Heme oxygenase system in myocardial infaction and cardio-renal syndrome

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The University of Toledo
A Dissertation

Entitled

Heme Oxygenase System in Myocardial Infarction and Cardio-renal syndrome

By Sumit Ranjan Monu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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(August) 2013
An Abstract of
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August 2013

Sumit Ranjan Monu
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<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>pACC</td>
<td>phospho Acetyl-CoA Carboxylase</td>
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<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
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<tr>
<td>ANOVA</td>
<td>One way analysis of variance</td>
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<tr>
<td>AoOFT</td>
<td>Aortic outflow tract diameter</td>
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<tr>
<td>Bach-1</td>
<td>BTB and CNC homology1</td>
</tr>
<tr>
<td>Cdk2</td>
<td>Cyclin dependent kinase2</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac index</td>
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<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
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<tr>
<td>CoPPIX</td>
<td>Cobalt ProtoporphyrinIX</td>
</tr>
<tr>
<td>CRS-1</td>
<td>Cardio-renal syndrome</td>
</tr>
<tr>
<td>CYP-450</td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Carnitine Palmitoyl Transferase-1</td>
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<tr>
<td>EDA</td>
<td>End diastolic area</td>
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<tr>
<td>EDD</td>
<td>End diastolic diameter</td>
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<tr>
<td>EDL</td>
<td>End diastolic length</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ESA</td>
<td>End systolic area</td>
</tr>
<tr>
<td>ESD</td>
<td>End systolic diameter</td>
</tr>
<tr>
<td>ESL</td>
<td>End systolic length</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelein-1</td>
</tr>
<tr>
<td>FAC</td>
<td>Fractional area change</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HO</td>
<td>Heme Oxygenase</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Keap-1</td>
<td>Kelch-like ECH associated protein 1</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left Ventricular Ejection Fraction</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MCP1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor-erythroid 2-related factor 2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-activated Receptor</td>
</tr>
<tr>
<td>RISK</td>
<td>Reperfusion injury salvage kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell derived factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SnMP</td>
<td>Tin Mesoporphyrin IX</td>
</tr>
<tr>
<td>SnPPIX</td>
<td>Tin protoporphyrin IX</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VF</td>
<td>Ventricular fibrillation</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>ZnPPIX</td>
<td>Zinc protoporphyrin IX</td>
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ABSTRACT

Myocardial infarction (MI) is a major cause of morbidity and mortality in the developing as well as developed world. Heart undergoes severe left ventricular remodeling, after MI which may eventually progress to heart failure. Newer strategies are required to protect the infarcted myocardium against ischemic injury and enhance repair of the infarcted heart. Cardio-renal syndrome (CRS) is another clinical problem which arises from the decompensated heart failure post-MI. CRS is characterized by acute kidney dysfunction and renal vasoconstriction and is mediated by renin angiotensin aldosterone (RAAS) axis and sympathetic nervous system (SNS) leading to renal failure. Recent findings implicate immune system in playing an important role in the development of CRS. The present studies were undertaken to investigate the role of Heme oxygenase-1 (HO-1) in protection and repair of infarcted heart and to examine the potential mechanisms mediating the protective effects of HO-1 in experimental mouse models. In addition, we also investigated the role of HO-1 as well as immune system in the development of CRS by using Lymphocyte suppressed SCID mice.

A total of 250 adult mice were used in the experimental studies (125 C57 mice and 125 SCID mice). An experimental MI model was induced by left anterior descending (LAD) artery ligation and effects of HO-1 induction on ventricular remodeling as well as on cardiorenal syndrome was examined. Echocardiography was used to assess the ventricular function and renal Doppler was used to record the renovascular resistance. Histological and Immunohistochemical techniques were used to assess cardiac angiogenesis, cardiac fibrosis, cardiac necrosis and renal fibrosis.
In the experimental studies, HO-1 expression was induced pharmacologically by Cobalt Protoporphyrin (CoPPIX) and Stannous Mesoporphyrin (SnMP) was used to inhibit the activity of HO and served as negative control. HO-1 induction promoted angiogenesis in infarcted hearts via vascular endothelial growth factor (VEGF) and Ets-1. In addition, HO-1 showed many beneficial effects on cellular and extracellular remodeling in the infarcted heart. We noticed improved cardiac energy metabolism with the treatment of CoPP. HO-1 favored fatty acid oxidation to meet the high energy demand of an infarcted heart.

We also investigated the role of HO-1 in CRS-1 in conjunction with immune system by using immunosuppressive and immunocompetent mice. We found that HO-1 has the potential to ameliorate CRS-1 in both SCID and C57 mice. Our study demonstrated that HO-1 dependent improvement in CRS-1 is more evident in immunosuppressed mice than in immunocompetent mice. This novel study indicates the modulatory role of immune cells in HO-1 dependent improvement of CRS-1 in experimental MI mouse model.

In conclusion, HO-1 in the experimental murine MI models played an important role in the recovery and repair of infarcted hearts by increasing angiogenesis, improving cardiac energy metabolism and decreasing myocardial fibrosis. Induction of HO-1 also ameliorated CRS in post MI mouse model.
1. INTRODUCTION

Cardiovascular disease accounts for 1 of every 2.9 deaths in the United States (Roger, Go et al. 2011). Myocardial infarction (MI) is one of the major causes of most of heart failures. Survival rates have improved dramatically in patients suffering an acute MI due to advanced medical management (Parikh, Gona et al. 2009). However it has resulted in a large number of individuals who, having survived the acute infarction, are at risk of developing heart failure (Parikh, Gona et al. 2009). Though advanced therapeutic strategies are adopted to treat these patients, the risk of developing heart failure remains high. Heart failure post MI is associated with alterations in cardiac geometry, function and structure i.e. ventricular remodeling (Cohn, Ferrari et al. 2000). Ventricular remodeling causes severe molecular and cellular changes in the heart affecting both the necrotic area as well as non-infarcted regions of the heart. This is clinically manifested as increased ventricular dilation, myocardial hypertrophy and deterioration in heart function(Cohn, Ferrari et al. 2000). The severity of ventricular remodeling depends upon the infarct size and the quality of cardiac repair (Cohn, Ferrari et al. 2000).

Heme oxygenase-1 (HO-1) is a stress-responsive and cytoprotective enzyme that catalyzes the degradation of heme into the biologically active reaction products biliverdin, carbon monoxide (CO) and free iron (Cao, Inoue et al. 2009). HO-1 plays a key role in maintaining cellular homeostasis (Otterbein, Bach et al. 2000), (Otterbein and Choi 2000), (Otterbein, Zuckerbraun et al. 2003). The cytoprotection is mediated by the antiapoptotic, anti-inflammatory, antioxidative, antiproliferative, and vasodilatory properties of HO reaction products (Otterbein, Zuckerbraun et al. 2003), (Brouard, Otterbein et al. 2000), (Otterbein, Bach et al. 2000), (Peyton, Reyna et al. 2002).
Interestingly, HO-1 is known to promote angiogenesis (Deramaudt, Braunstein et al. 1998), (Dulak, Deshane et al. 2008). HO-1 has been also acknowledged as a cardioprotective enzyme in various cardiovascular diseases but the underlying cardioprotective mechanism of HO-1 is still not well understood. We therefore propose that treatment with the HO-1 inducer Cobalt ProtoporphyrinIX (CoPP) will reverse heart dysfunction in the infarcted heart via the attenuation of fibrosis, enhanced angiogenesis, decreased inflammation and the reversal of ventricular remodeling in a murine model of experimental MI that involves the ligation of the left anterior descending artery (LAD). The possible role of a HO-1 inducer in ameliorating cardiac dysfunction by improving cardiac cellular and extracellular remodeling will offer a portal to new therapeutic interventions for surviving infarct patients. This will benefit not only the patient’s quality of life and their families but also the burgeoning health care costs associated with cardiac disease.

In addition we also investigated Type-1 cardiorenal syndrome (CRS-1) in this experimental MI model which is characterized by acute kidney dysfunction secondary to cardiac failure and renal arteriolar vasoconstriction. CRS-1 is mediated by renin-angiotensin and sympathetic nervous system (SNS) activation. HO-1 induction improves renal function in animal model of angiotensinII (AngII) induced hypertension. It has been reported that, by affecting the immune system by pharmacological interventions or by thymectomy, hypertension can be prevented (Geiger 2008). Therefore, in the present study, we evaluated the effects of post-ischemic heart failure on renal circulation and the differential effect of HO-1 induction in T- lymphocyte
suppressed severely combined immunodeficient mice SCID mice. We therefore propose that HO-1 induction will ameliorate CRS-1 in this experimental MI model.

2. REVIEW OF THE LITERATURE

2.1. History of HO-1

In recent years, science has witnessed an explosion in the elucidation of the role that the HO system plays in human physiology. HO is the rate-limiting enzyme in the catabolism of heme, a process that leads to the formation of equimolar amounts of the bile pigment biliverdin, free iron, and CO (Ibrahim, Friedland et al. 1983), (Abraham and Kappas 2008). Initially, it was incorrectly thought that cytochrome p-450 (CYP-450) was essential for the catabolism of heme (Tenhunen, Marver et al. 1969). However, this issue was conclusively settled by an elegant study in which the authors, Kappas and his Co-authors, showed clearly that HO could be induced in the liver by the inorganic element cobalt, independent of CYP-450, and that a complete separation of HO from CYP-P450 could be achieved (Maines and Kappas 1974). Heme degradation occurs mainly by oxidative cleavage of the α-methylene bridge of the heme molecule, eventually leading to the formation of the biliverdin IXα isomer along with trace amounts of IXβ, IXδ, and IXγ isomers. Biliverdin-IX formed in this reaction is rapidly reduced to bilirubin-IXα by biliverdin reductase. HO was purified to homogeneity from rat liver and pig and bovine spleen and was shown to have a molecular mass of 32 kDa (Jarstrand and Tunevall 1975), (Yoshida and Kikuchi 1978), (Yoshinaga, Sassa et al. 1982). Subsequently, the isolation and characterization of human HO-1 was reported by Abraham et al in 1988 (Abraham, Mitrione et al. 1988).

