Cardioprotective effects of growth hormone-releasing hormone agonist after myocardial infarction

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Whether the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis exerts cardioprotective effects remains controversial; and the underlying mechanism(s) for such actions are unclear. Here we tested the hypothesis that growth hormone-releasing hormone (GHRH) directly activates cellular reparative mechanisms within the injured heart, in a GH/IGF-1 independent fashion. After experimental myocardial infarction (MI), rats were randomly assigned to receive, during a 4-week period, either placebo (n = 14), rat recombinant GH (n = 8) or JI-38 (n = 8; 50 µg/kg per day), a potent GHRH agonist. JI-38 did not elevate serum levels of GH or IGF-1, but it markedly attenuated the degree of cardiac functional decline and remodeling after injury. In contrast, GH administration markedly elevated body weight, heart weight, and circulating GH and IGF-1, but it did not offset the decline in cardiac structure and function. Whereas both JI-38 and GH augmented levels of cardiac precursor cell proliferation, only JI-38 increased antiapoptotic gene expression. The receptor for GHRH was detectable on myocytes, supporting direct activation of cardiac signal transduction. Collectively, these findings demonstrate that within the heart, GHRH agonists can activate cardiac repair after MI, suggesting the existence of a potential signaling pathway based on GHRH in the heart. The phenotypic profile of the response to a potent GHRH agonist has therapeutic implications.

GH and IGF-1 Levels. To test the impact of rrGH and GHRH-A on the GH-IGF-1 axis, we measured circulating levels of these hormones (Fig. S2 A and B). Whereas treatment with GHRH-A did not increase serum levels of either GH or IGF-1 relative to placebo, treatment with rrGH led to marked increases in GH (679 ± 196 vs. 64 ± 23 ng/mL; P < 0.01) and IGF-1 (1,052 ± 91 vs. 553 ± 46 ng/mL; P < 0.01) compared with placebo.

Echocardiographic Measurements. Next, we measured the impact of GHRH-A and rrGH on cardiac structure and function after MI. Baseline echocardiography documented similar parameters for both; Fig. S1 A and Table S1). As expected, MI led to a time-dependent increase in LV chamber dimensions and a reduction in ejection fraction (EF) and fractional shortening (FS). Treatment with GHRH-A, but not with rrGH, attenuated the MI-induced increase in LV end-systolic diameter. In addition, the reduction in EF due to MI was ameliorated by GHRH-A (47 ± 4% vs. 38 ± 3%; P < 0.05)

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but not by rGH [44 ± 2%; \( P = \text{nonsignificant (NS)} \)], both compared with placebo. Similarly, a reduction in FS from 55 ± 1% to 18.5 ± 0.9% (\( P < 0.05 \)) due to MI was improved in the GHRH-A (28.7 ± 3.3%; \( P < 0.05 \)) but not in the rGH group (20.3 ± 1.3%; \( P = \text{NS} \)), both compared with placebo.

**Hemodynamic Measurements.** To directly assess the impact of these interventions on cardiac contractile performance and to separate the effects of GHRH-A on cardiac contractility and cardiovascular loading conditions, we performed in vivo hemodynamic analysis (Fig. 2 and Table 1). Treatment with GHRH-A but not rGH caused an increase in both stroke volume and cardiac output relative to placebo. This increase in cardiac performance was attributed, at least partially, to a reduction in ventricular afterload, measured as arterial elastance. Interestingly, arterial elastance was actually increased with rGH. LV end-systolic and end-diastolic pressures were similar in all groups. Consistent with the echocardiographic data, EF was higher in the GHRH-A than in the placebo or rGH groups. Similarly, stroke work was increased in the GHRH-A group vs. the placebo or rGH groups. With regard to myocardial contractility, the peak rate of pressure rise (dP/dt\(_{\text{max}}\)) was increased in the GHRH-A group in comparison with the placebo and rGH groups, whereas there were no significant differences in the peak rate of pressure decline (dP/dt\(_{\text{min}}\)) and the relaxation time constant; however, treatment with GHRH-A tended to increase preload-recruitable stroke work and the relationship between dP/dt\(_{\text{max}}\) and end-diastolic volume. Conversely, the ratio between dP/dt\(_{\text{max}}\) and end-systolic elastance tended to be lower in the GHRH-A group.

