Heme oxygenase-1 upregulation protects against intestinal ischemia/reperfusion injury: A laboratory based study

Nir Wasserberg a,*, Antonello Pileggi a, b, c, Shashikumar K. Salgar a, Phillip Ruiz a, Camillo Ricordi a, b, Luca Inverardi b, Andreas G. Tzakis a

Department of Surgery, University of Miami School of Medicine, Miami, FL 33136, United States
Diabetes Research Institute, University of Miami School of Medicine, Miami, FL 33136, United States

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Abstract
Objectives: Tissue damage caused by ischemia/reperfusion injury (IRI) of the intestine may lead to organ dysfunction in several clinical conditions, and is associated with increased incidence of chronic rejection after transplantation. Heme oxygenase-1 (HO-1) is a stress-inducible protein capable of modulating inflammation, oxidative stress, and cell death. The aim of the present study was to assess the effects of HO-1 upregulation on intestinal IRI.

Methods: Lewis rats (seven groups, n = 6 each) underwent intestinal warm ischemia induced by clamping the superior mesenteric artery and by ligating the inferior mesenteric artery for 60 min. After 120 or 240 min of reperfusion, tissue samples were collected for analysis. Cobalt protoporphyrin (CoPP) was administered IP at 10 or 20 mg/kg 24 h before IRI, to induce HO-1 upregulation. Control animals received vehicle alone. Tissue injury measurements included the following: histological changes, tissue myeloperoxidase (MPO) activity, nitrate/nitrite levels, and IL-6 levels.

Results: A significant HO-1 upregulation was demonstrated in pre-treated animals (p < 0.05, 95% CI: 0.84 to 0.05). Intestinal IL-6 mRNA expression levels were significantly reduced in animals treated with CoPP 20 mg/kg after 240 min of IRI (p < 0.05, 95% CI: 0.09–2.25). Significant reduction in MPO activity and NO products was observed in treated animals when compared to controls (p < 0.01, 95% CI: 0.07–0.24 and p < 0.01, 95% CI: 5.58–12.75, respectively).

Conclusions: Induction of HO-1 by CoPP administration before IRI was resulted in a significant reduction of intestinal tissue injury. Developing strategies to induce HO-1 upregulation before surgery will be important to reduce IRI in the clinical setting.

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* Corresponding author. Department of Surgery, University of Southern California Keck School of Medicine, 1200 North State Street, Room 18140, Los Angeles, CA 90033, United States. Tel.: +1 323 226 4222; fax: +1 323 226 4244.
E-mail addresses: nwasserberg@surgery.usc.edu, nwasserberg@gmail.com (N. Wasserberg).

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NW and AP equally contributed to this work.

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Introduction

Ischemia/reperfusion injury (IRI) is characterized by severe tissue damage that follows the local production of proinflammatory cytokines, chemokines, and reactive oxygen species (ROS). IRI profoundly influences the fate of the targeted organ, and it plays a critical role in the induction of organ dysfunction. Significant morbidity and high mortality rates have been described after IRI in many pathological conditions, including surgery for small bowel obstruction, abdominal aortic aneurysm, coronary bypass, cardiac infarct, mesenteric ischemia, trauma, and hemorrhagic and septic shock. In organ transplantation, IRI has been associated with early graft dysfunction and primary non-function, and with an increased incidence of chronic graft rejection episodes that affect graft survival. Although reperfusion is essential to restore intestinal function after ischemic events, it worsens the injury following ischemia. The complex mechanisms by which IRI results in tissue damage involve the activation of inflammatory mediators, including production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), upregulation of β2-integrins and endothelial adhesion molecules, triggering of the coagulation cascade, and complement activation. These events result in the amplification of local inflammation, with recruitment and accumulation of activated inflammatory cells, mainly polymorphonuclear (PMN) cells, which exert their cytotoxicity by releasing myeloperoxidase (MPO). Furthermore, the local production of oxidative stress mediators, including nitric oxide (NO) and ROS, contribute to extend tissue injury. The profound alterations of intestinal integrity and function include mucosal damage and increased intestinal permeability which associated with increased risk of bacterial translocation and with occurrence of multiple organ dysfunctions. Recent data point toward the role of the innate autoimmune response (namely natural IGM and the complement system) in IRI. The local production of oxidative stress mediators, including nitric oxide (NO) and ROS, contribute to extend tissue injury. The profound alterations of intestinal integrity and function include mucosal damage and increased intestinal permeability which associated with increased risk of bacterial translocation and with occurrence of multiple organ dysfunctions.

