Acute Humanin Therapy Attenuates Myocardial Ischemia and Reperfusion Injury in Mice

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Objective—Humanin (HN), an endogenous antiapoptotic peptide, has previously been shown to protect against Alzheimer’s disease and a variety of cellular insults. We evaluated the effects of a potent analog of HN (HNG) in an in vivo murine model of myocardial ischemia and reperfusion.

Methods and Results—Male C57BL6/J mice (8 to 10 week old) were subjected to 45 minutes of left coronary artery occlusion followed by a 24-hour reperfusion. HNG or vehicle was administered IP 1 hour prior or at the time of reperfusion. The extent of myocardial infarction per area-at-risk was evaluated at 24 hours using Evans Blue dye and 2-3,5-triphenyl tetrazolium chloride staining. Left ventricular function was evaluated at 1 week after ischemia using high-resolution, 2D echocardiography (VisualSonics Vevo 770). Myocardial cell signaling pathways and apoptotic markers were assessed at various time points (0 to 24 hours) following reperfusion. Cardiomyocyte survival and apoptosis in response to HNG were assessed in vitro. HNG reduced infarct size relative to the area-at-risk in a dose-dependent fashion, with a maximal reduction at the dose of 2 mg/kg. HNG therapy enhanced left ventricular ejection fraction and preserved postischemic left ventricular dimensions (end-diastolic and end-systolic), resulting in improved cardiac function. Treatment with HNG significantly increased phosphorylation of AMPK and phosphorylation of endothelial nitric oxide synthase in the heart and attenuated Bcl-2-associated X protein and B-cell lymphoma-2 levels following myocardial ischemia and reperfusion. HNG improved cardiomyocyte survival and decreased apoptosis in response to daunorubicin in vitro.

Conclusion—These data show that HNG provides cardioprotection in a mouse model of myocardial ischemia and reperfusion potentially through activation of AMPK-endothelial nitric oxide synthase-mediated signaling and regulation of apoptotic factors. HNG may represent a novel agent for the treatment of acute myocardial infarction. (Arterioscler Thromb Vasc Biol. 2010;30:1940-1948.)

Key Words: humanin ▪ myocardium ▪ ischemia-reperfusion

Heart disease is a major cause for morbidity and mortality and is the leading cause of death in the United States. In 2009, heart disease was projected to cost more than $304.6 billion, including health care services, medications, and lost productivity.1 Among heart diseases, coronary heart disease is the principal type accounting for 1 of every 5 deaths in the United States.2 Therefore, identifying novel therapeutic agents that improve survival in coronary vascular disease is of paramount significance considering the tremendous health care burden associated with this disease.

We have recently studied the role of a novel peptide called humanin (HN) in improving insulin action.3 HN is a 24-amino acid polypeptide that was initially characterized for its protective effects against various Alzheimer’s disease-associated insults.4,5 HN has been shown to enhance cell survival in prion-induced apoptosis,6 as well as in serum-deprived conditions in lymphocytes.7 The cytoprotective effects are broad, extending to both neuronal and nonneuronal cells,8–10 and the effects appear to be mediated both via an extracellular receptor, such as CNTFR-α/gp130/WSX-1 complex,11 as well as intracellular mechanisms.12 HN binds proapoptotic Bcl-2-associated X protein (Bax), a member of the B-cell lymphoma-2 (Bcl-2) family, inhibits its mitochondrial localization, and attenuates Bax-mediated apoptosis activation.12 The Bcl-2 family consists of both proapoptotic factors, such as Bid and Bim, 2 pro-apoptotic members of the BCL-2 family of proteins showing conservation only in the BH3 domain, and antiapoptotic proteins, including Bcl-2, The
ability of the Bcl-2 family to regulate cellular survival has been shown to be determined in part by a balance between its proapoptotic and antiapoptotic members. HN has also been shown to bind and inactivate other proapoptotic members of the Bcl-2 family, including Bid and Bim.\textsuperscript{13,14} In addition to direct protein-protein interactions, HN has been shown to activate signal transducer and activator of transcription-3 (STAT-3),\textsuperscript{15} c-Jun N-terminal kinase (Jnk),\textsuperscript{16} and tyrosine kinases,\textsuperscript{15} all of which have been implicated in its actions. Since its initial discovery, several cDNAs sharing similar sequence homology to HN have been identified in plants, nematodes, and rodents, demonstrating that HN is evolutionarily conserved.\textsuperscript{12} HN is transcribed from an open reading frame within the mitochondrial 16S ribosomal RNA.\textsuperscript{17} Endogenous HN is both an intracellular and secreted protein and has been detected in skeletal muscle, brain, liver, testis, and colon at specific stages of development.\textsuperscript{3,17} In addition, HN is present in cerebral spinal fluid, seminal fluid, and plasma.\textsuperscript{3}

