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Ghrelin improves disturbed myocardial energy metabolism in rats with heart failure induced by isoproterenol

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To explore the effects of ghrelin on disturbed myocardial energy metabolism during chronic heart failure (CHF). Rats were subcutaneously injected with isoproterenol (ISO) for 10 days with or without ghrelin for another 10 days. Enzyme immunoassay was to measure ghrelin concentrations. Compared with the control group, ISO-treated rats showed suppressed cardiac function with high ghrelin/GHS-R expressions. These rats also showed the decreases in food consumption and weight. The decreased levels of plasma glucose and myocardial glucogen, but the high lactate in blood and myocardium showed myocardial metabolic disturbance. Compared with the group given ISO alone, the rats with ghrelin (20 and 100 μg/kg/day) improved cardiac dysfunction and increased food intake by 13.5 and 14.2% (both $P < 0.01$), and rate of weight gain by 95% ($P < 0.05$) and 1.71-fold ($P < 0.01$), respectively. The plasma glucose were increased by 49.7 and 50.8% (both $P < 0.01$), and myocardial glucogen, by 40.5 and 51.7% (both $P < 0.01$), but blood lactate decreased by 1.56- and 1.96-fold (both $P < 0.01$), and myocardial lactate by 32.1 and 48.7% (both $P < 0.05$), respectively. Their MCT1 mRNA and protein expressions increased. The myocardial ghrelin/GHS-R pathway can be upregulated during CHF. The ghrelin can attenuate cardiac dysfunction and energy metabolic disturbance in CHF rats. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ghrelin; metabolism; heart failure; glucose; lactate; monocarboxylate transporter-1; GHS-R

Introduction

Homeostasis of myocardial energy metabolism plays a vital role in maintaining the normal cardiac performance. The notable characteristic of cardiac energy metabolism is that various metabolic fuels, such as fatty acids, glucose, and lactate, can be taken up and supply for the heart pumping function [1,2]. In prolonged cardiac diseases, particularly ischemic cardiac hypertrophy, myocardial fat oxidation is downregulated and glucose oxidation is accelerated, which contributes to energy metabolic disturbance [3]. Therefore, the heart turns from a net lactate consumer to a net lactate producer with decreased ATP production and over-release of hydrogen ion [H^+] from the ischemic myocardium. These changes disturb the cardiac excitation–contraction coupling and increase the susceptibility to ischemic hypoxia damage [4]. Metabolic remodeling occurs when heart disease develops into cardiac cachexia at the end-stage of chronic heart failure (CHF), further aggravating cardiac damage and dysfunction [5].

Ghrelin, an endogenous ligand of the growth hormone secretatogue receptor (GHS-R), isolated from the stomach, was first reported in 1999 [6]. Growing evidence suggests that ghrelin is involved in regulation of body energy metabolism. Ghrelin administrated either intracerebroventricularly or peripherally stimulates food intake and weight gain, thus inducing obesity and causing a positive energy balance through decrease in fat oxidation and increase in carbohydrate utilization by a growth hormone (GH)-independent mechanism [7,8]. With the hemodynamic and anabolic effects of GH/insulin-like growth factor (IGF-1), ghrelin may play a role in maintaining the myocardial growth and skeletal muscle mass via activation of the GH/IGF-1 axis [9]. Significantly, cardiovascular tissues are rich in GHS-R mRNA [10]. Therefore, ghrelin may be exerted directly on the heart and vasculature rather than being mediated by GH [11]. As an important autocrine/paracrine factor, ghrelin has various cardiovascular effects, including inotropic action, vasodilatation, anti-apoptosis, and anti-inflammation [12–16], which suggest that ghrelin may maintain cardiovascular homeostasis.

This study has shown that ghrelin has effective preventive and therapeutic effects on myocardial ischemia damage [17,18], ischemia/reperfusion injury [19], congestive heart failure [20], and cardiac dysfunction induced by shock [21]. This indicates that ghrelin has a powerful cardiovascular protective action. Ghrelin not only improved the cardiac performance but also ameliorated the cachectic state in rats with CHF [22]. Clinical trials with few subjects found that, in addition to beneficial hemodynamic effects, ghrelin improved muscle wasting and increased exercise capacity, through increased muscle strength and lean body mass of patients with CHF [20,23,24]. However, the cardioprotective mechanisms of ghrelin in cardiac cachexia with end-stage CHF are poorly

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understood. In particular, very little is known about the effects of ghrelin on myocardial metabolic remodeling.

