EXPERIMENTAL STUDY

The Relationship of Appetite-Regulating Hormones in the Development of Cardiac Cachexia

Can Wang,^1^ MD, Xiaoying Dong,^1^ PhD, Limu Wei,^1^ MD, Junfeng Sun,^1^ PhD, Fali Zhao,^1^ PhD, Choushuan Meng,^1^ MD, Dongdong Wu,^1^ MD, Ting Wang,^1^ MD and Lu Fu,^1^ PhD

Summary

The physiological control of appetite regulation involves circulating hormones with orexigenic (ghrelin) and anorexigenic (cholecystokinin) properties that induce alterations in energy intake via perceptions of hunger and satiety. We sought to investigate the relationship between appetite-regulating hormones and the cachexia associated with chronic heart failure.

We randomized male Sprague-Dawley rats into myocardial infarction (MI) or sham operation (SO) groups. The levels of brain natriuretic peptide (BNP), cholecystokinin (CCK) and ghrelin in the plasma of all rats were detected by enzyme-linked immunosorbent assay (ELISA); the expression of BNP, CCK, and ghrelin in the myocardial tissue of all rats were detected by western blotting, immunohistochemistry, real-time polymerase chain reaction (PCR); myocardial morphology was assessed by microscopy.

Plasma BNP and CCK levels in the cardiac cachexia (CC) groups and the heart failure non-cachexia (HF-nc) groups were significantly higher than those in the control groups (P < 0.01), and the expression of BNP and CCK in the myocardial tissue of rats in CC groups and HF-nc groups were increased compared with the corresponding control groups (P < 0.01). In contrast, plasma and cardiac expression of ghrelin decreased compared with the sham group (P < 0.01). Furthermore, plasma CCK levels were positively correlated with BNP concentrations (P < 0.001) and significantly negatively correlated with the ejection fraction (P < 0.001) in model animals; plasma ghrelin levels were negatively associated with BNP levels (P = 0.0023) and positively associated with ejection fraction (P = 0.0042).

The appetite-regulating hormones (ghrelin and CCK) may present as a potential significant biomarker for cachexia associated with chronic heart failure.

Key words: Heart failure, Cholecystokinin, Ghrelin, Myocardial infarction

As a major public health issue, chronic heart failure is one of the most common causes of death in western countries. At advanced states patients can develop cardiac cachexia (CC), a serious complication characterized by significant weight loss and body wasting. Since there is no universally accepted definition of CC, this condition is rarely identified or diagnosed and rarely treated. According to Anker, CC should be considered as a weight loss higher than 6% over a period of at least six months. Cachexia is not only related with poor outcomes, but also with an unfavorable response to drug treatment and poor quality of life. Despite the high morbidity and mortality associated to CC, there are no known specific biomarkers for the diagnosis of this condition. von Haehling, et al. reported that there are numerous appetite-regulating hormones contributing to the wasting process by altering appetite and energy expenditure. The imbalance in these hormone systems, may be responsible for the development of satiety without adequate food intake.

Cholecystokinin (CCK) is a classic intestinal hormone and a transmitter in the central and peripheral nervous systems. CCK decreases meal size and increases intermeal interval indicating an effect on satiation as well as satiety. A recent study demonstrated that CCK was expressed at high concentrations in cardiac myocytes despite being synthesized predominantly in the gastrointestinal tract and central nervous system. Previous studies have shown that CCK regulates blood pressure and increases cardiac contractility. These findings indicate that CCK has a variety of effects as a novel cardiovascular hormone.

Ghrelin is a novel growth-hormone-releasing peptide isolated from the stomach that has been identified as an endogenous ligand for the growth-hormone secretagogue receptor. This peptide results in a positive energy balance by stimulating food intake and inducing adiposity through growth-hormone independent mechanisms.
addition, ghrelin has several cardiovascular effects, as indicated by the presence of its receptor in blood vessels and ventricles of the heart.\textsuperscript{24} Infusion of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with heart failure.\textsuperscript{25} Furthermore, repeated administration of ghrelin improves cardiac structure and function, and attenuates the development of cardiac cachexia in rats with heart failure.\textsuperscript{26} These results suggest that ghrelin may be an important biomarker of cardiovascular disease and may have potential therapeutic capacity.

In the work described here, we aimed to investigate the expression of CCK and ghrelin in rats with cardiac cachexia and to analyze whether the plasma level of CCK and ghrelin are related to the clinical parameters in cachexia associated with chronic heart failure.

