The Gut Hormone Ghrelin Partially Reverses Energy Substrate Metabolic Alterations in the Failing Heart

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Background—The gut-derived hormone ghrelin, especially its acylated form, plays a major role in the regulation of systemic metabolism and exerts also relevant cardioprotective effects; hence, it has been proposed for the treatment of heart failure (HF). We tested the hypothesis that ghrelin can directly modulate cardiac energy substrate metabolism.

Methods and Results—We used chronically instrumented dogs, 8 with pacing-induced HF and 6 normal controls. Human des-acyl ghrelin [1.2 nmol/kg per hour] was infused intravenously for 15 minutes, followed by washout (rebaseline) and infusion of acyl ghrelin at the same dose. 3H-oleate and 14C-glucose were coinfused and arterial and coronary sinus blood sampled to measure cardiac free fatty acid and glucose oxidation and lactate uptake. As expected, cardiac substrate metabolism was profoundly altered in HF because baseline oxidation levels of free fatty acids and glucose were, respectively, >70% lower and >160% higher compared with control. Neither des-acyl ghrelin nor acyl ghrelin significantly affected function and metabolism in normal hearts. However, in HF, des-acyl and acyl ghrelin enhanced myocardial oxygen consumption by 10.2±3.5% and 9.9±3.7%, respectively (P<0.05), and cardiac mechanical efficiency was not significantly altered. This was associated, respectively, with a 41.3±6.7% and 32.5±10.9% increase in free fatty acid oxidation and a 31.3±9.2% and 41.4±8.9% decrease in glucose oxidation (all P<0.05).

Conclusions—Acute increases in des-acyl or acyl ghrelin do not interfere with cardiac metabolism in normal dogs, whereas they enhance free fatty acid oxidation and reduce glucose oxidation in HF dogs, thus partially correcting metabolic alterations in HF. This novel mechanism might contribute to the cardioprotective effects of ghrelin in HF. (Circ Heart Fail. 2014;7:643-651.)

Key Words: energy metabolism ■ hormones

The extrinsic regulation of cardiac energy substrate metabolism is effected by neural mediators and peptide hormones.1-3 Most studies on hormonal regulators have explored the effects of insulin and glucagon-like peptide, which are particularly interesting for their therapeutic use.4,5 Surprisingly, little attention has been paid to the potential control of cardiac metabolism by ghrelin, a gut-derived 28-amino acid peptide known as a major stimulator of growth hormone release and food intake.6 Ghrelin is mainly produced by X/A-like cells of the gastric oxyntic glands, although several other tissues can synthesize it.7 Since the report of its discovery in 1999,8 this hormone has been intensively investigated not only for its role in the regulation of appetite but also for its diverse, direct actions on organs and systems, including heart and blood vessels.9 Associations between single-nucleotide polymorphisms of ghrelin or ghrelin receptors and cardiovascular diseases have been described.10 Prompted by initial findings in a rat model of myocardial infarction,11 some authors tested the therapeutic effects of ghrelin infusion in chronic heart failure (HF) patients: 3 weeks of treatment improved left ventricular (LV) function, exercise capacity, and muscle mass.12 Ensuing experimental studies confirmed the potential curative action of this hormone in rodents subjected to myocardial infarction.13-15 The beneficial effects of ghrelin on the failing heart might be due to multiple mechanisms, thus far characterized mainly in vitro, encompassing antiapoptotic protection and direct inotropic action.16-18 However, because the foremost role of this hormone is the control of systemic metabolism, one possible, important mechanism might be the normalization of cardiac energy substrate consumption. At rest, the healthy heart obtains most of

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the energy from the oxidation of free fatty acids (FFA) and lactate. In failing hearts, FFA oxidation falls concomitantly with an abnormal elevation in glucose oxidation.19-21 The pathophysiological significance of this alteration is still debated; nonetheless, several investigators have proposed the use of modulators of cardiac metabolism for the treatment of HF.22 Ghrelin is an endogenously produced peptide; hence, it might prove a biocompatible candidate for the correction of cardiac metabolic alterations. To date, no studies have determined the direct effects of ghrelin on cardiac oxygen consumption and substrate oxidation in vivo. The present study was aimed at filling this gap of knowledge. Ghrelin, an orexigenic peptide, promotes systemic anabolism; therefore, we tested the hypothesis that high circulating levels of it can directly lower energy turnover both in normal and failing hearts, with possible differential effects on FFA and carbohydrates oxidation. We also evaluated potential repercussions of metabolic regulation by ghrelin on cardiac mechanical efficiency.