In this study, the model of isoproterenol (ISO)-induced myocardial injury and CHF was used to observe the changes in the endogenous ghrelin/GHS-R axis and the effects of ghrelin supplementation on lactate and glucose metabolism. The purpose of this study was to explore the effects of ghrelin on myocardial metabolic remodeling in cachectic rats with CHF.

Materials and Methods

Materials

All animal care and experimental protocols were in compliance with the PR China Animal Management Rule (Ministry of Health, PR China) (Document No. 55, 2001) and the rule of the Animal Care Committee of Capital Medical University. Male Wistar rats (220–250 g) were provided by the Animal Department, Capital Medical University. Ghrelin, antibodies for ghrelin and GHS-R1a, and the ghrelin enzyme immunoassay (EIA) kit were provided by Phoenix Pharmaceuticals (St Joseph, CA, USA). Monocarboxylate transporter-1 (MCT1) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sequences of oligonucleotide primers for amplifying cDNA were as follows: brain natriuretic peptide (BNP-S), 5′-TCTGCTCCTTTTCCTTA-3′; BNP-A, 5′-GAACTATGTGCCATCTTGGA-3′; ghrein-S, 5′-TTGAGCCCAGAGCACCAGAAA-3′; ghrelin-A, 5′-AGTTGCAGAGGAGGCAGAAGCT-3′; GHS-R1a-S, 5′-CTATCAGATGTCT-3′; GHS-R1a-A, 5′-GGAAGAGATGGCGAAGTAG-3′; MCT1-S, 5′-TTGG ACCCGAGGTTCTCC-3′; MCT1-A, 5′-AGGCGGCCTAATGTG-3′ and for sample loading, glyceraldehydes, phosphate, dehydrogenase (GAPDH-S), 5′-ACGGATTGGTCGTATTGGG-3′, GAPDH-A, 5′-CTGGAAGATGGTGATGGG-3′. The above sequences of oligonucleotide primers were synthesized by ShangaiShengwugongcheng Company (Shanghai, China). Other chemicals and reagents used were of analytical grade.

Experimental Protocols

The model of ISO-induced myocardial injury was produced as described previously [25] with minor modifications. Male rats were randomly divided into four groups ($n = 6$ each): (i) control group, in which the rats were subcutaneously injected with physiological saline (2 ml/kg) twice a day for 20 days; (ii) ISO group, in which the rats were subcutaneously given ISO by 20, 10, and 5 mg/kg/day for the first 3 days, then 3 mg/kg/day for the next 7 days. Then, the rats were given physiological saline (2 ml/kg) for the next 10 days; (iii) low-dosage ghrelin group; and (iv) high-dosage ghrelin group. The rats in these two groups were given the same doses of ISO and in the same way as the ISO group. Then, these rats were subcutaneously injected with ghrelin, 20 (the third group) and 100 (the fourth group) μg/kg/day, respectively, for the following 10 days. During this period, rats were housed under standard conditions (temperature $20 \pm 1\,^{\circ}C$, humidity $50 \pm 10\%$, light from 6 am to 6 pm) and given standard rodent chow and free access to water. The experiment was stopped 12 h after the last administration of the drugs, and echocardiography was performed. The rats were anesthetized with 20% urethane (1 g/kg, intraperitoneally). After hemodynamic parameters were measured, a blood sample was drawn from the carotid artery and mixed with 1 mg/ml EDTA-2Na and 10 μg/ml aprotinin. The blood was immediately centrifuged and stored at $-70\,^{\circ}C$. Hearts were removed and weighed immediately. Fresh rat myocardium (100 mg) was acidified by adding 1 M acetic acid (1 ml/100 mg myocardium), boiled for 10 min to inactivate intrinsic proteases, and stored at $-70\,^{\circ}C$ before homogenization [17] for measuring ghrelin by EIA. Histological staining of sections of the cardiac apex was performed by means of hematoxylin & eosin (H&E), periodic acid-schiff (PAS), and immunohistochemistry.

Echocardiography Assessment

Echocardiography was performed in a blind way. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system (Vevo-770, Toronto, Canada). Left ventricle wall thickness, dimensions of left ventricle chamber, and fractional shortening (FS) were measured from at least three consecutive cardiac cycles according to the recommendations of the American Society of Echocardiology. Left-ventricular end-diastolic dimension (LVEDV), LV mass (LVM), and LV ejection fraction (LVEF) were calculated using a modified version of Simpson's method [26].

