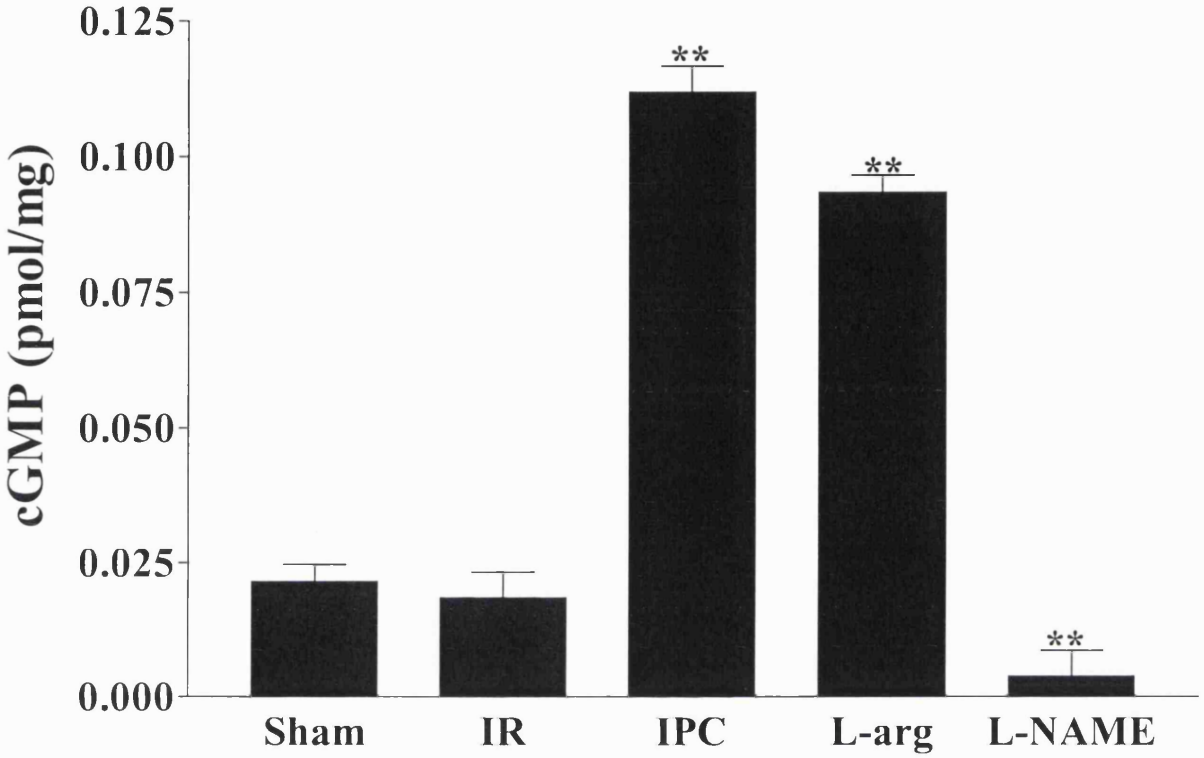

Fig. 5.5. Hepatic cGMP levels (pmol/mg) at the end of 2 hrs reperfusion phase. Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR, NS= not significant vs. sham, Students t test.

5.3.5 Correlation of hepatocellular injury and NO production with HM

At the end of 2 hrs of reperfusion there was a significant negative correlation between plasma transaminases and HM and significant positive correlations between hepatic ATP and HM and between NOx and HM (Table 5.2).

Table 5.2. Correlation between HM (x) and ALT, AST, ATP and NOx (y)

	Regression analysis	Spearmans Correlation coefficient	P value
HM vs. ALT	y = -55.62x + 7.1	r = 0.95	p< 0.001
HM vs. AST	y = -35.15x + 5.9	r = 0.91	p< 0.001
HM vs. ATP	y = 0.12x - 0.32	r = 0.91	p< 0.001
HM vs. NOx	y = 2.30x - 16.3	r = 0.95	p< 0.001

5.3.6 NADPH diaphorase histochemical stain

Nitric oxide synthase (NOS) appraised with NADPH diaphorase staining was associated with hepatocytes and vascular endothelium in centrilobular zone. The distribution of NOS was similar in both IPC and L-arginine treated groups (groups 3 and 4). IR and IPC + L-NAME groups (groups 2 and 5) did not show positive staining for NOS (Fig. 5.6).

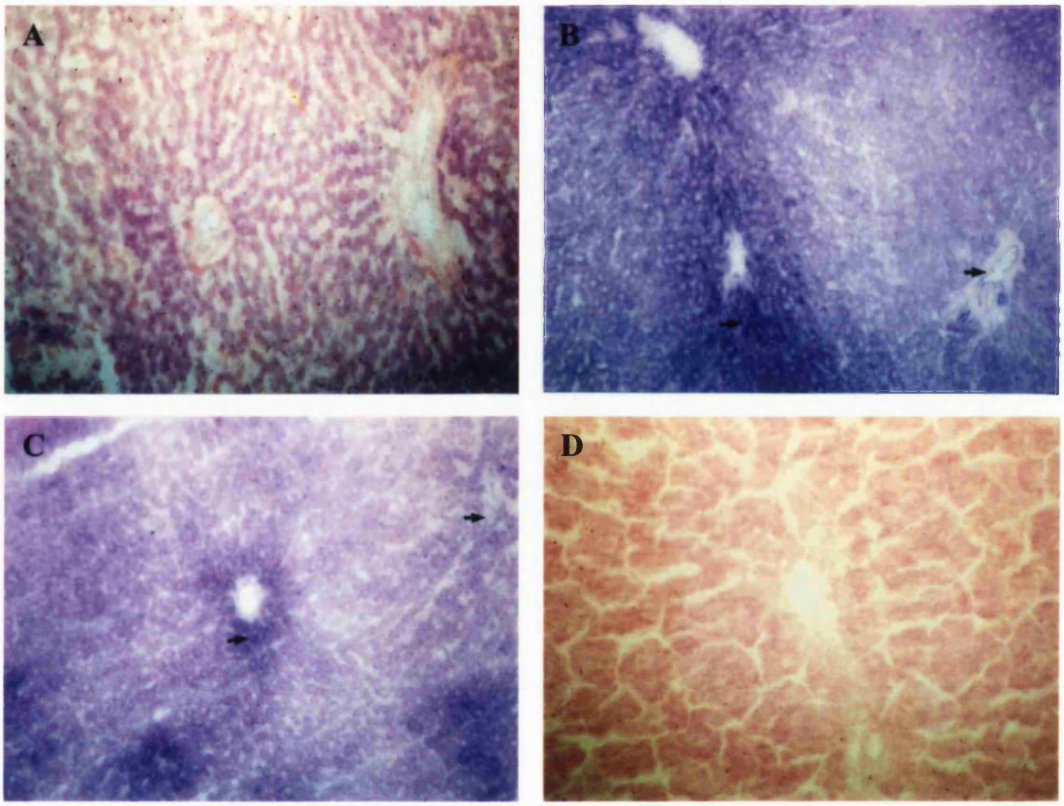

Figure 5.6 Representative photomicrographs of NOS staining using NADPH Diaphorase (in arrows) in hepatocytes in centrilobular zone and endothelium. The Groups were A) IR, B) IPC + IR, C) L-arginine + IPC + IR and D) L-NAME + IPC + IR

5.4 Discussion

This study has investigated the effect of ischaemic preconditioning on hepatic parenchymal perfusion and how it relates to nitric oxide metabolism. The rat model of lobar hepatic ischaemia reperfusion and, ischaemia and reperfusion times were identical to those used in chapter 4. In this study, the systemic hemodynamic parameters including mean arterial blood pressure, heart rate, body temperature and oxygen saturation did not change significantly during the experiments excluding any systemic contributions to the altered hepatic parenchymal perfusion. Laser Doppler flowmetry is a reliable method for the continuous measurement of tissue blood flow [167] and has been used to grade the severity of IRI by recording changes in the liver microcirculation. LDF allows a continuous in vivo recording of the HM without directly affecting the HM [170] and its use has been discussed in section 3.2. To evaluate the effect of NO on hepatic microcirculation following IRI, additional groups had L- arginine (amino acid substrate for NO biosynthesis) and L- NAME (non- specific inhibitor of NOS) administered prior to IR, as discussed previously.

Previously, improvement in microcirculation perfusion parameters following IPC has been shown by Zapletal et al [67] but the mechanism was not addressed. The results of the present study also indicate that IPC is associated with an improvement in the HM following reperfusion. NO may be the mechanism behind IPC as L-arginine treatment protected against IRI similar to preconditioning, whereas L-NAME administration aggravated microcirculatory impairment and cellular injury in preconditioned animals.

LDF measurements calculated as a mean of 1-minute data at each stage of experiment were expressed in Flux units. The preischaemic baseline recordings of HM were expressed as a standard 100% in each individual experiment. LDF reflects the real blood flow in the hepatic tissue microcirculation, which includes the collateral flow and backflow from hepatic veins in addition to portal vein flow and hepatic arterial flow. All groups subjected to 45 minutes of ischaemia showed impairment of HM indicated by similar biphasic curve of initial partial recovery of blood flow followed by decline during 2 hrs of reperfusion. The failure of recovery of blood flow to baseline values reflects the cascade of events associated with reperfusion injury. Several mechanisms contribute to this microcirculatory failure including narrowing of the sinusoid lumens by endothelial cell swelling [200] secondary to ischaemia-induced ATP deficiency and the consequent failure of ion transport through the cell membrane [201]. A significant reduction of leukocyte velocity with subsequent stasis and intrasinusoidal plugging has been suggested as a hindrance for blood perfusion [202]. Increased leukocyte adherence with increased permeability to macromolecules in postcapillary venules contributes to the reflow paradox [203].

The comparison of HM changes between the groups at different time points show that with IPC the HM significantly improved within 30 mins of reperfusion. This suggests that the mechanism of preconditioning modulating flow in the microcirculation occurs either during the ischaemic period or starts immediately following reperfusion. This could be further evaluated by studying NOS induction during the ischaemic period and

NO formation serially before or after reperfusion. The effect of preconditioning is likely to involve modulation of immediate microcirculatory events at the level of capillaries and post capillary venules, as these are the primary sites of IR induced microcirculatory failure. This data would support the hypothesis that IPC may act through release of NO which has vasodilator as well as an anti inflammatory effect. Recent studies have indicated the contribution of altered endothelin (ET-1)/NO balance in mediating the sinusoidal perfusion failure [204]. The importance of endogenously produced NO in counteracting the increased action of ET-1 has been demonstrated by the fact that blockade of endogenously produced NO during postischaemic reperfusion aggravates microvascular and hepatocellular injury [183, 204]. In this study, L- arginine administration before IR improved HM when compared with the IR group at the end of the initial 30 mins of reperfusion. This again suggests that NO formation is a key factor in microvascular perfusion following IR. Endogenous NO may limit IRI via inhibition of the vasoconstrictive effects of stellate cell activation [125]. NO has a vasodilator effect [6] and also inhibits neutrophil adhesion [126] and platelet aggregation [123], and has functions suggesting it to be a key player in the maintenance of the HM. In contrast, use of the NO synthesis inhibitor L-NAME with IPC in this study blocked the improvement in HM seen with IPC and further exacerbated the impairment in HM during the 2 hrs of reperfusion. Nitric oxide synthase inhibition has been shown to increase neutrophil accumulation and liver injury [126] and impair microvascular blood flow [183]. The role of leukocyte in IRI has been confirmed by the observation that the degree of leukocyte infiltration of the reperfused tissue correlates with postoperative liver function

impairment and hepatocyte injury [10]. Also, in neutropenic animals there is a substantial decrease in the severity of IR injury [205]. NOS inhibition promotes leukocyte adhesion and has been shown to decrease sinusoidal blood flow velocity using intravital fluorescence microscopy [206]. Both IPC and L-arginine treatment resulted in increased amounts of NOx and cGMP. Administration of L-NAME resulted in significant reductions in NOx and cGMP indicating effective inhibition of NOS. NO has been shown to activate cytosolic guanylate cyclase which in turn increases cGMP concentrations leading to vasodilatation. Recently, Ishikawa et al [207] have shown improved hepatic tissue blood flow with increased hepatic cyclic nucleotides in IRI. Thus, in this study the alterations in NOx measurements when considered together with the changes in HM between the groups would suggest that NO metabolism is strongly associated with modulation of hepatic parenchymal perfusion with preconditioning. This is demonstrated by the significant positive correlation between HM and NOx.

