

Redox therapeutics in hepatic ischemia reperfusion injury

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during hepatic ischemia reperfusion.

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Core tip: Reactive oxygen and nitrogen species play a central role in the pathology of ischemia-reperfusion injury. In this review we discuss the efforts of many groups to trial therapeutics to ameliorate this damage in animal models of disease as well as clinical trials in humans. The failure of some trials has served to highlight the complexity of timing and compartmentalization of Ischemia Reperfusion injury. Finally, we discuss the emerging potential of replenishing nitric oxide by nitrite therapy and the uniquely broad therapeutic profile of nitrite.

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Abstract

Ischemia-reperfusion plays a major role in the injury experienced by the liver during transplantation. Much work has been done recently investigating the role of redox species in hepatic ischemia-reperfusion. As animal models are better characterized and developed, and more insights are gained into the pathophysiology of hepatic ischemia reperfusion injury in humans the questions into exactly how oxidants participate in this injury are becoming more refined. These questions include effects of cellular location, timing of injury, and ability of therapeutics to access this site are increasing our appreciation of the complexity of ischemia reperfusion and improving attempts to ameliorate its effects. In this review, we aim to discuss the various methods to alter redox chemistry during ischemia reperfusion injury and future prospects for preventing organ injury

INTRODUCTION

Ischemia reperfusion injury (IRI) identified in animal models in the mid-70's was not a widely used clinical term until the mid-80's^[1-3]. Ischemia reperfusion is a process whereby the initial damage caused to tissue by compromised blood flow (and associated deficit in oxygen and nutrient delivery) is then compounded by additional and more severe injury caused by restoration of blood flow (*i.e.*, reperfusion). Multiple participants play a role in the post ischemic stress that is caused by restoration of blood flow and oxidant formation, the interplay of these participants form the initial layer of complexity to the problem of IRI. An excellent review by Jaeschke and

Woolbright discusses in greater detail the cellular and molecular participants in post ischemic stress of IRI^[4]. Adding to this complexity is the multiple and diverse situations in which IRI occurs in the clinic which we posit likely leads to unique signatures of IRI in different diseases. In this review we discuss IRI that occurs in liver transplantation and how our understanding of these redox mechanisms may be critical as we attempt to define and implement strategies aimed at expanding donation pools by utilizing marginal donors^[5]. The term marginal donor refers to grafts from donors of old age, steatosis, viral infection or other known insults to the liver graft. The added complexity resulting from each distinct clinical scenario has required further development of our scientific models to more closely mimic the problem^[6]. These models have revealed a more prominent role for IRI when marginal grafts are used. The goal of this review is to provide the reader a brief review of the pathophysiology of IRI and focus the discussion on attempts to ameliorate IRI and how these therapeutics are suggesting a direction forward for clinical strategies.

One emerging candidate for the inhibition of mitochondrial dysfunction and restoration of nitric oxide bioactivity during ischemia reperfusion injury is the Nitrite anion. Emphasis on nitrite within this review will discuss the unique profile of *in vivo* nitrite to address the multiple insults of IRI injury.

IRI

During the ischemic phase of injury liver tissue is left with anaerobic metabolism to keep up with the demand of various cellular processes for high energy phosphates. Eventually the supply of high energy phosphates becomes inadequate resulting in disruption of cellular homeostasis. Primarily disruption of Na⁺/K⁺ ATPase function results in loss of membrane gradients which then allows for Ca²⁺ influx to the cell. Further production of reactive oxygen and nitrogen species (ROS, RNS), H⁺ and toxic metabolites amplify injury and attract water into the cell and the resultant edema further impairs cellular function. As cell death begins within the hepatocytes, nearby endothelial cells and Kupffer cells begin to express adhesion molecules and chemokines, that recruit neutrophils to the site of injury and amplify tissue injury^[7]. Below we discuss individual cell types implicated in liver IRI during transplantation and their respective roles in controlling the redox milieu (Figure 1).

