Ghrelin Plays a Minor Role in the Physiological Control of Cardiac Function in the Rat

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We have previously reported that a 7-d pretreatment with hexarelin, a synthetic ligand of the GH secretagogue receptor (GHS-R), largely prevented damages induced by ischemia and reperfusion in isolated rat hearts. Our aim was to ascertain whether ghrelin, an endogenous ligand of the GHS-R, is physiologically endowed with cardioprotective activity. Hypophysectomized rats were treated in vivo for 7 d with either ghrelin (320 μg/kg) or hexarelin (80 μg/kg), and their hearts were subjected in vitro to the ischemia and reperfusion procedure. Ghrelin was far less effective than hexarelin in preventing increases in left ventricular end-diastolic pressure (15% and 60% protection for ghrelin and hexarelin, respectively), coronary perfusion pressure (10% and 45% reduction), and release of creatine kinase in the heart perfusate (15% and 55% reduction). In the second experiment, normal rats were passively immunized against ghrelin for 21 d before the ischemia and reperfusion procedure. In these isolated hearts, the ischemia-reperfusion damage was not significantly increased compared with control rats. After hypophysectomy, CD36 mRNA levels significantly increased, whereas those of atrial natriuretic factor significantly decreased. We conclude that: 1) ghrelin plays a minor role in the control of heart function; and 2) hexarelin effects are mediated in part by the GHS-R and largely by interactions with the CD36.

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EXARELIN IS A HIGHLY effective GH secretagogue (GHS), a family of small synthetic compounds that exhibit potent and dose-dependent GH-releasing activity (1). The GHSs bind to a specific G protein-coupled receptor, the GHS-R, that is expressed in the brain and several peripheral tissues, including the heart (2, 3). We have previously shown that the hearts of hypophysectomized rats are more sensitive to posts ischemic ventricular dysfunction, and both GH and hexarelin, given in vivo for 1 wk, were similarly competent in reverting the effects of GH deficiency (GHD) in hypophysectomized rats (4). Acute cardiotropic effects of hexarelin were demonstrated also in humans (5). Demonstrations of cardioprotective effects have also been provided for non-peptidyl GHS in rabbit and pig models (6, 7). Earlier studies had suggested that GH plays an important role in maintaining cardiovascular health, and alterations of the somatotropic function are frequently associated with abnormalities of cardiac structure and function (8). Given the direct cardiac effects of the GHSs and reversibility of cardiovascular abnormalities reported during GH treatment in hypopituitary patients (9), it is also possible that long-term therapy with GHS may be beneficial in adults with overt GHD. Recently, a gastric-derived peptide, named ghrelin, has been proposed as a natural ligand of the GHS-R (10). Ghrelin is endowed with a strong stimulatory effect on GH secretion in rats and humans (10–13), and recent studies have demonstrated that ghrelin has acute hemodynamic effects in healthy volunteers (14). On the basis of the foregoing, the administration of ghrelin should be able to revert the cardiac effects of hypophysectomy. Therefore, in the present study we have investigated whether ghrelin administration for 7 d might improve cardiac function in hearts of hypophysectomized rats undergoing low-flow ischemia and reperfusion in vitro to an extent comparable to hexarelin. Moreover, to ascertain whether ghrelin has a physiological role in the control of cardiac function, we have measured the effects of selective ghrelin deficiency, induced in vivo by passive immunization for 3 wk with specific antiserum, on cardiac damage induced in vitro by ischemia-reperfusion. Finally, we have also investigated by RT-PCR whether the effects of ghrelin and hexarelin are mediated by alteration of local synthesis of other known cardioactive factors, such as atrial natriuretic factor (ANF), endothelin-1 (ET-1), angiotensin-converting enzyme (ACE), cardioprotein-1 (CT-1), adrenomedullin (AM), and the scavenger receptor CD36.

