THE USE OF HYPERTONIC PRECONDITIONING IN HEPATIC ISCHEMIA-REPERFUSION INJURY

By

George D. Oreopoulos MD

A thesis submitted in conformity with the requirements
For the degree of Master of Science,
Institute of Medical Science,
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ABSTRACT

BACKGROUND: Hepatic ischemia-reperfusion (I/R) injury contributes to organ injury and dysfunction after hepatic surgery and transplantation. I/R induces Kupffer cell activation, leading to the release of pro-inflammatory cytokines that promote injury, increase adhesion molecule expression, and facilitate PMN-mediated injury. Studies suggest hypertonic saline (HTS) might exert beneficial effects on development of organ injury following shock on the basis of reduced PMN-endothelial cell (EC) interactions. **HYPOTHESIS:** We hypothesized that HTS might alter expression of cytokines responsible for adhesion molecule expression and thus minimize PMN-mediated injury. **METHODS:** An *in-vivo* model of rodent warm partial hepatic I/R in which rats were pretreated with HTS or normal saline was used to test our hypothesis. Liver injury was assessed by serum AST. DNA fragmentation and TUNEL staining were used to assess hepatic apoptosis. Hepatic PMN sequestration was measured by myeloperoxidase assay. Hepatic ICAM-1, VCAM-1, TNF-α, and IL-10 mRNA were assessed by northern blot and hepatic TNF-α protein was measured by ELISA. NF-κB activation was measured by EMSA. IL-10, HO-1, HSP-70, p38, JNK, and STAT3 proteins were assessed by western blot. Activated EC (HUVECs) were used to determine the effect of hypertonicity (HT) on EC ICAM-1 *in-vitro*. Peritoneal macrophages (PEMs) were used to examine the ability of HT to modulate macrophage cytokine production. **RESULTS:** HTS prevented liver I/R injury and reduced hepatic PMN sequestration. Expression of I/R-induced hepatic and PMN adhesion molecules was prevented in HTS animals. HT reduced HUVEC ICAM-1 without associated cytotoxicity. HTS pretreatment prevented I/R induced activation of NF-κB and reduced TNF-α expression. These changes correlated with increased IL-10 following HTS I/R. HTS prevented I/R-induced hepatic apoptosis. HT prevented LPS-induced PEM TNF-α and augmented IL-10. These results suggest that HTS protects against hepatic I/R by reducing PMN-EC interactions through the inhibition of both PMN and EC adhesion molecules. Reduced adhesion molecule expression correlates with reduced expression of TNF-α and augmented IL-10. Additional mechanisms of HTS protection include reduced apoptosis and augmented levels of anti-inflammatory molecules such as HO-1. **Thus:** Preconditioning with HTS may represent a new strategy in the prevention of regional I/R-induced organ dysfunction.

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LIST OF ABBREVIATIONS

AP-1	activating protein-1 transcription factor
ARDS	acute respiratory distress syndrome
CIT	cold ischemic time
CNS	central nervous system
CO	carbon monoxide
COX-2	cyclooxygenase-2
EC	endothelial cell
ET	endothelin
ERK	extracellular signal regulated kinase
HCC	hepatocellular carcinoma
HES	hydroxyethyl starch
HIF-1	hypoxia inducing factor-1
HO-1	heme oxygenase-1
HS	hemorrhagic shock
HSD	hypertonic saline dextran
HSF-1	heat shock factor-1
HSP	heat shock protein
HT	hypertonicity/hypertonic
HTS	hypertonic saline
HVE	hepatic vascular exclusion
ICAM-1	intercellular adhesion molecule-1
ICP	intracranial pressure
ICU	intensive care unit
IκB	inhibitory kappa b

IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IPF	initial poor function
I/R	ischemia-reperfusion
JAK	janus kinase
JNK	c-Jun N-terminal kinase
KC	Kupffer cells
LPS	lipopolysaccharide
LR	lactated ringer's solution
MAPK	mitogen activated protein kinase
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
NF-κB	nuclear factor kappa B
NLS	nuclear localization sequence
NO	nitric oxide
PAF	platelet activating factor
PEM	peritoneal exudative macrophage
PMN	neutrophil
PNF	primary non-function
PRBCs	packed red blood cells
PS	phosphatidyl serine
PTC	portal triad clamping/Pringle maneuver
ROS	reactive oxygen species
SAPK	stress activated protein kinase (= JNK)

SEC	sinusoidal endothelial cells
SOCS-3	suppressor of cytokine signaling-3
STAT	signal transducer and activator of transcription
TF	tissue factor
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1
WIT	warm ischemic time

CHAPTER 1: HEPATIC ISCHEMIA-REPERFUSION- CLINICAL ASPECTS

OBJECTIVES

The aim of this chapter is to describe the importance of hepatic ischemia-reperfusion injury (I/R) in a number of different clinical scenarios including: hepatic resection, hepatic operation for trauma, hemorrhagic shock resuscitation and liver transplantation.

HEPATIC RESECTION

The frequent use of radiologic imaging studies has been associated with an increased detection of mass lesions in the liver (1). A wide spectrum of conditions must be considered when a hepatic mass is noted, a number of which require resection for management and occasionally diagnosis. The most common indications for liver resection include: colorectal metastases, other metastases, hepatocellular carcinoma, Hydatid disease, focal nodular hyperplasia, and hemangiomas.

The safety and success of hepatic resection depends on effective control of bleeding during surgery and the ability of the non-resected lobe to sustain life in the immediate post-operative period. In addition to the general risks of disease transmission and reduced immune function the hepatobiliary surgeon must be concerned with the impact of intra-operative blood loss on the incidence of post-operative hepatic failure and tumor recurrence in patients operated on for hepatobiliary malignancy (2-4).

Perioperative blood loss and hepatic failure

Despite refined operative techniques, between 40% and 100% of patients undergoing hepatectomy require blood transfusions (5). Operative mortality is less than 10%, even in patients with cirrhosis. Hepatic failure after resection is a lethal complication in patients with cirrhosis (6). Post-operative hepatic failure is reported in 7-20% of patients (7-9). Nonami and others have reported that post-operative hepatic failure and mortality was closely related to blood loss during the operative procedure (8;10-14). The usual chain of events leading to in-hospital death in patients undergoing hepatectomy for HCC in-

tomy for HCC includes: suboptimal pre-operative liver function, massive intra-operative bleeding, post-operative septic complications and eventual hepatic failure (15).

Intra-operative blood loss and tumor recurrence

There is also evidence that intra-operative blood loss may be associated with an increased risk of recurrence and decreased survival in patients operated on for a hepatobiliary malignancy. Recurrence of hepatocellular carcinoma (HCC) after liver resection is common and most patients who die with HCC, die with recurrence(16). Thus, the prevention of recurrent HCC is important in order to improve the survival of patients with HCC. Several groups have noted an direct relation between perioperative blood transfusion and HCC recurrence after hepatic resection (17){Asahara et al 1999}. This adverse effect of perioperative blood transfusion on disease recurrence and survival does not seem to be limited to patients with HCC. Data from the National Cancer Institute indicate that patients undergoing hepatic resection for metastatic colorectal cancer and receive ≥ 11 units of PRBC's intraoperatively had a significantly decreased disease-free and overall survival compared with patients who received 3-10 units of blood (18). The mechanism of the effect of blood transfusion on tumor recurrence remains unclear. This effect may be secondary to a non-specific immunosuppressive effect of transfused blood. Thus, an operative approach that will minimize or avoid the need for blood transfusion should therefore be adopted.

Vascular Occlusion Techniques

The use of vascular occlusion techniques has allowed a definite margin of safety in hepatic resection. Vascular clamping techniques allow for a relatively bloodless operative field, contributing to an unhurried, meticulous, and accurate intrahepatic dissection. Surgeons must balance the ability to control blood loss on the one hand against I/R injury to the liver on the other. I/R injury is accentuated with prolonged periods of vascular occlusion and in patients with limited hepatic reserve secondary to chronic liver disease (19). The most widely applied methods of vascular control in hepatic resection are clamping of the hepatic pedicle (PTC) and hepatic vascular exclusion (HVE). PTC was first described by Pringle in

1908 (20). This technique reduces bleeding during parenchymal transection, but fails to prevent back bleeding via the hepatic veins. HVE however, combines hepatic pedicle clamping with occlusion of the inferior vena cava above and below the liver. Both methods are effective in limiting hepatic blood loss but controversy exists as to the relative benefits and drawbacks of each technique (21;22).

HEPATIC I/R IN TRAUMA

I/R injury to the liver occurs in the setting of trauma when vascular clamps are applied to arrest severe hepatic bleeding (regional I/R) and with resuscitation from hemorrhagic shock (global I/R). In patients who survive an initial severe injury, multiple organ failure (MOF) is the leading cause of death, occurring in 11.4% of trauma patients with an associated mortality rate of 61.5% (23). Sufficient blood loss to cause hypotension leads to systemic hypoperfusion, the clinical correlate for global I/R injury. This process activates multiple humoral and cellular cascades, leading to the release of proinflammatory cytokines. Thus, after major trauma, patients are resuscitated into a hyperinflammatory state, putting patients at risk for death and the complications of organ-injury and dysfunction. Champion et al had initially described the frequent occurrence of hepatic dysfunction within hours of resuscitation from hemorrhagic shock, characterized by an early elevation of serum transaminases, a later peak in serum bilirubin and evidence of ischemic liver injury on electron microscopy (24). An increased risk of organ failure is associated with thoracic, abdominal, and pelvic injuries and is likely secondary to a high incidence of massive bleeding in these compartments.

The liver is the intra-abdominal organ most commonly injured in trauma. Overall mortality rate from liver injuries is 10%. Most liver injuries are minor and can be managed non-operatively (50-80%) with close observation, serial hematocrit determination and bed rest. In contrast, major trauma to the liver, whether isolated or accompanied by additional injuries, is difficult to manage, and is associated with considerable morbidity and mortality. Hemodynamic instability, continual blood product requirement, or enlarging lesions on CT scan indicate the failure of non-operative management and the need for laparo-

tomy. Thus, the major indication for operative hepatic procedures in trauma is hemorrhage control. Total operative blood loss has a significant impact on the mortality of blunt hepatic injuries (25). The failure of simple techniques such as laparotomy pad compression, application of topical hemostatic agents, and suture hepatorrhaphy to control hepatic bleeding necessitates the application of PTC to allow time for advanced hemostatic techniques to be applied (26-30).

LIVER TRANSPLANTATION

Liver transplantation is a widely accepted treatment for end-stage liver disease (31-33). I/R injury to the liver remains as a significant obstacle to successful liver transplantation (34). Graft I/R injury is associated with abnormal liver function tests, prolonged hospital stay, an increased rate of biliary strictures, and impaired graft survival (35;36). Graft injury is a result of the fact that livers are often harvested from brain-dead, injured donors maintained in an intensive care setting as well as the exposure of liver grafts to both cold and warm I/R (37).

Brain-dead patients are often hemodynamically unstable and may experience multiple episodes of systemic hypotension that adversely affect the liver (35;38). The use of pressors is associated with reduced liver perfusion. Early graft function was shown to be impaired when > 15 μg/kg/min of dopamine had been used to support the donor in association with a period of hypotension (39).

Initial poor function (IPF) describes grafts that function poorly in the postoperative period, but have enough function to support life. IPF is a problem in 2-23% of patients (35). Primary nonfunction (PNF) refers to grafts that fail to support life in the early postoperative period, occuring in 6.9-8.5% of cases, resulting in the need for urgent retransplantation or death of the patient (36;40). Perioperative variables that affect the incidence of IPF and PNF include cold and warm ischemic times as well as the intraoperative use of blood products (34;36;41).

Cold preservation of the liver has been shown to result in a microvascular injury (37). Cold ischemia time (CIT) has been defined as the interval between the time from flushing the portal vein in the donor during harvest until the removal of the graft from cold UW solution just before implantation in the recipient (34). The absolute limit of cold ischemia time in humans is 30 hours after which PNF develops in all cases. However, shorter CIT's have also been identified as contributing to the incidence of IPF(35).

Warm ischemia time (WIT) has been defined by Bilbao et al as the time from putting the liver graft into the recipient until hepatic artery reperfusion(34). Warm ischemia can be well-tolerated by the normal liver for periods exceeding 1 hour (42;43). Strasberg has found an exponential relationship between WIT and postoperative transaminase levels with a steep increase at > 90 minutes (36). Piratvisuth et al demonstrated that graft survival was reduced when WIT exceeded 180 minutes compared to grafts with a WIT < 180 minutes (58% versus 80%). In the same series, there was a trend towards a 3.6-fold higher incidence of IPF when WIT exceeded 180 minutes (34).

To minimize the effects of ischemia-reperfusion injury on the transplanted liver with its resultant higher incidence of PNF and IPF, a strategy to decrease cold and warm ischemic times, intra-operative blood loss and blood transfusion, as well as donor ICU length of stay should be adopted.

CHAPTER 2: THE USE OF HYPERTONIC SALINE IN TRAUMA AND CRITICAL CARE

Introduction

The past decade has observed a renewed interest in the systemic administration of hypertonic salt solutions in clinical medicine. Experimental and clinical evidence has suggested a role for hypertonic saline (HTS) in resuscitation for traumatic hypotension, burn shock, declamping shock in aortic surgery, and hemodynamic instability following cardiopulmonary bypass. Transient hypertonicity (HT) may reduce the incidence of systemic complications following severe trauma or surgery. The objective of this review is to discuss the use of hypertonic salt solutions in trauma and critical care and to review possible mechanisms through which HTS may exert beneficial effects.

Trauma Resuscitation

Trauma is the leading cause of death in North-Americans under 45 years of age, and the fourth leading cause of death overall (44). Hemorrhage is the leading cause of shock in injured patients. Therefore, the rapid control of bleeding and restoration of intravascular volume are primary goals of trauma resuscitation. Conventional resuscitation from hemorrhagic shock involves aggressive administration of large volumes of isotonic crystalloid, and prompt surgery for the control of internal bleeding (45).

The prehospital care setting presents logistical difficulties to the provision of adequate, early volume resuscitation (46). Short intra-city transport times and the small intravenous cannulae used by medics in hypotensive patients with collapsed peripheral veins preclude administration of enough isotonic crystalloid to normalize systemic blood pressure in the time it takes to reach hospital. In the military context, the amount of resuscitation fluids that can be delivered to a forward combat area is limited (47). These considerations emphasize the need for a 'small-volume' resuscitation fluid of equal or superior performance to conventional isotonic fluids.

Experimental studies of hypertonic saline in hemorrhagic shock

HTS solutions restore normal hemodynamics more rapidly than isotonic crystalloids with as little as a tenth of the volume (48-50). Large volumes of isotonic crystalloid are required to restore hemodynamic stability owing to its rapid redistribution to the entire interstitial fluid volume allowing only 30-40% of the infused volume to remain in the intravascular space (51;52). Plasma volume expansion with HTS is secondary to translocation of fluid from the intracellular to the intravascular space. The tendency of HTS to decrease intracellular fluid volumes prevents cellular and interstitial edema that occurs following resuscitation from hemorrhagic shock. This effect may diminish the occurrence of the 'no-reflow phenomenon' after I/R injury and also protect against adverse effects of interstitial edema in the lungs and heart (53). In efforts to prolong the beneficial hemodynamic effects of HTS infusion, several authors have described positive effects of added colloids such as Dextran-70 or hydroxy-ethyl starch (HES) to hypertonic (7.5%) NaCl solutions (51;54).

In addition to rapid restoration of intravascular volume and blood pressure, HTS solutions increase heart rate and cardiac output, and decrease systemic vascular resistance (49;53;55-57). The ability of HTS to improve myocardial contractility is controversial (48;58). Improved myocardial performance after HTS infusion may be secondary to increased intracellular calcium concentrations that occur with cell shrinkage or to oxygen-free-radical scavenging abilities of dextran which is often used in combination with HTS (HSD) (53;58). HTS also dilates precapillary sphincters, thereby increasing microcirculatory flow. Several groups have shown improved splanchnic and hepatic microcirculatory flow after resuscitation from hemorrhagic shock with hypertonic solutions (55;57;59;60). Compared to isotonic solutions, resuscitation from hemorrhagic shock (HS) with HTS, reduced shock-induced liver injury in association with decreased hepatic leukostasis (59;60).

Clinical trials of hypertonic resuscitation for hemorrhagic shock

Studies of HTS resuscitation in animals have prompted a number of clinical trials in humans (61-67). Most involved the use of 7.5% HTS (4cc/kg) as a resuscitative adjunct to be the first fluid administered for the treatment of systemic hypotension with subsequent administration of additional isotonic fluids or blood. The amount of HTS administered to patients was approximately 250 ml, a volume that can easily be given during transport to hospital. Several studies made use of a hypertonic-hyperoncotic solution of HTS combined with 4.2-12% Dextran-70 to prolong the beneficial hemodynamic effects of HTS alone.

The efficacy of HTS in restoring intravascular volume was demonstrated when patients who received hypertonic resuscitation exhibited more rapid and larger increases in their systolic blood pressure on arrival to the emergency room compared to patients receiving isotonic fluids. Hemodynamic improvements were accompanied by reduced requirements for additional fluids during the first 24-hours of admission (66). Overall, clinical trials of HTS in trauma have demonstrated a trend towards improved survival with hypertonic resuscitation. This survival benefit was significant in subgroup analysis of more severely injured patients, such as those requiring immediate operation or with severe head injury (61;65). Of note, a tendency towards a decreased rate of post-traumatic complications such as ARDS, renal failure, and coagulopathy was observed in HTS-resuscitated patients (65;68). However, the apparent inability of these studies to reach convincing, statistically significant improvements in survival over conventional resuscitation may result from small patient numbers, and considerable variability in the nature and extent of injuries exhibited by trauma patients (66;69). Importantly, the use of HTS as a resuscitative adjunct was demonstrated to be safe, without a single complication being reported. In contrast to concerns regarding the risk of increased bleeding from uncontrolled vascular injuries with HTS infusion seen in animal models, patients resuscitated with HTS had a tendency to require fewer blood transfusions and exhibit lower intraoperative blood losses compared to isotonic controls (66). HTS patients exhibited mild increases in serum sodium and chloride levels as well as increased serum osmolarity levels during the first 24 hours of admission that subsequently normalized (65;66).

Head Injury and Hypertonic Saline

The head injured patient with multiple injuries represents a special challenge with respect to fluid resuscitation in which intravascular volume must be rapidly restored, but over aggressive fluid administration is detrimental (70). It is hypothesized that the ability of HTS to restore intravascular volume by shifting fluid from the intracellular and interstitial spaces along an osmotic gradient would restore cardiovascular stability and decrease cerebral edema, and may be of benefit to the head injured, hypotensive multiple trauma patient (69). Animal models of combined head injury and hemorrhagic shock in which HSD was compared to LR resuscitation have demonstrated reductions in ICP and improvements in cerebral perfusion (70-73). In humans, patients with head injury and hypotension who were initially treated with HSD were about twice as likely to survive to hospital discharge (74). This work prompted a number of trials to examine 'hypertonic maintenance therapy' for head injured patients in whom HTS is used to maintain patients in a mildly hypertonic state throughout the acute phases of treatment in hospital (68;75;76). Using this approach, some investigators have demonstrated an inverse correlation between serum sodium concentration and ICP in head injured patients. However, any benefits of this approach with respect to ICP control are transient, being lost after 24 hours of therapy.

Hypertonic saline and post-traumatic complications

Most late post-traumatic deaths are the result of multiple organ failure. Multiple organ dysfunction syndrome (MODS) is characterized by a progressive failure of multiple interdependent organs. The lungs, liver, and kidneys are principal targets. Moore proposed a 'two-hit' model to explain the development of MODS in which an initial insult (such as shock-resuscitation) may 'prime' inflammatory cells such that a second, modest insult (such as infection) may provoke an exaggerated systemic inflammatory response and progressive organ failure. Activated macrophages release pro-inflammatory cytokines resulting in secondary mediator release and PMN activation. Endothelial cell activation and swelling leads to microcirculatory disturbances, ischemia, PMN sequestration and organ injury.

Conventional resuscitation strategies may increase the adverse effects of I/R. LR solution may itself be detrimental in that PMN activation, oxidant production, and apoptosis following hemorrhagic shock-resuscitation compared to HTS (77;78). In severely injured patients, early sequestration of primed PMNs in organs renders patients susceptible to the development of MODS. The choice of resuscitation fluid may influence the development of complications after hemorrhagic shock by modulating the immune response during this early, vulnerable period (79).

The lung is the most common organ to fail in severely injured patients and is a common indication for admission to the ICU. Acute Respiratory Distress Syndrome (ARDS) may develop in up to 40% of severely injured patients. Hemorrhagic shock is thought to prime neutrophils for increased oxygen free-radical production when they are sequestered in the lung. *Rizoli et al* demonstrated that hypertonic resuscitation has a protective effect on the development of acute lung injury in rats subjected to hemorrhage-resuscitation followed by intratracheal LPS (80). HTS induced PMN shedding of L-selectin and also prevented LPS-induced upregulation of PMN CD11b, suggesting an ability of hypertonicity to modulate PMN activation and adhesion molecule expression (81). Furhter work by Rizoli et al has demonstrated *in-vitro* that cell volume is a critical factor in modulating PMN responses to HT. Cell volume regulation of surface L-selectin expression was dependent on the p38 mitogen-activated-protein-kinase pathway (MAPK) (82). HT prevented LPS-induced p38 phosphorylation and LPS-induced upregulation of CD11b (83). These effects of HT on intracellular signaling and PMN function may contribute to protective effects of HTS *in-vivo*.

Hypertonic saline and sepsis

Hemorrhagic shock is known to exert an inhibitory effect on leukocyte functions and have a synergistic effect on the development of infection (84;85). To explain the decreased rate of systemic complications observed with hypertonic resuscitation, several groups have examined the role of HTS in modulating immune responses (86). HTS reversed hemorrhage induced depression in T-cell functions in mice, an

effect that was attributed to a stimulatory effect of HT on the p38 pathway (87). In a '2-hit' rodent model of hemorrhage-resuscitation followed by cecal ligation and puncture, hypertonic resuscitation improved survival and was associated with decreased lung and liver injury, better containment of intra-abdominal infection, and a decreased rate of bacteremia (88). Others have demonstrated reduced gastrointestinal bacterial translocation after hypertonic resuscitation from hemorrhage (89). Experimental evidence suggests that some of the hemodynamic alterations associated with septic shock may be reversed with HTS with a restoration of cardiac output and improved oxygen extraction capabilities in early endotoxic shock (90). When septic animals were resuscitated to baseline cardiac outputs, significantly less fluid was required with hypertonic resusctiation compared to animals receiving isotonic fluids (91;92). This fluid-sparing effect may be secondary to reduced plasma leakage secondary to an ability of HTS to reduce leukocyte-endothelial cell interactions in post-capillary venules (93).

Effects of hypertonicity on endothelial cell function

Most work examining the effects of HT on endothelial cell (EC) function has occurred in the context of studies of the pathophysiology of diabetic retinopathy or accelerated atherosclerosis in diabetic patients and involved chronic endothelial exposure to hypertonic glucose solutions. Hypertonic glucose treatment of EC lengthens cell proliferation, disturbs the cell cycle, and induces apoptosis (94). HT may also modulate EC adhesion molecule expression. Hypertonic glucose or mannitol induces a concentration dependent increase in ICAM-1, VCAM-1, e-selectin expression, and PMN adhesion to EC exposed to hypertonic glucose or mannitol for 24 hours (95-97). These results would seem to contradict the observed effects of HTS on PMN-EC interactions in studies of hemorrhagic shock-resuscitation. However, the dose, timing of hypertonic exposure, and nature of the osmotic solute may all play a role in determining whether EC adhesion molecule expression is increased or inhibited in response to HT.

Adhesion molecules are not the only genes whose expression is regulated by HT in EC. Cytokines such as IL-1, IL-8, and IL-18 are released by EC following hypertonic treatment (98). Genes involved in cell

volume homeostasis are also active following hypertonic exposure. Exposure of EC to HT induces cell shrinkage. Full recovery of cell volume requires several hours following hypertonic exposure. Acute cell volume restoration is initially mediated by increased uptake of Na, K, and Cl with the activation of ion transporters such as the Na-K-2Cl (NKCC1) cotransport system (99). The mechanisms by which cell volume changes regulate ion transporter activity remain undefined. Recent work indicates that the stress-activated protein kinase (SAPK/JNK) phosphorylates NKCC1 in a cell-volume sensitive manner, although the mechanism by which cell shrinkage activates JNK is unclear (100).

Subacute cell volume restoration requires an increased concentration of intracellular neutral amino acids such as taurine, proline, and betaine (osmolytes). Hypertonic stress augments intracellular osmolyte concentrations through increases in the expression of amino acid transporters (transport system A) (101;102). Osmolytes such as taurine may have additional effects beyond that of cell volume restoration. Taurine may function as an anti-oxidant and regulator of intracellular calcium fluxes. Exogenous taurine and betaine administration is protective in a model of hepatic I/R (103). Taurine has also been shown to inhibit EC apoptosis induced by chronic hypertonic glucose exposure (94). Survival factors such as fibroblast growth factors (FGF) inhibit EC apoptosis. Basic fibroblast growth factor (bFGF) is released from EC when they are moved from isotonic to hypotonic or hypertonic conditions (104). The release of bFGF may be one endogenous defense that serves to protect cells exposed to rapidly changing environmental conditions.

The tolerance limit of EC to HT is controversial (105;106). In contrast to data showing increased apoptosis following prolonged exposure to hypertonic glucose, Shackford has demonstrated that relatively extreme HT of 460mOsM is tolerated by EC without a loss of viability or cell function (105). However, prolonged EC exposure to media with an osmolarity greater than 460 mOsM significantly reduced cell viability and function. This level of HT may represent the point at which sodium-proton exchange is overwhelmed (105).

Homeostatic systems that regulate cell volume may have additional roles in acute inflammation. The bumetanide-sensitive cotransporter BSC2, one of the two major isoforms of Na-K-Cl cotransporters is increased following EC shear stress and exposure to pro-inflammatory cytokines such as TNF-α and IL-1β (107). These results suggest an important link between cellular stresses such as pro-inflammatory stimuli and the regulation of cell volume (100). Thus, the induction of cellular mechanisms of volume homeostasis following stimulation by proinflammatory cytokines may serve to modulate subsequent organ injury by an undefined mechanism.

These results suggest an ability of HT to modulate EC gene expression. The induction of mitogen-activated protein kinases (MAPK) is a major mechanism by which eukaryotic cells transduce extracellular signals into phenotypic responses. Hypertonic treatment with NaCl, glucose, and mannitol all activated the MAPKs p38, ERK1/2, and JNK. However, hypertonic urea did not induce MAPK activation, implying an important role for cell shrinkage in HT-induced MAPK phosphorylation (100;108). Hypertonic activation of ERK in EC induces prostacyclin production (109). Augmented EC prostacyclin production following hypertonic exposure may explain some of the beneficial effects of HTS on I/R injury. NF-κB is also activated by hypertonic stimulation, and likely plays a role in HT-induced gene expression (98).

Effects of hypertonicity on macrophage function

Macrophages regulate many aspects of the pathogenesis of the Acute Respiratory Distress Syndrome (ARDS), hepatic I/R injury and other acute inflammatory states. Similar to EC, hypertonic exposure of macrophages increases their uptake of organic osmolytes such as betaine, taurine, and myoinositol via increased expression of osmolyte transporter genes (110-112). Osmosensitive signaling pathways resulting in augmented transporter expression in macrophages are poorly defined. Activation of p38 by HT has been shown to be important in the expression of osmolyte transporters (112). Transcription factors

such as TonEBP, and STAT proteins may also play a role in the expression of osmolyte transporters by macrophages (113;114).

HT has also been shown to modulate macrophage proinflammatory activity. Chronic exposure to hypertonic dextrose solutions decreased macrophages' ability to mount a respiratory burst and phagocytose bacteria, suggesting that chronic exposure to hypertonic dextrose may play a role in impaired peritoneal defense in peritoneal dialysis(115-117). Proinflammatory cytokine expression by macrophages is also modulated by HT (118). Preincubation of peripheral blood macrophages in hypertonic (405mOsM) medium delayed LPS-induced TNF-α mRNA expression. Similar effects were noted following a 24 hour hypertonic pretreatment of Kupffer cells, which caused a 90% inhibition of LPS-induced TNF mRNA and protein expression (119). Recent evidence suggests a role for augmented COX-2 expression and prostaglandin production in the inhibition of macrophage TNF expression by HT. Although the mechanism remains undefined, studies of macrophage cytokine production following hypertonic exposure suggest a role for cell-volume induced changes in the intracellular ionic environment in regulating posttranslational processing of proinflammatory cytokines such as IL-1β (120).

Heme oxygenase-1 (HO-1) is a stress-inducible enzyme that is also known as heat shock protein 32 (HSP32). HO-1 mediates the rate-limiting reaction in the degradation of heme to bilirubin resulting in the production of carbon monoxide (CO). In addition to second-messenger functions, CO has been identified as an important vasodilator that acts to maintain microvascular perfusion following I/R insults to the liver. Richmon et al were able to demonstrate that intra-carotid administration of hyperosmolar mannitol resulted in a sustained induction of HO-1 within the CNS (121). However, the precise mechanism of HO-1 induction by HT under these circumstances is unclear. HO-1 being a member of the heat shock group of proteins may be induced in a similar manner to that seen with HSP 70 (122-124).

Prostaglandins such as prostaglandin E_2 (PGE_2) exert vasodilatory effects and prevent platelet-adhesion to vascular endothelium *in-vivo*. Stimulation of cultured rat liver macrophages (Kupffer cells) with LPS or PMA in hypertonic culture medium increased PGE_2 synthesis in a time and osmolarity-dependent manner when compared to Kupffer cells stimulated with LPS or PMA under isotonic conditions (125).

CHAPTER 3: PATHOPHYSIOLOGY OF HEPATIC ISCHEMIA-REPERFUSION INJURY

Introduction

Impaired organ function secondary to I/R injury is an important problem in hepatic resection, solid organ transplantation, and hemorrhagic shock-resuscitation (126;127). Cellular alterations induced during ischemia prime tissue for subsequent injury during reperfusion. The injury induced by reperfusion may be more severe than the injury induced by prolonged ischemia alone (128). Patients with marginal hepatic reserve will benefit from efforts to reduce I/R injury. I/R may have a significant role in increasing alloreactivity after transplantation. Thus, efforts to reduce I/R injury may not only improve organ function but may also reduce acute and chronic rejection and extend the limits of transplantation allowing the use of organs that were previously considered marginal or unacceptable (129). Hepatic I/R is a complicated process with multiple operative mechanisms including the vascular endothelium, PMNs, and the microcirculation.

Experimental models of hepatic I/R

Various experimental models of hepatic I/R have been used to study this process. Models have included total hepatic ischemia with or without a portosystemic shunt, and regional hepatic ischemia models with varying degrees of partial ischemia. Spleno-caval shunts reduced hepatocellular injury associated with I/R with reduced serum TNF-α levels and apoptosis and was accompanied by a decreased NF-κB binding activity indicating the importance of splanchnic congestion as an additional mediator of injury (130). Splanchnic congestion introduces the confounding factors of bacterial translocation and endotoxemia, and augmented TNF-α production. In order to study hepatic I/R alone reliably, it has been suggested that models avoiding splanchnic congestion are most desirable.

Kupffer Cells

Kupffer cells (KC) are the largest population of fixed tissue resident macrophages. KC activation occurs within the first hour of liver reperfusion and is sustained throughout later reperfusion injury. Evidence for KC activation after hepatic I/R includes morphological changes detected by electron microscopy, as

well as the production of reactive oxygen species (ROS), TNF-α, IL-1, IL-6, proteases and platelet activating factor (PAF) (131;132). KCs are the predominant source of ROS in the hepatic vasculature in early reperfusion (133). KC-generated ROS modulate gene transcription in neighboring hepatocytes and EC via the activation of redox-sensitive transcription factors such as NF-κB and AP-1 (134). Selective inactivation of KCs using gadolinium chloride or methyl palmitate attenuates both early and later phases of hepatic I/R injury (135). Activated KCs modulate the activity of neighboring cells in a paracrine fachion by releasing proinflammatory mediators (131). The inhibition of KC activity indirectly modulates PMN accumulation within the liver by reducing adhesion molecule and chemokine expression following I/R (136).