Kupffer cells

Kupffer cells are perhaps the most important producer of ROS during the ischemic stress that occurs during the cold preservation of transplantation. A general lack of appreciation of the details of the hepatic IRI led to studies providing more detail into the timing and compartmentalization of IRI. The observation that glutathione was oxidized in the extracellular space but not within the hepatocyte drew attention to leukocytes. Given that very few neutrophils had infiltrated the liver during the early

phase of ischemia focus was directed at the resident macrophages of the liver, the Kupffer cells, as possible mediators of glutathione oxidation^[8,9]. Given that circulating leukocytes have not begun to infiltrate the liver in the early phase of ischemia and that donor blood is largely flushed from the liver during the initiation of cold preservation it is likely that the role of Kupffer cells in ROS production is relatively more important during the IRI experienced by the transplanted organ. The main ROS generated by Kupffer cells has been demonstrated to be the superoxide anion radical, and selective inhibition of Kupffer cells has been shown to ameliorate IRI further emphasizing the role of ROS derived from these cells^[8,10,11]. Targeting the Kupffer cells clinically is challenging given their multiple roles in normal liver function and defense. However, this work reveals the clinical relevance of inhibiting ROS within the more accessible extracellular or vascular space.

Neutrophils

As Kupffer cells contribute to the early ischemic phase, neutrophils infiltrate and cause much of the damage after reperfusion. Cytokines and chemokines produced by the activated Kupffer cells initiate expression of the cellular adhesion molecules such as ICAM-1 and VCAM-1. Neutrophils become activated, adhere, and begin to infiltrate the hepatic tissue. Once activated neutrophils are capable of producing large amounts of superoxide, hydrogen peroxide, and hypochlorous acid^[12,12]. These ROS are then capable of injuring the hepatocyte with the mitochondria being principal targets^[13,14].

Mitochondria

Mitochondria play a central and complex role in IRI as both sources and targets of reactive oxygen species (ROS). Mitochondria produce ROS such as the superoxide anion as a result of electron leak during normal respiration. This process is enhanced during ischemia leading to decreased ATP production and organelle dysfunction^[15]. Ultimately, mitochondria become central to the fate of the cell as opening of the mitochondrial permeability transition pore (MPT) instantly short circuits the membrane potential resulting in the cessation of ATP production and necrosis ensues^[16]. Interestingly, whilst opening of the MPT may occur if the ischemic phase is long enough, it has also been shown that the MPT opening can be triggered by oxidant stress that ensues with the restoration of oxygen supply thereby supporting the model of IRI in that the tissue injury is amplified during the post ischemic stress phase^[14].

ANTI-OXIDANT BASED THERAPIES TO INHIBIT IRI

As described above, elucidation of the mediators, timing, and location of IRI has proven complex and presented many potential targets for therapy. Indeed further experiments to evaluate the role of many therapeutics within

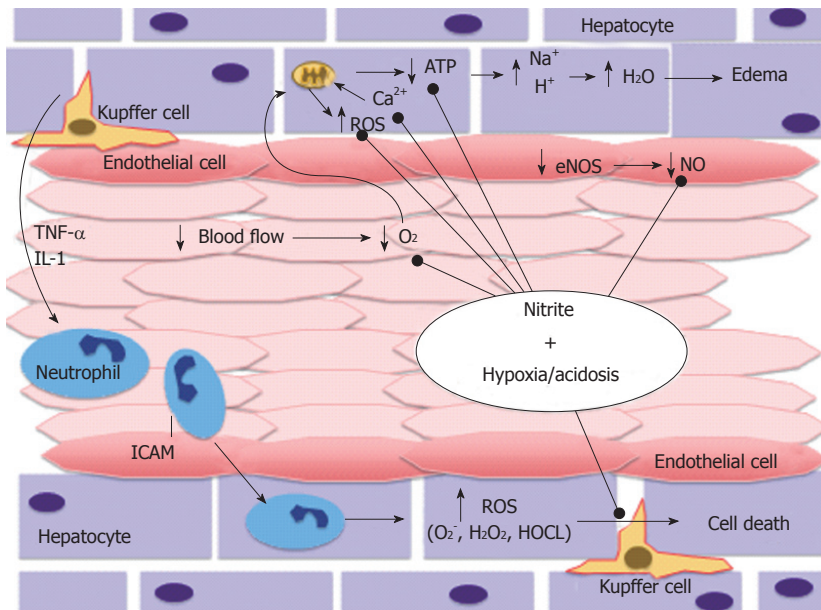


Figure 1 Ischemia initiates injury leading to reactive oxygen species formation from mitochondria and Kupffer cells. Restoration of blood flow introduces neutrophils and substrate that further amplify injury. Nitrite reduction in the setting of hypoxia and acidosis restores nitric oxide bioavailability and augments mitochondrial tolerance to ischemia if administered prior to injury. ROS: Reactive oxygen species; ATP: Adenosine triphosphate; TNF: Tumor necrosis factor; IL: Interleukin; ICAM: Intercellular adhesion molecule; O_2^- : Superoxide anion; H_2O_2 : Hydrogen peroxide; HOCL: Hypochlorous acid.

