**Renin-Angiotensin System**

**HO-1 Induction Improves The Type-1 Cardiorenal Syndrome in Mice With Impaired Angiotensin II–Induced Lymphocyte Activation**


Abstract—Type-1 cardiorenal syndrome, characterized by acute kidney dysfunction secondary to cardiac failure and renal arteriolar vasoconstriction, is mediated by the renin–angiotensin–aldosterone axis and sympathetic nervous system activation. Previous reports indicate that angiotensin II modulates immune function and causes recruitment and activation of T-lymphocytes. The goal of this study was to evaluate the effects of postischemic heart failure on renal morphology and circulation and the beneficial effects of heme oxygenase-1 (HO-1) induction in T-lymphocyte–suppressed severe combined immune deficiency (SCID) mice. Mice were divided into 4 groups: sham, myocardial infarction (MI), MI treated with an HO-1 inducer, cobalt protoporphyrin, and with or without stannous mesoporphyrin, an inhibitor of HO activity. Heart and kidney function were studied 30 days after surgery. Fractional area change was reduced 30 days after surgery in both the C57 and SCID MI–groups as compared with their respective controls (P<0.01). Renal Pulsatility Index and renal injury were increased in C57 and SCID MI–groups compared with the sham group. HO-1 induction improved renal vasoconstriction as well as ameliorated renal injury in both the SCID and C57 MI–groups (P<0.01). However, improvement was more evident in SCID mice. In addition, our results showed that plasma creatinine, angiotensin II, and renin were significantly increased in the C57 and SCID MI–groups as compared with their respective controls. HO-1 induction decreased these parameters in both MI groups. Stannous mesoporphyrin reversed the beneficial effect of cobalt protoporphyrin in both mouse strains. The study demonstrates that T-lymphocyte suppression facilitated the HO-1–dependent improvement in the attenuation of type-1 cardiorenal syndrome. *(Hypertension. 2013;62:310-316.)*

Key Words: angiotensin II ■ heart failure ■ heme oxygenase-1 ■ T-lymphocytes ■ vasoconstriction

Type-1 cardiorenal syndrome (CRS-1) is characterized by acute kidney dysfunction secondary to decompensated heart failure. Several pathophysiological mechanisms have been proposed to explain the interaction between heart and kidney during heart failure. A model based on 4 interrelated cardiorenal connectors: the renin–angiotensin–aldosterone system, the sympathetic nervous system, inflammation, and NO/reactive oxygen species balance has been proposed. Angiotensin II (Ang II) and sympathetic nervous system activation induce renal vasoconstriction, which can be noninvasively evaluated by renal Doppler sonography. The interaction between oxidative stress, inflammation, and activation of renin–angiotensin system (RAS) has been documented well.

Ang II increases in heart failure in an attempt to balance the hypoperfusion of the kidney because of decreased cardiac output. In addition, Ang II plays a key role in the development of cardiorenal syndrome because of its association with increased vascular reactive oxygen species production in kidney, which contributes to renal injury. Various experimental models of excess Ang II demonstrate myocardial and renal fibrosis secondary to increased inflammation. Recent studies on the role of Ang II and aldosterone in the pathogenesis of hypertension have shown that both compounds initiate vascular and tissue inflammation that leads to the activation of the immune response. More specifically, the accumulation of T-cells in the vasculature seems to play a critical role in the development of hypertension. Reports have also shown that Ang II–induced recruitment of T-lymphocytes in renal cortical interstitium aggravates renal damage.