Jaeschke et al have shown that KCs are activated by complement and that initial KC activation with reperfusion is potentiated by activated complement factors (131;137). KC inactivation protects against the later, PMN-mediated reperfusion injury phase, suggesting an interaction between KC and PMNs that contributes to PMN activation (138). Inflammatory mediators such as TNF-α and PAF prime both KC and PMNs, dramatically sensitizing the liver towards subsequent inflammatory stimuli such as LPS.

Neutrophils

With the restoration of blood flow to post-ischemic vascular beds, progressive accumulation of PMNs occurs. PMNs have a primary role in the pathogenesis of hepatic injury following I/R. Furthermore, hemorrhagic shock-resuscitation induces PMN-mediated injury to the lungs, liver, and intestinal mucosa, suggesting that PMNs have a role in the development of multiple organ dysfunction syndrome (MODS) following global or regional I/R insults (139;140). The presence of PMNs in the post-ischemic liver correlates with decreased animal survival (141). Neutropenia and antibodies directed against PMN adhesion molecules protect tissue against I/R injury (142). Functional and structural liver damage is directly associated with the presence and degree of PMN infiltration in liver tissue after I/R (133). PMNs start to accumulate within the hepatic sinusoids during the first hour of reperfusion, peaking at 6 hours (133;143).

This process continues for approximately 24 hours, when hepatic PMN accumulation has increased approximately 100 times compared to basal levels (142).

The accumulation of PMNs at inflammatory sites is mediated by the local generation of chemotactic agents that regulate PMN migration from the vascular compartment (144). PMN chemoattractants include C5a, formylmethionyl peptides, PAF, leukotriene B4, fibronectin, IL-8, and the C-X-C family of chemokines (145). TNF-α is associated with PMN-mediated liver injury but does not itself induce PMN chemotaxis. Instead, it stimulates the hepatic production of C-X-C chemokines (145). Chemokines also serve to activate PMNs, enhancing their respiratory burst activity and surface expression of $\beta 2$ integrins. Antibodies directed against chemokines such as KC and MIP-2 reduce hepatic PMN accumulation and diminish I/R injury only partially, indicating that while important, additional mechanisms are involved in PMN recruitment and injury (145).

PMN adhesion molecules are upregulated throughout reperfusion. Antibodies against the PMN β_2 integrin MAC-1 (CD11b/CD18) or its counter ligand, ICAM-1 attenuate subacute hepatic I/R injury, suggesting PMNs mediate their effects during this time. Interestingly, these antibodies had a very limited effect on PMN-accumulation during early reperfusion, suggesting that sinusoidal PMN accumulation occurs independently of ICAM-1 and MAC-1, and may be secondary to mechanical trapping. Endothelial swelling, vasoconstrictive mediators, and reduced PMN deformability may all contribute to PMN accumulation. Adhesion molecules may be more important in parenchymal infiltration and injury. Transendothelial extravasation of PMNs is dependent on ICAM-1 and MAC-1. PMNs also express VLA_4, suggesting a role for VCAM-1 in transmigration. I/R-induced endothelial damage may provide PMNs with direct access to hepatocytes, and therapies directed against CD11b or ICAM-1 may act to reduce PMN adherence to parenchymal cells. Studies show that PMN adherence is crucial to the occurrence of PMN-oxidant damage (126;146).

PMN-mediated injury occurs in the sub-acute reperfusion phase 4-24 hours after I/R (142;143). Activated hepatic PMNs produce ROS as well as inflammatory mediators such as LTB_4, 12-HETE, and elastase. Elastase is a neutral protease that degrades connective tissue matrix and plasma proteins. This protease is a primary cause of PMN toxicity and its activity has been implicated as a pathogenic mediator in I/R injury (147;148). Intracellular PMN elastase activity increases following I/R and PMN activation. Elastase has also been shown to be able to induce IL-8 gene transcription, suggesting that an inflammatory cycle exists whereby infiltrating PMNs release elastase and induce the production of further chemoattractants (144).

Lymphocytes

T-lymphocytes have been shown to mediate granulocytic inflammatory responses through several cytokines including TNF-α, IFNγ, IL-4, and GM-CSF. Relatively little work has examined the role of lymphocytes in the pathophysiology of hepatic I/R injury. Zwacka et al have convincingly demonstrated that T-lymphocytes accumulate in post-ischemic lobes within minutes of reperfusion and that they play a role in mediating upstream events critical to subsequent PMN-recruitment (149). T-lymphocytes are required for complete activation of post-I/R inflammatory responses. It remains to be determined whether T-lymphocytes mediate Kupffer cell activation or whether Kupffer cells mediate activation and recruitment of T-lymphocytes (134).

Oxidant-mediated injury

With I/R, ROS generation can exceed the capacity of endogenous redox degrading systems leading to pathophysiological events initating a cascade of hepatocellular injury, necrosis, apoptosis, and inflammation. ROS cause direct cellular damage through protein oxidation, degradation, lipid peroxidation, and DNA damage (134). The exact source and precise role of reactive oxygen species in hepatic I/R is unclear. In the acute phase of hepatic I/R, EC and KC are thought to be the main sources of ROS and PMNs are thought to be the main ROS source during the subactue injury phase. Acute redox damage

can activate signal transduction pathways and lead to the onset of subacute injury involving the production of cytokines, adhesion molecules, chemoattractants, and the recruitment of PMNs that amplify initial organ damage.

Adenine nucleotide catabolism results in the accumulation of hypoxanthine in ischemic cells. Xanthine oxidase generated within EC during ischemia metabolizes hypoxanthine to uric acid and generates superoxide, and hydrogen peroxide from molecular oxygen reintroduced with reperfusion. Superoxide may react to form highly reactive hydroxyl radicals (133). EC Xanthine oxidase may circulate throughout the body and contributes to ROS production and distant injury after hepatic I/R (134;150).

Mitochondria are a dominant site of ROS production since they are the major cellular compartment that consumes oxygen and generates energy (134). I/R-induced decoupling of oxidative phosphorylation in mitochondria leads to the excessive production and release of ROS into the cytoplasm. Mitochondrial ROS production is an important trigger in apoptosis and may contribute to overall organ damage (134).

Ischemia may impair the production of endogenous antioxidants such as reduced glutathione and render hepatocytes more susceptible to oxidant injury. I/R-induced impairment of cellular antioxidant production is most pronounced in the pericentral region where hypoxia is greatest (151). Thus, the ability to maintain and regenerate glutathione stores may be vital to preventing cell injury and death following the significant oxidant exposure associated with hepatic I/R (152). The administration of antioxidants such as N-acetylcysteine (NAC) is associated with significantly reduced liver injury following I/R (151;153;154).

The degree to which direct, oxidant-mediated injury contributes to the pathophysiology of hepatic I/R injury remains controversial (155). The ability of hepatocytes to detoxify intracellular reactive oxygen species (ROS) is highly effective, and exceeds the concentrations of ROS observed after hepatic I/R.

Thus, additional mechanisms of ROS-mediated injury have been proposed (151). ROS may directly regulate signal transduction, acting as 2nd-messengers and are capable of activating transcription factors such as NF-κB and AP-1 (155). I/R-induced activation of both NF-κB and AP-1 could be prevented through overexpression of the free radical scavenger mitochondrial superoxide dismutase, illustrating the importance of ROS in intracellular signaling cascades that affect proinflammatory gene expression. The ability of antioxidant treatments to modulate intracellular signaling cascades is supported by data showing that NAC decreased I/R-induced JNK activation (156).

Microcirculation

Maintenance of microcirculatory hepatic flow after hepatic I/R is essential to prevent hepatic dysfunction and failure. The degree of microcirculatory failure following hemorrhagic shock has been shown to determine the extent of hepatocyte injury (157;158). Microcirculatory disturbances following I/R have been characterized by two distinct mechanisms: perfusion failure of nutritive capillaries (no-reflow) and the sequelae of reflow-paradox associated consequences which include: accumulation of leukocytes, leukocyte-EC interactions, leukocyte transendothelial migration, and loss of endothelial integrity(159).

Prolonged ischemia results in perfusion failure of individual capillaries during reperfusion. Tissue blood flow shows a transient hyperemic response and then a gradual decline following I/R to organs with a single blood supply. This results in a heterogeneous distribution of blood flow and areas of focal hypoxia(158). Several mechanisms may account for this phenomenon including thrombosis of microvessels, capillary plugging, intravascular hemoconcentration, interstitial edema-induced extravascular compression and swelling of capillary EC (159). Capillary plugging may be secondary to accumulation of PMN and platelet aggregates. Whether PMN plugging in the liver contributes to microcirculatory failure is controversial (133;142;160). Microcirculatory failure may also be secondary to an altered balance of vasoconstrictors such as endothelin-1 and vasodilators such as nitric oxide and carbon monoxide (126;161;162). Scommotau et al have suggested that maintaining the balance between ET and vasodila-

tors such as nitric oxide should be the goal of pharmacologic therapy directed towards the hepatic microcirculation following I/R (161).

The role of NO in I/R is still controversial (157;161;163-166). Following hepatic I/R, NO is produced in large quantities. Kupffer cells are the major source of iNOS following reperfusion (165). NO has been reported to have both cytotoxic and cytoprotective effects. NO is capable of acting as a vasodilator, superoxide radical scavenger, and inhibitor of platelet aggregation and PMN adhesion (163;167). On the other hand, high concentrations of NO may contribute to I/R injury by reacting with superoxide to produce peroxynitrite which can induce sulfhydryl oxidation and lipid peroxidation (164;165).

Heme oxygenase

Carbon monoxide (CO) is a gaseous molecule that is generated during the degradation of heme to biliverdin by heme-oxygenase (HO). HO exists as three distinct isoenzymes, HO-1, HO-2, and HO-3. HO-1, also called heat shock protein 32 (HSP32), is induced by a variety of stresses including hyperthermia, cytokines, and heavy metals. Hepatic HO-1 expression increases following I/R (168). Increased HO activity allows for the removal of heme, the transmissible form of the potent pro-oxidant iron that can activate EC and increase their expression of adhesion molecules such as ICAM-1 (169). Bilirubin and biliverdin generated by HO are oxidant scavengers with significant anti-complement properties. HO-1's importance as an endogenous antioxidant is demonstrated by the fact that cells harvested from HO-1 deficient mice are highly susceptible to the free radical accumulation (170;171).

CO may perform second messenger functions by activating soluble guanylate cyclase or by hyperpolarizing membrane potentials through stimulation of potassium channels (172). CO can regulate the production of the second messenger cGMP which may in turn activate the AP-1 transcription factor (173).

Zinc-protoporphyrin IX, a potent HO inhibitor is known to increase hepatic vascular resistance and sinusoidal constriction. Hepatic Ito cells (stellate cells) are considered to be the CO sensing machinery for sinusoidal relaxation. In addition to maintaining sinusoidal perfusion, endogenously generated CO was found to maintain mitochondrial redox state, and hepatic secretory function following I/R (157). Hemorrhagic shock enhances hepatic HO-1 expression (151;157). The precise signaling mechanisms leading to I/R-induced hepatic HO-1 expression are uncertain, but are thought to involve activation of the transcription factor hypoxia-inducing factor-1 (HIF-1) (151). HO-1 increases in parallel with hepatocellular injury. HO-1 blockade prior to hemorrhagic shock resuscitation and hepatic I/R augmented liver injury. Furthermore, pharmacologic or gene therapy-induced overexpression of HO-1 prevented I/R injury and was associated with improved portal venous blood flow, increased bile production, and decreased hepatocyte injury (174).

Cytokines

Cytokines are principal effectors of the inflammatory response. These small peptides are generally less than 50 kDa in size and are produced by leukocytes and other cells that exert local effects via autocrine or paracrine activities or systemically in an endocrine fashion (175). Cytokines are pro- or anti-inflammatory and disruption of the balance between these types may lead to immune suppression or cardiovascular collapse and organ failure. Proinflammatory cytokines such as TNF and IL-1 serve important functions and their complete neutralization may render the host immune-compromised (176;177). Release of anti-inflammatory IL-10 is enhanced following hemorrhage-resuscitation and may account for hemorrhage-induced immunosuppression as neutralizing IL-10 antibody restored post-hemorrhage T-cell proliferation responses (178). The roles of distal cytokines such as IL-6 in the inflammatory response are less clear. The production of proinflammatory cytokines by the liver following I/R has been correlated not only with liver injury, but also injury to remote organs such as the heart and lungs (179;180).

Endotoxemia (LPS)

A high incidence of pre- and post- operative endotoxemia has been observed in patients undergoing major liver resections (181;182). The source of endotoxemia following hepatic I/R is likely the gastrointestinal tract. Systemic endotoxemia following hepatectomy can stimulate KC and elicit cytokine release, some of which are chemotactic, inciting an inflammatory response (183).

Tumor necrosis factor-alpha (TNF-α)

TNF-α is a powerful, proximal mediator of liver I/R injury. TNF primes PMNs for augmented cytotoxicity, induces the production of ROS and cytokines, and induces hepatocyte apoptosis. TNF-α mediates PMN adhesion to EC and hepatocytes by increasing their expression of ICAM-1 and VCAM-1 (184;185). TNF-α is released following hepatic I/R with peak levels corresponding to the duration of ischemia. Activated KC are the likely source of TNF-α as their specific blockade eliminates hepatic TNF-α production and early injury following I/R (131;177;186).

ROS likely play a role as 2nd messengers in the cascade of events leading to TNF production through activation of the transcription factor NF-κB (177). Complement cascade activation has also been implicated in hepatic I/R induced TNF-α production. The C5a complement fragment is known to induce monocyte TNF expression (177). *In-vitro*, hydrogen peroxide causes human monocytes to produce TNF and *in-vivo*, antioxidants prevent hepatic TNF expression following I/R (186). ROS induce NF-κB activation and cytokine gene transcription. Inhibitors of NF-κB prevented hepatic TNF-α expression following I/R (186).

Further evidence that TNF contributes to the pathophysiology of liver injury following I/R includes work demonstrating postoperative increases in TNF-α levels in patients who developed rejection after liver transplantation (181). Selective inhibition of TNF production improved hepatic blood flow, and reduced

hepatic PMN accumulation and injury in a severe hepatic I/R model (187). These results are consistent with a role for TNF-α in inducing adhesion molecule and chemoattractant expression following hepatic I/R (188). The heart is known to have TNF receptors and TNF results in myocardial depression, these results also support a role for systemic release of TNF-α following hepatic I/R in the development of remote organ injury and dysfunction (187;188). TNF-α produced by liver I/R may lead to the dysfunction of remote organs as in the case of pulmonary injury (189).

Interleukin-6 (IL-6)

IL-6 is an acute phase reactant cytokine with pleiotropic biological effects (190). Whether IL-6 is a predominantly pro- or anti-inflammatory cytokine is controversial. Certainly, it is a marker of injury and correlates with the degree of surgical stress to which patients are subjected (176;181;188). The liver is an important source of IL-6 and is the primary site for its clearance. IL-6 can be produced by a number of cells including hepatocytes and EC. Exogenous IL-6 administration reduced I/R-induced TNF expression and was associated with increased levels of soluble TNF-receptor (190). These results suggest a potential protective role of IL-6 in hepatic I/R.

Interleukin-10 (IL-10)

IL-10 has been identified as a potent anti-inflammatory cytokine. It is produced by macrophages, T-cells, B-cells, mast cells, and keratinocytes (191). IL-10 suppresses monocyte production of proinflammatory molecules such as IL-1, IL-6, IL-8, IL-12, TNF, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, granulocyte colony stimulating factor, MHC class II molecules, B7, chemokines such as MIP-1α and ICAM-1 (192;193). IL-10 may also exert anti-inflammatory effects indirectly by triggering the release of IL-1 receptor antagonist (IL-1ra) and soluble TNF receptors 1 and 2 which compete with membrane bound receptors for circulating proinflammatory cytokines and inhibit their activity (183).

IL-10 has also been implicated as an inhibitory factor with protective effects against LPS-mediated injury. Exogenous IL-10 administration protected against lethal endotoxemia and decreased LPS-induced TNF-α production (194). In a different model, exogenous IL-10 protected against intraabdominal sepsis and inhibited hepatic and pulmonary TNF-α expression (195). Sepsis-induced hypercoagulability may be inhibited *in-vivo* as IL-10 was shown to inhibit monocyte LPS-induced tissue factor (TF) expression (196). Furthermore, endogenous IL-10 modulates lung cytokine and chemokine production, macrophage ROS production, and PMN infiltration in several models of inflammatory acute lung injury(197;198).

The regulation of IL-10 production by activated macrophages is poorly defined (199). IL-10 is involved in an autoregulatory feedback loop with IL-1 and TNF. Recent work by Brightbill et al demonstrated that NF-κB, C/EBP (CCAAT/ enhancer-binding protein), or AP-1 do not contribute to IL-10 regulation and that Sp-1 may instead be a central mediator of IL-10 induction (200). The role of MAPKs such as ERK and p38 in IL-10 expression is unclear. While p38 activation is essential to LPS-induced IL-10 expression, ERK activation is not (199). In addition to a negative regulatory influence of IL-10 on proinflammatory gene expression, IL-10 is capable of downregulating its own expression (201).

IL-10 mediates its effects on cytokine expression at multiple levels by inhibiting cytokine transcription, decreasing mRNA stability, and increasing the expression of inactivating cytokine receptors (202). Evidence exists to support a role for IL-10 in modulating the activation of the transcription factor NF-κB (203). IL-10 is capable of prevented NF-κB activation at a whole organ level in association with reduced expression of proinflammatory cytokines such as TNF-α (204). Multiple mechanisms by which IL-10 is capable of inhibiting NF-κB activation have been described (203;205). Recent work by a number of investigators has highlighted a role for the the JAK/STAT signaling pathway in regulating anti-inflammatory effects of IL-10 (206-209). IL-10 receptor complexes are tetramers consisting of two IL-

10R1 polypeptide chains and 2 IL-10R2 chains. IL-10R1 mediates high affinity ligand binding and signal transduction. IL-10R2 is required only for signaling (207). IL-10 binding to the extracellular domain of IL-10R1 activates phosphorylation of receptor-associated Janus tyrosine kinases, JAK1 and Tyk2 (207). These kinases phophorylate specific tyrosine residues on the intracellular domain of the IL-10R1 chain, inducing a conformational change, allowing the latent transcription factor STAT3 (signal transducer and activator of transcription-3) to dock. STAT3 binds to these sites via its SH2 (Src homology 2) domain and is in turn phosphorylated by the receptor-associated JAKs. STAT3 homodimerizes and translocates to the nucleus where it binds to STAT-binding elements (SBEs) in the promoter regions of various IL-10-responsive genes. Studies in STAT3 and JAK1 deficient mice demonstrate that these molecules are absolute requirements for the ability of IL-10 to inhibit LPS-induced monocyte synthesis of TNF-α (207).

Suppressor of cytokine signaling family of proteins (SOCS)

The SOCS family of proteins is also known as cytokine-inducible SH2 domain-containing (CIS) proteins, JAK-binding protein (JAB), or STAT-induced STAT inhibitors (SSI) (210). These inhibitors mediate negative feedback of cytokine signaling pathways in a widespread manner. The ability of IL-10 to induce de novo synthesis of SOCS-3 in monocytes correlates with its ability to inhibit expression of pro-inflammatory genes (206). The mechanisms by which SOCS-3 inhibits cytokine signaling is unclear (210).

The liver has been identified as an important source of IL-10 following I/R, with resident macrophages (KCs) being the likely source (186). Peak levels of IL-10 depend on the duration of ischemia. When administered with reperfusion, IL-10 prevented TNF-α production and prevented injury to post-ischemic livers (186). IL-10 is released from human monocytes following stimulation with hydrogen peroxide and hepatic IL-10 release following I/R could be prevented with the administration of antioxidants (186).

This suggests a role for ROS as 2nd messengers in a signaling cascade leading to increased IL-10 expression. TPCK, a specific inhibitor of NF-κB translocation prevented increased expression of IL-10 following hepatic I/R (186). Thus, ROS are the likely stimuli to NF-κB translocation and IL-10 gene transcription following I/R.

Adhesion molecules

Adhesion molecules mediate leukocyte adherence, a classical post-reperfusion event. EC respond to pro-inflammatory cytokines by increased production and surface expression of adhesion glycoproteins. Promoter regions of genes for endothelial cell adhesion molecules have revealed a dependence on NF-κB for cytokine-induced transcription (211). I/R-induced increases in adhesion molecule expression are accompanied by changes in leukocyte-kinetics within the liver. The number of rolling and saltating leukocytes is significantly increased after reperfusion. The first peak in these changes occurs 30 minutes after reperfusion and is transient, lasting 60-120 minutes. The second peak increase in hepatic leukocyte rolling and adherence occurs 5 hours after reperfusion. These changes do not return to baseline until approximately 24 hours after reperfusion (211;212). Adhesion molecules can be divided into 3 families: the immunoglobulin supergene family, the selectin family, and the integrin family (213).

ICAM-1

ICAM-1, also known as CD54 is a 90 kD protein member of the Immunoglobulin supergene family (214). It is both a constituitively expressed and inducible molecule. Constituitive expression of ICAM-1 occurs in leukocytes, fibroblasts, dendritic cells, bone marrow progenitors, epithelial and EC. Transcriptional regulation is thought to account for most regulatory effects on ICAM-1 expression although possible post-translational effects of TPA and IFN-γ may be active in stabilizing mRNA. The ICAM-1 gene promoter region contains numerous regulatory enhancer elements including 2 TATA boxes, 3 transcription initiation sites, 2 κB-like elements, and an Sp1 sequence, suggesting a complicated regulation. The

Sp1 site probably regulates constitutive ICAM-1 transcription. The κB enhancer in the ICAM-1 promoter is the most important enhancer element (215).

The basic function of ICAM-1 is the induction of specific and reversible cell-cell adhesion resulting in intercellular communication. Cross-linking of ICAM-1 initiates outside-in signaling and interferes with calcium mobilization and induces tyrosine phosphorylation. Thus, ICAM-1 also has a signaling function (214). Cell-associated and soluble ICAM-1 binds Mac-1 and LFA-1. These ligands must be activated and undergo a conformational change in order to expose a high-affinity ICAM-1 binding epitope. The affinity of ICAM-1 binding domains for their ligands is regulated by glycosylation. ICAM-1 may be differentially glycosylated depending upon post-translational modification and cell-type, or by oxidant stress or inflammatory mediators such as PAF (214).

ICAM-1 is normally only expressed on the surface of sinusoidal EC and KCs in the liver (215). With warm ischemia, ICAM-1 expression by sinusoidal EC and hepatocytes increases. ICAM-1 (and VCAM-1) expression following hepatectomy has been correlated with a warm ischemia time greater than 40 minutes (216). Post-storage (24 hours) biopsy of human liver grafts show that ICAM-1 is upregulated in KC, sinusoidal EC and hepatocytes (217). *In-vivo* and *in-vitro* studies have suggested a coordinated induction of ICAM-1 in these cell types in response to the same factors. Hepatocytes and sinusoidal EC are anatomically exposed to high levels of cytokines that are normal components of the portal blood circulating in liver sinusoids. Important sources of these cytokines include resident hepatic macrophages (KCs) and the sinusoidal EC (215). TNF-α, IFN-γ, and LPS are potent inducers of ICAM-1 expression (215). Reperfusion induces a further augmentation of ICAM-1 expression by these cell types (143;217;218). ICAM-1 is further upregulated by a feedback cascade with inflammatory cells that infiltrate the liver. Thus, small differences in ICAM-1 mRNA and protein levels at early time points may be amplified to greater levels of expression as reperfusion and cell infiltration continue (129). The strongest areas of ICAM-1 staining occur in the more severely injured, necrotic areas of the liver with reperfusion

(219). In models of partial hepatic ischemia, similar changes do not occur in the non-ischemic lobes of the liver until later reperfusion, suggesting a role for circulating factors in the induction of ICAM-1 expression remote from post-ischemic areas of the liver (219). ICAM-1 and its PMN counter-ligand CD11b are both important in endothelial transmigration and adherence-dependent parenchymal injury by PMNs. Another counterligand to ICAM-1 is LFA-1, present on monocytes and lymphocytes (NK cells).

Anti-ICAM-1 blocking antibodies offer significant protection against PMN-induced reperfusion injury. In studies of partial hepatic I/R with the blocking antibody 1A29 against ICAM-1, Farhood et al noted a 32-36% decrease in PMN numbers in post-ischemic liver (219). In partial hepatic I/R, pretreatment with monoclonal antibodies against ICAM-1 and CD18, decreased PMN infiltration and reduced lipid peroxidation with subsequent improvements in hepatic energy metabolism and animal survival rates following 6 hours of reperfusion (143). Kuzume et al have shown that intraportal injections of 1A29 decreased hepatic necrosis, peri-central PMN accumulation, and improved hepatic blood flow after I/R. ICAM-1 knockout animals that had undergone 90 minutes of ischemia followed by 6 hours of reperfusion were significantly protected against hepatic injury (220). ICAM-1 knockouts had significantly lower serum transaminase values and decreased PMN infiltration compared to wild-type controls. Interestingly, beyond 6 hours of reperfusion, there was no difference in the number of infiltrating PMNs between mutant and wild type groups despite decreased serum transaminases in the mutant animals. Microcirculatory failure and sinusoidal congestion was decreased in mutant animals compared to wild type. These results suggest a very complex process of PMN-EC interactions. The fact that beyond 6 hours of reperfusion, PMN infiltration in the mutants was unchanged compared to wild-type controls despite significant hepatocellular protection suggests that alternate pathways for PMN recruitment may be involved in later PMN liver infiltration. The impact of the loss of these adhesion receptors in mutant mice undergoing hepatic I/R is not solely mediated through PMN adhesion. However, prevention of the early interaction between PMNs, adhesion receptors, and EC is essential for imparting a persistent attenuation of reperfusion injury (220). In human liver surgery, increased hepatic ICAM-1 expression has been associated

with an increased rate of post-operative complications following hepatic resection and correlated with more severe indices of post-preservation and I/R injury in liver transplantation (216-218).

Selectins

The selectin family of adhesion molecules is composed of P- (platelet), E- (endothelial), and L- (leukocyte) selectin (211). The selectin counterligands are a group of sialyated and fucosylated glycoconjugates. The most extensively characterized of these ligands is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a homodimeric mucin expressed on the surface of leukocytes and is capable of binding all 3 selectins (221). This group of adhesion molecules is responsible for the rapid capture of leukocytes and subsequent rolling along the endothelium before firm adhesion and transmigration can occur. L-selectin is constituitively expressed on the surface of quiescent PMN's. E- and P- selectin can be detected in KC, sinusoidal EC, and platelets.

In response to partial hepatic I/R, the expression of E-selectin is increased, with peak expression occurring at 5 hours reperfusion (211). P-selectin is a transmembrane glycoprotein associated with the alpha-granules in resting platelets and is also found in the granules of EC known as Weibel-Palade bodies (222). P-selectin molecules are not expressed on the surface of resting EC, but can be rapidly mobilized to the cell surface following EC activation by pro-inflammatory mediators (222). Following hepatic I/R, P-selectin expression follows a bimodal pattern with the first peak occurring at 20 minutes following reperfusion, and the second peak occurring at the 5 hour reperfusion time point. These two peaks correlated with mobilization of preformed P-selectin from Wiebel-Palade bodies and subsequent de-novo synthesis (222).

Protective effects against hepatic I/R have been demonstrated with anti-P-selectin blocking antibodies as well as in L-selectin and P-selectin deficient knockout mice (220;222). L-selectin deficiency has been shown to protect against hepatic I/R injury (220). While P-selectin deficiency altered hepatic leukocyte

kinetics following hepatic I/R and improved hepatic blood flow, it remains controversial whether strategies directed against P-selectin protect against tissue injury following hepatic I/R (221;223;224).

β_2-integrins

The β_2-integrin family of adhesion molecules consists of 3 members. All 3 are heterodimeric proteins with a common β_2 chain (CD18) but differ in their non-covalently associated α-chain (CD11a-c). Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) are involved in PMN adhesion to and migration through endothelium by binding to endothelial ICAM-1. Circulating lymphocytes primarily express LFA-1, whereas Mac-1 is expressed on PMNs, and monocytes (225). Mac-1 is constitutively expressed on PMN's but is transformed to an active conformation and is quantitatively increased after PMN activation (226). The interaction between PMN β_2-integrins and endothelial ICAM-1 sensitizes the PMN through the activation of intracellular signaling pathways mediated by β_2 -integrins. Subsequently, the production and release of cytolytic enzymes and ROS is potentiated. Mac-1 also plays a role in the adherence-dependent hydrogen peroxide production of PMNs, PMN chemotaxis, aggregation, and phagocytosis (142;226). Mac-1 blockade protects against hepatic I/R injury by reducing hepatic PMN accumulation and reducing PMN production of cytotoxic mediators (142). Mac-1 is known to be upregulated on PMNs following the use of hepatic vascular exclusion for human liver resection (227).

Coagulation system

Most experimental work in liver I/R has focused on the role of oxygen free radicals, cytokines and cellular mediators of injury. However, activation of the coagulation cascade has also been implicated as an important factor in I/R injury. The generation of thrombin or fibrin can contribute to the accumulation of leukocytes in post-ischemic tissues. Furthermore, activation of the coagulation cascade may exacerbate EC damage induced by activated leukocytes (144). Strategies to inhibit platelet aggregation such as the administration of prostacyclin analogues or thromboxane synthetase inhibitors have decreased liver in-

jury and improved survival following I/R (147). Administration of heparin or antithrombin III (AT-III) are protective in models of hepatic I/R (135;144). Heparin or AT-III administration reduced plasma concentrations of CINC, suggesting that microthrombus formation in the liver following I/R contributes to the production of cytokines and chemokines. Tissue factor (TF), also known as thromboplastin is a membranous glycoprotein that functions as a receptor to the coagulation factor VII/VIIa. TF is an initiation factor for blood coagulation. The formation of a complex between TF and factor VIIa leads to activation of factors IX and X which induces thrombin production and fibrin deposition. Pro-inflammatory mediators such as TNF-α, IL-1 and LPS increase surface expression of TF and promote coagulation, leading to microthrombi formation and later necrosis (228). Following hepatic I/R, TF is increased on the surface of monocytes and sinusoidal EC surrounding necrotic areas in post-ischemic regions (229). The intensity of TF staining is closely related to the grade of liver damage following I/R. Yoshimura et al have demonstrated significant liver protection with tissue factor pathway inhibitor following I/R injury (230).

Complement system

Complement is deposited in reperfused tissues. Complement activation by ischemic tissue generates C3a and C5a. These components induce vascular leakage, and enhance leukocyte activation. Complement components, particularly C5a have been implicated in KC activation during early reperfusion (137).

Heat shock proteins

Exposure to transient sublethal hyperthermia and the following recovery period is referred to as heat shock preconditioning. Heat shock preconditioning provides a transient state of tolerance not only to a second thermal stress but also to other types of environmental insults including oxygen radicals, heavy metals, or I/R injury, a phenomenon known as crosstolerance (231-233). The heat shock response is associated with the induction of a specific set of heat shock proteins (HSPs). The exact mechanism of the

protective effects provided by HSPs is poorly defined. Some families of HSPs are thought to assist in the folding and stabilization of other proteins allowing them to be described as 'molecular chaperones'.