HO exists in two isoforms, HO-1 and HO-2. HO-1 (32-kDa) is an inducible
isoform, also known as heat shock protein 32, and HO-2 (36-kDa) is a constitutively expressed isoform (Yoshinaga, Sassa et al. 1982), (Keyse and Tyrrell 1989). HO-3, a third isoform has also been described in rats, but it became evident that it is a pseudogene derived from the HO-2 transcript (McCoubrey, Huang et al. 1997), (Hayashi, Omata et al. 2004). HO-1 and HO-2 are products of two genes (Cruse and Maines 1988), (Maines 1997) and localized in chromosome 22 and chromosome 16 (Maines 1997) respectively. HO enzyme activity can be inhibited using synthetic metalloporphyrins of which Zinc Protoporphyrin IX (ZnPPIX) and Tin Protoporphyrin IX (SnPPIX) are most commonly used (Drummond and Kappas 1981), (Drummond and Kappas 1982). Down-regulating HO activity (Drummond and Kappas 1981), (Yoshinaga, Sassa et al. 1982) was first shown to be therapeutically important when a safe and effective method for transiently blocking bilirubin production in newborns was developed (Kappas, Drummond et al. 1988), (Kappas, Drummond et al. 1995), (Kappas, Drummond et al. 2001), (Valaes, Drummond et al. 1998), (Martinez, Garcia et al. 1999), (Kappas 2004). This method resolved the problem of progressive, unpredictable, and often undiagnosed jaundice in newborns especially those infants born in deprived socioeconomic settings who had a particular risk of brain damage resulting from uncontrolled hyperbilirubinemia (Abraham and Kappas 2008). HO-1 is ubiquitously present in almost every organ at low levels and under physiological conditions. High levels of HO-1 are found only in the spleen and other tissues/cells that degrade senescent erythrocytes, such as the reticuloendothelial cells of the liver and bone marrow (Tenhunen, Marver et al. 1968), (Tenhunen, Marver et al. 1969). HO-2 differs from HO-1 in tissue distribution and high levels of HO-2 are found in the testes, brain, central nervous system, liver, kidney, vasculature, and gut
Predominantly HO is localized in the microsomal fraction, or smooth endoplasmic reticulum (Tenhunen, Marver et al. 1969). However, HO-1 has recently been found in other subcellular compartments including the plasma membrane caveolae of endothelial cells together with biliverdin reductase and it is suggested that compartmentalization of HO-1 in the caveolae may play a role in cellular protection by modulating caveolae-mediated signaling cascades (Kim, Wang et al. 2004). A fraction of HO-1 is localized in the liver mitochondria along with biliverdin reductase and modulates mitochondrial heme metabolism and oxygen uptake and production of reactive oxygen species (ROS) (Converso, Taille et al. 2006). Mitochondrial localization of HO-1 could explain its protective effect under conditions characterized by increased mitochondrial ROS production, such as ischemia reperfusion injury, sepsis, and neurodegenerative disorders (Converso, Taille et al. 2006). Exposure to hypoxia or Hemin resulted in nuclear localization of a truncated form of HO-1 lacking the C-terminus of the protein (Lin, Weis et al. 2007). Despite the decreased enzyme activity of this truncated nuclear HO-1, it was equally cytoprotective, possibly via activation of transcription factors that are involved in the oxidative stress response, including activator protein-1(Lin, Weis et al. 2007). HO-1 is degraded via an endoplasmic reticulum-associated pathway and is mediated by the ubiquitin-proteasome system (Lin, Chiang et al. 2008).

2.2. Induction and regulation of HO-1

2.2. A. HO-1 inducing factors

HO-1 expression and HO activity are influenced by numerous factors. The induction of HO enzyme activity by its substrate heme was reported by Tenhunen et al. (Tenhunen,
Marver et al. 1970). Shibahara et al. showed that HO-1 expression was increased by heat shock in rats and suggested that HO-1 is a heat shock protein (Shibahara, Muller et al. 1987). Although HO-1 is up-regulated in response to heat shock in rats (Taketani, Kohno et al. 1988), (Raju and Maines 1994), it is not induced by hyperthermia in humans (Yoshida, Biro et al. 1988), (Shibahara, Sato et al. 1989), (Taketani, Kohno et al. 1989). Many HO-1-inducing stimuli have been characterized since these initial studies in the 1980s, and the role of HO-1 as a stress response protein has become evident (Otterbein and Choi 2000). Most of the HO-1-inducing factors and conditions cause oxidative stress by increasing the production of ROS and/or decreasing intracellular glutathione levels (Ryter, Alam et al. 2006). It is also important to note that several metalloporphyrins e.g. ZnPPIX and SnPPIX can paradoxically induce HO-1 expression, although they also inhibit HO enzyme activity (Sardana and Kappas 1987).

2.2. B. Regulation of HO-1 expression

The existence of a vast number of HO-1 inducers is suggestive of several response elements in the promoter of the HO-1 gene and numerous interactions between the components of different signaling pathways. HO-1 Induction involves activation of different protein phosphorylation cascades, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), tyrosine kinases, and protein kinases A, C, and G (Ryter, Alam et al. 2006). The MAPKs are major mediators of the HO-1 stress response. The MAPK system is comprised of three signaling pathways, the extracellular-regulated kinases (ERK pathway), the c-Jun N-terminal kinases (JNK pathway), and the p38 kinases (p38 pathway). HO-1 inducers may activate one or more of these MAPK pathways (Ryter, Alam et al. 2006), (Alam and Cook 2007). Among all
three MAPK pathways, p38 pathway is the major MAPK cascade activating HO-1 in response to various stresses. However, Sodium arsenite activates all three MAPK pathways in liver tumor cells and induces HO-1 (Elbirt, Whitmarsh et al. 1998). MAPK cascades are activated during cellular stress and regulate cell proliferation and differentiation (Cobb 1999). Nitric Oxide (NO) induced induction of HO-1 involves both the p38 MAPK and ERK pathways in HeLa cells (Chen and Maines 2000), whereas hypoxia induces HO-1 in rat cardiomyocytes via the p38 MAPK pathway alone (Kacimi, Chentoufi et al. 2000).

The HO-1 gene is comprised of a proximal promoter region at 0.3 kb upstream from the transcription initiation site and two distal enhancer regions at 4 and 10 kb upstream from the transcription initiation site (Alam, Cai et al. 1994), (Alam, Camhi et al. 1995), (Alam and Cook 2007). A stress-responsive element is the dominant regulatory element in the distal enhancers and contains the heme response element and the AP-1 binding site (Ryter, Alam et al. 2006). Several transcription factors including AP-1 factors (Jun and Fos), Maf proteins, and the cap’n’collar/basic-leucine zipper transcription factors Nrf2 (nuclear factor-erythroid 2-related factor 2) and Bach1( BTB and CNC homology 1) bind to the StRE as hetero- or homodimers (Alam and Den 1992), (Alam, Stewart et al. 1999), (Sun, Hoshino et al. 2002), (Sun, Brand et al. 2004). Nrf-2 positively regulates antioxidant, anti-inflammatory and cytoprotective genes containing ARE element (Motohashi and Yamamoto 2004), (Levonen, Inkala et al. 2007), (Jyrkkanen, Kansanen et al. 2008). Both Nrf2 and Bach1 form heterodimers with small Maf proteins. Nrf2 increases HO-1 transcription, while Bach1 competes with Nrf2 and represses HO-1 transcription (Alam, Stewart et al. 1999), (Sun, Hoshino et al. 2002),
(Sun, Brand et al. 2004). HO-1 induction could also be through other transcription factors too. Hyperopia and lipopolysaccharide induce HO-1 via AP-1 factors (Camhi, Alam et al. 1995), (Lee, Camhi et al. 2000). Heme dictates its action by binding with Bach1 and inhibits its DNA binding activity leading to nuclear export (Sun, Hoshino et al. 2002), (Suzuki, Tashiro et al. 2004). In addition, a cytoplasmic Kelch-like ECH-associated protein 1 (Keap1) binds Nrf2 under basal conditions, prevents its translocation to the nucleus, and facilitates its degradation, thus reducing HO-1 expression (Itoh, Wakabayashi et al. 1999), (Itoh, Wakabayashi et al. 2003). In cells exposed to oxidative stress, Nrf2 dissociates from Keap1 and moves from cytoplasm to the nucleus, and binds to its target genes (Dinkova-Kostova, Holtzclaw et al. 2002).

In addition to StRE, other regulatory elements like (CAAT)/enhancer-binding protein(C/EBP) site and the cadmium-responsive element (CdRE) are found in the promoter region of HO-1 (Ryter, Alam et al. 2006). There are binding sites present in the promoter region of the HO-1 gene for nuclear factor-NB (NF-NB), signal transducer and activator of transcription 3 (Stat3) and hypoxia-inducible factor 1α (HIF-1α) have also been identified, as well as IL( Interleukin)-6, heat shock and metalloporphyrin-responsive elements (Shibahara, Sato et al. 1989), (Lavrovsky, Schwartzman et al. 1994), (Lee, Jiang et al. 1997), (Deramaudt, da Silva et al. 1999), (Yang, Nguyen et al. 2001), (Ryter, Alam et al. 2006). The presence of several of the regulatory elements described above in the HO-1 promoter gene support the role of HO-1 as a stress response protein.

In humans the expression of the HO-1 gene is also influenced by polymorphisms in the HO-1 promoter region. The shorter GTn repeat allele and -413A allele enhance transcriptional activity of the HO-1 gene compared with the long GTn and
-413T alleles (Hirai, Kubo et al. 2003), (Ono, Goto et al. 2004), (Brydun, Watari et al. 2007). This HO-1 promoter polymorphisms could play an important role in clinical conditions as it has been associated with cardiovascular diseases including coronary artery diseases and restenosis (Kaneda, Ohno et al. 2002), (Schillinger, Exner et al. 2004). However, there are contradictory reports of no association between HO-1 polymorphism and disease (Tiroch, Koch et al. 2007), (Turpeinen, Kyllonen et al. 2007).

2.3. Protective role of HO-1 and its reaction products

Initially it was thought that the beneficial effects of HO lay in basic heme metabolism and that the heme degradation products were waste products with potential toxic effects. High levels of inhaled CO are known to impair O2 transport and cause tissue hypoxia, high levels of bilirubin cause neonatal jaundice and neurologic damage, and iron is a pro-oxidant. However, HO-1 emerged as a protective enzyme in various tissues and conditions, including the cardiovascular system (Otterbein, Soares et al. 2003), (Peterson, Frishman et al. 2009). The beneficial effects of HO-1 are presumably achieved by the degradation of the pro-oxidant called as heme and the heme degradation products. Biliverdin and bilirubin serve as powerful antioxidants (Stocker, Yamamoto et al. 1987) while CO mediates the antiapoptotic, anti-inflammatory, antiproliferative and vasodilatory properties of HO-1 (Thorup, Jones et al. 1999), (Brouard, Otterbein et al. 2000), (Otterbein, Bach et al. 2000), (Peyton, Reyna et al. 2002). Iron induces the synthesis of ferritin, which is also a cytoprotective molecule that sequesters free iron (Vile and Tyrrell 1993).
2.3. A. Antioxidant effect

Biliverdin and bilirubin play a major role in mediating HO-1 induced antioxidant effect. Antioxidant activity of bilirubin increases in hypoxic conditions (Stocker, Yamamoto et al. 1987). Biliverdin and bilirubin scavenge peroxyl radicals and other ROS, including superoxide, hydroxides, hypochlorous acid, and singlet oxygen (Nakamura, Uetani et al. 1987), (Stocker and Peterhans 1989), (Stocker 2004). Bilirubin can protect cells from a 10,000-fold excess of hydrogen peroxide (H2O2) (Baranano, Rao et al. 2002). Biliverdin and bilirubin also scavenge reactive nitrogen species such as peroxynitrite (Kaur, Hughes et al. 2003), (Mancuso, Bonsignore et al. 2003). The mechanism behind the
powerful scavenging of reaction oxygen/nitrogen species by bilirubin is explained by an amplification cycle, whereby bilirubin is oxidized to biliverdin and recycled back to bilirubin by biliverdin reductase (Baranano, Rao et al. 2002). However, it has recently been shown that the bilirubin-biliverdin redox amplification cycle has a limited role in anti-oxidant defense (Maghzal, Leck et al. 2009). This finding was further corroborated by recent research in which bilirubin is shown to be a more efficient antioxidant than biliverdin and conversion of biliverdin to bilirubin by biliverdin reductase protects the integrity of the cell (Jansen, Hortmann et al. 2010). HO-1 generated bilirubin also protects the cell against oxidative stress in vascular smooth muscle cells (SMCs) (Clark, Foresti et al. 2000). Both bilirubin and biliverdin protect kidneys and liver against ischemia reperfusion injury (Fondevila, Shen et al. 2004), (Adin, Croker et al. 2005).