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that degrades heme (a pro-oxidant) to generate carbon monoxide (a vasodilatory gas that also has anti-inflammatory properties), bilirubin (an antioxidant derived from biliverdin), and iron. HO-1 is present in heart and renal tissue, and low levels lead to cardiac and renal dysfunction. Upregulation of HO-1 has a cardio- and renoprotective function mediated...
Increased HO-1 expression has a protective effect against ischemia-reperfusion damage, by improving cardiac metabolism. Increased HO-1 expression has a protective effect against ischemia-reperfusion injury in the kidney, and can correct blood pressure elevation after Ang II exposure. The therapeutic effect of HO-1 induction during cardiac ischemic damage has been studied, but little is known about the beneficial effects of increased levels of HO-1 4 weeks after MI and the role of the enzyme in limiting CRS-1. Carbon monoxide reduces renal arteriolar vascular tone by its effect on Ca channels, and decreases phenylephrine induced vasoconstriction in mesenteric vessels. In addition, increased levels of HO-1 counteract aldosterone-elicited arterial injury through the inhibition of both oxidative stress and inflammation. HO-1 interacts with T-lymphocyte-mediated immunity, playing an immunosuppressive role. Thus, HO-1 induction could be considered as a therapeutic approach to the treatment of CRS-1.

This study demonstrates the role of T-lymphocytes in CRS-1 and the beneficial effects of HO-1 induction in attenuating cardiorenal syndrome in immunocompetent and immunosuppressed mice after postischemic heart failure.

Materials and Methods

The animal protocols were approved by the University of Toledo Animal Care and Use Committee and performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twenty-five SCID and 19 C57 male mice weighing 22 to 26 g, aged 8 to 10 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed under specific pathogen-free (SPF) conditions, in a single ventilated cage system, fed standard mouse pellets and water ad libitum.

Design of the Study

Both animal models, that is, C57 as well as SCID mice were divided into 4 groups: Control, MI, MI treated with cobalt protoporphyrin (CoPP), and MI treated with CoPP and stannous mesoporphyrin (SnMP).

Left Anterior Descending Coronary Artery Ligation Method

Animals were anesthetized using ketamine and xylazine (80 and 10 mg/Kg, respectively). Animal chests were shaved and intubation was done by the endotracheal method. After intubation, animals were kept on ventilation with 100% oxygen and isoflurane (0.75%–1.5%) done by the endotracheal method. After intubation, animals were kept on ventilation with 100% oxygen and isoflurane (0.75%–1.5%) at 180 breaths per minute using a MiniVent Mouse Ventilator (type 845, Harvard Apparatus). Body temperature was maintained at 37°C by using a Heating pad. Left thoracotomy was performed in the 3rd intercostal space under sterile conditions, and the pericardium was opened to expose the heart. An 8.0 prolene ligature (Ethicon) was tied around the proximal left coronary artery, just distal to the left atrial appendage border. Blanching of the anterolateral region of the left ventricle (LV) was used to confirm infarction. Chest was then closed with a single 5.0 silk suture between the 3rd and 4th ribs, and muscle layers were recombined. After closing the skin with a continuous suture, all mice were hydrated with saline and were given an analgesic, carprofen at the dose of 4 mg/Kg, for 2 days. At the end of the surgery, mice were housed for 24 hours in an incubator at 37°C and then allowed to recover in single cages and monitored twice a day for 7 days. Control surgery was performed without coronary artery ligation. All groups underwent echocardiography and renal echodoppler examination 30 days after surgery.

Echocardiographic Evaluation

Transthoracic echocardiography was performed using a Siemens Acuson Sequoia sonography machine with a 15-MHz linear probe. Animals were anesthetized with 3% isoflurane, and temperature controlled anesthesia was maintained with 1.5% isoflurane. Two-dimensional cine loops and M-mode cine loops of a long-axis view and a short-axis view of the LV were recorded. All mice were imaged by a single operator.

End-diastolic areas (EDA) and end-systolic areas, end-diastolic and end-systolic length were measured from the long-axis B-mode image, and end-diastolic diameter (EDD) and end-systolic diameter were measured from the short-axis M-mode image. Fractional Area Change (FAC), an index of LV contractile function, was determined using the following formula: FAC=(EDA–end-systolic areas)/EDA. Aortic Doppler flow was measured from B-mode images of a long-axis view, placing the sample volume at the level of the aortic eflux tract. Aortic velocity–time integral was measured from aortic Doppler flow curve. Aortic outflow tract diameter was measured from the same B-mode images of a long-axis view.