**Histopathology.** MI size (Fig. 3A) in rGH and placebo groups was similar (45 ± 2% vs. 41 ± 1%, respectively), whereas GHRH-A rats had reduced MI size (36 ± 3%; \( P < 0.05 \) vs. placebo and rGH). The reduced infarct burden was also reflected in the percentage of ventricular fibrosis (Fig. 3B), which was strikingly reduced with GHRH-A (20 ± 1%) in comparison with placebo (29 ± 1%) and rGH (27 ± 1%; \( P < 0.01 \) for both), whereas capillary density (Fig. S3A) was higher in rGH (0.02 ± 0.002/mm\(^2\)) than in the placebo or GHRH-A groups (0.01 ± 0.001/mm\(^2\) and 0.006 ± 0.001/mm\(^2\), respectively; \( P < 0.001 \) for both). The width of myocytes was not different among groups (Fig. S3B).

**GHRH Receptor.** The presence or absence of GHRHR was detected in frozen sections of pituitary, heart, and skeletal muscle under fluorescent and confocal microscopy (Fig. 4A and Table S2), and the intensity of the fluorescence of the GHRHR was measured in paraffin tissues of treated and nontreated rats (Fig. S4). The expression of GHRHR was confirmed by Western blotting (Fig. 4B and C), and the GHRHR was also detected within cardiomyocytes (Fig. 4D). In addition, using real-time quantitative PCR, we demonstrated the presence of mRNA for GHRHR in rat heart (Fig. S5A and B and Tables S3 and S4), and the radioligand binding studies revealed that the ischemic rat heart samples showed specific high-affinity binding sites for GHRH antagonist, JV-1-42 ligand, characterized by a K\(_d\) of 0.86 nM and a B\(_{\text{max}}\) of 51.28 fmol/mg protein.

**Impact on Cellular Division and Proliferation.** Immunostaining for Ki67\(^+\) myocytes and nonmyocytes revealed no differences between the border and infarct zones; however, in the remote zone, the expression of Ki67\(^+\) cells was higher in the rGH group (\( P < 0.01 \) for both) (Fig. 5A and B). Next we measured the proliferation of endogenous c-kit\(^+\) cardiac precursor cells. Importantly, the expression of c-kit\(^+\) cells (mast cells excluded) per cubic millimeter was higher (\( P = 0.02 \)) in both treated groups than in placebo (Fig. 6).

TUNEL staining (Fig. 5C) did not show differences between groups. On the other hand, real-time quantitative PCR revealed that the expression of an antiapoptotic gene (Bcl2) was up-regulated in GHRH-A (\( P = 0.07 \)), whereas the proapoptotic gene (Bax) trended to be down-regulated in the same group (\( P = 0.207 \)). Accordingly, the ratio between Bax and Bcl2 expression was significantly reduced in the GHRH-A group in comparison with placebo- or rGH-treated rats (\( P = 0.05 \)).

**Discussion**

The main finding of the present study is that GHRH-A has a cardioprotective role in vivo after acute MI. Animals receiving GHRH-A had improved cardiac structure and function and reduced infarct size. In addition, cardiac fibrosis, which is one of the main biologic determinants of poor prognosis in heart failure and strongly associated with severe arrhythmias, diastolic dysfunction, and sudden death (14), was markedly reduced in the GHRH-A group but not in the rGH group. The cardiac effects of GHRH agonist seem to be direct, not involving the GH/IGF-1 axis, because the circulating levels of these hormones were not increased by GHRH-A treatment.
graphs showing (2606) Impact of rrGH and GHRH-A on myocardial infarct burden. Bar
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dant human GH improves cardiac function and reduces LV
treatment (15). In most studies, early treatment with recombi-
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The present findings can be viewed in the context of previous
evaluations of the GH/IGF-1 axis that have yielded variable
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shown to be dependent on the timing of the treatment, the stage
of the disease at treatment initiation, and different dosing regi-
ments (8) and might be related to the heterogeneous origin of
treatment (15). In most studies, early treatment with recombi-
nant human GH improves cardiac function and reduces LV
remodeling (5, 16, 17), whereas other studies did not show
beneficial effects (15, 18). Similarly, treatment with rrGH did not
show beneficial effect in rats with large MI (19). Conversely, in
rats all studies starting late after MI showed improvement on
cardiac function (20–22). Importantly, all treatments with re-
combiant human GH in rats had a clear limitation, due perhaps
to the production of anti-GH antibodies after 2 weeks of treat-
ment (23). Therefore, the long-term effects (either beneficial or
deleterious) remain unknown in these models (8).

Our findings demonstrate that rrGH markedly increases BW,
HW, and circulating levels of GH and IGF-1 but does not
improve cardiac function or prevent remodeling; on the contrary,
rats treated with rrGH exhibited larger chambers and worse EF.
These results are in agreement with a study that showed that GH
caused adverse effects on the process of LV remodeling (18).

