The Heme Oxygenase System Selectively Enhances the Anti-Inflammatory Macrophage-M2 Phenotype, Reduces Pericardial Adiposity, and Ameliorated Cardiac Injury in Diabetic Cardiomyopathy in Zucker Diabetic Fatty Rats

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ABSTRACT
Cardiac function is adversely affected by pericardial adiposity. We investigated the effects of the heme oxygenase (HO) inducer, hemin on pericardial adiposity, macrophage polarization, and diabetic cardiopathy in Zucker diabetic fatty rats (ZDFs) with use of echocardiographic, quantitative real-time polymerase chain reaction, Western immunoblotting, enzymatic immunocassay, and spectrophotometric analysis. In ZDFs, hemin administration increased HO activity; normalized glycemia; potentiated insulin signaling by enhancing insulin receptor substrate 1 (IRS-1), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (PKB)/Akt; suppressed pericardial adiposity, cardiac hypertrophy, and left ventricular longitudinal muscle fiber thickness, a pathophysiological feature of cardiomyocyte hypertrophy; and correspondingly reduced systolic blood pressure, total peripheral resistance, and pro-inflammatory/oxidative mediators, including nuclear factor κB (NF-κB), cJNK, c-Jun-N-terminal kinase (cJNK), endothelin (ET-1), tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, activating protein 1 (AP-1), and 8-isoprostane, whereas the HO inhibitor, stannous mesoporphyrin, nullified the effects. Furthermore, hemin reduced the pro-inflammatory macrophage M1 phenotype, but enhanced the M2 phenotype that damps inflammation. Because NF-κB activates TNFα, IL-6, and IL-1β and TNF-α, cJNK, and AP-1 impair insulin signaling, the high levels of these cytokines in obesity/diabetes would create a vicious cycle that, together with 8-isoprostane and ET-1, exacerbates cardiac injury, compromising cardiac function. Therefore, the concomitant reduction of pro-inflammatory cytokines and macrophage infiltration coupled to increased expressions of IRS-1, PI3K, and PKB may account for enhanced glucose metabolism and amelioration of cardiac injury and function in diabetic cardiomyopathy. The hemin-induced preferential polarization of macrophages toward anti-inflammatory macrophage M2 phenotype in cardiac tissue with concomitant suppression of pericardial adiposity in ZDFs are novel findings. These data unveil the benefits of hemin against pericardial adiposity, impaired insulin signaling, and diabetic cardiomyopathy and suggest that its multifaceted protective mechanisms include the suppression of inflammatory/oxidative mediators.

Introduction
The inflammatory and metabolic systems have been evolutionarily well-conserved in species and are fundamental for survival (Hotamisligil, 2006). However, these systems can be offset by obesity or nutrition overload, leading to inflammation in metabolic sites, such as the adipose tissue and skeletal muscles. In general, obesity and insulin resistance are closely associated with a state of low-grade inflammation because of incessant activation of a wide variety of inflammatory mediators, including nuclear factor κB (NF-κB), tumor necrosis factor α (TNF-α), and c-Jun-N-terminal kinase (cJNK) (Feinstein et al., 1993; Hotamisligil et al., 1993; Hotamisligil and Spiegelman, 1994; Uysal et al., 1997; Permana et al., 2006; Tuncman et al., 2006; Sabio et al., 2008; Tilg and Moschen, 2008; Fernandez-Veledo et al., 2009; Karalis et al., 2009; Scaccuzchio et al., 2009; Ndisang, 2010). Moreover, NF-κB stimulates TNF-α, interleukin (IL)-6, and IL-1β, which in turn may activate cJNK to create a vicious cycle that may aggravate insulin resistance and tissue damage (Ndisang, 2010). These destructive processes may be further exacerbated by macrophage infiltration, an event characterized by elevated levels of ED-1 (ED-1 is the primary antibody for activated macrophage) (Bazan et al., 2012). In general, macrophages express distinct patterns of surface receptors when responding to different stimuli. At present, two distinct polarization states of macrophages, classical (M1) and alternative (M2), have been characterized (Gordon and Martinez, 2010; Ndisang, 2010). Although the M1 phenotype promotes inflammation, the M2 phenotype dampens inflammatory events. Therefore, the concomitant reduction of M1...
phenotype, NF-κB, TNF-α, cJNK, IL-6, and IL-1β would limit tissue insults and decrease the oxidative destruction of important metabolic regulators, such as adiponectin and insulin, in type-2 diabetes (T2D) (Kamigaki et al., 2006; Kaneto et al., 2006).

Emerging evidence indicates that adipocytes from different body compartments have distinct inflammatory phenotype based on their anatomic location (Hamdy et al., 2006). In general, pericardial or ectopic adiposity is more malignant than subcutaneous adiposity, although they are both implicated in the pathogenesis of obesity-related cardio-metabolic complications, such as insulin-resistant T2D and coronary artery disease in lean and obese individuals (Hamdy et al., 2006; Rosito et al., 2008). Although we recently reported the insulin-sensitizing effects of the heme oxygenase (HO) inducer, hemin, in Zucker diabetic fatty rats (ZDFs) (Ndisang et al., 2009), the effects of the HO system on pericardial adiposity remains largely unclear. Similarly, the effect of upregulating the HO system with hemin on macrophage polarization in cardiac tissue has not been reported. Whether hemin therapy will improve cardiac function in ZDFs after suppressing M1 phenotype, NF-κB, TNF-α, cJNK, IL-6, and IL-1β in the left ventricle will be investigated. Therefore, this study was designed to investigate the role of hemin on pericardial adiposity and the mechanisms by which hemin ameliorates diabetic cardiopathy in ZDFs, an insulin-resistant T2D model with diabetic cardiomyopathy (Poornima et al., 2006; van den Brom et al., 2010).

