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Heme Oxygenase and Carbon Monoxide: Medicinal Chemistry and Biological Effects
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Application of Heme Oxygenase-1, Carbon Monoxide and Biliverdin for the Prevention of Intestinal Ischemia/Reperfusion Injury

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Summary **Intestinal ischemia/reperfusion (I/R) injury occurs frequently in a variety of clinical settings, including mesenteric artery occlusion, abdominal aneurism surgery, trauma, shock, and small intestinal transplantation, and is associated with substantial morbidity and mortality. Although the exact mechanisms involved in the pathogenesis of intestinal I/R injury have not been fully elucidated, it is generally believed that polymorphonuclear neutrophils, pro-inflammatory cytokines, and mediators generated in the setting of oxidative stress, such as reactive oxygen species (ROS), play important roles. Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme into equimolar quantities of biliverdin and carbon monoxide (CO), while the central iron is released. An inducible form of HO (HO-1), biliverdin, and CO, have been shown to possess generalized endogenous anti-inflammatory activities and provide protection against intestinal I/R injury. Further, recent observations have demonstrated that exogenous HO-1 expression, as well as exogenously administered CO and biliverdin, have potent cytoprotective effects on intestinal I/R injury as well. Here, we summarize the currently available data regarding the role of the HO system in the prevention intestinal I/R injury.**

Key Words**: intestinal ischemia reperfusion injury, heme oxygenase, carbon monoxide, biliverdin, reactive oxygen species**

Introduction

Despite improvements in surgical technique and pharmacological strategies over the past several decades, intestinal ischemia/reperfusion (I/R) injury remains a clinically significant problem [*1–3*]. A delay in either the diagnosis of intestinal ischemia or in the implementation of effective treatment may cause gangrenous necrosis of the gut. Even after successful reintroduction of blood flow is obtained following bowel ischemia, reperfusion of ischemic gut can potentially worsen acute intestinal injury due to generation

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of reactive oxygen species (ROS), which are known to play a critical role in gut epithelial cell damage. Although the exact mechanisms involved in the pathogenesis of intestinal I/R injury have not been fully elucidated, intestinal I/R injury is a major cause of morbidity and mortality, as the intestine is very susceptible to I/R injury and I/R injury results in systemic complications, including bacterial translocation and multiple organ failure [*4*].

A number of therapeutic modalities have been used to attenuate intestinal I/R injury in both experimental and clinical studies. The heme oxygenase (HO) enzymes are catalysts for the rate-limiting step in the conversion of heme into biliverdin, carbon monoxide (CO) and free iron [*5*]. Recently, activity of the inducible form of HO, HO-1, as well as the byproducts of the HO system, including both CO and biliverdin, have been shown to exert potent cellular protective effects by virtue of their anti-inflammatory, anti-apoptotic and anti-oxidant actions against oxidative stress in various settings [*6*, *7*]. The optimal therapeutic approach is dependent upon an understanding of pathophysiology of intestinal I/R injury and mechanism of the potential strategies aimed at ameliorating I/R injury. Among these potential therapeutic options, application of the HO system might be one of the most promising approaches. The aim of this review is to provide an outline of current knowledge of the HO system and the feasibility of this approach for preventing intestinal I/R injury.

The Heme Oxygenase System

Initially, HO was recognized as the enzyme responsible for heme catabolism by opening its tetrapyrrole ring structure to yield CO, biliverdin, and free iron [*5*]. Biliverdin is rapidly reduced to bilirubin by biliverdin reductase (Fig. 1). Since inducible HO-1 was discovered and found to be a stress-responsive protein with broad cytoprotective properties, interest in the HO system has been renewed and it has attracted the attention of the scientific community worldwide [*8*, *9*].