A further level of complexity is given by the presence of 2 distinct forms of circulating ghrelin. To exert its main functions via the growth hormone secretagogue receptor 1a (GHS-R1a), ghrelin must first undergo acylation with octanoic acid at its third serine residue.6 However, solid evidence indicates that also the nonacylated form, named des-acyl ghrelin, is active at many levels, in some cases sharing the functions of the acylated form and in other cases antagonizing them.6,3 Des-acyl ghrelin receptors have been postulated, but not identified yet. Therefore, our hypothesis was tested using des-acyl ghrelin and acyl ghrelin, alternatively. The study was performed by simultaneously measuring hemodynamics, cardiac function and rate of myocardial oxygen, FFA, glucose, and lactate consumption in conscious dogs. Large animal models are particularly advantageous for this type of studies, in that they allow withdrawal of blood samples from coronary sinus to measure cardiac substrate metabolism in vivo.

Methods
Surgical Instrumentation and Hemodynamic Measurements
Nineteen adult, male, mongrel dogs (25–27 kg) were chronically instrumented as previously described.23 Briefly, anesthesia was induced with propofol (6 mg/kg IV) and maintained with 1.5% to 2% isoflurane during 40% oxygen/60% air ventilation, a thoracotomy was performed in the left fifth intercostal space, a catheter was placed in the descending thoracic aorta, a solid-state pressure gauge (P6.5; Konigsberg Instruments) was inserted into the left ventricle through the apex, a Doppler flow transducer (Craig Hartley) was placed around the left circumflex coronary artery, and a pair of pacing leads was fixed on the LV free wall. Wires and catheters were run subcutaneously to the intrascapular region, the chest was closed in layers, and the pneumothorax was reduced. Antibiotics were given after surgery and the dogs were allowed to fully recover. After 7 to 10 days of recovery from surgery, dogs were trained to lie quietly on the laboratory table. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Temple University and conform to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

Experimental Protocol
HF was induced in 13 dogs by pacing the LV at 210 beats/min for 3 weeks and at 240 beats/min for an additional week.23 Dogs were considered in congestive HF when LV end-diastolic pressure was ≥25 mm Hg, reflected by clinical signs such as dyspnea and ascites. Six chronically instrumented dogs were used as normal control. The experiments were conducted in intact, conscious animals placed on the laboratory table after overnight fasting. HF dogs were studied at spontaneous heart rate, with the pacemaker turned off. Control hearts were paced at 140 beats/min to match the highest predictable spontaneous heart rate occurring in our HF dog model. A catheter was inserted into the coronary sinus, through a peripheral vein, under x-ray fluoroscopic guidance. Therefore, we could withdraw paired blood samples from aorta and coronary sinus. Measurements and blood samples were taken at spontaneous heart rate, with the pacemaker turned off. After baseline hemodynamic measurements, the radioisotopic tracers [9,10-3H]-oleate (0.7 μCi/min) and [U-14C]-glucose (20 μCi) as a bolus, followed by 0.3 μCi/min were infused through a peripheral vein to track, respectively, the metabolic fate of FFA and glucose used by cardiac muscle as source of energy.24,25 In 8 HF and 6 control dogs, human des-acyl ghrelin (Bachem) was then infused at the dose of 4 μg/kg per hour (equivalent to 1.2 nmol/kg per hour) for 15 minutes. The infusion was then stopped for 60 minutes to re-establish the baseline condition, followed by 15 minutes infusion of 4 μg/kg per hour human acyl ghrelin (Bachem). Sequence of infusion, doses, and infusion times were selected to (1) avoid acyl ghrelin–induced increases in growth hormone during the experiment (thus the acylated form was infused later) and (2) to obtain measurable effects while remaining within the time window that precedes increases in growth hormone. Given the unavailability of previous similar dog studies in literature, we used as a reference a protocol of acute ghrelin infusion in humans26 at a dose unfeasible to elicit major growth hormone changes within the first 15 minutes.

Once we determined the functional and metabolic response to ghrelin, we tested its potential analogies or differences with changes occurring during a standard stress such as β-adrenergic stimulation: in 5 HF dogs, isotope-labeled substrates were infused and hemodynamic measurements and blood samples were taken at baseline and after 5 minutes of dobutamine infusion at 5 and 10 μg/kg per minute.