EIA for Ghrelin Determination

Myocardial tissue was homogenized and centrifuged at 12 000 rpm for 20 min, and the supernatant was used to determine protein concentration by Bradford's method [27,28]. Ghrelin was purified from rat myocardium as previously described [6]. After pre-equilibration with buffer A (1% TFA; 3 ml, 3 times) and then with buffer B (60% acetonitrile in 0.1% TFA; 1 ml, once), the supernatant extracted from myocardium was put into a 200-mg Sep-Pak C18 cartridge. Then, the cartridge was washed with buffer A (3 ml, twice), and slowly eluted with buffer B (3 ml, once). After that, the eluate was collected, evaporated, and lyophilized. The purified peptide was processed with the ghrelin EIA kit according to the manufacturer's instructions. Plasma was used directly for ghrelin EIA.

Measurement of Levels of Ghrelin, GHS-R, MCT1, and BNP mRNA

Total RNA from the LV myocardium (~100 mg) was extracted by Trizol reagent (Invitrogen, CA, USA) [17]. Two micrograms of total RNA was reverse-transcribed into single-strand cDNA. PCR was performed in a 0.2-ml tube containing 2 μl cDNA, 1 μl 100 pmol/l solutions of each S and A primers, 12.5 μl Taq Hotstart PCR Master Mix and 8.5 μl distilled water in a total volume of 25 μl. After denaturing at $95\,^{\circ}C$ for 5 min, the PCR for ghrelin, GHS-R, MCT1 occurred in the following conditions: 35 cycles at $98\,^{\circ}C$ for 10 s, $55\,^{\circ}C$ for 30 s, and $72\,^{\circ}C$ for 1 min; and a final extension for 7 min at $72\,^{\circ}C$; 30 cycles at $94\,^{\circ}C$ for 30 s, $55\,^{\circ}C$ for 30 s, and $72\,^{\circ}C$ for 40s; and a final extension for 7 min at $72\,^{\circ}C$; 30 cycles at $94\,^{\circ}C$ for 30 s, $51\,^{\circ}C$ for 30 s, and $72\,^{\circ}C$ for 40 s; and a final extension for 7 min at $72\,^{\circ}C$, respectively. The conditions for PCR for GAPDH were the same as above. After 1% agarose was dyed by ethidium bromide, the bands were separated through electrophoresis. Then, half-quantitation analysis was done using gel imaging and scanning. The independent tests were repeated three times. The optical density of the bands was measured and analyzed using Gel Documentation System (Bio-Rad, Hercules, CA, USA). The ratio of ghrelin (347 bp) mRNA to GAPDH mRNA was considered as the relative amount of

Table 1. Effects of ghrelin on heart weight, cardiac coefficient, and cardiac function in rats with chronic heart failure treated with ISO

	Controls	ISO	Ghrelin (20 μg/kg/day) + ISO	Ghrelin (100 μg/kg/day) + ISO
Body weight (kg)	0.36 ± 0.023	0.29 ± 0.027**	0.33 ± 0.010##	0.33 ± 0.020##
Heart weight (g)	1.14 ± 0.12	1.34 ± 0.16*	1.38 ± 0.18	1.27 ± 0.071#
Herart weight/body weight	3.20 ± 0.30	4.70 ± 0.53**	4.20 ± 0.46	3.90 ± 0.24##
LVEF (%)	66.72 ± 8.05	55.16 ± 8.98*	84.77 ± 5.26##	85.87 ± 5.36##
FS (%)	40.11 ± 3.04	30.86 ± 5.84*	55.47 ± 6.14##	56.62 ± 6.32##
LVEDV (μl)	302.75 ± 88.19	466.46 ± 128.24*	160.53 ± 53.60##	164.72 ± 28.47##
Protein (mg/ml)	2.80 ± 0.41	3.71 ± 0.45*	2.94 ± 0.74##	2.94 ± 0.52##

LVEF, ejection fraction; FS, fractional shortening; LVEDV, left-ventricular end-diastolic dimension.
Data are mean ± SD. $n = 6$.
* $P < 0.05$.
** $P < 0.01$ *versus* control group.
# $P < 0.05$.
$P < 0.01$ *versus* ISO group.

ghrelin mRNA. The relative mRNA amounts of GHS-R1a (199 bp), MCT1 (91 bp), and BNP (256 bp) were determined by the above method.