Transcription of heat shock genes requires activation of the transcription factor HSF-1 (233;234). HSF-1 is normally present in non-stressed cells in a monomeric, non-DNA binding form. Following heat shock, HSF-1 trimerizes and gains DNA binding activity. The level of HSP expression appears to be cell-type specific and cells from different organs in the same individual show different patterns of HSP expression (235). Recent evidence suggests that HSF binding to the promoter regions of pro- and anti-inflammatory cytokines may modulate their expression following injury.

Oxygen free radicals generated with I/R may result in intracellular calcium overload and protein malfolding and partial denaturation. When mitochondrial membrane proteins are denatured during ischemia, the integrity of mitochondria is irreversibly impaired and cells are unable to regenerate ATP following reperfusion. Beneficial effects of HSP 70 induction are thought to result from the refolding of damaged or denatured proteins during reperfusion and a more rapid return to cellular homeostasis (236;237). The fundamental action of heat shock proteins may be to salvage denatured proteins and prevent mitochondria from losing their membrane integrity (237). Terajima et al studied the hepatic microcirculation after I/R in animals that had undergone heat shock preconditioning 48 hours earlier (231). Hepatic microcirculatory failure was significantly decreased in preconditioned animals. Sinusoidal blood flow was improved, and leukocyte rolling and adhesion was reduced.

Heat shock preconditioning decreases the nuclear translocation of NF-κB, and prevents reperfusion-induced upregulation of endothelial ICAM-1 (238;239). The possibility that heat shock modulates leukocyte-EC interactions is supported by the work of Javadpour et al in which HSP 72 induction was associated with decreased PMN-mediated lung injury following I/R induced by infrarenal aortic cross-

clamping (240). HSPs also have been shown to upregulate bcl-2, an anti-apoptotic protein that regulates the release of cytochrome C from mitochondria (134).

HSP 70 represents an endogenous protective mechanism against I/R. High levels of HSP 70 expression correlated with a decreased incidence of acute rejection episodes(241). Recent work by Mizushima et al demonstrated cardio- and hepato-protective effects of heat shock preconditioning prior to resuscitated trauma and hemorrhage (242). Four hours after resuscitation, preconditioned animals exhibited elevated levels of HSP70 in the liver and had lower plasma levels of TNF-α, and IL-6. Furthermore, hepatic function was better preserved following resuscitation in preconditioned animals. These results are consistent with previous work demonstrating that HSP70 inhibits the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6.

Cell signaling pathways

Reperfusion of ischemic tissue can lead to a variety of different fates for affected cells depending on the genetic programs that are activated. Severely injured cells die, while surviving cells may dedifferentiate and proliferate to replace dead or irreversibly injured neighbors (243-245). A number of distinct cell signaling transduction pathways are activated by the stress of I/R injury and may play a role in modulating cell phenotype in response to injury. Particular interest has focused on the mitogen-activated-protein kinase family (MAPK) following I/R due to their possible roles in stress-induced apoptosis (245). MAPK members are characterized as proline-directed serine/threonine-protein kinases. Three main subgroups exist: the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases (SAPKs/JNKs), and the p38 MAPKs.

P38 mitogen activated protein kinase

The p38 MAPK pathway responds to stresses such as inflammatory cytokines, endotoxin, osmotic shock, heat shock, UV light, metabolic inhibitors and I/R (246). P38 activation in post-ischemic myocardium

plays a role in the apoptotic pathway of cell death with the inhibition of p38 activation resulting in decreased myocardial apoptosis in an isolated perfused model of cardiac I/R (247). Both tissue- and isoform-specific patterns of stress kinase activation following I/R have been described (245). In a study of rodent liver transplantation, cold storage and reperfusion failed to induce p38 activation in the liver (248). The lack of I/R-induced hepatic p38 activation likely reflects organ specific patterns of MAPK activation following I/R.

Stress-activated protein kinase (SAPK/JNK) subfamily of c-Jun kinases

The stress-activated protein kinases (SAPKs) are a subfamily of the extracellular signal regulated kinase (ERK) family of kinases. SAPKs are markedly activated in response to the inflammatory cytokine TNF-α, UV irradiation and cellular stresses including heat shock and protein synthesis inhibitors (243). JNKs can be activated by ROS generated with I/R (134). The role of JNK is to phosphorylate members of the AP-1 family of transcription factor proteins and mediate AP-1 activation (134). Together with NF-κB, AP-1 may be an early mediator of the subacute inflammatory response following I/R (249). It has been suggested that I/R-induced AP-1 binding activity is involved in promoting hepatic regeneration (249). Increased AP-1 binding activity has also been implicated in the modulation of apoptosis (134).

In a study of renal I/R injury, ischemia alone did not increase SAPK activity, whereas reperfusion markedly activated SAPKs, peaking at 20 minutes reperfusion, and remaining increased for 90 minutes (243). A similar pattern of SAPK activation in response to the restoration of cellular ATP levels following chemical anoxia, although the precise mechanism of SAPK activation under I/R conditions is unknown. SAPKs were activated in response to hepatic I/R and their activation was partially prevented by pretreating animals with IL-1 receptor antagonist, suggesting that IL-1 mediates at least in part, SAPK activation (250). Ischemia induces translocation of JNK1 from the cytoplasm to the nucleus. Only nuclear JNK1 becomes active following reperfusion. SEK1, an upstream kinase for JNK1 is localized in the nucleus (244). Interestingly, SEK1 is phosphorylated with reperfusion, suggesting that it may be involved in the

activation of JNK1 that has undergone nuclear translocation. C-Jun expression and activation rapidly follows hepatic reperfusion. As JNK1 can also phosphorylate transcription factors at the c-fos promoter. Mizukami et al speculate that c-Jun and c-fos act synergistically to activate the AP-1 complex during reperfusion (244). Mizukami's work is consistent with that of Bradham et al who have identified increased transcription of AP-1 components and increased AP-1 transcription, and binding activity following reperfusion in a model of rodent liver transplantation (248). The pattern of JNK activation following hepatic I/R is thought to reflect the impact of soluble factors such as TNF-α and its second messenger, ceramide which is capable of activating JNK (248).

Extracellular signal regulated kinase (ERK)

ERK activation is associated mainly with the cellular response to growth factors. ERKs are rapidly activated by stimulation of cell surface receptors and play a central role in proliferation and differentiation. ERKs regulate Elk1which controls expression of c-fos and other genes (250). While JNK/SAPK and ERK pathways are often activated concomitantly as in the case of hepatic I/R, they are believed to be involved in separate, but interconnected pathways. Hepatic I/R rapidly activates both ERK-1 and ERK-2 (248;250). In contrast, myocardial I/R does not seem to activate members of the ERK family (246). These differences may reflect an organ specific pattern of MAPK activation following I/R.

NF-κB activation

Various cellular stresses increase the binding activity of NF-κB, including LPS and pro-inflammatory cytokines such as TNF-α, as well as ROS such as superoxide (155;251). Increased hepatic NF-κB binding activity correlates with augmented proinflammatory cytokine and adhesion molecule expression (252;253). The NF-κB transcription factor family is composed of 5 different protein members, which form various homo- or hetero-dimers. NF-κB proteins are related through the Rel homology domain (RHD) which subjects them to regulation centered around nuclear-cytoplasmic shuttling. The RHD is

the dimerization and DNA binding domain. It contains a nuclear localization sequence (NLS), and the binding site for inhibitors of NF-κB, the IκBs (254). By binding to NF-κB dimers, IκBs mask their NLS and cause them to be retained in the cytoplasm. The IκB family of inhibitory proteins includes IκBα, IκBβ, and IκBγ. Some IκBs contain a nuclear export sequence, which allows them to combine with NF-κB dimers bound to gene promoter regions in the nucleus, and cause their transport to the cytoplasm. Potent NF-κB activators induce almost complete degradation of IκBs within minutes (254). This process is mediated by the 26S proteasome and depends on phosphorylation of two conserved serines in the N-terminal region of IκB. The kinase that phosphorylates them is serine-specific and called IκB kinase (IKK). Phosphorylation of IκB allows it to dissociate from NF-κB, unmasking the NLS. Phosphorylated IκBs undergo polyubiquitination, with major acceptor sites for ubiquitin at arginines 21 and 22 (in IκBα).

An alternative mechanism of NF-κB activation involves the phosphorylation of IκBα on Tyrosine 42, resulting in its reversible dissociation from NF-κB. This mechanism has been reported to occur when cells are exposed to anoxic conditions. This pathway of NF-κB activation has been reported to play a prominent role following hepatic I/R(249).

NF-κB binding activity is increased within 15 minutes of transplantation following cold storage (248). The timing of NF-κB activation following hepatic I/R is consistent with the activity of TNF-α and the production of ROS produced during reperfusion. In addition to the induction of proinflammatory mediators, NF-κB activation has been implicated in protection from TNF-α induced apoptosis (249;254). Whether NF-κB assumes a protective (ie-inhibiting apoptosis) or injurious/proinflammatory role following I/R likely depends on the status of additional transcription factors bound to gene promoters.

NF-κB plays a key role in the transcriptional regulation of adhesion molecules important in inflammation (253). Following partial hepatic I/R, NF-κB activation correlated with increased transcription of both ICAM-1 and VCAM-1 (253).

Hepatic apoptosis

Cells contain a cell-death program that results in their deletion. Under physiologic circumstances, rates of apoptosis are paired with rates of mitosis so that cell numbers are kept relatively constant. Apoptotic cells are phagocytosed by neighboring cells such as macrophages. Dysregulated apoptosis occurs in a number of pathophysiologic states including carcinogenesis, sepsis, and I/R injury.

Apoptosis is characterized by cell shrinkage, disappearance of microvilli, membrane blebbing, chromatin condensation, and nuclear fragmentation (255). In the final stages of apoptosis, the cell separates into small fragments, although the cytoplasmic membrane remains intact. These 'apoptotic bodies' are rapidly phagocytosed, making them difficult to identify by light microscopy.

A number of biochemical changes are associated with apoptosis. These include alterations in plasma membrane phospholipid orientation, shifts in intracellular ion homeostasis, activation of proteinases and endonucleases, the intracellular generation of ceramide, and the activation of transglutaminase (255). Early in apoptosis, phophatidylserine (PS) is exteriorized from the inner surface of the plasma membrane and acts as a marker for phagocytosis by neighboring cells and mononuclear cells. Fluorescence labeled annexin V can be used to bind PS residues on the cell surface and identify apoptotic cells. With apoptosis, proteinases are activated, in particular, those belonging to the caspase family. Caspases are cysteinyl aspartate-specific proteases. At least 10 distinct members of this family exist and certain caspases can activate others in a proteolytic cascade. Caspases mediate cell death by cleaving essential substrates. Caspases can be divided into initiator and executioner types. Initiator caspases are involved in the activation of executioner caspases that cleave specific death sustrates including poly(ADP-ribose) polymerae

(PARP), DNA polymerase kinase, and the inhibitor of the caspase activated Dnase (ICAD) (256). Caspase 3 and Caspase 7 are two main executioner caspases activated during apoptosis. Following caspase activation, an endonuclease is activated which cleaves DNA into fragments. DNA is cleaved into fragments measuring 180-200 base pairs. These small fragments can be identified in a ladder pattern on agarose gel electrophoresis.

Recently, it is thought that a mitochondrial event mediates the execution phase of apoptosis (255). Mitochondrial permeability is altered, with the inner mitochondrial membrane becoming permeable to ions, causing mitochondrial swelling, depolarization, and uncoupling of oxidative phosphorylation. This change in mitochondrial permeability occurs before the onset of apoptosis and inhibition of mitochondrial membrane permeability changes inhibits a number of apoptotic changes (255). Mitochondrial changes are followed by the release of cytochrome C, thereby activating caspase 9. Activated caspase 9 causes caspase 3 activation, resulting in DNA fragmentation. Thus, mitochondria appear responsible for apoptosis through the release of cytochrome C.

A number of exogenous ligands interact with cell surface receptors and cause apoptosis. Such ligands include Fas ligand (FasL), TNF, and TGFβ(255). Binding of these ligands to their receptor initiates a cascade of intracellular signals. So-called 'death factors' include FasL, TNF and TNF-related apoptosis-inducing ligand (TRAIL). Fas/CD95 is a member of the TNF-R superfamily and leads to lethal hepatitis. Fas has a 'death domain' that binds Fas-associated protein with death domain (FADD) which contains a death effector domain (DED) in its amino-terminal region. FADD-like interleukin converting enzyme (ICE) (FLICE), also known as pro caspase 8 binds to the DED of FADD, resulting in activation of this proteinase.

While FasL is expressed in the liver and is a potent inducer of hepatocyte apoptosis, the binding of TNF to TNF receptor -1, or -2 (TNFR1, TNFR2) may cause cell death or cell growth. The binding of TNF to

TNFR1 or Fas activates an apoptotic pathway whereas TNFR2 binding activates a pathway leading to NF-κB activation. Through the activation of NF-κB, TNFR2 induces the expression of survival genes and can prevent apoptosis (257;258). TNFR1 and Fas are linked to cytoplasmic proteins referred to as TNF receptor associated and Fas associated- death domains TRADD and FADD respectively. Receptor-interacting protein (RIP) mediates interaction between these domains and contains a kinase domain that communicates the signal. TNF binding to TNFR1 or Fas induces conformational changes in TRADD and FADD that allow RIP binding. RIP initiates intranuclear communication that activates endonucleases that destroy the cell's DNA (257).

A family of apoptosis regulating proteins exists and includes both anti-apoptotic and pro-apoptotic members. Bcl-2 and its homologs are anti-apoptotic. Bcl-2 prevents apoptosis at the level of the mitochondria by preventing the release of cytochrome C. Biliary tract obstruction and hypoxia have been shown to induce hepatocyte Bcl-2 and protect against apoptosis.

Apoptosis has been described in various models of I/R injury including the heart, kidney, lungs, and liver (255;259-261).The induction of hepatocyte apoptosis is a very early mechanism of hepatic I/R injury. After short periods of warm ischemia, hepatocyte apoptosis is detectable as early as one hour reperfusion (262). Apoptosis occurs during reperfusion and the longer the duration of ischemia, the greater the number of apoptotic hepatocytes and SEC (263). Interestingly, hepatocyte apoptosis was demonstrated to act as a signal to induce primed PMNs to accumulate within hepatic sinusoids and subsequently undergo transendothelial migration (264). However, the precise nature of the apoptosis-related signal leading to hepatic PMN accumulation is unknown.

Cold ischemic injury and subsequent reperfusion causes severe SEC injury following liver transplantation and the degree of SEC injury correlates with functional impairment of the liver graft. Clavien et al have shown that apoptosis of the SEC is the prime mechanism of injury under these circumstances and

that SEC apoptosis is dependent on the activation of caspase 3 (265;265). In addition to a role for caspase 3 in SEC apoptosis following hepatic I/R, the same group has implicated calpains, a group of nonlysosmal cysteine proteases, although the mechanisms by which calpain mediates apoptosis in the ischemic liver remain unclear (266;267).

I/R induces mitochondrial dysfunction directly or via the Fas/FasL pathway, resulting in the release of cytochrome C and the activation of caspases. The release of proinflammatory mediators with I/R such as ROS, and TNF-α is known to cause apoptosis. ROS produced by hepatocytes mediate apoptosis of SEC. Furthermore, antioxidants have been shown to protect against hepatic I/R-induced apoptosis (268). TNF-α expression has been demonstrated to have a central role in hepatic apoptosis associated with inflammatory injury (269). Necrosis appears to be a secondary phenomenon as hepatic apoptosis preceded elevations in serum transaminases (256). Thus, TNF-α induced apoptosis is an early and possibly causal event in hepatic dysfunction and failure (269). TGF-β is another cytokine known to facilitate apoptosis. However, this cytokine could not be detected in plasma or liver tissue following porcine hepatic I/R, suggesting that TNF-α may play a greater role in I/R-induced hepatic apoptosis (270).

Preconditioning the liver with a short period of ischemia prior to a more prolonged ischemic insult was protective against hepatocyte and SEC apoptosis and reduced liver injury following I/R through the inhibition of caspase-3 activity, and upregulation of Bcl-2 (271). Adenoviral transfer of the gene for the anti-apoptotic factor Bcl-2 has been a successful approach in the prevention of experimental warm hepatic I/R injury (272;273). These results suggest that pro-apoptotic pathways that are activated following hepatic I/R may be suitable targets for strategies to reduce liver injury (274).

CHAPTER 4: HYPOTHESIS AND OBJECTIVES

As outlined in the preceding discussion, hepatic I/R is an important problem in resection for tumor, trauma, and transplantation. Liver failure after resection is often lethal and reported in 7-20% of patients (6-9). Intraoperative blood loss correlates with post-operative liver failure and an increased risk of recurrence and decreased survival in patients operated on for hepatobiliary malignancy (8;11;17;275). In trauma, the liver is a major source of intra-abdominal hemorrhage, making hemorrhage control the main operative indication in this setting. Total operative blood loss adversely affects the mortality of blunt hepatic injuries (25). Actively bleeding liver injuries may require vascular clamping to achieve hemorrhage control. Vascular clamping techniques reduce intraoperative blood loss, but may contribute to liver injury. Thus, surgeons must balance the ability to control blood loss against I/R injury to the liver. The development of strategies to protect the liver prior to the application of vascular clamps or as part of a resuscitation strategy for hemorrhage may minimize adverse effects of I/R and improve patient outcome.

Hepatic I/R injury occurs in a biphasic pattern (276). The acute reperfusion phase is characterized by ROS generation and KC activation (277). KC activation leads to production of superoxide, chemokines and proinflammatory cytokines such as TNF-α(132). These events activate neighboring ECs and hepatocytes, increasing the expression of adhesion molecules, setting the stage for PMN infiltration during the subacute phase of reperfusion injury (143;278;279). In the subacute reperfusion phase, activated PMNs amplify the acute injury by transmigrating into the liver and releasing free radicals and proteolytic enzymes (276). Therefore, strategies to protect against hepatic I/R must target early upstream events in order to prevent subsequent PMN-mediated injury.

HTS has been used to resuscitate patients from traumatic hypotension (61-67). Benefits of HTS include the rapid restoration of intravascular volume, improved myocardial contractility and improved microcirculatory flow (53;56;57). Studies of HTS noted a trend towards a reduced incidence of ARDS, renal

failure and coagulopathy following resuscitated shock (65;68). While the mechanism is poorly defined, it is suggested that reduced PMN-EC interactions contribute to these effects (60;77;80-82;280).

We hypothesized that HTS pretreatment would prevent hepatic I/R injury by altering EC adhesion molecule expression and thus minimize PMN-mediated injury. We further hypothesized that HTS pretreatment may protect against subacute reperfusion phase PMN-mediated injury by modulating acute phase reperfusion events such as macrophage activation and proinflammatory cytokine production that contribute to EC adhesion molecule expression. The objectives of the present study were to determine the effects of HTS on: the degree of liver injury in a rodent model of hepatic I/R, as well as the expression of adhesion molecules *in-vivo* following hepatic I/R and *in-vitro* using an endothelial cell line, and to determine levels of both pro- and anti-inflammatory cytokines that are known to modulate injury and cell activation following I/R. To meet these objectives, an *in-vivo* model of warm, partial hepatic I/R injury as well as *in-vitro* models of activated EC and macrophage cultures were used.

CHAPTER 5: MATERIALS AND METHODS

Materials

Guanidine isothiocyanate (TRIzol reagent) was obtained from Life Technologies (Burlington, Canada). Triton X-100 from Caledon (Georgetown, Canada), and ^{32}P from Amersham (Oakville, Canada). EDTA was from BDH (Toronto, Canada). Sodium Chloride was purchased from BDH (Toronto, Canada) and was prepared in deionized water to a 4M stock solution which was then passed through a 0.22 μm filter unit (Millipore). The anaesthetic drugs used were ketamine from Rogar/STB (London, Canada), and xylazine from Bayer (Etobicoke, Canada). The rat neutrophil isolating kit NIM2 was purchased from Cardinal Associates (Santa Fe, NM). Lipopolysaccharide (Escherichia coli 0111:B4) was from Sigma. 3% thioglycollate (Life Technologies, Inc.) was prepared as per the manufacturer's instructions. Caspase 3 substrate AFC (7-amino-4(trimethylfluoromethyl)coumarin) was obtained from Calbiochem (San Diego, CA). The selective HO-1 inhibitor, zinc protoporphyrin-IX (Aldrich Chemical Company, Milwaukee, WI) was initially dissolved in 0.2N NaOH, pH adjusted to 7.4 and then a stock solution of 2.5mg/ml was made by adding isotonic saline solution. All chemicals used were of the highest purity available.

Solutions

Endotoxin-free DMEM, HBSS, PBS, and RPMI were obtained from Life Technologies (Burlington, Canada). PMNs were isolated in DMEM containing 10% fetal calf serum (FCS), (HyClone, Logan, UT) and penicillin/streptomycin (Life Technologies). TBS contained 100mM NaCl and 50mM Tris, pH 7.4. Isotonic 0.9 % NaCl solution was obtained from Abbott Laboratories (Montreal, Canada). The Toronto Hospital Pharmacy Department prepared HTS/7.5% NaCl for use in pretreatment of animals.

Antibodies and cDNA probes

A fluorescein isothiocyanate (FITC)-labeled anti-CD11b OX-42 monoclonal antibody was obtained from Serotec (Toronto, Canada). A polyclonal rabbit anti-rat IκBα antibody was purchased from New England Biolabs (Beverly, MA) and polyclonal rabbit anti-rat heme-oxygenase-1 (HO-1) from StressGen Biochemicals (Victoria, Canada). A mouse anti-rat Heat shock protein 70 (HSP-70) monoclonal anti-

body was obtained from NeoMarkers (Fremont, CA). Polyclonal rabbit anti-rat IL-10 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal rabbit anti-rat phospho-STAT3 (Tyr705) and polyclonal rabbit anti-rat STAT3 antibody was purchased from New England Biolabs (Beverly, MA). Polyclonal rabbit anti-phospho-p38, anti-phospho-ERK, and anti-phospho-JNK-1 antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-p38 and polyclonal rabbit anti-ERK were purchased from New England Biolabs (Beverly, MA). Murine monoclonal anti-human CD54 (ICAM-1) antibody was obtained from Zymed (San Francisco, CA). Anti-rat IL-10 neutralizing antibody (AF519) was purchased from R&D Systems (Minneapolis, MN). The IL-10 receptor antagonist, 1B1.2 was a kind gift from Dr. Alice Mui (University of British Columbia, Vancouver, Canada). The murine ICAM-1 cDNA probe, murine VCAM-1 cDNA probe, murine TNF-α cDNA, murine tissue factor cDNA probe and murine IL-10 cDNA probes were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Glyceraldehyde-3-phosphate dehydrogenase G3PDH cDNA probe was obtained from Clontech (Palo Alto, CA). Recombinant rat TNF-α was purchased from Biosource (Burlington, Canada).

Animals

Animals used for these experiments were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. The experimental protocol had undergone prior review and approval by the University Health Network (UHN) Animal Care Committee. Adult male Sprague-Dawley rats, weighing 300-350g (Charles River, St. Constant, Canada), were allowed to acclimatize for 1 week prior to being used in any experiments. Rats were housed in pairs, in clear plastic cages, kept on a 12 hour alternating light- dark cycle and were allowed access to standard laboratory rodent chow and water ad-libidum. Pathogen-free female BALB-c mice aged 6-7 weeks were obtained from Taconic Farms and were chow fed and allowed to acclimatize for 1 week prior to use. C57BL/10-Il10^{tm1Cgn} mice were obtained from the University of Cologne, Germany through Jackson Laboratories and housed under patho-

gen-free (SPF) conditions. Otherwise, these animals were cared for in a manner identical to that of the BALB-c mice.

Animal model of warm partial hepatic I/R injury

The experimental protocol is outlined in figure 1. Rats were anesthetized with intraperitoneal (i.p.) ketamine (80 mg/kg) and xylazine (8 mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and fluid administration. After a 10 minute stabilization period, animals received 4 ml/kg volume of isotonic (0.9% NaCl) or hypertonic (7.5% NaCl) saline via the arterial line. Sixty minutes later, rats underwent midline laparotomy. The xyphoid process was elevated and the arms of a self-retaining retractor were inserted into the flanks. The bowel was eviscerated temporarily and covered with warm saline-gauze. The liver was mobilized by sharply dividing its diaphragmatic attachments. The portal venous and hepatic arterial branches to the left and median hepatic lobes were dissected-out and occluded with a 3.5 mm microvascular clamp (Piling-Weck). The exteriorized bowel was returned to the abdominal cavity and the abdomen was closed with several interrupted 4-0 silk sutures. Following a 30 min period of warm-partial hepatic ischemia, the abdomen was re-opened and the liver was visually inspected for blanching. The microvascular clamp was removed, allowing the ischemic left and median lobes to reperfuse. The abdomen was then closed in 2 layers using running 4-0 silk suture. Animals were allowed to undergo reperfusion and recover for 0 - 240 minutes before they were re-anaesthetized and euthanized at which time blood and tissue samples were harvested. Sham-operated animals underwent the same surgical procedures with the exception of clamp placement and received isotonic (0.9%) saline as their initial pretreatment. Sham animals were maintained for equivalent times as ischemia-reperfusion (IR) rats prior to sample harvest. Control rats underwent no manipulation except for general anaesthesia prior to harvest of blood and tissue samples.

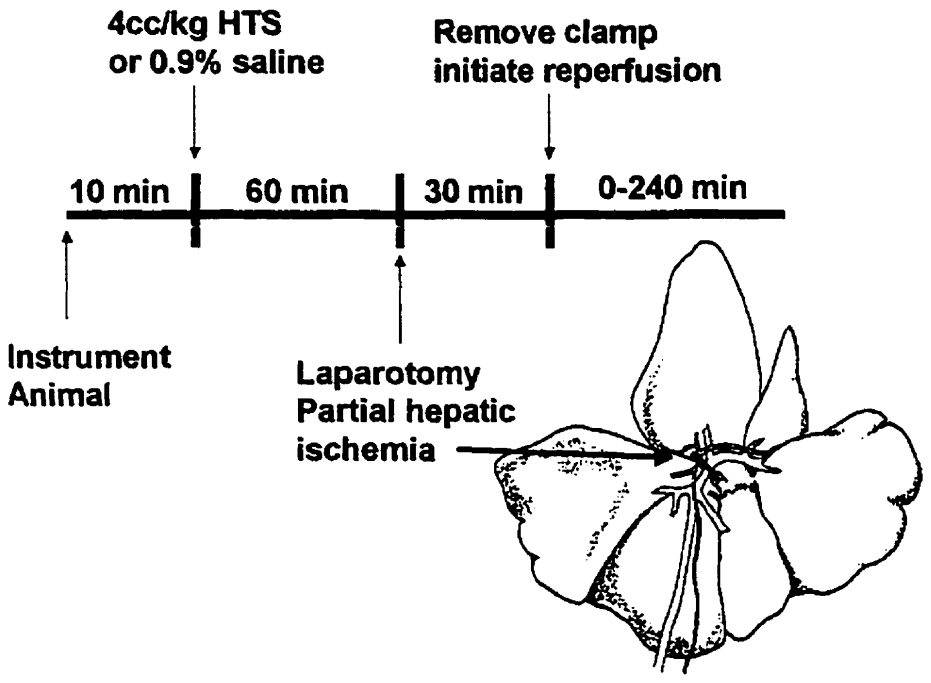

Figure 1: In-vivo model of rat partial warm hepatic ischemia-reperfusion.

Animals were instrumented and then allowed to stabilize for 10 minutes. Pretreatment consisted of a 4cc/kg dose of 7.5% saline (HTS) or an equal volume of isotonic saline. One hour later, rats underwent laparotomy, and partial hepatic ischemia for 30 minutes. Reperfusion for 0-240 minutes was initiated with clamp removal.

Assessment of liver injury

Animals were sacrificed at various intervals. At the end of the experimental protocol, approximately 3 ml of blood were withdrawn by cardiac puncture into heparinized syringes for evaluation of plasma aspartate aminotransferase (AST). Blood samples were analyzed by the clinical biochemistry laboratory (TML) and reported as IU/L. Liver tissue was harvested and snap frozen in liquid nitrogen for evaluation of myeloperoxidase (MPO) levels, mRNA, TNF or IL-10 protein, and NF-κB or AP-1 activation. Hepatic apoptosis was evaluated by DNA fragmentation and an in-situ end-labeling technique (TUNEL staining) carried out in the UHN histopathology laboratory.(281)

Assessment of serum sodium concentration and osmolarity

At the end of the experimental protocol, approximately 3 ml of blood were withdrawn by cardiac puncture into heparinized and plain vacutainer tubes (Becton Dickinson, Franklin Lakes NJ) and sent to the clinical biochemistry laboratory for analysis (Toronto Medical Laboratories, Canada).

Myeloperoxidase (MPO) assay

Liver samples were thawed, and approximately 0.5 g of tissue was homogenized in 25 ml of potassium phosphate (10mM; pH 7.4) for 1 min using a Brinkmann Polytron (model PT10/35, Brinkmann Instruments, Westbury NY). The homogenate was centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended and homogenized in 25 ml of potassium phosphate-buffered saline (50 mM; pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The homogenate was frozen overnight at -70°C, rehomogenized for 1 min, and sonicated (model VC 50T, Sonics & Materials, Danbury, CT) at 40 W for 1 min. After centrifugation as described above, the supernatant was collected and used for both MPO and protein assays. MPO activity was assessed by colorimetric assay using H2O2 as a substrate in a Cobas FARA II Chemistry System (Roche Diagnostic Systems, NJ) at A655nm over three minutes as previously described. The absorbance change per minute was used as a measure of MPO activity. Results are expressed as MPO activity per milligram of protein. Protein concentrations were determined using the Pierce bicinchoninic acid (BCA) protein assay (Pierce, Rockford IL).

Measurement of neutrophil surface CD11b

Flow cytometric analysis was used to evaluate PMN surface expression of CD11b. 100 µl of whole rat blood was incubated with FITC-labeled anti-CD11b Ab at 1/10 dilution at room temperature for 20 minutes. Erythrocytes were lysed with lysing buffer (0.84% NH4Cl). The remaining cells were washed twice with DMEM and analyzed on the FACScan (Becton Dickinson, Palo Alto, CA) using an FL1 de-

tector (488 nm excitation wavelengths). Typically, 5,000 cells were analyzed per sample and the results were expressed as the mean channel fluorescence (MCF).

Measurement of neutrophil Annexin-V binding

At the 4 hr reperfusion time point, rats were exsanguinated under anaesthesia and neutrophil isolation was performed according to the manufacturer's instructions using the NIM2 rat neutrophil isolation kit (Cardinal Associates, Santa Fe, NM). Briefly, the total blood volume of the animal was obtained by cardiac puncture and collected in a citrated glass tube. The blood was layered onto two discontinuous Ficoll-Hypaque gradients (NIM2). After centrifugation at room temperature (400 x g for 45 min) the pelleted cells were washed in HBSS once, and the erythrocytes were lysed in erythrocyte lysing buffer (0.84% NH_4Cl). Isolated cells consisted of > 90% PMN. Annexin-V binding to PMNs was determined flow-cytometrically using the Genzyme TACS Annexin V Apoptosis detection kit (Genzyme, Cambridge, MA). One million cells per animal were resuspended in 100 µl of Buffer A (containing Annexin V-FITC). After a 15 min, room temperature incubation in the dark, cells were centrifuged (1200 rpm x 5 min) and then resuspended in 100 µl of Buffer B. Annexin-V binding was analyzed on the FACScan (Becton Dickinson, Palo Alto, CA) using an FL1 detector (488 nm excitation wavelengths). Typically, 5,000 cells were analyzed per sample and the results were expressed as the % Annexin-V positive of the total number of cells analyzed.

Measurement of TNF-α

Hepatic tissue TNF-α was evaluated at the 2 hour reperfusion time point. Tissue homogenates (100 ul/sample) were evaluated by sandwich ELISA technique using a commercial kit from R&D Systems (Minneapolis, MN) and compared to a standard curve as described previously. Results are reported as ng TNF-α/ml.