An alternative approach for increasing systemic levels of GH
is the administration of GHS such as ghrelin (24) or a synthetic
GHS peptides such as hexarelin (20, 25). Nagaya et al. (24)
showed that ghrelin improved LV function and attenuated car-
diac remodeling in a chronic heart failure model; however, these
results were attributed to both GH/IGF-1–dependent and GH-
dependent vasodilatory effects of ghrelin. Similarly, Tivesten
et al. (6) showed that hexarelin increased stroke volume and
reduced total peripheral resistance. In contrast, Shen et al. (19)
reported increased survival rate but no hemodynamic beneficial
effect of GH-releasing peptide in dogs subjected to transient
coronary occlusion, suggesting that these effects were mediated
by GHS receptors rather than through the GH/IGF-1 axis (i.e.,
by a GH independent pathway). To date, only one study in vitro
has shown cardioprotective and a direct effect of GHRH (10). In
that study, GHRH cardioprotection was demonstrated in iso-
lated rat hearts subjected to ischemia–reperfusion injury,
whereas in our work, cardiac function was assessed by echo-
cardiography and in vivo closed-chest LV catheterization in rats
subjected to a permanent occlusion.

Table 1. Hemodynamic parameters and indices of systolic and diastolic function derived from
left-ventricular pressure–volume relationships

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo (n = 8)</th>
<th>rrGH (n = 6)</th>
<th>GHRH-A (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>256 ± 6.6</td>
<td>247 ± 6.8</td>
<td>270 ± 17</td>
</tr>
<tr>
<td>Integrated performance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF (%)</td>
<td>29.8 ± 1.4</td>
<td>26.2 ± 1.7</td>
<td>36.9 ± 2.6*†</td>
</tr>
<tr>
<td>SW (mmHg × μL)</td>
<td>9,424 ± 1158</td>
<td>6,920 ± 790</td>
<td>12,000 ± 866*†</td>
</tr>
<tr>
<td>SV (μL)</td>
<td>131 ± 20</td>
<td>98 ± 13</td>
<td>161 ± 12‡</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>30.5 ± 5.0</td>
<td>22.5 ± 3.4</td>
<td>40.1 ± 3.1*†</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>4.1 ± 0.7</td>
<td>4.4 ± 0.6</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

Afterload

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo (n = 8)</th>
<th>rrGH (n = 6)</th>
<th>GHRH-A (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDP (mmHg)</td>
<td>85 ± 1.8</td>
<td>91 ± 2.3</td>
<td>83 ± 1.1</td>
</tr>
<tr>
<td>Ea (mmHg/μL)</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.5 ± 0.004*†</td>
</tr>
<tr>
<td>LVEDV (μL)</td>
<td>413 ± 56</td>
<td>351 ± 38</td>
<td>421 ± 26</td>
</tr>
<tr>
<td>Contractility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dtmax (mmHg/s)</td>
<td>6,198 ± 194</td>
<td>6,243 ± 313</td>
<td>6,986 ± 163‡</td>
</tr>
<tr>
<td>dP/dtmax-EDV (mmHg/s per μL)</td>
<td>22.3 ± 8.1</td>
<td>17.5 ± 4.3</td>
<td>42.5 ± 12.9</td>
</tr>
<tr>
<td>Ees (mmHg/μL)</td>
<td>0.26 ± 0.09</td>
<td>0.33 ± 0.12</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>45 ± 3.6</td>
<td>48 ± 5.0</td>
<td>53 ± 2.1</td>
</tr>
<tr>
<td>Lusitropy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dtmin (mmHg/s)</td>
<td>3,986 ± 177</td>
<td>4,028 ± 334</td>
<td>3,989 ± 106</td>
</tr>
<tr>
<td>Tau-G (ms)</td>
<td>16.8 ± 0.9</td>
<td>19.9 ± 0.4</td>
<td>16.9 ± 0.5</td>
</tr>
</tbody>
</table>

All values represent mean ± SEM. SW, stroke work; SV, stroke volume; CO, cardiac output; Ea/Ees, ratio
between arterial elastance (Ea) and end-systolic elastance (Ees); LVEDP, left ventricular end-systolic pressure;
LVEDP, left ventricular end-diastolic pressure; LVEDV, left ventricular end-diastolic volume; dP/dtmax, peak rate
of the pressure rise; dP/dtmax-EDV, relationship between dP/dtmax and end-diastolic volume; PRSW, preload
recruitable stroke work; dP/dtmin, peak rate of pressure decline; Tau-G, relaxation time constant calculated
by Glantz method.

*P < 0.05 vs. placebo.
†P < 0.01 vs. rrGH.
‡P < 0.05 vs. placebo and rrGH.