Materials and Methods

Animal Treatment and Biochemical Assays. Our experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics, which is in conformity with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Twelve-week-old male ZDFs, a genetically obese leptin receptor-deficient (fa/fa) animal model of T2D, and their corresponding age- and sex-matched Zucker-lean (ZL) littersmates were purchased from Charles River (Willington, MA). The animals were housed at 21°C with 12-hour light/dark cycles, fed with standard laboratory chow, and had access to drinking water ad libitum. The drugs used for this study were hemin, an inducer of HO (30 mg/kg intraperitoneally; Sigma-Aldrich, St. Louis, MO), and stannous-mesoporphyrin (SnMP), a blocker of HO (2 mg/100 g body weight intraperitoneally; Porphyrin Products, Logan, UT). The doses of SnMP and hemin used in this study were shown to be effective in previous studies (Goodman et al., 2006; Li et al., 2008; Ndisang and Jadhav, 2009a; Ndisang et al., 2009, 2010). Hemin and SnMP were prepared as we previously reported and administered for use against porphyria (Anderson and Collins, 2006), and SnMP has successfully completed phase III clinical trials (Anderson et al., 2005). At 14 weeks of age, the animals were randomly assigned to five experimental groups (6–14 per group): controls (ZDFs and ZLs), hemin-treated ZDFs and ZLs, ZDFs + hemin + SnMP, ZDFs + SnMP, and ZDFs + vehicle dissolving hemin and SnMP. Extended methodology is available in Supplemental Methods.

During the treatment period, glucose levels were monitored weekly after 6 hours of fasting in metabolic cages with use of a glucose meter (BD, Franklin Lakes, NJ). Body weight was also measured on a weekly basis. At the end of the 8-week treatment period, the animals were 22 weeks of age. A day before sacrifice, the animals were fasted in metabolic cages for 24-hour urine collection and weighed. Systolic blood pressure was determined using noninvasive tail-cuff method (Model 29-SSP; Harvard Apparatus, Montreal, QC, Canada). Plasma samples were collected using intra-cardiac puncture, and the pericardial fat pad and the heart were isolated, cleaned, and weighed using an analytical balance (Precisa Instruments Ltd, Dietikon, Switzerland). The atria were removed from the heart, and the right ventricle free wall was separated from the left ventricle, including the septum, as we previously reported (Jadhav et al., 2008).

Hemin and SnMP were prepared as we previously reported and advanced into the left ventricular chamber to measure the left ventricular hemodynamic parameters. After positioning of the pressure transducer into the left ventricle, the rat was allowed to stabilize for 10 minutes before the left ventricular hemodynamic measurements were recorded. Arterial blood pressure was subsequently recorded by pulling the catheter out of the ventricular chamber into the aorta. Central venous pressure was measured by inserting the miniature tip sensor pressure transducer catheter into the superior vena cava through the right jugular vein. Data were acquired on a Biopac Data Acquisition system and assessed on AcqKnowledge software as we previously reported (Jadhav et al., 2008; Senanayake et al., 2012).

Echocardiography. Echocardiographic evaluation was done in rats with use of a Vevo 660 high-frequency ultrasound machine (VisualSonics, Markham, ON, Canada) equipped with B-mode imaging. For consistency, all measurements were done by the same investigator, and all ultrasound procedures did not exceed 30 minutes for each rat. Before ultrasound experiments, anesthesia was induced with 5% isoflurane, maintained at 0.5%–1% isoflurane (Abbott Laboratories, Saint-Laurent, QC, Canada), and the rats were placed with the ventral side up on an electrocardiogram (ECG) plate (VisualSonics, Markham, ON, Canada) with each paw covered with electrode cream (Signa Crème; Parker Laboratories, Fairfield, NJ) and secured to ECG contacts with surgical tape to monitor heart rate throughout each experiment. A temperature probe was inserted rectally to maintain internal body temperature at 37°C. To prevent artifact with this high-resolution ultrasound system, the animal was depilated by wiping from the chest area with deplaiy cream (Nair, New York, NY). Thereafter, EcoGel 200 (Eco-Med Pharmaceuticals, Mississauga, ON, Canada) was then applied to the thorax for ultrasound.

A RMV 710B scanhead (VisualSonics, Markham, ON, Canada) was used to gather all parasternal short and long-axis views of the rat ventricle in B-mode. The areas of three different short axis views along the ventricle were measured and designated as A1, A2, and A3. The ventricular length of one long axis view was measured and divided by four to give ventricular height. Use of different short axis views at different levels of the ventricle compensates for irregularities in ventricular shape and greatly increases accuracy of chamber volume measurements (Ram et al., 2011). All of these values were measured at both systole and diastole with use of VisualSonics software. With these values, end systolic and
Hemin Abates Pericardial Adiposity

Results

Hemin Therapy Abated Pericardial Adiposity and Restored Normoglycemia in ZDFs. ZDFs were severely hyperglycemic, with fasting glucose levels of 24.6 ± 3.1 mM (Table 1), whereas their age- and sex-matched littermate control-ZLs were normoglycemic (7.2 ± 0.8 mM). The 8-week regimen of hemin to ZDFs reduced the elevated glycemia to a physiologic level (24.6 ± 3.1 versus 6.8 ± 1.3 mM; \( P < 0.01 \)), whereas the cotreatment of hemin and the HO inhibitor, SnMP, abolished the effect of hemin, suggesting a role of the HO system on glucose homeostasis. Similarly, hemin treatment significantly reduced pericardial adiposity (1.85 ± 0.2 versus 0.79 ± 0.3 g/kg body weight; \( P < 0.01 \)) and cardiac hypertrophy (3.1 ± 0.3 versus 2.4 ± 0.14 g/kg body weight; \( P < 0.01 \)) in ZDFs, whereas the coadministration of hemin and SnMP nullified the effect, suggesting a role of the HO system on the regulation of pericardial adiposity and cardiac hypertrophy. The vehicle dissolving hemin and SnMP had no effect on blood glycemia, pericardial adiposity, and heart weight (Table 1).