RNA extraction and Northern blot analysis

Total RNA was extracted using the guanidium-isothiocyanate method.(282) Briefly, liver tissue was harvested from treated animals and immediately frozen in liquid nitrogen. Approximately 100mg of liver tissue was thawed and then homogenized in 10 ml of 4 M guanidine-isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM β-mercaptoethanol. Messenger RNA (mRNA) was isolated using a mRNA extraction kit (Quik Prep Micro Purification Kit, Amersham Pharmacia Biotech, Inc, Baie d'Urfe, Quebec). RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (NEN Life Sciences, Boston, MA). Hybridization was carried out using a ^{32}P-labeled, random-primed murine ICAM-1 cDNA probe, murine VCAM-1 cDNA probe, murine TNF-α cDNA probe, or murine IL-10 cDNA probe. Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens as previously reported. To control for loading, blots were then stripped and reprobed for G3PDH, a ubiquitously expressed housekeeping gene.

Nuclear Protein Extraction

Nuclear protein extracts were prepared from liver tissue by the method of Deryckere and Gannon.(283) Aliquots of 200-500 mg of frozen tissue were ground to powder with a mortar and pestle in liquid nitrogen. The thawed powder was homogenized in a Dounce tissue homogenizer with 4 ml of solution A (0.6% Nonidet P-40, 150 mM NaCl, 10mM HEPES pH 7.9, 1mMEDTA, and 0.5 mM PMSF). The cells were lysed with five strokes of the pestle. After transfer to a 15-ml tube, debris was pelleted by briefly centrifuging at 2000 rpm for 30 sec. The supernatant was transferred to 50-ml Corex tubes, incubated on ice for 5 min, and centrifuged for 10 min at 5000 rpm. Nuclear pellets were then resuspended in 300 μl of solution B (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2mM benzamidine, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and incubated on ice for 20 min. The mixture was transferred to microcentrifuge tubes, and

nuclei were pelleted by centrifugation at 14,000 rpm for 1 min. Supernatants containing nuclear proteins were aliquoted in small fractions, frozen in liquid nitrogen and stored at -70°C. Protein quantitation was performed using the BIORAD protein assay dye reagent (BIO-RAD, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was used to assess NF-κB binding activity to the ICAM-1 variant NF-κB site. The probe used for EMSA was a 25-bp double-stranded construct (5'-TAGCTTGGAAATTCCGGAGCTGAAG-3') corresponding to a sequence in the ICAM-1 variant κB binding site. End labeling was performed by T4 kinase in the presence of [^{32}P]ATP. Labeled oligonucleotides were purified on a Sephadex G-50 M column (Pharmacia Biotech, Inc., Piscataway, NJ). An aliquot of 5 μg of nuclear protein was incubated with the labeled double-stranded probe (50,000 cpm) in the presence of 5 μg of nonspecific blocker, poly(dI-dC) in binding buffer (10 mM Tris-HCL, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM DTT) at 25°C for 20 min. Specific competition was performed by adding 100 ng of unlabeled double-stranded oligonucleotide probe to the nuclear extract from the sample with the greatest nuclear binding (ie- t = 2 hours reperfusion), while for nonspecific competition, 100 ng of unlabeled double-stranded mutant oligonucleotide probe that does not bind NF-κB was added. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1 x Tris glycine EDTA buffer. Gels were vacuum dried and subjected to autoradiography.

Western blot analysis

To study ICAM-1, IL-10, HO-1, and HSP-70 expression in the liver, whole liver was homogenized in TBS/1% Triton X-100 solution. Equal amounts of liver tissue homogenate (50 μg protein/sample) were separated on 15% SDS-PAGE under nonreducing conditions. Separated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes and blocked overnight at 4°C with Tris-buffered saline containing 5% non-fat dried skim milk. The membranes were then incubated with a 1/1000 dilution

of antiserum against the protein of interest at room temperature for 1 hour. Ag-Ab complexes were identified with goat anti-rabbit IgG tagged with horseradish peroxidase (Sigma, St. Louis, MO) and detected using the enhanced chemiluminesence (ECL) detection system (Amersham, Arlington Heights, IL).

DNA fragmentation analysis

8 hr reperfusion liver samples were analyzed for DNA fragmentation and compared to samples from control rats (negative control) and apoptotic HL-60 cells (positive control). 100mg of liver tissue was homogenized in 1ml of lysis buffer containing (100mM Tris, 5mMEDTA, 200mM NaCl, 0.2% SDS, and Proteinase-K 400μg/ml, pH 8.5). The homogenate was incubated for 60 min at 50°C in a shaking water bath after which an equal volume of cold isopropanol was added and the mixture was then centrifuged at 14,000 x g for 30 min. The pellets were allowed to air-dry for 20 min and then resuspended in 100μl of TE buffer (10mM Tris, 1mM PMSP, pH 7.6) containing 80μg/ml of RNase and incubated overnight at 25°C. Samples were then electrophoresed in a 1% agarose gel to which 7μl of ethidium bromide had been added at 125mV for 1 hr. Gels were then photographed under UV light.

Caspase 3 isolation and activity

Four hour reperfusion post-ischemic or control liver samples were analyzed for caspase 3 activity. 0.1g of liver tissue was homogenized with 5 strokes of a Dounce tissue homogenizer in 1ml of isolation buffer (25mM Hepes, pH 7.5, 5mM $MgCl_2$; 1mM EDTA, 1mM PMSF, 10 μg/ml each of Leupeptin and Pepstatin A). Tissue homogenates were left on ice for 20 min, then vortexed and spun at 17,400 x g for 20 min at 4°C. Pellets were resuspended in 1ml of incubation buffer (100 mM HEPES, pH 7.5, 10% sucrose; 0.1% CHAPS, 1mM PMSF, 10 μg/ml each of Leupeptin and Pepstatin A, and 10 mM DTT). After centrifugation at 14,000 x g, the lysates were assayed for caspase 3 activity spectrofluorometrically using Caspase 3 AFC (7-amino-4(trimethylfluoromethyl)coumarin) as substrate in a fluorescence photometer with an excitation wavelength of 400 nm and emission of 505 nm. The fluorescence intensity

was calibrated with standard concentrations of AFC, and caspase activity was calculated from the slope of the recorder trace and expressed in picomoles per second per milligram of protein. In order to confirm that results obtained using the aforementioned caspase 3 assay were actually secondary to caspase 3 acting on the AFC substrate and did not represent non-specific substrate cleavage secondary to caspases other than caspase 3 or other proteases present in the tissue homogenate, we re-measured the caspase 3 activity in tissue lysates using a commercially-available kit from Boehringer Mannheim (cat # 2 012 952). Tissue lysates were prepared identically as described above, and then applied to a 96-well plate that had previously been coated with an anti-caspase 3 antibody, ensuring the specificity of substrate cleavage and measured caspase 3 activity.

Role of inducible heme-oxygenase-1 (HO-1) in protective effect of HTS

Whole liver tissue homogenates were first subjected to western blot analysis as described above to determine HO-1 protein expression. In a separate set of experiments, rats were pretreated with 20 mg/kg of zinc-protoporphyrin-IX (Zn-PP) or an equal volume of isotonic saline i.p. 24 hours before use. The following day, rats were subjected to the same experimental protocol as described above. Control animals were pretreated with Zn-PP or 0.9% saline and sacrificed the next day. Sham and IR animals were treated similarly. They were instrumented, pretreated with HTS or isotonic saline and underwent hepatic I/R 1 hour later. After 30 minutes of actual or simulated ischemia (shams), animals underwent 4 hours reperfusion and were sacrificed after blood samples for AST measurement and liver tissue samples were harvested.

In-vitro models

We made use of 2 *in-vitro* models to determine whether the effects of hypertonicity in-vivo at the whole organ level are secondary to effects on individual cell-types known to be important in hepatic ischemia-reperfusion injury. Both models involved sequential exposure to cellular stresses of hypertonicity and

endotoxin. The first model made use of peritoneal macrophages harvested from BALB-c mice. The second model used cultured endothelial cells harvested from the umbilical cords of healthy newborns.

Peritoneal exudative macrophages

Peritoneal exudative macrophages (PEM) were pretreated with hypertonic conditions and then resuspended in isotonic medium and activated with LPS to examine the ability of hypertonicity to modulate macrophage inflammatory responses and to elucidate the mechanism(s) by which hypertonicity may differentially regulate the expression of pro- and anti-inflammatory cytokines (figure 2).

Cell preparation

Peritoneal exudative macrophages (PEM) were harvested in ice-cold HBSS 5-6 days after the intraperitoneal injection of 2 ml of sterile thioglycollate. The cells were washed twice in cold HBSS and resuspended in RPMI, 10% FCS, L-Gln at 1 x 10^6 cells/ml. This procedure consistently yields a >96% macrophage cell population by Wright's stain, with >97% viability by trypan blue exclusion (24). Cells were divided into 4 groups: control; hypertonic pretreatment alone; isotonic pretreatment followed by LPS stimulation; hypertonic pretreatment followed by LPS stimulation. Cells were incubated for 60 minutes at 37 degrees C, 5% CO_2 prior to experimentation.

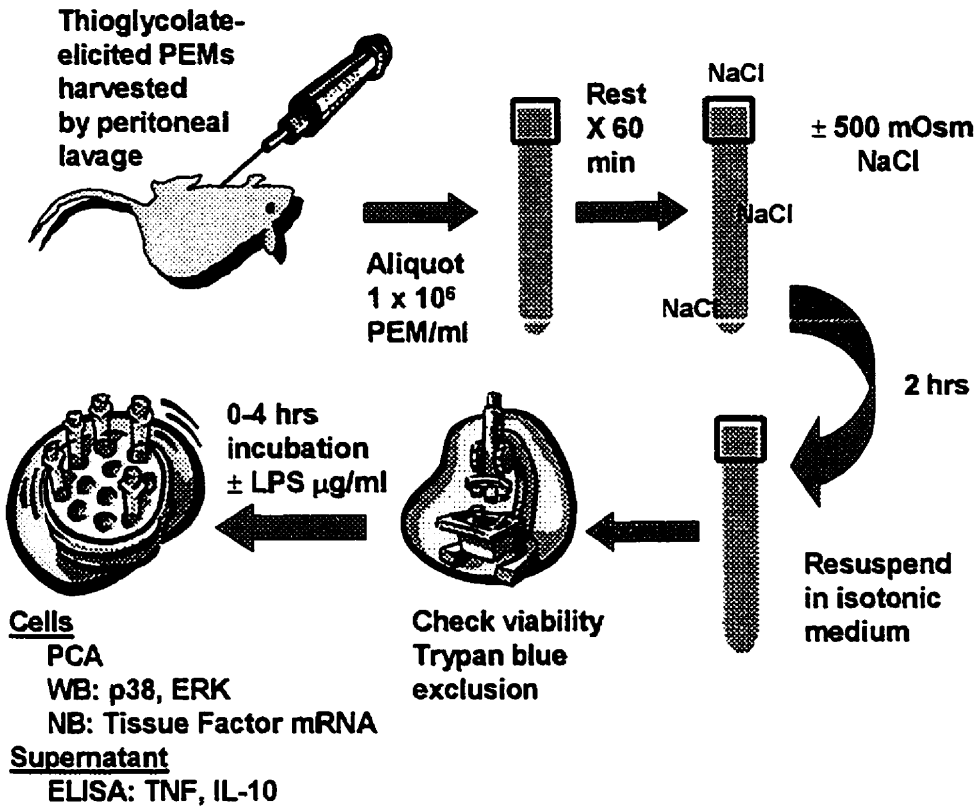

Figure 2: Hypertonic pretreatment of Peritoneal Exudative Macrophages (PEM) followed by activation by LPS.

Thioglycolate-elicited PEMs were harvested from BALB-c mice by peritoneal lavage and aliquoted in R10G medium at a concentration of 1 x 10^6/ml. After 1 hour, sterile NaCl was added to the medium to achieve a concentration of 500mOsm. Hypertonic conditions were maintained for 2 hours after which time, cells were resuspended in fresh isotonic medium and viability was assessed by trypan blue exclusion. After a 0-4 hour incubation in the presence of LPS 1µg/ml, cells were spun-down and harvested for procoagulant activity (PCA), Tissue factor mRNA by northern blot analysis, or assessment of MAPK activation (p38 or ERK) by western blot analysis. Supernatants were assayed for TNF and Il-10 concentrations by ELISA.

Hypertonic Pretreatment and Cell Stimulation

PEM were then incubated at 37 °C in 5% CO2 for 2 hours in either isotonic (290 mOsm) media (RPMI, 10% FCS, L-Gln at 1 x 10^6 cells/ml) or media that had been made hypertonic (500 mOsm) by the addition of sterile NaCl. Some groups were treated with a neutralizing antibody against IL-10 (25 µg/ml) or against the IL-10 receptor (10 µg/ml) prior to the addition of LPS. Cells were then stimulated with LPS 1µg/ml for 10-240 minutes.

Measurement of TNF-α

TNF-α was evaluated at 0, 30, 60, 120 and 240 minutes after LPS was added to cell suspensions with or without hypertonic pretreatment. Cells were pelleted and their supernatants were evaluated by sandwich ELISA technique using a commercially available kit purchased from R&D Systems (Minneapolis, MN) and compared to a standard curve.

Measurement of IL-10

IL-10 was evaluated at 240 minutes after LPS was added to cell suspensions with or without hypertonic pretreatment. Cells were pelleted and their supernatants were evaluated by sandwich ELISA technique using a commercially available kit purchased from R&D Systems (Minneapolis, MN) and compared to a standard curve.

Human umbilical vein endothelial cells (HUVECs)
HUVECs were exposed to sequential (or simultaneous) stresses of hypertonicity and LPS stimulation to determine the ability of hypertonicity to modulate endothelial cell adhesion molecule (ICAM-1) expression.

Tissue Culture

HUVECs were isolated as previously described (284). Briefly, fresh umbilical cord veins were cannulated and flushed with sterile saline, followed by a 10-minute incubation at 37°C in the presence of Collagenase Type IV (0.2%). Cells were collected, washed, resuspended in Medium 199, supplemented with 20% fetal calf serum, 100 U/ml K-penicillin G, 100 μg/ml streptomycin sulfate, 50 μg/ml endothelial growth factor and 100 μg/ml heparin and grown to confluence on gelatin-coated flasks. Passages 1 to 3 were used for all experiments.

ICAM-1 enzyme-linked immunosorbent assay (ELISA)

HUVECs were grown to confluence in gelatin-coated 96-well plates and stimulated with LPS (1 μg/ml) for 6 hours after hypertonic pretreatment (350-500 mOsm) through the addition of sterile, NaCl to the culture medium for 2 hours followed by the replacement of isotonic medium prior to the addition of LPS. In a separate series of experiments, the timing of the hypertonic exposure period was varied with respect to the 6-hour period of LPS stimulation. After incubation for various times at 37°C, cells were washed with phosphate-buffered saline and monolayers were incubated with the murine anti-human ICAM-1 antibody, at room temperature for 1 hour, washed again and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G for an additional hour. Cells were washed in phosphate-buffered saline and HUVEC monolayers were checked for confluence before color development with addition of the substrate, o-phenylenediamine hydrochloride. After 20 minutes, the reaction was stopped with 4.5 mol/L sulfuric acid and the optical density was read at 492 nm using a microtiter plate reader.

Lactate Dehydrogenase (LDH) measurement

In order to assess cell injury, LDH activity was measured in the culture medium of HUVECs following a 6- hour exposure to LPS alone, increasing levels of hypertonicity (350-500 mOsm), or LPS combined with hypertonicity, and compared to LDH release from control HUVECs. LDH measurement was carried out in the clinical biochemistry laboratory of Toronto Medical Labs (TML) on 5 ml aliquots of culture medium from cells exposed to the aforementioned conditions.

RNA extraction and Northern blot analysis

Total RNA was extracted using the single step guanidium-isothiocyanate method of Chomczynski and Sacchi (282). In brief, RNA from HUVECs (7×10^6 cells) was extracted by the addition of a solution containing 4 mol/L guanidine-isothiocyanate, 25 mmol/L sodium citrate, 0.5% sarcosyl and 100 mmol/L β-mercaptoethanol.. RNA was denatured and electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (NEN Life Sciences, Boston, MA). Hybridization was carried out

using a random 32P-labeled cDNA probe against human ICAM-1. Bolts were stripped and reprobed with the housekeeping gene glyceraldehydes-3-phosphate-dehydrogenase to ensure equal loading.

Statistical Analysis

Data are presented as the mean ± SE of the number of experiments indicated. When blots are shown, they are representative of at least three independent studies. Significance was assessed using Student's t test and one-way analysis of variance with post-hoc testing with the Tukey-Kramer multiple comparisons test. $P < 0.05$ was considered significant.

CHAPTER 6: RESULTS

In-vivo model of hepatic I/R

Mean arterial pressure

Mean arterial pressure (MAP) was assessed to exclude any gross differences in animals' macrohemodynamic state that could account for any differential effects of HTS on liver injury. The data shown in figure 3 are representative of at least 3 measurements (rats) per time point for each group. There were no significant differences in MAP between treatment groups at all time points examined, ($p > 0.05$).

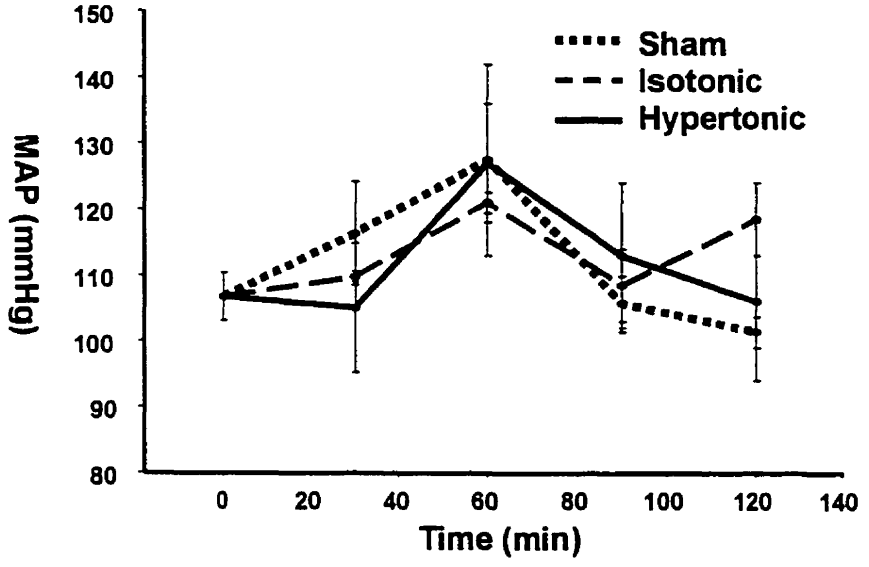

Figure 3: The effect of pretreatment on mean arterial blood pressure (MAP).

Following systemic administration of isotonic or hypertonic saline, MAP was monitored via an arterial line and expressed as mmHg. At least 3 measurements for each treatment group (Sham, Isotonic, Hypertonic) at each time point were recorded. Sham animals received isotonic saline but did not undergo vascular clamp placement.

Plasma sodium and osmolality

To ensure that HTS was mediating the expected changes in plasma [Na] (Fig. 4A) and osmolality (Fig. 4B), these were measured following HTS or isotonic saline administration. Three measurements of plasma [Na] were taken for each time point in each group. Baseline plasma [Na] for all animals was 142 ± 1.5 meq/L. The highest measured plasma [Na] was 162 meq/L, 10 min after HTS. Plasma [Na] peaked in HTS animals 10 min after HTS and remained significantly elevated compared to isotonic I/R

animals for the duration of the protocol. Serum osmolality was 301.6 ± 2.3 mmol/kg in controls. Following HTS, serum osmolality peaked within 5 min at 337 ± 3.2 mmol/kg and normalized over the subsequent 30 min.

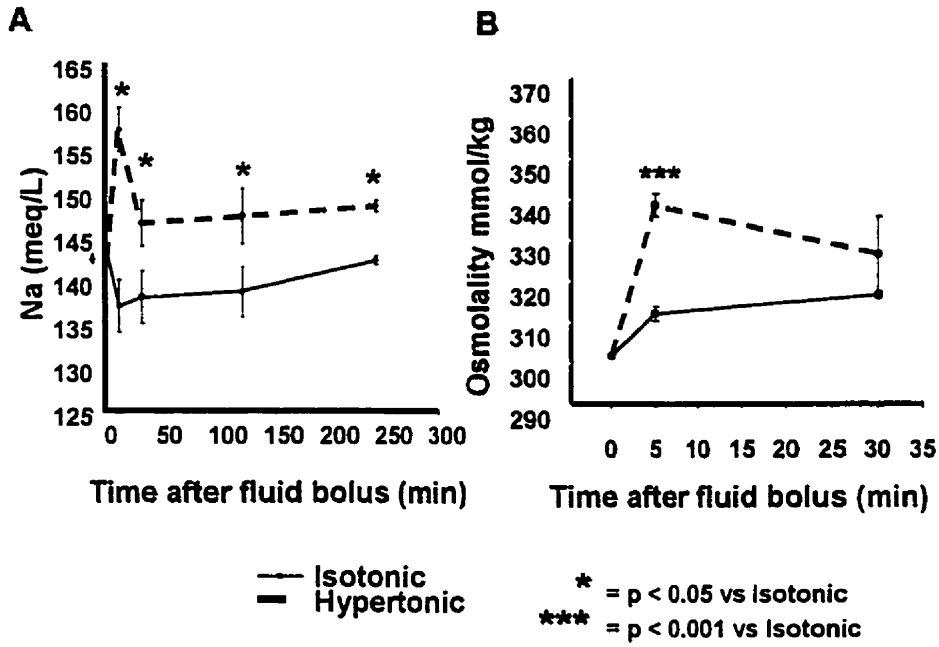

Figure 4: Plasma sodium and osmolality.

Plasma sodium concentration and osmolality was measured from blood obtained by cardiac puncture at various intervals following administration of HTS or isotonic saline. Na concentration (panel A) is expressesed as meq/L and osmolality (panel B) in mmol/kg. At least 3 measurements per group (Isotonic pretreatment, Hypertonic pretreatment) were taken for each time point. * = $p < 0.05$, *** = $p < 0.001$.

Assessment of liver injury

Plasma AST level has been shown to be a sensitive marker of liver injury in the rat (285). We measured plasma AST at the early 30 min (Fig. 5A) and later 4 hr reperfusion (Fig. 5B) time points to determine whether HTS pretreatment exerted any protective effect during the acute, PMN-independent and later, PMN-dependent subacute reperfusion phase.

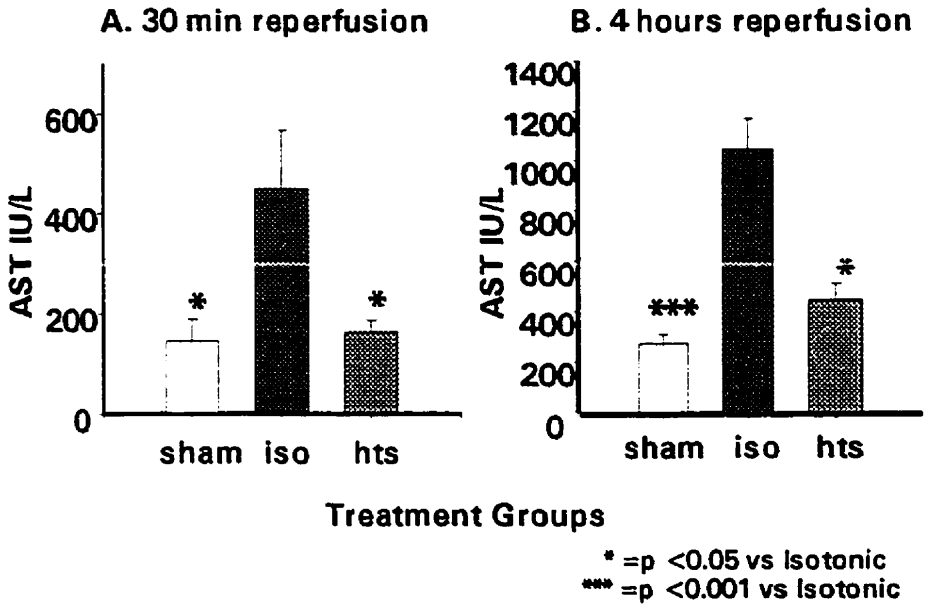

Figure 5: Plasma AST concentrations.

Plasma AST concentration was measured from blood obtained by cardiac puncture at 30 min (A) and 4 hrs (B) reperfusion as an index of hepatic injury. Data are expressed as AST IU/L. The data represent the mean and SEM of at least 5 animals per group. *** =p <0.001 versus isotonic, * =p <0.05 versus isotonic.

I/R following pretreatment with isotonic saline caused an approximate 3-4 fold increase in plasma AST levels compared to sham animals at both time points, indicating the occurence of significant injury following partial I/R. Preconditioning with HTS offered significant protection against I/R at both the early and later time points as indicated by AST levels that were similar to those of the sham group.

Myeloperoxidase (MPO) levels

To determine whether reduced hepatic injury with HTS preconditioning was accompanied by decreased hepatic PMN sequestration, we measured liver MPO, a PMN marker enzyme (Fig. 6).

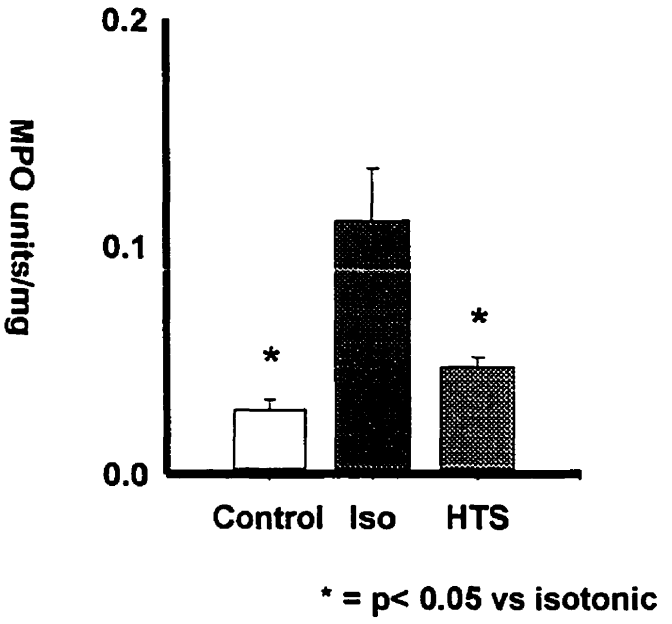

Figure 6: Myeloperoxidase (MPO) activity.

Myeloperoxidase activity as a marker of hepatic PMN infiltration was measured at 4 hrs reperfusion. The data are expressed as MPO units/mg of protein and represent the mean and SEM for at least 8 animals per group. * = $p < 0.05$ versus isotonic.

Baseline MPO in controls (n = 8) was 0.028 ± 0.005 units/mg protein. I/R following isotonic pretreatment caused a significant accumulation of hepatic PMNs as measured by MPO. In contrast, preconditioning with HTS (n = 16) prevented PMN sequestration in the liver with hepatic MPO levels that were no different from control.

Neutrophil CD11b expression

CD11b/CD18 mediates firm adhesion and transmigration of PMNs into the liver following I/R (225). Activation of PMNs by proinflammatory cytokines induces a rapid increase in surface CD11b (226). Thus, CD11b levels are an index of the PMN activation state (227). In order to explain reduced hepatic PMN sequestration and injury observed with HTS, circulating PMN surface CD11b was measured at 30 min reperfusion (Figure 7). Baseline CD11b in controls (n = 4) were 0.785 ± 0.031 MCF units. I/R following isotonic pretreatment (n = 4) significantly increased PMN surface CD11b. In contrast, pretreat-

ment with HTS (n =5) caused a modest but significant inhibition of I/R induced PMN CD11b, indicating that HTS modulates the PMN activation state and decreases PMN adhesion molecule expression.

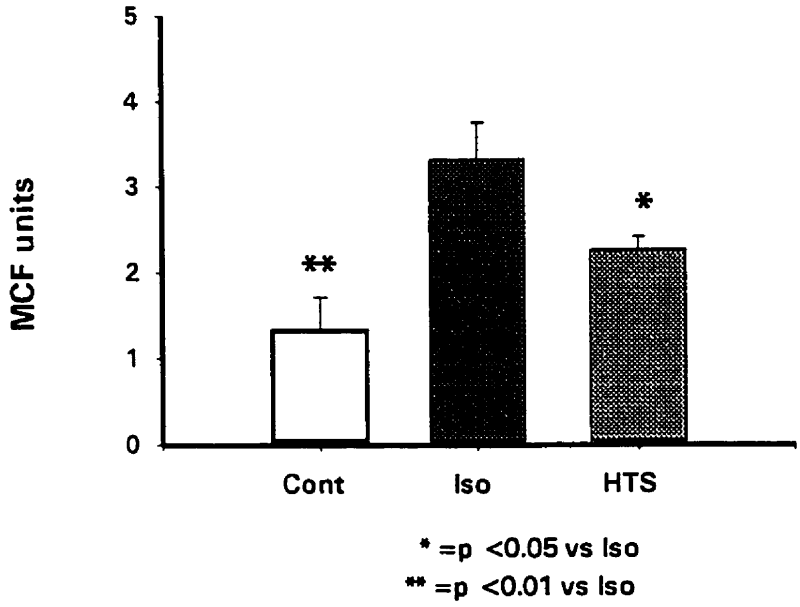

Figure 7: Circulating PMN surface CD11b expression.

Rats were pretreated with isotonic saline (Iso) or HTS and underwent 30 min partial hepatic ischemia 1 hr later followed by 30 min reperfusion when blood was withdrawn for evaluation of PMN CD11b expression by flow cytometry. Control rats did not undergo hepatic I/R. The data are expressed as mean channel fluorescence (MCF) units and represent the mean and SEM of at least 4 animals per group. * =p <0.05 versus isotonic, ** =p <0.01 versus isotonic.

Hepatic adhesion molecule expression

The modest reduction in PMN CD11b by HTS seemed unlikely to account for the marked reduction in hepatic injury and PMN sequestration. An alternative explanation for reduced hepatic PMN accumulation would be the inhibition of hepatic endothelial cell adhesion molecules such as ICAM-1 and VCAM-1, both of which have been shown to be important in the adherence, transmigration, and adhesion-dependent cytotoxicity of PMNs during reperfusion injury (220;278;279). The expression of hepatic ICAM-1 (Figure 8) and VCAM-1 (Figure 9) were examined by measuring whole liver mRNA levels at

30 min and 4 hrs reperfusion by Northern blot analysis. These time points were chosen to examine adhesion molecule expression during the acute phase of reperfusion injury when reactive oxygen species and pro-inflammatory cytokines induce their expression and during the subacute phase of reperfusion injury when they contribute to hepatic PMN sequestration.

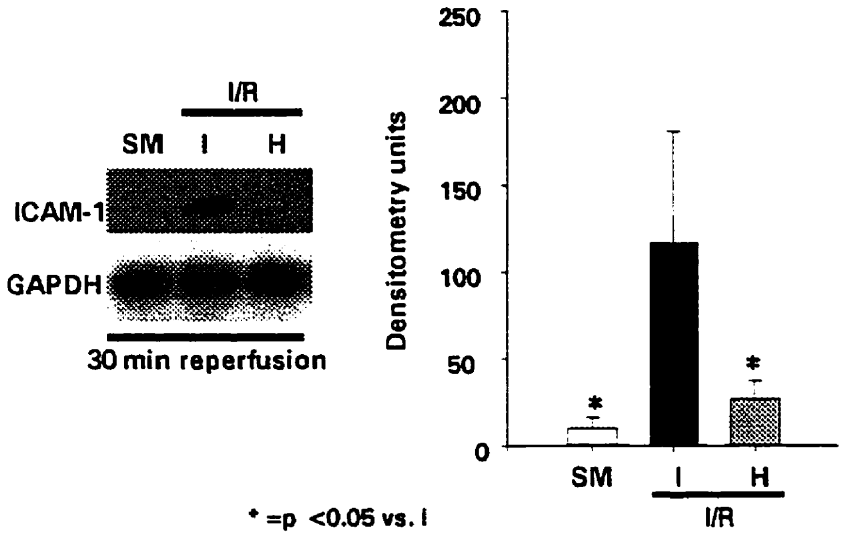

Figure 8: ICAM-1 mRNA expression at 30 minutes reperfusion.