At the end of the protocol, the HF dogs were euthanized with 100 mg/kg of sodium pentobarbital.

Hemodynamic Recordings, Blood Gas Analysis, and Calculated Parameters
The aortic catheter was attached to a strain-gauge transducer to measure aortic pressure. LV pressure was measured using the solid-state pressure gauge. Blood flow in the left circumflex coronary artery was measured with a pulsed Doppler flowmeter (model 100, Triton Technology). LV diameter was measured by connecting the implanted piezoelectric crystals to an ultrasonic dimension gauge. All signals were digitally stored via an analog-digital interface (National Instruments) at a sampling rate of 250 Hz. Digitized data were analyzed off-line by commercially available software (Notocord hem evolution, Notocord). The parameters were determined during one respiratory cycle and comprised heart rate; mean aortic pressure; LV end-diastolic, peak systolic, and end-systolic pressure; mean blood flow in the left circumflex coronary artery; and the maximum and minimum of the first derivative of LV pressure. The difference between end-diastolic and end-systolic LV internal diameters was used as a surrogate of stroke volume and multiplied by heart rate to obtain a surrogate of cardiac output. The inverse of this latter was then multiplied by mean arterial pressure to calculate total peripheral resistance. Finally, the area of LV pressure–diameter loops (PDA) was calculated to obtain an index of stroke work.25,26

Blood gases tension was determined using a blood gas analyzer (ABL 800 Flex, Radiometer), and oxygen concentration was measured using a hemoglobin analyzer (OSM3, Radiometer). LV myocardial oxygen consumption (MVO2) per beat was calculated by multiplying the arterial–coronary sinus difference in oxygen content by total mean coronary blood flow, assumed to be twice the mean blood flow in the left circumflex coronary artery, and divided by heart rate.23 Finally, LV external mechanical efficiency was calculated as the ratio PDA/MVO2/beat.25,26
Circulating Hormones

Hormones were measured in arterial plasma previously collected and stored at −80°C. The concentration of the 2 forms of ghrelin was measured in duplicate by Enzyme Immunoassay kit (SPI-Bio, Bertin Pharma, France), using a monoclonal antibody specific to the C-terminal part of either acylated ghrelin or des-acyl ghrelin (Des-Octanoyl-Ser3), respectively. Measurements were performed in peptides in plasma enriched with HCl immediately after separation from blood cells to preserve acylated ghrelin levels, according to the manufacturer’s instructions.

Plasma growth hormone concentrations were measured in duplicate by Dog Growth Hormone Enzyme-Linked ImmunoSorbent Assay (ELISA) Kit (CUSABIO, China).

Plasma insulin was assessed by Insulin (Porcine/Canine) ELISA (ALPCO Diagnostics, Salem, NH) according to the manufacturer’s instructions.

Total and Labeled Metabolites

The concentrations of total and labeled FFA, glucose, and lactate, which are the 3 main cardiac energy substrates,3,20 were determined in arterial and coronary sinus blood samples, as previously described.23 Rapid measurements of total glucose and lactate were obtained from the same blood samples used for blood gas analysis tested with multipurpose cartridges (ABL 800 Flex, Radiometer). These measurements were repeated 2 to 3 times per sample to verify their consistency. Total FFA concentration in plasma was determined spectrophotometrically. 3H-oleate activity was measured in plasma, whereas 14C-glucose activity was determined in blood deproteinated with ice-cold 1 mol/L perchloric acid (1:2 vol/vol). 3H2O and 14CO2 activities were also measured in plasma and whole blood, respectively.

Mean coronary blood flow and the specific activities of 3H-oleate and 14C-glucose were multiplied, respectively, by the arterial–coronary sinus difference of 3H2O and 14CO2 blood content and by mean coronary blood flow and then divided by heart rate to calculate the rate of FFA and glucose oxidation per beat. Arterial and coronary sinus concentration of lactate were multiplied by mean coronary blood flow and then divided by heart rate to calculate net chemical lactate uptake per beat.