It is unlikely that the immediate increase in HM with IPC is a result of increased collateral flow to the postischaemic lobes since the rat liver is multilobed with individual blood inflow to each lobe. Studies on the myocardium have demonstrated that preconditioning decreased the infarct size with no significant difference on collateral blood flow in the preconditioned and control groups [23]. The results of this study also show that with IPC flow in the HM did not increase after 30 mins of reperfusion. That the microcirculatory benefit of IPC is evident within 30 mins of reperfusion would suggest that the mechanism is influencing the liver parenchyma

during or immediately after liver ischaemia. It would also suggest that IPC causes only a limitation of the microcirculatory damage in early IRI. The severity of the sinusoidal perfusion failure is dependent on the ischaemia time [175] and experimental studies using intravital microscopy have shown incomplete recovery of microvascular perfusion 24 hrs following prolonged ischaemia [208].

ATP levels measured at the end of reperfusion phase were significantly higher in preconditioned as well as L-arginine treated groups. This could either be a result of reduced cellular metabolism in the preconditioned livers or an increased ATP synthesis once oxygen levels have returned on reperfusion. Wang et al [209] have shown that during myocardial ischaemic preconditioning NO which is generated upon ischaemic stress triggers the opening of mitochondrial ATP-sensitive potassium channels which augments ATP synthesis in the ischaemic myocytes. In a speculative review Stefano et al [210] have surmised that the drop in ATP levels during ischaemic episode leads to an increase in intracellular calcium and release of NO. The NO so produced downregulates the cells excitatory state to protect itself until return of oxygen levels and increase in ATP levels, akin to physiological processes such as exercise [210]. A slowed down or suspended cell metabolism with slowing of ATP depletion and reduced accumulation of ischaemic catabolites is a feature of the preconditioned myocardium [23]. Further, ATP itself can release NO via P_{2y} purinoreceptors located on vascular endothelial cells, to induce vasodilatation of the hepatic vasculature [211]. In rabbits, ATP- induced vasodilatation in the hepatic vascular bed was readily attenuated by both L-NMMA and

L-NAME [212]. The ATP induced vasodilatation may contribute to improving the microcirculation in the preconditioning response. The association of preconditioning induced improvement in HM with reduced hepatocellular injury is demonstrated by the significant negative correlation between HM and plasma transaminases and the significant positive correlation between HM and ATP.

The results of NADPH diaphorase histochemical stain demonstrated a differential regional distribution of nitric oxide synthase (NOS). This is a rapid technique that localizes NOS on tissue sections. Diaphorases are a group of redox enzymes, which are able to reduce various chromagen in the presence of a reduced co-factor. A diaphorase with absolute dependence on NADPH previously used to stain neurons in the brain was found to be related to NOS [174]. All isoforms of NOS have since been found to have NADPH diaphorase activity. Non- NOS NADPH diaphorase activity is present in tissues. However, when tissues are cross linked with fixative as done in this study, the non- NOS NADPH diaphorase is denatured and what remains is usually specific for NOS isoenzymes. NADPH diaphorase is therefore recognized as a simple, convenient and reliable marker for detecting NOS. In liver, as in many other organs, NO has many cellular sources. In the liver NO is produced by most cell types including endothelial cells [213], hepatocytes [214], hepatic stellate cells [125], and macrophages [215]. In this study light microscopy demonstrated positive NADPH diaphorase staining in the hepatocytes and vascular endothelium with IPC and L-arginine treatment. There was absence of positive staining with IR and NO inhibition. These data suggest an increased NOS

content in the liver with IPC and emphasize the participation of NO in IRI and IPC. Considering the hypothesis presented, the NO is likely to be derived from the constitutive isoform of NOS since induction of inducible isoform of NOS requires several hours.

In conclusion, IPC improves flow in the HM and reduces hepatocellular injury. Inhibition of NO synthesis blocks the HM improvement seen with preconditioning and exacerbates liver injury. These observations are in keeping with the known association between the severity of IR and reduction in HM post reperfusion and would support NO metabolism as a key mediator of the microcirculatory effect of IPC.

Chapter 6

Nitric Oxide Synthase Distribution and Expression with Ischaemic Preconditioning of the Liver

6.1 Introduction

In chapters 4 and 5, the association of NO with improved hepatic oxygenation [69] and microcirculation [68] after IPC has been demonstrated. Peralta et al have also have suggested NO is a central mediator of IPC both in normal [41, 45, 49, 129]and steatotic livers [73]. In addition, other NO donors had similar effects to IPC in increasing post transplant survival in a rat model of cold preservation and reperfusion injury [31]. Thus, there is increasing evidence for the role of NO in the IPC effect and it is attractive to hypothesize this experimental evidence may eventually lead to pharmacological strategies using NO for protecting the liver from ischaemic injury. NO in the liver is produced by various isoforms of the enzyme nitric oxide synthase and so far isoform specific studies in relation to NO production with IPC have not been studied. Defining the isoform specific pathway for NO production with IPC will allow a better understanding for the development of drug targeting to induce or enhance the preconditioning response.

In this chapter, the study described has attempted to identify the NOS isoforms responsible for generation of the cytoprotective effect of NO during liver IPC.

6.2 Material and Methods

6.2.1 Animal preparation, surgical procedure and experimental protocols

These methods were identical to those used in chapters 4 and 5. Briefly, the animals were anaesthetised and laparotomy was carried out through a midline incision as described in section 3.11. The ligamentous attachments from the liver to the diaphragm were severed and the liver was exposed. Ischaemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and hepatic artery branches using an atraumatic microvascular clamp. The other hepatic lobes were not handled during the procedure. At the end of the ischaemia period the vascular clamp was removed and reperfusion was allowed. The experimental protocols were as described in section 3.12, and the study groups of 6 animals each were 1) Sham, 2) ischaemia reperfusion (IR), 3) Ischaemic preconditioning (IPC) + IR, 4) L-arginine + IR and, 5) L-NAME + IPC + IR). At the end of the experiment the animals were killed by exsanguination.

6.2.2 Measurements of hepatocellular injury and nitric oxide production

At the end of 2 hrs of reperfusion, 2ml blood sample each were taken from the inferior vena cava for measurements of liver enzymes alanine aminotransferase (ALT) in plasma (See section 3.3) and plasma nitrite + nitrate (NOx) measurements [171]. (See section 3.4).

6.2.3 NOS immunohistochemistry and western blotting

At the end of 2 hrs of reperfusion biopsies of the ischaemic lobes of liver were collected on dry ice, frozen and immediately stored at -70^{o} C. NOS immunohistochemistry was performed on Cryostat-cut sections thaw-mounted onto polylysine-coated slides according to the method described in section 3.8. The preparation of protein extracts and, SDS gel electrophoresis and Western blotting were performed according to the method described in section 3.9.

6.2.4 Statistical analysis

As described in section 3.15, the experimental results are expressed as mean ± SD of 6 animals in each group. Data was analyzed using analysis of variance (ANOVA) for multiple comparisons. Analysis between two groups was performed using unpaired Student's t test (two- tailed) where ANOVA indicated significance for the multiple comparison. Statistical significance was accepted when $p < 0.05$.

6.3 Results

The changes in ALT and NOx are shown in fig. 6.1. Both IPC and L-arginine treatment produced similar decrease in plasma ALT and increase in NOx. L-NAME treatment with IPC aggravated the increase in ALT and decreased NOx.

The expression of NOS was investigated in liver biopsy samples taken at the end of 2 hrs reperfusion period. With eNOS and iNOS immunohistochemistry, the negative controls had no positive staining. Both immunohistochemistry (fig.6.2.) and western blotting (fig.6.3.) showed increased eNOS expression with IPC and L-arginine treatment. The immunostaining was localized to hepatocytes and vascular endothelium. There was no eNOS expression in IR and L-NAME + IPC groups. There was no iNOS expression within the experimental groups (fig.6.3.).

6.4 Discussion

This study has addressed the relationship of endothelial isoform (e-NOS) and inducible isoform (i-NOS) of nitric oxide synthase with the ischaemic preconditioning effect on early (< 2 hrs) ischaemia reperfusion injury of the liver. The major finding of the study is that cytoprotective nitric oxide is produced by eNOS during ischaemic preconditioning of the liver.

In liver, as in most tissues, NO production is complex, in that NO is produced by various cell types and its rate of production can vary enormously [216]. NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of the enzyme exist and all three types are present in liver tissue [216]. The chemistry of NO is also complex in that it reacts with many cellular components such as oxyhaemoglobin (HbO_2), superoxide (O_2^-), thiols and various intracellular enzymes [217]. The steady-state concentration of NO in liver will therefore depend on the rate of NO production,

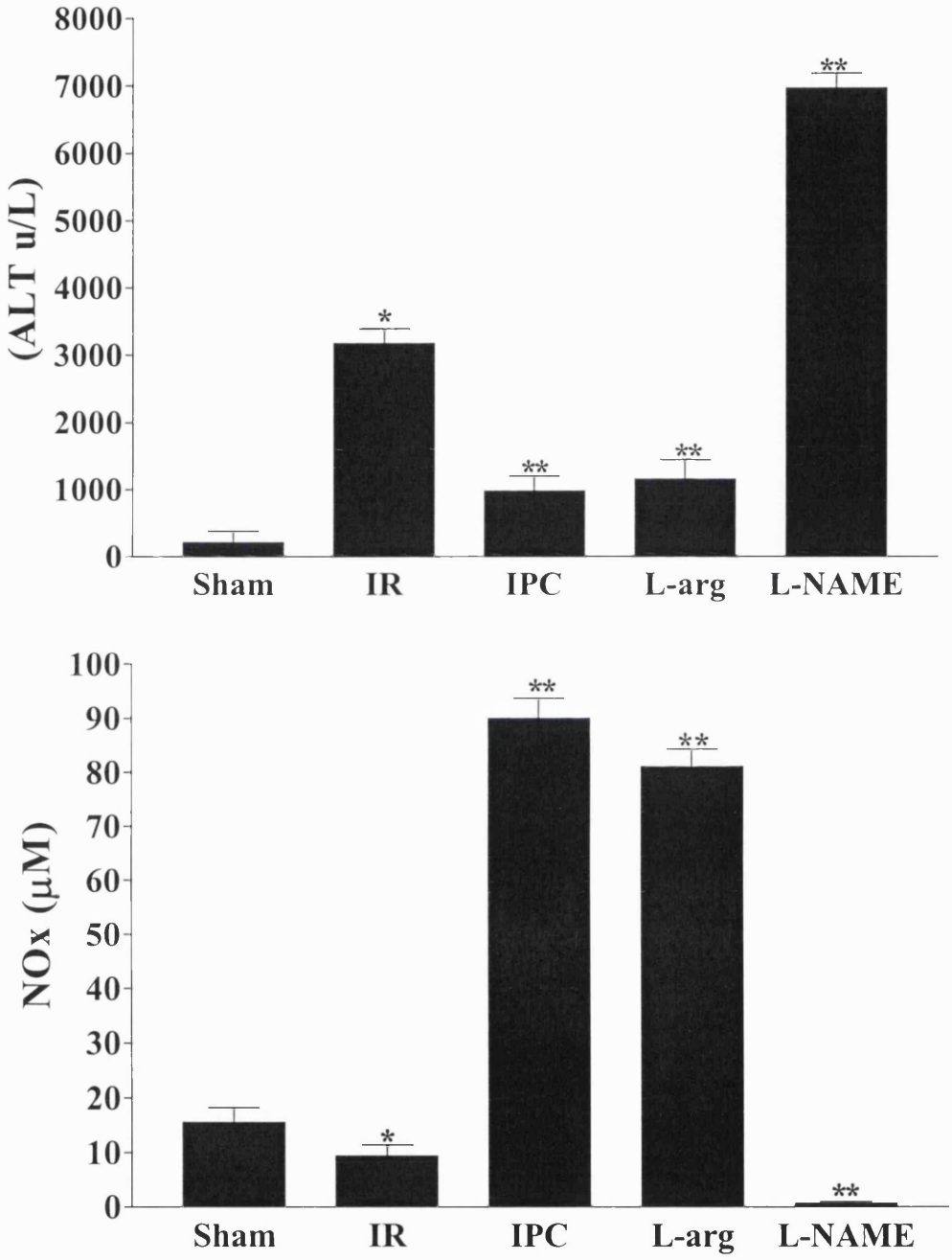

Fig. 6.1. Plasma Alanine aminotransferase (ALT) and nitrite + nitrate, NOx (μM). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR, Students t test.