Animals were pretreated with hypertonic (H) or isotonic (I) saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 30 minutes of reperfusion, liver samples were harvested for northern blot analysis for ICAM-1 mRNA. Representative blot for ICAM-1 is displayed on the left. SM =sham, I =Isotonic pretreatment, H =hypertonic pretreatment. The lower panel on the left displays a northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for ICAM-1 mRNA at 30 min reperfusion. Measurements are expressed in arbitrary densitometry units. * =p <0.05 vs. I.

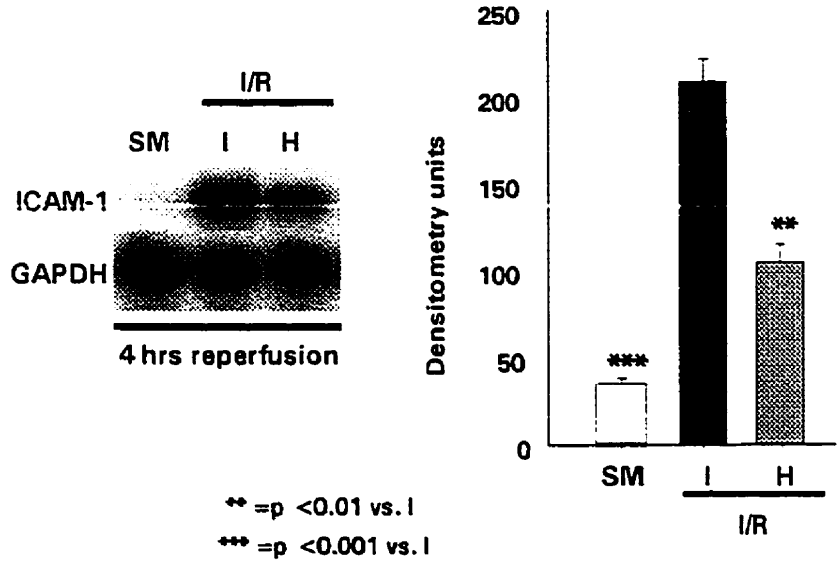

Figure 9: ICAM-1 mRNA at 4 hours reperfusion.

Animals were pretreated with hypertonic (H) or isotonic (I) saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 4 hours of reperfusion, liver samples were harvested for northern blot analysis for ICAM-1 mRNA. Representative blot for ICAM-1 is displayed on the left. SM =sham, I =Isotonic pretreatment, H =hypertonic pretreatment. The lower panel on the left displays a northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for ICAM-1 mRNA at 4 hrs reperfusion. Measurements are expressed in arbitrary densitometry units. ** =p < 0.01 vs. I, *** =p <0.001 vs. I.

Sham animals (SM) did not exhibit any significant induction of ICAM-1 at the early 30 min (Figure 8, blot-upper panel) or later 4 hr (Figure 9, blot-upper panel) time points. ICAM-1 mRNA expression was significantly increased following I/R at both the early and the later time points in animals receiving isotonic (I) pretreatment fluids (Figure 8, 9 respectively). In contrast, HTS pretreatment (H) completely prevented early I/R-induced ICAM-1 mRNA (Figure 8) and significantly reduced its expression at 4 hrs reperfusion (Figure 9). Sample loading was shown to be equal by GAPDH analysis (Figures 8, 9 blot-lower panel).

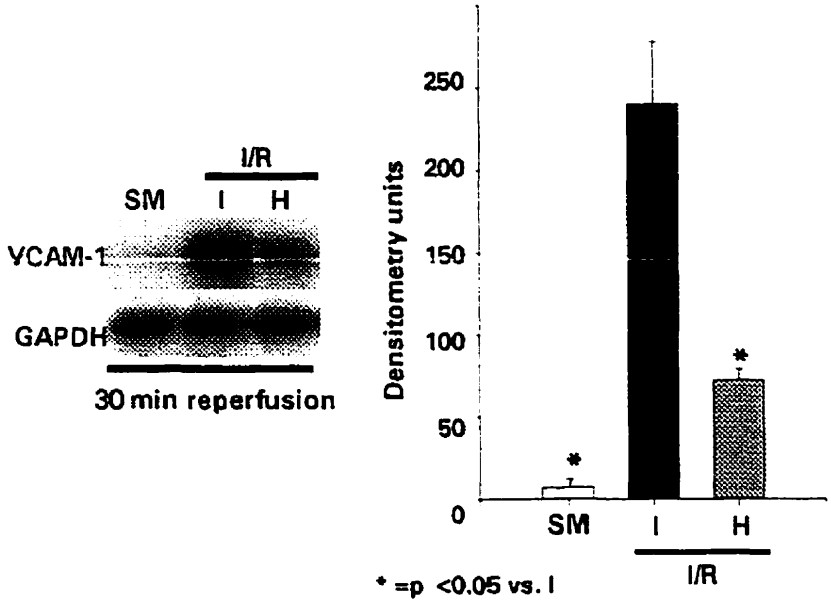

Figure 10: VCAM-1 mRNA at 30 minutes reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 30 minutes of reperfusion, liver samples were harvested for northern blot analysis for VCAM-1 mRNA. Representative blot for VCAM-1 is displayed in the upper panel on the left. SM =sham, I = Isotonic pretreatment, H =hypertonic pretreatment. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for VCAM-1 mRNA at 30 min reperfusion. Measurements are expressed in arbitrary densitometry units. * =p <0.05 vs. I.

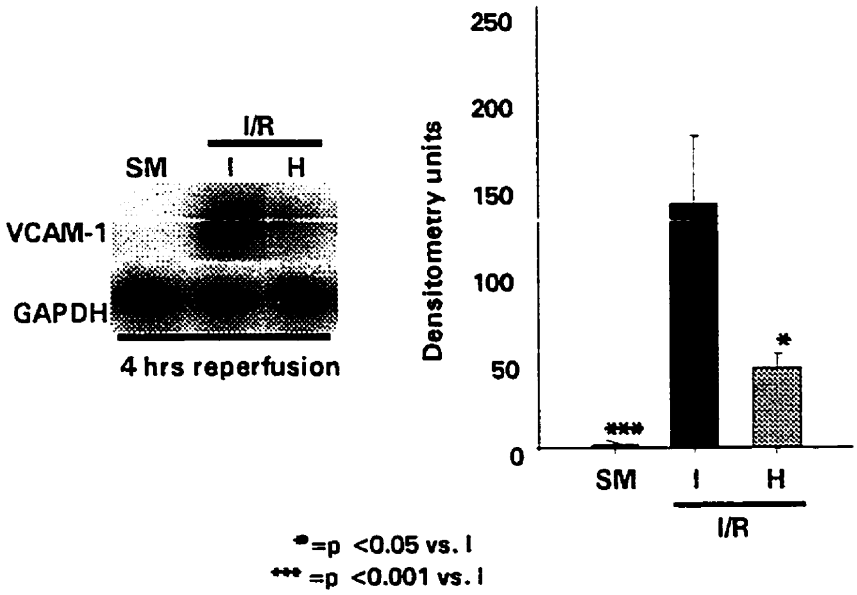

Figure 11: Hepatic VCAM-1 mRNA at 4 hours reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 4 hrs of reperfusion, liver samples were harvested for northern blot analysis for VCAM-1 mRNA. Representative blot for VCAM-1 is displayed in the upper panel on the left. SM =sham, I =Isotonic pretreatment, H =hypertonic pretreatment, I/R =ischemia-reperfusion. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for VCAM-1 mRNA at 4 hrs reperfusion. Measurements are expressed in arbitrary densitometry units. * =p <0.05 vs. I, *** =p <0.001 vs. I.

Minimal levels of VCAM-1 mRNA were detected in sham (SM) animals at 30 min and 4 hrs reperfusion (Figures 10, 11). Similar to ICAM-1, isotonic I/R (I) strongly induced early and subacute phase VCAM-1 mRNA while HTS (H) significantly reduced VCAM-1 mRNA expression at both time points. Sample loading was shown to be equal by GAPDH analysis (Figures 10, 11 blot-lower panel). This data suggests that pretreatment with HTS prevents the expression of endothelial adhesion molecules that are normally induced in early reperfusion. This reduction in PMN and hepatic adhesion molecule expression correlates with reduced hepatic PMN sequestration and injury in the subacute phase of reperfusion injury.

To assess whether changes in ICAM-1 protein expression were mirrored by changes at the protein level, hepatic ICAM-1 protein levels were assessed by Western blot analysis at the 4 hr reperfusion time point, when differences in ICAM-1 protein expression would be expected to translate into differences in hepatic PMN accumulation (Figure 12).

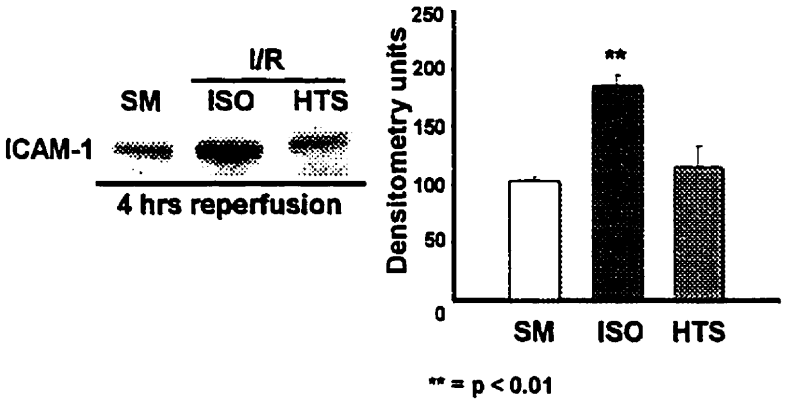

Figure 12: Hepatic ICAM-1 protein expression at 4 hours reperfusion.

Animals were pretreated with hypertonic (HTS) or isotonic (ISO) saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 4 hours of reperfusion, liver samples were harvested for western blot analysis for ICAM-1 protein. Representative blot for ICAM-1 is displayed on the left. SM = sham, ISO = Isotonic I/R, HTS = hypertonic I/R. Sample loading was shown to be comparable between groups by coomassie blue gel staining. The graph on the right displays the densitometric analysis for ICAM-1 protein at 4 hrs reperfusion. Measurements are expressed in arbitrary densitometry units. ** = $p < 0.01$.

In contrast to ICAM-1 mRNA levels, hepatic ICAM-1 protein expression in sham animals was constitutively expressed. Similar to ICAM-1 mRNA levels, isotonic I/R significantly increased hepatic ICAM-1 protein expression as compared to sham animals. HTS preconditioning prevented I/R-induced increases in hepatic ICAM-1 protein expression.

NF-κB translocation

The inducible expression of adhesion molecule genes is largely under the control of the transcription factor NF-κB (253). Thus, NF-κB binding to the ICAM-1 promoter following reperfusion was evaluated to

determine whether HTS pretreatment modulates the activation of this intracellular signaling pathway (Figure 13).

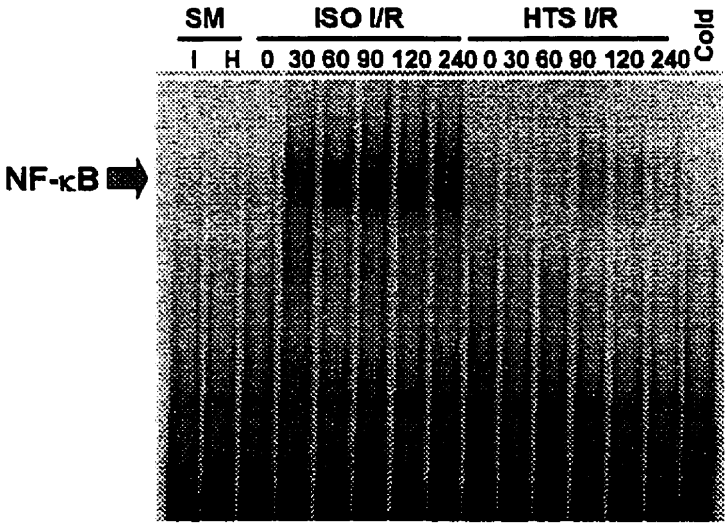

Figure 13: Electromobility shift assay (EMSA) for NF-κB binding to ICAM-1 promoter.

A representative autoradiograph of EMSA is shown demonstrating NF-κB binding to κB consensus sequence of ICAM-1 promoter region. Liver tissue was obtained from isotonic and hypertonic saline pretreated sham animals (SM I, SM H) as well as HTS (HTS I/R) or isotonic (ISO I/R) saline pretreated animals following ischemia and 0-240 min reperfusion. The probe for EMSA was a ^{32}P-ATP-end labeled double-strand construct corresponding to a sequence in the ICAM-1 proximal promoter region containing the NF-κB motif. Cold competition using an excess of unlabelled probe is also shown for the 60 min ISO I/R sample on the right.

Sham operated animals that had received isotonic saline or HTS Reperfusion of isotonic I/R animals increased NF-κB binding to the ICAM-1 promoter. In contrast, I/R-induced NF-κB binding to the ICAM-1 promoter was prevented by hypertonic pretreatment. Controls using cold and mutant probes for specific and non-specific competition revealed the specificity of the ICAM-1 oligonucleotide. These results suggest an ability of HTS to modulate the activation of intracellular signaling pathways responsible for hepatic endothelial adhesion molecule expression.

TNF-α expression

TNF-α is an important proximal mediator of hepatic I/R injury. The major source of hepatic TNF is the resident hepatic macrophage (Kupffer cell). This proinflammatory cytokine acts in an autocrine and paracrine manner to activate neighboring ECs and hepatocytes with the initiation of intracellular signaling events such as NF-κB translocation. TNF-α also contributes to a cytokine-mediated injury in the early phase of reperfusion through the induction of hepatic apoptosis (132;276). To determine whether HTS pretreatment modulates proinflammatory cytokine expression following hepatic I/R as one mechanism for reduced hepatic adhesion molecule expression, TNF-α mRNA levels were assessed by Northern blot analysis of 30 and 60 min reperfusion liver samples (Figures 14, 15 respectively).

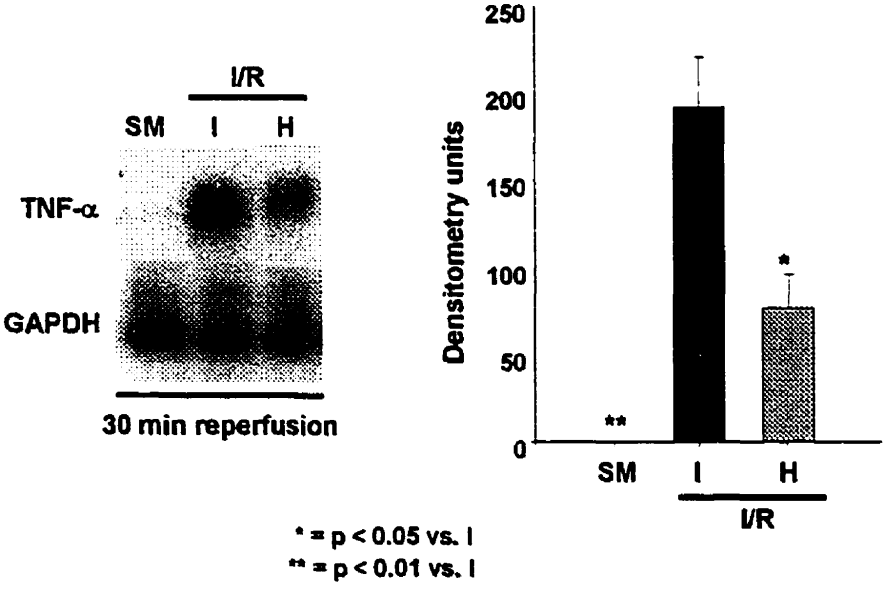

Figure 14: Hepatic TNF-α mRNA expression at 30 minutes reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 30 minutes of reperfusion, liver samples were harvested for northern blot analysis for TNF-α mRNA. Representative blot for TNF-α is displayed in the upper panel on the left. SM = sham, I = Isotonic pretreatment, H = hypertonic pretreatment. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for TNF-α mRNA at 30 min reperfusion. Measurements are expressed in arbitrary densitometry units. * = $p < 0.05$ vs. I, ** = $p < 0.01$ vs. I.

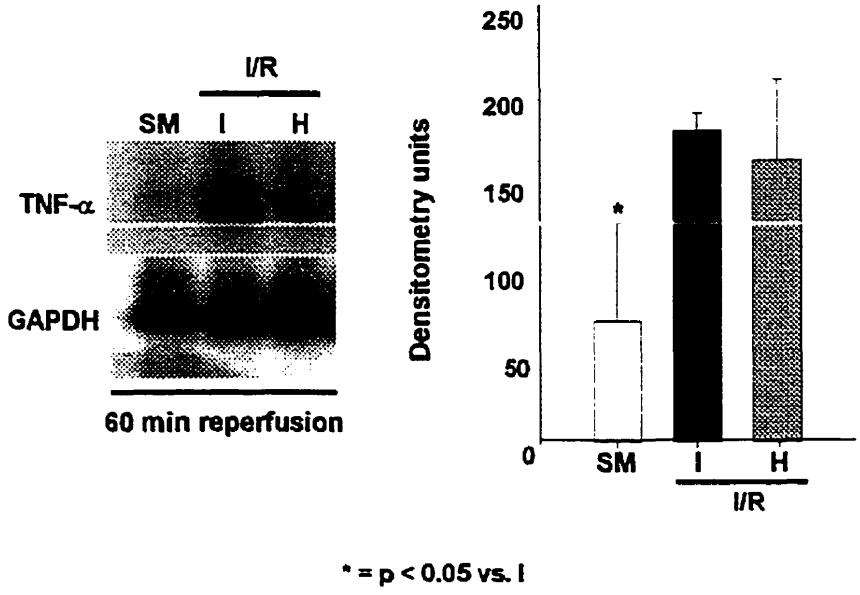

Figure 15: Hepatic TNF-α mRNA at 60 minutes reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 60 minutes of reperfusion, liver samples were harvested for northern blot analysis for TNF-α mRNA. Representative blot for TNF-α is displayed in the upper panel on the left. SM = sham, I = Isotonic pretreatment, H = hypertonic pretreatment. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for TNF-α mRNA at 60 min reperfusion. Measurements are expressed in arbitrary densitometry units. * = $p < 0.05$ vs. I.

TNF-α mRNA expression was minimal in the sham group (SM), while I/R induced a significant increase in TNF-α mRNA at both the 30 and 60 min time points in isotonic pretreated (I) animals. Administration of HTS to sham operated animals did not induce TNF-α expression (data not shown). HTS pretreatment (H) reduced I/R-induced TNF-α expression at the 30 min time point compared to isotonic animals (Figure 14). This difference in I/R-induced hepatic TNF-α mRNA levels was not present at the 60 min reperfusion time point, with similar levels of TNF-α mRNA expression between I and H groups (Figure 15, densitometry graph).

To determine whether early differences in TNF mRNA expression between HTS and isotonic pretreated animals correlated with similar changes at the protein level, TNF-α protein expression was assessed at the 2 hr reperfusion time point by ELISA (Figure 16).

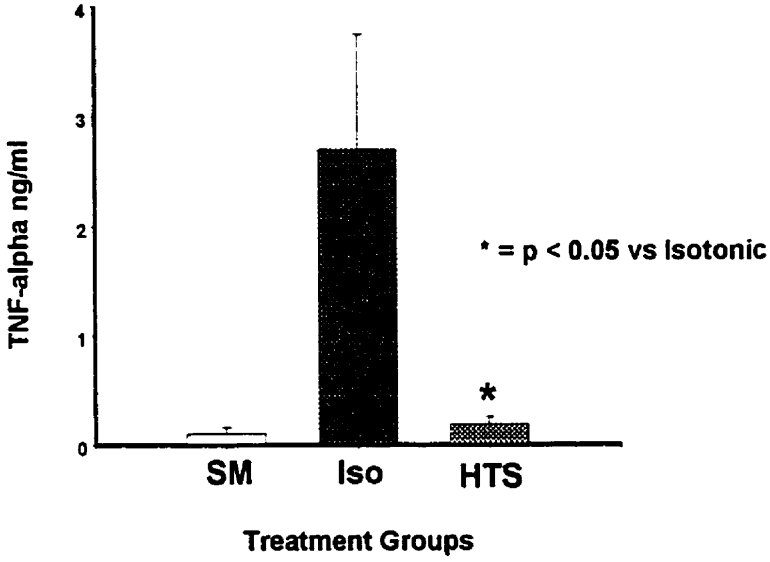

Figure 16: Hepatic TNF-α protein expression at 2 hours reperfusion.

Rats were pretreated with 4cc/kg of hypertonic (H) or isotonic (I) saline one hour before 30 minutes of warm, partial hepatic ischemia. Following 120 minutes of reperfusion, ischemic lobes were harvested and tissue lysates subjected to ELISA for TNF-α. Sham (SM) animals received isotonic saline but did not undergo ischemia-reperfusion. Sham livers were harvested at identical time points to the I/R groups. Data shown are represent the mean and SEM of 3 animals per group and are expressed as ng/ 4 mg of liver tissue of TNF-α. * = $p < 0.05$ versus isotonic.

TNF-α in shams (n= 4) was 0.104 ± 0.058 ng/ 4 mg hepatic tissue. I/R significantly increased hepatic TNF-α protein levels in isotonic pretreated rats (Iso) (n= 6). However, HTS pretreatment (HTS) (n= 6) prevented the I/R-induced increase in TNF-α protein seen in the isotonic I/R animals. There was no significant difference in TNF-α levels between HTS and sham (SM) groups ($p>0.05$). These results suggest that HTS-mediated differences in TNF-α mRNA expression in early reperfusion are amplified at the protein level at later time points. Furthermore, the ability of HTS pretreatment to modulate hepatic pro-

inflammatory cytokine expression suggests that HTS may alter the response of hepatic Kupffer cells to I/R.

Reversal of HTS protective effect by exogenous TNF-α administration

Early reductions in hepatic TNF-α mRNA levels correlated with reduced hepatic TNF-α protein expression following I/R in HTS preconditioned animals. To determine whether the inhibition of hepatic TNF-α expression plays a direct role in HTS' protective effect against I/R injury, exogenous TNF-α (15 μg/kg) was administered intra-arterially to HTS preconditioned rats (n = 3) at the time of reperfusion and serum AST levels were determined at the 4 hr reperfusion time point (Figure 17).

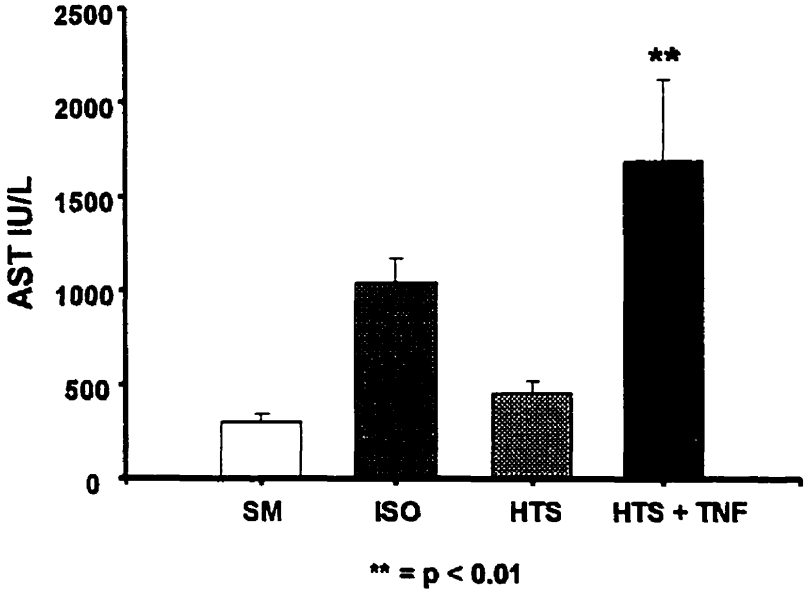

Figure 17: Reversal of HTS protective effect by exogenous TNF-α administration.

Rats were pretreated with isotonic saline (ISO) or HTS and underwent 30 min partial hepatic ischemia 1 hr later followed by 4 hrs of reperfusion when blood was withdrawn for evaluation of serum AST as a marker of liver injury. Recombinant rat TNF-α (15 μg/kg ia.) was administered at the time of reperfusion to HTS preconditioned rats (HTS + TNF) to determine the role of TNF-α inhibition in HTS protection against hepatic I/R injury. SM = sham operated rats that had received isotonic saline pretreatment. ** = $p < 0.01$.

Systemic administration of exogenous TNF-α to HTS preconditioned I/R rats reversed the protective effect of HTS on liver injury as assessed by serum AST levels ($p < 0.01$ vs. HTS). There was no signifi-

cant difference in serum AST between isotonic I/R (ISO) and HTS I/R + TNF-α (HTS + TNF) groups. These results suggest an important role for the early inhibition of hepatic TNF-α expression in protection against I/R-induced liver injury by HTS preconditioning.

Hepatic apoptosis

Programmed cell death, or apoptosis contributes to liver injury following I/R (270;274). This process is mediated by TNF-α (269). With reduced hepatic expression of pro-apoptotic TNF-α following I/R in HTS pretreated animals, hepatic apoptosis was measured to determine whether reduced apoptosis could be another protective mechanism of HTS preconditioning. DNA fragmentation, detected by a 'ladder-ing' pattern in gel-electrophoresis is a relatively late marker of apoptosis. Thus, DNA fragmentation analysis of post-ischemic liver tissue was carried out following 8 hrs reperfusion (Figure 18).

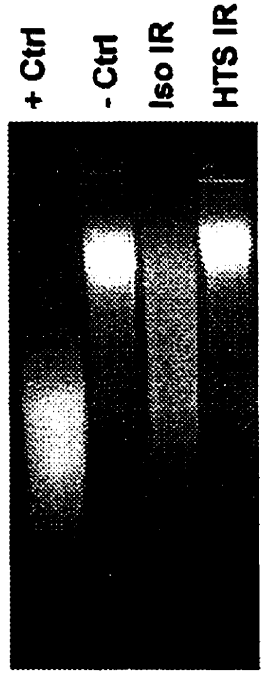

Figure 18: Hepatic DNA fragmentation analysis.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following an 8 hr reperfusion period, ischemic lobes were harvested and subjected to DNA fragmentation analysis as an indication of apoptosis. + Ctrl = DNA laddering pattern of HL-60 cells rendered apoptotic by retinoic acid treatment; - Ctrl = DNA from livers of control rats; Iso IR = DNA from 8 hr reperfusion ischemic lobes of rats pretreated with isotonic saline; HTS IR = DNA from 8 hr reperfusion ischemic lobes of rats pretreated with HTS. Shown is a representative gel from 3 independent experiments.

DNA fragmentation was evident in the pattern of DNA laddering seen with retinoic acid treated (apoptotic) HL-60 cells (+Ctrl). DNA laddering was not present in the livers of control rats (-Ctrl). Isotonic I/R caused significant DNA fragmentation (Iso IR). HTS prevented I/R-induced DNA fragmentation (HTS IR), with a gel pattern similar to that of the negative control.

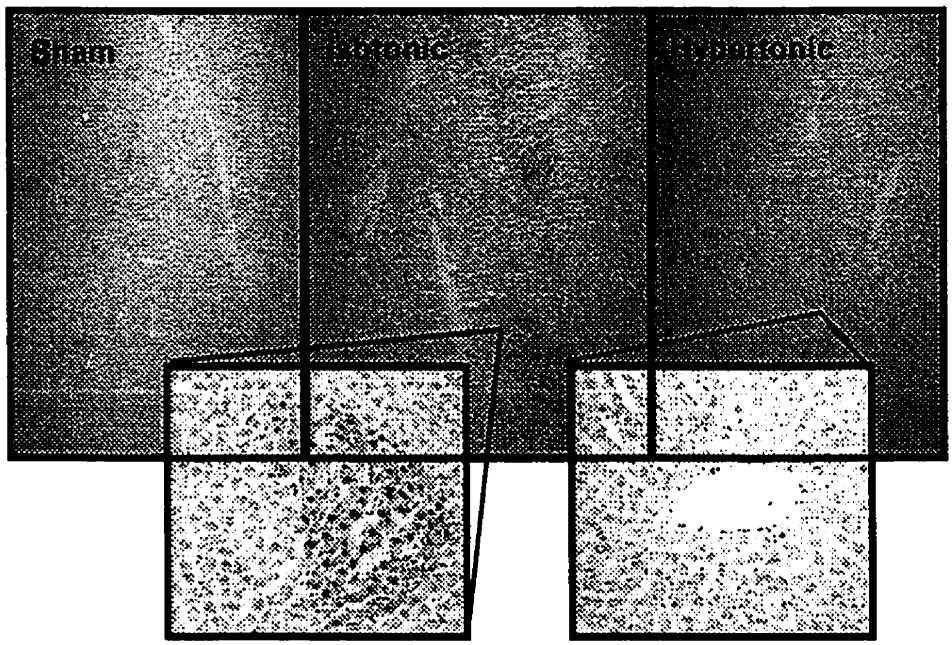

Figure 19: In-situ end labelling for apoptosis.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 4 hrs reperfusion, ischemic lobes were harvested and sections embedded in paraffin for in-situ end labeling of fragmented DNA in apoptotic cells. Left panel- representative section from sham rat as seen under low power magnification; middle panel- representative section from isotonic I/R ischemic lobe as seen under low power magnification with positively-stained hepatocytes, inset shows view as seen under high power magnification; right panel- representative section from hypertonic I/R ischemic lobe as seen under low power magnification, inset shows view as seen under high power magnification.

To confirm this protective effect of HTS in modulating hepatic I/R-induced apoptosis, we used an in-situ end labeling technique on hematoxylin and eosin stained sections of 4 hr reperfusion ischemic lobes (Figure 19). Sham livers (n =4) exhibited no positively stained cells for apoptosis (Figure 19, left panel) whereas isotonic I/R (n =3) induced a patchy distribution of apoptotic hepatocytes representing 5-10%

of their total (Figure 19, middle panel). Sections from ischemic lobes of HTS treated animals (n =3) showed only occasional apoptotic cells, representing <1% of the total number of hepatocytes (Figure 19, right panel).

Caspase 3 activity

With apoptosis, proteinases are activated, including those belonging to the caspase family (255). Caspases mediate cell death by cleaving essential substrates. Caspase 3 belongs to the 'executioner' subtype of caspases that cleave specific death substrates including poly(ADP-ribose) polymerase (PARP). Thus, caspase 3 activation is considered a major effector of apoptosis. Caspase 3 activity was measured at the 4 hr reperfusion time point to determine whether HTS modulates the activity of this major pro-apoptotic pathway (Figure 20).

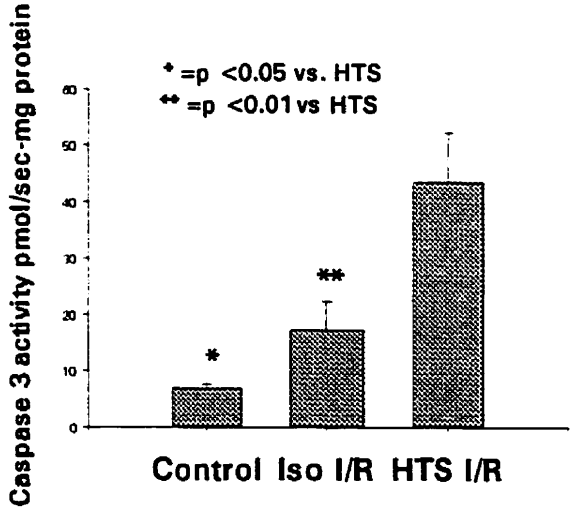

Figure 20: Post-ischemic lobe 4 hour reperfusion caspase 3 activity.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 4 hrs reperfusion, ischemic lobes were harvested and hepatic caspase 3 activity was measured by means of a fluorometric assay. Control liver samples, n =3; Isotonic I/R (Iso I/R), n =4; Hypertonic I/R (HTS I/R), n =3. * =p <0.05 vs. HTS, ** =p <0.01 vs. HTS.