Materials and Methods

Animals

Adult male intact and hypophysectomized Sprague Dawley rats weighing 155–160 g (Charles River Laboratories, Inc., Calco, Como, Italy) were housed under controlled conditions (22 ± 2°C, 65% humidity, and artificial light from 0600–2000 h). In the first experiment, hypophysectomized rats were randomly assigned to three experimental groups (12 animals each) and treated sc once a day for 7 d with: 1) saline

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Abbreviations: ACE, Angiotensin-converting enzyme; AM, adrenomedullin; ANF, atrial natriuretic factor; CK, creatine kinase; CPP, coronary perfusion pressure; CT-1, cardioprotein-1; ET-1, endothelin-1; GHD, GH deficiency; GHS, GH secretagogue; GHS-R, GHS receptor; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular-developed pressure; LVP, left ventricular pressure; PGI2, prostacyclin.
hundred nanograms of total RNA from each sample were subjected to acid guanidium thiocyanate-phenol-chloroform extraction method (18).

The isolated hearts were perfused retrograde fashion through the aorta with gassed Krebs Henseleit solution (37 C) as previously described (17). The perfusion rate was adjusted to yield a coronary perfusion pressure (CPP) of 55–60 mm Hg with a flow rate of 12 ml/min. Left ventricular pressure (LVP) was measured by inserting a small latex balloon into the ventricular cavity and filling it with saline until left ventricular end-diastolic pressure (LVEDP) stabilized in the range of 5–7 mm Hg. The preparations were electrically paced at a frequency of 300 beats/min with rectangular pulses (1 msec duration, voltage 10% above threshold) by a Grass stimulator (model S-88, Grass Instruments, Quincy, MA). The hearts were allowed to stabilize for 20 min and subsequently were exposed to the low-flow ischemia and reperfusion protocol.

Ischemia was induced by reducing the coronary flow to 2 ml/min (CPP, 4–6 mm Hg) for a period of 40 min. At the end of this period, reperfusion was resumed at the preischemic flow rate of 12 ml/min for another period of 20 min. In this study, CPP and LVP were monitored with Statham transducers (HP-1280C) connected to a dynograph (HP-7754A, Hewlett-Packard Co., Waltham, MA). LVEDP and postischemic left ventricular-developed pressure (LVDP, calculated as the peak LVP minus LVEDP) were also evaluated. Furthermore, the reactivity of the coronary vasculature to angiotensin II was evaluated to assess the integrity of endothelium-dependent relaxant mechanisms. Angiotensin II activity and concentrations of 6-keto-PGF1α were determined by the rat 6-keto-PGF1α ELISA (Amersham Pharmacia Biotech, Milan, Italy, respectively).

Statistical analysis

Results are expressed as the mean ± SEM of six determinations. Data were analyzed for statistical significance by one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. A P value below 0.05 was considered significant.

Effects of ischemia-reperfusion in hearts from hypophysectomized rats

In the hearts from hypophysectomized rats, the values of LVEDP gradually increased during the ischemic phase and remained significantly more elevated than in the normal controls (P < 0.05) until the end of reperfusion (Fig. 1).

Similar results were obtained after calculating the LVDP values. In fact, at the end of reperfusion, heart preparations from control rats recovered about 50% of preischemic values, whereas in the hypophysectomized this level was down to only 13% (Fig. 2).