Fig. 3. Impact of rrGH and GHRH-A on myocardial infarct burden. Bar graphs showing (A) circumferential infarct size and (B) percentage of fibrosis. (C) Representative Masson's trichrome-stained histologic sections for infarct size measurement. The circumferential infarct size (MI%) was significantly attenuated in the GHRH-A group (*P = 0.011 vs. placebo and rrGH). Similarly, the percentage of fibrosis was reduced in the GHRH-A group (*P = 0.0002 vs. placebo and rrGH).
The mechanism underlying the differences between GHRH and GH effects is unclear. Postreceptor signaling cascades can be one reason for differences in activity between GHRH and GH. GHRH actions involve the stimulation of GHRHR, a G protein–coupled receptor that activates at least two transduction pathways, the adenyl cyclase/cAMP/protein kinase A pathway via the G\(\alpha\) subunit (26) and the Ras/MAPK pathway through the \(\beta\gamma\) subunits (27).

The activation of the ERK1/2 signaling pathway has been connected with several cellular activities, such as proliferation, differentiation, and survival, and ghrelin has previously been shown to activate both ERK1/2 and the serine threonine kinase Akt (28). GHRH induces activation of cAMP and a significant activation of the Akt and ERK1/2 survival pathways, as has been demonstrated by Western blotting after GHRH administration. The PI3k/Akt pathway is a well-known signaling pathway for cell protection, and recently Granata et al. (10) reported that ERK1/2 and PI3k/Akt are involved in survival effects induced by GHRH and found that GHRH increased ERK1/2 and Akt phosphorylation, cAMP, and phosphorylation on serine 133 of cAMP response element–binding protein. Recently, Lorenz et al. (29) proposed that specific phosphorylation events on ERK 1/2 integrate differing upstream signals to induce hypertrophy. Hexarelin has also previously been shown to promote neuroprotection through activation of the PI3/Akt pathway (30). Moreover, the PI3k/Akt pathway controls cell size, including cardiomyocyte size (31), and is associated with cardiomyocyte hypertrophy and apoptosis (30, 32).

Traditionally, the adult heart has been considered a postmitotic organ where the cardiac myocytes were terminally differentiated without ability to divide. However, several investigators (33–35) have suggested that at least a subpopulation of myocytes re-enters the cell cycle and divides, and that a pool of cardiac stem cells may reside in the myocardium. In the present study, the expression of Ki67+ cells was significantly higher at the remote zone but only in the rrGH group, and this was accompanied by an increase in capillary density in the same group. Previous study has docu-

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**Fig. 4.** Presence of GHRHR in the heart and cardiomyocytes. (A) Cryosections of pituitary (Top), heart (Middle), and skeletal muscle (Bottom) incubated with GHRHR (green). (Scale bars, 50 \(\mu\)m.) GHRHR specificity is demonstrated by intense immunohistochemical reactivity in pituitary (positive control) and heart; negative results are observed in skeletal muscle (negative control). (B) Western blotting detected a 47 kDa protein corresponding to GHRHR. Molecular weight markers are indicated on the left side of the panel and NC corresponds to negative control. (C) GHRHR protein abundance measured by Western blotting analysis and expressed in arbitrary units. (D) Representative confocal micrograph image showing the presence of GHRHR (green) on cardiomyocyte sarcolemmal membrane. (Scale bar, 10 \(\mu\)m.)

**Fig. 5.** Impact of rrGH and GHRH-A on cell division and apoptosis. (A) Bar graph showing the expression of cells positively stained for Ki67 in the remote zone. *\(P < 0.05\) vs. placebo and GHRH-A. (B) Representative confocal micrograph image of Ki67+ cells (cyan, white arrows), tropomyosin (red), and DAPI (blue). (Scale bar, 20 \(\mu\)m.) (C) Bar graph showing expression of TUNEL+ cells per unit area.
Brüel et al. (36) also reported that the number of c-kit+ cells is associated with improvement in cardiac performance showing the increased expression of c-kit+ cells per unit area in rrGH- and GHRH-A infarct zone in both treated groups; recruitment of c-kit+ stem cells (mast cell tryptase (red), c-kit (green), and nuclei (blue) obtained from pla-

under confocal microscopy. Panels depict representative triple staining for which showed increased c-kit+ cells expression (clusters) in the Bax and Bcl2 supported an antiapoptotic effect of GHRH-A.