Interestingly, single amino acid substitutions of HN can lead to significant alterations in its potency and biological functions. HNG (HN in which the serine at position 14 is replaced by glycine), is a highly potent analogue of HN. HNG has been shown to reverse the learning and memory impairment induced by scopolamine in mice\textsuperscript{18} and has rescue activity against memory impairment caused by Alzheimer’s disease-related insults in vivo.\textsuperscript{19} HNG has also been shown to protect against neuronal cell death in an animal model of stroke.\textsuperscript{20}

Considering the role HN plays in promoting cell survival, we hypothesized that HNG may protect the heart against myocardial ischemia and reperfusion (MI-R) injury. We tested this hypothesis using a mouse model of MI-R and analyzed potential cytoprotective pathways and downstream signaling mechanisms.

**Materials and Methods**

**Animal Preparation for In Vivo Studies**

Male, 8- to 10-week-old C57BL/6J mice from The Jackson Laboratory were used in the present study. HNG peptide\textsuperscript{18–20} was custom prepared by the Laboratory Animals. HNG was reconstituted in sterile saline and administered by intraperitoneal (IP) injection. Mice were injected with either vehicle (sham) or HNG IP and were killed at 15 minutes, 30 minutes, 4 hours, or 24 hours later. The animals were killed by decapitation without sedation. This method was specifically chosen to avoid the confounding effects of anesthesia on signaling mediators. Whole hearts were rapidly excised, rinsed thoroughly with saline, snap frozen in liquid nitrogen, and stored at \textasciitilde 80°C. For signaling studies after MI-R, mice were treated with either HNG or vehicle at the time of reperfusion and were killed either 30 minutes, 4 hours, or 24 hours after reperfusion. The ischemic portion of the heart comprising primarily the LV was carefully dissected away from the nonischemic area of the heart, frozen in liquid nitrogen, and stored at \textasciitilde 80°C. Immunoblots were performed as described before.\textsuperscript{18,24,25} Briefly, frozen heart tissue was pulverized in a liquid-nitrogen-cooled mortar and pestle, and protein was extracted from powdered tissue using an ice-cold 0.15 mmol/L NaCl/0.05 mmol/L Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS (RIPA buffer) and quantified using the bicinchoninic acid assay method. For Western blot analysis, 30 μg of total protein was electrophoresed on Bis-Tris 4 to 12% gradient gels (Bio-Rad), and wet transferred onto polyvinylidene fluoride membrane (PVDF) membranes. Bands were visualized by chemiluminescence (Super West Dura kit; Pierce Biotechnologies) with a 16-bit camera imaging system (Fuji LAS-3000; Fujifilm) until a saturated pixel was observed and densitometry performed using multigauge software. Antibodies to STAT-3, pSTAT-3 Tyr705, Akt, pAKT Ser473, endothelial nitric oxide synthase (eNOS), pE NOS Ser1177, AMP-activated protein kinase (AMPK), pAMPK Thr172, mitogen-activated protein kinase (MAPK), pMAPK Thr202/Tyr204, Bcl-2, and GAPDH were all obtained from Cell Signaling, and anti-Bax antibody was obtained from Abcam. Protein levels of endogenous mouse HN were measured using a rabbit polyclonal antibody to rat (1:500, GeneTex), which is the rat homolog to HN.

**MI-R Protocol**

Surgical ligation of the left coronary artery (LCA) was performed as described previously.\textsuperscript{21–23} Briefly, mice were anesthetized with ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg), orally intubated, and connected to a rodent ventilator. A median sternotomy was performed. The LCA was visualized and ligated using a 7 to 0 silk suture mated to a BV-1 needle along with a short segment of PE-10 tubing. Mice were subjected to 45 minutes of LCA ischemia followed by varying periods of reperfusion. For determination of infarct size (Inf), a 24-hour reperfusion was performed. Left ventricular (LV) area-at-risk (AAR) and Inf were determined at 24 hours by methods previously described.\textsuperscript{21–23} For studies investigating signaling mediators, the time of reperfusion was based on the study groups with reperfusion periods ranging from 30 minutes to 24 hours (ie, 30 minutes, 4 hours, 12 hours, and 24 hours after reperfusion). A 7-day reperfusion was performed in a group of animals that had cardiac function assessed by echocardiogram.