Livers from control animals exhibited low levels of caspase 3 activity (6.95 ± 1.31 caspase 3 activity units/mg protein). I/R under isotonic pretreatment conditions increased caspase 3 activity 2-3 times above that of control. I/R following HTS pretreatment conditions increased caspase 3 activity approximately 2.5 times greater than that seen with isotonic I/R.

These results are unexpected given that HTS reduced hepatic I/R-induced apoptosis as measured by two separate methods (DNA fragmentation and in-situ end labeling of fragmented DNA). To confirm that results obtained using the caspase 3 assay were secondary to cleavage of the substrate by caspase 3 and did not represent non-specific substrate cleavage secondary to other caspases or proteases present in the tissue homogenate, the experiment was repeated and caspase 3 activity was measured using a specific, fluorometric immunosorbent enzyme assay (FIENA) technique (Figure 21).

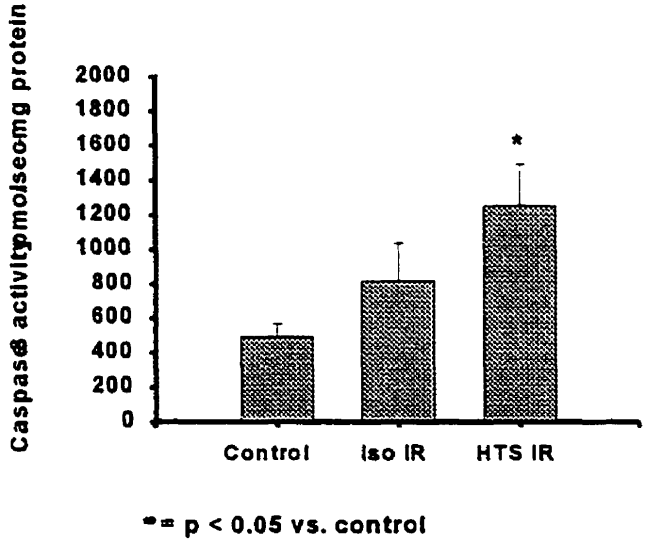

Figure 21: Post-ischemic lobe 4 hour reperfusion caspase 3 activity measured by FIENA technique.
Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 4 hrs reperfusion, ischemic lobes were harvested and hepatic caspase 3 activity was measured by means of a fluorometric immunosorbent enzyme assay (FIENA). Control liver samples, n = 3; Isotonic I/R (Iso IR), n = 3; Hypertonic I/R (HTS IR), n = 3. * = p < 0.05 vs. control.

Using the second, more specific assay, a similar pattern of caspase 3 activity was obtained, suggesting that the elevated caspase 3 activity measured in the previous assay was in fact secondary to cleavage of the substrate by caspase 3. As caspase 3 activation in most models of inflammatory liver injury results in the induction of apoptosis, these results suggest a downstream blockade of the caspase 3 pathway by hypertonicity, or the upregulation of anti-apoptotic molecules by HTS. Alternatively, elevated hepatic caspase 3 activity in HTS pretreated animals represents a delay in I/R-induced caspase 3 activation as compared to isotonic saline pretreated I/R animals that may have been missed by measuring caspase 3 activity at the 4 hr reperfusion time point.

JNK and p38 activation

As hepatic apoptosis is increased in isotonic I/R animals, a role for a pro-apoptotic, caspase 3-independent pathway is implicated. Both JNK and p38 MAPKs have been implicated in the induction of apoptosis in other models of regional I/R injury (247;286). As HT also activates these pathways, HTS could activate one or both of them, rendering them refractory to activation by I/R, preventing I/R-induced apoptosis. Thus, JNK and p38 activation were examined by western blot analysis for the active, phosphorylated forms of JNK (Fig. 22, upper panel) and p38 (Fig. 22, middle panel) using phospho-specific antibodies to probe liver tissue harvested from sham, isotonic I/R (Iso I/R), and hypertonic I/R (HTS I/R) animals at 0-90 min. Sample loading was assessed by measurement of unphosphorylated JNK1 protein (Fig. 22, lower panel).

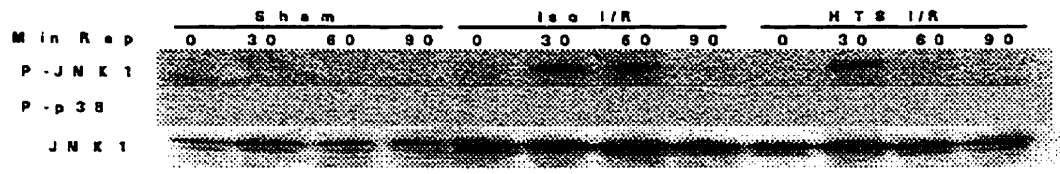

Figure 22: Hepatic JNK and p38 activation following I/R.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 0-90 min of reperfusion, liver samples were harvested for western blot analysis for phospho-JNK or phospho-p38 protein. Representative blots from 3 independent experiments for P-JNK1, P-p38, and JNK1 are displayed. Sham, Iso I/R =Isotonic pretreatment, HTS I/R =HTS pretreatment.

P38 was not activated at any time between 0-90 min in sham or I/R treatment groups. JNK was activated in isotonic I/R animals within 30 min reperfusion and then decreased over the subsequent 60 min, consistent with previous published reports of JNK activation following hepatic I/R (244). Hepatic I/R following HTS pretreatment resulted in a lower level and more transient activation of JNK, suggesting that HTS modulates JNK activation following hepatic I/R.

Apoptosis of circulating PMNs

The ability of HTS to inhibit I/R-induced hepatocyte apoptosis as shown by TUNEL staining provides evidence to support a role for transient HT in modulating apoptosis. Hypertonic glucose solutions are known to induce apoptosis in PMNs (287). Thus, HTS may differentially modulate apoptosis in a cell type specific manner. We hypothesized that an alternative explanation for reduced hepatic PMN accumulation and injury may be the induction of apoptosis of circulating PMNs. PMN apoptosis was therefore assessed by annexin-V binding. Annexin-V binds to misaligned phosphatidyl-serine residues on the surface of apoptotic cells. Circulating PMNs were isolated from blood withdrawn from rats at the 4 hr reperfusion time point and annexin-V binding was assessed using flow cytometric analysis (Figure 23). Treatment groups included unmanipulated control rats (CTRL, n =6), sham rats (SM, n =8), isotonic I/R animals (ISO, n =9), and HTS I/R rats (n =10).

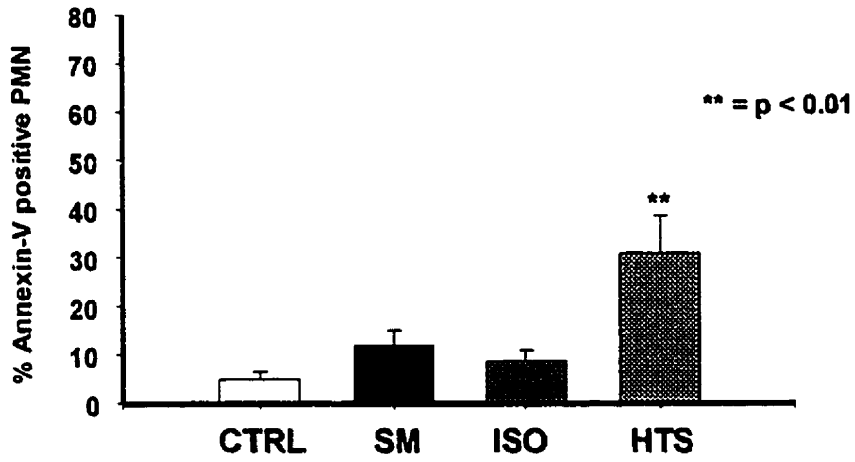

Figure 23: Circulating PMN apoptosis.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 4 hrs of reperfusion, PMNs were harvested from blood samples and annexin-V binding was assessed by flow cytometry. Treatment groups include control rats (CTRL, n = 6), sham rats (SM, n = 8), isotonic I/R animals (ISO, n = 9), and HTS I/R rats (n = 10). Data are expressed as % Annexin-V positive PMNs. ** = $p < 0.01$.

PMNs isolated from control rats exhibited low levels of spontaneous apoptosis (5.0 ± 3.9 % annexin-V positive). PMNs from sham and isotonic I/R had similar levels of apoptosis (11.8 ± 8.7 and 8.6 ± 6.8 % annexin-V positive respectively, $p > 0.05$). Circulating PMNs from I/R rats that had undergone HTS pretreatment 5.5 hours earlier had the highest levels of apoptosis (31.0 ± 24.2 % annexin-V positve). These results suggest an ability of a single agent (HTS) to differentially modulate apoptosis according to cell type in a manner that preserves organ function (reduced hepatocyte apoptosis) and reduces cell-mediated injury (increased PMN apoptosis) following I/R injury.

Induction of endogenous cellular defenses by transient exposure to hypertonicity

Exposure to a transient, sublethal stress induces intracellular protective systems that may inhibit cellular responses to subsequent stimuli. This process is exploited in ischemic preconditioning and heat shock

preconditioning when an initial exposure to transient, sublethal environmental stressors such as a short period of I/R or heat, induce the expression of a characteristic set of protective proteins that are capable of mitigating the injury resulting from a second, normally lethal stress such as I/R injury. To examine whether HTS preconditioning induces anti-inflammatory molecules that may contribute to its protective effects in hepatic I/R, 3 different anti-inflammatory/protective molecules were assessed: Heat shock protein 70 (HSP 70), Heme oxygenase-1 (HO-1), and Interleukin-10 (IL-10).

HSP 70 expression

Members of the HSP-70 family are thought to act as 'molecular chaperones' by stabilizing protein structure following cellular insults such as I/R, allowing them to refold and for cellular homeostasis to be restored. HT has been shown to induce HSP 72 in hepatocytes (123). Thus, HSP 70 protein expression was assessed by western blot analysis to determine whether pretreatment with HTS induced an earlier expression of HSP 70 during reperfusion as compared to isotonic I/R that may protect against early injury events (Figure 24).

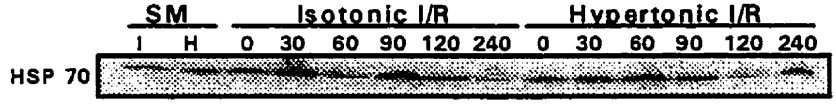

Figure 24: Hepatic Heat shock protein 70 (HSP70) expression following I/R.

Representative western blot analysis for HSP 70 protein. Liver tissue from sham animals treated with isotonic saline or HTS (30 min time point), as well as HTS and isotonic I/R animals at 0, 30, 60, 90, 120, 240 min reperfusion were subjected to western blot analysis and probed with antibody against HSP 70.

Isotonic and hypertonic sham animals (SM I, SM H) exhibited low levels of HSP 70 by western blot. I/R increased HSP 70 protein expression in both the isotonic and HTS I/R groups that was most pronounced between 0-90 min reperfusion. There were no significant differences in hepatic HSP 70 protein expression between isotonic and hypertonic I/R groups.

HO-1 expression

Heme oxygenase (HO) generates carbon monoxide (CO) and bilirubin in the degradation of heme. CO is a gaseous molecule that mediates hepatic sinusoidal relaxation and maintains sinusoidal perfusion pressure following hepatic I/R (157). Bilirubin is an endogenous antioxidant that may prevent reperfusion-induced oxidant damage (171). Heme oxygenase-1 (HO-1), also known as HSP 32 is the stress-inducible form of HO. HO-1 induction occurs with I/R and also following HT exposure (121). Thus, hepatic HO-1 protein expression following I/R was measured to determine whether HTS modulates levels of HO-1. Western blot analysis of liver tissue from was performed using an antibody against rat HO-1 (Figure 25). HO-1 expression was first examined during the 1 hr preconditioning period after isotonic saline or HTS infusion (Figure 25 A) to determine whether HTS infusion alone alters hepatic HO-1 expression. At 1 hr reperfusion, hepatic HO-1 expression was determined to examine the combined effects of preconditioning and I/R on HO-1 levels (Figure 25 B).

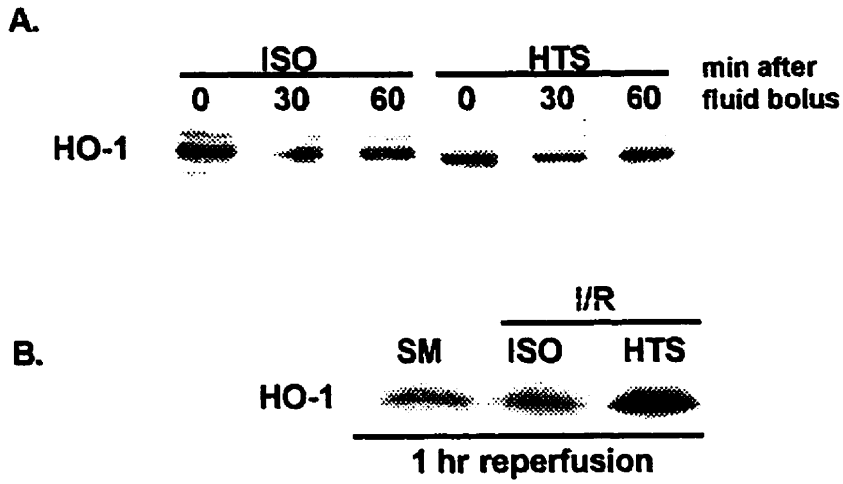

Figure 25: Hepatic HO-1 protein expression.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia 1 hr later. Following 60 minutes of reperfusion, liver samples were harvested for western blot analysis for HO-1 protein. Representative blot for HO-1 are displayed. In panel A, HO-1 expression is determined immediately, 30 min, or 60 min after isotonic saline or HTS infusion. In panel B, HO-1 expression at the 1 hr reperfusion time point is displayed. 50 μg of tissue lysate were loaded per sample and equal loading confirmed with coomassie blue staining. SM = sham, I = Isotonic pretreatment, H = hypertonic pretreatment, I/R = ischemia-reperfusion.

During the preconditioning period (ie- for the first hr after isotonic saline or HTS infusion, prior to laparotomy and I/R), liver samples from both isotonic saline and HTS pretreated animals displayed equal levels of constitutive HO-1 expression (Figure 25 A). Western blot analysis of liver tissue from animals sacrificed at 1 hr reperfusion demonstrated that isotonic sham animals have low levels of hepatic HO-1 protein. Analysis of post-ischemic lobes from isotonic pretreated animals (I/R, ISO) reveals HO-1 protein levels similar to that of sham at 1 hr reperfusion whereas HTS I/R (I/R, HTS) increased HO-1 expression in post-ischemic lobes. Analysis of later reperfusion time points showed a more variable pattern of HO-1 protein expression with no obvious differences between HTS and Iso I/R treatment groups for

the duration of the protocol (data not shown). These results suggest that HO-1 protein expression is augmented in HTS preconditioned animals in the first hour of reperfusion following hepatic I/R.

To determine the role of this differential expression of HO-1 in early reperfusion in the protective effect of HTS on hepatic I/R, we made use of the selective HO-1 inhibitor, zinc-protophorphyrin-IX (ZnPP). Rats were pretreated with ZnPP (20 mg/kg ip.) 24 hrs prior to their use in the hepatic I/R protocol. Rats were pretreated as usual with 4cc/kg ia. of HTS or isotonic saline, and were subjected to partial hepatic ischemia 1 hr later. After a 30 min ischemic period, rats were reperfused for 4 hrs when they were sacrificed, and blood for serum AST was taken (Figure 26). Treatment groups included: sham (SM, n =4), ZnPP pretreated sham (Zn-SM, n =2), isotonic I/R (Iso IR, n =14), ZnPP pretreated rats who received isotonic saline before I/R (Zn Iso IR, n =5), HTS pretreated I/R animals (HTS IR, n =8), and ZnPP pretreated rats who received HTS before I/R (Zn HTS IR, n =7).

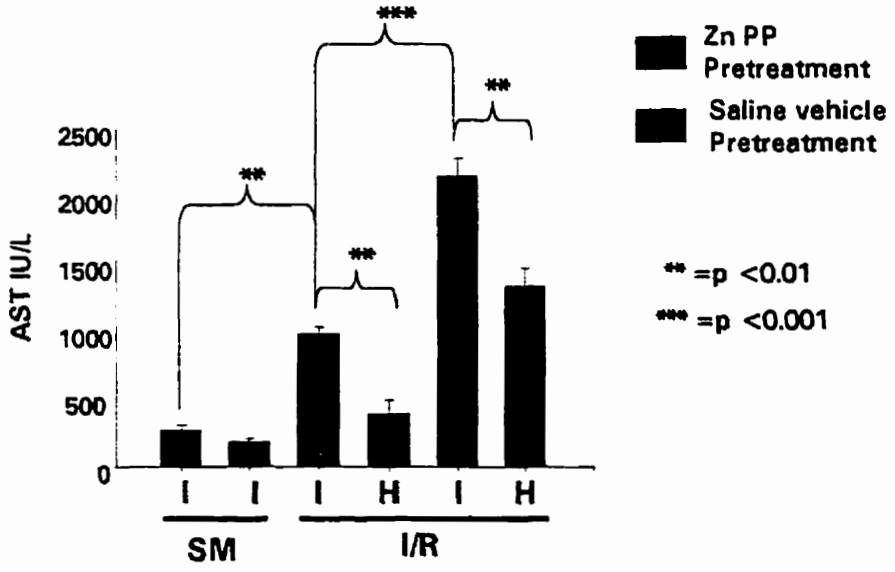

Figure 26: Role of HO-1 in protection from I/R by HTS pretreatment.

To determine the role of augmented early reperfusion HO-1 protein expression in the protection from hepatic I/R injury, rats were pretreated with the selective HO-1 inhibitor, zinc protoporphyrin IX (ZnPP) 24 hrs before hepatic I/R protocol and serum AST was measured after 4 hours reperfusion with or without HO-1 inhibition, with hyper-

tonic (H) or isotonic (I) pretreatment. Treatment groups: sham (SM, n =4), ZnPP pretreated rats are represented by hatched bars. Rats treated with intraperitoneal saline vehicle 24 hrs before I/R are shown in black. I/R =ischemia-reperfusion injury groups. ** =p <0.01 , *** =p <0.001.

ZnPP pretreatment of sham animals did not increase serum AST levels, indicating that ZnPP by itself does not induce liver injury. Pretreatment of isotonic I/R animals with ZnPP more than doubled liver injury as measured by serum AST compared to isotonic I/R without ZnPP, indicating the importance of HO-1 activity in preventing I/R induced liver injury. Pretreatment of HTS I/R animals with ZnPP increased liver injury when compared to HTS I/R animals alone, but still prevented liver injury when compared to ZnPP-pretreated animals who received isotonic saline prior to hepatic I/R, suggesting that while HTS preconditions for augmented HO-1 in early reperfusion, mechanisms other than HO-1 induction are likely responsible for HTS-mediated protection from hepatic I/R injury.

IL-10 expression

IL-10, a potent anti-inflammatory cytokine has been shown to suppress the production of proinflammatory molecules such as TNF and ICAM-1, and prevent PMN activation (192). As the effects of IL-10 are similar to observed effects of HTS *in-vivo*, hepatic IL-10 expression was assessed by Northern blot analysis for IL-10 mRNA at 30 and 60 min reperfusion (Figure 27, 28) and IL-10 protein levels were measured by western blot analysis at 60 min reperfusion (Figure 29). IL-10 mRNA expression was not increased in Sham (SM) animals until the 60 min time point (Figure 28). However, HTS pretreatment (H) significantly increased IL-10 mRNA at both 30 and 60 min reperfusion. Compared to HTS pretreated animals, IL-10 mRNA expression in isotonic I/R animals (I) was significantly delayed, being almost absent at 30 min reperfusion (Figure 27). By 60 min reperfusion, IL-10 mRNA expression in isotonic I/R livers was comparable to that of HTS pretreated animals. IL-10 mRNA was not expressed in sham animals that received HTS (data not shown). Notably, the early differences in hepatic IL-10 mRNA expression between isotonic and HTS pretreated animals at 30 min reperfusion were correlated with differences at the protein level when IL-10 was assessed by western blot analysis at 60 min reperfu-

sion (Figure 29). Hepatic IL-10 protein was augmented in HTS I/R animals (H) compared to isotonic I/R (I) rats. Thus, HTS increases the hepatic expression of the potent anti-inflammatory cytokine, IL-10 following I/R. These results suggest an ability of HTS to shift the profile of cytokine expression in early reperfusion away from I/R-induced TNF expression towards augmented anti-inflammatory IL-10 as a novel mechanism that may be contribute to reduced- TNF, endothelial adhesion molecule expression, PMN sequestration, apoptosis, and injury.

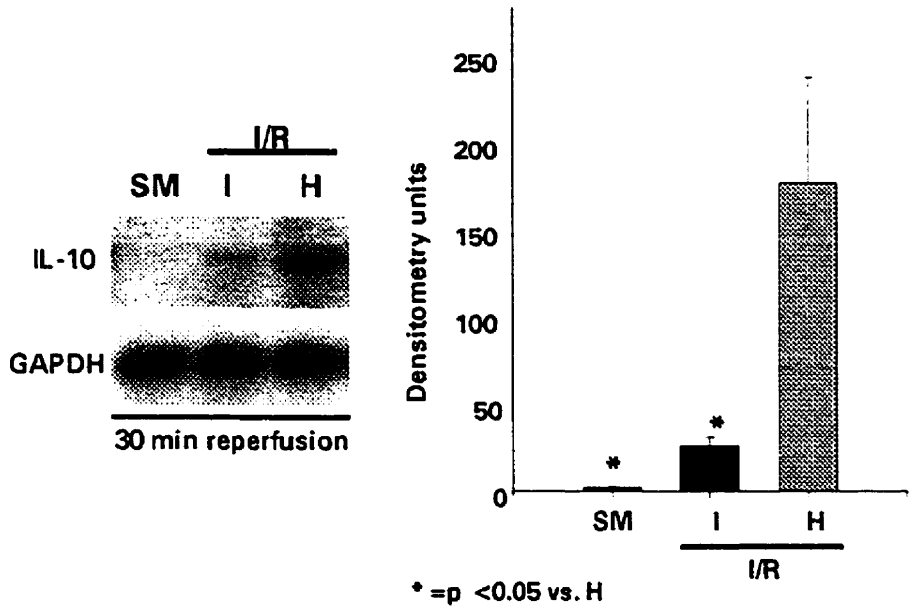

Figure 27: Hepatic IL-10 mRNA expression at 30 minutes reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 30 minutes of reperfusion, liver samples were harvested for northern blot analysis for IL-10 mRNA. A representative blot for IL-10 is displayed in the upper panel on the left. SM =sham, I =Isotonic pretreatment, H =hypertonic pretreatment. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for IL-10 mRNA at 30 min reperfusion. Measurements are expressed in arbitrary densitometry units. * =p < 0.05 vs. I.

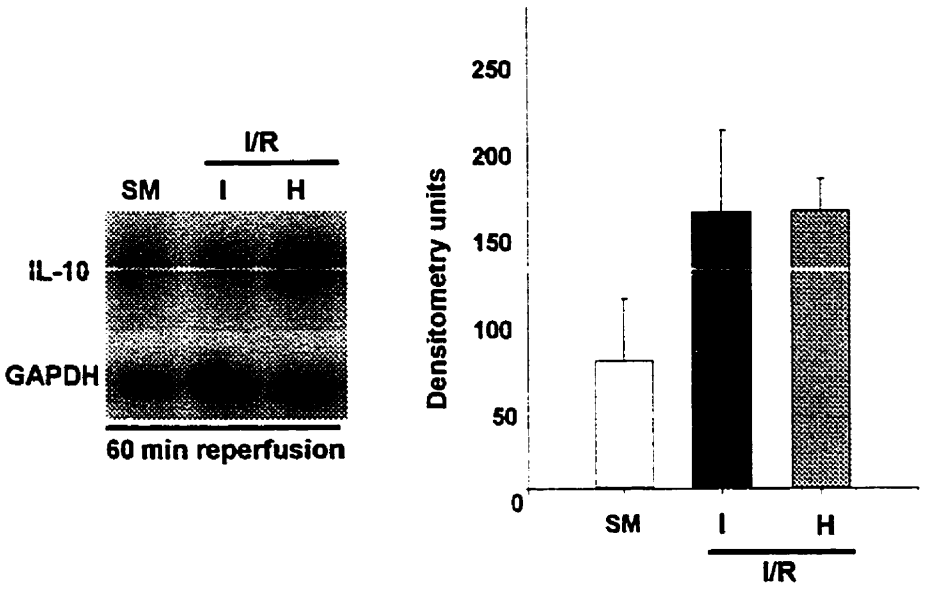

Figure 28: Hepatic IL-10 mRNA expression at 60 minutes reperfusion.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 60 minutes of reperfusion, liver samples were harvested for northern blot analysis for IL-10 mRNA. A representative blot for IL-10 is displayed in the upper panel on the left. SM = sham, I = Isotonic pretreatment, H = hypertonic pretreatment. The lower panel displays northern blot for G3PDH, a ubiquitously expressed housekeeping gene to control for sample loading. The graph on the right displays the densitometric analysis for IL-10 mRNA at 60 min reperfusion. Measurements are expressed in arbitrary densitometry units.

Figure 29: Western blot analysis for hepatic IL-10 protein.

Animals were pretreated with hypertonic or isotonic saline (4cc/kg) and underwent 30 minutes of warm partial hepatic ischemia. Following 60 minutes of reperfusion, liver samples were harvested for western blot analysis for IL-10 protein. A representative blot for IL-10 is displayed. 50 μg of tissue lysate were loaded per sample and equal

loading confirmed with coomassie blue staining. SM =sham, I =Isotonic pretreatment, H =hypertonic pretreatment, I/R =ischemia-reperfusion groups.

STAT 3 activation

The JAK-STAT signaling pathway transduces IL-10 receptor mediated intracellular signaling events (208). Two members of the Janus kinase family of kinases (Jak1, Tyk2) are associated with the IL-10 receptor complex. With IL-10 binding to its receptor, Jak1 and Tyk2 engage the transcription factor-signal transducer and activator of transcription 3 (STAT3), activating it by phosphorylating a tyrosine residue, allowing it to disengage from the IL-10 receptor and translocate to the nucleus where it can modulate transcription events. A key effector of IL-10 activity, suppressor of cytokine signaling-3 (SOCS-3) requires STAT3 activation for its synthesis. Thus, STAT3 activation was examined as an index of IL-10 activity that would support the concept that IL-10 is not only differentially expressed following HTS pretreatment and I/R, but is also biologically active. STAT3 activation was measured by western blot analysis using a specific antibody to phospho-STAT3 protein (Figure 30).

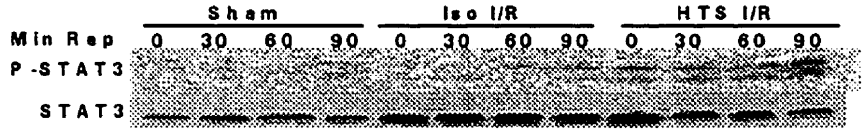

Figure 30: Hepatic STAT3 activation following I/R.

Animals were pretreated with HTS or isotonic saline (4cc/kg) and underwent 30 min of warm partial hepatic ischemia. Following 0-90 min of reperfusion, liver samples were harvested for western blot analysis for phospho-STAT3 protein. Representative blot for P-STAT3 is displayed. Blot was reprobed with antibody to plain STAT3 to control for sample loading. Sham, Iso I/R =Isotonic pretreatment, HTS I/R =hypertonic pretreatment.

STAT3 was not activated in sham animals during 0-90min reperfusion. In contrast, STAT3 activation was increased in HTS pretreated animals between 0-90 min reperfusion, whereas only minimal STAT3

phosphorylation was seen in isotonic I/R animals at 90 min reperfusion. These results are consistent with increased activity of IL-10 in HTS-pretreated I/R animals, although the downstream effects of STAT3 activation remain to be defined in this model.

In-vitro models of hypertonic immune modulation

We made use of 2 *in-vitro* models to determine whether the effects of hypertonicity *in-vivo* at the whole organ level are secondary to effects on individual cell-types important in hepatic I/R injury. Similar to our in-vivo model, our in-vitro models involved cellular exposure to sequential stresses of hypertonicity and an inflammatory stimulus (LPS) to determine how the first stimulus modulates cellular responses to the second.

Modulation of endothelial cell adhesion molecule expression by hypertonicity
Having demonstrated reduced ICAM-1 in the liver following HTS pretreatment, we asked whether HT might exert direct effects on expression of ICAM-1 in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were exposed to sequential (or simultaneous) stresses of hypertonicity and LPS stimulation to determine the ability of hypertonicity to modulate endothelial cell adhesion molecule (ICAM-1) expression.

Dose-response effect of hypertonic pretreatment on HUVEC ICAM-1 expression

We first examined the effect of HT pretreatment on HUVEC ICAM-1 protein expression in response to LPS stimulation for 6 hrs (Fig. 31). HUVEC surface expression of ICAM-1 protein was measured using a cell-ELISA technique. Regardless of treatment group, all cells' media was replaced with fresh isotonic medium prior to LPS stimulation.

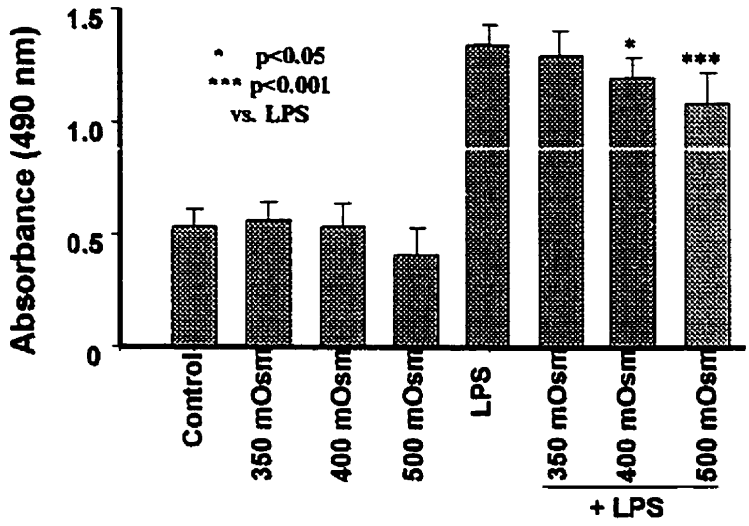

Figure 31: Dose-response effect of hypertonic pretreatment on HUVEC ICAM-1 expression.

HUVECs were preincubated with hypertonic medium (350, 400, 500 mOsM) for 2 hrs prior to LPS (1 μg/ml) for 6 hrs. Treatment groups include control, hypertonic pretreatment for 2 hrs followed by isotonic medium alone for 6 hrs (350 mOsm, 400 mOsm, 500 mOsm), 2 hrs isotonic pretreatment followed by LPS (LPS), hypertonic pretreatment for 2 hrs followed by LPS for 6 hrs in isotonic medium (350 mOsm +LPS, 400 mOsm + LPS, 500 mOsm + LPS). * = $p < 0.05$ vs LPS, *** = $p < 0.001$ vs. LPS.

Treatment groups included control cells which were un-manipulated except for a change of fresh isotonic medium 2 hours after the beginning of the experiment; hypertonic pretreated cells (350, 400, 500 mOsM) without subsequent LPS stimulation; isotonic-LPS in which cells rested in isotonic medium for 2 hours prior to replacement of the medium and addition of LPS (1 μg/ml); hypertonic-LPS groups that underwent 2 hours of hypertonic exposure (350, 400, 500 mOsm) prior to changing to isotonic medium, and the addition of LPS (1 μg/ml).