lower and not statistically signi-

cant when assessed by TUNEL analysis; however, at a molecular level, changes in the expression of Bax and Bcl2 supported an antiapoptotic effect of GHRH-A. We also examined the abundance of cardiac precursor cells, which showed increased c-kit+ cells expression (clusters) in the infarct zone in both treated groups; recruitment of c-kit+ stem cells is associated with improvement in cardiac performance (37). Bruel et al. (36) also reported that the number of c-kit+

cells in a GH-treated group was 31% higher than that of the control group, but it was not statistically significant. Given the observation of similar increases in c-kit cells with GH and GHRH, yet greater reverse remodeling with GHRH, it is attractive to speculate that GHRH may stimulate cardiopoiesis to a greater extent. An alternate explanation is that the c-kit cells may traffic and/or proliferate to a greater or earlier extent. Finally, the findings of an antiapoptotic milieu might suggest improved survival of differentiation cardiac precursor cells (CPCs). Future work is required to evaluate the direct effects of GHRH on CPCs. Besides, CPCs possess the IGF-1/IGF-1 receptor system (38), which potentiates their survival and growth (39). Further studies are needed to ascertain whether GHRH-A or rrGH stimulated existing cardiac stem cells to differentiate into mature cardiac myocytes.

In summary, the present findings document that GHRH activation in the heart leads to MI size reduction, favorable hemodynamics, and recovery of functional performance to a greater degree than that due to GH following myocardial injury, and that this occurs without stimulation of BW or HW. These findings support ongoing basic and translational research into GHRH signal transduction mechanisms within the heart.

Materials and Methods

Animal Model. MI induced by coronary artery ligation was performed in female 6-month-old Fisher-344 rats, as described previously (40). Animals were randomly assigned to receive placebo, GHRH-A (JI-38, 50 μg/kg), or rrGH (0.5 mg/kg), starting 2 h after surgery. All treatment was given s.c. twice daily for 4 weeks. The Institutional Animal Care and Use Committee of University of Miami approved all protocols and experimental procedures.

Drugs. The rrGH was supplied by Dr. A. F. Parlow from the National Hormone and Pituitary Program (University of California, Los Angeles-Harbor), and GHRH-A (JI-38) was made in the laboratory of one of us (A.V.S.) (12, 13).

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8. Mill JG, et al. (2005) [The early administration of growth hormone results in deleterious effects on ventricular remodeling after acute myocardial infarction]. Arch Bras Cardiol 84:115–121.
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Rats were anesthetized by intra−

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The expression of GHRHR was measured by
fi

fi

in noninfarcted remote myocardium.

hematoxylin and eosin (H&E) and Masson’s trichrome stain to assess myocardial infarct size. The impact of MI was quantified in two ways: circumferential extent of scar and percentage area fibrosis. NIH ImageJ version 1.30v for Windows was used to quantify the circumferential extent of scar to total LV tissue. Image-processing software (Imaging Processing Toolkit 5.0; Reindeer Graphics) and Adobe Photoshop CS2 were used to assess the percentage area of fibrosis as previously described with minor modifications (3). The percentage of fibrosis was calculated by using the following formula: %fibrosis=fibrotic area /

/ (fibrotic area + healthy area).

H&E-stained sections of hearts from midventricular level were used to measure the myocyte width. At least 35–50 cardiomyocytes were counted and averaged at the level of the nuclei in noninfarcted remote myocardium.

Total RNA Isolation. Total RNA from heart tissue was extracted using TRZol (Invitrogen). The quality of RNA isolated was tested using NanoDrop1000 (Thermo Fisher Scientific). OD 260/280 ratio was in the range of 1.8–2.1 for all samples.

Myocyte Isolation. The isolation of myocytes was performed as previously described (4). Briefly; the rats were anesthetized with pentobarbital (100 mg/kg; Sigma) with heparin (4,000 U/kg; APP Pharmaceuticals). For the isolation of myocytes, the hearts were cannulated and perfused through the aorta with CaCl2-free bicarbonate buffer containing 120 mM NaCl, 5.4 KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 20 mM NaHCO3, 10 mM 2,3-butanediol monoxime, 5 mM taurine, and 5.6 mM glucose, gassed with 95% O2/5% CO2. This was followed by enzymatic digestion with collagenase type-2 (1 mg/mL; Worthington Biochemical) and protease type-XIV (0.1 mg/mL; Sigma).

Cardiomyocytes were obtained from digested hearts followed by mechanical disruption, by filtration, centrifugation, and resuspension in a Tyrode solution containing 0.125 mM CaCl2, 144 mM NaCl, 1 mM MgCl2, 10 mM Hepes, 5.6 mM glucose, 1.2 mM NaH2PO4, and 5 mM KCl, pH 7.4.