To date, three isoforms (HO-1, HO-2 and HO-3) have been identified. HO-2 is for the most part a constitutively synthesized protein existing in the brain and testis [*8*, *10*]. HO-3, a recently cloned gene, is also involved in heme degradation, but is a less efficient heme catalyst than HO-2 [*11*]. In contrast, the "inducible" isoform, HO-1, is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions [*12*]. It is upregulated in response to oxidative stress, hyperthermia, and proinflammatory stimuli in a variety of tissues, and has been shown to exert potent cytoprotective and anti-apoptotic properties, providing generalized endogenous anti-inflammatory protection against oxidative stress [*13*]. Although the beneficial roles of HO-1 are not completely understood, there is accumulating evidence that endogenous induction of HO-1 in the setting of various stressors is indispensable for the survival of organisms. Poss and Tonegawa generated HO-1 deficient mice by targeted deletion of the mouse HO-1 gene. Mice lacking the HO-1 gene frequently die *in utero*, and the mice that survive to adulthood exhibit growth failure, anemia, chronic inflammation characterized by hepatosplenomegaly, leukocytosis, glomerulonephritis, and histological hepatoportal cellular infiltration [*14*, *15*]. A human patient that was HO-1-deficient, who unfortunately died in his childhood, demonstrated clinical manifestations similar to those observed in HO-1 deficient mice, including growth failure, anemia, tissue iron deposition, lymphoadenopathy, leukocytosis and increased sensitivity to oxidative injury [*16*]. These observations strongly support the evolving paradigm that HO-1 serves to provide cytoprotection against oxidative stress and is necessary in mammals.

One possible explanation for the protective role of HO-1 may be the removal of free heme. Free heme is highly lipophilic and will rapidly intercalate into the lipid membranes of adjacent cells, and activate vascular endothelial cells resulting in an upregulation of adhesion molecules or E-selectin [*17*]. More importantly, free heme derived from degraded hemoglobin protein during I/R injury has been implicated as the source of catalytic iron that would participate in the Fenton reaction, converting H_2O_2 to more reactive hydroxyl radicals and promoting more severe tissue damage by propagating lipid peroxidation. Thus, the HO system is equipped to cope with the problems caused by intracellular high protein-unbound, free heme concentrations. Furthermore, because HO-1 functions by catabolizing heme to biliverdin, iron, and CO, these byproducts of heme degradation are believed to be effector molecules underlying the potent cytoprotection observed with the HO system [*10*, *18*, *19*]. Thus, in addition to removal of the pro-oxidant heme, in turn, the breakdown of heme to three byproducts has its own significance in essential cellular metabolism and contributes to the suppression of oxidative stress [*20–22*]. Also, the beneficial role of the HO-1 system was substantiated when exogenous HO-1 was demonstrated to ameliorate tissue injury and induce cytoprotection [*23–26*].

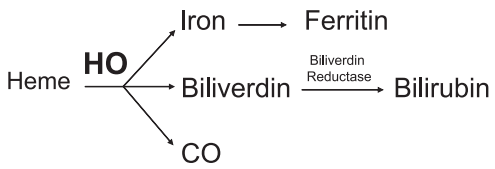

Fig. 1. Heme Oxygenase System

Intestinal Ischemia/Reperfusion (I/R) Injury

Clinical significance of intestinal I/R injury

Intestinal I/R injury occurs frequently in a variety of clinical settings, including surgical treatment for abdominal aortic aneurysm, mesenteric artery occlusion, cardiopulmonary bypass, bowel strangulation, neonatal necrotizing enterocolitis and small intestinal transplantation [*27–31*]. Of note, severe hemorrhagic shock also causes intestinal I/R injury. The systemic response against a decrease in blood pressure or blood volume is characterized by the preferred maintenance of the centralized blood flow, leading to disproportionate splanchnic vasoconstriction, and with resuscitation, the reperfused gut initiates a proinflammatory response that contributes to both local gut and remote organ injury [*32*, *33*]. This vascular phenomenon may partially explain the susceptibility of intestine to I/R injury.