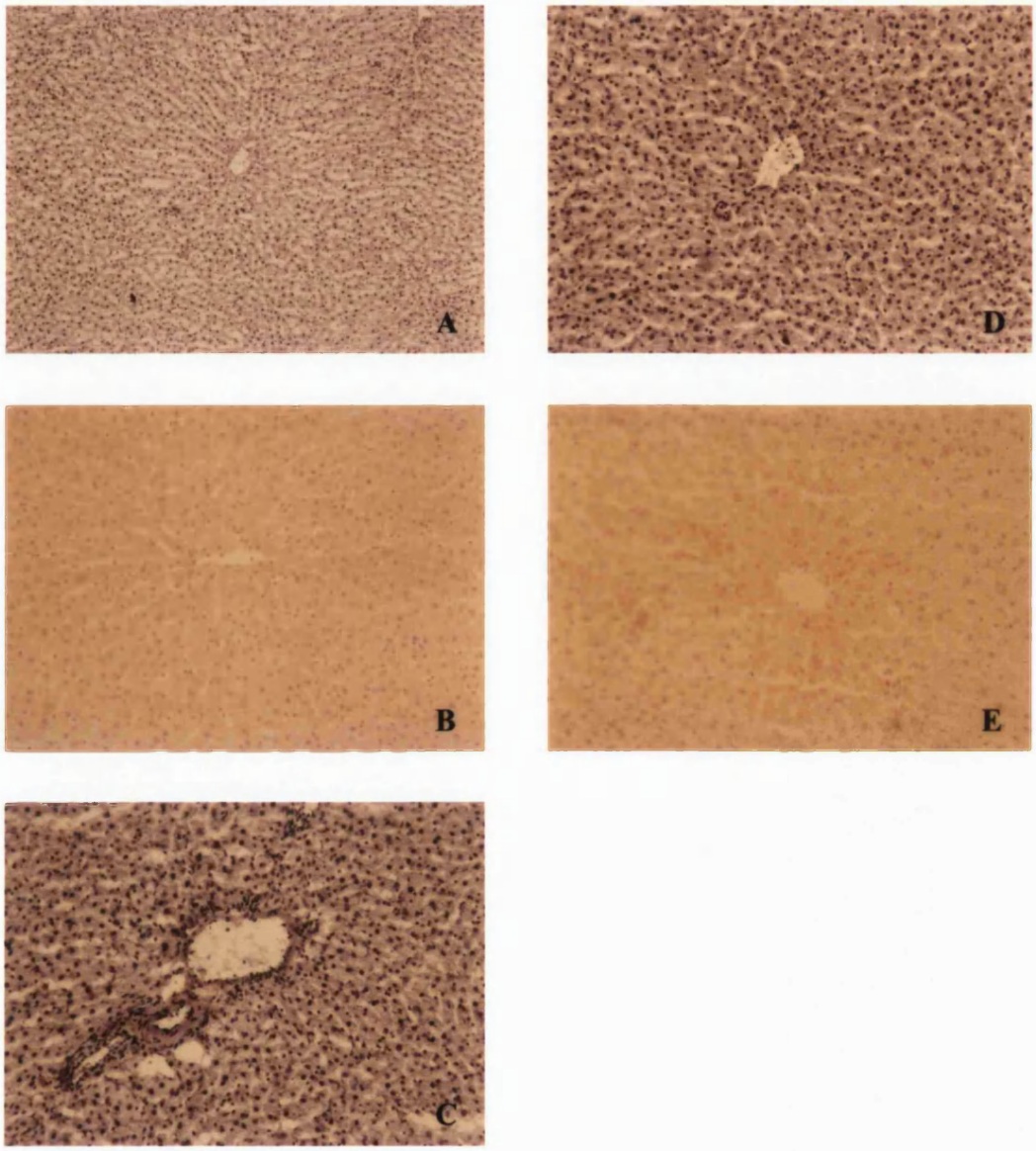

Figure 6.2. Representative photomicrographs of eNOS immunostaining. The brown stain in hepatocytes and vascular endothelium indicates eNOS expression. The groups were A) Sham, B) IR, C) IPC + IR, D) L-arginine + IR, and E) L-NAME + IPC + IR

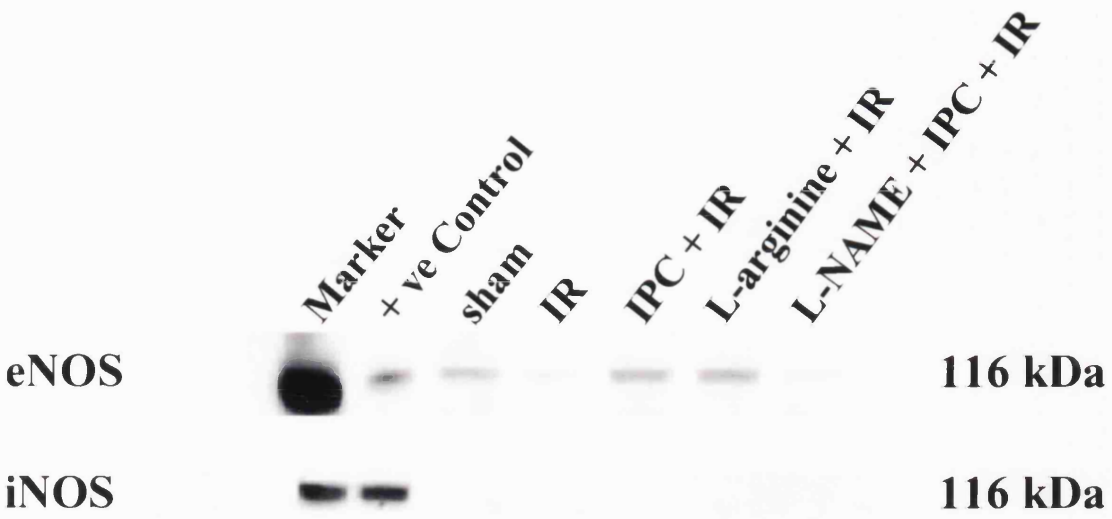

Figure 6.3. Western blotting for eNOS and iNOS

the cellular location of that production and also the rate of NO metabolism as it combines with other species. NO production in liver comes mainly from endothelial NOS (e-NOS) and inducible NOS (i-NOS). e-NOS derived NO is mainly produced in sinusoidal endothelial cells [218] where it may contribute to local perfusion distribution [219] and portal pressure [220]. NO from i-NOS can generally be produced from most liver cell types, including endothelial cells [213], hepatocytes [214], hepatic stellate cells [221] and macrophages [215].

This study has suggested that eNOS activity was upregulated and iNOS activity was absent in the context of IPC during early ischaemia reperfusion injury. This is not surprising since the potential levels of NO resulting from NOS isoforms can be very different in that e-NOS is constitutively expressed and directly regulated by calcium [222] and therefore NO production from e-NOS is quicker but the production is generally short-term. eNOS mRNA or western blot at variable time periods were not measured in this study, but these could determine the time points during ischaemia or the reperfusion phase at which eNOS is expressed. i-NOS activity is protein expression dependent and requires several hours for induction [223] but produces larger quantities of NO and for longer periods of time because NO production continues until the i-NOS protein is degraded or the substrate becomes limiting. The transient ischaemia of IPC induces protection for subsequent prolonged ischaemia; given this short time course for triggering IPC, it is therefore not surprising that transient ischaemia should result in up regulation of e-NOS activity. The short time course suggests that IPC events are

triggered at posttranslational level since transcriptional activity takes several hours. A precedent exists for this hypothesis; in the myocardium, transcription of new proteins and subsequent i-NOS generation has been linked to the late effect of preconditioning [224].

The ALT and NOx measurements indicate that the IPC induced hepatoprotection was effective for a time course of 2 hrs reperfusion following 45 mins ischaemia. Given the fact that e-NOS production is only short term, the sustained protection found in this study raises the possibility that molecules in addition to e-NOS contribute to the preconditioning signal. The possibility of compensatory up-regulation of i-NOS isoform is excluded by the results. The other possibility is enzymatic products of e-NOS activity may contribute towards the multi factorial trigger/ mediator of IPC. Exogenous nitric oxide (L-arginine treated group) produced similar rise in NOx and up-regulation of e-NOS as with IPC. NO is not the sole product of e-NOS; under conditions of L-arginine depletion free radicals may be generated [225]. Recent evidence suggests that free radicals are essential for preconditioning [75]. Whilst L-arginine depletion is unlikely to occur during the short duration of these experiments, free radicals were not measured in this study. The possibility of free radicals synthesized as a by product of e-NOS activity contributing to the preconditioning effect cannot be excluded in this study.

The NOS inhibition used in this study was not isoform specific. It was assumed that the role of iNOS would be negligible since the enzyme takes several hours to be induced

and the results indicate that NOS inhibition was complete and effective for eNOS as well as iNOS. The observation from this study that eNOS may contribute to early preconditioning may have potential importance in the clinical application of preconditioning in man. Patients who develop acute myocardial infarction have increased eNOS polymorphisms [226]. Such polymorphism may adversely affect the nitric oxide generation resulting from a transient ischaemic insult and may therefore attenuate protection potentially afforded by IPC. There is no evidence that such polymorphism exists in liver ischaemia reperfusion injury so far and this area warrants further investigation. Nonetheless, eNOS may prove to be a useful target in hepatoprotection from ischaemia reperfusion injury.

Chapter 7

The Effect of Ischaemic Preconditioning on Hepatic Oxygenation, Microcirculation, and Function in a Rat Model with Moderate Hepatic Steatosis

7.1 Introduction

The steatotic liver is particularly susceptible to ischaemia reperfusion injury (IRI) resulting in poor outcome following liver surgery [70] and transplantation [71, 72]. Impaired microcirculation [199], decreased mitochondrial ATP synthesis [227]and increased neutrophil adhesion [228] are some of the postulated mechanisms of injury in steatotic livers. Further, increased reperfusion injury in livers of fatty rats was associated with a change from apoptotic form of cell death to necrosis [229]. Excessive fat accumulation in the liver (steatosis) is a common metabolic disorder seen in humans with an incidence ranging from 6% to 24% in autopsy series [230, 231]. It is caused by a wide variety of conditions and diseases such as alcohol, obesity, malnutrition, hyperalimentation, diabetes, pregnancy and hepatitis but many are idiopathic [232, 233]. There is an increased risk of initial poor function and primary non-function of the graft after transplantation of a fatty liver and the risk increases with the severity of steatosis [234, 235]. With the increasing numbers of orthotopic liver transplantations and the concomitant lack of suitable donors, many liver transplant programs increasingly use donor livers of "marginal" quality such as fatty livers [236, 237]. The graft as well as patient survival is diminished after use of such organs [19, 20]. There is therefore, an urgent need for strategies against IRI to increase the number of organs available for liver transplantation and moreover, improve the outcome after transplantation of fatty livers.

Clavien and co-workers have demonstrated significantly decreased serum transaminase levels after IPC in a group of patients with steatotic livers (20 to 50% steatosis) undergoing hemihepatectomy under inflow occlusion [29]. In a recent report, Serafin et al [73] have shown decreased liver injury and increased animal survival in ischaemically preconditioned obese Zucker rats. Clearly, the effect of preconditioning response on fatty livers will have potential clinical implications for the future.

In previous chapters, relationship of IPC with improved hepatic oxygenation, microcirculation and function has been demonstrated. In this chapter, the aim of the study was to investigate whether IPC has similar effects in the steatotic liver. Since this was only a preliminary study designed to evaluate the effects of IPC on hepatic oxygenation and microcirculation in steatotic livers, the mechanism has not been addressed.

7.2 Material and Methods

7.2.1 Animal model, anaesthesia and surgical procedure

Male Sprague- Dawley rats, each weighing 250- 300g were used for the experiments. To induce fatty liver, all animals were fed with commercial high cholesterol (2%) diet, for 12 weeks. The changes in body weight of the animals were monitored weekly. The development of the steatosis was examined macroscopically and confirmed by histological examination.