GH and IGF-1 Measurements. At the end of the study, blood was drawn 1 to 2 h after the last rGH or GHRH-A injection, and the serum was stored at −80 °C until the measurements were done. All of the samples were assayed together, and each sample was assayed in duplicate. Rat serum GH was measured using a rat GH ELISA Kit (DSL-10-72100; Diagnostic Systems Laboratories), following the manufacturer’s recommendations. This test is an enzymatically amplified “one-step” sandwich-type enzyme immunoassay, where standards, controls and unknown samples are incubated in microtitration wells precoated with the anti-rat GH antibody. The standard curve of the assay was established with samples provided by the manufacturer. Rat serum IGF-1 was measured using a rat IGF-1 RIA Kit (DSL-2900; Diagnostic Systems Laboratories), after extraction with acid ethanol, according to the manufacturer’s recommendations. The IGF-1 assay included quality controls provided by the manufacturer. The standard curve of the assay was established with samples provided by the manufacturer.

GHRHR Receptors. The expression of GHRHR was measured by immunofluorescence and real-time PCR. The detection of GHRHR protein was carried out by Western blotting using the method of Schulz et al. (5). The binding affinity of GHRHR was demonstrated by radioligand binding assay (see details in each section, respectively).

Immunostaining. Cardiomyocytes were stained as described. Briefly, after isolation, 150 μL of cardiomyocytes in suspension were allowed to sediment and then fixed for 10 min (2% paraformaldehyde). Cells were stained with rabbit polyclonal antibody against human GHRHR at 4 °C for 24 h, followed by the secondary antibody at 37 °C for 1 h (Table S2). Frozen sections were used for positive controls (pituitary) and negative controls (skeletal muscle).

Paraffin sections were deparaffinized and rehydrated by immersion in xylene and a graded series of ethanol. Antigen retrieval was performed by a heat-induced method with citrate buffer (Dako). After blocking with 10% normal donkey serum, sections were incubated with a primary antibody (Table S2) at 37 °C for 1 h, followed by application of secondary antibody. Omission of the primary antibodies on parallel sections was used as negative control. Nuclei were counterstained with DAPI (Invitrogen). The total numbers of positively stained cells were quantified per slide to calculate the number of cells per unit volume (cubic millimeter) on each sample. Morphometric analysis was performed by using Adobe Photoshop CS3.

To quantify apoptosis of cardiac cells, TUNEL staining was performed on paraffin-embedded tissue sections according to the manufacturer’s protocol using a commercially available kit (In Situ Cell Death Detection Kit, POD; Roche Diagnostics). Slides were analyzed by fluorescent microscopy under 40x magnifications. Apoptotic nuclei were identified by green fluorescence staining and expressed as a percentage per cubic millimeter from tissue sections per animal. All images were obtained with both fluorescent (Olympus IX81; Olympus America) and an LSM710 Zeiss confocal laser scanning module (Carl Zeiss MicroImaging).
Quantification of Immunohistochemistry Staining for GHRHR. All images were obtained using a 40x objective, and the settings were kept the same for the entire study. Ten high-power fields of confocal images were taken from each sample (n = 3 for each group) (Fig. S4). The quantification of the fluorescence intensity was performed after deconvolution, using Huygen Essential software, version 3.4 (Scientific Volume Imaging). An optical density plot of the selected area was generated using the histogram tool in Image Pro plus version 6.3 (Media Cybernetics), and the mean staining intensity (intensity per pixel) was recorded.

Real-Time PCR. The expression of GHRHR was measured using real-time quantitative PCR, as described previously by Havt et al. (6).

We evaluated the mRNA expression of rat GHRHR and β-actin. The probes designed to evaluate the expression of GHRHR and β-actin are 5′-GCC ACC CCC GAC TTT CTC AGT TTG ATG CCC/BHQ_1/-3′ and 5′-6-FAM/ATCCG CGT CTG GAC CTG CTC GCC/BHQ_1/-3′, respectively. Gene-specific primer sequences were the following: GHRHR (sense) 5′-TCTGCTTTCCTAGGTCCCTGT-3′ and 5′-TGGTTCCTGGGCTTGG-3′ (antisense) with a product size of 110 bp; β-actin: (sense) 5′-GGGT TACGCCGTCCTCAT-3′ and 5′-GTCACGCAGATTTCCCC TCTC-3′ (antisense) with a product size of 133 bp.

All real-time PCR reactions were performed in the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Thermal cycling conditions comprised an initial denaturation step at 95 °C for 3 min, followed by 45 cycles at 95 °C for 30 s and an annealing temperature at 60 °C for GHRHR and β-actin for 1 min. As final steps, we included two cycles: one at 95 °C and the other at 60 °C, both for 1 min. All samples were run in triplicate, and each well of PCR reactions contained 25 μL as final volume, including 2 μL of cDNA, 200 nM of gene-specific primers, and 400 nM of probes. iQ Supermix (Bio-Rad) was used in the PCR. The efficiencies for all primers (Invitrogen Life Technologies) and probes (Integrated DNA Technologies) were tested before the experiments, and they were all efficient in the range of 95–105%.