**Methods**

**Animals and experimental designs:** Male Sprague-Dawley rats (280-300 g) were purchased from the Laboratory Animal Center of the First Affiliated Hospital of Harbin Medical University. Animal use was in accordance with the Chinese council on animal care guidelines, and the protocol for this study was reviewed and approved by the ethics committee of our hospital. After a 7-day acclimation period, the animals were randomly subjected to LAD ligation (ML, n = 20) or sham operation (SO, n = 20). Rats were anesthetized with 10% chloral hydrate (3 mL/kg, intraperitoneal injection), endotracheally intubated with a 14-gauge angiocath, and then mechanically ventilated with a ventilator. LAD ligation was performed through the fourth intercostal space, and the proximal LAD was ligated with 6-0 sutures. Successful ligation of the LAD was verified visually by the color change in the ischemic area and ECG leads I and aVL S-T segment elevations after the occlusion. SO animals underwent the same procedure but did not undergo ligation. Sterile techniques were used to perform all the surgeries. After 4 weeks, the model was determined based on weight variation. Samples from each rat were tested in duplicate, according to the detailed protocol provided by the manufacturers.

**Tissue preparation:** Rats were killed at 4 weeks after the operation and their hearts were rapidly removed and irrigated with cold saline solution. The weight of each heart was measured, and the ratio of heart weight to body weight (HW/BW) was calculated. A portion of the myocardium was fixed in 4% paraformaldehyde for further histological analysis, while duodenal samples, which ultimately served as a positive control in subsequent protein and gene expression analyses, and the remaining myocardial samples were quickly frozen in liquid nitrogen and stored at −80°C.

**Morphological changes in the myocardium:** Transverse sections of the cardiac apex were cut into 5-µm-thick sections and stained with hematoxylin and eosin (H&E) to analyze LAD ligation-induced changes in myocardial morphology and Masson’s trichrome to evaluate the severity of myocardial fibrosis. Three slides each were randomly selected from three rats in each group for morphological analysis.

**Real-time PCR:** Total RNA was prepared from the infarcted border with an RNA Extraction Kit (Takara Bio, Otsu, Japan), and cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Otsu, Japan), according to the manufacturer instructions. The expression levels of candidate genes were measured with SYBR Green on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The following primer sequences were used for this experiment: CCK: forward: 5’-CCC GAT ACA TCC AGG CTG-3’ and reverse: 5’-AAA TCC ATC CAG CAC ATT TAG CTG-3’; Ghrelin: forward: 5’-CCA AGG CCA TGG TGT CTT CA-3’ and reverse: 5’-CTG CAG TTT AGC TGG TGG CTT C-3’; BNP: forward: 5’-CAG TCA GTC GCT TGG GCT GTT-3’ and reverse: 5’-GCA GAG TCA GAA GCC GGA GT-3’; GAPDH: forward: 5’-GCC ACA GTG AAG GCT GAG AAT G-3’ and reverse: 5’-ATG GTG AGA ACA GGA GCC-3’. The 2−ΔΔCT method was used to calculate the mRNA levels of each gene.

**Western blotting:** Noninfarcted left ventricular tissue were homogenized and suspended in lysis buffer containing phosphatase inhibitor and Phenylmethylsulfonyl fluoride. Extracts were incubated for 30 minutes on ice and stirred every 5 minutes, after which the samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The protein concentrations were determined by a protein assay kit (BCA, Beyotime Institute of Biotechnology, Haimen, China). Then, the proteins were subjected to heat denaturation before being separated on 12% SDS-polyacrylamide gels and then transferred to PVDF membranes, which to obtain plasma. Plasma CCK, ghrelin and BNP levels were evaluated by using a commercial specific ELISA kit (RayBiotech, Inc., Norcross, GA). The detection limits for CCK, ghrelin, and BNP were 0.2 pg/mL, 48.2 pg/mL, and 4.7 pg/mL, respectively. The coefficient of variation was < 10%. Samples from each rat were tested in duplicate, according to the detailed protocol provided by the manufacturers.

**Laboratory investigation and immunoassay:** After the above measurement, blood was collected from the abdominal aorta and centrifuged for 15 minutes at 3000 xg
were blocked for 1 hour at room temperature before being incubated with primary antibodies against CCK (1:800; Sigma-Aldrich, Saint Louis, USA), ghrelin (1:1,000; Abcam, Boston, USA), and GAPDH (1:4,000; WanLeiBiotechnology, ShengYang, China) overnight at 4°C. Then, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG 1:2,000 or anti-rabbit IgG 1:2,000; ZhongShan, Beijing, China) for 1 hour at room temperature. All images were captured and analyzed using Image Lab software (Bio-Rad Universal Hood II, USA). The expression levels of the above proteins were normalized to those of GAPDH. All the experiments were performed three times.