Animals were anaesthetised by inhalation of 4-5% isoflurane in 50% oxygen and air in an induction chamber and then maintained by inhalation of 1-2% isoflurane in oxygen and nitrous oxide (1:2) via a face mask in a standard anaesthetic circuit, and prepared for aseptic surgery. The surgical procedure was carried out as described in section 3.3. Hepatic tissue oxygenation and hepatic microcirculation were continuously measured over the surface of median and left lateral lobes respectively, throughout the procedure as described in sections 3.1 and 3.2, respectively.

7.2.2 Measurement of hepatocellular injury

At the end of 2 hrs reperfusion period, 2 mls blood sample was taken from the inferior vena cava for measurement of plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT) (see section 3.3) as markers of hepatocellular injury.

7.2.3 Hepatic tissue ATP measurement and histology

At the end of 2 hrs reperfusion period biopsies of ischaemic lobes of liver were freeze clamped in liquid nitrogen for adenosine triphosphate (ATP) determination (See section 3.5). Separate liver biopsies of ischaemic lobes were fixed in 10% formalin for histological examination (see section 3.10).

7.2.4 Experimental groups and protocol

Three groups of animals (n= 6, each) were used

Group 1: Sham: The liver was exposed for 3 hours. There was no liver ischaemia.

Group 2: Ischaemia-reperfusion (IR): Ischaemia was induced in the median and left lateral hepatic lobes for 45 minutes, followed by a 2 hour period of reperfusion.

Group 3: Ischaemic preconditioning (IPC) + IR: The median and left lateral lobes were preconditioned with 5 minutes ischaemia followed by 10 minutes of reperfusion. This was followed by IR (group 2 procedure).

7.2.5 Data collection and Statistical analysis

As described in section 3.15 the data were collected continuously on a laptop computer and a NIRS algorithm specifically developed for liver NIRS (149) was used. One way analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated where unpaired Student's t test was used for statistical analysis between groups. The relationship of hepatocellular injury with Cyt ox Cu_a redox changes and HM was tested using Spearmans correlation coefficient.

7.3 Results

7.3.1 Induction of fatty liver

All animals tolerated the high cholesterol diet with no mortality. The animals maintained a normal body weight during the cholesterol feeding periods, with no significant difference between the groups. The liver weight and the liver weight/ body weight ratio were not significantly different between the groups.

At laparotomy the animals showed fat deposition in the skin, liver and spleen. The liver was enlarged, yellowish in colour, with rounded edge and firm consistency (Fig. 7.1). Some animals had mimimal ascites but abdominal varices were not seen.

7.3.2 Liver histological examination

Moderate grade, macrovesicular steatosis was seen under light microscopy examination of liver biopsies after 12 weeks of high cholesterol feeding (Figure 7.2).

7.3.3 Systemic hemodynamic parameters

In all animals in the three experimental groups the heart rate, mean arterial blood pressure, body temperature and oxygen saturation did not change significantly throughout the experiments.

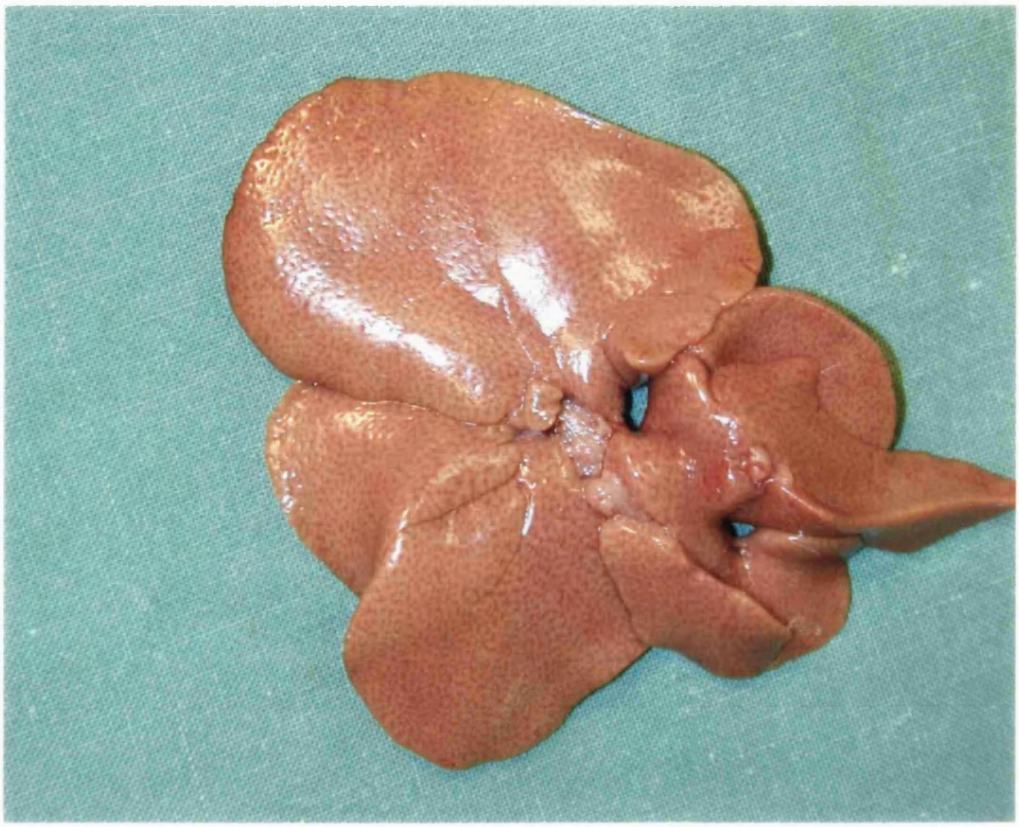

Figure 7.1 With steatosis the liver was enlarged, yellowish in colour with a rounded edge

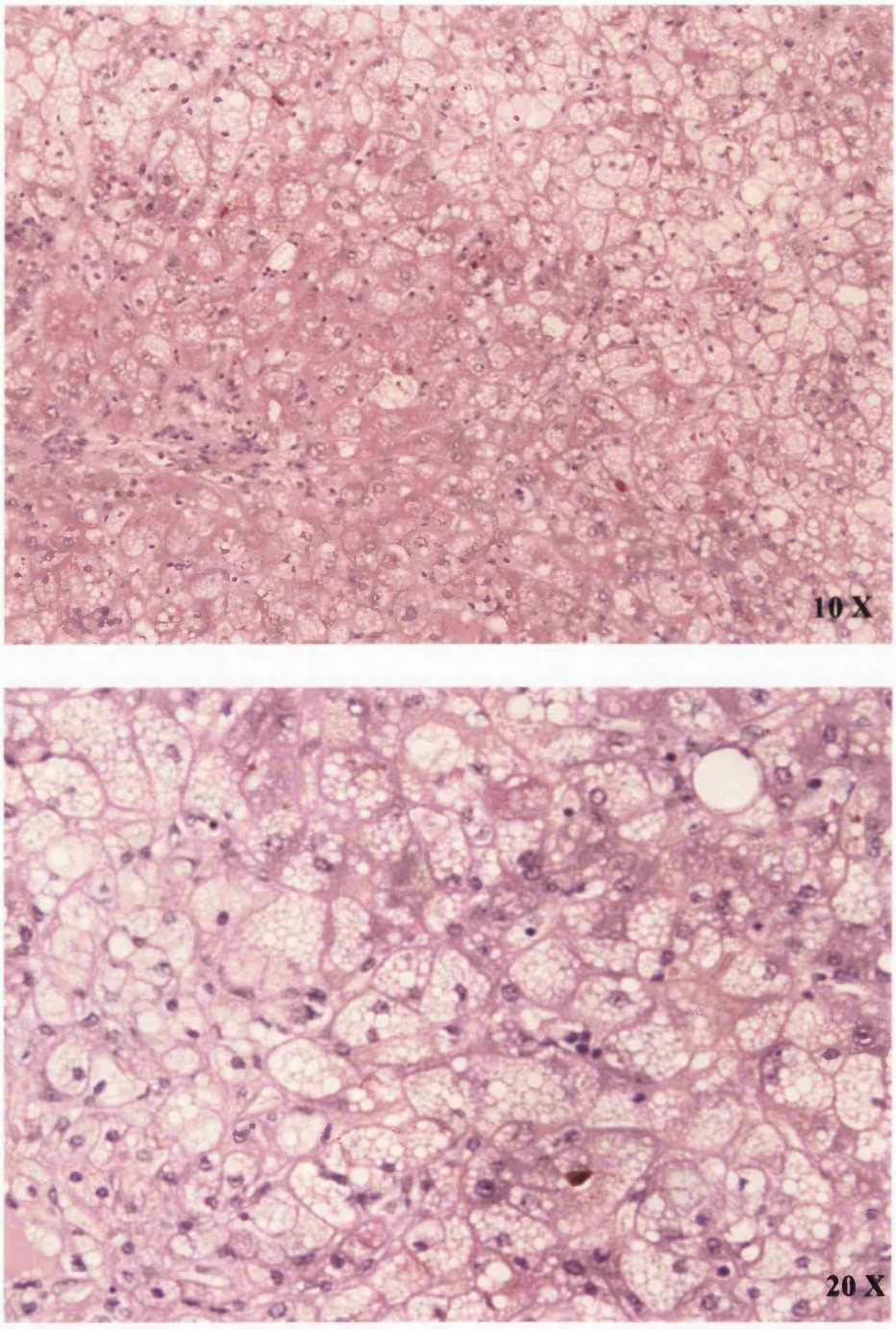

Figure 7.2. Photomicrographs of liver demonstrating macrovesicular fat accumulation in hepatocytes

7.3.4 Hepatic tissue oxygenation

Figure 7.3 illustrates the pattern of changes and Tables 7.1 and 7.2 list the changes in extracellular oxygenation indicated by hepatic oxyhaemoglobin (HbO2), deoxyhaemoglobin (Hb) and total haemoglobin (HbT, which indicates blood volume) and, mitochondrial oxygenation indicated by reduction oxidation (redox) changes of the copper centre (Cu_A) of cytochrome oxidase (Cyt Ox Cu_A redox state) in all experimental groups.

In the sham laparotomy (group 1) there were no significant differences in HbO2, Hb and Cyt Ox Cu_A redox state during the 3-hour period of recording (all $p > 0.05$ vs. baseline) (Fig.7.3. and Tables 7.1 and 7.2)

In the IR group (group 2), at the end of 45 mins of ischaemia (before unclamping) IR resulted in significant decreases in HbO2 and Cyt Ox Cu_A redox state and HbT (Fig.7.3. and Table 7.1) with no significant difference in Hb when compared with respective sham group values. On reperfusion (after unclamping) HbO2 further decreased and Hb increased and, there were significant differences in these values when compared with respective sham group values at the end of 2 hrs of reperfusion (Fig.7.3. and Table 7.2). HbT failed to improve and Cyt Ox Cu_A redox state remained significantly reduced following reperfusion when compared with sham group value at the end of 2 hrs of reperfusion.

In the IPC group (group 3), at the end of 45 mins of ischaemia (before unclamping) the HbO2 and HbT levels were significantly different from sham group but not significantly different from IR group. Hb was not significantly altered (Fig.7.3. and Table 7.1). Cyt Ox Cu_A redox state, was significantly decreased when compared with sham group but not significantly different from IR group at the end of 45 mins of ischaemia (before unclamping). On reperfusion (after unclamping), the HbO2, Hb and HbT levels improved significantly. HbO2 and HbT were significantly higher and Hb significantly lower when compared to IR group at the end of 2 hrs of reperfusion (Fig.7.3. and Table 7.2). Cyt Ox Cu_A redox state showed a downward trend in the initial 30 minutes of reperfusion, but later showed significant increase to above baseline values and at the end of 2 hrs of reperfusion was significantly higher then the sham and IR groups (Fig.7.3. and Table 7.2).