It is generally accepted to conceptually divide I/R injury into two categories of those following warm ischemia and cold ischemia [*34*]. Warm I/R injury usually occurs after temporal reduction or cessation of blood flow to the intestine in warm condition (usually body temperature). On the other hand, cold ischemia is only experienced by some hibernators or by cadaveric donor organs being preserved at low temperature, which is widely-used technique to preserve organs prior to transplantion during the time required to transfer the graft from the donor to the recipient. Hypothermia (cold temperature; usually <4°C) is employed to slow the rate of cellular metabolism and delay the onset of cell death; however hypothermia introduces unique and new forms of cellular damage. Hypothermia suppresses the Na^+/K^+ pump, leading to cellular swelling. Also, cellular damage is accelerated by an increase in chelatable iron and mediated by mitochondrial permeability transition, so-called "chilly injury" [*35*].

Warm and cold I/R injury cause similar injury after reperfusion [*36*]. In both types of I/R injury, the intestine with I/R injury can be a source of pro-inflammatory mediators that enhances local intestinal injury and dysfunction, and activates circulating neutrophils that cause remote organ injury. If severe enough, the inflammatory response after I/R may result in the systemic inflammatory response syndrome and multiple organ failure (MOF), which account for 30–40% of the mortality in tertiary referral intensive care units [*37*]. Thus, I/R injury may extend beyond the ischemic area at risk to include injury of remote, nonischemic organs. Interestingly, the colon is more resistant to ischemia than small intestine in both warm and cold I/R canine models, although the reasons for the difference between the susceptibility of the colon and the small intestine to I/R injury have not been elucidated [*38*].

Experimental models of bowel I/R injury

A simple and reliable animal model can be used to study the pathophysiology of intestinal I/R injury, as well as evaluate potential treatments for this problem. Superior mesenteric artery occlusion (SMAO) and revascularization have been used as reproducible models of warm I/R injury in small or large animals. In this model, the magnitude of I/R injury is time-dependent. The intestinal function of absorption-secretion, the blood flow, the cellular integrity, the mucosa wall, its motility and the hormonal function of the intestinal tract are impaired. Hemorrhagic shock and resuscitation is another well-defined model where intestinal I/R injury is observed. Typically, anesthetized animals are subjected to hemorrhagic shock by shedding blood with a mean arterial pressure (MAP) of ~40 mmHg or less, and then the animals are resuscitated with the fluid or blood. A clinically relevant model of cold I/R injury is intestinal transplantation. Intestinal transplantation procedures necessitate cold preservation and are followed by warm reperfusion of the grafts, resulting in some degree of cold I/R injury in the intestinal grafts. Harvested organs are subjected to further damage at the time of reperfusion when warm oxygenated blood is reintroduced into the graft (warm reperfusion).

Mechanisms of intestinal ischemia/reperfusion injury

Multiple factors have been shown to be involved in the process of intestinal I/R injury. However, intestinal I/R injury basically consists of two specific stages; the ischemic stage, or hypoxemia, and the revascularization stage, or the reintroduction of oxygen into the ischemic intestine. During ischemic stage, the lack of oxygen results in decreased production of adenosine triphosphate (ATP). As the consumption of ATP continues, ATP is degraded into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and further to adenosine, which is then degraded to inosine and hypoxanthine. While hypoxanthine is metabolized to uric acid in the normoxic condition, hypoxanthine is not converted to uric acid and accumulates in the ischemic tissues during hypoxia [*39*]. ATP depletion leads to alterations in intracellular calcium and sodium concentrations and activation of cytotoxic enzymes, such as proteases or phospholipases, ultimately resulting in cell damage.

On reperfusion, as molecular oxygen is reintroduced in the hypoxic intestine, oxygen reacts with hypoxanthine and xanthine oxidase to generate superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2). The superoxide anion requires an additional electron to make it more stable, so it steals an electron from the nearest source, such as mitochondrial DNA or the mitochondrial membrane, causing mitochondrial damage via lipid peroxidation. These initial ROS ($\cdot O_2^-$ and H_2O_2) are not only detrimental, but also signal transduction molecules involved in several signaling cascades