**TABLE 1.** Nucleotide sequence of primers used in the RT-PCR assay

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>GenBank accession no.</th>
</tr>
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<tr>
<td>ACE</td>
<td>GTTCGTTGAGGAAGGTAGTGACGC</td>
<td>CCGTGGAGGCTGTGGCAGATCTTG</td>
<td>NM012544</td>
</tr>
<tr>
<td>ANF</td>
<td>CAGAGGATGGCCGCAGAC</td>
<td>GAGCAAGGCGCCGACCCTTA</td>
<td>M27498</td>
</tr>
<tr>
<td>ET-1</td>
<td>TCGTGTGTTTGGGTGCTTCCC</td>
<td>GTGTTCCCTTGTGCTGTCGTTG</td>
<td>NM012548</td>
</tr>
<tr>
<td>CT-1</td>
<td>CACCTGTGCCGGCCTCTTT</td>
<td>GTTACCCCTTTCCTTCCC</td>
<td>D78591</td>
</tr>
<tr>
<td>AM</td>
<td>TGTTTCCGGCTGCTCTCATC</td>
<td>CGGTTGATGTGGCCCTTCCC</td>
<td>U15419</td>
</tr>
<tr>
<td>CD36</td>
<td>GGATACACATAAGCCAAGGT</td>
<td>CATAAAAGCAACAAAACAT</td>
<td>AF072411</td>
</tr>
</tbody>
</table>

Enzyme and hormone assay

Completeness of hypophysectomy was determined by measuring plasma GH concentrations using a standard double-antibody RIA and reagents provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Child Health & Human Development, and United States Department of Agriculture.

Heart perfusates were collected in an iced-cooled beaker before flow reduction and during the 20 min of reperfusion. Creatine kinase (CK) activity and concentrations of 6-keto-PGF1α were evaluated using commercially available kits (Boehringer Mannheim Italia, Milan, Italy, and Amersham Pharmacia Biotech, Milan, Italy, respectively).

**Statistical analysis**

Results are expressed as the mean ± SEM of six determinations. Data were analyzed for statistical significance by one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. A P value below 0.05 was considered significant.

**Results**

**Effects of ischemia-reperfusion in hearts from hypophysectomized rats**

In the hearts from hypophysectomized rats, the values of LVEDP gradually increased during the ischemic phase and remained significantly more elevated than in the normal controls (P < 0.05) until the end of reperfusion (Fig. 1). Treatment with hexarelin for 7 d largely protected the heart of hypophysectomized rats from ischemia-reperfusion damage, and at the end of the reperfusion periods LVEDP values were not statistically different from those of control rats (Fig. 1). In contrast, heart preparations from hypophysectomized rats given ghrelin for 7 d generated LVEDP values that were consistently greater than the corresponding preischemic values (Fig. 1).

Similar results were obtained after calculating the LVDP values. In fact, at the end of reperfusion, heart preparations from control rats recovered about 50% of preischemic values, whereas in the hypophysectomized this level was down to only 13% (Fig. 2). Treatment with ghrelin was not really beneficial, whereas hexarelin induced a recovery of LVP up to values almost superimposable to those of controls (Fig. 2).
The level of CK activity released in the perfusates during the 20 min of reperfusion, a biochemical marker of myocardial cell lesions, was almost 3-fold higher ($P < 0.05$) in the heart of hypophysectomized animals compared with that of control rats (Fig. 3). Treatment with hexarelin reduced almost 50% ($P < 0.05$) the amount of CK released by the hearts of hypophysectomized rats during reperfusion. In contrast, heart preparations from hypophysectomized rats given ghrelin generated levels of CK activity similar to those released by hearts from saline-treated hypophysectomized rats (Fig. 3).

Because prostacyclin (PGI$_2$) generation plays an important role in maintaining flow within vessels and protecting the heart against ischemia, PGI$_2$ release in the heart perfusates was measured by assaying the levels of its stable metabolite, 6-keto-PGF$_{1\alpha}$. The rate of 6-keto-PGF$_{1\alpha}$ production in hearts from hypophysectomized rats was significantly reduced ($P < 0.05$) in both the preischemic and reperfusion periods compared with control rats (Fig. 4). Treatment with hexarelin prevented this fall in the rate of 6-keto-PGF$_{1\alpha}$ production during the preischemic period. At reperfusion, the rate of formation of the eicosanoid in hearts from hexarelin-treated hypophysectomized rats was diminished only by 16%, and was not significantly different from that of control rats. In contrast, in hearts from hypophysectomized rats given ghrelin for 7 d, the rate of formation of 6-keto-PGF$_{1\alpha}$ was still reduced by 47% in the reperfusion period (Fig. 4).