Stroke volume was calculated using the following formula:

\[ \text{Stroke volume}=3.14 \times (\text{Aortic outflow tract diameter}/2)^2 \times \text{Aortic velocity time interval} \]

Cardiac index was calculated using the following formula:

\[ \text{Cardiac index}=(\text{Stroke volume} \times \text{heart rate})/\text{Body weight} \]

Renal EchoDoppler Evaluation

Immediately after echocardiographic evaluation, renal echoDoppler was performed using the same probe (Siemens Acuson Sequoia echo machine with a 15-MHz linear probe). Abdomens of the mice were shaved, and Doppler analysis of interlobar arteries blood flow was performed from a transversal B-mode image of both kidneys placing the sample volume in the renal cortex. All mice were imaged by a single operator. Peak velocity, end-diastolic velocity, and mean velocity were measured, and Pulsatility Index was determined using the following formula:

\[ \text{Renal Pulsatility Index}=(\text{peak velocity}–\text{end-diastolic velocity})/(\text{mean velocity}) \]

Renal Histology

Formalin-fixed, paraffin-embedded kidney sections were cut 5 μm thick, deparaffinized, and rehydrated. For collagen detection, slides were incubated in saturated picric acid containing 0.1% of Direct Red (Sigma) for 1 hour in the dark. Images were captured on a Nikon Eclipse 80i microscope equipped with a Nikon camera head DS-F11 (Niko, Tokyo, Japan). For quantitative analysis, 24 randomly chosen fields from each animal were digitized. Collagen volume was determined using the Image J software (http://rsweb.nih.gov/ij).

Plasma Renin and Ang II levels

The plasma levels of renin and Ang II level were measured in plasma using an ELISA assay (Assay Gate, Inc, Ijamsville, MD). Briefly, the Ang II measurements are based on the competitive method of enzyme-linked immunoassays. The antisera is captured by antibodies coated on a 96-well plate. The Ang II concentration in the sample is determined directly from the standard curve. The mouse renin immunoassay is based on a solid phase sandwich enzyme-linked immunoassay (ELISA) method. Samples, calibrators, and controls are added to the wells coated with monoclonal antibody to mouse renin. After incubation step, the mouse renin binds to the monoclonal antibody on the well. A standard curve is prepared relating color intensity to the concentration of renin on each plate. Positive and negative controls on a plate are used. The tests are end point measurements and in duplicates.

Plasma Creatinine Levels

Plasma Creatinine levels were measured by an enzyme-linked immunoassay (Cayman Chemical Co, Ann Arbor, MI) according to manufacturer instructions.
Drug Administration

CoPP Administration
CoPP, a potent HO-1 inducer, was administered via intraperitoneal injection 5 days after left anterior descending ligation and then every 5 days for 4 weeks at a dose of 3 mg/Kg body weight.

Tin Mesoporphyrin Administration
Tin Mesoporphyrin (SnMP), an inhibitor of HO activity, was administered at a dose of 20 mg/Kg body weight via intraperitoneal injection every 2 days for 15 days before euthanasia.

Statistical Analysis
Data are expressed as mean±SEM. Significance of difference in mean values was determined using 1-way analysis of variance followed by the Newman–Keul post hoc test. *P<0.05 was considered to be significant.

Results

Body Weight Measurement
Mice were examined 30 days after MI, and their body weights were not significantly different in any of the treated groups of both C57 and SCID animals when compared with sham-operated controls (Table).

Echocardiographic Examination
Thirty days after MI, EDA and EDD were increased in both C57 and SCID mice when compared with sham-operated control (P<0.01, respectively). CoPP treatment reduced both EDA and EDD to sham-operated levels (Table). EDA was reduced significantly (P<0.05) in both C57 and SCID when compared with MI mice. A similar result was seen with EDD, a (P<0.01) decrease was seen in C57 mice, and a (P<0.05) decrease in SCID mice when compared with MI mice. SnMP abolished the effects of CoPP, and the values of EDA and EDD were similar to those in the MI mice (Table). Thirty days after MI, cardiac index was significantly reduced in both C57 and SCID mice because of reduced LV systolic function (Table). Administration of CoPP improved cardiac index compared with the MI group in both C57 and SCID mice. Concurrent administration of SnMP reversed the beneficial effects of CoPP. MI resulted in a significant decrease in FAC in both C57 (P<0.05) and SCID (P<0.01) mice when compared with sham-operated animals. CoPP restored FAC levels to those of the sham-operated animals. SnMP reversed the effects of CoPP (Figure 1).