Hemin therapy also affected ZLs, although less intensely. A slight but significant reduction in blood glucose level, cardiac hypertrophy, and pericardial adiposity were observed in ZLs. These effects were abolished by SnMP (Table 1). In hemin-treated ZDFs, blood glucose level, cardiac hypertrophy, and pericardial volumes in units of cubic centimeters (equivalent to milliliters) were each calculated for the left ventricle with use of Eq. 1:

\[
V = (A_1 + A_2)h + (\pi h^3/6) + (A_1h/2)
\]

End systolic volume was subtracted from end diastolic volume to give stroke volume (\( V_s \)) in milliliters. Heart rate in beats per minute for each rat was recorded at three different times throughout one imaging period and then averaged. Cardiac output (CO) in milliliters per minute was then determined using heart rate (\( f_H \)) and stroke volume (\( V_s \); Eq. 2):

\[
CO = f_H \times V_s
\]

Ejection fraction (%) for each rat was also calculated using stroke volume (\( V_s \)) and end diastolic volume (EDV; Eq. 3):

\[
E_F = V_s / EDV
\]

To measure left ventricular free wall thickness, a clip in parasternal long axis view was obtained for all experimental groups. At least three individual images were exported from the clip at both systole and diastole with use of Premiere Elements 2.0 (Adobe, San Jose, CA), and then left ventricular wall thickness was determined using ImagePro 6.0 software (Bethesda, MD). Heart rate (\( f_H \) in beats per minute) was calculated from ECG traces during the blood pressure experiment.

Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction for p65-NFκB, Activating Protein 1 and cJNK. The left ventricle was homogenized, and quantitative real-time polymerase chain reaction was done as we previously reported (Jadhav et al., 2008; Ndisang et al., 2009, 2010). In brief, triplicate samples of 1 μl of cDNA each were run using a template of 3.2 pmol of primers for NFκB (forward 5' CGCGTCTTGCCTAACAGTGCCG-3' and reverse 5' TGGTGGCTTATCTGATGTTGTCG-3'), activating protein 1 (AP-1) (forward 5'AGCAAGATGCTTGGAGCCAC3' and reverse 5' TTCCATGGGTCCCTGCTTTGAGAT-3'), HO-1 (forward 5'AGCAACAGAAGGCTACTGAGAGCAAGA-3' and reverse 5'TTCCATGGGTCCCTGCTTTGAGAT-3'), glyceraldehyde-3-phosphate dehydrogenase (forward 5'AGCAGATGCGTTTCCGTTACAAGTGCGA-3' and reverse 5'AGAAGGATACTGAGAGCAAGA-3'), phosphatidylinositol-3-kinase (PI3K) (sc67306), protein kinase-B (PKB) (sc9118) and insulin receptor substrate 1 (IRS-1) (8299) were used. Denstometric analysis was done with UN-SCAN-IT software (Silk Scientific, Orem, Utah). Glyceraldehyde-3-phosphate dehydrogenase antibody (Sigma-Aldrich) was used as a control to ascertain equivalent loading.

Left Ventricular Histology. The middle portion (midpapillary level) of the left ventricle of heart was separated, fixed in 10% formalin phosphate buffer for 48 hours, processed, and paraffin embedded as we previously reported (Jadhav et al., 2008). Then, sections of 5-μm thicknesses were cut and stained with hematoxylin and eosin for histologic analysis. The left ventricular sections were obtained from the middle portion of the left ventricle to avoid differences in regional cardiomyocyte size in different regions of the left ventricle. The cardiac sections were scanned using a microscope (Aperio Scan Scope Model CS; Aperio Technology Inc, Vista, CA) and analyzed using Aperio Image Scope V11.2.0.780 software (e-Pathology Solution; Aperio Technology). Left ventricular myocyte width and longitudinal myocyte thickness were measured randomly in 20–30 cardiac muscle fibers from each left ventricular tissue section. Muscle fiber thickness was quantified and analyzed among different groups. For consistency, myocytes were positioned perpendicularly to the plane of the section, with a visible nucleus and cell membrane clearly outlined. Unbroken areas were selected for measurement. All sections were imaged at 40× zoom (40×: 0.50 μm/pixel) in Aperio Image Scope using length measurement tool (micrometers).

Statistical Analyses. All data were expressed as means ± S.E.M. from at least six independent experiments, unless otherwise stated. Statistical analyses were done using unpaired Student's \( t \) test and two-way analyses of variance in conjunction with Bonferroni test for repeated measures when appropriate. Group differences at the level of \( P < 0.05 \) were considered to be statistically significant.

**TABLE 1**

Effect of hemin and SnMP on physiologic variables in ZDFs and ZLs (eight animals per group)

<table>
<thead>
<tr>
<th>Physiologic Variables</th>
<th>Control ZL</th>
<th>ZL + Hemin</th>
<th>Control ZDF</th>
<th>ZDF + Hemin</th>
<th>ZDF + Hemin + SnMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>363.7 ± 5.4</td>
<td>354.5 ± 9.5</td>
<td>383.6 ± 5.4</td>
<td>363.4 ± 6.5</td>
<td>352.5 ± 8.2*</td>
</tr>
<tr>
<td>Fasting glucose level (mM)</td>
<td>7.2 ± 0.5</td>
<td>6.4 ± 0.3*</td>
<td>24.6 ± 3.1</td>
<td>6.8 ± 1.3**</td>
<td>19.2 ± 2.8</td>
</tr>
<tr>
<td>Cardiac hypertrophy (g/kg body weight)</td>
<td>2.7 ± 0.2</td>
<td>2.3 ± 0.1*</td>
<td>3.8 ± 0.3</td>
<td>2.4 ± 0.14**</td>
<td>3.4 ± 0.3*</td>
</tr>
<tr>
<td>Pericardial adiposity (g/kg body weight)</td>
<td>1.3 ± 0.1</td>
<td>0.97 ± 0.05*</td>
<td>1.85 ± 0.2</td>
<td>0.79 ± 0.13**</td>
<td>1.72 ± 0.5*</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) versus control ZDFs or control ZLs; ** \( P < 0.01 \) versus control ZDFs or control ZLs; † \( P < 0.05 \) versus controls; †† \( P < 0.05 \) versus ZDF + Hemin.
pericardial adiposity were reduced by 72.3, 36.8, and 57%, respectively, whereas in ZLs, these same parameters were reduced by 11.1, 14.8, and 25.4%, respectively, suggesting greater selectivity of the actions of hemin in diseased conditions, such as the situation in ZDF and less active in healthy status, as in the case of ZLs.