Heme oxygenase-1 (HO-1) is a stress-inducible protein present in many mammalian cell types. Various stimulants can induce upregulation of HO-1 protein expression, including hemolysis, inflammatory cytokines, and oxidative stress. Heme oxygenase-1 is the rate-limiting enzyme of heme degradation into free iron, carbon monoxide and biliverdin, which is readily converted into bilirubin. Induction of HO-1 upregulation has been reported to reduce tissue damage after IRI in various organs, such as brain, lung, and liver; however, data on the protective effect of HO-1 upregulation on small bowel IRI are still limited. Although the mechanisms by which HO-1 upregulation exerts its cytoprotective effects have not been completely elucidated, the central hypothesis focuses on its anti-inflammatory, anti-apoptotic, and antioxidant effects mediated by the by-products of heme degradation, carbon monoxide (CO), biliverdin, and bilirubin, and possibly via MAPK-p38. In the present study, we evaluated the effects of HO-1 induction before IRI on intestinal damage. Intestinal IRI was induced in rats treated with cobalt protoporphyrin (CoPP), a powerful HO-1 inducing agent. Our data demonstrate that CoPP-mediated HO-1 upregulation can significantly reduce IRI-related intestinal tissue damage.

Materials and methods

Animals

Eight-week-old inbred male Lewis rats weighting 200–250 g (Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in the animal facility at the University of Miami School of Medicine, and had free access to water and rat chow. All animal manipulations were conducted under protocols approved by the University of Miami School of Medicine Animal Care and Use Committee.

Rat model for the induction of intestinal IRI

Animals were fasted overnight before surgery. A midline laparotomy was performed under general anesthesia (Isoflurane, Abbott Labs-Animal Health, Abbot Park, IL). The inferior mesenteric artery (IMA) was isolated and ligated at its origin from the aorta. The superior mesenteric artery (SMA) was dissected, free at its aortic origin, and occluded with an atraumatic micro-vascular clamp. The abdominal incision was sutured, and animals were kept under anesthesia during the following 60 min of warm intestinal ischemia. At this time, the incision was reopened and the arterial clamp was removed. The intestine was inspected for complete reperfusion (e.g. pulse, color, peristalsis, and warmth). The abdominal muscular layer and skin were then sutured, and the animals were awakened. After either 120 or 240 min of reperfusion, animals were sacrificed, and tissues were harvested for the analysis of tissue damage.

Experimental design

Cobalt protoporphyrin IX chloride (CoPP; Sigma, St. Louis, MO) was freshly prepared by dissolving it with 0.1 N NaOH and diluting 1:1 v/v in 0.9% NaCl; the pH was adjusted to 7.4 and the solution was sterilized by filtration (0.22 μm). Animals were given a single intraperitoneal (IP) injection of either 10 mg/kg (n = 12) or 20 mg/kg (n = 12) of CoPP 24 h before the induction of intestinal warm ischemia. Animals receiving vehicle alone served as controls (n = 12).

Tissues obtained from additional animals that were treated with the same schedule of CoPP 10 mg/kg (n = 5), 20 mg/kg (n = 5), or vehicle alone (n = 5) but not subjected to IRI, were evaluated for HO-1 protein expression 24 h after injection, by western blot (WB) analysis. Tissue samples were fixed in either 10% buffered formalin or snap-frozen and were stored at −80 °C. Serum samples were
collected at selected time points before and after injury, and were stored at −80 °C until assay.