The functional integrity of the vascular endothelium was evaluated by measuring the reactivity of the coronary vessels to angiotensin II. The vasoconstriction induced by angiotensin II was significantly higher in hearts of hypophysectomized rats compared with those from intact rats (Fig. 5). In fact, injection of angiotensin II into the perfusion system of hearts from hypophysectomized saline-treated rats caused a CPP rise almost 4-fold higher ($P < 0.05$) than that recorded in hearts from control rats (Fig. 5). Treatment with hexarelin significantly reduced the effect of angiotensin II in hypophysectomized rats. In contrast, ghrelin failed to protect the vascular endothelium from the ischemic damage (Fig. 5).

**Effects of ischemia-reperfusion in hearts from rats passively immunized against ghrelin**

The induction of selective ghrelin deficiency for 3 wk had very limited effects on cardiac performance. In fact, all hemodynamic parameters measured in the heart of ghrelin-Ab-treated rats undergoing the ischemia-reperfusion procedure remained similar to those of rats treated with preimmune serum (Table 2). Also, biochemical markers such as CK release and 6-keto-PGF$_{1\alpha}$ generation did not indicate any major worsening in ghrelin-Ab-treated rats (Table 2). Passive immunization against ghrelin did not induce any measurable modification in basal GH plasma levels (data not shown).
Hypophysectomy induced a significant reduction of ANF cardiac mRNA levels (27%; \( P < 0.05 \) vs. control) that was not counteracted by treatment with ghrelin and hexarelin. In fact, in hypophysectomized rats treated for 7 d with ghrelin, ANF mRNA levels decreased by 41% compared with control, and those treated with hexarelin decreased by 40% (Table 3; \( P < 0.05 \)). The opposite pattern was determined concerning CD36 mRNA levels. In fact, hypophysectomy induced a sharp 50% increase (\( P < 0.05 \)) in cardiac CD36 mRNA levels, an effect that was only partially counteracted by ghrelin (36% increase) and hexarelin (38% increase; Table 3). Hypophysectomy and GHS treatment had nonsignificant effects on cardiac mRNA levels of ACE, ET-1, CT-1, and AM (Table 3).

**Discussion**

In the present study, we demonstrate for the first time that ghrelin does not have relevant roles in the physiological regulation of cardiovascular function in the rat. In fact, the induction of a selective ghrelin deficiency for 3 wk in normal rats very slightly aggravated the effects of the ischemia-reperfusion procedure on their isolated hearts. Moreover, ghrelin administration for 7 d only minimally improved cardiac performance in isolated hearts of hypophysectomized rats undergoing ischemia and reperfusion. The low effectiveness of ghrelin is perhaps surprising, because in the same hypophysectomized model hexarelin displayed a very important protective effect against the ischemic damage.

It is well known that hexarelin activates a G protein-coupled receptor, the GHS-R, which is expressed in the pituitary gland, hypothalamus, and several other nonendocrine tissues (2, 3). Because it has been shown that ghrelin is a very effective ligand of the GHS-R (10), it was expected that it could share the same cardiac activities of hexarelin. Indeed,
Interestingly, Ong’s research group described an unexpected vasoconstrictive effect elicited by hexarelin in perfused heart preparations (26). Hexarelin dose-dependently increased the CPP by direct interaction with CD36 expressed on endothelial cells of the microvasculature, but was devoid of any effect in CD36 knockout mice and in rats genetically deficient of CD36. This cardiovascular effect of hexarelin appears distinct from that of ghrelin, which was reported to decrease vascular resistance (14, 19). Thus, ghrelin and hexarelin would have different cardiovascular effects based mainly on their ability to bind the GHS-R (ghrelin and hexarelin) and the CD36 (hexarelin).

Even if the discovery of hexarelin ability to bind the CD36 brings new light on its mechanism of action, many aspects still need to be investigated further.