Table. C57 and SCID Mice: In Vivo Heart Measurements by Echocardiography and Renal EchoDoppler 30 Days After MI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHAM</th>
<th>MI</th>
<th>MI+CoPP</th>
<th>MI+CoPP+SnMP</th>
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<tr>
<td>Body weight, g</td>
<td>n (C57,SCID)=8.7</td>
<td>n (C57,SCID)=6.4</td>
<td>n (C57,SCID)=6.4</td>
<td>n (C57,SCID)=5.4</td>
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<tr>
<td>C57</td>
<td>26.5±2.4</td>
<td>26.2±1.5</td>
<td>25.8±1.2</td>
<td>25.7±1.8</td>
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<tr>
<td>SCID</td>
<td>27.4±2.8</td>
<td>27.8±2.2</td>
<td>26±1</td>
<td>25±0.8</td>
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<tr>
<td>Heart rate, bpm</td>
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<td></td>
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<tr>
<td>C57</td>
<td>409±64</td>
<td>397±10</td>
<td>410±52</td>
<td>465±38</td>
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<tr>
<td>SCID</td>
<td>421±52</td>
<td>412±93</td>
<td>419±93</td>
<td>355±22</td>
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<tr>
<td>Echocardiography</td>
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<tr>
<td>EDA, cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C57</td>
<td>0.154±0.02</td>
<td>0.216±0.02§</td>
<td>0.172±0.03†</td>
<td>0.224±0.03§†</td>
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<tr>
<td>SCID</td>
<td>0.15±0.02</td>
<td>0.18±0.02§</td>
<td>0.14±0.01†</td>
<td>0.17±0.011</td>
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<tr>
<td>EDD, cm</td>
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<tr>
<td>C57</td>
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<td>0.471±0.02§#</td>
<td>0.437±0.02*</td>
<td>0.498±0.05§#</td>
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<tr>
<td>CI, ml/min per Kg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C57</td>
<td>2034±3720</td>
<td>1392±65‡</td>
<td>1904±307‡</td>
<td>1439±123‡</td>
</tr>
<tr>
<td>SCID</td>
<td>2165±431</td>
<td>1451±43‡</td>
<td>2037±227§</td>
<td>1560±193§*</td>
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<tr>
<td>PI</td>
<td></td>
<td></td>
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<tr>
<td>C57</td>
<td>0.95±0.005#</td>
<td>1.16±0.096‡</td>
<td>1.14±0.092‡</td>
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<td>0.72±0.08</td>
<td>1.38±0.20‡</td>
<td>0.90±0.19*</td>
<td>1.53±0.1‡</td>
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</tbody>
</table>

Values are expressed as mean±SD. CI indicates cardiac index; CoPP, cobalt protoporphyrin; EDA, end-diastolic area; EDD, end-diastolic diameter; MI, myocardial infarction; n, number of animals tested; PI, Pulsatility Index; and SnMP, stannous mesoporphyrin.

*P<0.05 vs MI.
†P<0.01 vs MI.
‡P<0.05 and vs sham.
§P<0.01 vs sham.
|$P<0.05$ vs MI+CoPP.
||$P<0.01$ vs MI+CoPP.
#P<0.05 vs SCID.
Renal EchoDoppler Examination

Renal arterial resistance indices were significantly lower in SCID mice than in C57 mice (Figure 2). Both C57 and SCID mice developed significant renal vasoconstriction 30 days after MI (Figure 2). In CoPP-treated C57 and SCID mice, Pulsatility Index decreased in comparison with the MI group (Figure 2C). However, the reduction was more pronounced in SCID mice administered CoPP (30% reduction in SCID mice versus 18% decrease in C57 mice in renal vasoconstriction Figure 2D; \( P < 0.05 \)). Concurrent administration of SnMP with CoPP increased renal vasoconstriction in both C57 and SCID MI mice treated with CoPP (Figure 2C and 2D).

