Linking proximal and downstream signalling events in hepatic ischaemia/reperfusion injury

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Abstract

Hepatic I/R (ischaemia/reperfusion) injury occurs in a variety of clinical settings including transplantation, elective liver resections and trauma. One of the challenges in studying the pathophysiology of I/R injury is the fact that the liver plays a central role in a variety of metabolic pathways in addition to governing aspects of immune surveillance and tolerance. The pathways activated in response to insults as varied as toxins, microbial and endogenous ligands and I/R may share common elements. The multiple intracellular signalling cascades involved in this process and the initiating events are still under investigation. Recent work on the role of TLRs (Toll-like receptors) in I/R injury has elucidated some of the more proximal signalling events in the pathway. In addition to the well-established role of signalling molecules such as NO (nitric oxide) in mediating damage or protection following hepatic I/R, more recent studies have focused on the participation of endogenous danger signals or DAMPs (damage-associated molecular patterns) such as HMGB1 (high-mobility group box 1). The complex interplay between HMGB1, TLRs and the many intracellular signalling molecules and pathways is illustrative of how our understanding of hepatic I/R injury is continually evolving.

NO in hepatic I/R (ischaemia/reperfusion)

Depending on the enzymatic source {eNOS [endothelial NOS (NO synthase)] compared with iNOS (inducible NOS)} and the type of I/R (cold compared with warm), NO can play either a protective or harmful role in hepatic I/R injury. It appears that the low level of constitutively expressed eNOS-derived NO is primarily beneficial. In a model of partial warm hepatic I/R, eNOS-knockout mice displayed worse injury compared with wild-type counterparts, suggesting that eNOS-derived NO may be protective [1]. Vasodilation and subsequent improvement in hepatic microcirculation are the proposed mechanism of protection. Injury was attenuated in this model in iNOS-knockout mice, suggesting a potentially harmful role for iNOS in this setting. However, giving selective iNOS inhibitors in a liver transplant model seems to worsen graft apoptosis and necrosis [2]. Furthermore, adenoviral iNOS treatment of donors in an orthotopic syngeneic rat liver transplant model ameliorated liver graft injury and improved post-transplant survival [3]. Thus the hepatoprotective role compared with the potentially harmful role of NO in the context of hepatic I/R is unclear based on currently available evidence and appears to vary depending on whether the model is transplant or warm I/R. In addition to effects on total hepatic blood flow, some of the other potentially beneficial downstream effects of increased NO expression include up-regulation of cytoprotective haem oxygenase-1 and down-regulation of cellular adhesion molecules and subsequent neutrophil infiltration [4–6]. This recruitment of neutrophils and increase in pro-inflammatory mediators may be due to a combination of increased iNOS and eNOS expression.

Many of the toxic effects as compared with the protective effects of NO in the liver may depend on the local tissue redox state. The products of NO reaction with reactive oxygen species during reperfusion can further modify other proteins via S-nitrosation of thiol groups or nitration of tyrosine residues [7]. The anti-apoptotic effects of NO are at least partly accomplished by its modification of effector caspases via S-nitrosation of cysteine residues on these proteases [8]. Other anti-apoptotic effects of NO are mediated via haem oxygenase-1, heat-shock protein and Bcl-2 [9,10]. Previous work has shown that hepatic I/R injury occurs in two distinct phases with an early phase consisting primarily of Kupffer cell-induced oxidant stress followed by a later phase involving inflammatory cell infiltration and cytokine production. NO reacts with superoxide anion to yield peroxynitrite. Peroxynitrite, which is highly reactive with many targets, can lead to cellular damage or be protective in other settings [11]. The exact role of peroxynitrite in I/R injury has yet to be fully determined. Furthermore, the role of nitrite, formed by oxidation of NO, is being explored in multiple models of I/R injury. In the setting of hypoxic organ damage, it appears that nitrite may play a cytoprotective role via reduction to NO or nitrosated proteins depending on the local tissue pH and oxygen tension. In *in vivo* models of heart and hepatic I/R injury, nitrite delivery appears to reduce injury as measured by serum ALT (alanine aminotransferase)

Key words: damage-associated molecular pattern (DAMP), dendritic cell, high-mobility group box 1 (HMGB1), ischaemia/reperfusion injury, nitric oxide synthase, Toll-like receptor (TLR).
Abbreviations used: DAMP, damage-associated molecular pattern; DC, dendritic cell; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; GM-CSF, granulocyte/macrophage colony-stimulating factor; HMGB1, high-mobility group box 1; IL-6, interleukin-6; I/R, ischaemia/reperfusion; iNOS, inducible NOS; NPC, non-parenchymal cells; TLR, Toll-like receptor.
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and histopathological changes of hepatocellular necrosis [12].