Western Blot Analysis

Sample preparation

Myocardial tissue (100 mg) was homogenized in 2 ml of buffer A [210 sucrose, 2 EGTA, 40 HEPES, 5 EDTA, and 2 PMSF, pH 7.4, in mM] for two interrupted 15 s bursts, using a homogenizer [29]. Then, 3 ml of buffer B (1.17 M KCl, 58.3 mM tetrasodium pyrophosphate) was added, mixed briefly. After the solution was set on ice for 15 min, and centrifuged (75 min at 230 000 g) at 4 °C, the supernatant was discarded and the pellet was washed with 2 ml of buffer C (10 mM Tris base and 1 mM EDTA, pH 7.4). The pellet was resuspended in 600 μl of buffer C and homogenized for two interrupted 10 s bursts. Two hundred microliters of 16% SDS was added, and then samples were removed from ice, vortex mixed, and centrifuged (20 min at 1100 g) at room temperature. The supernatant was divided into three aliquots and stored at −70 °C for immunoblot detection of MCT1.

Western blot analysis of MCT1

Protein concentrations of the supernatant were determined by the bicinchoninic acid method [30]. Myocardial samples (100 μg) and prestained molecular weight markers were separated on 12% SDS–PAGE (90 V for 3.5 h). Proteins were then transferred to polyvinylidene difluoride membranes (15 V for 35 min), which were blocked with 0.5% nonfat dried milk in Tris-buffered saline (TBS) for 2 h and then incubated with MCT1 antibody (1 : 200 dilution) at 4 °C overnight. After being washed three times for 10 min each in TBS, membranes were incubated with goat anti-rabbit IgG antibody (1 : 3000 dilution) for 1 h. Then, the membranes were washed four times for 15 min each in Tris-buffered saline Tween-20 and underwent enhanced chemiluminescence detection. Autoradiographs were scanned and relative densities were quantified.

Immunohistochemistry

Apexes of hearts were rapidly removed from rats, fixed in 10% buffered formalin for 24 h, dehydrated, and embedded in paraffin by standard procedures. Sections that were 4 μm thick were dewaxed and rehydrated. Sections were then incubated in 3% H_2O_2 to block endogenous peroxidase for 30 min. After being washed three times for 3 min each in phosphate-buffered saline (PBS) and 0.05% Triton X, sections were immersed in 10 mM citrate buffer and submitted to antigen retrieval in a microwave oven (92–98 °C, 15 min). Sections were cooled to room temperature, and then blocked with 10% normal serum. After that sections were incubated in (i) anti-ghrelin serum (diluted 1 : 1000), or anti-GHS-R1a antibody (diluted 1 : 1000), or anti-MCT1 antibody (diluted 1 : 200) at 4 °C overnight; (ii) diluted goat anti-rabbit IgG antibody for 30 min; (iii) 3,3′-diaminobenzidine tetrahydrochloride solution. Between steps, sections were washed three times for 3 min each in PBS, and washed with distilled water after step 3. Negative controls were run routinely in parallel by replacing the primary antibody with PBS. In addition, positive controls for ghrelin, GHS-R1a, and MCT1 immunostaining were assayed.

Statistical Analysis

Statistical analyses involved the application of SPSS v11.5 (SPSS Inc., Chicago, IL, USA). All data presented as mean ± SD among groups were analyzed by one-way analysis of variance and Student–Newman–Keuls test. Linear regression analysis was used to assess the correlation between two variables. A value of $P < 0.05$ was considered statistically significant.

Results

ISO-Induced Myocardial Injury and Inhibition of Cardiac Function

Compared with the control rats, the rats given ISO alone showed markedly higher heart weight, heart coefficient (ratio of heart weight to body weight), and myocardial protein concentrations by 17.54 ($P < 0.05$), 46.88, and 32.52% (both $P < 0.01$), respectively (Table 1). The values for LVEF and FS were decreased significantly by 20.96 ($P < 0.05$) and 29.97% ($P < 0.05$), respectively; and LVEDV was increased by 54.08% ($P < 0.05$). Histological sections of ISO-treated hearts showed widespread subendocardial necrosis, hypertrophia, and abundant fibroblastic hyperplasia with capillary dilatation and leukocytic infiltration (Figure 1). The level of myocardial BNP mRNA increased by 89.13% ($P < 0.01$) in the