**Determination of HO-1 expression by western blot analysis**

Frozen tissues were thawed in lysis buffer (330 mM NaCl, 50 mM Tris–HCl pH 7.6, 0.5% Triton X-100, leupeptin 10 μg/ml, aprotenin 10 μg/ml, phenyl-methyl-sulphonyl fluoride 1 mM, iodoacetamide 1.8 mg/ml, all were from Sigma), mechanically homogenized, and incubated on ice for 30 min, with intermittent vortexing. Tissue lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, and pellets were discarded. Protein concentration of the supernatants was assessed by a bicinchoninic acid-based colorimetric quantitation assay (micro BCA kit; Pierce, Rockford, IL). Twenty micrograms of proteins was resuspended in loading buffer 1:5 v/v (0.5 M Tris–HCl with 0.4% sodium dodecyl sulfate pH 6.8, 4 M glycerol, 600 mM dithiothreitol, 0.001% bromophenol blue), boiled for 3 min, and were loaded in a 1.5-mm thick 12% polyacrylamide separating gel. Prestained standards (Kaleidoscope; Biorad, Hercules, CA) were used as molecular weight markers. PAGE was performed at 100 V for 2 h. Recombinant rat HO-1 protein (StressGen Biotechnologies Corp., Victoria, BC, Canada) and bovine muscle actin (Sigma) for 1 h. A horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000; Biorad) was used as secondary antibody, in a 1-h incubation. Positive signals were revealed with the addition of a chemiluminescent substrate (SuperSignal West Pico; Pierce), and exposure for 30 s to 5 min on X-ray films (Pierce). Relative quantities of HO-1 protein were determined using densitometry analysis (Alpha Innotech Co., FluorChem digital imaging system, Alphaeasy-FC 32-bit 1-D analysis software, San Leandro, CA) and normalized according to the actin content of individual samples.

**Real-time quantitative RT-PCR analysis**

Cytokine and HO-1 mRNA steady state levels were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the LightCycler™ instrument (Roche–Boehringer Mannheim, Indianapolis, IN). Extractions of mRNA was performed using the RNA NOW-LM kit (Biogentex, Seabrook, TX). Synthesis of first strand cDNA was performed from DNase-treated RNA (DNase I, Gibco–Life Technology, Carlsbad, CA), utilizing the SuperScript II RT kit (Gibco–Life Technology), with 25 ng/μl oligo(dT)12–18 as primer. The RT product was amplified by PCR in the LightCycler™ that allows real-time quantification of the PCR product, based on the incorporation of a fluorescent dye into the neosynthesized DNA and its measurement at the end of each PCR cycle. Relative levels of the initial transcript copy number in each cDNA sample were calculated as previously described. Primer sequences were created as follows: HO-1 forward 5′-CGCAACCCCCA AACGTAA-3′, reverse 5′-CACAGGTCGGCAACAG-3′; IL-6 forward 5′-AATCTGTCTTGTCTTGGAGTTCCG-3′, reverse 5′-AGC TTCAGGATTTGGGTAAGGAAGC-3′; TNF-α forward 5′- CACCCCTTCTTGCTAATCTCTG-3′, reverse 5′-CACC AGCTGTCCTCTTGGTGTTG-3′; GAPDH forward 5′-TTCC GTGTTCTACCACCC-3′, reverse 5′-TCTTACTCTCTGGAGGC-3′; actin forward 5′-GACTACCTCATGAGATCC-3′, reverse 5′- TCTCTTCTGATCATCCTGTC-3′. Data were analyzed as arbitrary units calculating the ratio to the reference housekeeping message (actin or GAPDH, as specified).