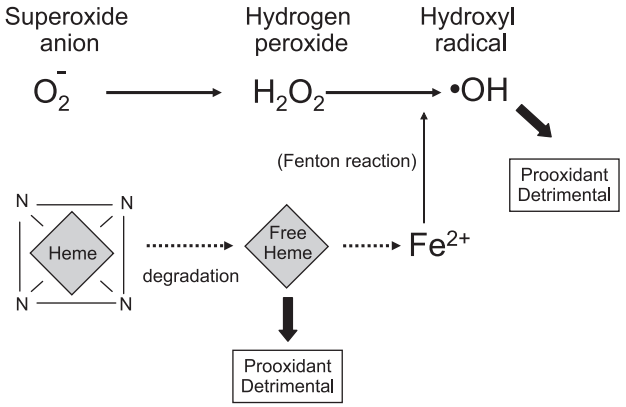

Fig. 2. Reactive oxygen species involved in I/R injury

affecting numerous cellular processes including inflammation and apoptosis [*40*]. The hydroxyl radical, the most detrimental ROS in I/R injury process, is generated in biological systems from superoxide anion and hydrogen peroxide by the Haber-Weiss reaction or from hydrogen peroxide by the Fenton reaction in the presence of iron [*41*, *42*]. Hydroxyl radicals easily react with cellular macromolecules, including DNA, proteins and lipids, and give rise to I/R injury-originated intestinal damage (Fig. 2). In addition, both $\cdot O_2^-$ and H_2O_2 can interact with nitric oxide (NO) and form highly reactive peroxynitrite (ONOO-) [*43*].

Pathophysiology of intestinal I/R

Typically, the intestinal damage by I/R injury starts at the villus layer of the mucosa. There are several explanations for the exquisite susceptibility of intestinal mucosa to I/R injury. Physiologically, the high baseline rate of oxygen use by intestinal mucosa renders the intestine relatively incapable of increasing oxygen transport in the face of hypoxic stress and thus more susceptible to ischemic injury [*44*]. Anatomically, the villous microcirculation is characterized by a peculiar vascular network consisting of centrally located arterial vessels and a subepithelial network of small veins and capillaries. This unique communication of vessels may result in regional hypoxia at the villous tip, even though the overall oxygen extraction efficiency of the gut may be high [*45*]. Furthermore, leukocyte-endothelial cell interaction and capillary perfusion failure occur [*46*]. The malperfusion of intestine is also associated with a marked alteration in lymphatic capillary drainage of the villi.

Microscopic criteria for grading of intestinal tissue injury was reported by Park *et al.* and has been frequently used for evaluating the efficacy of various pharmacological manipulations aimed at modulating intestinal ischemia and its consequences [*29*, *47*]. Grade 0 is defined as normal mucosa and grades 1 to 5 indicate increasing degrees of villous damage. The pathological feature in grade 1 is the development of a subepithelial space at the tip of the villi (Gruenhagen's space). This space is more pronounced in grade 2, and turns to massive epithelial lifting down the side of the villi in grade 3. The villi are denuded of epithelium in grade 4, and lost in grade 5. In grade 6, the crypt layer infarction is seen. In grade 7, the entire intestinal mucosa is necrotic and grade 8 depicts transmural infarction [*29*].

Apart from necrosis, programmed cell death, or apoptosis, is a common cellular response to oxidative injury. Although resultant epithelial cell death has been attributed to necrosis, it is obvious that apoptosis occurs after I/R injury [*48*]. We have previously demonstrated that I/R injury induces apoptosis in the intestinal mucosa [*49*]. Only the procedure of cold storage results in villous and crypt apoptosis [*50*].

Inflammation

Various inflammatory mediators have been implicated in intestinal I/R injury. Of these mediators, the inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β are known to play an important role in the early period of inflammatory response of warm I/R injury. Yamamoto *et al.* demonstrated that plasma TNFα and IL-1β levels were significantly elevated and this occurred in association with increased blood endotoxin levels and mucosal injury. These changes were inhibited by FR167653, a specific IL-1β and TNF inhibitor, suggesting a critical role of TNFα and IL-1β in the pathophysiology of intestinal I/R injury [*51*]. Interestingly, the elevation of TNFα is not dramatic in a cold I/R model [*52*]. These inflammatory reactions are related to the expression of adhesion molecules such as P- and E-selectins [*53*].