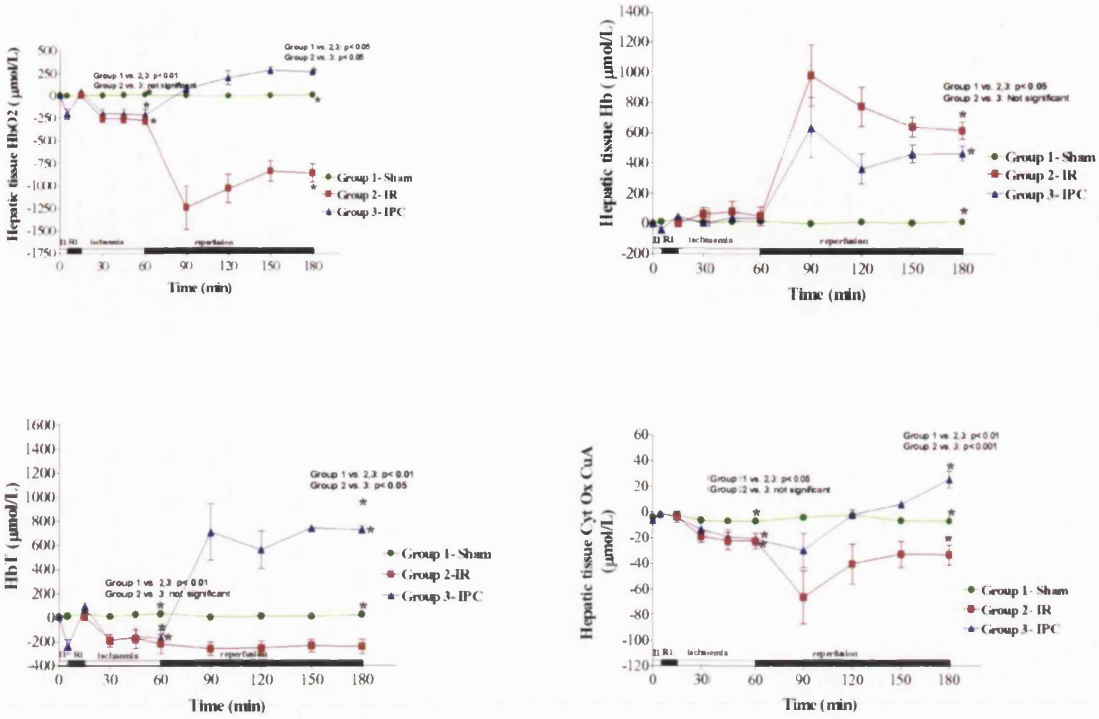

Figure 7.3. Hepatic tissue oxygenation during 45 min of ischaemia and 2 hrs of reperfusion, measured by NIRS. Values are mean ± SD of 6 animals in each group. I1= 5 min ischaemia; R1= 10 min reperfusion. Keys: HbO2, Oxyhaemoglobin; Hb, Deoxyhaemoglobin; HbT, Total haemoglobin; Cyt Ox CuA , Cytochrome oxidase CuA redox state.

Table 7.1. Hepatic tissue oxygenation (μmole/L) and Hepatic Microcirculation (Flux units, absolute values) end of 45 min of warm ischaemia (pre-reperfusion). Values are mean ± SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC + IR)
HbO_2	11.6 ± 11.5	-269.2 ± 46.4*	-209.9 ± 57.7 * ^{NS}
Hb	20.4 ± 7.6	51.7 ± 6.0 ^{NS}	41.9 ± 3.8 ^{NS}
HbT	32.0 ± 16.1	-217.4 ± 82.0*	-168.5 ± 63.5*‡
Cyt Ox Cu_A	-6.8 ± 1.7	-22.2 ± 6.2*	20.9 ± 4.5*‡
HM	84.9 ± 5.7	19.9 ± 4.5*	15.5 ± 2.8* ^{NS}

*$p<0.05$ vs. Group 1; $^{‡}p<0.05$ vs. Group 2; ^{NS}Not significant ($p>0.05$ vs. group 2) using unpaired t test.

Keys: HbO_2 : Oxyhaemoglobin; Hb: Deoxyhaemoglobin; HbT: Total haemoglobin, Cyt Ox Cu_A : Cytochrome oxidase Cu_A redox state; HM: Hepatic microcirculation

Table 7.2. Hepatic tissue oxygenation (μmole/L) and Hepatic Microcirculation (Flux units, absolute values) end of 2-hrs of reperfusion. Values are mean ± SD of 6 animals in each group.

	Group 1 (Sham)	Group 2 (IR)	Group 3 (IPC + IR)
HbO_2	10.3 ± 9.8	173.9 ± 85.3*	264.6 ± 29.7 *^{NS}
Hb	14.2 ± 5.7	615.0 ± 57.3*	465.0 ± 47.8 *^{NS}
HbT	24.4 ± 13.8	789.8 ± 127.1*	730.0 ± 30.5* ^{NS}
Cyt Ox Cu_A	-6.9 ± 1.8	-33.2 ± 8.0*	25.5 ± 6.7*‡
HM	76.0 ± 4.3	17.4 ± 4.0*	50.5 ± 2.2*‡

*$p<0.05$ vs. Group 1; $^{‡}p<0.05$ vs. Group 2; ^{NS}Not significant ($p>0.05$ vs. group 2) using unpaired t test.

Keys: HbO_2 : Oxyhaemoglobin; Hb: Deoxyhaemoglobin; HbT: Total haemoglobin, Cyt Ox Cu_A : Cytochrome oxidase Cu_A redox state; HM: Hepatic microcirculation

7.3.5 Hepatic microcirculation

Figure 7.4 illustrates the changes in mean percentage (standard deviation) of HM with respect to the preischaemic baseline level (100%). Tables 7.1 and 7.2 lists the changes in HM in absolute values.

In the Sham laparotomy group (group 1), mean HM did not change significantly with respect to baseline throughout the 3 hour period of recording.

In IR group (group 2), at the end of 45 mins of ischaemia (before unclamping) mean HM had decreased to 21.7 % (SD: 10.1). On reperfusion (after unclamping) mean HM failed to recover and at the end of 2 hrs of reperfusion the mean HM recorded was 21.1 % (SD: 14.4). There were significant differences between this group and group 1 ($p< 0.001$) (Fig.7.4.)

In IPC group (group 3), mean HM during the preconditioning period of 5 mins of ischaemia, decreased to 40.2 % (SD: 15.0) and after 10 mins reperfusion recovered to 134.4 % (SD: 30.9). At the end of subsequent 45 mins of ischaemia (before unclamping) mean HM decreased to 22.3 % (SD: 11.3). On reperfusion, mean HM progressively recovered to reach a final value of 70.9 % (SD: 17.1) at the end of 2 hrs of reperfusion. There were significant differences between this group and groups 1 and 2 at the end of 2 hrs of reperfusion ($p< 0.001$).

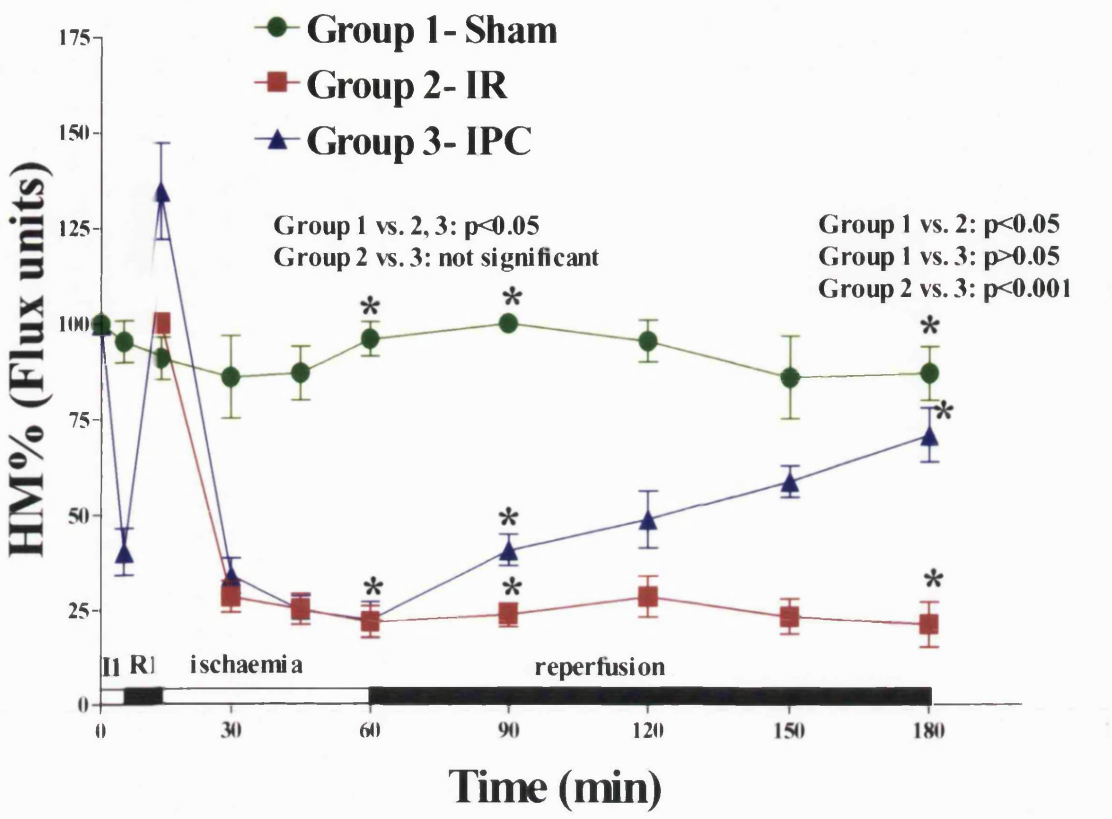

Fig. 7.4. Hepatic microcirculation in Flux units (%) during 45 min of ischaemia and 2 hrs of reperfusion, measured by laser Doppler flowmetery (LDF). Values are mean ± SD of 6 animals in each group. I1= 5 min ischaemia; R1= 10 min reperfusion.

7.3.6 Hepatocellular injury

IR resulted in significant increased ALT and AST levels measured at the end of 2 hrs of reperfusion (both $p < 0.05$ vs. sham). IPC resulted in decreased ALT and AST levels (both $p < 0.05$ vs. IR; $p > 0.05$ vs. sham). (Fig. 7.5)

7.3.7 Hepatic tissue ATP

IR resulted in significantly decreased ATP levels measured at the end of 2 hrs of reperfusion ($p < 0.05$ vs. sham). IPC resulted in increased ATP levels in comparison to IR ($p < 0.05$). (Fig. 7.6.)

7.3.8 Correlation of hepatocellular injury with Cyt ox Cu_a redox changes and HM

At the end of 2 hrs of reperfusion there was a significant negative correlation of plasma transaminases with cyt ox cu_A redox changes and HM (Table 7.3.).

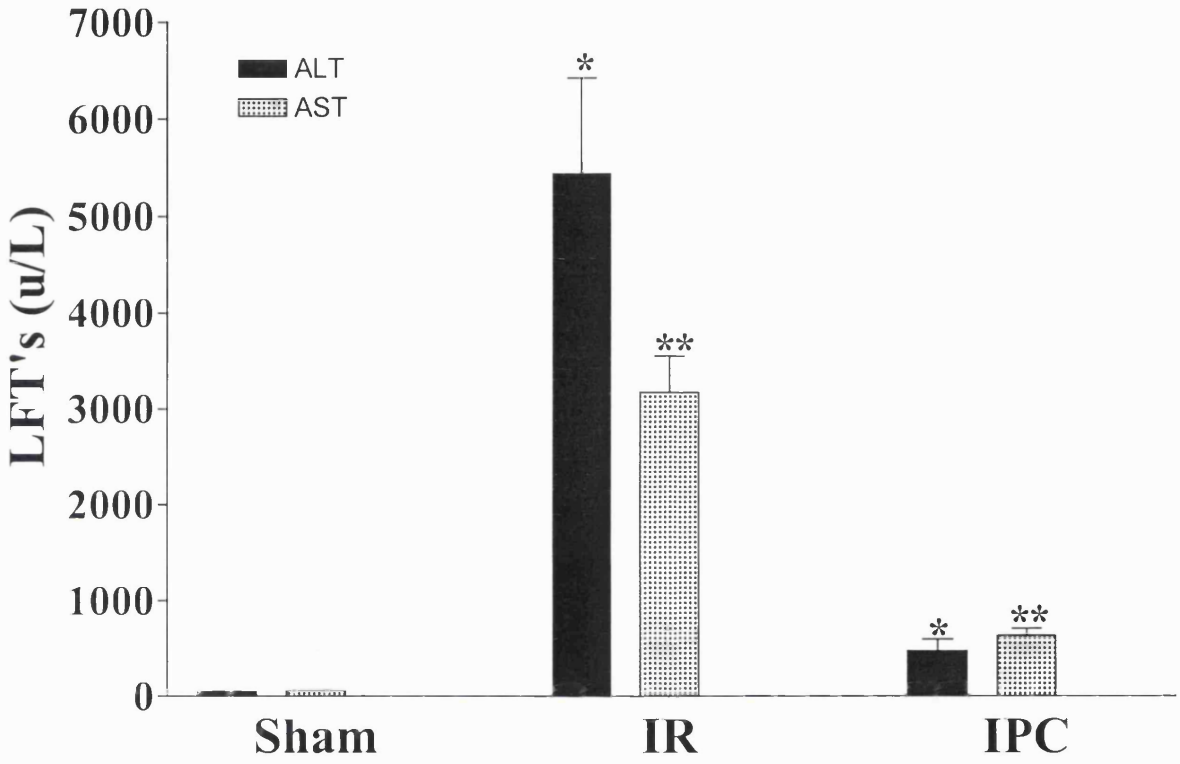

Fig. 7.5. Serum Alanine aminotransferase (ALT) and aspartate aminotransferase levels (U/L). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR, Students t test.