**Histopathological assessment of tissue damage**

Histopathological examination was performed in a blinded manner by single pathologist (P.R.), on 4-μm-thick paraffin-embedded sections stained with hematoxylin and eosin. A score of 0 to 8 described by Park et al.6 was used for the evaluation of intestinal mucosal injury. Score 0 indicated normal mucosa; score 1: subepithelial space at the villi tip; score 2: extended subepithelial space; score 3: epithelial lifting along villous side; score 4: denuded villi; score 5: loss of villus tissue; score 6: crypt layer infarction; score 7: transmucosal infarction; and score 8: transmural infarction. Inflammatory infiltrate was graded from 0 to 3, according to the percentage of inflammatory cells detected on a high power field of the histopathological specimen. Score 0: no signs of infiltration above normal; score 1: mild infiltrate above normal (10–20%); score 2: moderate infiltrate above normal (20–60%); and score 3: severe cellular infiltrate above normal (>60%).

**Myeloperoxidase activity assay**

Myeloperoxidase (MPO) is an enzyme specific of polymorphonuclear neutrophil leukocytes (PMN) and assessment of MPO activity provides an indirect measure of tissue PMN cells infiltration. Tissues were collected at selected time points were snap-frozen and stored at −80 °C until MPO activity was assessed on protein extracts. Briefly, the frozen samples were homogenized with a mortar and pestle in potassium phosphate buffer 0.05 M pH 5.4, containing 0.5% hexadecyl-trimethyl-ammonium bromide (Sigma). They were sonicated at 40 W for 30 s on ice before and after three cycles of freezing (in liquid nitrogen) and thawing (in a water bath at 37 °C), and then incubated for 2 h at 60 °C to inactivate other peroxidase activities. Supernatants were collected after ultracentrifugation of the protein extract at 40,000 × g for 15 min at 4 °C, and were assayed for MPO activity. Protein concentration of the supernatants was assessed by a bicinchoninic acid-based colorimetric quantitation assay (Pierce). Myeloperoxidase activity was measured with a reaction in which tissue supernatants (as source of MPO enzyme) were mixed with 0.004% hydrogen peroxide (H2O2) in 80 mM sodium phosphate buffer, and 16 mM 3,3′,5,5′-tetramethylbenzidine was dissolved in dimethylsulfoxide (all from Sigma). The change in absorbance (ΔA) at 652 nm during a 3-min reaction was acquired using a Beckman
Nitrate/nitrite serum levels

Nitric oxide (NO) plays a major role in mediating oxidative stress. It is practically cumbersome to measure NO production in biological samples because its half-life is very short (seconds), but its by-products nitrates (NO$_3^-$) and nitrites (NO$_2^-$) are stable and can be measured in biological fluids, providing a surrogate marker and quantitative indicator of NO production.

Blood samples from control and treated animals were collected at selected time points before the procedure, after 60 min of ischemia, and after either 120 or 240 min of reperfusion. They were centrifuged at 1000 × g for 10 min at room temperature, and the serum was stored at −80°C until assessment. Nitrate/nitrite levels were measured as previously described, using a modified Griess reaction. Briefly, serum was first centrifuged at 13,500 rpm for 10 min at room temperature, and then the supernatant was deproteinized via filtration through Ultrafree-MC 10,000 MW filter units (Millipore, Billerica, MA). Samples diluted in distilled water were incubated at 37°C for 20 min with 0.2 mM FAD, 2 mM NADPH (Sigma), and 8 U/ml nitrate reductase (Roche) in 96-well flat bottom plates (Corning). After a second 5-min incubation at 37°C with 5 mg/ml L-lactate dehydrogenase (Roche) and 181 mM sodium pyruvate, the plates were chilled on ice, incubated at 4°C for 15 min in the presence of 12.3 mM sulfanilamide dissolved in 0.1 M HCl (Sigma). A 10-min incubation at room temperature in the dark with 1 mM naphthylenediamine was followed, and absorbance was measured at 550 nm. An NaN$_2$O$_3$ standard curve that was run in parallel and data were expressed as total concentration of NO$_2^-$ in micromolar.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance for individual groups. A correction for multiple comparisons was performed using Bonferroni adjustment. Differences were considered significant for \( p < 0.05 \).