Fractional Shortening and Ca2+ Transient Measurements in Isolated Cardiomyocytes

Canine LV myocytes were isolated from 4 failing and 4 normal dog hearts, and fractional shortening and Ca2+ transients were measured as previously described by us25,30 (see Data Supplement). Myocytes were chosen on the basis of their morphology (rod shaped) and absence of spontaneous contractions. The maximal magnitude of contraction was normalized to resting cell length and expressed as a percent shortening. For [Ca2+]i fluorescence measurements, the F0 (or F unstimulated) was measured as the average fluorescence of the cell 50 ms prior to stimulation. The maximal Fluo-4 fluorescence (F) was measured at peak amplitude, and background fluorescence was subtracted from both parameters. Human des-acyl ghrelin or acyl ghrelin (Bachem) was added to the perfusate at the concentration of 50 nmol/L. Using the same selected cell, the measurements were performed following this sequence: baseline, des-acyl ghrelin, rebaseline (after ≥3 minutes washout time), and acyl ghrelin. At least 15 contraction cycles were analyzed in each condition. Data were acquired and analyzed using Clampex 10.2 electrophysiology software.

Real-Time Polymerase Chain Reaction and Western Blot to Measure the Expression of Cardiac GHS-R1a

RNA extraction from dog heart tissue and reverse transcription were performed as described by us81 (see Data Supplement). The following primer sequences were used: dog GHS-R1a, Fwd 5’-CACGCAAATCTGCAACCTTG-3’ and Rev 5’-CACCCGTTACTCTTTGGACA-3’ (accession no. NM_001099945.1); 18s rRNA; Fwd 5’-CCCCATCGAACGTGCTGCCCATC-3’ and Rev 5’-TGCTGCCTTCCTTGATGTGTA-3’ (accession no. NR_003278.3). Real-time polymerase chain reaction was performed with 50 ng cDNA, 150 nmol/L of each primer, and the IQ-SYBR-green mastermix (BioRad, Milan, Italy) using the ABI-Prism 7300 (Applied Biosystems), as previously described by us.55

Proteins (60 μg) were extracted from cardiac tissue of normal and HF dogs, resolved in 10% SDS-PAGE, and incubated with specific GHS-R1a antibody (Santa Cruz, 1:500). Blots were reprobed with actin antibody (Santa Cruz, 1:500) for normalization.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed by using commercially available software (SPSS Statistics, IBM). Hemodynamic and metabolic changes at different time points were compared by 1-way ANOVA for repeated measures and comparisons between groups of 2-way ANOVA, in both cases followed by Student–Newman post hoc test. When samples were not normally distributed, a nonparametric test was used and data presented as dot plots. The sample size used for statistical analysis of PDA, coronary flow, and cardiac metabolism was in some cases lower than the total number of dogs per group because of random technical problems, including distorted pressure–diameter loops, defective coronary flow probes, and difficult blood sampling from the coronary sinus. For all the statistical analyses, significance was accepted at P<0.05.

Results

Circulating Ghrelin, Growth Hormone, and Insulin

As shown in Figure 1, circulating des-acyl ghrelin was markedly increased after 15 minutes of infusion, both in normal
and HF dogs. The washout period after the first infusion stop was able to establish rebaseline levels not significantly different from baseline. The ensuing 15 minutes infusion of acyl ghrelin produced a similar effect, although less pronounced compared with des-ghrelin infusion.

Because acyl ghrelin has a secretagogue function, it was important to rule out possible increases in growth hormone. In fact, growth hormone was not significantly changed throughout the experimental protocol in both groups (Table 1). We also evaluated possible insulin changes because they could be a confounding factor in a study on cardiac metabolism. Also, in this case, we did not observe significant changes (Table 1).

**Hemodynamics and Cardiac Function**

The degree of hemodynamic and cardiac function impairment in HF compared with control was consistent with previous studies in this dog model. Values of heart rate, LV systolic pressure, \( dP/dt_{\text{max}} \), LV end-diastolic pressure, mean aortic pressure, and left circumflex coronary artery blood flow in the normal and HF dogs are reported in Table 2. Neither des-acyl nor acyl ghrelin caused any significant changes. As regards cardiac function, LV end-diastolic diameter and \( dP/dr_{\text{max}} \) were not significantly affected by the 2 forms of ghrelin (Table 2). Cardiac output and total peripheral resistance in normal dogs did not display significant changes during des-acyl and acyl ghrelin administration (Figure 2). However, in HF dogs, cardiac output significantly increased by \( \approx 20\% \); whereas, consistent with stable values of mean aortic pressure, total peripheral resistance significantly decreased by \( \approx 20\% \) in response to both des-acyl and acyl ghrelin (Figure 2). Also, PDA did not change significantly in normal dogs, whereas, in HF dogs, it significantly increased by \( \approx 24\% \) in response to both des-acyl and acyl ghrelin (Figure 3A). Because LV end-diastolic pressure and diameter were not significantly altered by des-acyl or acyl ghrelin, the changes in PDA could not be attributed to increased preload (Table 2).