Given its diverse biological effects as a signalling molecule, it is not surprising that NO plays both protective and potentially harmful roles in the setting of hepatic I/R injury. In addition to the apparent benefit of constitutively expressed eNOS-derived NO, iNOS-derived NO is also protective in several models of cold I/R. NO, through its reaction with reactive oxygen intermediates generated in the course of reperfusion injury, can further modify other proteins and prove to be anti-apoptotic. The generation of reactive nitrogen and oxygen intermediates can also mediate much of the hepatocellular damage depending on the intracellular ratio of these intermediates to NO. iNOS and NO production is closely tied to multiple other inflammatory mediators in the liver. NF-κB (nuclear factor κB), IL-6 (interleukin-6) and activation of STAT (signal transducer and activator of transcription) may be a few of the inflammatory downstream signals through which NO mediates I/R injury in the liver [13,14].

Given that L-arginine and L-homoarginine are the sole substrates for the NOSs, modulating their availability in the context of hepatic I/R injury becomes a potentially useful strategy. Circulating arginine is quickly depleted following hepatic I/R secondary to the release of a large amount of arginase, the other major catabolic enzyme of arginine, from necrotic hepatocytes [2]. Due to the rapid enzymatic degradation of circulating arginine by arginase, administering supplemental arginine to animals that have undergone hepatic I/R appears to be an inefficient method [15]. Inhibiting arginase may prove to be a more effective strategy for increasing substrate availability for NOS (K.M. Reid, A. Tsung, T. Kaizu, G. Jeyabalan, A. Ikeda, L. Shao, G. Wu, N. Murase and D.A. Geller, unpublished work). In animal models of warm and cold hepatic I/R, it appears that arginase inhibition is protective. This benefit is most likely mediated via NO-dependent effects [16]. Increasing arginine availability locally may also have NO-independent effects.

TLR4 (Toll-like receptor-4) function in hepatic I/R

More recent work on hepatic I/R injury has focused on the link between innate immunity and the proximal events involved in cell damage. DAMPs (damage-associated molecular patterns) released from damaged cells appear to govern part of this process by engaging certain cell-surface receptors. One of the key receptor families identified in this recognition and signalling process is the family of TLRs [17–20]. Although TLRs recognize and respond to various ligands, TLR4 has been studied extensively in the context of hepatic I/R injury. This particular TLR recognizes both microbial and endogenous molecules. TLR4 responds to several endogenous ligands, or DAMPs, including heparin sulfate, fibrinogen, hyaluronic acid, heat-shock proteins and HMGB1 (high-mobility group box 1) [20–25]. Emerging evidence suggests that DAMPs initiate inflammation in I/R through activation of TLR4-dependent signalling. We and others have shown that animals deficient in TLR4 signalling exhibit reduced damage and a decreased inflammatory response [26–28]. Because both hepatocytes and NPCs (non-parenchymal cells) [Kupffer cells, sinusoidal endothelial cells, stellate cells and hepatic DCs (dendritic cells)] in the liver express TLR4, initial experiments were conducted to elucidate which cell type contributed significantly to the damage following hepatic I/R. A chimaeric mouse model was used to explore these differences. Both wild-type and TLR4-mutant strains of mice underwent irradiation to eradicate bone marrow cells followed by syngeneic bone marrow transplantation with populations of cells with either intact or mutant TLR4 signalling components. In this model, the host's parenchymal cells retained the host phenotype, while the NPCs exhibited the donor phenotype. After subjecting these mice to partial hepatic I/R, it was evident that animals expressing the TLR4 mutant phenotype on NPCs exhibited less damage compared with those expressing intact TLR4 signalling on NPCs following I/R [28]. Further evidence linking NPCs to TLR4-mediated damage included depleting the liver of phagocytic cells using gadolinium chloride. Depletion of these cell populations, which included Kupffer cells and DCs, reduced I/R damage in wild-type mice. Additional protection after phagocyte depletion was not seen in TLR4-mutant mice undergoing I/R. Thus our results suggest that NPCs, rather than hepatocytes, expressing TLR4 are responsible for mediating much of the inflammation and organ damage after I/R.