The HO inducer, hemin, and HO blocker, SnMP, also affected body weight. A slight body weight decrease (<10%) was observed in hemin- and SnMP-treated animals (Table 1). In ZL+hemin, ZDF+hemin, and ZDF+hemin+SnMP, the percentage decrease in body weight was 2.5, 5.3, and 8.1%, respectively. Although body weight decrease can affect blood glucose levels, it is unlikely in this case, because the slight body weight decrease in hemin- and SnMP-treated rats were accompanied by opposite effects on glucose levels (Table 1). Accordingly, we observed a decrease in glucose levels in hemin-treated animals, but an increase in SnMP-treated animals, suggesting that the HO system may be endowed with intrinsic anti-diabetic effects. The decrease in body weight may not be attributable to toxicity, because we recently showed that several indices of toxicity, including plasma gamma-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase levels were within normal range (Ndisang et al., 2009).

Hemin therapy also improved cardiac hemodynamics (Table 2). In hemin-treated ZDF, systolic blood pressure was reduced by 13.95%, whereas cardiac output increased by 8.2%. Of interest, the decrease in systolic blood pressure was associated with a 12.2% decrease in total peripheral resistance, suggesting reduced afterload to the left ventricle (Boron and Boulpaep, 2009). Correspondingly, a reduction of 8.9% in the rate of left ventricular pressure development was observed (Table 2). Furthermore, hemin increased the left ventricular ejection fraction by 5.4%, and this effect was accompanied by a 2.2% reduction of left ventricular systolic pressure. Therefore, increased cardiac output coupled to the concomitant reduction of total peripheral resistance, left ventricular pressure development, and left ventricular systolic pressure are indicative of improved cardiac function in hemin-treated ZDFs.

**Hemin Therapy Enhanced HO-1 and HO Activity but Abated Endothelin-1 and 8-Isoprostane in the Left Ventricle of ZDFs.** To investigate the role of the HO system in the improved cardiac function and insulin-signaling in ZDFs, we measured HO activity, endothelin-1 (ET-1), and 8-isoprostane. Our results indicate that the basal levels of HO-1 and HO activity in ZDFs were 2.7-fold higher than in control ZLs. Of interest, hemin therapy greatly attenuated the elevated levels of left ventricular ET-1 in ZDFs, whereas SnMP abolished the effect of hemin (Fig. 1D). Hemin therapy also reduced 8-isoprostane level in ZDFs, although less intensely, compared with ZDFs, because only a 28.9% reduction was observed in hemin-treated ZLs, compared with 57.6% in hemin-treated ZDFs.

Hemin therapy markedly increased the levels of HO-1 and HO activity in ZDFs by 8.1- and 10.56-fold, respectively (Fig. 1, A and B), whereas the cotreatment with the HO inhibitor, SnMP, nullified the effects of the HO inducer, hemin. Similarly, treatment with SnMP alone depleted the basal levels of HO-1 and HO activity (Fig. 1, A and B). Hemin therapy also enhanced the levels of HO-1 and HO activity in ZLs, although a greater increment was observed in hemin-treated ZDFs (Fig. 1, A and B). The higher magnitude of HO signaling may be responsible for the more intense reduction of glycemic levels in ZDFs, compared with ZLs (Table 1). Alternatively, the less preponderant increase in HO activity in ZL rats may suggest greater stability of the HO system in healthy conditions.

Because elevated oxidative stress is among the causative factors of insulin resistance and cardiac dysfunction, we measured 8-isoprostane, an important marker of oxidative stress (Delanty et al., 1997). In ZDFs, the basal levels of left ventricular 8-isoprostane were markedly elevated, suggesting enhanced oxidative stress (Fig. 1C). Of interest, hemin therapy significantly reduced 8-isoprostane level by 57.6%. Contrarily, in SnMP+ZDF–treated animals, the effect of hemin on 8-isoprostane level was annulled, and 8-isoprostane was reversed to levels comparable to those observed in control ZDFs. On the other hand, in SnMP-treated animals, the levels of left ventricular 8-isoprostane were further increased, suggesting that oxidative stress is further potentiated by blockade of basal HO activity (Fig. 1C). Hemin therapy also reduced 8-isoprostane level in ZLs, although less intensely, compared with ZDFs, because only a 28.9% reduction was observed in hemin-treated ZLs, compared with 57.6% in hemin-treated ZDFs.

Because 8-isoprostane stimulates ET-1 (Fukunaga et al., 1995) and both ET-1 and 8-isoprostane are involved in the oxidative destruction of tissue, we also assessed ET-1 in the left ventricle. Our results indicate that the levels of ET-1 in ZDFs were 2.7-fold higher than in control ZLs. Of interest, hemin therapy greatly attenuated the elevated levels of left ventricular ET-1 in ZDFs, whereas SnMP abolished the effect of hemin (Fig. 1D). Hemin therapy also reduced ET-1 levels in ZLs, although to a lesser extent, compared with ZDFs. Accordingly, a reduction of 25.2% in ET-1 level was observed in hemin-treated ZLs, compared with 54.2% in hemin-treated ZDFs.

Therefore, the preponderant increase in HO activity in hemin-treated ZDFs, compared with hemin-treated ZLs (Fig. 1A), coupled to the more accentuated reduction in left

**TABLE 2**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Systolic BP (mm Hg)</th>
<th>Cardiac Output (ml/min)</th>
<th>Ejection Fraction (%)</th>
<th>LVSP (mm Hg)</th>
<th>+dP/dt (mm Hg/s)</th>
<th>Total Peripheral Resistance (mm Hg.mm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ZDF</td>
<td>137.6 ± 3.7</td>
<td>82.06 ± 9.36</td>
<td>62.35 ± 0.93</td>
<td>135.05 ± 7.31</td>
<td>2828.74 ± 194.41</td>
<td>1.32 ± 0.14</td>
</tr>
<tr>
<td>ZDF+Hemin</td>
<td>118.4 ± 2.5*</td>
<td>88.76 ± 4.67</td>
<td>65.70 ± 1.21</td>
<td>131.97 ± 2.66</td>
<td>2575.86 ± 103.35</td>
<td>1.16 ± 0.08*</td>
</tr>
<tr>
<td>Percentage</td>
<td>−13.95*</td>
<td>8.26</td>
<td>5.37</td>
<td>−2.28*</td>
<td>−8.94*</td>
<td>−12.16*</td>
</tr>
</tbody>
</table>