**Immunohistochemistry:** For the CCK and ghrelin immunostaining experiments, myocardial tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin and then sectioned at a thickness of 4 μm. The sections were subsequently de-paraffinized, rehydrated and then sectioned at a thickness of 4 μm. The sections were then incubated with a rabbit anti-rat antibody against CCK overnight at 4°C. Then, the sections were counterstained with hematoxylin and observed under a fluorescence microscope (OLYMPUS DP73, Japan).

**Statistical analysis:** The results are presented as the mean ± standard deviation (SD). The group mean values were compared with an independent sample t-test. Pearson’s correlation coefficient was used to study the associations between appetite-regulating hormones expression and heart functional parameters. SPSS 20.0 software (SPSS Inc, Chicago, IL, USA) was used to perform statistical analysis. To reduce subjective bias when assessing the results, the data collectors, outcomes adjudicators, and data analysts were blinded by labeling the groups with non-identifying terms (A or B) until the entire analysis was completed. In all the tests, a value of P < 0.05 was considered significant.

### Table. Data of Heart Function and Weight in Each Group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>sham</th>
<th>post-op</th>
<th>HF-nc</th>
<th>post-op</th>
<th>CC</th>
<th>post-op</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (mm)</td>
<td>2.849 ± 0.079</td>
<td>3.016 ± 0.089</td>
<td>2.943 ± 0.088</td>
<td>3.333 ± 0.081**</td>
<td>2.964 ± 0.128</td>
<td>3.368 ± 0.161**</td>
</tr>
<tr>
<td>LVSTd (mm)</td>
<td>0.821 ± 0.033</td>
<td>0.859 ± 0.031</td>
<td>0.813 ± 0.033</td>
<td>0.733 ± 0.009**</td>
<td>0.814 ± 0.029</td>
<td>0.738 ± 0.038**</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>5.735 ± 0.513</td>
<td>5.949 ± 0.495</td>
<td>6.188 ± 0.287</td>
<td>7.125 ± 0.257*</td>
<td>5.742 ± 0.672</td>
<td>7.756 ± 0.640**</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.719 ± 0.793</td>
<td>4.556 ± 0.915</td>
<td>3.890 ± 0.470</td>
<td>5.825 ± 0.702*</td>
<td>3.664 ± 0.601</td>
<td>6.404 ± 0.619**</td>
</tr>
<tr>
<td>BW (g)</td>
<td>286.500 ± 3.665</td>
<td>331.125 ± 8.593</td>
<td>286.750 ± 3.500</td>
<td>317.750 ± 8.617*</td>
<td>286.125 ± 4.611</td>
<td>264.125 ± 5.270**</td>
</tr>
</tbody>
</table>

Body weights and cardiac functional and structural parameters in the rat models at the end of the study. LA diameter indicates left atrial diameter; BW, body weight; IVSTd, end-diastolic interventricular septal thickness; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; LVFS, left ventricular short-axis fractional shortening; pre-op, preoperation group; post-op, postoperation group; sham, sham operation group; HF-nc, heart failure non-cachexia group; and CC, cardiac cachexia group. Data are expressed as the mean ± standard deviation (SD). Comparred with post-op, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, compared with post-op1, **P < 0.01, compared with post-op2, *P < 0.05.

**Results**

**The success rate of animal models:** After surgery intervention, twenty-eight surviving animals (twelve in MI group and sixteen in SO group) completed this study. Four weeks after ligation of the anterior descending artery, eight adult male Sprague-Dawley rats were divided into CC group, the rest for the heart failure non-cachexia group, according to the changes of body weight and the echocardiography result. There was no significant difference in heart function parameters and body weight before surgery in all groups. After four weeks, compared with the sham operation group, LA, LVDd and LVDs were significantly increased in the HF-nc group (P < 0.01, P = 0.05, P = 0.05 respectively) and CC group (P < 0.01); LVEF was significantly lower (P < 0.01), and IVSTd was thinner (P < 0.01) in the HF-nc group and CC group, the difference was statistically significant (Table).

**Myocardial degeneration and fibrosis:** H&E and Mason’s trichrome staining showed (Figure 1) that significant inflammatory infiltrates, cardiomyocyte derangement, and collagen fiber deposition were present in the HF-nc group and CC group.

**Measurement of plasma BNP, CCK and ghrelin levels:** Plasma BNP, CCK and ghrelin levels were measured by ELISA (Figure 2), which showed that plasma BNP levels were significantly higher in the HF-nc group and CC group than in the corresponding SO group (P < 0.01), while there was no difference in the HF-nc group and CC group. Plasma CCK concentrations were also increased in the HF-nc group and CC group compared with the SO group (P < 0.01). Furthermore, relative to the HF-nc group, plasma levels of CCK in the CC group increased more significantly (P < 0.01). In contrast, compared with the control group and HF-nc group, plasma ghrelin levels were declined dramatically in the CC group (P < 0.01).