**Myocardial AAR and Inf Determination**

LV AAR and Inf determination was performed using Evans Blue dye and 2,3,5-triphenyltetrazolium chloride (Sigma Chemical) staining method. All of the procedures for the AAR and Inf determination have been previously described.\textsuperscript{21–23} AAR and Inf determinations were performed in a blinded fashion.

**Echocardiographic Assessment of LV Structure and Function**

Baseline echocardiography images were obtained 1 week before LCA ischemia to avoid any confounding effects of anesthesia as previously described using a VisualSonics VEVO 770 high-resolution ultrasound system.\textsuperscript{21} One week after MI-R, a repeat echocardiography was performed, and images were analyzed in a blinded manner.

**Signaling Studies and Protein Analysis**

For acute signaling studies, mice were injected with either vehicle (sham) or HNG IP and were killed at 15 minutes, 30 minutes, 4 hours, or 24 hours later. The animals were killed by decapitation without sedation. This method was specifically chosen to avoid the confounding effects of anesthesia on signaling mediators. Whole hearts were rapidly excised, rinsed thoroughly with saline, snap frozen in liquid nitrogen, and stored at \textasciitilde 80°C. For signaling studies after MI-R, mice were treated with either HNG or vehicle at the time of reperfusion and were killed either 30 minutes, 4 hours, or 24 hours after reperfusion. The ischemic portion of the heart comprising primarily the LV was carefully dissected away from the nonischemic area of the heart, frozen in liquid nitrogen, and stored at \textasciitilde 80°C. Immunoblotting was performed as described before.\textsuperscript{18,24,25} Briefly, frozen heart tissue was pulverized in a liquid-nitrogen-cooled mortar and pestle, and protein was extracted from powdered tissue using an ice-cold 0.15 mmol/L NaCl/0.05 mmol/L Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS (RIPA buffer) and quantified using the bicinchoninic acid assay method. For Western blot analysis, 30 μg of total protein was electrophoresed on Bis-Tris 4 to 12% gradient gels (Bio-Rad), and wet transferred onto polyvinylidene fluoride membrane (PVDF) membranes. Bands were visualized by chemiluminescence (Super West Dura kit; Pierce Biotechnologies) with a 16-bit camera imaging system (Fuji LAS-3000; Fujifilm) until a saturated pixel was observed and densitometry performed using multigauge software. Antibodies to STAT-3, pSTAT-3 Tyr705, Akt, pAKT Ser473, endothelial nitric oxide synthase (eNOS), pE NOS Ser1177, AMP-activated protein kinase (AMPK), pAMPK Thr172, mitogen-activated protein kinase (MAPK), pMAPK Thr202/Tyr204, Bcl-2, and GAPDH were all obtained from Cell Signaling, and anti-Bax antibody was obtained from Abcam. Protein levels of endogenous mouse HN were measured using a rabbit polyclonal antibody to rat (1:500, GeneTex), which is the rat homolog to HN.

**Cell-Specific Expression of Endogenous HN in Heart**

Cardiomyocytes were isolated from 10-week-old C57BL/6J mice using a modification of Alliance for Cellular Signaling Procedure Protocol PP0000125. Briefly, hearts were digested with modified Joklik’s media (11g/L Joklik’s medium/34.5 mmol/L NaHCO3/0.49 mmol/L MgSO4/10 mmol/L CaCl2, 2.3-butane dione monoxide, pH 7.2) containing collagenase (Worthington Type II; Worthington Biochemical Corp), bovine serum albumin (1 mg/mL), and 12.5 µmol/L CaCl2 for 10 to 15 minutes. Isolated cardiomyocytes were pelleted and then resuspended in myocyte culture medium (medium 199 with Earle’s salts/L-glutamine/2.2 g/L NaHCO3 con
Western blot analysis performed as described earlier for detection protocol and when confluent, were lysed, protein extracted, and were kindly provided by D. Casper at Albert Einstein College of Medicine, observed using a Zeiss Axioscop Light microscope with 10 magnification for analysis (using Axiocam and Zeiss Axiovision software).