Control HUVECs had low basal levels of ICAM-1 expression. While LPS stimulation increased surface ICAM-1 protein expression approximately 2.5 times basal levels (Fig. 31), pretreatment with 400 mOsM or 500 mOsM HT resulted in a dose-dependent inhibition of ICAM-1 expression following LPS com-

pared to LPS alone. Hypertonicity of 350 mOsM had no effect on LPS-stimulated ICAM-1 expression. Hypertonic pretreatment at all levels for 2 hours did not alter the level of ICAM-1 protein measured 6 hours later compared to control. Thus, pretreatment of HUVECs with transient HT resulted in a dose-dependent inhibition of LPS-induced ICAM-1 expression.

Dose-response effect of simultaneous hypertonic exposure and LPS stimulation on HUVEC ICAM-1 expression

Our previous studies demonstrated that a single dose of HTS in vivo caused sustained elevation in serum hypertonicity for at least 4 hrs.(80) We therefore examined the effect of simultaneous HT and LPS to determine whether sustained HT might suppress LPS-induced ICAM-1 (Fig. 32).

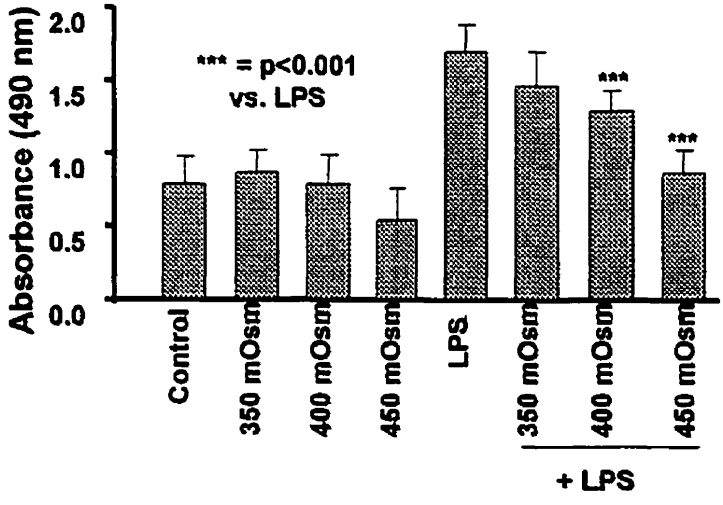

Figure 32: Dose-response effect of simultaneous hypertonic exposure and LPS stimulation on HUVEC ICAM-1 expression.

HUVEC were exposed to hypertonic medium (350, 400, 500 mOsm) and LPS (1 μg/ml) simultaneously for 6 hrs. Treatment groups include control, 6-hr HT alone (350 mOsm, 400 mOsm, 450 mOsm), LPS alone in isotonic medium for 6 hrs (LPS), simultaneous HT and LPS for 6 hrs (350 mOsm +LPS, 400 mOsm + LPS, 450 mOsm + LPS). *** = $p < 0.001$ vs. LPS.

We evaluated sustained HT at doses of 350, 400, and 450 mOsM on LPS stimulated ICAM-1. Treatment groups for this series of experiments included: control, 6-hr Hta lone, LPS alone in isotonic medium for 6 hrs (LPS), and simultaneous HT and LPS exposure for 6 hrs.

Similar to the pretreatment experiment, hypertonicity (350, 400, 450 mOsM) alone did not increase HUVEC surface ICAM-1 expression when compared to control. LPS stimulation under isotonic conditions caused a 2.5-fold increase in ICAM-1 over basal levels. HT inhibited LPS-induced ICAM-1 expression in HUVECs in a dose dependent fashion, losing effectiveness at 350 mOsM. Maximal inhibition of LPS-induced ICAM-1 by hypertonicity occurred at the 450 mOsM level ($p < 0.001$ vs. LPS), decreasing ICAM-1 by approximately 40% relative to the isotonic-LPS group.

Release of LDH as a marker of cellular injury by HUVECs in response to hypertonicity

To rule out cytotoxicity related to hypertonic exposure, two approaches were taken. First, microscopic appearance of cells appeared normal at 350-450 mOsM while sustained treatment with 500 mOsM caused cell detachment. Second, we examined lactate dehydrogenase (LDH) release into the culture medium as an indicator of HUVEC injury. Cells were exposed to 400, 450, or 500 mOsm HT for 6 hrs with or without the presence of LPS (1 μg/ml) (Fig. 33).

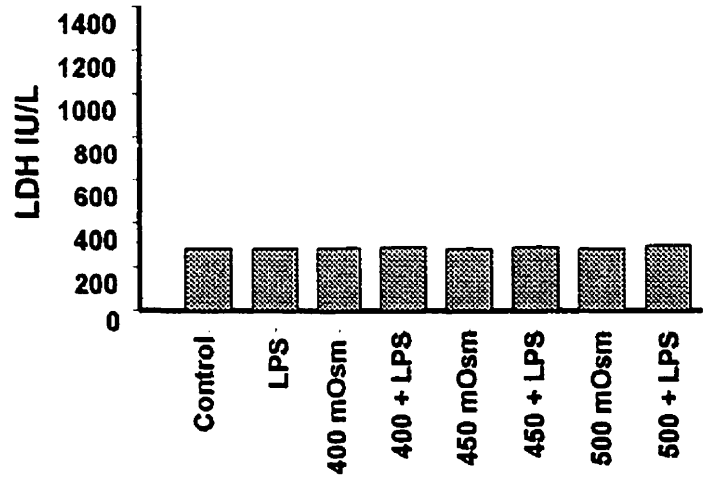

Figure 33: Release of LDH as a marker of cellular injury to HUVECs in response to hypertonicity.
LDH was determined at the end of a 6 hr incubation period in the presence or absence of HT (400, 450, 500mOsm) with or without LPS (1 ug/ml). Treatment groups included: Control, isotonic-LPS (LPS), HT alone (400 mOsm, 450 mOsm, 500 mOsm), HT + LPS (400 + LPS, 450 + LPS, 500 + LPS). Results are expressed as LDH IU/L.

LDH release was not significantly different between any of the treatment groups assessed at the end of 6 hours. These results suggest that the degree and duration of HT (350-450 mOsM for up to 6 hrs) used in these experiments does not induce a cellular injury, or aggravate any injury induced by LPS stimulation.

Effect of timing of HT exposure relative to LPS on HUVEC LPS-induced ICAM-1 expression

As HT exposure during the period of LPS stimulation was more effective than pretreatment in reducing HUVEC ICAM-1, we hypothesized that a vulnerable period exists during the 6 hr LPS incubation during which time transient HT would be as effective as continuous HT. Thus, we measured HUVEC ICAM-1 expression at the end of a 6 hr LPS incubation period in which cells had been exposed to 400 mOsM HT for 2 hrs (Figure 34). Fresh isotonic medium containing LPS was replaced at the end of the 2 hr HT exposure. Treatment groups included: control, 2 hrs HT (400 mOsm) pretreatment followed by 6 hrs of

isotonic medium alone, 2 hrs of HT for the first two hours of the 6 hr LPS period (0-2 hrs), 2 hrs of HT for the second two hours of the 6 hr LPS period (2-4 hrs), and 2 hrs of HT for the last two hours of the 6 hr LPS period (4-6 hrs).

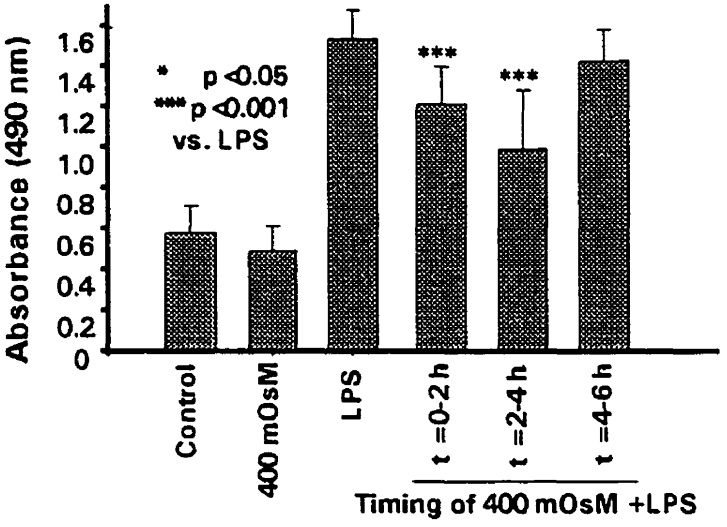

Figure 34: Importance of timing of HT exposure relative to LPS on HUVEC LPS-induced ICAM-1.

HUVEC were exposed to HT (400 mOsM) for 2 hrs (0-2 hrs, 2-4 hrs, 4-6 hrs) during LPS (1 μg/ml) stimulation for 6 hrs. Treatment groups included: control cells, 2 hrs HT (400 mOsM) pretreatment followed by 6 hrs of isotonic conditions, 2 hrs of HT for the first two hrs of the 6 hr LPS period (0-2 hrs), 2 hrs of HT for the second two hrs of the 6 hr LPS period (2-4 hrs), and 2 hrs of HT for the last two hrs of the 6 hr LPS period (4-6 hrs). * =p <0.05 vs. LPS, *** =p <0.001 vs. LPS.

Similar to previous experiments, hypertonic pretreatment did not increase HUVEC ICAM-1 expression compared to control cells. Maximal inhibition of LPS-induced ICAM-1 occurred when a 2 hr HT exposure was applied during the first 4 hrs of LPS stimulation. HT applied for 2 hrs for the last 2 hrs of the 6 hr LPS stimulation period had no effect on HUVEC ICAM-1 when compared to isotonic-LPS. Thus, transient HT applied during the first 4 hrs of LPS stimulation inhibits HUVEC ICAM-1 expression.

Effect of transient hypertonicity on HUVEC LPS-induced ICAM-1 RNA expression
To evaluate the mechanism whereby HT applied during the first 4 hrs of LPS stimulation might affect LPS-induced ICAM-1 expression, we performed northern blot analysis for ICAM-1 RNA expression at the end of a 4 hr LPS stimulation (1 μg/ml) period with or without 2 hr HT (400mOsM) for the first or the second half of the LPS stimulation period (Figure 35, upper panel). Treatment groups included control, isotonic-LPS (LPS), hypertonicity + LPS (LPS + HTS 0-2h, LPS + HTS 2-4h).

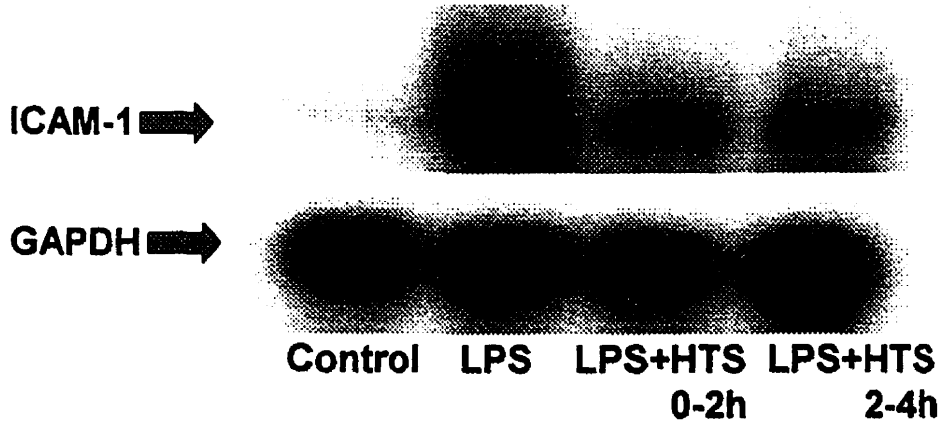

Figure 35: Effect of transient hypertonicity on HUVEC LPS-induced ICAM-1 mRNA expression.
Representative northern blot analysis for ICAM-1 RNA expression after cells were treated for 2 hrs of 400mOsM HT for the first or second half of a 4 hr LPS (1μg/ml) incubation period. Treatment groups included control, isotonic-LPS (LPS), HT + LPS (LPS + HTS 0-2h, LPS + HTS 2-4h). Lower panel shows northern blot for GAPDH to control for sample loading.

Control cells demonstrated little expression of ICAM-1 RNA, while LPS stimulation under isotonic conditions markedly augmented ICAM-1 RNA expression. In contrast, transient HT (400 mOsM for 2 hrs) during the first or the second half of the LPS stimulation period significantly reduced LPS-induced ICAM-1 RNA expression. Sample loading was shown to be comparable as assessed by northern blot

analysis for GAPDH (Figure 29, lower panel). These results suggest that modulation of ICAM-1 RNA is one mechanism by which HT inhibits LPS-induced ICAM-1 expression.

Hypertonic modulation of cytokine production by macrophages

Having demonstrated that HTS preconditioning modulates TNF-α and IL-10 expression following I/R at the whole organ level *in-vivo*, we examined the ability of HTS to modulate the production of these cytokines by macrophages *in-vitro*. Murine peritoneal thioglycollate-elicited macrophages were preconditioned with a 2 hr exposure to 500 mOsM HT and then stimulated with LPS (1μg/ml) for 4 hrs under isotonic conditions when TNF-α (Fig. 36, A) and IL-10 (Fig. 36, B) were measured in the culture supernatant by ELISA.

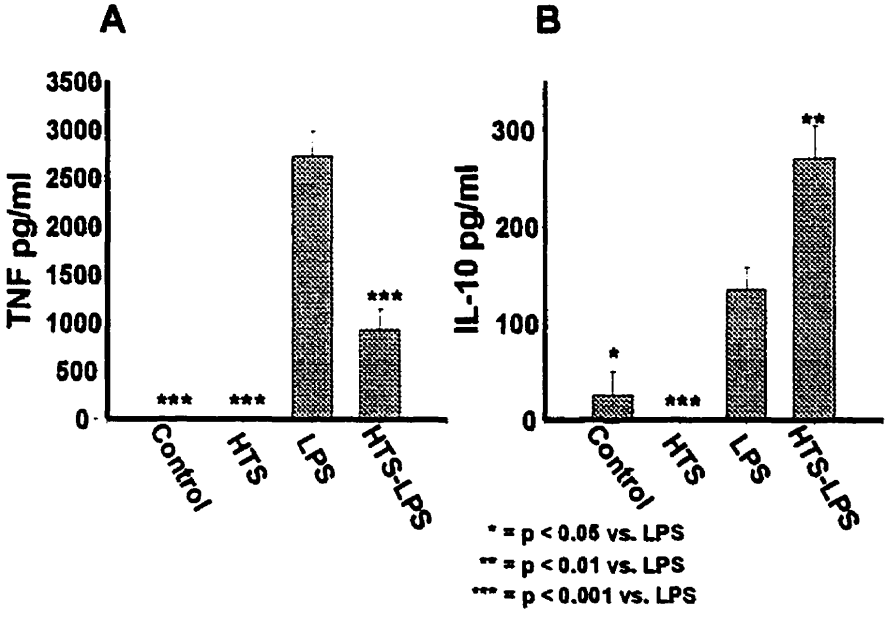

Figure 36: Hypertonic modulation of LPS-induced PEM TNF-α and IL-10.

PEMs were incubated in the presence of LPS (1ug/ml) for 4 hrs under isotonic conditions with or without pretreatment with 500 mOsM HT for 2 hrs. TNF-α and IL-10 (A and B respectively) were measured in the culture supernatants by ELISA at the end of the LPS incubation period. Groups included: unmanipulated control cells (control), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), and cells that were precondi-

tioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS-LPS). * = $p < 0.05$ vs. LPS, ** = $p < 0.01$ vs. LPS, *** = $p < 0.001$ vs. LPS.

Treatment groups included unmanipulated control cells (control), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS-LPS).

Control and HTS groups did not produce any detectable TNF-α. In contrast, LPS induced a significant increase in supernatant TNF-α protein (Fig. 36, A). HTS preconditioning inhibited LPS-induced TNF-α production by approximately 65%. Control and HTS macrophages produced little IL-10. Stimulation of macrophages with LPS induced IL-10 (Fig. 36, B). Preconditioning with HT for 2 hrs augmented LPS-induced IL-10 production, resulting in a 2-fold increase in IL-10 levels compared to the isotonic LPS group. These results suggest that HT modulates the profile of stimulated macrophage cytokine production *in-vitro*, and support the concept that HTS preconditioning modulates I/R-induced KC cytokines *in-vivo*.

Role of augmented IL-10 in hypertonicity mediated inhibition of macrophage TNF-α production *in-vitro*
A number of different investigators have demonstrated that IL-10 is capable of inhibiting TNF-α expression. The current work describes an ability of HT to augment IL-10 expression in response to a second inflammatory stimulus (I/R or LPS) both *in-vivo* and *in-vitro*. This effect was correlated with reduced hepatic TNF-α expression *in-vivo* and an inhibition of LPS-induced macrophage TNF-α production *in-vitro*. It was therefore hypothesized that augmented IL-10 in response to hypertonic preconditioning plays a direct role in the inhibition of TNF-α expression with secondary effects on liver injury, apoptosis, and adhesion molecule expression *in-vivo*. This hypothesis was examined in-vitro using the previously described macrophage model in which cells are initially preconditioned with hypertonic medium (500 mOsM) for 2 hrs and then stimulated with LPS (1μg/ml) for 4 hrs under isotonic conditions. TNF-

α protein expression was then measured by ELISA at the end of the 4 hr LPS incubation period. Three separate approaches were taken to determine IL-10's role. In the first series of experiments, IL-10's biologic activity was blocked through the addition of anti-IL-10 neutralizing antibody (25 μg/ml) to the culture medium immediately prior to LPS stimulation (Figure 37).

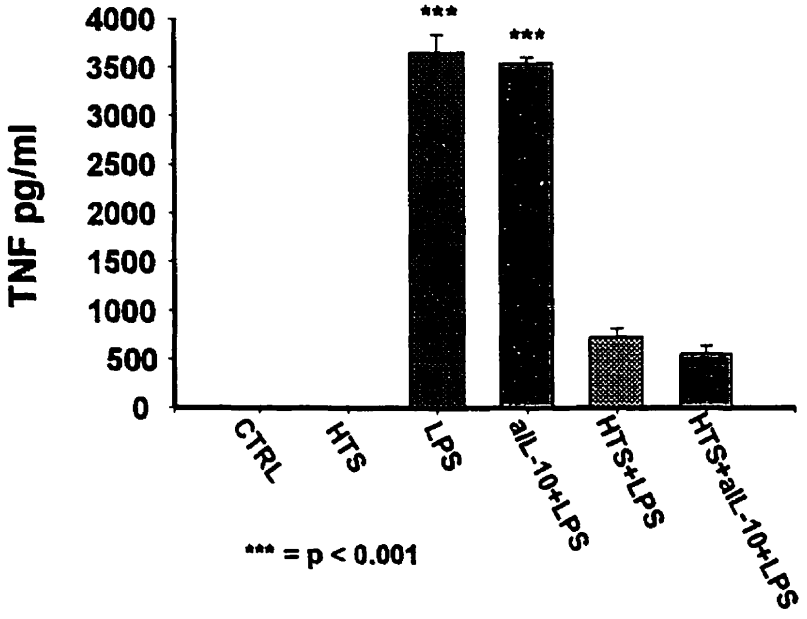

Figure 37: The effect of anti-IL-10 neutralizing antibody on the inhibition of LPS-induced PEM TNF-α production by hypertonicity.

PEMs were incubated in the presence of LPS (1μg/ml) for 4 hrs under isotonic conditions with or without pretreatment with 500 mOsM HT for 2 hrs. TNF-α was measured in the culture supernatants by ELISA at the end of the LPS incubation period. Groups included: unmanipulated control cells (CTRL), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS) in the presence of anti-IL-10 neutralizing antibody (25 ug/ml) (aIL-10 + LPS), cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS+LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium with anti-IL-10 neutralizing antibody (25 ug/ml) and then stimulated with LPS for 4 hrs. *** = $p < 0.001$.

While control (CTRL) and hypertonic preconditioned cells (HTS) exhibited no detectable TNF-α production, LPS alone significantly increased macrophage TNF-α production (LPS). The addition of IL-10 neutralizing antibody to isotonic cells stimulated with LPS (aIL-10 + LPS) did not alter their production of TNF-α. Similar to previous studies, hypertonic preconditioning inhibited LPS-induced TNF-α pro-

duction (HTS + LPS). The addition of IL-10 neutralizing antibody to hypertonic preconditioned macrophages prior to stimulation with LPS (HTS + aIL-10 + LPS) did not result in any significant changes to LPS-induced TNF-α production as compared to HTS+LPS cells alone. The results of these initial studies demonstrating the ability of HT to inhibit LPS-induced TNF-α production in the presence of a neutralizing antibody against IL-10 would suggest that IL-10 does not play a direct role in the hypertonic inhibition of TNF-α. To avoid the possibility that concentrations of the neutralizing antibody against IL-10 were inadequate, a second approach was taken to inhibit IL-10's biologic activity by treating cells with the IL-10 receptor antagonist 1B1.2 (10 μg/ml) prior to activation with LPS (Figure 38) (288). Treatment groups included: unmanipulated control cells (CTRL), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS) in the presence of the IL-10 receptor antagonist 1B1.2 (10 μg/ml) (LPS + I), cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS+LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium with the IL-10 receptor antagonist 1B1.2 (10 μg/ml) and then stimulated with LPS for 4 hrs.

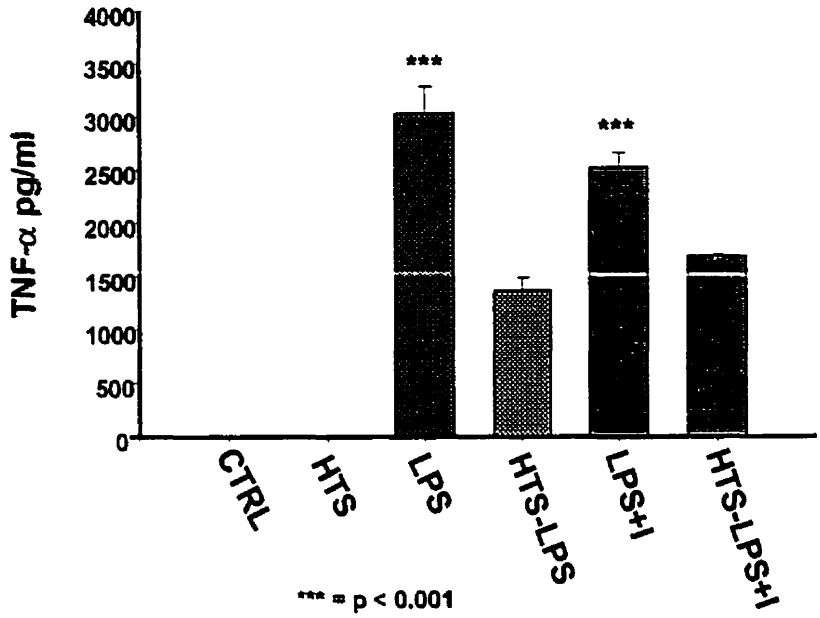

Figure 38: The effect of IL-10 receptor blockade on the inhibition of LPS-induced PEM TNF-α by hypertonicity.

PEMs were incubated in the presence of LPS (1μg/ml) for 4 hrs under isotonic conditions with or without pretreatment with 500 mOsM HT for 2 hrs. TNF-α was measured in the culture supernatants by ELISA at the end of the LPS incubation period. Groups included: unmanipulated control cells (CTRL), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS) in the presence of the IL-10 receptor antagonist 1B1.2 (10 μg/ml) (LPS + I), cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS+LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium with the IL-10 receptor antagonist 1B1.2 (10 μg/ml) and then stimulated with LPS for 4 hrs. *** = $p < 0.001$.

Similar to the experiments using the IL-10 neutralizing antibody, IL-10 receptor blockade failed to reverse the inhibitory effect of HT on LPS-induced macrophage TNF-α production (Figure 38). These results would be consistent with the concept that augmented IL-10 expression following hypertonic preconditioning of macrophages does not play a direct role in the inhibition of TNF-α production by HT. To definitively address the hypothesis and avoid technical concerns regarding the active concentrations of IL-10 blocking antibodies used above, a final set of studies, examined the ability of HT to block LPS-induced TNF-α production in the complete absence of IL-10 by using macrophages harvested from mice homozygous for a targeted deletion of the IL-10 gene (Figure 39). As in the initial PEM experiments,

treatment groups included unmanipulated control cells (control), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS-LPS).

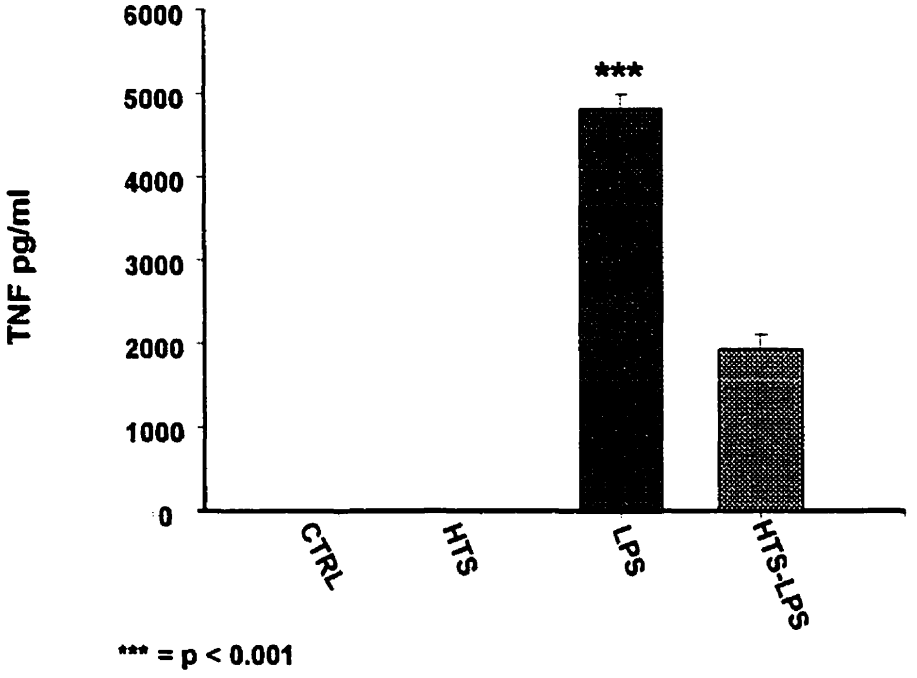

Figure 39: The effect of IL-10 deficiency on the inhibition of LPS-induced PEM TNF-α production by hypertonicity.

PEMs were harvested from mice homozygous for a deletion of the IL-10 gene and incubated in the presence of LPS (1μg/ml) for 4 hrs under isotonic conditions with or without pretreatment with 500 mOsM HT for 2 hrs. TNF-α was measured in the culture supernatants by ELISA at the end of the LPS incubation period. Groups included: unmanipulated control cells (CTRL), macrophages exposed to HT (500 mOsM) for 2hrs and then resuspended in isotonic medium alone for a 4 hr period (HTS), macrophages stimulated with LPS for 4 hrs under isotonic conditions (LPS), and cells that were preconditioned with HT for 2 hrs and then resuspended in isotonic medium and stimulated with LPS for 4 hrs (HTS+LPS). *** = $p < 0.001$.

Control and HTS cells did not produce TNF-α at detectable levels. While baseline production of TNF-α following LPS stimulation alone in IL-10 deficient cells was approximately double the level produced by wild-type macrophages harvested from BALB-c mice, hypertonic preconditioning still resulted in a more

than 50% inhibition of LPS-induced TNF-α production, providing definitive evidence excluding a direct role for IL-10 in the inhibition of macrophage TNF-α production *in-vitro*.

CHAPTER 7: DISCUSSION

Recent interest in HTS has focused on the resuscitation of humans and experimental animals from hemorrhagic shock (48-50;61-63;65;66). There is clear evidence to suggest beneficial hemodynamic effects of HTS administration in the setting of traumatic hypotension. In addition to beneficial hemodynamic effects, a tendency towards fewer systemic complications such as ARDS and renal failure in patients given HTS has been observed (65;68). These effects suggest that HTS has immune-modulating properties that may have a beneficial impact on the development of post-traumatic organ dysfunction.

Previously we reported on the use of HTS in a rodent model of post-traumatic lung injury. HTS induced shedding of PMN L-selectin and prevented LPS-induced CD11b expression. These changes were accompanied by reduced alveolar neutrophilia and lung injury (80). Thus, HTS inhibits PMN-EC interactions following resuscitated hemorrhagic shock, a form of global I/R injury. While these studies focused on the modulation of PMN adhesion molecules by HT, there have been no previous reports on the potential of HTS to inhibit EC adhesion molecule expression.

Hepatic I/R is relevant to the operative management of hepatic tumors, severe hepatic injuries, hemorrhagic shock-resuscitation, and liver transplantation. Post-operative hepatic failure is often lethal. When operating for hepatic trauma, it is unlikely that injured livers, subjected to previous episodes of hypoperfusion would be able to tolerate I/R as well as in the situation of healthy patients undergoing elective hepatic resection. Furthermore, hepatic dysfunction has been regarded as a critical element in the development and sustained existence of MODS in critically ill patients. Thus, the ability to reduce shock-resuscitation induced injury to the liver following trauma may have a beneficial impact on the incidence of post-traumatic organ dysfunction. Prevention of hepatic I/R injury may decrease the incidence of graft primary non-function, acute rejection, and biliary strictures after transplantation. Therapies directed against I/R events that could be administered prior to application of vascular clamps in hepatic surgery or as part of a strategy for resuscitation from hemorrhagic shock may have a positive impact on patient outcome.

Given the previously documented, beneficial effects of HTS by our group and others, as well as the clinical importance of hepatic I/R injury, we chose to examine the effects of HTS in modulating I/R injury in a rodent model of partial hepatic I/R. The specific objectives of the present study were to determine the effects of HTS on the degree of liver injury, as well as its effects on EC adhesion molecule expression and on the balance of pro- and anti-inflammatory cytokines known to modulate injury and cell activation following I/R.

A rodent model of partial hepatic I/R injury was chosen for several reasons. Partial hepatic I/R models in rats are well described, allowing us to compare our results with those of others. Selective clamping of the hepatic vasculature prevents splanchnic pooling and the breakdown of gut barrier function, allowing the effects of I/R to be examined in isolation. Portosystemic shunting procedures are difficult to perform in rodents and would unneccessarily complicate the experimental protocol. A pretreatment model was chosen as one that may be relevant to the elective hepatic resection or transplant scenario. Furthermore, the pretreatment model would allow us to examine the effects of HTS as a form of stress preconditioning that may confer delayed ischemic tolerance, the reverse effect of priming events described in Moore's '2-hit hypothesis' (289;290).

The most significant finding of the present study is that preconditioning with HTS prevented I/R injury to the liver. HTS prevented I/R-induced elevations in AST during both the acute and subacute phases of reperfusion injury. The ability of HTS to reduce injury at the 30 min reperfusion time point suggests that it may modulate the production of ROS and KC activation, while reduced injury at the 4 hr reperfusion time point suggests that PMN-mediated injury is prevented. Measurements of hepatic myeloperoxidase levels, as a marker of hepatic PMN sequestration indicated that protection by HTS during the subacute reperfusion phase was accompanied by reduced hepatic PMN sequestration. PMN-EC interactions play a critical role in hepatic I/R injury (143;278). Production of ROS species and proinflammatory cytokines

such as TNF-α during the acute reperfusion phase activate neighboring ECs and increase their expression of ICAM-1 and VCAM-1, facilitating PMN-mediated amplification of the original early phase injury during the subacute reperfusion phase.