Results

CoPP administration results in HO-1 upregulation

In order to assess the HO-1 upregulation that was induced with the utilized treatment protocols, western blot analysis was performed on tissue samples obtained 24 h after injection of saline, CoPP 10 or 20 mg/kg of body weight. Western blot analysis of spleen extracts showed a substantial, dose-dependent upregulation of HO-1, in animals treated with 10 and 20 mg/kg of CoPP (Fig. 1A). Densitometry analysis performed on WB films showed that HO-1 protein expression was 1.4- and 1.7-fold higher than baseline in animal that received 10 and 20 mg/kg of CoPP, respectively (Fig. 1B).

Heme oxygenase-1 mRNA steady state expression was analyzed by real-time quantitative RT-PCR performed on intestinal samples obtained from control animals that did not undergo IRI protocol (baseline), and after 240 min of reperfusion in animals receiving either vehicle or CoPP 20 mg/kg 24 h before treatment. Heme oxygenase-1 baseline levels in untreated animals were 0.11 ± 0.02 arbitrary units; after 240 min of reperfusion they raised to 0.62 ± 0.24 in vehicle-treated animals (\( p < 0.05 \), 95% CI: −0.84 to −0.05 vs. baseline), and 1.22 ± 0.32 in animals receiving 20 mg/kg of CoPP (\( p < 0.001 \) 95% CI: −1.49 to −0.59 vs. baseline; \( p < 0.01 \) 95% CI: −1.07 to −0.15 vs. controls at same time point) (Fig. 2). These data showed that with our intestinal IRI protocol a substantial upregulation of HO-1 mRNA steady state level was induced in control...
Ischemic insult results in a series of morphological alterations of the injured intestine after reperfusion, including mucosal damage and inflammatory cell infiltrate that can be assessed to evaluate the extension of the tissue damage. Mucosal damage and intestinal inflammatory infiltrate assessed on specimens collected after 240 min of reperfusion were reduced in the intestine of animals receiving 20 mg/kg of CoPP, when compared to controls (Table 1) (mucosal damage: 2.0 ± 0.9 and 0.2 ± 0.2, respectively) and (intestinal inflammatory infiltrate: 3.9 ± 0.5 and 1.1 ± 0.4, respectively). However, these results were not statistically significant (p = 0.8, ANOVA, and p = 0.2, ANOVA, respectively). Fig. 3 demonstrates the histological difference between study and controls groups after 240 min of reperfusion. Reduced mucosal damage was not observed after 120 min or in the animals receiving lower CoPP dose.

**COPP induced HO-1 results in reduced histopathological pattern of tissue injury**

IL-6 production is induced upon injury as a response to inflammation and stress. Local synthesis and release of IL-6 may result in amplification of the inflammatory reaction via its chemotactic properties. After induction of ischemia and 240 min of reperfusion IL-6 steady state mRNA expression in the intestine of vehicle-treated animals was 18.2-fold higher than basal levels (no ischemia) (2.24 ± 0.79 vs. 0.12 ± 0.14, respectively; p ≤ 0.01, 95% CI: −3.5 to −0.88). In contrast, IL-6 mRNA levels 240 min after reperfusion were only 8.7-fold higher than baseline when HO-1 upregulation was induced by CoPP administration before IRI (1.06 ± 0.59; p = 0.12, 95% CI: −2.19 to 0.29 vs. baseline, p ≤ 0.05, 95% CI: 0.09−2.25 vs. controls, p < 0.002, ANOVA; Fig. 4). This statistically significant difference in IL-6 reduction was not observed after 120 min of reperfusion.

**Myeloperoxidase activity is significantly reduced in treated animals**

Basal MPO activity in non-manipulated animals was 0.07 ± 0.02 U/mg of protein, and significantly increased 120 min (0.24 ± 0.01) and 240 min (0.22 ± 0.06) after reperfusion in vehicle-treated animals (p < 0.001, 95% CI: −0.21 to −0.10 and p < 0.001, 95% CI: −0.21 to −0.09 vs. baseline, respectively). Pre-treatment of the animals with 10 mg/kg of CoPP to induce HO-1, consistently resulted in a clinical reduction of MPO levels after both 120 min (0.17 ± 0.9, p = 0.2) and 240 min of reperfusion (0.08 ± 0.06, p < 0.01, 95% CI: 0.06−0.23) when compared to their non-treated counterparts. Similarly, the administration of 20 mg/kg of CoPP showed lower MPO levels after