**Legend:**
- BP: blood pressure; LVSP, left ventricular systolic pressure.
- *The negative percentage changes in LVSP and left ventricular pressure development (+dP/dt) are well correlated to the reduction of total peripheral resistance and systolic blood pressure, all of which are indicative of improved cardiac function (Boron and Boulpaep, 2009).
- *P < 0.05 versus control ZDFs; 6-8 animals per group.
ventricular 8-isoprostane (Fig. 1C) and ET-1 (Fig. 1D) levels may account for the greater anti-diabetic effect (Table 1) and improved cardiac effects (Table 2) in hemin-treated ZDFs.

**Hemin Therapy Suppressed Pro-Inflammatory Cytokines that Deregulate Glucose Metabolism Cardiac Function.** TNF-α, IL-6, and IL-1β are cytokines that impair cardiac function and glucose metabolism (Li et al., 2006; Burgess et al., 2010; Ndisang, 2010); therefore, we investigated whether the improvement in cardiac function and glucose metabolism in hemin-treated ZDFs would be accompanied by reduction of these cytokines. Our results indicate that the levels of TNF-α, IL-6, and IL-1β in the left ventricle of control ZDFs were significantly elevated by 4.5-, 9.1-, and 2.5-fold, respectively, compared with the levels in control ZLs (Fig. 2). Treatment with hemin markedly reduced TNF-α, IL-6, and IL-1β levels by 71.2, 51.3, and 56.8%, respectively. In contrast, the coapplication of the HO inhibitor, SnMP, with hemin reversed the effects of hemin (Fig. 2A–C), suggesting a role of the HO system in the regulation of these inflammatory cytokines. Hemin therapy also reduced the levels of TNF-α, IL-6, and IL-1β in the ZLs, although less intensely. A reduction of 35.4, 37.0, 28.5% in TNF-α, IL-6, and IL-1β levels, respectively, was observed in hemin-treated ZLs, compared with 71.2, 51.3, and 56.8%, respectively, in hemin-treated ZDFs.

**Hemin Abated Transcription Factors that Impair Insulin Signaling and Cardiac Function.** Many inflammatory and oxidative transcriptional factors, including NF-κB, AP-1, and cJNK, are implicated in tissue damage and insulin resistance (Bennett et al., 2003; Kaneto et al., 2006). In ZDFs, quantitative real-time polymerase chain reaction analyses indicated that the levels of NF-κB, AP-1, and cJNK in the left ventricle were strikingly elevated (Fig. 3). Treatment with hemin reduced NF-κB and AP-1 levels by 2.5- and 2.9-fold respectively, whereas the HO inhibitor, SnMP, nullified the effects of hemin (Fig. 3, A and B). Moreover, treatment with SnMP alone further enhanced NF-κB and AP-1 in ZDF rats by 20 and 31.9%, respectively, suggesting the involvement of basal HO activity in the regulation of these oxidative/inflammatory mediators. Furthermore, in ZDFs, the basal expression of left ventricular cJNK, a substance that suppresses insulin biosynthesis (Kaneto et al., 2006), was markedly increased by 4.75-fold, but was abated by hemin (Fig. 3C). Hemin therapy also reduced NF-κB, AP-1, and cJNK levels in ZL rats by 30.5, 24.8, and 26.1%, respectively, which were less intense, compared with reductions of 59.3, 65.6, and 57.8%, respectively, in hemin-treated ZDFs, suggesting greater selectivity of hemin in diseased condition.

**Hemin Abated Inflammation but Potenti-ated Insulin-Signaling Agents.** Because macrophages are among the fundamental sources of many of the circulating inflammatory molecules in obesity and are postulated to be causal in the development of insulin-resistant T2D (Gordon and Martinez, 2010; Ndisang, 2010), we used specific markers
(ED1 and ED2) to quantify the M1 pro-inflammatory phenotype (ED1) and the M2 anti-inflammatory phenotype (ED2). Our Western immunoblotting and relative densitometric analyses indicated that the expression of the pro-inflammatory M1 phenotype in control ZDFs was significantly elevated (Fig. 4A) but, of interest, was abated by 40.1% by hemin therapy. On the other hand, the anti-inflammatory phenotype, M2, was significantly reduced in control ZDFs (Fig. 4B) but, of interest, was enhanced by 61.3% by hemin therapy, suggesting that hemin may preferentially favor macrophage polarization toward the M2 anti-inflammatory phenotype as an alternative mechanism to counteract tissue insult.

Because IRS1, PI3K, and PKB are important proteins implicated in the insulin signal transduction pathway (Ndisang, 2010), we investigated the effect of hemin therapy on these proteins. Our results indicate that the expression of IRS1 in control ZDFs was depressed (Fig. 4C). However, hemin therapy greatly enhanced the expression of IRS1 by 2.3-fold. Similarly, hemin therapy significantly increased the expressions of PI3K (Fig. 4D) and PKB (Fig. 4E) by 3.5- and 2.8-fold, respectively.

**Hemin Therapy Reduced Longitudinal Muscle Fiber Thickness in ZDFs.** Longitudinal muscle fiber thickness is a common pathophysiological feature of cardiac myocyte hypertrophy (Conrad et al., 1995; Rodriguez et al., 2005; Jadhav et al., 2008). In untreated ZDFs, enlarged cardiomyocytes with increscent nuclei were evident, compared with normal cardiomyocytes in control ZLs (Fig. 5A). The inter-myofibril space was reduced in ZDF controls, compared with the
age- and sex-matched ZLs (Fig. 5A). Indeed, a 44% increase in cardiomyocyte longitudinal fiber thickness was observed in the ZDF controls, compared with ZLs (21.9 ± 0.89 versus 15.2 ± 0.49 μm; P < 0.01; n = 6) (Fig. 5B). Of interest, hemin treatment reduced cardiomyocyte longitudinal fiber thickness by 25%, compared with ZDF controls (18.1 ± 0.76 versus 21.9 ± 0.89 μm; P < 0.05; n = 6). However, this reduction did not reach the basal level of cardiomyocyte longitudinal thickness of the ZLs (15.2 ± 0.49 μm) (Fig. 5B).