**Results**

**HN Is Expressed in the Heart**

To confirm the presence of HN, we analyzed the expression level of HN protein in the heart. HN is highly expressed in the heart as demonstrated by Western blot analysis (Figure 1A). Analysis of endogenous HN protein in different cell types shows that HN is predominantly present in the cardiomyocytes (Figure 1B) with minimal expression in smooth muscle and endothelial cells.

**Endogenous HN Increases After MI-R**

To assess changes in endogenous HN in the heart after MI-R, we analyzed the expression level of HN protein in the LV at different time points after MI-R. Endogenous HN levels increase after MI-R and persist as long as 24 hours (Figure 1C).

**HNG Decreases Infarct Size in a Dose-Dependent Fashion**

Eight-week-old male C57BL6/J mice were subjected to 45 minutes of LCA ligation followed by a 24-hour reperfusion, at which time the extent of myocardial infarction was evaluated. HNG or vehicle was administered (IP) 1 hour before ischemia. The total AAR was ~60% of the LV, similar in both vehicle and HNG-treated animals (P=NS) (Figure 2B). The LV Inf in mice receiving vehicle was 58±4% of AAR. There was a decrease in Inf relative to the AAR in the heart after MI-R, we analyzed the expression level of HN protein in the LV at different time points after MI-R. Endogenous HN levels increase after MI-R and persist as long as 24 hours (Figure 1C).
to evaluate for therapeutic potential, we administered HNG at the time of reperfusion and studied the effects on Inf. Intracardiac administration of HNG (2 mg/kg) at the time of reperfusion reduced Inf by 47% ($P < 0.01$) (Figure 2C). The Inf compared with AAR was $48 \pm 4\%$ in controls compared with $26 \pm 3\%$ in HNG-treated animals ($P < 0.01$). When Inf is expressed as percent of LV, it was $32 \pm 3\%$ among controls compared with $17 \pm 2\%$ in HNG ($P < 0.01$), demonstrating a significant reduction in Inf following treatment with HNG. Representative photomicrographs from vehicle and HNG-treated animals are shown in Figure 2B.

**HNG Results in Improved Cardiac Function After MI-R**

The effects of HNG on LV structure and function following 45 minutes of ischemia and 7 days of reperfusion were assessed by in vivo transthoracic echocardiography. These data are depicted in Figure 3A through 3E. After MI-R, there was a significant increase in LV end-systolic diameter (from baseline) in the vehicle-treated groups but not in the HNG-treated group (2.1 ± 0.1 to 2.4 ± 0.4 mm in I/R vehicle, $P < 0.001$; 2.3 ± 0.1 to 2.7 ± 0.2 mm in I/R + HNG) (Figure 3A). Similarly, LV end-diastolic diame-
ter was increased in vehicle (compared with baseline) but was unchanged in HNG (3.6 ± 0.1 to 4.6 ± 0.3 mm in I/R + vehicle, \(P < 0.001\); 3.7 ± 0.1 to 3.7 ± 0.1 mm in I/R + HNG, \(P = \text{NS}\)) (Figure 3B). Ejection fraction was significantly decreased in both vehicle and HNG-treated groups (78.0 ± 2.2 to 25.2 ± 3.2% in vehicle and from 75.5 ± 1.6 to 56 ± 3.8% in HNG, \(P < 0.0001\)) (Figure 3C). Stroke volume decreased from 33.3 ± 2.0 to 18.1 ± 1.9 mL/min in I/R + vehicle group and from 34.7 ± 2.5 to 22.8 ± 1.4 mL/min in I/R + HNG (\(P < 0.001\)) (Figure 3D). Cardiac outputs were significantly decreased after MI-R in both groups with a decrease from 19.2 ± 1.4 to 10.1 ± 1.0 mL/min in I/R + vehicle group and a decrease from 17.2 ± 1.1 to 12.4 ± 0.8 mL/min in I/R + HNG (\(P < 0.001\)) (Figure 3E).