2.3. B. Antiproliferative effect

In several cell types such as VSMCs, T cells, and fibroblasts, HO-1 inhibits their proliferation (Peyton, Reyna et al. 2002), (Song, Mahidhara et al. 2002), (Song, Mahidhara et al. 2004), (Zhou, Song et al. 2005), (Liu, Pachori et al. 2006). Similar to the anti-apoptotic effect, the antiproliferative effect of HO-1 is predominantly driven by CO (Morita, Perrella et al. 1995), (Morita, Mitsialis et al. 1997). Interestingly, signaling pathways involved in the antiproliferative effect of CO differ in different cell types. CO down regulates the ERK MAPK pathway (Song, Mahidhara et al. 2002) in airway SMC and thus inhibits proliferation while in VSMCs, CO exerts its antiproliferative effect via activation of the p38 MAPK pathway, inhibition of the cell cycle transcription factor E2F-1 and activation of the cell cycle inhibitor p21Cip1 (Morita, Mitsialis et al. 1997), (Otterbein, Zuckerbraun et al. 2003). Similarly, in T cells the antiproliferative
effect of CO is associated with increased expression of p21Cip1 and decreased caspase-8 activity and is independent of the cGMP and MAPK pathways (Song, Mahidhara et al. 2004). Although CO plays a major role in regulating cell proliferation, biliverdin and bilirubin also regulate cell proliferation. Bilirubin decreases SMC proliferation by inhibiting cyclins A, D1, and E, and cyclin-dependent kinase 2 (cdk2) via the p38 MAPK pathway, and decreases neointimal formation after balloon injury (Ollinger, Bilban et al. 2005). In addition, HO-1 and CO also increase the proliferation of cell types, such as endothelial cells and regulatory T cells (Li Volti, Wang et al. 2002), (Brusko, Wasserfall et al. 2005), (Lee, Gao et al. 2007).

2.3. C. Anti-inflammatory effect

Conners et al. were the first to demonstrate that induction of HO-1 has an anti-

**HO-1 and Inflammation**

![Diagram of HO-1 and Inflammation](image)

**Figure.2. Anti-inflammatory action of HO-1**
inflammatory effect (Conners, Stoltz et al. 1995). An anti-inflammatory role of HO-1 is supported by the fact that HO-1 null mice suffer from chronic inflammation (Poss and Tonegawa 1997). The only case of HO-1 deficiency in humans also showed severe inflammatory syndrome (Poss and Tonegawa 1997), (Poss and Tonegawa 1997), (Yachie, Niida et al. 1999), (Kawashima, Oda et al. 2002). CO predominantly imparts the anti-inflammatory effect of HO-1 and is mediated by the MAPK pathway (Otterbein, Bach et al. 2000) and not by NO. HO-1 suppress the production of proinflammatory cytokines, such as monocyte chemotactic protein 1 (MCP-1), IL-1, IL-6, and TNF-alpha. HO-1 also enhance the anti-inflammatory response by increasing the expression of the anti-inflammatory cytokine IL-10 (Otterbein, Bach et al. 2000), (Morse, Pischke et al. 2003). IL-10 increases the expression of HO-1, and the anti-inflammatory effect of IL-10 is dependent on HO-1 (Lee and Chau 2002) suggesting a positive feedback loop. Additionally, HO-1, via the NF-NB pathway (Sarady, Otterbein et al. 2002), decreases production of pro-inflammatory granulocyte macrophage colony-stimulating factor (GM-CSF). Down-regulation of adhesion molecules, such as E- and P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) may partly contribute to the anti-inflammatory effect of HO-1 (Hayashi, Takamiya et al. 1999), (Rucker, Schafer et al. 2001), (Soares, Seldon et al. 2004), (Song, Bergstrasser et al. 2009). Down-regulation of these vascular adhesion molecules decreases infiltration of leukocytes in the injured tissue and thereby attenuates inflammation.

2.3. D. Antiapoptotic effect: - CO mediates the antiapoptotic effects of HO-1 in most cellular conditions. HO-1 and CO protected inflammation-induced apoptotic liver damage in mice (Sass, Soares et al. 2003) and also inhibited apoptosis in transplanted
lungs and hearts (Song, Kubo et al. 2003), (Akamatsu, Haga et al. 2004). HO-1 prevents tumor necrosis factor alpha (TNF-α) induced apoptosis, presumably via CO in fibroblasts (Petrache, Otterbein et al. 2000). CO uses different signaling pathways in different cells and conditions e.g. in VSMCs, the antiapoptotic effect of CO were mediated partly by guanosine 3’5’-cyclic monophosphate (cGMP) (Liu, Chapman et al. 2002). CO uses the p38 MAPK pathway to inhibit TNF-α induced apoptosis in endothelial cells (Brouard, Otterbein et al. 2000) that also require the activation of NFkB (Brouard, Berberat et al. 2002). Interestingly, HO-1 and CO increase apoptosis in endothelial cells (Thom, Fisher et al. 2000). Further, excessive expression of HO-1 in rat aortic smooth muscle cells enhances apoptosis and bilirubin/biliverdin were involved in this effect (Liu, Chapman et al. 2002).

![Figure-3 HO-1 and apoptosis](image-url)
HO-1 can exert its antiapoptotic effect through heme degradation reaction products other than CO. HO-1 overexpression increases the efflux of iron from cells and inhibits apoptosis (Ferris, Jaffrey et al. 1999). In Jurkat T cells HO1 over expression inhibits Fas mediated apoptosis through an iron-dependent mechanism (Choi, Pae et al. 2004). Bilirubin also has the potential to protect against peroxynitrite and bile acid-induced apoptosis by its antioxidant action (Foresti, Sarathchandra et al. 1999), (Granato, Gores et al. 2003). In contrast, bilirubin has toxic effects on brain and increases apoptosis of neurons in vitro (Silva, Rodrigues et al. 2002).

2.3. E. Vasoactive effects

Regulation of vascular tone by HO-1 is highly dependent upon CO. CO causes vasodilatation via the activation of soluble guanylate cyclase (sGC) thereby increasing cGMP levels (Morita, Perrella et al. 1995) (Sammut, Foresti et al. 1998), (Duckers, Boehm et al. 2001). Sylvester and McGowan reported the vasodilatory property of CO (Sylvester and McGowan 1978). A controversy arose as to whether CO acts in this manner through cGMP which was clarified by Hussain et al. (Hussain, Marks et al. 1997) who showed in rabbit aortic rings that CO-dependent vasodilatation is abolished by the specific sGC inhibitor 1H-[1,2,4] oxadiazolo [4,3-I]quinoxalin-1-one (ODQ). However CO has been also found to activates calcium dependent potassium channels which may contribute to the CO vasodilatory effect (Wang, Wang et al. 1997), (Jaggar, Leffler et al. 2002). In addition, smooth muscle cell derived CO may also have paracrine effects on endothelial cells and can regulate vascular tone by modulating the expression of endothelin 1 (ET-1) and platelet-derived growth factor E (PDGF-E) in endothelial cells (Morita, Perrella et al. 1995). In addition, there are reports’ suggesting that the
vasoregulatory role of CO is dependent on NO (Foresti, Hammad et al. 2004). However, CO also behaves as a vasoconstrictor molecule in some model systems and this effect of CO is due to the inhibition of NO formation (Johnson, Teran et al. 2002), (Johnson and Johnson 2003).

2.3. F Proangiogenic effect

HO-1 plays an important role in promoting angiogenesis which could be defined as the formation of new capillaries from pre-existing capillaries by increased migration and proliferation of endothelial cells (sprouting). Abraham et al demonstrated that induction of HO-1 in coronary endothelial cell culture enhanced angiogenesis (Deramaudt, Braunstein et al. 1998). Further studies showed that the proangiogenic effect of HO-1 in endothelial cells is primarily mediated by CO (Jozkowicz, Huk et al. 2003), (Li Volti, Sacerdoti et al. 2005). In addition, CO also inhibits endothelial cell apoptosis (Soares, Usheva et al. 2002). The proangiogenic mechanism of HO-1 involves the induction of angiogenic growth factors and cytokines including vascular endothelial growth factor (VEGF) expression in endothelial cells and VSMCs (Dulak, Jozkowicz et al. 2002), (Jozkowicz, Huk et al. 2003). HO-1 increases neovascularization in both rat and mouse hind limb ischemia models through the induction of VEGF and stromal cell-derived factor 1 (SDF-1) (Suzuki, Iso-o et al. 2003), (Tongers, Knapp et al. 2008). HO-1 mediates prostaglandin and H2O2 induced VEGF synthesis (Jozkowicz, Huk et al. 2002), (Cisowski, Loboda et al. 2005). HO-1 and CO are involved in the downstream response of cells to VEGF and SDF-1 stimulation as both VEGF and SDF-1 induce HO-1 expression (Bussolati, Ahmed et al. 2004), (Deshane, Chen et al. 2007)suggesting a positive feedback loop. The effects of HO-1 on angiogenesis also depend upon the
cellular condition. HO-1 inhibits inflammation-induced angiogenesis by preventing leukocyte infiltration, but enhances VEGF-induced non-inflammatory angiogenesis (Bussolati, Ahmed et al. 2004). Deletion of the constitutive isoform HO-2 results in endothelial cell activation and massive inflammation-driven angiogenesis which was reversed by the addition of biliverdin to the HO-2 (−/−) endothelial cells (Bellner, Martinelli et al. 2009). The evidence described above establishes that the HO system suppresses inflammation-induced angiogenesis.

There are other putative factors which influence HO-1/CO regulated angiogenesis. These include HIF-1, a major transcription factor which drives hypoxia induced angiogenesis (Kelly, Hackett et al. 2003) and has recently been shown to up-regulate HO-1 expression (Chin, Jiang et al. 2007). Upregulation and stabilization of HIF-1 alpha by CO indicates the existence of a feedback loop between HO-1/CO and HIF-1 alpha. (Chin, Jiang et al. 2007), (Faleo, Neto et al. 2008). HO-1 increases proangiogenic cytokine IL-8 (Pae, Oh et al. 2005). HO-1 not only increases angiogenic factors but the over expression of HO-1 down-regulates the antiangiogenic factors VEGF receptor 1 (VEGF-R1) and soluble endoglin (Cudmore, Ahmad et al. 2007).