**Discussion**

The present study demonstrates, for the first time to our knowledge, that upregulating the HO system with hemin preferentially favors macrophage polarization toward the M2 phenotype that dampens inflammation and suppresses the M1 pro-inflammatory phenotype. Another novel observation is that hemin therapy suppresses pericardial adiposity in ZDFs, a model of insulin resistance, dyslipidemia, hyperglycemia, and diabetic cardiomyopathy (Poornima et al., 2006; Ndisang et al., 2009; van den Brom et al., 2010). Pericardial adiposity and cardiac hypertrophy are associated with elevated inflammatory and/or oxidative insults and dyslipidemia that adversely affect cardiac function, especially in individuals comorbid with obesity and insulin resistance (Hamdy et al., 2006; Jadhav et al., 2008; Rosito et al., 2008; McAuley et al., 2011). Of interest, hemin therapy significantly reduced pericardial adiposity and cardiac hypertrophy and attenuated macrophage infiltration and several pro-inflammatory and/or oxidative mediators, such as NF-κB, AP-1, cJNK, TNF-α, IL-6, and IL-1β (Bennett et al., 2003; Kaneto et al., 2006; Li et al., 2006; Burgess et al., 2010; Ndisang, 2010), but enhanced important proteins involved in the insulin signal transduction...
pathway, such as IRS-1, PI3K, and PKB (Ndisang, 2010), with corresponding reduction of hyperglycemia in ZDFs.

The hemin-dependent preferential enhancement of the M2-phenotype may be considered to be a novel and alternative anti-inflammatory mechanism through which the HO system counteracts inflammatory insult. Although one study had previously reported the role of HO-1 promoter in macrophage polarization (Weis et al., 1998), the expression levels of M1 and M2 phenotypes were not measured; thus, our study provides the first solid evidence on the role of the HO system on macrophage polarization. In addition to macrophage infiltration, the HO system may reduce tissue inflammation through other mechanisms, including the suppression of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β (Li et al., 2006; Burgess et al., 2010; Ndisang, 2010). Consistently, in hemin-treated ZDFs, TNF-α, IL-6, and IL-1β were markedly reduced. Similarly, other pro-inflammatory and/or oxidative mediators, such as 8-isoprostane, NF-κB, AP-1, and cJNK (Bennett et al., 2003; Kaneto et al., 2006) were greatly attenuated by hemin therapy, whereas the HO inhibitor, SnMP, nullified the hemin effects and exacerbated oxidative and/or inflammatory insults causing further impairment in glucose metabolism.

Another important observation from our study is the attenuation of cardiac hypertrophy and the corresponding reduction of left ventricular longitudinal muscle fiber thickness, a common pathophysiological feature of cardiomyocyte hypertrophy (Conrad et al., 1995; Rodriguez et al., 2005; Jadhav et al., 2008). Other mechanisms that may be responsible for the improvement of cardiac hemodynamic parameters by the HO system include vascular contractility (Sammut et al., 1998), ventricular contractility (Achouh et al., 2005), and the reduction of cardiac injury (Conrad et al., 1995; Rodriguez et al., 2005; Jadhav et al., 2008; Jadhav and Ndisang, 2009; Ndisang and Jadhav, 2009b), which would result in a healthier heart with improved ventricular contractility and improved hemodynamics (Achouh et al., 2005). Furthermore, the HO system generates a vasodilator-like carbon monoxide, a stimulator of cGMP that modulates both vascular contractility (Sammut et al., 1998; Achouh et al., 2005) and ventricular contractility and, thus, hemodynamics (Achouh et al., 2005). Of interest, in the present study, the abrogation of cardiac hypertrophy and longitudinal muscle fiber thickness were also associated with improved cardiac hemodynamics. In particular, systolic blood pressure was significantly decreased and cardiac output increased by 8.3% in hemin-treated ZDFs. The decrease in systolic blood pressure and left ventricular systolic pressure observed were accompanied by the reduction of total peripheral resistance, and these effects would reduce the afterload to the left ventricle and, thus, prevent the onset of ventricular dysfunction (Awan et al., 1981; Boron and Boulpaep, 2009; Pingitore et al., 2011). An abnormal left ventricular function would affect the cardiac performance and contributes to the symptoms associated with cardiac failure (Heidenreich et al., 2012).

Hemin therapy also enhanced the HO system and abated NF-κB, AP-1 cJNK, TNF-α, IL-6, and IL-1β in ZL controls, although the magnitude was smaller, compared with ZDFs with depressed HO activity. The reasons for this selective effect of HO are not fully understood. However, it is possible that, because ZLs are healthy animals with normal and/or functional insulin signaling, the HO system may be more stable, compared with ZDFs, which have depressed HO...
activity. Of importance, the selectivity of the HO system in diseased conditions could be explored against the comorbidity of insulin-resistant diabetes and obesity. Nevertheless, future studies will be done to investigate the selective effects of the HO system on ZL controls. Although we previously reported the insulin-sensitizing effect of hemin in the gastrocnemius muscle of ZDF, tissue-specific response is a well-known phenomenon in the pathophysiology of insulin resistance and impaired glucose metabolism, and different tissues may respond distinctly to the same stimuli, indicating that a physiologic response in one tissue may not necessarily be the same in another tissue (Farret et al., 2006; Zhang et al., 2010). Whether the reported effects were unique for the gastrocnemius muscle or universal for other tissues is critical for understanding the role of hemin in insulin resistance and glucose metabolism. Therefore, studying the effect of an upregulated HO system in the left ventricle of ZDFs is important for the advancement of knowledge in this area. Moreover, the effects of hemin therapy on left ventricular IRS-1, PI3K, and PKB in ZDFs, a model with diabetic cardiomyopathy (Poornima et al., 2006; van den Brom et al., 2010), remains poorly understood. Of interest, the present study unveils that the restoration of normoglycemia in hemin-treated ZDFs was accompanied by the concomitant potentiation of left ventricular IRS-1, PI3K, and PKB and the improvement of cardiac hemodynamics, particularly the reduction of total-peripheral resistance and systolic blood pressure.