We have previously demonstrated that hexarelin has cardioprotective effects in the calcium subtraction-repletion model (calcium paradox) that were not shared by GH (29). In fact, a 7-d in vivo administration of hexarelin induced in perfused hearts a clear-cut inhibition of the steep ventricular contracture that is the mechanical expression of calcium paradox, whereas treatment with GH was ineffective. Moreover, hexarelin effect needed multiple exposures of the heart to the hexapeptide, because both the in vitro direct stimulation and the in vivo 3-d treatment were ineffective (29). Thus, we argued that hexarelin was acting by modifying the functional status of other cardioactive factors. For this reason, in the present study we measured modifications of cardiac mRNA levels after a 6-d treatment with hexarelin or ghrelin. Our results clearly demonstrate that a large decrease in ANF mRNA levels and, conversely, a significant increase of CD36 mRNA may be among those alterations responsible for the increased susceptibility of hypophysectomized rats to ischemia-reperfusion damage. Although hexarelin and ghrelin did not restore ANF and CD36 mRNA levels, hexarelin (not ghrelin) may have exerted its beneficial effects by competing with oxidized long-chain fatty acids for binding on CD36. A lower level of oxidized long-chain fatty acid may have protected the endothelial cells of cardiac microvasculature from the ischemia-reperfusion stress, thus eliciting the better pattern of PGI2 generation and CK release observed in hypophysectomized rats treated with hexarelin compared with the other two hypophysectomized groups. We are convinced that the ability of hexarelin to bind the CD36 is the key, or a very important clue, to explain why this hexapeptide is endowed with much more pronounced cardioprotective effects compared with ghrelin and other GHS.

In conclusion, the results of this study demonstrate that

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**TABLE 3.** Effects of ghrelin and hexarelin treatment on the mRNA levels of cardioactive factors in normal and hypophysectomized (HYPOX) rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HYPOX + saline</th>
<th>HYPOX + ghrelin</th>
<th>HYPOX + hexarelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF</td>
<td>1.493 ± 0.178</td>
<td>1.129 ± 0.175</td>
<td>1.133 ± 0.074</td>
<td>1.058 ± 0.072</td>
</tr>
<tr>
<td>ET-1</td>
<td>2.288 ± 0.219</td>
<td>1.679 ± 0.143</td>
<td>1.339 ± 0.226</td>
<td>1.381 ± 0.141</td>
</tr>
<tr>
<td>CT-1</td>
<td>0.442 ± 0.0047</td>
<td>0.0483 ± 0.0071</td>
<td>0.0450 ± 0.0043</td>
<td>0.0386 ± 0.0019</td>
</tr>
<tr>
<td>AM</td>
<td>0.089 ± 0.058</td>
<td>0.999 ± 0.122</td>
<td>0.972 ± 0.093</td>
<td>1.049 ± 0.050</td>
</tr>
<tr>
<td>CD36</td>
<td>0.153 ± 0.017</td>
<td>0.109 ± 0.017</td>
<td>0.097 ± 0.013</td>
<td>0.105 ± 0.012</td>
</tr>
<tr>
<td>ACE</td>
<td>0.071</td>
<td>0.017</td>
<td>0.012</td>
<td>0.033</td>
</tr>
<tr>
<td>ANF</td>
<td>1.195 ± 0.043</td>
<td>1.081 ± 0.071</td>
<td>0.781 ± 0.043</td>
<td>0.795 ± 0.033</td>
</tr>
</tbody>
</table>

Results were determined by RT-PCR and have been corrected for the relative glyceraldehyde-3-phosphate dehydrogenase value to normalize RNA loading. Values are arbitrary units and are expressed as the mean ± SEM of six hearts.

a P < 0.05 vs. relative control.
ghrelin is endowed with scarce, if any, physiological relevance in the control of cardiac function. Nonetheless, other GHS molecules, and notably hexarelin, may play important beneficial effects in protecting the heart from ischemia-reperfusion damage.

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**References**