2.4. HO-1 and its reaction products in cardiovascular diseases

Myriad studies have shown the protective effect of HO-1 and its reaction products in the heart and vasculature in pathological conditions. Increased expression of HO-1 has been demonstrated in vivo in the heart and vasculature and in vitro in cardiomyocytes, endothelial cells, and VSMCs in response to various stimuli, such as hyperthermia, I/R, hypoxia, cytokines, hemin, NO and angiotensin II (Raju and Maines 1994), (Maulik, Sharma et al. 1996), (Motterlini, Foresti et al. 1996), (Durante, Kroll et al. 1997), (Lee,

2.4. A. HO-1 in myocardial infarction and heart failure

HO-1 and its reaction products have been intensely investigated to elucidate their long-term and short term effects. The importance of HO-1 as a cardioprotective enzyme was demonstrated by the presence of increased right ventricular dilatation, right ventricular infarction, and mural thrombi in HO-1 null mice after chronic hypoxia (Yet, Perrella et al. 1999). In addition, cardiac-specific over expression of HO-1 improves post ischemic cardiac function, decreases infarct size, and reduces cardiac apoptosis, inflammatory cell infiltration, and oxidative damage in I/R mouse hearts (Yet, Tian et al. 2001), (Vulapalli, Chen et al. 2002)). CO exposure via perfusion buffer decreases infarct size, improves post ischemic cardiac function, and decreases the incidence of I/R-induced ventricular fibrillation (Bak, Varadi et al. 2005). In addition, pretreatment of rat hearts with CORM-3, a CO donor, protected the heart during I/R, and the protective effect involved the regulation of cardiac Na+, K+, and Ca2+ levels (Varadi, Lekli et al. 2007). Additionally, CORM-3 exerts a positive inotropic effect on isolated perfused rat hearts, and this effect involves cGMP and Na+/H+ exchange (Musameh, Fuller et al. 2006). Prolonged exposure to 30–100 parts per million (ppm) of CO by inhaled air worsens myocardial I/R injury, increases the severity of post ischemic ventricular arrhythmias, impairs post ischemic cardiac function, and increases infarct size in rats. The cytoprotective and prongiogenic effects of HO-1 suggest a potential role for HO-1 in cardiac cell therapy and cardiac regeneration after MI.
It has been demonstrated that cardiac-specific HO-1 overexpression has beneficial effects in failing mice hearts (Wang, Hamid et al. 2010). HO-1 improved post-MI survival, ameliorated left ventricular dilatation and dysfunction, decreased apoptosis, hypertrophy, interstitial fibrosis, oxidative stress, and increased neovascularization and these effects were mediated, at least partially, by CO (Wang, Hamid et al. 2010). HO-1 induction inhibited angiotensin II-induced cardiac hypertrophy in rats (Hu, Chen et al. 2004).

Elevated serum bilirubin levels are associated with a decreased risk of coronary artery disease suggesting a protective role for HO-1 (Hopkins, Wu et al. 1996). The protective effect of higher serum bilirubin levels against ischemic heart disease occurs in patients with Gilbert syndrome, a genetic disorder characterized by above-normal levels of circulating unconjugated bilirubin (Vitek, Jirsa et al. 2002). Conversely, low serum bilirubin concentrations were associated with coronary artery calcification (Tanaka, Fukui et al. 2009).

HO-1 polymorphisms in humans may also modulate the susceptibility to coronary artery disease. Short GTn repeat length protects against coronary artery disease (Chen, Lin et al. 2002), (Kaneda, Ohno et al. 2002), (Dick, Schillinger et al. 2005), (Brydun, Watari et al. 2007) (Chen, Chau et al. 2008). In contrast, no association between HO-1 polymorphisms and coronary artery disease especially in the large study of over 3000 patients has been reproted (Endler, Exner et al. 2004), (Lublinghoff, Winkler et al. 2009).

2.4. B. HO-1 in Atherosclerosis

The immune system and ROS play critical role in the development of atherosclerosis and
there is considerable evidence that supports a beneficial role for HO-1 in atherosclerosis. Oxidized LDL is also a major inducer of HO-1 (Agarwal, Balla et al. 1996). Endothelial activation by oxidized LDL and TNFα are crucial in the development of atherosclerotic lesions (Chen, Liu et al. 2006), (Gao, Belmadani et al. 2007). Endothelial HO-1 overexpression attenuates the production of inflammatory mediators and reverses the decrease in eNOS by oxidized LDL and TNFα (Kawamura, Ishikawa et al. 2005). HO-1 induction attenuates monocyte chemotaxis after exposure to mildly oxidized LDL both in vitro and in vivo (Ishikawa, Sugawara et al. 2001). Several proatherogenic factors, including LDL, increased blood pressure, and smoking induces HO-1 (Wang, Lee et al. 1998), (Ndisang and Wang 2003), (Fukano, Oishi et al. 2006). HO-1 is present in atherosclerotic lesions in both humans and animals, and is detected at high levels in the lesions macrophages/foam cells (Wang, Lee et al. 1998), (Ishikawa, Sugawara et al. 2001). HO-1 is expressed in endothelial cells in the early stage of lesion formation and found in SMCs in advanced lesions HO-1 (Wang, Lee et al. 1998), (Ishikawa, Sugawara et al. 2001). Induction of HO-1 reduces lesion size in LDL-receptor knockout mice, whereas inhibition of HO activity increases atherosclerotic lesions in heritable hyperlipidemic rabbits (Ishikawa, Sugawara et al. 2001), (Ishikawa, Sugawara et al. 2001). Furthermore, mice deficient in both HO-1 and apolipoprotein E (ApoE) develop larger and more advanced lesions than mice deficient in ApoE alone (Yet, Layne et al. 2003). It is clinically of note that HO-1 reverses atherosclerotic plaque progression from vulnerable plaque to a more stable phenotype by reducing the size of the necrotic core and intraplaque lipid accumulation and increasing the thickness of the fibrous cap and accumulation of VSMCs in the intima in apoE-null mice (Cheng, Noordeloos et al.
HO-1 modulates the development of atherosclerosis in multiple ways. Hemin an inducer of HO-1 reduces monocyte chemotaxis in response to oxidized LDL (Low density lipoprotein) (Ishikawa, Navab et al. 1997). The levels of HO-1 expression in macrophages is important for the antiatherogenic effect of HO-1 (Orozco, Kapturczak et al. 2007). Both reduced and the absence of HO-1 expression in macrophages increased the generation of ROS, production of inflammatory cytokines, and formation of foam cells when treated with oxidized LDL (Orozco, Kapturczak et al. 2007). In addition, the absence of HO-1 resulted in greater macrophage content in atherosclerotic lesions (Orozco, Kapturczak et al. 2007).

The protective role of bilirubin against atherosclerosis and coronary artery disease was first reported by Wu and his colleagues who showed that bilirubin prevents oxidation of LDL (Wu, Fung et al. 1994). In addition, a low serum bilirubin concentration is associated with an increased risk of coronary artery disease (Schwertner, Jackson et al. 1994). Bilirubin inhibits lipid peroxidation in LDL-receptor knockout mice, and protects VSMCs against oxidative stress which is manifested by decreased plasma lipid hydroperoxide levels (Clark, Foresti et al. 2000) (Ishikawa, Sugawara et al. 2001). Elevated bilirubin levels suppress the production of proinflammatory mediators and ameliorate endothelial dysfunction (Kawamura, Ishikawa et al. 2005). Bilirubin and biliverdin also reduce the proliferation of SMCs in vitro and decrease balloon injury-induced neointima formation in hyperbilirubinemia Gunn rats (Ollinger, Bilban et al. 2005).

CO contributes to the antiatherogenic effect of HO-1 by several mechanisms. CO
suppresses arteriosclerotic lesions associated with chronic graft rejection by inhibiting leukocyte infiltration, SMC proliferation, and the expression of proinflammatory cytokines in transplanted aortic segments (Otterbein, Soares et al. 2003). CO reduced neointimal hyperplasia and prevented vascular stenosis following balloon angioplasty (Otterbein, Zuckerbraun et al. 2003). Recently, it has been shown that HO-1 induction reduced in-stent stenosis in rats and rabbits and a similar effect was observed in rats after CORM-2 treatment suggesting a role for CO (Hyvelin, Maurel et al. 2010). The anticoagulative, antiapoptotic, and vasodilatory effects of CO contribute to protection against atherosclerosis and vascular injury. CO prevents human platelet aggregation in vitro and platelet-dependent thrombosis in mice (Brune and Ullrich 1987), (Peng, Mundada et al. 2004).

2.4. C. HO-1 in hypertension

The effect of increased levels of HO-1 on the development of hypertension was pioneered by Sacerdoti et al who showed that induction of HO-1 prevented the development of hypertension in the spontaneously hypertensive rats (SHR) (Sacerdoti, Escalante et al. 1989). Subsequently, other inducers of HO-1 including Heme arginate, Hemin, L-arginate were shown to lower blood pressure in the SHR model (Levere, Martasek et al. 1990), (Martasek, Schwartzman et al. 1991). The transfer of the human HO-1 gene to SHR also reduced blood pressure (Sabaawy, Zhang et al. 2001). AngII induced hypertension and renovascular hypertension were prevented by HO-1 induction (Botros, Schwartzman et al. 2005), (Yang, Quan et al. 2004). Increased levels of HO-1 lowered blood pressure in established AngII hypertension (Vera, Kelsen et al. 2007). HO plays an important regulatory role in the maintenance of intrarenal blood flow under
basal conditions as well as in response to increased levels of vasoconstrictors including 20-HETE (20-Hydroxy-eicosatrienoic acid) (Kaide, Zhang et al. 2004), (Zou, Billington et al. 2000). The products of the CYP-450 dependent arachidonic acid pathway have been implicated in the development of hypertension. HO regulates several cytochrome P450s, including those responsible for the formation of 20-HETE (potent vasoconstrictor). Interestingly, the targeting of biliverdin reductase with siRNA in TALH (Thick ascending loop of henle) cells increases Ang II-dependent superoxide production, as well as sodium reabsorption (Kelsen, Patel et al. 2008). These findings highlight the role of the kidney in the long term regulation of blood pressure.

2.5. Renal injury and HO-1

The protective effects of elevated levels of HO-1 and of heme degradation products in renal injury reflect the vasorelaxant, anti-inflammatory, and antiapoptotic effects of the HO system (Nath 2006). Chronic administration of angiotensin II induces systemic hypertension, proteinuria, oxidative stress, and also elevated levels of HO-1 (Ishizaka, de Leon et al. 1997) (Aizawa, Ishizaka et al. 2000). In the kidney such upregulation occurs largely in the renal tubular epithelium, whereas in the vasculature it occurs largely in endothelial and adventitial cells (Aizawa, Ishizaka et al. 2000). The upregulation of HO-1 is functionally significant because inhibition of HO activity worsens proteinuria and GFR (Glomerular filtration rate) while hemin attenuates hypertension, proteinuria, the reduction in GFR, and apoptosis induced by angiotensin II (Nath 2006), (Aizawa, Ishizaka et al. 2001). In addition, overexpression of HO-1 by gene transfer reduces the pressor response to angiotensin II (Aizawa, Ishizaka et al. 2001).
The vasorelaxant effects of elevated levels of HO activity are likely mediated through CO and bilirubin. In the isolated perfused kidney, angiotensin II exerts pressor effects and generates CO via increased levels of HO-1; inhibition of HO activity reduces CO levels and exaggerates the pressor effect of Ang II (Li, Jiang et al. 2004). Bilirubin also attenuates the pressor effects of angiotensin II, as demonstrated by studies utilizing the hyperbilirubinemic Gunn rat (Pflueger, Croatt et al. 2005). This animal model exhibits resistance to the pressor effect of angiotensin II, less impairment in endothelium-dependent vascular relaxation, and greater preservation of vascular content of tetrahydrobiopterin, the latter representing an essential co-factor for endothelial nitric oxide synthase activity (Pflueger, Croatt et al. 2005). By scavenging oxidants, bilirubin preserves the vascular content of tetrahydrobiopterin, thereby optimizing endothelial nitric oxide synthase activity and, in turn, vascular relaxation (Pflueger, Croatt et al. 2005). HO-1 also attenuates the cytotoxic effects of angiotensin II, tubular epithelial cells derived from either the proximal tubule or the thick ascending limb (Bhaskaran, Reddy et al. 2003), (Quan, Yang et al. 2004).