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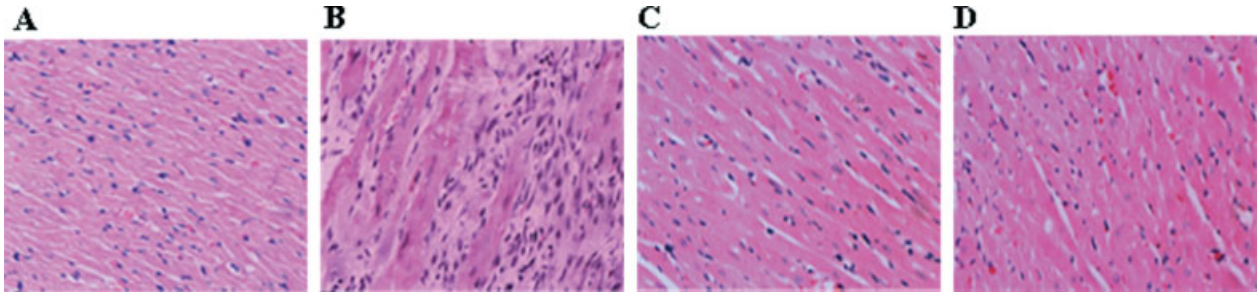

Figure 1. Pathology examination of cardiac apex in rats with chronic heart failure induced by ISO and ghrelin (hematoxylin and eosin staining, ×200). (A) Control group. (B) Group given ISO. (C) Group treated with ISO and ghrelin of low dosage. (D) Group treated with ISO and ghrelin of high dosage.

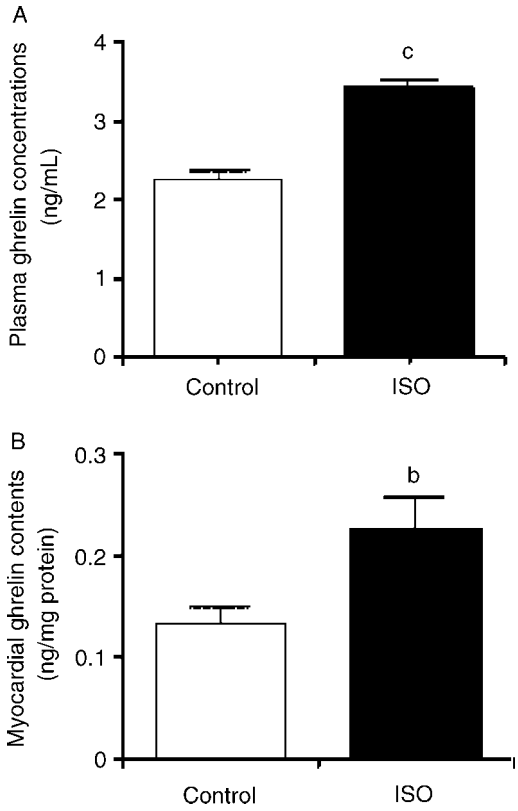

Figure 2. The levels of ghrelin in plasma and left ventricle of rats with chronic heart failure induced by ISO. (A) Levels of plasma ghrelin in control and ISO-treated groups. (B) Levels of myocardial ghrelin in control and ISO-treated groups. $n = 6$. Data are mean ± SD and compared by Student–Newman–Keuls test. $^{b}P < 0.05$, $^{c}P < 0.01$ *versus* control group.