When 1 week after MI-R cardiac function was compared between the vehicle and HNG treated groups, LV end-systolic diameter (4.1 ± 0.4 versus 2.7 ± 0.2 mm in I/R + vehicle versus I/R + HNG, respectively; \(P < 0.001\)) (Figure 3A) and LV end-diastolic diameter were significantly lower in the HNG-treated group compared with the vehicle-treated animals (4.6 ± 0.3 versus 3.7 ± 0.1 mm, I/R + vehicle versus I/R + HNG, respectively; \(P < 0.001\)) (Figure 3B). The animals that received HNG treatment demonstrated a significantly higher ejection fraction as compared with the vehicle group (57 ± 3% in the HNG-treated group as compared with 24 ± 2% in the vehicle group, \(P < 0.001\)) (Figure 3C). Stroke volume was increased in the HNG-treated group (22.7 ± 1.4 versus 18.0 ± 1.9 mL/min in I/R + HNG versus I/R + vehicle, respectively; \(P = 0.05\)) (Figure 3D) with a tendency toward increased cardiac output (12.4 ± 0.8 versus 10 ± 1.0 mL/min in I/R + HNG versus I/R + vehicle, respectively; \(P = 0.09\)) (Figure 3E). Representative baseline and post-MI-R M-Mode images from vehicle and HNG-treated animals are shown in Figure 3F.

**HNG Activates AMPK-eNOS Signaling in the Heart**

To understand mechanisms through which treatment with HNG improved cardiac survival, we studied changes in signaling pathways relevant to energy metabolism and cell survival in the heart following a single IP dose of HNG (2 mg/kg) by Western blot analysis. There was a significant activation of AMPK in the heart within 15 minutes of administration of HNG as evidenced by the increased phosphorylation of AMPK (pAMPK\(^{Thr172}\)) and increased pAMPK\(^{Thr172}/\text{AMPK}\) ratio. This activation persisted up to 24 hours (\(P < 0.001\)) (Figure 4A). Phosphorylation of eNOS (p-
eNOSSer1177) was significantly higher at 30 minutes and 24 hours (P<0.01) (Figure 4B) in response to HNG. Activation of STAT-3 (pSTAT-3Tyr705 normalized to total STAT-3) tended to be elevated at 30 minutes compared with sham but returned to normal at 4 hours (P=0.07) (Figure 4C). The ratio of pAKTSer473 to total Akt protein tended to decrease but was not statistically significant at 15 minutes after HNG and returned to normal by 4 hours (Figure 4D). There were no differences in activation of pAS160Thr642 (Figure 4E), mTOR, or MAPK pathways following HNG treatment.

To test the relevance of these pathways in cardioprotective effects of HNG following ischemia and reperfusion, signaling pathways were assessed after MI-R at different time points. Following MI-R, there was a significant increase in AMPK activation in both vehicle and HNG-treated groups. However, the vehicle and HNG-treated after MI-R groups were not different from each other, although a tendency for higher levels of AMPK activation with HNG was observed at 12 hours (P=0.07) (supplemental Figure I, available online at http://atvb.ahajournals.org). We also observed increased levels of pSTAT-3 and eNOS (supplemental Figure II) following MI-R in both groups. There were no significant changes in eNOS, Akt, STAT-3, MAPK, or vascular endothelial growth factor between the vehicle and HNG-treated groups.

**HNG Attenuates Expression of Bax After Ischemia and Reperfusion**

As determined by Western blot analysis, there was a significant increase in Bax with MI-R in saline-treated animals at 30 minutes and 4 hours after reperfusion (Figure 5A). In parallel with an increase in Bax, Bcl-2 expression was also increased up to 24 hours (Figure 5B). In the HNG-treated group, Bax and Bcl-2 expression were significantly attenuated compared with vehicle-treated groups (P<0.05) (Figure 5A and 5B). Interestingly, there were no significant differences in the ratio of Bcl-2 to Bax in both the groups (Figure 5C). Furthermore, no changes in cleaved caspase-3 levels were observed between the groups (data not shown).

**HNG Improves Cardiomyocyte Survival and Decreases Apoptosis In Vitro**

In vitro, a cell viability assay showed that HNG conferred a 15% survival protection against daunorubicin-induced apoptosis in cardiomyocytes. Daunorubicin alone, at 1 μmol/L concentration, killed 52% (48% survival) of cells. In presence of HNG, 37% cells were killed (63% survival) (Figure 6A). CaspASE FITC-VAD-FMK in situ apoptosis assay substantiated the effect of HNG on apoptosis in H9c2 cells. In the presence of HNG, there was a significant decrease in FITC-stained apoptotic cells as compared with cells exposed to daunorubicin alone (Figure 6B). This was also evident in phase-contrast images where cells looked normal and viable in the presence of HNG+daunorubicin, whereas daunorubicin-only-treated cells were visibly stressed and apoptotic.