Ileus (intestinal dysmotility)

Ileus, defined as impaired propulsive bowel motility, frequently occurs in association with intestinal I/R injury [*54–56*]. An inverse correlation between post-ischemic gastrointestinal motility and the duration of intestinal warm ischemia was reported by Udassin *et al.* [*57*]. The development of ileus results in abdominal distention, nausea, and emesis. In some instances, ileus can contribute to the development of more serious complications, including acute gastric dilatation, pulmonary aspiration, respiratory compromise, cardiac arrhythmias, or intestinal perforation. Furthermore, because ileus precludes oral intake of food or fluids, patients recovering from severe disease status are routinely provided with water, essential electrolytes, glucose and analgesics *via* parenteral routes of administration. However, in the absence of ileus, intravenous fluids and/or parenteral analgesics would be unnecessary in many cases, and, thus, the typical period of hospitalization after abdominal surgery could be greatly shortened.

The pathogenesis of ileus is multifactorial, but inflammation of the gut wall plays a major role. A rat model of intestinal I/R injury after hemorrhagic shock demonstrated

that the development of ileus is accompanied with intercellular adhesion molecule expression and neutrophil infiltration in the intestine [*58*]. The cellular responses after warm ischemia were preceded by the molecular activation of nuclear factor kappa B, upregulation of granulocyte colony-stimulating factor and IL-6 mRNA and phosphorylation of the downstream signaling and transcription factor Stat3 [*59*]. Inducible nitric oxide synthase is upregulated after severe intestinal I/R injury insult and mediates ileus development [*60*].

Bacterial translocation

An important function of the intestinal mucosa is to provide a selective barrier that facilitates the absorption of nutrients while preventing translocation of bacteria and absorption of their toxic products. Intestinal I/R injury has been shown to increase mucosal permeability, which is associated with the development of MOF in patients [*61–63*]. Damage of the mucosal layer by I/R injury allows enhanced uptake of proteolytic enzymes, bacteria, and endotoxin from the intestinal lumen into circulation.[1] This damage adversely affect cardiac and respiratory function, and together with the release of cardiodepressant substances, may create a vicious circle in which impaired heart function leads to a progressive deterioration of intestinal perfusion. This in turn, results in a further reduction of flow to outer layers of intestine, leading ultimately to total bowel infarction [*64*]. The increase in intestinal permeability caused by I/R injury is one of the important factors leading to bacterial translocation [*65*]. Bacterial translocation was seen in 44% of pediatric patients that underwent small intestinal transplantation [*2*].

The Efficacy of the HO-1 System for the Prevention of Intestinal I/R Injury (Table 1)

HO-1 induction protects the intestine from I/R injury

As discussed above, activation of the HO-1 system confers cytoprotective effects during the cascade of events caused by I/R injury [*66*, *67*]. In this regard, HO-1 overexpression can be achieved by either exogenously administration of HO-1 or enhancement of endogenous HO-1.

Exogenous administration of HO-1 by gene transfer could be a specific and attractive approach [*23*]; however, the use of viral vectors in clinical practice is currently very limited and more studies are necessary to show the efficacy and safety of selectively delivering HO-1 in this manner. As far as we know, HO-1 gene transfer as a therapeutic strategy for intestinal I/R injury has not been reported.