Other groups have examined the hepatic microcirculation following resuscitation from hemorrhagic shock with HTS (59;60). HTS improved small bowel and hepatic microcirculatory flow following hemorrhagic shock (57;59). Corso showed that HTS prevented leukocyte stasis in hepatic sinusoids as well as leukocyte adherence to the endothelial lining of post-sinusoidal venules following hemorrhagic shock (60). Improved resuscitation of the microcirculation with HTS may explain improvements in hepatic energy metabolism observed shortly after I/R in rats given HTS (291).

To our knowledge, this is the first report to show that EC adhesion molecule expression in the liver can be modulated by HTS. The inhibition of I/R-induced ICAM-1 and VCAM-1 expression shown in our model may explain improved resuscitation of the hepatic microcirculation with HTS described by Corso. ICAM-1 is both constituitively expressed and inducible. Constituitive expression occurs on sinusoidal endothelial cells (SEC) and KC. *In-vitro* and *in-vivo* studies have suggested a coordinated induction of ICAM-1 in response to local inflammatory mediators such as TNF-α. Important sources of these mediators include KCs and Ecs (215). With ischemia, ICAM-1 increases on SEC and hepatocytes. Following reperfusion, ICAM-1 is further increased in a feedback cascade with infiltrating PMNs (217). Thus, small differences in ICAM-1 mRNA and protein expression early in reperfusion may be amplified to greater levels as reperfusion and PMN infiltration continue (129). In partial hepatic I/R models, adhesion molecule expression in non-ischemic lobes does not increase until reperfusion, suggesting a role for circulating factors in the induction of ICAM-1 remote from post-ischemic areas of the liver (219). We observed a similar pattern for both ICAM-1 and VCAM-1 mRNA expression following I/R. Notably, HTS prevented the induction of ICAM-1 and VCAM-1 mRNA in both the ischemic and non-ischemic lobes.

Therapeutic strategies directed against ICAM-1 and VCAM-1 have reduced liver injury in other experimental models of I/R. Anti-ICAM-1 blocking antibodies offer significant protection against PMN-induced reperfusion injury (143). In studies of partial hepatic I/R an ICAM-1 blocking antibody (1A29) decreased PMN infiltration of the liver (219). Intraportal injections of 1A29 decreased hepatic necrosis, PMN accumulation, and improved hepatic blood flow after I/R. Notably, 1A29 conferred marked protection in terms of plasma transaminase levels as early as 6 hours after reperfusion (278). It has also been suggested that anti-ICAM-1 therapies may also protect against KC-dependent early reperfusion injury, possibly by interfering with KC-hepatocyte or KC-EC interactions (278). ICAM-1 knockout animals that had undergone hepatic I/R were significantly protected. ICAM-1 knockouts had lower serum transaminase values and decreased PMN infiltration compared to wild-type (WT) controls. However, beyond 6 hours of reperfusion, there was no difference in the number of infiltrating PMNs between mutant and WT groups despite hepatic protection in mutants. Microcirculatory failure and sinusoidal congestion were decreased in mutants, suggesting a role for ICAM-1 in regulating microcirculatory flow. These results suggest a complex process of PMN-EC interactions. The fact that beyond 6 hours of reperfusion, PMN infiltration in mutants was unchanged compared to WT controls despite significant hepatocellular protection suggests alternate pathways for PMN recruitment may be involved in later PMN infiltration. Evidently, the impact of the loss of these adhesion receptors in mutant mice undergoing hepatic I/R is not solely mediated through PMN adhesion. However, prevention of the early interaction between PMNs, adhesion receptors, and endothelium is essential for imparting a persistent attenuation of reperfusion injury (220). The inhibition of I/R induced ICAM-1, VCAM-1 and PMN CD11b expression by HTS may play a role in preventing 'early reperfusion events' such as capillary no-reflow, firm adhesion of PMNs, cell activation, PMN transmigration, and PMN adhesion-dependent injury to both hepatocytes, and EC.

Although the data is limited, the accumulation of CD4 T-helper lymphocytes in post-ischemic lobes during the early reperfusion phase plays a critical role in modulating subsequent PMN-mediated injury

(149). It remains to be determined whether decreased hepatic adhesion molecule expression during early reperfusion following HTS preconditioning contributes to alterations in hepatic lymphocyte sequestration that could affect subacute phase PMN-mediated injury. ICAM-1 cross-linking induces cell activation and intracellular signaling events (214). Decreased ICAM-1 expression may therefore reduce cell-cell adhesion events leading to cell activation following I/R.

Our *in-vivo* studies addressed the effect of HTS on I/R-induced adhesion molecule expression at the whole organ level. Following I/R, EC ICAM-1 expression is increased from basal levels and ICAM-1 is induced in hepatocytes and KC (215-217). Our results indicating decreased hepatic ICAM-1 expression may indicate an effect of HTS on one or all of these cell types. However, as VCAM-1 is only induced in SECs following hepatic I/R, and therefore, effect of HTS on EC adhesion molecule expression is likely (216).

To further test our hypothesis that HTS modulates EC adhesion molecule expression, we made use of an *in-vitro* EC model. LPS was chosen as the inflammatory stimulus used to activate HUVECs, since it has been reported to increase rapidly with reperfusion following hepatic I/R, activates similar cell-signaling pathways and is a consistent activator of EC, with respect to adhesion molecule expression (182). Moreover, LPS was previously used in our model of post-traumatic lung injury (80).

HT pretreatment of HUVECs reduced LPS-induced ICAM-1, albeit to a lesser extent than anticipated from the *in-vivo* study. This difference in pretreatment effect between models may be a reflection of cell-specific differences in the response of HUVECs as compared to the combined responses of different cell types *in-vivo*, or may be related to differences in the exact nature and timing of the second inflammatory stimulus (I/R or LPS) relative to the period of HT exposure. An additional explanation for the reduced effect of HT pretreatment on HUVECs may be the presence of additional cell types *in-vivo* such as hepatic KC. It is well known that KC pro-inflammatory cytokine production plays an important role in

upregulation of EC adhesion molecules (134). Thus, an effect *in-vivo* directed at KC activation by HTS would not have been evident in a HUVEC culture. Further studies are warranted in this regard. However, our studies employing simultaneous HT exposure and LPS stimulation of HUVECs revealed a more significant dose-dependent inhibition of ICAM-1 by HT providing a 'proof of principle' with respect to the ability of transient HT to modulate EC adhesion molecule expression.

The timing of HT exposure relative to HUVEC activation seems important, as only a 2 hr, transient HT exposure during the first 4 hrs of LPS stimulation was required to exert ICAM-1 inhibition to the level observed with continuous, 6 hr HT. Furthermore, transient HT applied during the first 4 hrs of LPS prevented LPS-induced ICAM-1 RNA expression, suggesting that HT modulates ICAM-1 both *in-vivo*, and *in-vitro* at the gene level. These results may have both clinical and mechanistic relevance. The data suggests that early HT exposure relative to the inflammatory stimulus (I/R or LPS) is important. Furthermore, these findings would suggest that the effects of HT are more likely to be on the initial pathways leading to ICAM-1 gene induction rather than later post-transcriptional effects.

Reduced levels of ICAM-1 gene expression may reflect an ability of HT to modulate the activation of upstream signaling pathways. HT was previously reported to modulate LPS-induced activation of the MAPK p38 in PMNs with secondary effects on PMN CD11b expression (83). The induction of ICAM-1 expression following LPS stimulation or I/R is largely under the control of the transcription factor NF-κB (253;292). The ability of transient HT to modulate ICAM-1 expression raises the possibility that HT may inhibit NF-κB activation. Alternatively, osmotically induced activation of additional transcription factors such as heat shock factor-1 (HSF-1) may play a role. HT is known to rapidly induce HSF-1 activation (293). HSF-1 is both an activator of heat shock genes and also a repressor of non-heat shock genes, including ICAM-1 (294). Thus, the differential activation or repression of transcription factors by HT may explain altered ICAM-1 gene expression. The timing of HT exposure *in-vivo* or *in-vitro* relative

to the second inflammatory stimulus (I/R or LPS) may influence the activation of different transcription factors with combined effects at the gene level.

The ability of HT exposure to modulate HUVEC ICAM-1 expression at both the gene and protein levels is supportive of our *in-vivo* finding that HTS administration modulates hepatic ICAM-1 expression and supports our hypothesis that HTS modulates EC adhesion molecule expression as part of its spectrum of potentially beneficial immune-modulating properties.

It is important to note that HT itself can induce EC ICAM-1 expression in models of prolonged (2-14 day) exposure (95-97). The results of these previous experiments suggest that high doses (> 600 mOsM) and prolonged exposure to HT (hrs-days) is not innocuous. Combined with our own data on transient hypertonic EC exposure, this work highlights the concept that the degree and timing of sequential cellular stresses can augment or inhibit subsequent cellular responses. Short periods of sublethal heat stress, hypothermia, and ischemia may all protect against lethal injury from a later ischemic insult (231;232;295). Furthermore, different cellular stresses can induce cross-tolerance to each other (233). In contrast, hemorrhagic shock-resuscitation followed by low-dose LPS augments cellular activation and injury ('2-hit hypothesis') (296). What remains unknown is the critical cellular event(s) that determines whether a particular stress will augment or inhibit subsequent cell responses. Results from our *in-vitro*, HUVEC model demonstrating variable effects of HT on ICAM-1 depending on the time of exposure relative to activation by LPS suggests that the timing of this form of cellular stress is important.

HTS pretreatment prevented I/R induced upregulation of PMN β_2-integrin, CD11b/CD18 (Mac-1). Mac-1 is constituitively expressed on PMNs but is transformed to an active conformation and quantitatively increased after PMN activation, mediating the firm adhesion of PMNs to ICAM-1 (226). This interaction sensitizes PMNs through the activation of intracellular signaling pathways. Mac-1 also plays a role in PMN adherence-dependent hydrogen peroxide production, chemotaxis, aggregation, and phago-

cytosis (142;226). Thus, adhesion is a prerequisite for the killing of EC or hepatocytes by PMNs. Changes in the surface expression of PMN adhesion molecules are sensitive indicators of the PMN activation state (227). In human liver resection, hepatic vascular exclusion leads to CD11b upregulation on circulating PMNs (227). Similar changes follow reperfusion of liver grafts. Similar to studies by Thiel et al, we were able to detect changes in PMN CD11b expression as early as 30 min reperfusion (227). Therefore, the ability of HTS to reduce PMN CD11b expression following I/R suggests that HTS can inhibit PMN activation. Previous work in our own laboratory suggests this to be the case. In a two-hit model of hemorrhagic shock-resuscitation followed by LPS, HTS prevented LPS-induced upregulation of CD11b (80). Kuroda has raised concerns that HT-induced inhibition of PMN function may predispose patients to an increased risk of infectious complications (297). However, we have demonstrated that HT-induced PMN inhibition is reversible, making this less of a concern (81). Thus, systemic administration of HTS may be considered for use as a reversible form of immunosuppression, possibly with broader application to other forms of PMN-mediated injury.

Numerous cell types are involved in the pathophysiology of hepatic I/R injury including PMNs, KC, hepatocytes, EC, lymphocytes, and hepatic stellate (Ito) cells. Interactions between different cell types contribute to the cascade of events leading to I/R injury and blocking these interactions with therapies directed against adhesion molecules or pro-inflammatory cytokines protects against injury. One of our objectives was to examine the effects of HTS preconditioning on levels of proinflammatory cytokines following hepatic I/R. We chose to examine hepatic TNF-α levels because it is a proximal mediator in the early phase of reperfusion injury, and because TNF blockade has been shown to have similar effects to those we observed with HTS, with impaired I/R-induced ICAM-1 and VCAM-1, and reduced PMN priming, hepatic PMN accumulation, and reperfusion injury (184;185;187). Our results demonstrating reduced hepatic TNF-α mRNA and protein levels during the early phase of reperfusion injury are consistent with reduced 30 min reperfusion plasma AST, PMN CD11b, hepatic ICAM-1 and VCAM-1 expression and suggest that HTS pretreatment modulates cytokine expression at a proximal point in the cascade

of reperfusion injury. Reversal of the HTS protective effect through the administration of exogenous TNF-α supports the concept that inhibition of I/R-induced hepatic TNF-α is an important mechanism of protection by hypertonic preconditioning.

While HTS prevented the full expression of I/R-induced TNF-α mRNA at the 30 min reperfusion time point, the effect was relatively small compared to the almost complete inhibition of hepatic TNF-α protein at 2 hrs. HT was previously shown to inhibit ATP-induced post-translational processing of the immature form of IL-1β in monocytes (120). These results suggest that HTS may exert post-translational effects in addition to modulating gene expression at the mRNA level which may both contribute to the inhibition of I/R-induced hepatic TNF-α expression.

As the major source of hepatic TNF-α following I/R is the resident macrophage, or KC, reduced TNF-α expression would suggest an ability of HTS to modulate KC activation in response to I/R (130;131;177;186). Haussinger has demonstrated *in-vitro* that HT inhibits the production of IL-6 by stimulated KC, and that this effect may be secondary to altered MAPK activation (298). There may be additional effects of reduced hepatic TNF that we did not examine directly in the present study, including protection against remote organ injury. For example, others have shown that TNF inhibition in hepatic I/R models reduced I/R-induced cardiac and pulmonary dysfunction (187-189). Analysis of non-ischemic lobes of the liver for ICAM-1 mRNA in our own study showed a delayed time course of ICAM-1 expression in non-ischemic lobes compared to ischemic lobes. This is consistent with the effects of soluble mediators such as TNF on adhesion molecule expression at sites remote from the original injury and is consistent with work by Meyer et-al showing increased remote organ ICAM-1 mRNA following a similar I/R insult to the one used in our model (299).

NF-κB binding to a consensus sequence of the ICAM-1 promoter region was examined to determine whether impaired NF-κB activation could be responsible for decreased ICAM-1 expression seen in HTS preconditioned I/R animals. HTS preconditioning reduced NF-κB binding to the ICAM-1 promoter region, providing an explanation for decreased hepatic ICAM-1 mRNA levels with HTS I/R. As the inducible regulation of ICAM-1 is largely under the control of NF-κB, a similar mechanism likely accounts for reduced ICAM-1 mRNA and protein levels seen in our *in-vitro* HUVEC model, although LPS instead of TNF would be responsible for NF-κB activation (215). Thus, HTS preconditioning has the ability to modulate intracellular signaling pathways important in the regulation of adhesion molecule gene expression. Following hepatic I/R, activation of KC by the complement cascade (specifically C5a), as well as by ROS leads to NF-κB activation and increased TNF-α expression (177;177). TNF-α itself can induce ICAM-1 expression through the activation of NF-κB (215). Thus, the inhibition of NF-κB activation and hepatic ICAM-1 expression by HTS is consistent with the finding of reduced TNF-α expression in early reperfusion.

The potent anti-inflammatory cytokine, IL-10 is capable of inhibiting the synthesis of proinflammatory cytokines such as TNF as well as adhesion molecules such as ICAM-1 (192;193). IL-10 is produced by cells of the monocyte/macrophage lineage as well as by lymphocytes (191). Hepatic IL-10 expression was examined following I/R to determine whether its expression was increased by HT preconditioning. Our results showing that hepatic IL-10 expression is augmented in HTS preconditioned I/R animals at both the mRNA and protein levels may account for reduced adhesion molecule and proinflammatory cytokine expression following I/R. The precise mechanism of IL-10's inhibitory effects are still being defined although results suggest that it prevents NF-κB activation, which would also be consistent with our findings (203-205). The shift in the profile of pro- and anti-inflammatory cytokines towards the expression of IL-10 by HT preconditiong represents a novel mechanism by which HT may protect against the devolopment of inflammatory organ injury. The binding of IL-10 to its cell surface receptor results

in the activation (by phosphorylation) of the signal transducer and activator of transcription-3 (STAT3) transcription factor (206-208). Therefore, phosphorylated STAT3 was measured as an index of hepatic IL-10 activity *in-vivo*. STAT3 activation in hypertonic I/R animals but not in isotonic I/R animals is consistent with the augmented IL-10 in HTS I/R animals and further suggests that IL-10 is biologically active under these circumstances.

Future studies to determine suppressor of cytokine signaling-3 (SOCS-3) gene expression, are warranted. SOCS-3 is thought to mediate some of the inhibitory effects of IL-10 on proinflammatory cytokine activity and is itself a gene whose transcription is regulated by STAT3 binding to its promoter region (206;210). While the inhibitory effects of HTS on liver injury, TNF and adhesion molecules are consistent with IL-10's documented effects, studies making use of neutralizing IL-10 antibodies or IL-10 receptor blockers *in-vivo* will be required to determine the precise role of augmented IL-10 expression by HTS in our model.

The main source of both TNF-α and IL-10 following hepatic I/R is the resident hepatic macrophage, or KC. Resident tissue macrophages are thought to play a prominent role in modulating the tissue injury associated with hepatic I/R as well as in ARDS (131;135;300-302). Data from our *in-vivo* model showing that HTS preconditioning induces a shift in the profile of pro- and anti-inflammatory cytokines towards increased anti-inflammatory IL-10 suggests that HTS modulates the response of KC to I/R. The ability of macrophages to produce both pro- and anti-inflammatory cytokines as well as the mechanisms by which they are regulated are currently a subject of keen interest (199;200). Based on our experience with HTS' beneficial effects *in-vivo* in our models of post-hemorrhage lung injury and hepatic I/R, we evaluated the effect of HT on macrophages *in-vitro* (80).

Similar to the pattern of TNF-α and IL-10 expression we observed *in-vivo* with HT preconditioning, hypertonic exposure of murine peritoneal macrophages prior to LPS activation inhibited TNF-α and aug-

mented LPS-induced IL-10. These results confirm an ability of HT to modulate the profile of pro- and anti-inflammatory cytokine expression. Studies involving hypertonic dextrose or amino acid exposure of human macrophages in the context of peritoneal dialysis have also described inhibition of proinflammatory cytokine expression by macrophages (118). The mechanism(s) underlying this effect are unclear. HT inhibition of LPS-induced macrophage TNF expression could be prevented by the addition of indomethacin, suggesting a role for HT-induced prostaglandin production in the inhibitory effect (119). In a study of KC stimulated with LPS under HT conditions, Zhang et-al found that prostaglandin production by KC was increased in a dose dependent manner with increasing HT and was accompanied by a strong induction of COX-2 mRNA (125).

Others have shown that the LPS-induced induction of TNF-α gene transcription is dependent on NF-κB activation while LPS-induced IL-10 depends on activation of the transcription factor Sp-1 (200;303). We have shown that HT prevents NF-κB activation *in-vivo*. Whether HT pretreatment prevents NF-κB activation in LPS stimulated PEMs and its effects on Sp-1 remain to be determined as differential activation of these two transcription factors could also account for increased IL-10 in the face of reduced TNF. We hypothesized that IL-10 may be acting to inhibit TNF synthesis. However, studies employing IL-10 neutralizing antibodies, IL-10 receptor blockade, as well as macrophages harvested from IL-10 deficient mice did not support a direct role for IL-10 in the inhibition of LPS-induced macrophage TNF-α production *in-vitro*.

Most of the beneficial immune-modulating effects of transient HT described *in-vivo* and *in-vitro* are inhibitory in nature. The best-studied effects of HT-induced gene transcription relate to enhanced expression of osmolyte transporters that contribute to the restoration of cell-volume homeostatsis through the accumulation of intracellular neutral amino acids (101;102;304). Enhanced transporter gene transcription is secondary to activation of transcription factors such as TonE-binding protein (113). Recent work by Haussinger and others indicates that HSF-1 is also activated by HT and supports the concept that dif-

ferent kinds of cellular stress may induce a generic set of cellular defenses that may the include the heat shock proteins (123;124;305).

Members of the heat shock protein-70 family of heat shock proteins protect against oxidant-mediated injury, inhibit the activation of NF-κB, and prevent ICAM-1 expression following hepatic I/R injury (231;238;239;306). Despite evidence demonstrating the induction of HSP70 by HT *in-vitro*, hepatic HSP70 levels were not augmented by HT preconditioning (123).

A different member of the heat shock group of proteins is HSP32, also known as heme-oxygenase-1 (HO-1). HO-1 activity results in the production of carbon monoxide (CO) and bilirubin. HT has been shown to induce macrophage HO-1 expression (121). CO acts as a second messenger and vasodilator to maintain sinusoidal perfusion pressure following hepatic I/R, and bilirubin functions as an endogenous antioxidant (169;231) (151;171;174). In contrast to hepatic levels of HSP70, HTS pretreatment significantly increased HO-1 protein expression in post-ischemic lobes when compared to isotonic I/R rats. This suggests that heat shock protein expression following transient HT is differentially regulated depending on the particular HSP.

Using the selective HO-1 inhibitor, zinc-protophorphyrin IX (Zn-PP-IX), we were able to recapitulate the results of Amersi et al demonstrating that inhibition of HO-1 enhances I/R injury (174). However, HO-1 inhibition prior to HTS administration and hepatic I/R failed to restore AST levels to those of isotonic I/R controls, indicating that augmented HO-1 expression following HTS pretreatment is at most, only partially responsible for the protective effects of HTS on hepatic I/R, and that alternative mechanisms play a greater role.

Recently, it has been recognized that apoptosis, or programmed cell death is a significant component of liver injury following I/R (262;263;270;272;274;307). ROS produced by hepatocytes under hypoxic conditions are known to mediate SEC apoptosis. TNF-α has also been demonstrated to play a central

role in hepatic apoptosis and associated liver failure (269). As HTS inhibited I/R induced hepatic TNF expression, and was shown by others to prevent apoptosis of the liver following hemorrhagic shock, it seemed likely that the prevention of hepatic apoptosis would be a mechanism of protection in our model (78). Furthermore, HTS has been shown to protect against LPS-induced thymic cell apoptosis(308). Cellular resistance to apoptosis correlates with cells'ability to regulate their volume in a hypertonic environment (309). Thus, a relationship between mechanisms of cell volume homeostasis and apoptosis exists (310). It is conceivable that transient HT following HTS administration induces cell volume regulatory mechanisms that protect against I/R-induced hepatocyte apoptosis. CD95-receptor mediated apoptosis is decreased with moderate HT (309).

Using two distinct methods, DNA laddering, and in-situ end-labelling (TUNEL staining), hepatic apoptosis was shown to be prevented in HTS preconditioned livers following I/R. Furthermore, TUNEL staining revealed hepatocytes to be the cells undergoing apoptosis in isotonic I/R animals. The lack of necrosis seen in liver sections from I/R animals despite significant apoptosis and AST release in isotonic I/R animals is consistent with the results of Kohli et al who showed that apoptosis is the predominant mechanism of cell death with ischemic times less than 60 minutes (263). Furthermore, our results demonstrating a measurable increase in hepatic apoptosis as early as 4 hrs reperfusion are consistent with those of Sasaki et al who observed apoptosis at 3 hrs reperfusion following a similar, 30 min ischemic period (262). Surprisingly, caspase 3 was significantly elevated in HTS preconditioned livers. As caspase 3 is a distal, 'executioner' caspase that mediates hepatic apoptosis following I/R, this result seemed paradoxical to the substantial protection against apoptosis by HTS (271). To exclude the possibility that the AFC substrate used in the caspase 3 assay was being non-specifically cleaved by other caspases or proteases liberated during I/R, we employed a second, immunologically specific caspase 3 assay, confirming that the increased activity was in fact secondary to caspase 3. One possible explanation for reduced hepatocyte apoptosis in the face of augmented caspase 3 activity may be that increased hepatic caspase 3 activity in HTS animals may actually represent a delay in the peak activity as compared to iso-

tonic saline pretreated animals that had peaked earlier. This possibility remains to be confirmed in future studies.

Assuming augmented hepatic caspase 3 activity in HTS pretreated I/R animals does not simply represent a delay in I/R-induced caspase 3 activation mediated by HTS, HT may induce a blockade of caspase-mediated hepatic apoptosis distal to caspase 3. Alternatively, HT may prevent hepatic apoptosis through the induction of anti-apoptotic molecules such as p53 (311).

P38 and JNK activation have both been associated with increased apoptosis following I/R injury (247;312). Unlike JNK, p38 was not activated in the liver following reperfusion. This may represent a tissue-specific pattern of MAPK activation that occurs in response to I/R (245). While JNK was rapidly activated in isotonic I/R animals on reperfusion, reperfusion induced JNK activation was inhibited in HTS preconditioned livers. While it remains uncertain whether the JNK-AP-1 pathway is the dominant pathway of apoptosis following hepatic I/R, reduced induction of this pro-apoptotic pathway may be one mechanism by which hepatic I/R induced apoptosis is prevented following HTS.

Finally, while HTS protected against I/R-induced hepatocyte apoptosis, it increased apoptosis of circulating PMNs. These results are consistent with those of Jin et al who reported on the ability of HT to induce PMN apoptosis *in-vitro* (287). Thus, augmented PMN apoptosis after preconditiong with HTS may be another mechanism by which hepatic PMN accumulation and injury are reduced following I/R.

In summary, the major finding of the present study was that HTS preconditioning prevented I/R injury to the liver. This protective effect was associated with reduced hepatic and PMN adhesion molecule expression and decreased hepatic PMN sequestration. The ability of HTS to inhibit EC adhesion molecule expression *in-vivo* was supported by studies demonstrating that transient HT reduced ICAM-1 expression by activated EC *in-vitro*. Preconditioning with HTS was associated with an altered cytokine profile fol-

lowing I/R, in which TNF-α expression was inhibited and expression of IL-10 was augmented. The ability of HT to shift the balance of pro- and anti-inflammatory cytokine production by activated macrophages towards increased IL-10 suggests that HTS modulates the activation of resident hepatic macrophages following I/R. Reduced TNF-α expression was associated with decreased I/R-induced hepatocyte apoptosis. In contrast, apoptosis of circulating PMNs was increased following HTS infusion. Thus, HT may act to modulate cellular responses to an I/R insult in a manner that reduces injurious interactions between different cell types responsible for liver injury. HTS preconditioning may represent a novel strategy in the prevention of organ injury associated with global or regional I/R insults.

CHAPTER 8: DIRECTIONS FOR FUTURE RESEARCH

The current study provides new insights into the immune-modulatory effects of HTS as applied to a regional model of I/R injury. The demonstration that transient hypertonicity (HT) can inhibit endothelial adhesion molecule expression represents a novel finding that compliments previous work showing that HTS modulates PMN adhesion molecule expression. The finding that TNF is reduced and IL-10 increased following HT preconditioning *in-vivo* and *in-vitro* suggests that HTS can induce a shift in the profile of macrophage cytokine production. These results open new avenues for research in the areas of HT resuscitation and immune modulation. Importantly, the demonstration that HT preconditioning provides a form of delayed ischemic tolerance may lead to new clinical indications for HTS administration and improve patients' ability to sustain I/R insults associated with elective surgery, trauma, or transplantation.

Future studies using the current *in-vivo* model of HT preconditioning and hepatic I/R may initially focus on confirming the role of IL-10 in mediating the beneficial inhibitory effects of HTS preconditioning and characterizing the mechanism by which HT modulates IL-10 expression.

Determining the expression of IL-10 induced SOCS-3

Many of IL-10's anti-inflammatory effects have been ascribed to enhanced expression of SOCS-3. The ability of IL-10 to induce SOCS-3 expression correlates with its ability to inhibit LPS induced TNF and IL-1 expression (206). The transcription factor STAT3 mediates SOCS-3 gene transcription. Given that IL-10 and STAT3 phosphorylation are both increased in HTS preconditioned animals, assessment of SOCS-3 expression is warranted (210).

Confirmation of the protective role of IL-10 following HTS preconditioning and I/R

While many of the beneficial effects we have observed with HTS preconditioning such as reduced TNF-α expression, decreased ICAM-1, and inhibition of PMN activation are consistent with possible activity of augmented IL-10 by HTS, a direct relationship between IL-10 and these effects of HTS needs to be

confirmed. Several approaches lend themselves to the clarification of this issue. Neutralizing antibodies against IL-10 could be administered *in-vivo* prior to HTS. Alternatively, an antagonist to the IL-10 receptor could be used to neutralize the biologic activity of endogenous IL-10 *in-vivo*. Following IL-10 neutralization, plasma AST, as well as hepatic ICAM-1 and TNF-α expression could be measured. Alternatively, the development of a murine model of HTS preconditioning and hepatic I/R would permit the use of IL-10 knockout mice (313).

Induction of IL-10 gene expression by hypertonicity

Studies by Brightbill et al have indicated the critical importance of Sp-1 binding to the IL-10 promoter in LPS induced expression of IL-10 by macrophages (200). The activation of Sp-1 in HT preconditioned macrophages could be determined by EMSA. Similar experiments could be performed to determine NF-κB binding to the TNF-α promoter consensus sequence. The differential activation of NF-κB and Sp-1 by LPS in HT preconditioned macrophages would provide one explanation for reduced TNF and enhanced IL-10 expression following LPS or I/R. The murine IL-10 promoter fragment containing the Sp-1 binding sequence has been identified (200). RAW264.7 cells could be transfected with a series of IL-10 promoter mutants containing a luciferase reporter gene and stimulated with HT +/- LPS to determine if a fragment of this promoter is responsible for HT modulation of IL-10 expression.

Mechanism of HTS protection against I/R induced hepatic apoptosis

While we have demonstrated that I/R induced hepatocyte apoptosis is prevented by HTS, the mechanism by which this protection occurs remains unclear, particularly in light of the data demonstrating significant elevations in caspase 3 in HTS I/R animals. Several possible mechanisms lend themselves to future study. Firstly, the time course of caspase 3 activation in our model needs to be clarified to address the possibility that augmented activity in HTS preconditioned rats does not actually represent a delay in caspase 3 activity compared to isotonic I/R animals. Additionally, the role of specific pro-apoptotic pathways in hepatocyte apoptosis following I/R should be determined to ascertain which of these may be

relevant to the protective effect of HTS on apoptosis. To evaluate the hypothesis that reduced hepatic TNF-α is important in the protection of hepatocytes from apoptosis by HTS, HT preconditioned rats could be given exogenous TNF with reperfusion and DNA laddering could be assessed to determine whether HTS' protective effect is reversed.

Effect of HTS preconditioning on remote organ injury following hepatic I/R

I/R injury to the liver induces injury to remote organs (180;204;314-316). Other investigators have documented increased ICAM-1 mRNA in the heart, lungs, spleen, pancreas, kidney, and small bowel following 45 minutes of partial hepatic ischemia and 6 hours of reperfusion (299). These results suggest a mechanism by which PMNs could sequester and induce injury in these organs. The beneficial effects of HTS on reduced proinflammatory cytokine expression and impaired PMN-EC interactions in the liver following I/R provide a rationale for studies into the ability of HTS to protect against remote organ injury as well.

Effect of HTS preconditioning in a model of cold hepatic I/R

Cold I/R injury to the liver as may occur following revascularization of a hepatic allograft is characterized by a marked apoptosis of sinusoidal EC (265). Presently, it is unclear whether HTS preconditioning is capable of inhibiting EC apoptosis. The ability of HTS to protect against cold I/R injury would suggest a particular relevance of this treatment to hepatic transplantation. HTS could be used to precondition donor organs prior to harvest. While technically challenging, data supporting the use of HTS preconditioning in a rodent hepatic transplant model would provide a clear rationale for proceeding with studies of HTS in human transplantation (317).

Human trials of HTS for I/R injury

The ultimate goal of research in this area should be the improved care of patients. Preconditioning may induce anti-inflammatory molecules capable of protecting against I/R injury. Heat shock precondition-

ing of patients is impractical. Studies of pharmacologic preconditioning or the induction of LPS tolerance must deal with concerns regarding potential toxicity of the preconditioning agent. Ischemic preconditioning, used in hepatic surgery unnecessarily complicates the operation and increases operative time. Thus, preconditioning with HTS as a single bolus en-route to the operating room, or as part of trauma field resuscitation by pre-hospital care personnel is simple and has been shown to be safe after a large number of clinical trials of HTS in experimental animals and humans. It is hoped that data from the present and future studies will form the basis of a rationale for clinical trials into the use of HTS as a preconditioning agent that may improve patient outcome.

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