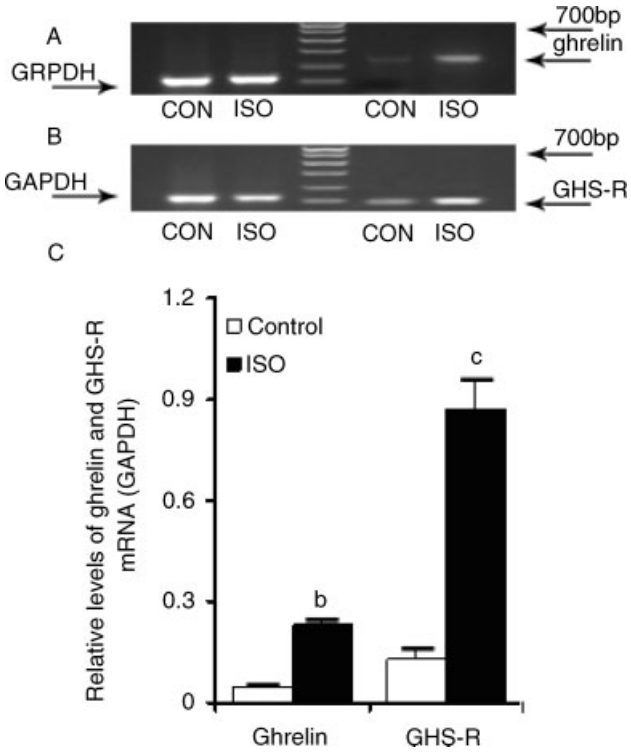

Figure 3. Gene expression levels of myocardial ghrelin, GHS-R1a in left ventricles of rats with CHF induced by ISO. A and B represent myocardial ghrelin, GHS-R1a, and mRNA, respectively. (C) Relative mRNA levels of myocardial ghrelin, GHS-R1a in rats with CHF induced by ISO. $n = 3$. Data (as ratio of target gene and GAPDH gene level) are mean ± SD and compared by Student–Newman–Keuls test. $^{b}P < 0.05$, $^{c}P < 0.01$ *versus* control group.

group given ISO alone compared with that in the control group (Figure 5).

ISO-Induced Increase in Ghrelin/GHS-R Levels in Plasma and Myocardium

EIA detected higher levels of ghrelin in plasma and ventricular tissues of CHF rats by 50.22 ($P < 0.01$) and 72.46% ($P < 0.05$), respectively, than those in the control group (Figure 2). Plasma ghrelin concentration was negatively correlated with LVEF and FS (both $P < 0.05$). Myocardial ghrelin content was negatively correlated with LVEF ($P < 0.01$) and FS ($P < 0.05$). The mRNA relative amount of ghrelin and GHS-R1a, in the myocardium of ISO-treated rats was increased by 172 ($P < 0.05$) and 270% ($P < 0.01$; Figure 3), respectively. The protein expression levels of myocardial ghrelin and GHS-R in the rats given ISO alone were higher by 22 and 20.5% (both $P < 0.01$), respectively, than those in the control group (Figure 4).

The Protective Effects of Exogenous Ghrelin on ISO-Induced Myocardial Injury and Inhibition of Cardiac Function

Compared with the rats given ISO alone, CHF rats treated with ghrelin (100 μg/kg/day) showed that the heart coefficient (ratio of heart weight to body weight) decreased by 20.5% ($P < 0.01$; Table 1). Ghrelin (20 and 100 μg/kg/day) decreased the gross cardiomyocyte protein contents by 25.9 and 26.1%, respectively (both $P < 0.05$). Compared with the group given ISO alone,