**Discussion**

In a series of experiments, we demonstrate that HNG offers cardioprotection in a mouse model of MI-R injury, including a decrease in Inf by 50% and significant improvement in LV function. We demonstrate that this protective effect of HNG is dose dependent, with a maximum effect at 2 mg/kg, is independent of the route of administration (IP or intracardiac), and is elicitable independent of the timing of delivery (1-hour pretreatment or at the time of reperfusion).

HN protein is endogenously expressed in the heart with the greatest levels found in cardiomyocytes. Furthermore, we show that the expression of the protein is regulated, with levels increasing in the LV in response to a severe stress, such as MI-R. This increase is consistent with its role in conferring robust cytoprotection in animal models of Alzheimer’s disease, serum deprivation, and stroke.5,6,11,20,27 In studies de-
scribed here, we demonstrate for the first time a potential role for HNG in offering protection against MI-R injury in vivo in a clinically relevant model system. Considering that HNG is a potent analog of HN (1000 times more potent than native HN), we propose that augmenting the physiological response (an increase in endogenous HN in response to MI-R) by administration of exogenous HNG leads to enhanced cardioprotection.

It is especially interesting that the beneficial effects are elicitable when given 1 hour before MI-R or during reperfusion. This suggests that the treatment with HNG initiates protective signaling cascades that aid survival when exposed to a severe insult (ie, cardiac ischemia). Moreover, the demonstration that HNG affords cardioprotection even when given during reperfusion highlights clinical therapeutic potential in acute coronary ischemia and myocardial infarction. Treatment with HNG resulted in preservation of LV dimensions after MI-R and resulted in significant improvement in cardiac function as evidenced by better ejection fraction, stroke volume, and cardiac output. The improvements in cardiac function seen in HNG-treated animals could be directly related to the smaller size of infarct.

HN has been shown to act through CNTFR-α/gp130/WSX-1 receptor complex in offering protection against apoptosis; many downstream signaling pathways, such as MAPK, phosphoinositide-3 kinase, Janus kinase-STAT could be activated in response to signaling through this complex. In fact, HN has been shown to induce its cytoprotective effects through activation of Jak-Stat, Akt, JNK, and p38 MAPK signaling pathways. All components of this receptor and signaling complex are present in the heart and skeletal muscle, and signaling through gp 130 receptors has been shown to activate MAPK and Jak-STAT in the heart and induce AMPK activation in skeletal muscle.

In our study, significant activation of AMPK was noticed in the heart within 15 minutes of treatment with HNG and persisted up to 24 hours. This could be potentially important because activation of AMPK can work through multiple mechanisms that include: (1) offering a metabolic advantage through switch to an energy conservation mode by stimulating energy-yielding processes, such as promoting glucose transport and accelerating glycolysis while inhibiting anaerobic processes, such as triglyceride and protein synthesis; (2) activating additional cardioprotective pathways, such as eNOS, which promotes vasodilation, decreases oxidative stress, and increases peroxisome proliferator-activated receptor-γ coactivator α, an important regulator of mitochondrial biogenesis and function; and (3) decreasing apoptosis, as has been shown in cardiomyocytes, endothelial cells, thymocytes, astrocytes via improved glucose utilization, and inhibition of cytochrome C release from the mitochondria. Indeed, we demonstrate that HNG treatment is associated with p-eNOS at serine residue 1177 (eNOSSer1177). Phosphorylation of this residue is a critical requirement for eNOS activation and has been reported to be mediated by AMPK during MI, and AMPK-eNOS signaling has been shown to mediate the cardioprotective effects of metformin. The increased generation of nitric oxide by eNOS could offer cardioprotection through its effects on vasodilation, inhibition of oxidative stress, platelet aggregation, leukocyte chemotaxis, and apoptosis. Concerns have been raised regarding the increased fatty acid oxidation with activation of this pathway, which could exacerbate acidosis during the reperfusion phase. Nevertheless, the role of AMPK in this process still seems likely beneficial as exemplified in AMPK-DN mice subjected to MI-R, which demonstrate a larger Inf, more apoptosis, and worse cardiac function than wild type.
Activation of STAT-3 has been shown to be a crucial step in the neuroprotective effects as well as the insulin-sensitizing effects of HN. A protective role of STAT-3 activation following ischemia and reperfusion injury has been shown earlier. Indeed, activation of STAT-3 promotes cardiomyocyte survival and hypertrophy, as well as cardiac angiogenesis in response to various pathophysiological stimuli. Mice with cardiomyocyte-restricted deletion of STAT-3 (cardiac-specific STAT-3-knockout) show enhanced susceptibility to injury caused by MI, further highlighting the role for this pathway in cardiac survival after MI-R. Activation of Akt has been shown to be important in the protective role of HN in stroke. However, we demonstrate that there is no significant change in pAkt or pSTAT-3 in the heart, suggesting that the cardioprotective effects of HNG in this model are not mediated through these pathways.