2.6. RISK PATHWAY

Protecting the myocardium after coronary artery occlusion is one of the approaches used in cardioprotection and in this regard pro-survival kinase signaling cascades, phosphatidylinositol-3-OH ( PI(3) K)-AKT and p42/p44 extra-cellular signal-regulated kinases (Erk1/2) are rapidly activated. This pathway has been termed the “Reperfusion injury salvage kinase (RISK)” pathway (Hauserloy, Tsang et al. 2005). Pharmacological activation of the RISK pathway protects the heart against ischemia-reperfusion injury (Hauserloy, Tsang et al. 2005), (Yellon and Baxter 1999) and reperfusion protects
against both apoptotic and necrotic cell death, as evidenced by a reduction in infarct size (Yellon and Baxter 1999). It is thought that protection is conferred downstream of the RISK pathway, at the mitochondria and nuclear level. Survival is promoted when activity of apoptotic proteins such as p53, caspases, GSK3β (Gross, Hsu et al. 2004) and the members of the Bcl family, BIM, BAD, BAX is decreased (Gross and Gross 2006). In addition, activation of the RISK pathway activates pro-survival factors including AKT, murine double minute 2 (Mdm2), Bcl survival protein Bcl2/BclxL, endothelial nitric oxide Synthase (eNOS), and 70kDa ribosomal protein S6 kinase (p70S6K) to confer protection (Gross and Gross 2006), (Mocanu, Field et al. 2006), (Hausenloy and Yellon 2004). The protective effects of the RISK pathway are attributed to many signaling molecules. However, research suggests that the end target of this pathway may be the mPTP (Hausenloy and Yellon 2004), (Davidson, Hausenloy et al. 2006) which is believed to open during the first few minutes of reperfusion in response to mitochondrial calcium overload, oxidative stress and ATP depletion (Hausenloy and Yellon 2003). The recruitment of the RISK pathway induces many protective mechanisms which act in concert to inhibit the opening of mitochondrial Permeability Transition Pore (mPTP). These include the phosphorylation and inactivation of various pro-apoptotic factors such as BAD and Bcl-2 associated X protein (Tsuruta, Masuyama et al. 2002) which are believed to exert their apoptotic action via the opening of the mPTP, phosphorylation and inhibition of GSK3h, which has been demonstrated to mediate inhibition of mPTP opening (Juhaszova, Zorov et al. 2004).

2.6. A. AKT pathway: - The serine/threonine kinase, also known as protein kinase B (PKB), is a central node in cell signaling downstream of growth factors, cytokines, and
other cellular stimuli. Once AKT is phosphorylated, it becomes active influencing many factors that regulate growth, proliferation and survival (Vanhaesebroeck and Alessi 2000). An important function of activated AKT in cells is the inhibition of programmed cell death. AKT enhances the survival of cells by blocking the function of pro-apoptotic proteins and processes. Several downstream targets of AKT, including GSK-3, BAD, caspase-9 and transcription factors such as CREB and NFκB, are potential mechanisms by which AKT promotes cell survival and blocks apoptosis (Al-Khouri, Ma et al. 2005), (Datta, Brunet et al. 1999), (Franke and Cantley 1997). For instance, AKT directly phosphorylates and inhibits BH-3-only protein, BAD (Datta, Dudek et al. 1997), (del Peso, Gonzalez-Garcia et al. 1997). Survival factors stimulate AKT mediated phosphorylation of BAD which triggers the release of BAD from its target protein (Datta, Katsov et al. 2000). In addition, AKT increases the activity of the pro-survival protein, Bcl2, which sequesters and inhibits pro-apoptotic signals (Fulton, Gratton et al. 1999). Furthermore insulin activation by the PI3K/AKT pathway delays the time to mPTP opening thereby reducing cell death in an eNOS dependent manner. AKT also plays an important role in both physiological and pathological angiogenesis through effects in both endothelial cells and cells producing angiogenic signals, such as tumor cells. In endothelial cells, the PI3K/AKT pathway is activated by VEGF (Olsson, Dimberg et al. 2006) and phosphorylation of the AKT targets contribute to the survival, growth and proliferation of endothelial cells. In addition, AKT activates endothelial Nitric Oxide Synthase (eNOS) (Dimmeler, Fleming et al. 1999), (Fulton, Gratton et al. 1999). The release of NO, produced by activated eNOS can stimulate vasodilation, vascular remodeling and angiogenesis (Morbidelli, Donnini et al. 2003).
2.7. **Myocardial energy metabolism:** - Heart is an intrinsically aerobic organ, constantly requiring and producing energy to meet the needs of contraction and maintenance of ionic homeostasis (Giordano 2005). It utilizes various substrates to produce high energy compounds, adenosine triphosphate, ATP. Under normal conditions, fatty acids are the main substrates used by mitochondria to provide myocardial energy.

In contrast, due to decreased blood supply, glucose becomes a preferential substrate for metabolism and ATP generation under ischemic conditions (Giordano 2005). However, the energy generated by glucose oxidation may not be sufficient enough to meet the energy requirements of the infarcted heart (Giordano 2005). Unlike under conditions of normal blood flow, during ischemia, Pyruvate produced by glycolysis is not so readily oxidized in the mitochondria but rather is reduced to lactate in the cytosol (Giordano 2005). This causes dramatic disruption in cell homeostasis and a decrease in contractile work, ATP concentration decreases, lactate and H+ accumulate, and intracellular pH falls that may lead to death of cardiomyocytes (Giordano 2005). The alterations in myocardial energy substrate metabolism that occur in heart failure, and the causes and consequences of these abnormalities, are poorly understood. Modulations of these energy pathways have the potential to serve as a tool to ameliorate cardiac remodeling.
3. AIMS OF THE RESEARCH

**AIM (1).** To determine whether HO-1 induction improve the myocardial function of the infarcted heart the LAD ligation model of MI.

**AIM (2).** To determine whether HO-1 induction ameliorates left ventricular remodeling after LAD ligation in murine infarcted heart.

**Aim (3).** To determine whether HO-1 induction modulates cardiac energy metabolism, angiogenesis and ameliorates left ventricular remodeling in experimental MI model.

**Aim (4).** To determine whether HO-1 induction ameliorates cardiorenal syndrome in experimental murine MI model.

**Aim (5).** To dissect the role of T-lymphocytes in cardio-renal syndrome in experimental murine MI model.
4. MATERIALS and METHODS

Procedures 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).

4.1. Animals

One hundred and twenty five Balb/SCID and 125 C57BL6 male mice weighing 22-26g, aged 8 to 10 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed under SPF conditions, in a single ventilated cages system, fed standard mouse pellets and water ad libitum.

4.2. Left anterior descending coronary artery ligation (LAD) method

We created experimental myocardial infarction in left ventricle by ligation of left anterior descending artery (supplies blood to left ventricle) to study left ventricular remodeling. Animals were anesthetized using ketamine and xylazine (80 mg/Kg and 10 mg/Kg respectively). Animal’s chest was shaved and intubation was done by endotracheal method. After intubation, animals were kept in ventilation with 100% oxygen and isoflurane (0.75% to 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 Heating pad. Left thoracotomy was performed in the 3rd intercostal space under sterile conditions and pericardium was opened to expose the heart. An 8.0 prolene ligature (Ethicon) was passed and 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 3\textsuperscript{rd} and 4\textsuperscript{th} ribs and muscle layers were recomposed. After closing the skin with continuous suture, all mice were hydrated with saline and was given an analgesic, carprofen at the dose of 4 mg/Kg, for two days. At the end of the surgery mice were housed for 24 hours in an incubator at 37\textdegree C and then recovered in single cages and monitored twice a day for 7 days. Control surgery was performed following the same procedure without coronary artery ligation. All groups underwent echocardiography and renal echoDoppler examination 30 days after surgery.

\textbf{Figure-4 Outline of experimental steps}
4.3. **Echocardiographic evaluation**: - We evaluated left ventricular function by the non-invasive echocardiographical method. Transthoracic echocardiography was performed using a Siemens Acuson Sequoia sonography machine with a 15 MHz linear probe. Animal’s chest was shaved. 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 and end-systolic areas (EDA, ESA), end-diastolic and end-systolic length (EDL, ESL) were measured from the long axis B-mode image and end-diastolic and end-systolic diameter (EDD, ESD) were measured from the short axis M-mode image. Fractional Area Change, an index of LV contractile function, was determined using the following formula FAC = (EDA-ESA)/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 efflux tract. Aortic velocity-time integral (Ao VTI) was measured from aortic Doppler-flow curve. Aortic outflow tract diameter (Ao OFT) was measured from the same B-mode images of a long axis view.

Stroke volume (S.V.) was calculated using the following formula

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

Cardiac index (C.I.) was calculated using the following formula

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

4.4. **Renal echo Doppler evaluation**: - Renal arterial resistance is a marker of renal dysfunction and we wanted to examine the effect of HO-1 induction on post-myocardial ischemic renal dysfunction. 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 (Vmax), End Diastolic Velocity (Vmin) and Mean Velocity (VM) were measured and Pulsatility Index (K-PI) was determined using the following formula

Renal pulsatility index= (Peak velocity- End diastolic Velocity)/ (Mean velocity) ^4.

Increased renal pulsatility index indicates higher renal arterial resistance.

4.5. Drugs administration: - CoPP, a potent HO-1 inducer, was administered via intraperitoneal injection 5 days after LAD ligation and then every 5 days for 4 weeks at the dose of 3 mg/Kg body weight to study the effect of HO-1 induction on left ventricular remodeling as well as post-myocardial ischemic renal dysfunction. SnMP, an inhibitor of HO activity, was administered via intraperitoneal injection every 2 days for 15 days before euthanasia at the dose of 20 mg/Kg body weight and it served as negative control for CoPP treated group.

4.6. Renal Histology: - We also assessed pathological changes in the kidney by performing histology on renal tissues. 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-Fi1 (Niko, Tokyo, Japan). For quantitative analysis at least 4 randomly chosen fields from each animal were digitized. Collagen volume was determined using the Image J software (http://rsbweb.nih.gov/ij).
4.7. Plasma Renin and AngII levels

To assess the Renin-angiotensin system after myocardial infarction we measured plasma renin and AngII levels. The plasma levels of Renin and Ang II level were measured in plasma using an ELISA assay (Assay Gate, Inc. Ijamsville, MD).