animal models of IRI have displayed promise^[6]. Further appreciation for the complexities of IRI within humans will develop as we attempt to intervene with therapies in clinical trials. Further, attempts to expand organ donation pools to decrease mortality within the waiting list for organ transplantation will result in a greater need to optimize graft function by inhibiting IRI. The following will discuss several potential therapies of relevance to ROS and RNS during IRI and their translational potential.

Ischemic preconditioning

Since initial observations that exposure of organs to brief periods of non-lethal ischemia provides protection from injury against a subsequent lethal ischemic event^[17], many studies have established the concept of ischemic preconditioning (IP) in multiple organs^[18-20] and across various species, including humans^[18,21,22]. Unlike many pathologies characterized by a period of ischemia, liver transplantation presents an ideal model for therapies such as ischemic preconditioning as liver is exposed to a defined, scheduled, and relatively controlled period of cold ischemia.

Animal models of IP have shown that IRI can be inhibited by a defined therapy period of multiple^[3-5], brief (5-10 min) bouts of ischemia followed by a reperfusion phase of equal time. As alluded to above although organ transplantation may represent an ideal model for IP it also emphasizes that the greatest limitation to IP in other human diseases, such as myocardial ischemia, is the inability to predict when the injury may occur and provide the therapeutic IP. This limitation has led to the search for the mechanism and mediators of IP in hopes of duplicating its effects pharmacologically. Interestingly, this

research has led to the finding that the effects of IP are not completely limited to the timing or location of the ischemic therapy in order to provide a protective effect and have led to the advent of ischemic post conditioning^[23] and remote ischemic preconditioning^[24].

The hypotheses to explain the phenomena of ischemic conditioning generally fall into the general categories of IP causing changes directly on the organ subjected to the repeated IP therapy or that a humoral factor is produced by the tissue undergoing IP that then provides a systemic effect of protection. Given the advent of remote IP the evidence would suggest the latter mechanism predominates. Further, blood taken from rabbits that have undergone preconditioning confer their protective effects during myocardial infarction in rabbits that have not undergone a preconditioning phase^[25]. Mechanistic studies have revealed that adenosine, bradykinin, or perhaps other circulating factors signal through G-protein coupled receptors^[26]. This initiates a signaling cascade involving activation of protein kinase C^[27], heat shock factor 1^[28], and mitogen-activated protein kinase^[29]. Activation of these receptors during preconditioning result in up regulation of multiple systems capable of attenuating IRI including superoxide dismutase, heat shock proteins, and eNOS^[30-32]. Interestingly, the mild oxidation associated with the sub-lethal insult of IP as opposed to the hypoxia itself has been shown to confer protection against a subsequent lethal insult. Specifically, inducing a mild oxidative insult of infusing a peroxide analog into the portal vein of a mouse protected the liver from a subsequent ischemic insult enforcing the concept that timing and amount of exposure to oxidants results in either protection by inducing anti-oxidant de-

fenses, or overwhelm the cell and initiate cell death^[33].

Attempts to translate preconditioning whether pre-, post-, or remote into human disease has had mixed results^[34]. In part this is due to variance in the research and clinical models such as the timing of preconditioning. Mechanistic studies into IP have led to many potential candidates to confer the protective effects of IP pharmacologically. Some of these molecules will be discussed further below and include redox active effectors. Ongoing work in humans will further assess the clinical relevance of IP on liver transplantation outcomes^[35].