Normal rat pituitary was used as positive control and rat β-actin as housekeeping gene. Negative samples were run in each reaction, consisting of no-RNA in reverse transcriptase reaction and no-cDNA in PCR. Two microliters of each amplification reaction was electrophoretically separated on 1.5% agarose gel, stained with SYBR Green I (Lonza), and visualized under UV light.

The mathematical method described by Pfaffl (7) was used to evaluate the relative expression ratio for GHRHR compared with β-actin, with the efficiencies for each set of real-time PCR reactions and the threshold cycle (CT).

The monitoring of proapoptotic and antiapoptotic genes was also assessed by real-time PCR. First-strand cDNA was synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse-Transcription Kit (Applied Biosystems), and ribosomal 18S RNA served as the housekeeping gene. We used TaqMan probes labeled with 6-carboxyfluorescein (FAM) for real-time RT-PCR reactions, according to the manufacturer’s protocol (Applied Biosystems). Data were analyzed by the CT relative quantification method.

Western Blotting. The detection of GHRHR protein immunoblot analysis was performed as described with minor modifications (4). Equal amount of proteins (80 μg) from rat pituitary, brain, heart, and for negative control (liver) were resolved in 12% SDS-PAGE and incubated overnight with rabbit polyclonal anti-human GHRHR antibody (Abcam; 1:1000) at 4 °C.

Radioligand Binding Studies. Radiolabeled derivatives of GHRH antagonist JV-1-42 were prepared by the chloramine-T method as described by Halmos et al. (8, 9). Preparation of membrane fractions from ischemic rat heart samples was performed as reported by Halmos et al. (8). Binding characteristics of GHRH binding sites were determined by in vitro ligand competition assays based on the binding of radiolabeled JV-1-42 to heart membrane fractions. Binding affinity (Kd) and capacity (Bmax) were calculated by Prism 4.0.1 (GraphPad Software).

Statistical Analysis. All values are shown as mean ± SEM. Echocardiographic parameters during a 4-week follow-up were compared within and between groups using one-way ANOVA for repeated measurements and two-way ANOVA followed by post hoc tests, respectively. For a given parameter, P < 0.05 was considered significant. All tests were carried out using Sigma Stat 3.5 (Jandel).

Fig. S1. (A) Changes over time in BW after MI. *P < 0.05 vs. baseline (BSN); †P < 0.01 vs. baseline and placebo at week 4. (B and C) Effects of 4-week treatment with rrGH or GHRH-A on (B) HW and (C) HW/BW ratio. *P < 0.0001 vs. placebo and GHRH-A. All values represent means ± SEM.

Fig. S2. Serum concentration (ng/mL) of (A) GH and (B) IGF-1 measured after 4-week treatment with placebo, rrGH, or GHRH-A. All values represent means ± SEM. *P < 0.01 vs. placebo and GHRH-A.

Fig. S3. Bar graphs showing (A) capillary density and (B) myocyte width measurements. All values represent means ± SEM. *P < 0.001 vs. placebo and GHRH-A.
Fig. S4. Representative confocal micrograph of the expression of GHRHR on cardiomyocytes from rats treated with placebo, rrGH, or GHRH-A. (Scale bars, 50 μm.) Bar graphs show the intensity of fluorescence (intensity per pixel). *P < 0.01 vs. placebo and GHRH-A.

Fig. S5. RT-PCR analysis of GHRHR and β-actin expression in representative samples of rat hearts treated with rrGH (A) and GHRH-A (B). DNA molecular marker is presented in lane M. Negative control, no cDNA in real-time PCR is shown in lane NC.

Table S1. Echocardiographic measurements at baseline (BSL) and at 4 weeks (W4) after MI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo (n = 10)</th>
<th>rrGH (n = 8)</th>
<th>GHRH-A (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSL</td>
<td>6.1 ± 0.1</td>
<td>5.9 ± 0.1</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>W4</td>
<td>7.9 ± 0.1</td>
<td>8.6 ± 0.1*</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSL</td>
<td>2.7 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>W4</td>
<td>6.4 ± 0.1</td>
<td>6.8 ± 0.2</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>FS (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSL</td>
<td>55.5 ± 1.2</td>
<td>58.2 ± 0.7</td>
<td>53.1 ± 1.0</td>
</tr>
<tr>
<td>W4</td>
<td>18.5 ± 0.9</td>
<td>20.3 ± 1.3</td>
<td>28.7 ± 3.3</td>
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<tr>
<td>EF (%)</td>
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<td></td>
<td></td>
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<tr>
<td>BSL</td>
<td>84.5 ± 1.4</td>
<td>89.2 ± 0.4</td>
<td>85.7 ± 1.6</td>
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<tr>
<td>W4</td>
<td>38.3 ± 3.4</td>
<td>44.3 ± 2.3</td>
<td>47.2 ± 4.0³</td>
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</tbody>
</table>

All values represent mean ± SEM. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter.