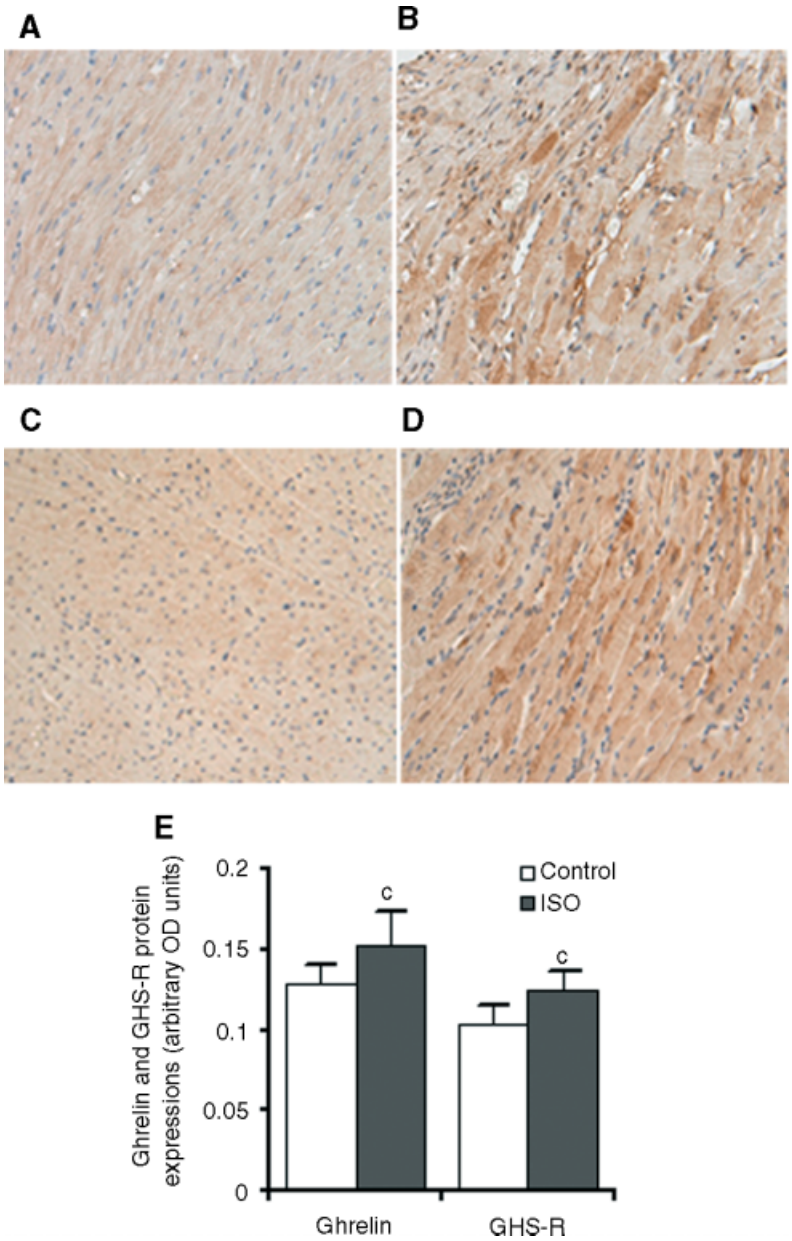

Figure 4. Protein expression levels of myocardial ghrelin and GHS-R1a in left ventricles of rats with CHF induced by ISO (immunohistochemical staining, 200×). (A and B) Myocardial ghrelin protein expressions in control group and ISO-treated group (ghrelin antibody dilution: 1 : 1000). (C and D) Myocardial GHS-R protein expressions in control group and group given ISO (GHS-R antibody dilution: 1 : 1000). (E) Relative amount of myocardial ghrelin and GHS-R1a protein expressions in CHF rats with ISO. $n = 6$. Data (optical density, OD) are mean ± SD and compared by Student–Newman–Keuls test. $^{c}P < 0.01$ *versus* control group.

the two dosages of ghrelin elevated LVEF and FS by 55.67 and 53.69%, 83.47 and 79.72% (all $P < 0.01$), and lowered LVEDV by 183 and 191% (both $P < 0.01$), respectively, suggesting that cardiac dysfunction was ameliorated by ghrelin treatment. Ghrelin treatment (20 and 100 μg/kg/day) decreased mRNA levels of myocardium BNP by 50 and 77.55% (both $P < 0.01$; Figure 5) compared with the group given ISO alone. Histological sections showed that the subendocardial necrosis, capillary dilatation, and leukocytic infiltration were significantly improved with ghrelin treatment (Figure 1).

In 20 days, food consumption and rate of weight gain of the rats given ISO alone were decreased by 63.2 ($P < 0.01$) and 293% ($P < 0.05$) per day, respectively, compared with those in the control group (Figure 6). Compared with the rats given ISO alone, the rats treated with ghrelin (20 and 100 μg/kg/day) increased food consumption by 13.5 and 14.2% (both $P < 0.01$), and rate of weight gain by 95 ($P < 0.05$) and 171% ($P < 0.01$) per day, respectively.

Alleviation of Metabolic Disturbance of Lactate and Glucose in Rats Treated With Ghrelin

Decrease in the levels of lactate in blood and myocardium

Compared with the control group, the rats given ISO alone showed increased lactate concentrations in blood by 159 and in myocardium by 73.6% (both $P < 0.01$), respectively. Ghrelin

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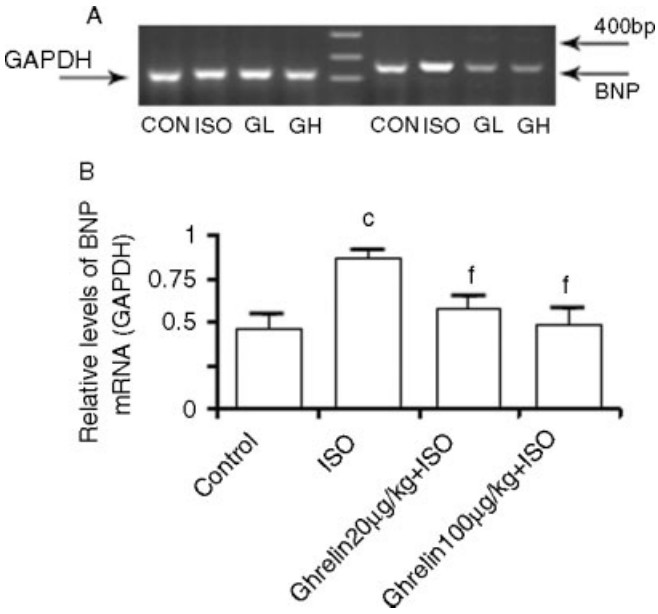