Cardiac myocyte death during MI-R occurs through necrosis as well as apoptosis. The activation of AMPK, as well as the ability of HN to inactivate proapoptotic peptides, such as Bax, Bid, and Bim, suggested that modulation of apoptosis pathway could be a mechanism through which HNG offers cardioprotection. Indeed, Bax was significantly downregulated in the myocardium of HNG-treated animals. The sustained rise of Bax seen in controls following MI-R was significantly attenuated in animals treated with HNG, suggesting Bax downregulation as another potential mechanism through which HNG offers better survival. This is similar to the mechanism implicated in cardioprotection via ischemic preconditioning and intermittent hypoxia, where downregulation of Bax decreases myocardial apoptosis and therefore Inf. A role for Bax in regulating cardiac apoptosis following MI-IR is illustrated in Bax−/− mice that demonstrate significantly smaller Inf compared with wild type, whereas Bax+/− demonstrate an intermediate Inf following MI-R. Along with upregulation of Bax, there is also upregulation of the antiapoptotic Bcl-2 protein in the vehicle-treated animals that underwent MI-R. In the HNG-treated animals, the rise in Bcl-2 levels is also attenuated, resulting in a similar Bax/Bcl-2 ratio as controls. This is not consistent with studies that showed favorable change in Bax/Bcl-2 ratio as one of the mechanisms that mediates cardiac survival. In addition, we observed no differences in cleaved caspase-3 expression between vehicle and HNG-treated groups. A role of HNG in attenuating apoptosis is also demonstrated in vitro, where cardiomyocytes exposed to daunorubicin, an anthracycline chemotherapeutic drug known to induce apoptosis, show significantly decreased apoptosis and improved survival in the presence of HNG.

The lack of a significant difference in AMPK, eNOS between controls, and HNG-treated animals after MI-R is intriguing especially considering the significant activation of AMPK and eNOS in the heart in acute signaling experiments. It is possible that the activation of these signaling pathways in response to a severe stressor, such as MI-R, overwhelms the difference attributable to HNG. It is also plausible that we may have missed a response because of the time points we chose or potential differences in these and other members of the apoptosis pathway may become apparent if this data could be interpreted in the context of the size of the infarct. It also raises the point that in addition to the proposed intermediate pathways, other mechanisms may be involved in the cardioprotection offered by HN. Future studies in Bax−/− and AMPK-DN mice should shed light on the relative role of each pathway in offering cardioprotection in response to HNG.

In summary, these studies demonstrate a novel role for HNG in offering cardioprotection in a mouse model of MI-R. Our data suggest that this protection may be mediated through activation of AMPK-eNOS signaling as well as alteration in proapoptotic factors. The significant decrease in Inf accompanied by improvement in cardiac function following a single treatment with HNG demonstrates clinical utility in the treatment for acute MI. Considering that diabetes mellitus increases the risk for cardiovascular disease and exacerbates the severity of acute myocardial infarction, and our recent work showing the salubrious role of HN and analogs on insulin sensitivity and diabetes, future studies may reveal a role for HNG in the treatment of ischemic heart disease in the setting of diabetes.

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Disclosures
R.H.M. and D.J.L. are both co-inventors on a US patent (Patent Cooperation Treaty 2008-006720) for treatment of type 2 diabetes mellitus, metabolic syndrome, myocardial injury, and neurodegeneration with humanin and analogs thereof.

References


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Supplemental Figures

Figure I. Effects of HNG on cardiac AMPK phosphorylation of threonine 172 (i.e., pAMPK$_{\text{Thr}172}$) following *in vivo* MI-R in mice.

Figure II. Effects of HNG on phosphorylation levels of serine 1177 on eNOS (i.e., phospho-eNOS$_{\text{Ser}1177}$) following *in vivo* MI-R in mice.
**Supplemental Figure I**

![Graph showing pAMPKThr172/Total AMPK ratio](image)

**Supplemental Figure II**

![Graph showing p-eNOSSer1177/Total eNOS ratio](image)