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Ischemia (min)</th>
<th>Reperfusion (min)</th>
<th>Mucosal damage (score)</th>
<th>Inflammatory infiltrate (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>0 (baseline)</td>
<td>0 (baseline)</td>
<td>1.4 ± 0.7</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle</td>
<td>60</td>
<td>120</td>
<td>3.0 ± 0.6</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>60</td>
<td>120</td>
<td>3.2 ± 0.4</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>Vehicle</td>
<td>60</td>
<td>120</td>
<td>2.9 ± 0.3</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>Vehicle</td>
<td>60</td>
<td>240</td>
<td>3.9 ± 0.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>CoPP 10 mg/kg</td>
<td>60</td>
<td>240</td>
<td>3.1 ± 0.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>CoPP 20 mg/kg</td>
<td>60</td>
<td>240</td>
<td>2.0 ± 0.9</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

A reduction in mucosal injury and inflammatory infiltrate scores was demonstrated after 240 min of reperfusion, however, this reduction was not statistically significant.
both 120 min (0.15 ± 0.06; \( p = 1.1 \) vs. CoPP 10 mg/kg and untreated groups) and 240 min (0.07 ± 0.06; \( p < 0.01, 95\% \ CI: 0.07–0.24 \) vs. untreated, and \( p = 0.85 \) vs. CoPP 10 mg/kg-treated group) of reperfusion (Fig. 5).

Treated animals show a reduction of NO metabolites serum levels

Assessment of NO by-products was performed on sera collected at selected time points, in order to determine the effects of the treatments on NO production in this model. Basal nitrate/nitrite levels in sera collected before IRI were 7.9 ± 1.2 µM/ml in controls, 8.4 ± 0.3 and 7.5 ± 1.0 µM/ml in animal receiving 10 or 20 mg/kg of CoPP, respectively (\( p = 1.6 \)). Serum nitrate/nitrite levels significantly increased following IRI in all non-treated groups (\( p < 0.0001, \) ANOVA) (Fig. 6). A statistically significant reduction in NO by-products level was observed only in the group that received 20 mg/kg of CoPP 240 min after reperfusion when compared to untreated animals (8.2 ± 1.6 and 17.3 ± 4.2 µM/ml, respectively; \( p < 0.01, 95\% \ CI: 5.58–12.75 \)) (Fig. 6).

Discussion

Induction of HO-1 upregulation has been documented in a variety of cell subsets and tissues following stress conditions.15,32–34 Additional evidence on the important role of HO-1 in the modulation of inflammation is provided by the observation that mice lacking HO-1 protein have increased sensitivity to oxidant injury,35 and share some pathological features with HO-1 deficiency in humans.36 Amongst other stimulants, NO, cytokines, heme, and heavy metals can induce HO-1 upregulation as a result of modulating HO-1 gene expression.15–18,32

In the present study we report on the protective effect of CoPP-induced HO-1 to reduce intestinal IRI. Expression of HO-1 mRNA steady state levels in the intestine of control animals was increased after injury, suggesting that HO-1 upregulation might be one of the constitutive mechanisms of defense of the organ during IRI. These data are
including the proinflammatory cytokine IL-6.28 In our experi-
animals.38,39
in vitro
CoPP treatment both 

Pharmacological induction of HO-1 upregulation by means of CoPP has been widely used to prevent or reduce inflamm-
mediated injury.16,19,38,39 It is conceivable that both systemic and local upregulations of HO-1 might con-
tribute to the observed protective effects from intestinal IRI in our model of chemically induced HO-1 upregulation. 
This assumption is further supported by previous data, which demonstrated that the inhibition of HO-1 by means of 