On the other hand, there are several pieces of evidence that suggest that induction of endogenous HO-1 through various pharmacological approaches can ameliorate intestinal I/R injury. Ferric protoporphyron (Hemin) is a substrate of HO-1. Attuwaybi *et al.* showed that treatment with Hemin

Table 1. The Efficacy of the HO-1 System for the Prevention of Intestinal I/R Injury

Author	Year	Journal	treatment	Animal	I/R model	reference
HO-1						
Wasserberg	2007	Int J Surg	Cobalt protoporphyrin (CoPP)	rat	warm ischemia (60 min)	[70]
Mallick	2005	World J Gastroenterol	pyrrolidine dithiocarbamate (PDTC)	rat	warm ischemia (30 min)	[72]
Mallick	2005	Microcirculation	Ischemic preconditioning	rat	warm ischemia (30 min)	[79]
Sakamoto	2005	Int J Hyperthermia	hyperthermia (42–43°C)	rat	warm ischemia (30 min)	[80]
Attuwaybe	2004	J Trauma	Hypertonic saline	rat	warm ischemia (60 min)	[73]
Attuwaybe	2004	J Surg Res	hemin	rat	warm ischemia (60 min)	[68]
Nussler	2003	Ann Surg	IL-2	rat	warm ischemia (60 min)	[74]
Attuwaybe	2003	J Surg Res	hypothermia (15°C locally)	rat	warm ischemia (75 min)	[81]
Tamion	2002	Am J Physiol	Ischemic preconditioning	rat	hemorrhagic shock followed by resuscitation	[78]
Tamion	2001	Am J Respir Crit Care Med	hemoglobin	rat	hemorrhagic shock followed by resuscitation	[69]
Tamaki	1999	Transplant Proc	glutamine	rat	warm ischemia (60 min)	[73]
Carbon monoxide						
Nakao	2006	Am J Transplant	ex vivo (5% CO bubbling)	rat	intestinal transplants (6 hrs cold ischemia)	[52]
Nakao	2003	Am J Pathol	inhalation (250 ppm)	rat	intestinal transplants (6 hrs cold ischemia)	[87]
Nakao	2003	GUT	inhalation (250 ppm)	rat	intestinal transplants (1 hr cold ischemia)	[86]
Nakao	2003	Surgery	inhalation (250 ppm)	rat	intestinal transplants (6 hrs cold ischemia)	[85]
Biliverdin/bilirubin						
Nakao	2004	Gastroenterology	biliverdin (ip)	rat	intestinal transplants (6 hrs cold ischemia)	[62]
Ceran	2001	J Pediatr Surg	bilirubin (iv)	rat	warm ischemia (45 min)	[101]
Hammerman	2002	J Pediatr Gastroenterol Nutr	bilirubin (iv)	rat	warm ischemia (45 min)	[102]

(50 μmol/kg, subcutaneously) 2 hours prior to warm I/R injury resulted in increased HO-1 protein expression, reduced mucosal injury, decreased myeloperoxidase (MPO) activity, and improved intestinal transit following gut I/R [*68*]. Likewise, the pretreatment with hemoglobin induces HO-1 and significantly reduced systemic inflammations associated with plasma concentrations of TNFα levels due to intestinal I/R injury in one study [*69*].

Administration of a chemical inducer of HO-1, cobalt protoporphyrin (CoPP), at 24 hours prior to ischemic insult significantly reduced intestinal tissue injury due to warm I/R injury [*70*]. Pyrrolidine dithiocarbamate (PDTC), which is used as an antioxidant and a metabolic inhibitor of a variety of biological reactions, including activation of transcription factor NF-kB, is known to induce HO-1. Mallick *et al.* have reported that PDTC treatment improved intestinal microvascular perfusion and attenuated I/R injury [*71*, *72*]. Glutamine and IL-2 have been also reported to induce HO-1 and prevent intestinal I/R injury, respectively [*73*, *74*]. Recently, interest in hypertonic saline (HTS) has resurfaced, as recent laboratory studies documented that HTS resuscitation profoundly modulates inflammation after hemorrhagic shock partly due to its ability to induce HO-1 [*75*, *76*]. Although several clinical randomized trials designed to test the efficacy of HTS in resuscitation have failed to document a consistent outcome advantage, the use of HTS for hemorrhagic shock may open new therapeutic windows because of its unique mechanisms [*77*].