Volatile anesthetics

Further evidence to support the principle of providing a pharmacologic agent that imparts the benefits of ischemic preconditioning is the finding that volatile anesthetics impart a similar profile of protection against ischemia reperfusion injury in the heart^[36]. Termed anesthetic preconditioning, this phenomenon has been reproduced in multiple species and by multiple volatile anesthetics. Mechanistically, it appears that anesthetic preconditioning does closely parallel ischemic preconditioning and interestingly *via* ROS species induces a minute stress that activates similar pathways of ischemic preconditioning within target cells. The net effect is the up regulation of anti-oxidant systems such as heme oxygenase, and eNOS that help protect the cell from an ensuing IRI^[37,38]. Human studies have revealed that patients preconditioned with sevoflurane experienced a reduction in peak transaminase levels, an improvement in clinical outcomes, and enhanced benefit in those with steatotic livers. Interestingly, iNOS mRNA was significantly increased in the preconditioned group suggesting a role for NO although further investigation into mechanism or eNOS expression was not performed^[39]. As the role of nitric oxide in IRI and protection is more complex a detailed discussion is saved for a later sub-section.

Glutathione/NAC

Glutathione (GSH) is an important intracellular anti-oxidant and reductant found in high concentrations within hepatocytes. The protective role of glutathione and its importance in intracellular detoxification is emphasized by the model of glutathione oxidation and depletion found in acetaminophen overdose. Glutathione may react directly with oxidants such as peroxynitrite and hydrogen peroxide, but also provides reducing equivalents to maintain catalytic antioxidant systems (*e.g.*, glutathione peroxidase) that provide protection against these ROS and RNS in intracellular compartments^[40,41]. Additionally, hepatocytes export glutathione and thereby detoxify the important ROS produced by Kupffer cells within the vascular space as described above during the early phases of IRI. GSH can either be administered itself or reduced GSH levels can be replenished by N-acetylcysteine (NAC) administration. Both GSH and NAC have been shown to reduce ROS production and oxidant stress after IRI^[42,43]. Additionally, clinical trials of NAC and GSH

have shown a reduction in biochemical markers of liver injury. However, few have reported differences in clinical outcomes associated with GSH or NAC infusion^[44]. It should be noted that GSH or NAC therapy is limited in that non-specific effects are likely, and neither target oxidants in specific compartments. Emerging and exciting recent findings indicate that targeted expression of antioxidants for example in the mitochondria may result in more effective and safer strategies^[45].

α -Tocopherol

α -Tocopherol is an orally administered analogue of vitamin E that limits lipid peroxidation. In general, α -tocopherol therapy studies have led to mixed results. In IRI of the liver, pre-treatment of α -Tocopherol in mice was shown to be protective. In humans, α -Tocopherol had no effect on biomarkers of hepatic damage after hepatic ischemia; however, patients in the α -Tocopherol treatment group had a reduction in ICU stay. Clearly, the implications on ICU length of stay are complex but suggest the potential for α -Tocopherol in human liver transplantation remains and require further testing.

Allopurinol

Xanthine oxidase has long been considered one of the major producers of ROS during IRI largely due to evidence of the protective effect of the xanthine oxidase inhibitor, allopurinol^[46]. However, multiple studies have demonstrated the limited contribution of xanthine oxidase mediated generation of ROS to the post ischemic stress of IRI. These findings are further supported by the dose and length of pretreatment required to convey the protective effects of allopurinol relative to the much smaller dose and time demonstrated to effectively inhibit xanthine oxidase^[47,48]. Additionally, the length of time required (d) for pretreatment with allopurinol to be effective will limit its potential clinically due to the limited lead time a patient has from notification until transplantation due to the nature of organ donation.

Superoxide dismutase

One of the problems with targeting ROS in IRI is the separation of intracellular and extracellular sources of the oxidant stress during IRI as well of the timing of ROS generation within these locations. Generally, Kupffer cells produce ROS early within the ischemic phase that injures intracellular targets within the hepatocytes. Later, during the reperfusion phase previously damaged and dysfunctional mitochondria in addition to infiltrating neutrophils contribute to the oxidant stress within the intra and extracellular spaces. This delineation of timing and location was emphasized by the failure of one of the early attempts of scavenging free radicals as a means to ameliorate IRI. In these studies catalase and superoxide dismutase were administered intravenously either in combination or alone^[49]. This study found only partial protection from IRI implicating a significant role for other ROS or highlighting the weakness that this therapy

cannot target the intracellular effects of ROS. Subsequent studies have utilized carbohydrate modifications of these enzymes or gene delivery to up regulate the intracellular activity of these enzymes and have shown potential benefit and cause for further investigation^[50,51].