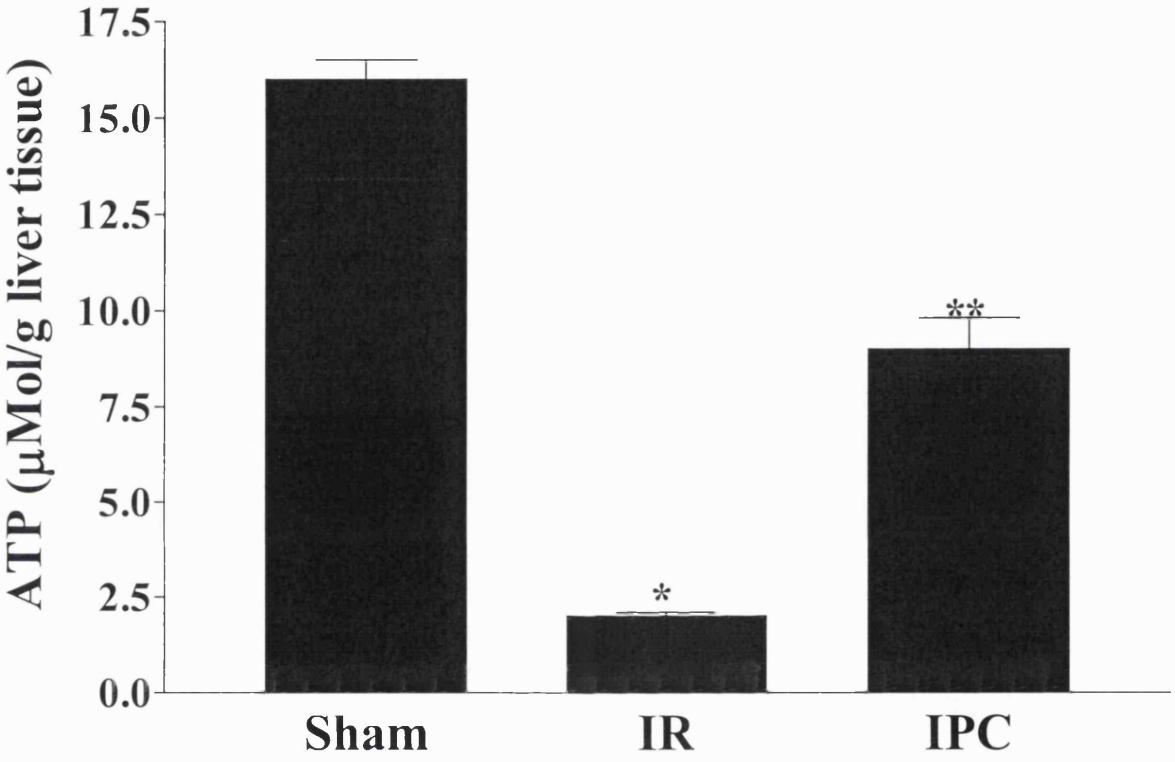

Fig. 7.6. Hepatic tissue ATP levels (μMol/g liver tissue). Values are mean ± SD of 6 animals in each group. *p<0.05 vs. sham, **p<0.05 vs. IR, Students t test.

Table 7.3. Correlation between hepatic tissue Cytochrome oxidase Cu_A redox state (Cyt Ox Cu_A) and hepatic microcirculation (HM) (x) and, liver enzymes (y)after 2 hrs of reperfusion following 45 mins of ischaemia.

	Regression analysis	Spearmans Correlation coefficient	P value
CytOx Cu_A vs. ALT	y = -67.094x + 1656.5	R = 0.9531	p< 0.01
CytOx Cu_A vs. AST	y = -34.697x + 1115.1	R = 0.9457	p< 0.01
HM vs. ALT	y = -65.762x + 5912.6	R = 0.9722	p< 0.001
HM vs. AST	y = 38.032x + 3556.4	R = 0.9038	p< 0.001

7.4 Discussion

This study has investigated the effects of ischaemic preconditioning in fatty livers subjected to ischaemia reperfusion injury. Rabbits given a high cholesterol diet together with diethylstilbestrol develop a rapid and extensive hepatic fibrosis with fatty infiltration, but this model is associated with 27% mortality after 8 weeks treatment [238, 239]. We simplified the method by feeding the rats with cholesterol rich diet alone for 12 weeks. All animals tolerated high cholesterol feeding with no mortality. At laparotomy the liver was enlarged, yellowish in colour with rounded edge and firm consistency suggestive of fatty change. High cholesterol feeding induces the formation of cholesterol fatty liver in which there is accumulation of triglyceride and cholesterol in the liver [238-240]. Morphological changes in cholesterol fatty livers have been investigated in rabbits [240] and in this species high cholesterol diet for 12 weeks led to macrovesicular fat accumulation with periportal inflammation and necrosis [241]. In the present study model, rats fed with high cholesterol diet developed moderate grade steatosis with macrovesicular fat accumulation. The patchy distribution of inflammation around centrilobular veins mainly in IR and IPC groups would be consistent with hepatocyte injury following ischaemia and reperfusion.

All animals had a normal body temperature throughout the experiments. The systemic hemodynamic parameters including mean arterial blood pressure, heart rate, body temperature and oxygen saturation were not significantly different in the experimental groups thus excluding any systemic contributions to IRI. The model employed was of

partial ischaemia and involved clamping the arterial and portal inflow to the median and left lateral lobes and maintaining the blood flow to the caudate and right lateral lobes. This prevents mesenteric ischaemia thus circumventing hemodynamic instability due to portal congestion and subsequent bacteremia [33].

Both tissue oxygenation and HM were measured in this study. Good tissue oxygenation is vital for liver graft function and survival [176]. Liver graft tissue oxygenation correlated with early graft function and survival in both experimental animals [177] and human liver transplantation [242]. Furthermore, tissue oxygenation has been shown to correlate significantly with the microcirculatory impairment and liver dysfunction induced by IRI [176]. Thus, direct measurement of hepatic tissue oxygenation and HM would be good indicators of hepatocyte viability. The use of NIRS to monitor liver oxygenation and LDF to monitor HM have been discussed previously in sections 3.1 and 3.2.

This study showed significant differences in extracellular and intracellular oxygenation with IPC. It is noteworthy that with IPC the intracellular oxygenation (Cyt Ox Cu_A redox state) showed a full recovery at the end of reperfusion phase. This result suggests an improvement in mitochondrial function or a decreased mitochondrial metabolism and ATP preservation in the preconditioned livers. Further insight may have been obtained by tissue ATP levels at earlier time points through the study. In studies on myocardial preconditioning, slowing of metabolism was a feature of the preconditioned

myocardium [23]. The results also show an increase in blood volume (HbT) with IPC, which was significantly different from IR group. The concomitant failure of improvement in Cyt Ox Cu_A redox state and HbT in the IR group suggests decreased or no flow in the hepatic parenchyma with decreased tissue oxygen availability. This could occur due to tissue oedema, decreased ATP and impaired mitochondrial function or hepatocyte death following IR. Whereas the increased Cyt Ox Cu_A redox state with IPC indicates viable hepatocytes suggesting a reduced hepatocellular injury in the preconditioned livers. Viable hepatocytes could be confirmed by methods such as TUNEL assay demonstrating decreased apoptosis.

All animals had a reduction in the baseline HM when compared with normal livers (data not shown). The reduction in HM associated with steatosis has been reported in other studies with fatty livers [243, 244]. The reduction in HM occurs due to sinusoidal compression and decrease in sinusoidal diameter by the enlarged fat laden hepatocytes [243]. This alteration in HM by the enlarged hepatocytes is augmented by an increase in fibroblast numbers with the formation of collagen bundles in the perisinusoidal space which causes further narrowing of the sinusoids [239, 240]. Vascular congestion in the narrow and irregular sinusoids, and leukocyte adhesion to the sinusoidal walls contributes significantly to the reduction of HM in fatty liver grafts [245]. In this study, HM changes were measured at different time points. LDF signal was recorded during ischaemia despite total lobar ischaemia. This has been reported in other studies and can be caused by random motion of residual blood cells, the influence of breathing

movements and back flow from hepatic veins. With 45 minutes ischaemia there were no differences between IR and IPC groups. In IR group, flow in the HM during the 2-hrs reperfusion phase was not significantly different from ischaemic levels, consistent with failure of the microcirculation. The pathophysiological mechanisms of primary microcirculation failure are no reflow and reflow paradox [203, 208]. No reflow is a result of postischaemic capillary perfusion failure due to sinusoidal endothelial cell swelling[208]. Recent evidence also supports the contribution of endothelin /nitric oxide balance in mediating sinusoidal perfusion failure [204]. Reflow paradox is a result of leukocyte adherence and increased macromolecular permeability in post capillary venules [203]. The fatty liver tolerates ischaemic insult poorly [246] and this evidence is supported by the results of this study. On the other hand with IPC, the increase in HM was evident within initial 30 mins of reperfusion following which there was linear increase in HM until the end of reperfusion phase. This suggests that IPC has an immediate effect on reperfusion.

This study has not investigated the mechanism for the preconditioning effect. Since the effect of IPC were evident immediately on reperfusion, the possible mechanism is likely to be triggered during the preconditioning phase. The preservation of intracellular oxygenation during the subsequent sustained ischaemic phase in this study would support this hypothesis [204]. Based on the findings of chapters 4 and 5 we tentatively support that preconditioning might be mediated through nitric oxide modulation of microcirculation during IRI.

In this study plasma liver enzymes measured at the end of reperfusion phase were markers of hepatocellular injury. The enzyme levels correlate with the severity of hepatocyte injury [182]. IR resulted in a severe increase in serum ALT and AST. Since the fatty liver is particularly susceptible to IRI severe hepatocyte injury is to be expected. In this study the failure of microcirculation and tissue oxygenation with IR is consistent with the severe hepatocyte injury or hepatocyte death indicated by the significant increases in liver enzyme levels. With IPC there was a significant decrease in liver enzyme levels. This suggests a reduced hepatocellular injury in the preconditioned livers. This result is consistent with the decreased cellular hypoxia secondary to increased flow in HM with IPC and viable hepatocytes indicated by increased intracellular oxygenation in the preconditioned livers.

In conclusion, this study has shown modulation of severe ischaemia reperfusion injury in moderately fatty livers with ischaemic preconditioning. This study provides evidence that ischaemic preconditioning has a hepatoprotective mechanism in the ischaemic fatty liver and this is associated with modulation of liver oxygenation and microcirculation akin to normal livers. These data may have important clinical implications in liver surgery and transplantation, as the technique is easily applicable in clinical situations.

Chapter 8

General Discussion of the Thesis and Future Plans

8.1 Methodological considerations

8.1.1 The Experimental model

The study of the cascade of phenomena related to ischaemia and reperfusion in the liver is of particular interest due to the importance of liver ischaemia to the results of liver surgery and transplantation. The analysis of liver ischaemia in the light of information obtained from other organs must be careful, due to the particular physiology of the blood circulation to the liver. The complexity of the hepatic circulation is also a major obstacle to reproduce and analyze-pathophysiological mechanisms under experimental conditions.

The experimental model employed in this study offered the opportunity of assessment of both haemodynamic and functional parameters in the rat liver, both under normal conditions and also when it is submitted to ischaemia and reperfusion.