Collectively, our study unveils the beneficial effect of the HO system on pericardial adiposity, impaired insulin signaling, and diabetic cardiomyopathy and suggests that the suppression of cardiac hypertrophy, left ventricular longitudinal muscle fiber thickness, and the reduction of inflammatory and/or oxidative mediators are among the multifaceted mechanisms by which the HO system maintains homeostasis in physiologic milieu. Because NF-κB activates TNFα, IL-6, and IL-1β (Ndisang, 2010) and TNF-α, cJNK, and AP-1 impair insulin signaling (Ndisang, 2010), the high levels of these cytokines and inflammatory and/or oxidative mediators in the chronic conditions of obesity and diabetes would create a vicious cycle that, when added to the oxidative insults generated by 8-isoprostane and ET-1 (Yura et al., 1999), would exacerbate cardiac insult and compromise cardiac function. Therefore, the concomitant reduction of cardiac hypertrophy, left ventricular longitudinal muscle fiber thickness, pro-inflammatory cytokines, and macrophage infiltration coupled to the potentiation of insulin signal transduction agents, such as IRS-1, PI3K, and PKB, may account for enhanced glucose metabolism and improved cardiac hemodynamics in hemin-treated ZDFs. Of importance, the novel findings of our study includes (1) the preferential polarization of macrophages toward anti-inflammatory M2-phenotype in cardiac tissue, as evidenced by increased expression levels of the M2-phenotype and the parallel reduction of the M1-proinflammatory phenotype; (2) the suppression of pericardial adiposity; and (3) the hemin-induced improvement of cardiac hemodynamic, particularly the reduction of total-peripheral resistance in ZDFs, a model of obese insulin-resistant T2D with cardiomyopathy (Poornima et al., 2006; van den Brom et al., 2010).

With the escalation of obesity, diabetes, and hypertension in industrialized and developing countries, the incidence of cardio-metabolic complications, including diabetic cardiomyopathy and heart failure, will increase. Cardio-metabolic complications are multifactorial diseases, and a wide variety of different pathophysiological factors, including inflammatory and/or oxidative insults, are involved. The present study highlights the ability of hemin therapy to suppress inflammatory and oxidative mediators and improve insulin signaling in T2D (Fig. 6). Impaired insulin signaling is not only an important etiological factor in the pathogenesis of type-1 and type-2 diabetes, but also an important pathophysiological driving force that is capable of dictating the dynamics and progression of the disease and its ultimate evolution into complications, such as diabetic cardiomyopathy. Therefore, the findings reported here could serve as a useful tool for the formulation of novel therapeutic agents against diabetes, pericardial adiposity, and related complications, such as diabetic cardiomyopathy. Of interest, our study may have great translational potential, because the drugs used (hemin and SnMP) may have therapeutic application. Both hemin and SnMP may have application in clinics, because hemin has been approved by the Food and Drug Administration for use against porphyria (Buck, 1995, http://www.medicine.virginia.edu/clinical/departments/pediatrics/education/pharm-news/1995-2000/199502.pdf; Anderson and Collins, 2006), and SnMP has successfully completed phase III clinical trials for possible use against neonatal jaundice (Alexander, 2004; Kappas, 2004; Anderson, 2005, http://www.clinicaltrials.gov/ct/show/NCT00004396).

![Fig. 6. Schematic representation of the actions of hemin. Hemin therapy enhances HO-1, which in turn reduces adiposity and pro-inflammatory cytokines, including IL6, IL-1β, TNF-α, cJNK, and M1-phenotype macrophage infiltration. Correspondingly, insulin sensitivity increases and normoglycemia is restored.](image-url)
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Authorship Contributions

Participated in research design: Ndisang.
Conducted experiments: Jadhav, Tiwari, Lee, Ndisang.
Performed data analysis: Jadhav, Tiwari, Ndisang.
Wrote or contributed to the writing of the manuscript: Jadhav, Tiwari, Lee, Ndisang.

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The heme oxygenase system selectively enhances the anti-inflammatory macrophage-M2 phenotype, reduces pericardial adiposity and ameliorated cardiac injury in diabetic cardiomyopathy in Zucker diabetic fatty rats

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Online supplemental

Extended methodology

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MATERIALS AND METHODS

Animal treatment and biochemical assays

Our experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics, which is in conformity with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Twelve-week-old male ZDF rats, a genetically obese leptin receptor-deficient \( (fa/fa) \) animal model of T2D and their corresponding age/sex-matched Zucker-lean (ZL) littermates were purchased from Charles River (Willington, MA, USA). The animals were housed at 21°C with 12-hour light/dark cycles, fed with standard laboratory chow and had access to drinking water \textit{ad libitum}. At 14 weeks of age, the animals were randomly assigned to five experimental groups \((n=6-14\) per group): \( \textbf{(A)} \) controls (ZDF and ZL), \( \textbf{(B)} \) hemin-treated ZDF and ZL, \( \textbf{(C)} \) ZDF+hemin+SnMP, \( \textbf{(D)} \) ZDF+SnMP and \( \textbf{(E)} \) ZDF+vehicle dissolving hemin and SnMP. Hemin, a HO-inducer (30 mg/kg i.p., Sigma, St Louis, MO) and stannous-mesoporphyrin (SnMP), a HO-blocker (2 mg/100 g body weight ip, Porphyrin Products, Logan, UT), were prepared as we previously reported (Jadhav et al., 2008). The drugs were given biweekly for eight weeks. Hemin has been approved by the FDA against porphyria (Anderson and Collins, 2006), while SnMP has successfully completed phase-III clinical trials (Anderson et al., 2005).