**MVO\(_2\), Mechanical Efficiency, and Energy Substrate Oxidation**

MVO\(_2\) increased significantly by \( \approx 10\% \) in response to both des-acyl and acyl ghrelin, but only in the HF group, with no significant differences at any time point between the 2 groups (Figure 3B). Consequently, the ratio PDA/MVO\(_2\), an index of mechanical efficiency, did not display significant changes during the experiment (Figure 3C).

Des-acyl and acyl ghrelin infusion did not significantly affect the arterial concentration of FFA, lactate, and glucose (Table 1). Nonetheless, cardiac FFA oxidation significantly increased by \( \approx 40\% \) and 30% in response to des-acyl and acyl ghrelin, respectively, but only in the HF group (Figure 4A). After acyl ghrelin infusion, cardiac FFA oxidation was no longer significantly different compared with the control group. However, this was only an apparent normalization of FFA metabolism, simply due to the significant drop of rebaseline values in control, which narrowed the differences between the 2 groups. Interestingly, in HF, the FFA oxidation at rebaseline displayed the opposite change, that is, it remained significantly higher compared with baseline. In contrast to FFA, cardiac glucose oxidation was significantly decreased by \( \approx 30\% \) and 40% in response to des-acyl and acyl ghrelin, respectively, but only in the HF group, and remained significantly higher compared with the control group (Figure 4B). Net cardiac lactate uptake was significantly higher in HF compared with control and was not affected by ghrelin in any of the 2 groups (Figure 4C).

**Comparison With Metabolic and Functional Changes Induced by \( \beta\)-Adrenergic Stimulation**

To test whether the partial normalization of energy substrate oxidation in response to ghrelin was simply the consequence of enhanced PDA and MVO\(_2\), we used \( \beta\)-adrenergic stimulation as a term of comparison. Dobutamine, a widely used selective \( \beta\)-receptor agonist, was infused at 2 different doses, and we found that 10 \( \mu \)g/kg per minute best matched the effect of des-acyl and acyl ghrelin on MVO\(_2\). The comparison is presented in Figure 5 as percent changes in metabolic and functional parameters. Although the percent increase in MVO\(_2\) was not significantly different in failing hearts stimulated with des-acyl ghrelin compared with failing hearts stimulated with dobutamine, glucose oxidation in the 2 groups changed in opposite directions. Moreover, PDA was more enhanced in dobutamine group due to a combination of more

**Table 1. Arterial Concentrations of Growth Hormone, Insulin, Glucose, Free Fatty Acids, and Lactate After 15 Minutes of 4 \( \mu \)g/kg per h Des-Acyl or Acyl Ghrelin Infusion in Control and HF Dogs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Des-Acyl Ghrelin</th>
<th>Rebaseline</th>
<th>Acyl Ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH, ng/mL</td>
<td>Control (n=4)</td>
<td>70.15±12.95</td>
<td>71.77±13.24</td>
<td>68.54±11.32</td>
</tr>
<tr>
<td></td>
<td>HF (n=4)</td>
<td>67.49±19.12</td>
<td>68.47±21.88</td>
<td>76.30±20.52</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>Control (n=4)</td>
<td>0.20±0.003</td>
<td>0.21±0.01</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td></td>
<td>HF (n=4)</td>
<td>0.20±0.002</td>
<td>0.22±0.02</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>Control (n=5)</td>
<td>92.29±1.35</td>
<td>91.44±2.42</td>
<td>85.21±3.43</td>
</tr>
<tr>
<td></td>
<td>HF (n=7)</td>
<td>90.30±1.64</td>
<td>91.30±2.75</td>
<td>88.70±1.66</td>
</tr>
<tr>
<td>Free fatty acids, mmol/L</td>
<td>Control (n=5)</td>
<td>0.62±0.04*</td>
<td>0.65±0.06*</td>
<td>0.68±0.05</td>
</tr>
<tr>
<td></td>
<td>HF (n=7)</td>
<td>0.83±0.04</td>
<td>0.85±0.06</td>
<td>0.76±0.10</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>Control (n=5)</td>
<td>0.91±0.08</td>
<td>0.88±0.09</td>
<td>0.97±0.07</td>
</tr>
</tbody>
</table>
|                     | HF (n=7)          | 1.02±0.16        | 1.09±0.17  | 1.15±0.19    | 1.05±0.15    