4.8. ELISA-inflammatory cytokines

Myocardial infarction is followed by activation of inflammatory cytokines and therefore we measured inflammatory cytokines in plasma using ELISA technique. Serum samples were frozen at -20 degree Celsius for TNF-α, IL-6 and IL-10 were determined using enzyme-linked immunosorbent assay (ELISA) kits (Bender Medsystems, Burlingame, CA, USA). The day before the experiments, ELISA plates (Corning, USA) were coated with the antigens overnight at 4 degree celsius. The day after, plates were washed with PBS and blocked 1 hour with polypep (Sigma,USA) 1% in PBS at room temperature. After blocking, plates were incubated 1 hour at room temperature with different polyclonal antibodies. For competition studies, soluble antigens were added to the plates contemporary to the primary antibody. Plates were then washed five times with PBS and incubated with secondary antibody conjugated with AV-HRP. After another set of washes, plates were developed with TMB for 15 minutes at room temperature. Sample absorbance was read at 450nm with a microplate reader.

4.9. Plasma Creatinine levels

Since plasma Creatinine level is a good indicator of renal function, we measured it by an enzyme-linked immunoassay (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions.
4.10. Protein extraction and Western blot: To explore the effect of HO-1 induction on cellular signaling we did perform immunoblotting studies. Frozen hearts and kidneys were pulverized under liquid nitrogen and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% tergitol, pH 7.5). Protein quantification was done using a commercial assay (Bio-Rad, USA). Homogenates were centrifuged at 27,000g for 10 minutes at 4 degree celsius, the supernatant was isolated, and protein levels were visualized by immunoblotting with antibodies. Briefly, proteins (35 µg/sample) were separated using sodium dodecyl sulfate (SDS) polyacrylamide gel (12%) under denaturing conditions and electrotransferred onto nitrocellulose (Bio-Rad) for 1 h at 100V. Membranes were blocked with 5% nonfat milk overnight at 4 degree celsius. Immunodetection of actin was performed as an internal control. Dye 800/680 secondary antibodies were added at an appropriate concentration of 1:1500 for one hour at room temperature (LI-COR Biosciences, NE, USA). Detection was carried out with LI-COR odyssey machine. Protein levels were visualized by immunoblotting with antibodies against HO-1 downstream signaling proteins. The image was analyzed by densitometry using image J software. Protein bands were quantified and values were normalized to those of actin.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
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<tbody>
<tr>
<td>Heme Oxygenase-1</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Thromboxane Synthase</td>
<td>Abcam</td>
</tr>
<tr>
<td>Beta actin</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>Acetyl Co-A carboxylase (ACC)</td>
<td>Abcam</td>
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<tr>
<td>Carnitine Palmitoyl transferase-1 (CPT-1)</td>
<td>Abcam</td>
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<tr>
<td>Acetyl Co-A carboxylase (ACC) and Phospho acetyl Co-A (pACC)</td>
<td>Abcam</td>
</tr>
<tr>
<td>gP91Phox</td>
<td>Abcam</td>
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<tr>
<td>3-Nitrotyrosine</td>
<td>Abcam</td>
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<tr>
<td>Angiotensinogen</td>
<td>Abcam</td>
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<td>Angiotensin Converting enzyme (ACE)</td>
<td>Abcam</td>
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<tr>
<td>PPAR-delta, Beta catenin</td>
<td>Cell Signaling</td>
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<tr>
<td>VEGF, Ets-1, Adiponectin</td>
<td>Cell Signaling</td>
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<tr>
<td>AKT, pAKT</td>
<td>Cell Signaling</td>
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</tbody>
</table>
4.11. **Statistical Analysis**: Data will be presented as mean ± standard error (SEM). For comparison between treatment groups, the null hypothesis was tested by a single factor analysis of variance (ANOVA) for multiple groups or unpaired t-test for two groups. Statistical significance (p < 0.05) between the experimental groups will determined by the Fisher method of analysis for multiple comparisons.
5. Results

5.1 Effect of CoPP administration on cardiac function: - Mice were divided into groups using a random process. We evaluated the left ventricular function by using echocardiography and found no significant differences in these functional parameters between sham groups as well as at baseline level. 5 days after LAD ligation, CoPP was administered intraperitoneally for the induction of HO-1. The effect of HO-1 on post-ischemic cardiac function was studied in vivo 30 days after LAD surgery. We evaluated the left ventricular function 30 days after surgery by calculating fractional area change. Fractional area change is percent change in left ventricular cross-sectional area between diastole and systole and is an ejection phase index of contractile function. We found that, LAD ligation significantly reduced the fractional area change in MI group compared to sham group. CoPP treatment significantly increased the fractional area change in comparison to MI treatment group. Inhibition of HO activity by SnMP treatment reduced the fractional area change significantly in comparison to CoPP treatment group alone indicating that the enhancement of fractional area change was due to HO-1 induction. (Table.2, Figure. 5).
We also evaluated left ventricular cardiac End diastolic area which is a measure of cardiac preload 30 days after MI. End diastolic area as well as End diastolic diameter was significantly increased in mice (Table 2 and Fig.6). In CoPP treated mice, End diastolic area and End diastolic diameter were significantly smaller compared to MI groups (Table 2 and Figure. 6). SnMP treatment reversed CoPP effect on FAC and on left ventricle dilatation (End diastolic area, End diastolic diameter) in both groups (Tab 2, Fig. 6). Cardiac index (cardiac output divided by body weight), which is a measure of standardized cardiac output. Cardiac output is the volume of blood being pumped by the
heart in the time interval of one minute. 30 days after MI, cardiac index was significantly reduced in mice because of the reduced LV systolic function (Table 2). Administration of CoPP improved cardiac index as compared to MI group. Concurrent administration of SnMP reversed these beneficial effects of CoPP. We found that there is HO-1 dependent improvement in cardiac function after 30 days of LAD ligation surgery.

Figure 6. Effect of CoPP in C57 mice on End Diastolic Area measured by Echocardiography 30 days post MI; Results are means±SE. *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP

5.2 Effect of CoPP administration on cardiac HO-1 expression

Since, we wanted to investigate the cardiac function upon CoPP treatment; we investigated HO-1 expression in the heart. 30 days after LAD surgery, we sacrificed the mice and collected hearts. Western blot analysis of HO-1 in cardiac tissue, normalized against actin, showed significant decrease of HO-1 expression in MI groups. CoPP is a strong inducer of HO-1 and its treatment significantly increased HO-1 expression in
MI+CoPP groups (Figure 7; p<0.05). In addition, SnMP also increased HO-1 expression confirming previous results (Sardana and Kappas 1987).

**Figure 7. Western blot of HO-1.** Effects of CoPP in C57 mice on the expression of HO-1 protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of HO-1 expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
5.3 Effect of CoPP administration on cardiac angiogenesis

Therapeutic angiogenesis in heart is an exciting area of research with the potential of improving care of patients with ischemic coronary artery disease. Angiogenesis refers to the extension of already formed primitive vasculature by budding off capillary network. As a result, new capillary networks are formed from pre-existing vessels through migration and proliferation of previously differentiated endothelial cells. VEGF is a key mediator in the natural process of ischemia induced myocardial neovascularization.

![Western blot of VEGF](image)

**Figure 8. Western blot of VEGF.** Effects of CoPP in C57 mice on the expression of VEGF protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of VEGF expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
Another protein E26 transforming sequence (Ets-1) is induced by VEGF in cultured endothelial cells which subsequently transactivates the VEGF called as VEGF Ets-1 cascade. These properties of VEGF Ets-1 cascade prompted us to investigate VEGF and Ets-1 expression upon HO-1 induction. We collected the heart tissues 30 days post LAD surgery and performed immunoblotting studies in peri-infarcted areas. We found that induction of HO-1 significantly enhanced the expression of VEGF as well as Ets-1 (Figure.8 and Figure.9). This enhanced expression of VEGF, Ets-1 was reversed in SnMP treated group. These results entail HO-1 dependant angiogenesis via VEGF, Ets-1 cascade in MI.

**Figure.9. Western blot of Ets-1.** Effects of CoPP in C57 mice on the expression of Ets-1 protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of Ets-1 expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
PPAR-δ is another regulator of endothelial cell proliferation and angiogenesis through VEGF. Thus, we examined the protein expression of this regulatory protein and found its expression to be increased in CoPP treated group.

Figure 10. Western blot of PPAR-δ. Effects of CoPP in C57 mice on the expression of PPAR-δ protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of PPAR-δ expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP

In addition to VEGF, Ets-1 and PPAR-δ we also examined the expression of β-catenin in heart tissues. β-catenin is known to be involved in the modulation of angiogenesis in
ischemic tissues. We found enhanced β-catenin expression in heart of CoPP treated mice which was reversed in SnMP treated groups.

**Figure 11. Western blot of beta catenin.** Effects of CoPP in C57 mice on the expression of beta catenin protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of beta catenin expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP
Figure 12. Western blot of Thromboxane synthase. Effects of CoPP in C57 mice on the expression of Thromboxane synthase protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of Thromboxane synthase expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.

Additionally, Thromboxane synthase expression was measured to assess vascular tone within heart. We found increased expression of this protein in MI treatment group while its expression was significantly reduced after CoPP treatment. Inhibition of HO activity significantly increased the expression of this protein suggesting that the reduced expression of Thromboxane synthase was due to induction of HO-1. Thromboxane
synthase increases the formation of Thromboxane which is a known vasoconstrictor. Thus, HO-1 may not be only involved in angiogenesis but also dilation of blood vessels within heart to increase blood supply to the infarcted heart.

5.4 Effect of CoPP administration on cardiac energy metabolism

Since our results indicated enhanced angiogenesis after HO-1 induction, we were interested to see if HO-1 induction modulates fatty acid oxidation. We examined two key elements, Carnitine Palmitoyl Transferase -1 (CPT1) and Phosphoacetyl Co-A Carboxylase (pACC). CPT1 plays a key regulatory role in controlling the rate of free fatty acids uptake by the mitochondria. CPT1 is inhibited by malonyl CoA and Acetyl CoA carboxylase is the enzyme that catalyzes the formation of malonyl CoA from acetyl CoA (Figure.13). Thus, increased Acetyl CoA carboxylase activity in turn decreases fatty acid transport by inhibiting CPT1 through increased formation of Malonyl CoA. Phosphorylation of Acetyl CoA carboxylase inactivates its function.
Figure 13. Myocardial energy metabolism
In our results, we observed increased CPT1 expression and decreased phosphorylation of ACC in CoPP treated group. This is an indication that probably HO-1 via enhanced angiogenesis normalized the blood supply to the infarcted heart and thus, heart is able to use free fatty acids like normal healthy heart.

PPAR delta is a transcriptional activator of fatty acid uptake and oxidation. In another interesting finding, CoPP treated group induced PPAR delta expression. It further
corroborates the noble finding that induction of HO-1 may be able to sustain myocardial fatty acid oxidation as normal healthy heart does. Possibly, through increased blood flow via enhanced angiogenesis.

**Figure 15. Western blot of pACC.** Effects of CoPP in C57 mice on the expression of pACC protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of pACC expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
Adiponectin is another major player which is involved in increasing fatty acid oxidation in muscles and that led us to measure its expression in the heart and found that HO-1 induce adiponectin expression in the heart. These results corroborate HO-1 induced fatty acid oxidation within heart after MI.