The current model has several advantages over a liver transplant model for the study of warm IRI. In contrast with preservation of organs at low temperatures, normothermic ischaemia produces rapid injury to the liver, with the possibility of in-vivo study without the systemic effect of transplantation itself, which represents a major disturbance for physiological investigation. The technical difficulty of reestablishing portal and arterial blood flow, the denervation of the organ, artifacts due to organ

cooling, and the possibility of immunological involvement make experimental liver transplantation a complex experimental model. Normothermic ischaemia of the liver is, to some extent, a simulation of the main circulatory and metabolic changes during transplantation, whilst remaining a simple, reproducible and efficient experimental method, with low mortality and morbidity.

The use of the liver as a whole organ in an in vivo model is ideal to study the relationship between post ischaemic liver blood flow and post ischaemic liver function. The experimental reproduction of these phenomena at others levels is only possible with the application of sophisticated methods of isolated perfused organs or cell and tissue cultures.

The rat was selected as the experimental animal due to its resistance to surgical trauma and infection, the advantage of size and liver anatomy which makes the technical procedure possible, compared with other laboratory animals; the availability and the relative economy of maintenance. Sprague Dawley is a very docile outbred albino rat (Rattus norvergicus), originated in Madison, Wisconsin in 1925, by R. Dawley. The original colony was closed shortly after its development and no new stock has been introduced since then, producing stable colonies which are descended directly from the original stock.

The rat fatty liver model was developed in-house by feeding the rats with cholesterol

rich diet for 12 weeks. The fatty liver is more susceptible to warm IRI than normal livers. The exact reasons for the increased IRI seen with the fatty livers is poorly understood. Further progress in understanding has been hampered by the lack of suitable experimental models. Further, in humans with fatty liver, the spectrum of histological changes varies widely and animal models may not closely simulate these changes. The model used in this study was simple, reproducible and with no associated mortality. A shortcoming of this model as with other diet induced animal models of steatosis including ethanol ingestion and choline-deficient diet [247], is the development of inflammatory changes in addition to steatosis [248]. This would make the model unsuitable to study inflammatory changes secondary to IR. In the present study, inflammatory changes on histology as a marker of ischaemic injury was not used.

The rat model of segmental liver ischaemia and reperfusion is a useful procedure for the study of the local and systemic effects of IRI. This model offers a well defined volume of tissue that can be easilv rendered ischaemic with little or no alteration of systemic haemodynamics. The anatomy of the rat liver, with separate lobes makes easy the identification of vascular elements and selective interruption of the blood supply. The down side is that it may overlook changes found with global ischaemia as a healthy well perfused liver lobe remains.

In this study, blood flow to the medial and left lateral lobes was interrupted, leaving the right and caudate lobes with normal circulation. In this procedure, approximately 70%

of the liver becomes ischaemic [33]. During the occlusion period, the right lateral and caudate lobes, not subjected to ischaemia, accommodate the splanchnic blood flow without major changes in the portal pressure. After the clamp is removed, the ischaemic lobes are therefore exposed to a constant, normal portal blood pressure. This is a particular feature of liver lobar ischaemia, which allows the assessment of small changes in the liver vascular bed due to ischaemia and reperfusion. An important aspect of this model of lobar ischaemia is that the portal blood flow is not totally interrupted. Total crossclamping of the portal vein produces haemodynamic deterioration of the animal, which often dies upon reperfusion after 20 or 30 minutes of ischaemia [249]. The down side again is that the conclusions may not be relevant to global ischaemia which is of more clinical relevance.

The ischaemic times (preconditioning + subsequent ischaemia) utilized in this study were selected on the basis of published information. Repeated experiments using different ischaemic times showed consistent time-related changes in the post-reperfusion liver transaminases values and thus providing an indicator of any beneficial (or adverse) effects of ischaemia [30,40]. It must be emphasized that this is rodent data and may not be directly extrapolated to human IR. The relevance of this data to human IR has been shown in only one clinical study by Clavien et al [29] where the preconditioning times established in a mouse model were used for the human study with a beneficial effect. More clinical studies will be required to prove unequivocally the optimal preconditioning time. The same range of ischaemic times has also been reported

in the literature as useful in a model of normothermic rat liver ischaemia [30,40]. 45 minutes of partial hepatic ischaemia is nonlethal but induces substantial liver injury [181]. The periods of observation after ischaemia were defined originally from published information, and evolved thereafter in the course of the study. A period of 2 hrs of reperfusion following ischaemia was chosen to assess changes in the early phase of reperfusion injury. The changes during the early reperfusion period do not reflect late changes, but have been shown to modulate the development of late reperfusion injury. This technique of liver ischaemia and reperfusion in the rat is easily performed and can always be reproduced; the initial ischaemic changes in the liver are evident and leave no doubt of the proper positioning of the clamp. In addition, the procedure can be performed rapidly, and the mortality is low.

The pharmacological manipulations of nitric oxide utilized in this study were selected from published information. Nitric oxide is produced by Nitric oxide synthase, the mammalian enzyme catalyzing the oxidation of L-arginine to L-citrulline and nitric oxide. Therefore the use of L-arginine (amino acid substrate for NO biosynthesis) is ideal to produce effects of NO stimulation [250]. Non specific NOS inhibition was chosen to fit in with the design of the study since both constitutive and inducible isoforms were being evaluated in the study. L-NAME is effective against eNOS and has variable inhibition of iNOS [250], but considering the hypothesis presented, the NO was likely to be derived from the constitutive isoform of NOS since induction of inducible isoform NOS requires several hours and there was no evidence of upregulation of iNOS.

The next section addresses the application of various tests to assess the pathophysiology of liver ischaemia and reperfusion in this in vivo rat model.

8.1.2 Near infrared spectroscopy

NIRS is a non-invasive technique to assess hepatic tissue oxygenation, blood volume and function. A commercially available near infrared spectrophotometer that was developed for measurement of brain tissue oxygenation was used [151]. An algorithm designed specifically to measure hepatic tissue oxygenation was used.

During the application of NIRS a few problems were encountered. Variations in measurements were found with the reapplication of the probes even on the same site. This may be caused by variation of the optical properties of the area under investigation which are tested and accounted for by the spectroscopy with the initial setting. This could also be due to light scattering when probe is taken off tissues. This problem restricts the NIRS application as the probes must be applied and maintained in the same site without movement during the whole procedure. This may be tedious in clinical situation but with patience can be applied in the operating room. Absolute quantitation of the measurement will make use of NIRS easier.

The volume of blood passing through the liver tissue changes during ischemia and

reperfusion which may affect the amount of light scattering. In theory, this may influence both NIRS as well as LDF measurements. As far as we know there are no reports of studies investigating the relationship between changes in light scattering and changes in blood volume in the rat liver in the context of liver ischemia reperfusion. In the brain however, large changes in the cerebral energy state and cerebral perfusion do not cause large changes in light scattering in hypoxic-ischemic piglet brains. NIRS has been compared with other techniques for assessing tissue oxygenation including Magnetic resonance spectroscopy and partial oxygen pressure (chapter 4, discussion and references included therein) and reported significant correlation. We have assumed in these studies that the observed optical density changes are caused by altered chromophore absorbance and not light scattering. To determine absolute changes in chromophore concentration, the optical path length in the tissue must be known as a function of wavelength. The differential path length factor has been determined specifically for the liver by measuring the absorption coefficient as a function of wavelength. The differential path length factor of the liver is 2.7, and this value was used to adjust the NIRS algorithm for calculating the changes in the chromophore concentration [149].

8.1.3 Laser Doppler Flowmetry

The estimation of microcirculatory blood flow is of crucial interest for the analysis of the events that occur during organ ischaemia and reperfusion. Changes in the tissue

blood flow of different organs during reperfusion have been described, and in case of the liver, there is now increasing evidence that impairment of hepatic microcirculation is a major determinant of ischaemia reperfusion injury.

Laser Doppler Flowmetry is a reliable method for the continuous measurement of tissue blood flow and has been used in the study of the microcirculation in a number of different organs and under a variety of different conditions [170, 251]. In this study, LDF was utilized to measure blood flow in capillaries and sinusoids rather than in major vessels running deeply inside the liver substance, thus the described range of penetration was considered sufficient to study blood flow in a liver lobe with a maximum thickness of less than 10 mm. This method does not measure the microcirculatory blood flow of the liver as a whole, which would be ideal, however, the location of the probe in a fixed point of the liver surface throughout the experiment allows the continuous record of the flow signal to be considered representative of the microcirculatory changes on that point during the different stages of the procedure, using the same place as a control. We have previously looked at the intersite variability of LDF measurements and found a co-efficient of variation of $\approx 4\%$ [165,170]. This would suggest that one point is satisfactory to reflect what is happening over the affected lobe. Parenchymal perfusion measured by LDF correlates well with total liver blood flow and surface LDF measurements are representative of deep parenchymal perfusion [163, 252]. A baseline recording of flow as a standard (100%) was used in each individual

experiment; nevertheless, the absolute perfusion values in pre-ischaemic, anaesthetized rats are rather constant, being around 220 LDF Flux Units.

A few problems were encountered with use of LDF. Motion artifact remains a problem. The Laser Doppler Monitor can produce artifact signals due to vibration or other movements of the fiberoptic probe itself and due to relative movements between the probe and the explored tissue. In the case of the rat liver, there is a considerable amount of respiratory movement. In the experimental model described in this work, it was possible to obtain reasonably artifact-free readings with a good level of anaesthesia, good mobilization of the liver to avoid diaphragmatic movements and leaving the probe loosely in contact with the liver (instead of firmly attached to a manipulator), allowing it to move with the liver if there is any slight movement.

The low Backscattered Light Level due to excessive light absorption in dark coloured organs is a further characteristic feature in liver ischaemia. During the initial reperfusion period in this model of rat liver ischaemia, the liver can become dark to an extent were the level of backscattered light drops below the critical level of detection and the system stops reading the blood flow. This effect often occurs when the period of ischaemia is longer than 90 minutes.

The value of the blood flow measured with this instrument was expressed in Flux units. The backscattered light varies from one organ to another depending on factors including light absorption and red cell fraction of different tissues and it is not possible to translate

the values into a unit of flux that can be utilized for different organs; nevertheless, when applied to the same organ the signal is reproducible within a narrow range of variation. Laser Doppler flowmetry is now recognized as a well validated method for the estimation of liver blood flow.

8.1.4 Liver transaminases

The quantitative assessment of hepatic function remains a problem due to the complexity of the multiple metabolic and excretory mechanisms of the liver. The functional reserve of this organ allows it to sustain even severe damage without evidence of metabolic derangement. Quantitative measurement of liver function is not possible fundamentally as it entails measurement of all processes which take place in the liver.

True function tests quantitate specific hepatic functions, such as the synthesis or degradation rate of a product metabolized exclusively by the liver.

Serum levels of albumin and clotting factors are important indicators of liver function; clearance of indocyanine grren, aminoacids, antipyrine, galactose, lignocaine metabolites and rate of urea synthesis have been proposed as quantitative function tests. Serum levels of bilirubin, transaminases and alkaline phosphatases are of unquestioned

value in defining the severity of liver injury, but these are measures of liver dysfunction rather than function.

ALT (alanine aminotransferase, glutamate pyruvate transaminase, GPT) occurs virtually exclusively in the liver and AST (aspartate aminotransferase, glutamate oxalate transaminase, GOT) occurs partly in the liver. They are only present in the cytoplasm of the parenchymal cells, and provide valuable diagnostic information on the presence of severe liver parenchymal cell damage. The level of intracellular enzyme released into plasma is an index of integrity of the cell membrane, and hence, indirectly, of damage to hepatocytes. ALT and AST may be of value to estimate the extent of the liver cell destruction but give no information about the function of the living liver cells.

Despite their limitations, ALT and AST are an indicator of major alterations of liver integrity, and were utilized in this study as a marker of hepatocellular injury.

8.1.5 Hepatic adenosine triphosphate

Ischaemia is associated with progressive loss of ATP. This is accompanied by only a small rise in ADP as the adenine nucleotides are degraded to nucleosides and base [12, 253]. There is therefore a net loss of adenine nucleotides. It has been shown that conditions of low ATP, high Ca2+ and an oxidised redox state unfold during ischaemia and reperfusion and may have an important role in the pathogenesis of necrotic cell

death following ischaemia and reperfusion [254]. In this study ATP measurements were used as marker of hepatocellular integrity. The assay used in this study for measuring ATP is well established and of proven efficacy [254]. The limitation is its analyzing ATP alone and not evaluating precursors and breakdown products which would provide indication of mechanism e.g., is low ATP due to lack of precursors or increased ATP metabolism?