Data are presented as mean±SEM. GH indicates growth hormone; and HF, heart failure. *P<0.05 HF vs control.
pronounced drop in peripheral resistance and an increase in contractility as indicated by the positive percent change in $dP/dt_{\text{max}}$. This change led to higher cardiac output and mechanical efficiency after β-adrenergic stimulation compared with des-acyl ghrelin stimulation.

**Fractional Shortening and Ca²⁺ Transients in Isolated Cardiomyocytes**

Ghrelin has been shown to exert positive inotropic actions in isolated rodent cardiomyocytes. Although we did not observe an increase in $dP/dt_{\text{max}}$ in conscious dogs, we tested the sensitivity of canine cardiomyocytes to ghrelin in vitro and the potential contractile response to it. As shown in Figure 6, HF cardiomyocytes were characteristically elongated. Fractional shortening increased significantly in response to both des-acyl ghrelin and acyl ghrelin in HF, but not in control cardiomyocytes, which was mirrored by changes in peak cytosolic calcium.

**GHS-R1a Gene and Protein Expression in Cardiac Tissue**

The gene expression of ghrelin receptor GHS-R1a has been found to be upregulated in human failing hearts. We tested a possible analogy between the human disease and our HF model. In the hearts of HF dogs, GHS-R1a mRNA increased by 85.7±16.7% compared with hearts of normal dogs ($P<0.05, n=5$ per group), whereas protein expression was increased by 31±4.8% ($P<0.05, n=3$ per group, see also Figure I in the Data Supplement).

**Discussion**

The novel finding of the present study is that acute increases of circulating des-acyl or acyl ghrelin partially reverse energy substrate metabolic alterations in failing hearts by lowering glucose oxidation while stimulating FFA oxidation. This metabolic change occurred in response to higher...
myocardial need of oxygen consequent to increased stroke work and cardiac output. Interestingly, despite its systemic anabolic effects, at the organ level, ghrelin promoted myocardial oxidation of fat, a less energetically efficient substrate. Nonetheless, oxygen demand matched cardiac performance; therefore, mechanical efficiency, that is, the ratio between LV stroke work and oxygen consumption, did not display significant changes. Acyl and des-acyl ghrelin bind different receptors while sharing some peripheral actions. We now provide evidence that one of the shared activities is the modulation of myocardial metabolism. On the other hand, neither form of ghrelin produced significant effects on the metabolism of normal hearts.

Other investigators have explored, at the biochemical level, the control exerted by acyl and des-acyl ghrelin on uptake of glucose and medium-chain fatty acids in cultured HL-1 and primary cardiomyocytes: only des-acyl ghrelin stimulated fatty acid uptake, whereas only acyl ghrelin prevented insulin-induced glucose uptake. The discordance between their results and ours, the latter rather indicating identical metabolic effects of the 2 forms of ghrelin, might be due to phenotypic disparities and the peptide concentrations they found to be effective in vitro (1–3 μmol/L) compared with the blood levels achieved during infusions in dogs (0.9–1.8 pmol/L). Based on the present data and on the known competition between energy substrates, we can only generically speculate that, in failing hearts, acyl and des-acyl ghrelin possibly blocked or, vice versa, potentiated one of the key limiting steps of, respectively, the carbohydrate and the FFA oxidative pathway.

Figure 3. Changes in pressure–diameter area (PDA, A), myocardial oxygen consumption (MVO₂, B), and mechanical efficiency indexed by the ratio PDA/MVO₂ (C) after 15 minutes of 4 μg/kg per h des-acyl or acyl ghrelin infusion in control (n=4 in A and C, n=6 in B) and heart failure (HF; n=6 in A and C, n=7 in B) dogs. *P<0.05 vs baseline; **P<0.05 vs rebaseline; #P<0.05 HF vs control.