Various inflammatory mediators are released by the injured endothelium and by recruited PMNs upon IRI,41 in-
cluding the proinflammatory cytokine IL-6.28 In our experi-
mental model HO-1 upregulation induction by CoPP was paralleled by a statistically significant reduction of inflam-
mation markers. Following IRI, a substantial increase in intestinal mRNA steady level of IL-6 was measured in control animals, while it was significantly reduced in the intestine of animals receiving 20 mg/kg of CoPP, when measured 240 min after reperfusion. Intestinal MPO activity, assessed as an indirect measure of PMN cell infiltrate, was markedly reduced in animals receiving CoPP treatments, when com-
pared to vehicle-treated controls. The reduction of MPO ac-
tivity in treated animals was more dramatic 240 min after reperfusion. Corresponding to MPO activity reduced pattern of inflammatory cell infiltrate and mucosal damage was ob-
served by histopathological analysis.

Oxidative stress plays a major role in the extension of organ injury upon IRI.1,6–11 Nitric oxide might exert a dual effect during intestinal inflammation, being both beneficial in reducing tissue damage, and deleterious in the course of its reaction with superoxide anion to form peroxynitrite anion.42 In the present study, increased NO metabolites (NO2/NO3) were measured in the sera of control animals 240 min after reperfusion. A significant reduction of NO2/NO3 was observed in the animals treated with 20 mg/kg of CoPP at the same time point. This reduction in NO by-
products correlated well with the observed reduction in intestinal tissue damage, suggesting a beneficial effect pos-
sibly caused by the inhibition of NO production through CoPP-mediated HO-1 upregulation.

Collectively, our data suggest that CoPP-induced HO-1 upregulation substantially reduces intestinal damage fol-
lowing IRI in a rat model. This was paralleled by a decreased inflammatory reaction which was more profound when high 
dose of CoPP (20 mg/kg) was given 24 h before IRI insult. Consistent with previous reports,40,43 the present study 
supports the fact that longer time of reperfusion is associ-
ated with increased tissue injury in untreated animals. In 
a model of intestinal warm IRI similar to the one utilized in 
the present study, Koksoy et al.42 reported mortality rates of 28% and 46% after 2 h and 4 h of reperfusion, re-
spectively. Interestingly, our data indicate that the benefi-
cial effects of the pre-treatment with CoPP are more 
pronounced when the analysis is performed after 240 min 
of reperfusion. It is therefore conceivable that the CoPP-
moved HO-1 upregulation may be capable of mediating the earlier recovery of the intestine following IRI observed in 
our study. Our results are in agreement with recent data published during the preparation of the current study. In 
that article, Attuwaybi et al.43 described the protection effect of Hemin induced HO-1 upregulation by showing sig-
nificant reduction in the intestinal mucosal injury and im-
proved intestinal transit following 60 min of ischemia and 
6 h of reperfusion. Their results failed to demonstrate a de-
crease in MPO activity which was attributed to the ‘opti-
mal’ time of reperfusion. The increasing body of evidence 
supporting the key role of HO-1 in inflammation and cyto-

Figure 6 Serum nitric oxide metabolites analyzed by using a modified Griess reaction. Basal levels in sera collected before ischemia reperfusion injury (IRI). Serum nitrate/nitrite levels 
significantly increased following ischemic protocol and reper-
fusion in all non-treated groups (p < 0.0001, ANOVA). A statistically significant reduced nitrate/nitrite level was observed after 240 min of reperfusion in the group receiving 20 mg/kg 
of cobalt protoporphyrin (CoPP) when compared to untreated animals. *p < 0.01, 95% CI: 5.58–12.75; †p < 0.0001, ANOVA. Data are expressed as µM/ml (average ± SD) of NO2/NO3.
of the transplanted organ. Furthermore, cytoprotection induction may minimize graft immunoreactivity, potentially resulting in improved graft function. Further understanding of the molecular mechanisms by which CoPP-induced HO-1 upregulation exerts its protective effects might allow for the implementation of targeted strategies clinically applicable.

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Ethical Statement: All animal manipulations for this study were conducted under protocols approved by the University of Miami School of Medicine Animal Care and Use Committee.

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