**Figure 16. Western blot of Adiponectin.** Effects of CoPP in C57 mice on the expression of Adiponectin protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of Adiponectin expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
5.5 Effect of CoPP administration on myocardial protection

Apoptosis plays a vital role in the process of tissue damage after myocardial infarction. Since, activation of the terminal apoptotic machinery includes caspase activation. We examined effect of HO-1 induction on expression of cleaved caspase-3 protein 30 days post MI. In MI treatment group, caspase-3 expression was significantly higher than the sham group. HO-1 induction brought back its expression comparable to sham group and concurrent administration of SnMP aggravates its expression (Figure 13).

Figure 17. Western blot of Caspase-3. Effects of CoPP in C57 mice on the expression of caspase-3 protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of caspase-3 expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP
We also examined the prosurvival kinase signaling pathway called as RISK pathway which includes AKT pathways. Once AKT is phosphorylated it becomes active influencing many factors that regulate survival and this prompted us to examine the phosphorylation of AKT in the infarcted myocardium upon HO-1 induction. We observed increased phosphorylation of AKT which was dependent upon HO-1 induction (Figure. 14).

**Figure. 18. Western blot of pAKT.** Effects of CoPP in C57 mice on the expression of pAKT protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of pAKT expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
5.6 Effect of CoPP administration on myocardial oxidative damage

In MI, ROS are generated in ischemic myocardium. ROS directly injure the cell membrane and cause cell death. NADPH and NADH oxidase is an inducible electron transport system found in cells that transfer reducing equivalents from NADPH or NADH to oxygen which results in formation of superoxide ions. In our present study, we examined the role of gp91phox containing NADPH oxidase in the development of left ventricular remodeling after MI.

**Figure 19. Western blot of gp91phox.** Effects of CoPP in C57 mice on the expression of gp91phox protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of gp91phox expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
In addition, we also measured the tyrosine nitrated proteins, accumulation of which reflects a loss of balance between oxidant formation and antioxidant defense mechanism. We showed that induction of HO-1 reduces the expression of both gp91phox as well as 3NT which in turn indicates that one of the mechanisms by which HO-1 protects the infarcted heart is via reduction of oxidative damage (Figure 15 and Figure 16).

![Western blot of 3-NT](image_url)

**Figure 20. Western blot of 3-NT.** Effects of CoPP in C57 mice on the expression of 3-NT protein in heart tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of 3-NT expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP
5.7 Effect of CoPP administration on renal arterial resistance in both C57BL6 mice and SCID mice

Renal dysfunction is a common clinical event that occurs post Myocardial Infarction. Arterial underfilling with consequent neurohormonal activation, systemic and intra-renal vasoconstriction are the main clinical features of renal dysfunction after Myocardial Infarction. Since, there is a strong correlation between renal arterial resistance and renal dysfunction; we measured the renal arterial resistance by calculating renal pulsatility index. Studies also indicate that immune cells in particular T-lymphocytes play major role in the post myocardial ischemic renal dysfunction. Hence, to elucidate the role of T-lymphocytes we used T-Lymphocyte suppressed SCID mice. These immune deficient mice were also divided into same four experimental groups as of C57 mice and underwent LAD ligation surgery. Renal Pulsality index is pulse-wave doppler measurement of downstream renal artery resistance. As expected, we found elevated renal pulsality index in MI treatment group. Both C57 and SCID mice developed a significant renal vasoconstriction 30 days after MI (Fig. 17). In CoPP treated C57 as well as SCID mice, K-PI got decreased in comparison to MI group (Fig.17). The SnMP treatment group showed increased renal arterial resistance confirming HO-1 dependent decrease in renal arterial resistance. Renal arterial resistance indices were significantly lower in SCID mice than in C57 (Fig. 17). Both C57 and SCID mice developed a significant renal vasoconstriction 30 days after MI (Fig. 17). In CoPP treated C57 as well as SCID mice, K-PI got decreased in comparison to MI group (Fig.17). However, the reduction was more pronounced in SCID mice in comparison to C57 mice (30% reduction in SCID mice vs. 18% decrease in C57 mice in renal vasoconstriction Fig.17,
p<0.05). Concurrent administration of SnMP administration worsened renal vasoconstriction in C57 and SCID mice with MI treated with CoPP (Fig. 17).

Figure-21. Renal echodoppler showing renal vascular resistance
A) schematic representation of the kidney and the renal interlobar arteries; B) schematic representation of Doppler waveform measured in a low resistive and in a high resistive vessel; C) Renal Pulsatility Index of C57 and SCID mice. Renal pulsatility index was used to measure renal vasoconstriction. PI was increased in MI groups of both strains. CoPP treatment induced renal vasodilatation (i.e. PI decrease). SnMP treatment increased renal vasoconstriction.
5.8 Effect of CoPP administration on post myocardial renal function

Serum creatinine is an important indicator of renal function and a rise in serum creatinine level is associated with renal dysfunction. Therefore, we did measure serum creatinine levels in our experimental mice 30 days post surgery. As expected, 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 to 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.18). These findings suggested that induction of HO-1 is not only able to ameliorate left ventricular remodeling but also renal dysfunction.

Figure-22 Plasma Creatinine.

Effect of CoPP in C57 and SCID mice on the levels of Plasma Creatinine 30 days post MI. Results are shown as means±SE. (A, B) Plasma Creatinine level (mg/dl) of C57 and SCID mice respectively; *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
5.9 Effect of CoPP administration on renal fibrosis

Since renal dysfunction is strongly correlated with renal fibrosis and damage to nephrons, we examined the renal histology using picrosirius staining in our studies. Collagen I & III

![Image](image_url)

**Figure 23 Renal histology (Picrosirius Staining)**

Effect of CoPP in C57 and SCID mice renal fibrosis 30 days post MI. Results are shown as means±SE, (A, B) fibrillar collagen value in percentage of C57 and SCID mice respectively; *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.

staining of both C57 and SCID kidney tissue revealed presence of perivascular and peritubular interstitial fibrosis in cortico-medullary junction. Fibrosis was significantly higher in MI groups in both C57 and SCID mice as compared to Control mice (Fig 19A
and Fig 19 B). However, the level of collagen accumulation in SCID MI mice was significantly lower than C57 MI mice (p<0.02). CoPP treatment significantly reduced renal fibrosis in both SCID and C57 mice as compared to MI group alone, the effect being more evident in SCID mice. Concurrent administration of HO-activity inhibitor SnMP reversed the beneficial effects of CoPP.

**5.10 Effect of CoPP administration on renin angiotensin system**

Myocardial infarction decreases the cardiac output due to decreased myocardial contractility. This reduction in cardiac output decreases the blood flow to the kidney and rennin angiotensin system gets activated. Renin is secreted by the kidney and it acts upon angiotensinogen which is normally produced by the liver. Once the renin acts upon angiotensinogen, it produces angiotensin I which is circulating the blood stream. This angiotensin-I is being acted upon by angiotensin converting enzyme which is present in the endothelial surface of the vascular system and produces angiotensinII. Angiotensin II is a potent vasoconstricctor and is responsible for renal dysfunction. Since HO-1 induction decreased the renal arterial resistance and also improved renal functional parameters, we examined this vasoconstrictor system in our present study. We examined the serum levels of renin as well as angiotensin II (Figure 20). We also did immunoblotting studies of renal tissues and looked for protein expression of angiotensinogen as well as angiotensin converting enzyme (Figure 21 and Figure 22). We observed increased serum renin and angiotensin II levels in MI treatment group, we also observed elevated protein expression of angiotensinogen as well as angiotensin converting enzyme in renal tissues of MI group. HO-1 induction decreased all the players of renin angiotensin system including serum renin, AngII, renal angiotensinogen and angiotensin converting enzyme. These
beneficial effects were reversed in SnMP treated group confirming the role of HO-1 in decreasing these parameters.

**Figure-24 Plasma Renin and AngiotensinII level**

Effect of CoPP in C57 and SCID mice on plasma Renin and AngII levels 30 days post MI. Plasma Renin level (ng/ml) *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP; Plasma AngII level (ng/ml) of C57 and SCID mice respectively; *p<0.05 vs. Control, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
Figure 25. Western blot of Angiotensinogen. Effects of CoPP in C57 mice on the expression of Angiotensinogen protein in kidney tissue 30 days post LAD ligature surgery. Western blot and densitometry analysis of Angiotensinogen expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
Figure 26. Western blot of ACE. Effects of CoPP in C57 mice on the expression of ACE in kidney tissue 30 days post LAD ligation surgery. Western blot and densitometry analysis of ACE expression in C57 mice. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.

5.11 Effect of CoPP administration on systemic inflammation

Myocardial infarction is accompanied by systemic inflammation and this was examined in the current study by assessment of circulating inflammatory markers. Plasma IL-6 (Figure 23, A, B) and TNFα (Figure 24 C, D) were higher in MI mice as compared to the Control mice (p<0.05).
The administration of CoPP 5 days after MI significantly decreased the inflammation in both strains and this effect was reversed by SnMP (p<0.05).

**Figure 27. ELISA for Inflammatory cytokines.** Effects of CoPP in C57 and SCID mice on the serum level of TNF-alpha and IL-6 30 days post LAD ligation surgery. Data are shown as mean band density normalized to β-actin. Results are means±SE, n=6/group; *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI+CoPP.
TABLE 2

<table>
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<th>SHAM</th>
<th>MI</th>
<th>MI+CoPP</th>
<th>MI+CoPP+SnMP</th>
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<tr>
<td>Body</td>
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<tr>
<td>rate (bpm)</td>
<td>SCID</td>
<td>421±52</td>
<td>412±93</td>
<td>419±93</td>
</tr>
</tbody>
</table>

Echocardiography

| EDD(cm) | C57   | 0.387±0.03 | 0.471±0.02§§ † | 0.437±0.02* | 0.498±0.05§§# † |
|         | SCID  | 0.36±0.03  | 0.35±0.04§§   | 0.33±0.01** | 0.39±0.03#     |
| C.I.(ml/min/Kg) | C57   | 2034±372 0 | 1392±65§      | 1404±307§   | 1439±123*##    |
|          | SCID  | 2165±431  | 1451±43§     | 2037±227§§  | 1560±193§§*   |

C57 and SCID mice: in vivo heart measurements by echocardiography 30 days after MI. Values are expressed as mean ± SD; n= number of animals tested; EDA= end diastolic area, EDD= end diastolic diameter; PI= pulsatility index; * p< 0.05 vs MI; ** p< 0.01 vs MI; § p< 0.05 and §§ p< 0.01 vs sham; # p< 0.05 vs MI+CoPP; ## p< 0.01 vs MI+CoPP; † p<0.05 vs SCID.
Discussion

Discussion of the research is divided into two parts. In the first part we discuss the role of HO-1 Induction in the improvement of Cardio-renal syndrome type-1 (CRS-1) in conjunction with the presence and the absence of the intact immune system. The second part will focus on the role of HO-1 Induction in the amelioration of post-ischemic cardiomyopathy and its underlying mechanism.