Non-pharmacological induction of HO-1 is also known to be protective for intestinal I/R injury. Ischemia preconditioning (IPC) refers to the phenomenon by which exposure of tissues to brief periods of ischemia protects them from the harmful effects of prolonged I/R injury. Although the mechanisms underlying the protective actions of IPC have not been fully elucidated, recent evidence has suggested that the protective effects of ischemic preconditioning are attributable to expression of HO-1 [*78*, *79*]. Tamion *et al.* have demonstrated that intestinal IPC with four cycles of 1 min ischemia and 10 min of reperfusion induced intestinal HO-1 and significantly reduced systemic inflammatory reactions related to intestinal I/R injury. These effects were abolished by tin protoporphyrin (SnPP), a HO-1 inhibitor.

Whole body hyperthermia to a core temperature of 42–43°C for 15 min significantly induced the production of heat shock protein-70 (HSP70) and HO-1 in intestinal mucosa and significantly reduced I/R-induced mucosal injury. The combination of zinc protoporphyrin (ZnPP, an HO-1 inhibitor) with hyperthermia extinguished the protective effects of hyperthermia on I/R injury, suggesting that the protective effects of hyperthermia are mediated by HO-1 expression [*80*]. Interestingly, Attuwaybi *et al.* revealed that regional hypothermia induces HO-1 and protected intestine from warm I/R injury [*81*]. Likewise, these protective effects were abrogated by SnPP.

Carbon monoxide (CO) prevents intestinal I/R injury

In accordance with the increasing number of studies about HOs, a new paradigm has been emerging that CO, one of byproducts through heme degradation by HO, functions as a signaling molecule that exerts significant cytoprotection due to its anti-inflammatory, vasodilating, and anti-apoptotic properties [*82–84*]. Similar to what has been observed with HO-1, CO has been demonstrated to mediate potent cytoprotective and anti-inflammatory effects during oxidative stress, hyperoxic lung injury, endotoxemia, and rejection of the liver, heart and lung [*18*, *19*].

We have shown the efficacy of CO gas inhalation for the prevention of cold intestinal I/R injury using a small intestinal transplantation model. Recipient treatment with CO at 250 ppm 1 hour before and 24 hours after reperfusion effectively inhibited an early up-regulation of proinflammatory mediators, yielded less severe histopathological changes, and resulted in significantly improved animal survival compared to air-treated controls [*85*, *86*]. CO also significantly reduced mRNA for proapoptotic Bax, while it up-regulated anti-apoptotic Bcl-2. The protective effects of CO in this study were mediated via soluble guanylyl cyclase, as 1H-(1,2,4)oxadiazole (4,3-alpha) quinoxaline-1-one (ODQ; a soluble guanylyl cyclase inhibitor) completely reversed the beneficial effect conferred by CO, suggesting that the effects of CO was mediated by activation of soluble guanylyl cyclase [*87*].

Since CO is gaseous molecule, inhalation of CO is straightforward delivery method for utilization of the HO systems that can be applied in the clinical setting. However, CO has long been known as a toxic gas due to its ability to interfere with oxygen delivery. To overcome these problematic aspects and minimize the concerns associated with *in vivo* CO gas administration to patients, *ex vivo* application of CO gas might be another way to deliver CO. Our group has previously shown that cold storage in a preservation solution that was bubbled with 5% CO significantly reduced I/R injury associated with intestinal transplantation [*52*].

Biliverdin/bilirubin prevent intestinal I/R injury

Similar to CO, recent evidence has indicated that biliverdin and bilirubin, synthesized as byproducts of the enzymatic reaction of catalyzed by HO, serve as a key mediators that help maintain the integrity of the physiological function of organs [*88*, *89*]. In particular, it is well known that these bile pigments have antioxidant activities [*90*]. Bilirubin is the most abundant endogenous antioxidant and accounts for the majority of the anitioxidant activity in human serum [*91*]. In the general population, high serum bilirubin levels correlate with a reduced risk of ischemic

heart disease [*92–95*] and a lower incidence of retinal damage in newborns [*96–98*]. Although both biliverdin and bilirubin possess anti-oxidant activities, most of the actions are attributed to bilirubin via H-donation to an incipient radical, such as a lipid peroxyl radical (LOO·), to form lipid hydroperoxide (LOOH) and bilirubin radical [*99*]. Oxidation of bilirubin by ROS results in the conversion of bilirubin to biliverdin, which is a precursor of bilirubin in the heme degradation and is recycled to bilirubin by biliverdin reductase in mammals. This recycling between bilirubin and biliverdin is believed to be behind one of the explanations for bilirubin's powerful antioxidant effects in the redox cycle [*100*].