During the treatment period glucose was monitored weekly after 6 hrs of fasting in metabolic cages with a glucose-meter (BD, Franklin Lakes, NJ, USA). Body weight was also measured on a weekly basis. At the end of the 8-week treatment period, the animals were 22 weeks of age. A day prior to sacrifice, the animals were fasted in metabolic cages for 24-hr urined collection and weighed. Systolic blood pressure was determined by non-invasive tail-cuff method (Model 29-SSP, Harvard Apparatus, Montreal, Canada) (Senanayake et al.), (Jadhav et al., 2008).
Plasma was collected by intra-cardiac puncture and the pericardial fat pad and the heart were isolated, cleaned and weighed using an analytical balance (Precisa Instruments Ltd, Switzerland). The atria were removed from the heart and the right ventricle free wall separated from the left ventricle including the septum as we previously reported (Jadhav et al., 2008).

**Determination of HO-1 and HO activity**

HO activity was evaluated as bilirubin production by our established method (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009). The tissues were homogenized on ice in 4 volumes of 5:1 K/Na 100 mmol/L phosphate buffer with 2 mmol/L MgCl2 (HO-activity buffer), centrifuged at 13,000 rpm for 15 minutes. From the supernatant, aliquots of 100 μl was to measure HO enzyme activity in a reaction volume of 500 μl, containing, 0.8 mmol/L nicotinamide dinucleotide phosphate, 20 μmol/L hemin, 2 mmol/L glucose-6-phosphate, 0.002 U/μl glucose-6-phosphate dehydrogenase and 100 μl liver cytosol as source of biliverdin reductase. The reaction was carried out in dark at 37ºC for 1 hour and stopped by adding 500 μl of chloroform. To extract bilirubin, the tubes were vigorously agitated and centrifuged at 13,000 rpm for 5 minutes. The chloroform layer was collected and read on a spectrophotometer at 464 nm minus the background at 530 nm. The amount of bilirubin in each sample was determined spectrophotometrically (extinction coefficient for bilirubin 40 mM⁻¹cm⁻¹), and expressed as nmole/mg protein/hour. As a positive control, spleen tissue was used.

HO-1 concentration was determined by enzyme-linked immunosorbent assay (ELISA) (EKS-810A, Stressgen-Assay Design, Ann Arbor, MI, USA) as previously reported following the manufacturer’s instructions (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009; Ndisang and Jadhav, 2010).
Western Immunoblotting

Left ventricular tissues were homogenized (1:10, w:v) in 10 mM Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of a cocktail of protease inhibitors, and centrifuged as we previously reported (Jadhav et al., 2008; Ndisang et al., 2008; Jadhav et al., 2009). Briefly, proteins were extracted and quantified by Bradford assay, and aliquots of 50 μg were loaded on a 10% SDS-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to nitrocellulose paper, non-specific bindings blocked with 3% non-fat milk, and incubated overnight with primary antibodies (Santa Cruz Biotechnology, CA, USA) including ED-2 (CD163) (sc-58956), ED-1 (CD68) (sc-59103), phosphatidylinositol-3-kinase (PI3K) (sc67306), protein kinase-B (PKB) (sc9118) and insulin-receptor-substrate-1 (IRS-1) (8299) were used. Densitometric analysis was done with UN-SCAN-IT software (Silk Scientific, Utah, USA). GAPDH antibody (Sigma St Louis, MO, USA) was used as a control to ascertain equivalent loading. After washing, blots were incubated with anti-rabbit IgG conjugated to horseradish peroxide (Bio-Rad, CA, USA), and the immuno-reactivity visualized using enhanced horseradish peroxide/luminol chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA).

Total RNA Isolation and Quantitative RT-PCR for p65-NF-κB, AP-1 and JNK

This was performed as we previously reported (Ndisang et al., 2008). In brief, left ventricular tissues were homogenized in 0.5 ml Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s specifications. Reverse transcription was carried out using First Strand cDNA Synthesis Kit (Novagen, Madison, WI, USA) with 0.5 μg Oligo (dT)6, 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 75 mM KCl, 3 mM MgCl2, 50 mM DTT, 10 mM each free dNTP and 100 U of MMLV reverse transcriptase according to manufacturer’s instruction. Quantitative PCR was performed using Applied Biosystems 7300 Real
Time PCR system (Foster City, CA, USA) and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM each free dNTP, hot start enzyme iQ Taq DNA polymerase (25 U/ml), 3 mM MgCl2, SYBR Green 1 and 10 nM fluorescein as passive reference (Katavetin et al., 2006). Triplicate samples of 1 µl of cDNA were ran using a template of 3.2 pmol of primers for p65-NF-κB (forward, 5'CATGCGTTTCGTTACAAGTGCGA-3' and reverse 5'TGGGTGCGTCTTAGTGTATCTGT-3'), AP-1 (forward, 5'AGCA GATGCTTGAGTTGAGAGCA3' and reverse, 5'TTCCATGGGTCCCTGCTTTGAGAT-3'), JNK (forward 5'AAGCAGCAAGGCTACTCCTTTCA-3' and reverse 5'ATCGAGACTGCTGTCTGTGTCTGA-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'AGCAAGGA TACTGAGAGCAAGA-3' and reverse 5'TCTGGGATGGAATTGTGAGGAGA-3') in a final volume of 25 µl. Sequences of all primers were confirmed by the National Research Council of Canada, Saskatoon.

**Determination of TNF-α, IL-1 and IL1-β**

TNF-α, IL-1 and IL1-β were quantified by ELISA (Immuno-Biological Laboratories Co Ltd, Takasaki-shi, Gunma, Japan) according to the manufacturer's instructions and read at 450 nm in a plate reader (SpectraMax 340PC, Molecular Device, CA, USA).

**Determination of Endothelin-1**

ET-1 was quantified by EIA (Cayman Chemical, Ann Arbor, MI, USA) as we previously reported (Ndisang and Jadhav, 2010). This immunometric assay is based on a double-antibody 'sandwich' technique that detects ET-1 within the range of 0-250 pg/ml. Briefly, supernatants from
homogenized left-ventricular tissues were purified by cold spike extraction, concentrated and the absorbance read at 405 nm in a plate reader (SpectraMax 340PC, Molecular Device, CA, USA).

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