Augmentation of endogenous nitric oxide and application of exogenous nitric oxide and Nitrite

Nitric oxide (NO) produced at low levels by endothelial nitric oxide synthase (eNOS) is associated with protection against IRI *via* multiple possible mechanisms including preventing leukocyte adhesion and limiting reactive oxygen species production by mitochondria. Moreover, deficits in eNOS-derived NO have been documented in numerous inflammatory disorders and IRI, although how eNOS activity is altered in human liver transplantation remains to be clearly defined^[52,53]. Consistent with protective effects of eNOS are gene therapy studies that show overexpression of this enzyme in the liver protects against IRI in mice^[54].

An alternate strategy to gene therapy is to augment NO using NO-repletion strategies. Many NO-donors exist but suffer from a lack of compartmentalized release that can result in unwanted effects (*e.g.*, hypotension)^[55]. Recent studies are beginning to address this limitation. As alluded to above, targeting drugs to the mitochondria is now a possibility. This has also been demonstrated with NO, with a mitochondrial targeted S-nitrosothiol showing protection against IRI in the heart^[56,57] in part by limiting ROS production in this organelle. The potential for this strategy in liver transplantation remains to be tested. In addition, recent studies have shown that nitrite administration can replete NO-signaling in areas of ischemia^[58]. The underlying concept is that nitrite will only be reduced to NO by ischemia sensitive mechanisms and thus only produce NO in the environments where needed and avoid unwanted systemic effects (Figure 1). This concept has been validated in numerous experimental models, including hepatic IRI and lung and liver transplantation providing a rationale for testing in humans^[59-64]. Supporting this rationale is the protective effects of inhaled NO in preventing IRI and improving graft function in liver transplant patients^[52]; the protective effects of inhaled NO was posited to be mediated by increased circulating nitrite levels. In addition to a therapy that can be administered during the ischemic phase, nitrite may also be a candidate therapeutic to mimic IP exemplified by studies showing nitrite administration to normal rats, resulting in protection against myocardial and hepatic IRI 24h thereafter *via* mechanisms that involved limiting mitochondrial dysfunction during the IRI period^[61].

CONCLUSION

Further appreciation of the time course and mediators of IRI has led to the discovery of many potential

therapeutics. Each of these faces the hurdle of increased complexity and other unknowns when trying to translate to the pathology seen in human disease. Take for instance the IRI of liver transplantation and the question of when best to administer a proposed therapeutic to: the donor, the graft after harvest, the graft during flushing prior to reperfusion, or after reperfusion. Now, clinical trials must utilize the best data available to choose timing of administration and the most valuable targets to investigate whether the therapy is working by the proposed mechanism. These studies will provide the mechanistic insights currently needed into the IRI of human pathology.

Additionally, the demand for a better understanding of IRI is increasing as we try to reduce the significant wait list mortality caused by demand outpacing supply. This push is causing a closer look at marginal grafts deemed as such because they are steatotic or come from donors of advanced age. These conditions are important to the field of IRI as many of the therapeutics outlined above show an enhanced benefit in steatotic livers. In order to optimize these grafts system changes are occurring within the organ donation network primarily to reduce cold ischemic times associated with transportation. These proposed changes will result in donors being transported to specialized centers more experienced with organ harvest which should minimize organ harvest time and as the recipient will be located at the same medical center this will significantly limit cold ischemic time. Importantly, the advent of donation centers will also create an opportunity to further study these processes. As our clinical trials are often controlled for safety by regulatory boards it has been logistically difficult to administer a therapeutic to a donor that will conceivably affect multiple recipients at multiple institutions. Although donation centers do not remove all logistical barriers of such studies they will create more opportunities to address IRI prior to the ischemic phase and certainly allow for tissue collection to improve our understanding of the human disease.

Given the outlined complexity of IRI it seems that the ideal candidate therapeutic will function by multiple pathways. By preventing ROS production, scavenging ROS in the vascular space and preventing the intracellular damage and mitochondrial dysfunction caused by ROS in the reperfusion phase we may envision an ideal therapeutic against IRI. It is unlikely a single candidate will fulfill the multitude of needs of the ideal therapeutic but as we gain further information on the mechanisms of human IRI it will be possible to identify a combination of therapeutics to best ameliorate the effects of IRI.

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