Ceran *et al.* demonstrated that bilirubin has potent protective effects in an experimental of small intestinal warm I/R injury in rats [*101*]. The intravenous injection of 20 mg/kg of bilirubin completely prevented mucosal injury and was associated with less lipid peroxidation and fewer histological changes. Similar results were also reported by Hammerman *et al.*, who also demonstrated that hyperbilirubinemia (14.9 ± 5.7 mg/dl) derived from intravenous injection of bilirubin attenuated intestinal warm I/R injury [*102*].

Administration of biliverdin to rodents has been shown to provide anti-oxidant protective effects in a rat intestinal cold I/R model. Biliverdin administration attenuates transplantation-induced I/R injuries to the small bowel by its anti-inflammatory and anti-oxidant actions [*62*]. The potential toxic actions of bile pigments range from itching in jaundiced patients to severe neuronal damage, primarily in the basal ganglia, as observed in kernicterus. Further examination will be required to assess the long-term outcomes after treatment with bile pigment; however, short term follow has not revealed any adverse effects [*103*].

Conclusions

As shown here, accumulating experimental evidence has clearly revealed that HO-1 overexpression and/or its enzymatic byproducts, including CO or biliverdin, confer protective effects against intestinal I/R injury. HO-1 related pharmacological research is a rapidly emerging field, which is likely to yield a number of therapeutic possibilities for patients in the clinical setting. Therefore, it will be important to explore the therapeutic potential of HO-1 inducers and byproducts of the HO system.

As far as we know, there have been no definitive trials designed to evaluate the efficacy chemical HO-1 inducers in the clinical setting. However, there are increasing reports showing that several drugs that are already in use clinically can induce HO-1 [*104*]. IPC with brief periods of intermittent ischemia, a known potent anti-oxidant intervention, might be used as a tool to prevent the local intestinal I/R injury and the associated systemic inflammatory response. Similarly, several clinical trials designed to evaluate the efficacy and safety of inhaled CO for various disease states have been initiated [*105*]. CO inhalation at a low concentration is applicable for in number clinical settings, and may be particularly useful in patients that are mechanically ventilated. An alternative and promising approach for CO delivery may include the use of CO-releasing molecules, which have been shown to exert vascular and cytoprotective activities that are reminiscent of the HO-1/CO pathway [*106*]. Intraperitoneal injection of CO-saturated solutions might be applicable as a simple procedure [*107*]. Alternatively, biliverdin/bilirubin may be more applicable for clinical use than a pharmacologic HO-1 inducer or CO. Indeed, a preliminary clinical report revealed the effectiveness of Goou and Yutan, traditional Chinese drug containing biliverdin, for the treatment of chronic liver disease [*108*]. Furthermore, it has been shown that therapy involving the combination of CO and biliverdin has additive effects in preventing I/R injury following heart or renal transplantation [*21*]. Considering the sensitivity of the small bowel to I/R injury, the severity of its consequences, I/R injury in intestinal transplantation may be one of the best candidates for early clinical application of these therapeutics.

In conclusion, HO-1 system including CO and biliverdin/bilirubin may serve as promising potential therapeutic options for intestinal I/R injury, although further toxicological studies will be required before they can be employed as drugs in the clinical setting. A more comprehensive understanding of the toxicity, pharmacokinetics, and biology of HO-1-related molecules will certainly help to allow us to harness the protective potential the HO-1 system prior to clinical application of theses therapeutics for the prevention intestinal I/R injury. The scientists working in HO-related field should not lose sight of the clinical patients, for whom the research is being conducted.

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