*P < 0.001 vs. placebo and GHRH-A.

†P < 0.05 vs. placebo.

‡P < 0.001 vs. placebo and rrGH.

§P < 0.05 vs. placebo and rrGH.

‖P < 0.01 vs. placebo.
Table S4. Real-time PCR values of the relative expression of mRNA for GHRHR and \( \beta \)-actin in rat heart tissue samples after treatment with GHRH-A (JI-38)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>rrGH</th>
<th>Ratio</th>
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<tr>
<td></td>
<td>( C_T ) value,</td>
<td>( C_T ) value,</td>
<td></td>
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<tr>
<td></td>
<td>(mean ( \pm ) SEM)</td>
<td>(mean ( \pm ) SEM)</td>
<td></td>
</tr>
<tr>
<td>GHRHR</td>
<td>32.38 ( \pm ) 0.42</td>
<td>32.96 ( \pm ) 0.18</td>
<td>1.91</td>
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<tr>
<td>( \beta )-actin</td>
<td>20.72 ( \pm ) 0.61</td>
<td>22.33 ( \pm ) 0.03</td>
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</table>

Ratio represents the gene expression level in the treatment group compared with the placebo group.

Table S3. Real-time PCR values of the relative expression of mRNA for GHRHR and \( \beta \)-actin in rat heart tissue samples after treatment with rrGH

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>rrGH</th>
<th>Ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( C_T ) value,</td>
<td>( C_T ) value,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mean ( \pm ) SEM)</td>
<td>(mean ( \pm ) SEM)</td>
<td></td>
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<tr>
<td>GHRHR</td>
<td>28.74 ( \pm ) 0.12</td>
<td>27.81 ( \pm ) 0.05</td>
<td>2.10*</td>
</tr>
<tr>
<td>( \beta )-actin</td>
<td>17.87 ( \pm ) 0.11</td>
<td>17.84 ( \pm ) 0.12</td>
<td>—</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \) vs. placebo.

Table S2. Antibody list

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Labeling</th>
<th>Fluorochromes</th>
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<tr>
<td>GHRHR</td>
<td>Rabbit polyclonal</td>
<td>Indirect</td>
<td>F</td>
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<tr>
<td>c-kit</td>
<td>Goat polyclonal</td>
<td>Indirect</td>
<td>F</td>
</tr>
<tr>
<td>Mast cell tryptase</td>
<td>Mouse polyclonal</td>
<td>Indirect</td>
<td>T</td>
</tr>
<tr>
<td>Ki67</td>
<td>Rabbit polyclonal</td>
<td>Indirect</td>
<td>F</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>Mouse polyclonal</td>
<td>Indirect</td>
<td>T</td>
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<tr>
<td>Nuclear DNA</td>
<td>DAPI</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt/dUTP</td>
<td>Direct</td>
<td>F</td>
</tr>
</tbody>
</table>

Direct labeling: primary antibody conjugated with the fluorochrome. Indirect labeling: species-specific secondary antibody with the fluorochrome. F, fluorescein isothiocyanate; T, tetramethyl rhodamine isothiocyanate.

Table S52. Antibody list

<table>
<thead>
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<th>Protein</th>
<th>Antibody</th>
<th>Labeling</th>
<th>Fluorochromes</th>
</tr>
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<tr>
<td>GHRHR</td>
<td>Rabbit polyclonal</td>
<td>Indirect</td>
<td>F</td>
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<tr>
<td>c-kit</td>
<td>Goat polyclonal</td>
<td>Indirect</td>
<td>F</td>
</tr>
<tr>
<td>Mast cell tryptase</td>
<td>Mouse polyclonal</td>
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<tr>
<td>Ki67</td>
<td>Rabbit polyclonal</td>
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<td>F</td>
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<tr>
<td>Tropomyosin</td>
<td>Mouse polyclonal</td>
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<tr>
<td>Nuclear DNA</td>
<td>DAPI</td>
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<td>N/A</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt/dUTP</td>
<td>Direct</td>
<td>F</td>
</tr>
</tbody>
</table>

Ratio represents the gene expression level in the treatment group compared with the placebo group.

*\( P < 0.05 \) vs. placebo.