After uncovering that functional TLR4 on NPCs was a key component in initiating the inflammatory cascade following hepatic I/R, the next question was which type of NPC was primarily involved in TLR4-mediated damage. In several other models, DCs have been shown to play a key role in recognizing endogenous danger signals [29–31]. In order to study the role of DCs in hepatic I/R, the previously mentioned murine model of partial hepatic I/R was employed. To overcome the challenge of small numbers of DCs in the liver, a technique of hydrodynamic tail vein injection of GM-CSF (granulocyte/macrophage colony-stimulating factor) was used to expand the population of liver DCs [32]. Mice with an expanded population of DCs that underwent I/R had worsened injury compared with controls. Levels of pro-inflammatory cytokines, including tumour necrosis factor and IL-6, were increased in animals with increased numbers of DCs undergoing I/R compared with controls. In order to further explore how I/R damage and DCs are related, the effect of I/R on hepatic DC phenotype was examined. Inflammatory cytokine production, mature phenotype and induction of allogenic T-cell proliferation were all increased with DCs following I/R (A. Tsung, N. Zheng, G. Jeyabalan, K. Izuishi, J.R. Klune, D.A. Geller, M.T. Lotze, L. Lu and T.R. Billiar, unpublished work). Since it was established previously that TLR4 activation on NPCs was critical in mediating I/R injury, the expression of this receptor on DCs was examined. TLR4 mRNA levels in hepatic DCs were increased significantly following I/R.

TLR4-mutant mice treated with GM-CSF to expand their DC population did not exhibit increased injury compared with mutant mice without GM-CSF treatment undergoing I/R. Inflammatory cytokine production was also greater in TLR4 wild-type DCs after I/R. Thus it appears that hepatic I/R injury requires functional TLR4 on the DCs.

Uncovering which ligand engages and activates TLR4 on liver DCs and initiates this inflammatory cascade was another key question in understanding hepatic I/R injury. HMGB1 is a nuclear factor that is released or secreted extracellularly by necrotic or stimulated cells as an early mediator of inflammation. Initial experiments in hepatic I/R with HMGB1 elucidated that hypoxic hepatocytes showed increased levels of HMGB1 expression and that inhibiting HMGB1 with a neutralizing antibody decreased hepatic damage following I/R [33]. Furthermore, giving recombinant HMGB1 to mice undergoing partial hepatic I/R worsened injury [33]. Evidence that this was a TLR4 dependent process was that, whereas the wild-type animals given HMGB1 exhibited increased injury, this was not the case in TLR4-mutant mice undergoing I/R and given exogenous HMGB1 [33].

Taken together, these results illustrate some of the proximal events involved in hepatic I/R injury. The innate immune system, through TLR4-dependent pathways, and the distal inflammatory cascade appear to be linked through HMGB1 engagement on DCs during the course of hepatic I/R. Multiple downstream pathways from TLR4 activation have been explored in the context of both endotoxin signalling and activation via DAMPs. How these downstream mediators are interrelated and participate in I/R injury is complex and under investigation. Activation of iNOS and whether iNOS-derived NO is protective or harmful appears to depend on the cell type, *in vivo* compared with *in vitro* models, the specific insult involved and whether the injury is an acute or chronic process. Future directions in this area will be aimed at evaluating the direct relationship between the innate immune system, which is involved in sensing local tissue damage, and inflammatory mediators, including iNOS, NO and HMGB1, which may serve to perpetuate or inhibit further local and systemic injury.

NIH (National Institutes of Health) funding source grant number for T.R.B. is GM-5044109.

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Received 10 August 2006