Figure 4. Changes in cardiac free fatty acids (FFA, A) and glucose (B) oxidation and in lactate uptake (C) after 15 minutes of 4 μg/kg per h des-acyl or acyl ghrelin infusion in control (n=5) and heart failure (HF; n=7) dogs. *P<0.05 vs baseline; **P<0.05 vs rebaseline; #P<0.05 HF vs control; §P<0.05 rebaseline vs baseline.
The causes of the different sensitivity of healthy versus diseased hearts are likely various and complex. One of them is perhaps the overexpression of acyl ghrelin receptor GHS-R1a that we found in canine failing hearts, consistent with previous reports in humans. Conceivably, HF might also upregulate the still unidentified des-acyl ghrelin receptor(s). The biological significance of augmented responsiveness to ghrelin warrants further investigations that might lead to important insights in the pathophysiology of HF. Of note, ghrelin is also synthesized by cardiomyocytes, where it may exert autocrine/paracrine functions, and is downregulated in failing hearts; therefore, GHS-R1a upregulation is likely a compensatory mechanism perhaps triggered, in part, by intracellular ligand–receptor cross talk.

Moderate improvements in stroke work and, consequently, in cardiac output were the only significant functional changes observed in response to acyl and des-acyl ghrelin and occurred only in dogs with HF. Consistent with previous findings in humans infused with ghrelin, larger stroke volumes were not associated to changes in heart rate and were possibly due to decreased afterload rather than increased cardiac contractility. The absence of significant changes in \(\frac{dP}{dt}\)max during ghrelin infusion further supports this interpretation. Because other authors have documented a direct positive inotropic effect of ghrelin in cardiomyocytes isolated from normal rodent hearts, we repeated similar experiments in dog cardiac cells to rule out the possible lack of sensitivity due to species differences. Both acyl and des-acyl ghrelin enhanced cytosolic calcium release and contractility during electric stimulation; however, this effect was present only in pathological cardiomyocytes. Our findings confirm in canine cardiomyocytes that ghrelin is a positive inotrope, in vitro, and provide additional evidence that the failing heart displays an augmented sensitivity to it. Prior ghrelin functional studies ex vivo have not compared normal with failing hearts. It is also important to consider that, to obtain measurable changes, the in vitro concentrations of acyl and des-acyl ghrelin used by us and by others were 10- to 25-fold higher than those achieved during the infusion in vivo, prompting questions about their pathophysiological or even therapeutic relevance.

A critically important problem for our experiments was to avoid the confounding interference of changes in circulating hormones or metabolic substrates concentration. In fact, any of those can potentially alter cardiac metabolism. We therefore designed our protocol based on a previous study in humans that tested different doses of ghrelin ≤1.5 nmol/kg per minute. Those authors found no significant changes in growth hormone after 15 minutes of infusion. In addition, we first infused des-acyl ghrelin, which lacks the growth hormone secretagogue function and, as shown by blood analysis, was not converted into circulating acyl ghrelin. Our approach proved successful, in that we did not observe significant changes in circulating...
growth hormone as well as insulin and FFA, glucose, and lactate; hence, we can safely conclude that the effects of acyl and des-acyl ghrelin on cardiac metabolism were direct. Of note, the metabolic changes occurred in response to peaks of ghrelin concentration 7- to 8-fold higher than physiological, preprandial peaks. The functional and metabolic response of the failing heart to this gut hormone is peculiar because it differs substantially from the one triggered by β-adrenergic or cholinergic receptor stimulation, that is, by the activation of the sympathetic or parasympathetic system. In fact, compared with ghrelin infusion, a moderate adrenergic stress with 10 μg/kg per minute of dobutamine increased MVO2 significantly, whereas functional performance was more markedly enhanced and myocardial glucose oxidation was increased rather than decreased. On the other hand, we previously showed that the stimulation of vagal cardiac efferents lowers, via muscarinic receptors, both myocardial glucose consumption and MVO2.

We did not test long-term effects of high ghrelin levels on cardiac metabolism, which is an obvious limitation of our study. Previous literature provides solid data documenting that chronic administration of ghrelin attenuates the symptoms and signs of HF, and we cannot exclude that the modulation of cardiac metabolism contributed to the therapeutic benefits. Patients who benefited from acyl ghrelin administration were injected intravenously with 2 μg/kg twice a day for 3 weeks; therefore, their heart likely experienced repeated phases of metabolic changes similar to those described by us.