**BNP, CCK and ghrelin expression:** As shown in Figure 3, the BNP to GAPDH ratio was higher in the HF-nc group and CC group than in the corresponding SO group (P < 0.01), while there was no statistical difference between the HF-nc group and CC group. The CCK to GAPDH ratio was also increase in the HF-nc group and CC group compared with the SO group (P < 0.01); the CC group had an increased tendency compared with the
HF-nc group, but the difference was not significant. Nevertheless, the ghrelin to GAPDH ratio was lower in the CC group than in the control group (P < 0.01) and HF-nc group (P < 0.05). Quantitative analysis of candidate genes mRNA expression levels in the myocardium, which was performed by real-time PCR, showed that CCK expression levels in the HF-nc group and CC group were significantly increased compared with the corresponding SO group (P < 0.01); in addition, the CC group was significantly higher than the HF-nc group (P < 0.05). Compared with the SO group, the expression levels of ghrelin in the HF-nc group was lower (P < 0.01); while ghrelin mRNA expression levels were lower in the CC group than in the control group (P < 0.01) and HF-nc group (P < 0.01).

Detection of CCK and ghrelin expression by immunohistochemical staining: CCK was expressed at low levels in the myocardial tissues of the SO groups. Staining for CCK was significantly increased in the infarcted and non-infarcted areas of the left ventricle in the HF-nc group and CC group compared with those in the corresponding SO group. However, the SO group myocardium showed a stronger immunoreactivity for ghrelin than in the HF-nc group and CC group (Figure 4).

The relationships between plasma CCK levels, ghrelin levels and heart failure parameters: The correlations between CCK levels, ghrelin levels, BNP levels and cardiac functional parameters are shown in the indicated figure (Figure 5). Plasma CCK levels were positively correlated with BNP levels (P < 0.001) and significantly negatively correlated with the LVEF (P < 0.001) in all test groups. However, plasma ghrelin levels were negatively associated with BNP levels (P = 0.0023) and positively associated with ejection fraction (P = 0.0042).

Discussion

CC is a serious complication of chronic heart failure, characterized by significant weight loss and body wasting. Chronic heart failure-related muscle wasting results from a chronic imbalance in the activation of anabolic or cata-
Figure 3. The noninfarcted area of the left ventricle were collected at the end of treatment and analyzed by RT-PCR and Western blotting. A: Analysis of CCK, ghrelin, BNP, and GAPDH protein expression from the noninfarcted area of the left ventricle samples by Western blot. B-C: Bar graphs show relative intensity of CCK to GAPDH, and the CCK mRNA levels; D: The bars show relative intensity of BNP to GAPDH. E-F: Bar graphs show relative intensity of ghrelin to GAPDH, and the ghrelin mRNA levels. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; sham, sham operation group; HF-nc, heart failure non-cachexia group; and CC, cardiac cachexia group. The dates were summarized from 3 independent experiments. All values are represented as the mean ± SD. Compared with sham operation group, **P < 0.01, ##P < 0.01; Compared with HF-nc group, &P < 0.05, &&P < 0.01.

Figure 4. CCK and ghrelin expression levels were detected by immunohistochemical staining (×400 magnification). Sham indicates sham operation group; HF-nc, heart failure non-cachexia group; CC, cardiac cachexia group; PC, positive control group; and NC, negative control group.

bolic pathways, caused by a series of immunological, metabolic, and neurohormonal processes. In spite of the high morbidity and mortality associated with this condition, there is no universally accepted definition or specific biomarkers for CC, which makes its diagnosis and treatment difficult. In the present study, we demonstrated that plasma CCK levels and the myocardium expression of CCK increased in cardiac cachexia rats and positively correlated with BNP concentrations and significantly negatively correlated with the LVEF. Compared with the control group and HF-nc group, plasma ghrelin levels in the CC groups dramatically decreased, and the expression of ghrelin in myocardium was also reduced, which associated negatively with BNP levels and positively associated with ejection fraction.