8.1.6 Nitric oxide studies

The transient and volatile nature of nitric oxide (NO) makes it difficult to measure directly. However, since most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions (NOx) are often used as a quantitative measure of NO production [171]. In this study NOx measurements were used as marker of NO production and to affirm the response to pharmacological manipulation with L-arginine and L-NAME. The assay described by Zeballos [171] is well established and of proven efficacy.

NADPH diaphorase histochemistry is a rapid technique that localizes NOS on tissue sections. Diaphorases are a group of redox enzymes, which are able to reduce various chromagen in the presence of a reduced co-factor. A diaphorase with absolute dependence on NADPH previously used to stain neurons in the brain was found to be related to NOS [174]. All isoforms of NOS have since been found to have NADPH diaphorase activity and no other enzyme unrelated to NOS have shown the same

activity. NADPH diaphorase is therefore recognised as a simple, convenient and reliable marker for detecting NOS.

NADPH diaphorase staining, however, does not differentiate between the 3 isoforms of NOS. Polyclonal antibodies against the specific NOS isoforms are commercially available (e.g. Santa Cruz Biotechnology) and can identify the isoforms on tissues sections and these were used for immunohistochemistry and western blotting in this study. Immunohistochemistry was used to study the distribution and localisation of NOS isoforms, western blotting to study protein expression. Both methods are qualitative analyses and hence no attempt was made to quantify these. Although the protein levels can be quantified by semiquantitative methods, these are not reliable for their absolute values given the qualitative nature of molecular biology techniques. A further limitation of molecular biology techniques is that they only indicate the presence of protein and as such may indicate synthesis of new protein. These techniques do not give an indication of protein phosphorylation and therefore enzymatic activity (173). The other option is to use enzyme phosphorylation assays, but these are indirect measurements and again do not help in establishing a direct link between protein and enzymatic activity [173]. In theory, a combination of specific NOS isoform genetic knockout model and molecular biology techniques would give a better understanding of NOS activity in a given setting.

8.1.7 Histology

In the fatty liver model, light microscopy examination was chosen because histopathological examination allows excellent appraisal of degree of steatosis. Many studies choose the end points of hepatocyte necrosis, endothelial injury and neutrophil infiltration as histologic markers, realizing that more detailed ultrastructural studies may be useful either to confirm or to explain histological findings in some cases. In this study histology was not used to appraise ischaemic tissue injury because the obvious limitation is that a lethally damaged cell may appear normal on gross histology at an early stage. Also, histology was performed at 2 hrs post reperfusion and would overlook the late phase changes of IR. In this study, clear differences were found between groups, and histology was of great help to understand the nature of steatosis.

8.2 Overall conclusions arising from the thesis and future plans

The results of the study have been discussed in detail in each chapter detailing the experiments (Chapters 4 to 7). This section addresses an appraisal of the overall conclusions and the neccessity and design of future studies are discussed.

The major findings of this study in the rat model of lober liver IRI were:

1. The hepatoprotective effect of ischaemic preconditioning was associated with an

increase in nitric oxide production.

2. The increase in nitric oxide was observed immediately after preconditioning period and continued through the subsequent ischaemia and reperfusion periods.
3. With ischaemic preconditioning, nitric oxide synthase was expressed by hepatocytes and vascular endothelium; eNOS was upregulated whereas iNOS expression remained absent suggesting that eNOS derived nitric oxide was associated with the ischaemic preconditioning effect.
4. The protective effect of ischaemic preconditioning was applicable to the fatty liver.

The above conclusions support the hypothesis presented in this study and strongly suggest a role for eNOS derived NO in hepatoprotection by IPC in early ischaemia reperfusion induced liver injury. The potential clinical application of the IPC effect is during liver surgery for tumors and during liver transplantation if the lobar rat data correlates with global human data. Although the technique of IPC is simple and easily applicable there would be concerns such as the increased operative time (due to period of time involving brief ischaemia and reperfusion) which may not be tolerated well in many operating theatres. Also other concerns such as, what is the ideal preconditioning time?, could IPC exacerbate damage in diseased liver (such as post chemotherapy, hepatic artery thrombosis, shock states), these questions largely remained answered since critical ischaemia times for the liver particularly with the trauma of surgery and disease are not known. Perhaps a better option is to use IPC to evaluate the mechanism

and allow the development of pharmacological manipulation for targeted drug intervention. Although it may be debatable whether preconditioning will ever fulfill its clinical expectations, the hope that a new therapeutic modality may emerge from this fascinating phenomenon has given great impetus to the search for its underlying mechanism. Clearly, identifying the mechanism of IPC will allow development of pharmaceutical agents that conduct the IPC reponse. Such agents will vastly assist resection of liver for tumors and preservation of livers for transplantation. The overall conclusions of this study suggest a central role for NO in the IPC cascade. Further mechanistically descriptive studies would allow the development of therapeutic regimens involving NO e.g., regime for NO donors administration prior to hepatectomy or liver preservation. The disadvantage of this strategy is clearly the diverse functions of NO and that NO donors may have many unwanted systemic side effects. There are no clinical studies on therapeutic evaluation using NO donors at present but it should be realized that it just might turn out to be the case where it may be better to stick with simple and reproducible technique of IPC.

This study has shown that liver eNOS is upregulated during the IPC effect. There are many questions which require further investigation. Firstly, this study has addressed effects in early IRI (2 hour reperfusion period). Since the effect of eNOS is short lasting, how will NO contribute if at all, to the protection in late IRI? It is therefore likely that if NO is responsible for late preconditioning in liver then the source of NO is likely to be iNOS derived. Since the induction of iNOS takes upto 4 to 6 hours [173], it

could be that iNOS is important in the late phase of IRI and this would fit in with the time course of late IRI. Further studies require to analyse iNOS and eNOS expression in late IRI. Survival models would further clarify this issue. In the heart there is increasing evidence for role of NO in the late phase of preconditioning.

The other question that will require further investigation is if there is any evidence that NOS induction with IPC is a liver alone effect. Could this be systemic NOS induction secondary to IPC? Biopsy from other organs at both early and late phase including contralateral liver biopsy would clarify whether the induction is a direct or indirect effect of IPC and whether there is systemic induction of NOS.

Another important avenue is the long term effect of IPC on microvascular perfusion. This study demonstrated that IPC caused only a limited or delayed microcirculatory damage in early IRI. Also, there was only a partial recovery of oxygenation parameters and microcirculation at the end of the 2 hrs reperfusion phase. Experimental studies by other researchers using intravital microscopy in rat liver IR have shown incomplete recovery of HM 24 hrs after prolonged ischaemia [208]. Therefore the question that remains unanswered is whether the protective effect of IPC is continued over several hours and days. IPC is associated with improved survival in animals. However the link between IPC and the improved survival remains unexplained. Although whether these explanations would ever be discovered remains debatable, but would be of enormous value in predicting patient outcome in the clinical situation. Recovery models after IR

looking at liver oxygenation and microcirculation and, NO activity would therefore be an interesting study for the future. The results of such a study would directly influence the potential clinical application of IPC. Another limitation of the present study was the direct measurement of HM. Although this study showed that IPC modulated HM, it would be of new interest to elucidate why the microcirculation is altered by IPC and which componenets of the HM are modulated by IPC. Experimental studies using intravital or confocal microscopy would allow vessel measurements and assessment of various perfusion parameters including sinusoidal perfusion, leukocyte adhesion, capillary permeability and shunting. This would clarify the effect of IPC on HM and help in development of drug targeting strategies. Recently developed physio-pharmacological models such as the dual-perfused rat liver [255] may allow assessment of the overall HM in finite detail to evaluate the role of NO [256] following IRI and IPC.

There is also evidence that human liver tissue can be preconditioned. To date, in the English literature, one study has demonstrated clinical benefits of hepatic IPC in humans [29]. Research in liver IPC is now 10 years old and although little is known about the mechanisms involved, it would be widely accepted that there is now enough animal data to justify conducting clinical research into this phenomenon.More human studies alone will prove unequivocally that IPC exists in human liver.

Three aspects can be distinguished in the process of preconditioning. The initial trigger (1), that is included in the short periods of ischemia and reperfusion, activates signalling

pathways (2), which in turn act upon an end-effector inducing the delay of lethal ischemic damage during sustained ischemia (3). To mechanistically define the causal relationship between these processes and NO, studies could be done using genetic knockout models and, eNOS and iNOS deficient models are now readily available[257].

Finally, although current research on the mechanisms of preconditioning seems to diverge more and more, it is possible that all these mechanisms converge into an as yet unidentified final common pathway. This conclusion is based not so much on the negative studies and observations discussed above but on the belief that a powerful adaptive phenomenon that is induced so easily and reproducibly in so many models, laboratories, and species is almost certain to be mediated by a universal mechanism. The data from this study suggests that nitric oxide is the key factor in hepatic ischaemic preconditioning.

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Appendix

Publications arising out of work described in this thesis

Papers

1. Ischaemic preconditioning of the liver: a review of mechanisms and clinical applications.

   Rahul S. Koti, Alexander M. Seifalian, Brian R. Davidson

   Digestive Surgery (in press)

2. The relationship of hepatic tissue oxygenation with nitric oxide metabolism in ischaemic preconditioning of the liver

   R. S. Koti, A. M. Seifalian, A. McBride, W. Yang, B. R. Davidson.

   FASEB Journal. 2002 Oct; 16(12): 1654-6

3. The effect of ischaemic preconditioning on hepatic microcirculation and function in a rat model of ischaemia reperfusion injury

   Rahul S. Koti, Wenxuan Yang, Michael R. Dashwood, Brian R. Davidson, Alexander M. Seifalian.

   Liver Transplantation 2002 Dec;8(12):1182-91

4. Nitric Oxide Synthase expression and distribution in Ischaemic preconditioning of the liver

Rahul S. Koti, Wenxuan Yang, Janice C. Tsui, Alexander M. Seifalian, Brian R. Davidson.

Hepatology (submitted)

5. The effect of ischaemic preconditioning on hepatic oxygenation, microcirculation, and Function in a Rat model of Moderate hepatic Steatosis

Rahul S. Koti, Wenxuan Yang, Alexander M. Seifalian, Brian R. Davidson.

Gastroenterology (submitted)

Abstracts

1. The effect of preconditioning on liver ischaemia reperfusion injury.

Rahul Koti, Alan McBride, Alexander Seifalian, Brian Davidson.

8th United European Gastroenterology Week, Brussels 2000.

Gut 2000; 47 (Suppl III); A158.

2. Hemodynamic Consequences of Preconditioning in Liver Ischaemia-Reperfusion injury.

R. Koti, W. Yang, A.M. Seifalian, B.R. Davidson.

British Society of Gastroenterology, Annual Meeting, Glasgow 2001

Gut 2001;48 (Suppl I):A23.

3. Role of Nitric Oxide in Ischaemic Preconditioning of the Liver.

R.S. Koti, W. Yang, J. Tsui, A.M. Seifalian, B.R. Davidson.

British Transplantation Society, 4th Annual Congress, Oxford, March 2001.

Proceedings.

4. Nitric Oxide in Ischaemic Preconditioning of the Liver. (Patey prize paper)

R.S. Koti, M.R. Dashwood, A.M. Seifalian, B.R. Davidson.

Surgical Research Society, Annual Meeting, Birmingham, May 2001.

British Journal of Surgery May 2001;88 (Suppl 1):3.

5. Nitric oxide synthase distribution and expression in ischaemic preconditioning of the liver

Rahul S. Koti, Wenxuan Yang, Janice Tsui, Alexander M. Seifalian, Brian R. Davidson.

The American Association for the Study of Liver Diseases, 52nd Annual Meeting, Dallas, Texas, November 2001.

Hepatology 2001; 34(4): 286A

6. The effect of ischaemic preconditioning on hepatic oxygenation, microcirculation and function in a rat model of moderate of hepatic steatosis

Rahul S. Koti, Wenxuan Yang, Alexander M. Seifalian, Brian R. Davidson

Digestive Diseases Week, May 19-22, 2002, San Francisco, California.

Gastroenterology 2002;7(suppl 1):34

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