Role of HO-1 Induction in the improvement of Cardio-renal syndrome type-1 in conjunction with the presence and the absence of the immune system

This is the first study to demonstrate the role of T-lymphocytes and HO-1 induction in improving cardio-renal syndrome (CRS)-1 in immuno-competent and immuno-compromised mice (SCID). We demonstrate that ANGII induced inflammation and T lymphocytes recruitment is decreased by the upregulation of HO-1 in a model of post ischemic heart failure. HO-1 induction decreased renal vasoconstriction and fibrosis and improved renal function in both immuno-competent and T lymphocyte suppressed mice, the effect being more efficacious in SCID mice.

Thirty days after left anterior descending coronary ligation, mice developed heart failure characterized by a significant dilatation of the left ventricle and reduction of fractional area change. The latter leads to a functional hypovolemia resulting in activation of SNS and RAAS, and the release of anti-diuretic hormone. CoPP treatment improved cardiac function as evidenced by increased fractional area change and decreased EDA in both SCID and C57 mice. Previous reports demonstrated that AngII modulates immune function and stimulates T lymphocyte activation and proliferation (Marvar, Thabet et al.)
As a result of the well documented role of T-lymphocytes in hypertension (Schiffrin 2010); we expected that SCID mice would be resistant to CRS-1 development. As expected, these animals had lower basal renal arterial resistance in comparison to C57 mice due to the absence of T-lymphocyte-RAAS interaction. In the MI treatment group, both mouse strains had a significant increase in renal arterial resistance which was reversed by CoPP. However, the CoPP mediated improvement was more evident in SCID mice suggesting a critical role of T-lymphocytes. This finding is in agreement with a previous report showing that T-lymphocytes play an important role in the development of hypertension (Marvar, Thabet et al. 2010). This suggests that renal vasoconstriction is not strictly related to heart failure but is influenced by other mechanisms including the interaction of HO-1, RAAS and T-lymphocytes.

The role of RAAS is well established in the initiation and maintainance of vasculature, myocardial and renal dysfunction in CRS-1 (Ronco, Cicoira et al. 2012). Consistent with these reports, our results show that ANGII and renin levels were increased in circulation in both the C57 and SCID MI groups and were decreased by CoPP. Additionally, we also observed downregulation of Angiotensinogen and Angiotsensin converting enzyme expression in CoPP treated C57 mice. One of the pathological abnormalities observed in CRS-1 is renal damage with increased plasma creatinine levels (Ronco, Cicoira et al. 2012). Our results demonstrate that both the C57 and SCID MI groups have not only altered metabolic homeostasis but also exacerbated renal damage, as evident by increased collagen deposition and the detioration of renal function. The production of inflammatory cytokines as a result of ischemic cardiac damage results in systemic inflammatory activation including in the kidney. Systemic
inflammation together with RAAS activation causes vascular T-lymphocyte recruitment (Geiger 2008). RAS activation is responsible for further renal damage (Kim, West et al. 2013), (Guo, Zhou et al. 2009). Activated T-lymphocytes amplify the ANGII effect stimulatory vascular ROS production in kidney contributing to a further ROS increase and more damage and renal dysfunction. Consistent with these reports, our results showed that plasma creatinine levels and renal damage was less in SCID mice when compared to C57 mice due to the absence of the T-lymphocyte-RAAS interaction suggesting that T lymphocytes contribute to the pathological effects of RAS deregulation in CRS-1. HO-1 induction is associated with a decrease in T lymphocyte proliferation(Pae, Choi et al. 2004). Our results demonstrate that the beneficial effects of HO-1 in improving CRS-1 are more prominent in SCID mice as compared to the C57 mice suggesting that a decrease of the T lymphocyte immune response amplified the effects of HO-1 in attenuating CRS-1. SnMP reduced the beneficial effect of HO-1 induction on left ventricle contractile function (FAC) and dilatation (EDA, EDD) in both C57 and SCID mice and worsened renal vasoconstriction and renal function. Thus, in heart failure, renal resistance and renal injury appears to be highly dependent on the inhibition of HO activity.

In conclusion (Figure.24), we demonstrate that a decrease of the T-lymphocytes immune response reduced kidney damage and renal vasoconstriction in a model of post ischemic heart failure when compared to immune-competent mice, and suppression of T lymphocytes amplified the beneficial effects of increased levels of HO-1 in improving CRS-1.
Figure 28. Scheme showing role of Immune system in renal injury during CRS-1
Role of HO-1 induction in the amelioration of post-ischemic cardiomyopathy and its underlying mechanism

Post-ischemic cardiomyopathy and progressive decline in cardiac function has been ascribed to chronic inflammation, increased oxidative injury, lack of significant response in angiogenesis (blood flow) and altered cardiac energy metabolism, (Neubauer, Horn et al. 1995) (Frangogiannis, Smith et al. 2002). HO-1 is an established powerful antioxidant and increased HO-1 gene expression plays an important role in the restoration of vascular function, reduction of inflammation, enhancing angiogenesis and in decreasing endothelial cell death. Also, mice deficient in HO-1 express severe cardiac dysfunction and abnormal ventricular remodeling (Liu, Wei et al. 2005).

Our present study demonstrates that pharmacological induction of HO-1 can rescue the infarcted myocardium by improving perturbed fatty acid oxidation via its antioxidant angiogenic properties. CoPP treated MI mice demonstrate increased levels of the angiogenic markers—VEGF, beta-catenin and Ets-1. We also observed an increased expression of pACC, PPARδ, Adiponectin and CPT-1—positive regulators of myocardial fatty acid oxidation. This was paralleled by the decreased expression of the oxidative stress markers—3NT and gp91Phox, and an improvement in cardiac function. The reversal of these beneficial effects by SnMP corroborates the role of HO-1 expression and HO activity in mediating the beneficial effects of CoPP. To our knowledge, HO-1 is a unique molecule possessing a dual antioxidant-angiogenic role in directly regulating the myocardial fatty acid oxidation in the infarcted heart, and, as such, establishes a novel mechanism for cardioprotection after the occurrence of a MI.
Though glucose oxidation has the advantage of being more oxygen efficient compared with fatty acid oxidation, it is not as carbon efficient and thus produces less ATP per molecule. In addition, the acidic environment generated by anaerobic glucose oxidation further exacerbates the usual grim prognosis of MI. The rate limiting step of fatty acid oxidation is the import of long chain fatty acids across the mitochondrial membrane through the action of Carnitine Palmitoyl Transferase I (CPT1). This is inhibited by up regulation of Acetyl CoA Carboxylase (ACC2) due to the increased formation of malonyl CoA, an inhibitor of CPT1. Surprisingly, we observed an increase in phosphorylated-ACC 2 (inactive form of ACC2) expression along with an increased CPT1 expression in the heart of CoPP treated MI mice. We measured the expression of PPARδ- an important protein in the maintenance of cardiac fatty acid oxidation (Cheng, Ding et al. 2004). HO-1 up regulation was associated with an increase in PPARδ expression in CoPP treated MI mice hearts. These effects were reversed by SnMP treatment suggesting the HO-1 mediated upregulation of fatty acid oxidation.

Cellular acidosis during prolonged ischemia is associated with increased oxidative damage which is not adequately counteracted by cellular antioxidant defense systems (Abdullah, Arnold et al. 2007). Therefore, we measured gp91Phox and 3NT–oxidative stress markers in our experimental model and sound that these oxidative markers were lower in CoPP treated MI mice and that this effect was reversed by SnMP suggesting that HO activity plays a crucial role in this process.

We measured key players regulating angiogenesis that include VEGF-Ets-1 cascade and determine an increase in these protein expression upon CoPP treatment. These effects were reversed by concomitant treatment with SnMP substantiating the role
of HO-1 in angiogenesis. Because myocardial energetic disturbances were present in the hearts of MI mice, we examined cardiac performance in hearts by echocardiography after 4 weeks of infarction. FAC was exacerbated in the MI group which improved with CoPP. Although heart rates were similar, left ventricular end diastolic area was greater in the hearts of the MI group. This was ameliorated by CoPP. Therefore, it happens that induction of fatty acid oxidation, enhanced angiogenesis along with reduction in pro-oxidant system in the group administered CoPP led to an improvement in the left ventricular myocardial functional parameter as assessed by echocardiography. Additionally, HO-1 induction also decreased the vasoconstrictor protein, Thromboxane synthase expression. It indicates that HO-1 induction not only increased angiogenesis but also increased blood supply by its vasodilatory effect in the infarcted heart and increased oxygen supply. This increased blood supply favors fatty acid oxidation within the infarcted heart and thus heart is able to perform efficiently.

In conclusion, our findings indicate that induction of HO-1 plays a major role in maintaining normal cardiac energy metabolism by inducing functional angiogenesis and reducing oxidative damage in ischemic myocardium. Our data is supportive of the concept that in an infarcted heart, impaired myocardial fatty acid oxidation is caused by a reduction in angiogenesis (less O2 supply) along with the lack of a responsive antioxidant system. Thus HO-1 induction in the failing heart opposes pathological ventricular remodeling, and this effect is mediated, at least in part, by normalizing FAO via the restoration of neoangiogenesis in addition to the stabilization of the redox microenvironment. Thus, HO-1 induction may prove viable as a strategic therapeutic modality in the treatment of heart failure.
Figure- 29. Possible mechanisms of cardioprotection by HO-1 after MI.

- Angiogenesis $\uparrow$
- Improve Cardiac Energy Metabolism $\uparrow$
- Decreases Inflammation and Apoptosis $\downarrow$

![Diagram showing possible mechanisms of cardioprotection by HO-1 after MI.]
Figure-30. Summarizing scheme

Myocardial infarction → Heart Failure → Administration of CoPPIX (5 days after MI) → Improvement in Cardiac function

- Systemic inflammation

- Renin/AngII

- Renal injury

- Cardiorenal syndrome-1

Angiogenesis → Improve Cardiac energy metabolism → Inflammation → Apoptosis

Mechanisms of improved cardiac function
6. Future prospects

Present study shows that HO-1 induction 5 days after experimental MI in animal models not only protected the infarcted heart but also ameliorated cardio-renal syndrome-1. Despite increasing knowledge of HO-1 and its protective role in cardiovascular and renal diseases, further studies are needed to evaluate the possible therapeutic potential of HO-1 and even its reaction products in cardiovascular diseases. The possible roles of HO-1 in post-MI cardiac regeneration could be of immense importance and require further research work. Recruitment of stem cells as well as activation of stem/progenitor cells by HO-1 could open new possibilities not only in heart and kidney but also in other injured or damaged organs. Role of immune system and its interaction with HO-1 after myocardial infarction could bring new advancements in the field of transplantation immunology. In addition, long-term effects of HO-1 induction as treatments for myocardial infarction and cardio-renal syndrome should be investigated.

Beneficial effects of HO-1 on cardiac cellular and extracellular remodeling open numerous therapeutic possibilities. HO-1 gene therapy in combination with stem cell therapy could improve the disease outcome. HO-1 can also play an important role in stem cell based therapy as it increases the survival and engraftment of stem cells. Preconditioning of various types of stem cells with CoPP could improve survival of transplanted cells in not only the infarcted heart but also in other injured organs.
References


tissue by a constitutively active form of hypoxia-inducible factor 1." Circ Res 93(11): 1074-1081.


incidence and case fatality in the National Heart, Lung, and Blood Institute's Framingham Heart study."

Circulation 119(9): 1203-1210.