Since the initial report in 1997 of an extremely high risk of death in patients with non-intentional weight loss, other authors have investigated the exact role of nutritional status in CHF patients. As a classical brain-gut peptide, CCK also appears to have several effects on the cardiovascular system, although the mechanisms underlying these effects remain unknown. Cholecystokinin octapeptide (CCK-8) has been considered a minor biologically active fragment and has been shown to significantly improve cardiac function and hypotension in rats with lipopolysaccharide-induced endotoxic shock, most likely through the CCK1 and CCK2 receptor, which are expressed on cardiac myocytes. Ghrelin is another appetite-regulated hormone that is widely distributed throughout various organs and tissues. Nagaya, et al. have shown that a specific receptor for ghrelin exists not only in the hypothalamus and pituitary but also in blood vessels and the heart and that intravenous injection of ghrelin causes beneficial hemodynamic effects via reducing cardiac afterload and increasing cardiac output without an increase in heart rate. Thus, it is possible that plasma
CCK and ghrelin levels may have important hemodynamic effects in patients with CHF. Because cardiac cachexia is a strong independent risk factor for mortality in patients with CHF, it would be interesting to investigate the expression of CCK and ghrelin in the development of cachexia in the most severe forms of CHF.

In the present study, we assessed CCK and ghrelin expression levels in rats with cardiac cachexia induced by LAD ligation. The results show that CCK protein expression levels and plasma CCK levels were significantly increased in the CC group compared with the control group. Goetze, et al. reported that proCCK measurement in plasma contains prognostic information in patients with stable heart failure. They also demonstrated that cardiac CCK expression is not regulated by hypoxia, but is markedly regulated by isoproterenol, which suggests that the expression represents adaptation to increased sympathetic activity.16) Therefore, we speculate that upregulated CCK may have autocrine or paracrine effects in the pathogenesis of heart failure.33) In this study, we also observed that ghrelin expression decreased in myocardium as well as the expression of its plasma levels. It is in conflict with the previous studies, in which ghrelin was elevated in cachectic patients with CHF.34) The mechanism by which ghrelin protein expression and plasma ghrelin concentrations reduce in rats with CC is unclear. We speculate that there are several possibilities. First, previous research showed that the negative interaction between CCK and ghrelin could be initiated directly on the vagus nerve, since both GHS-R1a and CCK1 have been detected on vagal afferents by immunohistochemistry34) and ghrelin injected intravenously suppresses, whereas intravenous CCK stimulates vagal afferent activity.35) Besides, CCK2 receptor knockout mice have an increased hypothalamic expression of GHS-R1a most likely contributing to the increased food intake and body weight observed in those mice36) indicating an interaction also on the level of the hypothalamus. In addition, CCK-8S injected intravenously reduces circulating ghrelin in healthy volunteers37) suggesting a regulatory action of CCK on ghrelin release, possibly directly exerted on X/A-like cells or through the release of somatostatin.38) Therefore, we speculate that the down-regulation of ghrelin expression levels may be due to the negative regulation of CCK up-regulation. Second, Friberg, et al. reported that GH secretion was stimulated in patients with AMI, especially in patients with severe cardiac damage, furthermore, the larger the myocardial damage, the higher were the growth hormone levels and the slower the decline.39) Since ghrelin is a GH-releasing peptide, increased GH levels may decrease ghrelin secretion due to the negative feedback loop of GH. Similarly, Matsumoto, et al. reported that serum ghrelin levels are significantly decreased in association with myocardial infarct size and cardiac function in patients with AMI.40)

Ischemic heart disease is the most prevalent cause of HF in humans.41) After MI, various inflammatory reactions lead to the formation of scar tissue, left ventricular wall remodeling, ventricular wall thinning, and continuous deterioration of heart function. Considering the limitations of single biomarkers and the inexistence of exclusive
molecules for CC, our studies focused in the use of a combination of multiple biomarkers. Therefore, our study on the expression of appetite-regulated hormones in the pathogenesis of CC caused by acute myocardial infarction is helpful in providing new biomarkers for the diagnosis of cachexia associated with heart failure and may play a major role in early prevention. In our opinion, future work should elucidate the exact mechanisms and physiological targets of cardiac CCK and ghrelin expression. However, our study still has several limitations. First, the current study is limited to describe infarcted size, which may influence the expression levels of CCK and ghrelin. Furthermore, we lacked monitoring of CCK and ghrelin expression at multiple time points during the development of cachexia associated with heart failure.

In conclusion, the appetite-regulating hormones (CCK and ghrelin) may play an important role in cachexia associated with chronic heart failure and present as a potential significant biomarker for cardiac cachexia.

Disclosures

Conflicts of interest: The authors declare no conflict interest.

References

17. Rehfeld JF. Cholecystokinin-from local gut hormone to ubiquitous messenger. Front Endocrinol (Lausanne) 2017; 8: 47.
35. Clerc P, Coll Constats MG, Lulka H, et al. Involvement of cholecystokinin 2 receptor in food intake regulation: hy-