Renal Histology Examination

Collagen I and III staining of both C57 and SCID kidney tissue revealed the presence of perivascular and peritubular interstitial fibrosis at the corticomedullary junction. Fibrosis was higher in MI groups in both C57 and SCID mice as compared with control mice (Figure 3A and 3B). However, the level of collagen accumulation in SCID MI mice was significantly lower than in C57 MI mice (\( P < 0.02 \)). CoPP reduced renal fibrosis in both SCID and C57 mice as compared with the MI group, the effect being more evident in SCID mice (Figure 3A and 3B). Concurrent administration of SnMP reversed the beneficial effects of CoPP (Figure 3A and 3B).
Plasma Creatinine Levels
As seen in Figure 4A and 4B, plasma creatinine was significantly higher in the MI group of both C57 and SCID mice ($P<0.05$). However, the plasma creatinine level in SCID MI mice was significantly lower than in C57 MI mice ($P<0.05$). CoPP significantly decreased plasma creatinine levels in both C57 and SCID mice, the effect being more evident in SCID mice (26% reduction in plasma creatinine levels compared with 17% reduction in C57 mice), and this effect was reversed by the concurrent administration of SnMP in both C57 and SCID mice ($P<0.05$; Figure 4C and 4D).

Plasma Renin and Ang II Levels
Myocardial infarction is accompanied by the activation of the RAS system and was examined by the measurement of plasma renin and Ang II levels. Plasma renin (Figure SA and SB in the online-only Data Supplement, respectively) and Ang II levels (Figure SC and SD, respectively) were significantly higher in C57 and SCID MI–groups as compared with their respective control groups ($P<0.05$). CoPP significantly decreased these RAS markers in both strains, and this beneficial effect of HO-1 induction was reversed by concurrent administration of SnMP ($P<0.05$; Figure SA–SD).

Discussion
This study demonstrates for the first time the beneficial effects of HO-1 induction in improving CRS-1 in immunocompetent and immunosuppressed mice. We demonstrate that Ang II mediated recruitment of T-lymphocytes, and that increased oxidative stress is decreased by the upregulation of HO-1 in a model of postischemic heart failure. Our results show that HO-1 induction decreased renal vasoconstriction and fibrosis, and improved renal function in both immunocompetent and T-lymphocyte–suppressed mice, the effect being more prominent in the latter SCID mice.

Thirty days after left anterior descending coronary ligation, C57 and SCID mice developed heart failure characterized by a significant dilatation of the LV with a reduction in FAC. A reduction in FAC leads to a functional hypovolemia and the activation of sympathetic nervous system and renin–angiotensin–aldosterone system, and the release of an antidiuretic hormone. CoPP improved cardiac function, as evidenced by increased FAC and decreased EDA in both SCID and C57 mice. Previous reports have shown that Ang II modulates immune function and causes T-lymphocyte activation and proliferation. Attributing a role of T-lymphocytes in hypertension, we expected that SCID mice would be resistant to the development of CRS-1. As expected, SCID mice had lower basal renal arterial resistance in comparison with the C57 mice probably because of the absence of T-lymphocyte–Ang II interaction. In the MI group, both mouse strains exhibited a significant increase in renal arterial resistance, which was attenuated by the upregulation of HO-1. The CoPP-mediated improvement was more evident in SCID mice, suggesting the role of T-lymphocytes in CRS-1. Thus, it seems that renal vasoconstriction is not related strictly to heart failure but is influenced by other mechanisms that involve interactions between HO-1, renin–angiotensin–aldosterone system, and T-lymphocytes.