In conclusion, our study revealed a new cardiovascular component of the multifaceted functions accomplished by the gut hormone ghrelin, namely, the direct modulation of myocardial metabolism of FFA and glucose associated with MVO2 enhancement under pathological conditions. Such metabolic modifications might contribute to the therapeutic effects of ghrelin in HF.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

Ghrelin, a gut-derived 28-amino acid peptide, is known as a major stimulator of growth hormone release and food intake. Experimental and clinical research on this peptide is constantly growing. Besides its actions on systemic metabolism, ghrelin also displays cardioprotective effects in animal models and patients with heart failure. The underlying mechanisms are likely multiple; however, because the foremost role of this hormone is the control of systemic metabolism, an important possibility to consider is the normalization of cardiac energy substrate consumption. In fact, the oxidation of free fatty acid and glucose is profoundly altered in failing hearts and metabolic modulators have been proposed for the treatment of this pathological condition. The present study tested the hypothesis that both the acylated and nonacylated ghrelin exert direct effects on cardiac metabolism in a preclinical large animal model of dilated cardiomyopathy. We found that acute infusions of acyl or des-acyl ghrelin partially normalize myocardial free fatty acids and glucose oxidation while enhancing oxygen consumption. These findings suggest that ghrelin might function as an endogenous modulator of cardiac metabolism and cast new light on the potential mechanisms responsible for its therapeutic effects in heart failure.
The Gut Hormone Ghrelin Partially Reverses Energy Substrate Metabolic Alterations in the Failing Heart

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Supplemental Methods

Fractional shortening and Ca\textsuperscript{2+} transient measurements in isolated cardiomyocytes

Explanted hearts were placed in ice-cold Ca\textsuperscript{2+} free Krebs-Henseleit buffer (KHB) containing (in mmol/L) 12.5 glucose, 5.4 KCl, 1 lactic acid, 1.2 MgSO\textsubscript{4}, 130 NaCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 25 NaHCO\textsubscript{3}, and 2 Na-pyruvate (pH 7.4 with NaOH). A small catheter was placed into the lumen of the LAD coronary artery that supplied the free-wall region of the left ventricle. The perfused myocardial region was cut out from the heart and rinsed for 15 minutes with non-recirculating KHB with 10 mM taurine and the perfusion maintained for 30–45 minutes with recirculating KHB containing 180 U/mL collagenase (Worthington, type 2), 20mM 2,3 butanedione monoxime (BDM), 20mM taurine, and 0.05mM CaCl\textsubscript{2}. The softened myocardial tissue was then removed from the cannula and minced to dissociate myocytes from the mid-myocardial layer. The resulting cell suspension was filtered and the myocytes were allowed to settle by gravity. The supernatant was removed and the cells were resuspended in KHB containing 1% weight/volume bovine serum albumin (BSA), 10 mM taurine, and 0.25 mM CaCl\textsubscript{2} and then oxygenated for 2–3 hours with 95% O\textsubscript{2} + 5% CO\textsubscript{2}. The temperature of all solutions was kept at 37\textdegree C throughout the isolation procedure and all solutions were equilibrated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. All experiments were conducted within 3 hours from cell isolation.

Fractional shortening and Ca\textsuperscript{2+} transients were measured as previously described (29-30). Myocytes were loaded with 5-10 \textmu M Fluo-4 AM (Molecular Probes) and placed in a heated chamber (35\textdegree C) on the stage of an inverted microscope and perfused with a normal
physiological Tyrode’s solution containing (in mM): 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 10 glucose, 2 Na-pyruvate, 1 CaCl₂ and 5 HEPES, pH 7.4. They were paced at 0.2 Hz and fractional shortening data was collected using edge detection (Crescent Electronics).

*Real-time PCR*

RNA Primer pairs were designed with Primer-3 software and all had an optimal annealing temperature at 61 °C. The housekeeping gene 18S rRNA was used as an internal standard for gene expression screening. RT-PCR primers were synthesized by Sigma-Aldrich (Milan, Italy).
Figure Legends

Figure 1. Ghrelin receptor (GHSR-1a) protein expression determined by Western blot in normal and HF heart samples. The bar graph represents the densitometric quantification of GHSR-1a normalized to total proteins and reported as percent of control (n=3 per group, P<0.05 vs. normal).