The role of RAS is well established in the initiation and maintenance of vascular, myocardial, and renal dysfunction in CRS-1. Consistent with these reports, Ang II and renin levels were increased in the circulation in both C57 and SCID MI–groups and were decreased by CoPP.

One of the pathological abnormalities observed in CRS-1 is renal damage manifested by increased plasma creatinine levels. Our results demonstrate that C57 and SCID MI–groups have not only altered metabolic homeostasis but also increased renal damage, as evidenced by increased collagen deposition and the deterioration in renal function. The production of inflammatory cytokines, attributable to ischemic cardiac damage, causes a systemic inflammatory activation in which the kidney is involved. Systemic inflammation, together with Ang II activation, results in T-lymphocyte recruitment.
and further renal damage. Activated T-lymphocytes amplify the Ang II effect stimulating vascular reactive oxygen species production in kidney contributing to a further increase in reactive oxygen species and added renal damage and dysfunction. Consistent with these reports, we show that plasma creatinine levels and renal damage were less in T-lymphocyte-suppressed mice (SCID) when compared with C57 mice because of the absence of a T-lymphocyte–RAS interaction. Thus, T-lymphocytes contribute to the pathological effects of RAS dysregulation in CRS-1. HO-1 induction is associated with a decrease in T-lymphocyte proliferation (7393). Our results demonstrate that the beneficial effects of increased levels of HO-1 in improving CRS-1 are more apparent in SCID mice than in C57 mice, suggesting that the decrease of the T-lymphocyte immune response amplified the effects of HO-1 in attenuating CRS-1. SnMP reduced the beneficial effects of HO-1 induction on LV contractile function (FAC) and dilatation (EDA, EDD) in C57 and SCID mice, and caused a deterioration in renal vasoconstriction and renal function. The fact that SnMP reversed the beneficial effects of CoPP is a clear indication that increased levels of HO activity plays a central role in preventing MI-induced cardiac and renal damage in this animal model. These observations offer a portal in the development of therapeutic approaches to prevent the irreversible damage that occurs after MI.

In conclusion (Figure 5), we demonstrate that a decrease of the T-lymphocyte immune response reduces kidney damage and renal vasoconstriction in a model of postischemic heart failure, and that suppression of T-lymphocytes amplified the beneficial effects of HO-1 in improving CRS-1.

**Perspectives**

We show that T-lymphocyte suppression facilitated the HO-1–dependent improvement in attenuating CRS-1 after postischemic heart failure. This is of clinical benefit because it highlights potential approaches to reverse the detrimental cardiac and renal perturbations associated with MI by pharmacological induction of HO-1.

**Acknowledgments**

All authors had full access to the data and take responsibility for its integrity. All authors have read and agreed with the article as written.

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**Disclosures**

None.

**References**

What Is New?

- After myocardial infarction, immunosuppressed mice have an increase in renal interlobar arterial resistance and renal damage.
- Beneficial role of heme oxygenase-1 in decreasing renal vasocstriction and renal dysfunction is evident in T-lymphocyte–suppressed mice.

What Is Relevant?

- Our data demonstrate that T-lymphocyte–related immunity plays a role in the maintenance of renal morphology and vasocostriction after post-ischemic heart failure.

Novelty and Significance

This is the first study demonstrating that T-lymphocyte suppression allowed heme oxygenase-1–dependent improvement in renal function and renal damage.
HO-1 Induction Improves The Type-1 Cardiorenal Syndrome in Mice With Impaired Angiotensin II–Induced Lymphocyte Activation
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HO-1 Induction Improves Type-1 Cardiorenal Syndrome in Mice with Impaired Ang II-induced Lymphocyte Activation (SCID Mice)

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Running Title: T-lymphocyte and HO-1 in cardiorenal syndrome

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RESULTS

Plasma Renin and Angiotensin II levels:

Figure S1. Effect of CoPP in C57 and SCID mice on plasma Renin and Angiotensin II levels 30 days post MI. (A, B) Plasma Renin level (ng/ml) *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP; (C, D) Plasma Angiotensin II level (pg/ml) of C57 and SCID mice respectively; *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.