Figure 5. Effects of ghrelin on myocardial BNP mRNA expressions in left ventricles of rats with CHF induced by ISO. (A) Lanes 1–4, myocardial GAPDH mRNA expressions; lanes 5–8, myocardial BNP mRNA expressions. (B) Relative amount of myocardial BNP mRNA expressions in rats with CHF given ghrelin. $n = 6$. Data (as ratio of level of target gene to that of GAPDH gene) are mean ± SD and compared by Student–Newman–Keuls test. $^{c}P < 0.01$ *versus* control group and $^{f}P < 0.01$ *versus* ISO group.

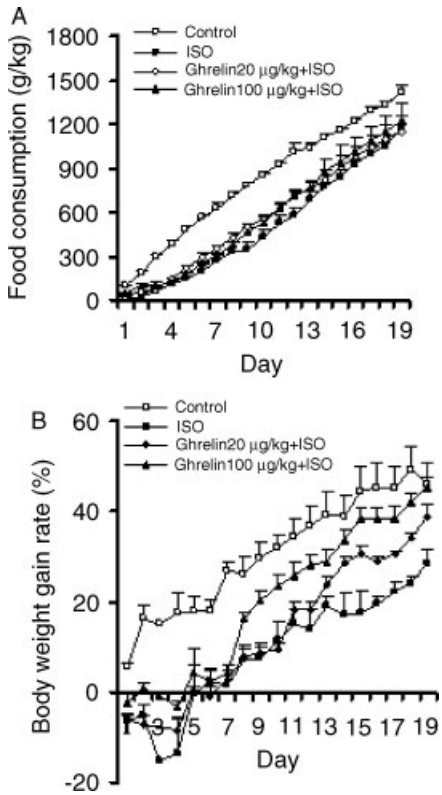

Figure 6. Effects of ghrelin on food consumption and rate of body weight gain in rats with chronic heart failure induced by ISO. (A) Food consumption. (B) Rate of body weight gain. $n = 6$. Data are mean ± SD and compared by Student–Newman–Keuls test.

administration (20 and 100 μg/kg/day) significantly decreased lactate levels in blood by 156 and 196% (both $P < 0.01$) and in myocardium by 32.1 ($P < 0.05$) and 48.7% ($P < 0.01$), respectively, compared with the rats given ISO alone (Figure 7).

Increase in myocardial MCT1 mRNA and protein expressions in the rats treated with ghrelin

The rats given ISO alone showed higher levels of myocardial MCT1 mRNA and protein expressions by 52.9 and 29% (both $P < 0.01$), respectively, than the rats in the control group (Figures 8–10). The rats treated with ghrelin (20 and 100 μg/kg/day) showed still higher levels of MCT1 mRNA by 73.3 and 102% (both $P < 0.01$), and protein expressions by 26.2 and 42.4% (both $P < 0.01$), respectively, than the rats given ISO alone.

Increase in the levels of plasma glucose and myocardial glycogen in the rats treated with ghrelin

Compared with the rats in the control group, the rats given ISO alone showed the decrease in plasma glucose concentration by 24.4% ($P < 0.05$), and in content of myocardium glycogen by 71.7% ($P < 0.01$). Ghrelin injections at 20 and 100 μg/kg/day significantly increased plasma glucose concentrations by 49.7 and 50.8% (both $P < 0.01$), and myocardial glycogen contents by 40.5 and 51.7% (both $P < 0.01$), respectively. Myocardial glycogen content in the rats treated with ghrelin 100 μg/kg/day was higher than that in those given ghrelin 20 μg/kg/day ($P < 0.01$) (Figure 11).

Discussion

Ghrelin is an important hormone in regulating energy metabolism in the body. Studies have shown that ghrelin may have beneficial effects on left ventricle function and energy metabolism in CHF through GH-dependent mechanisms due to the hemodynamic and anabolic effects of GH/IGF-1 [9]. A recent study showed that ghrelin could induce a positive energy balance and weight gain by stimulating appetite, decreasing fat oxidation and increasing carbohydrate utilization through a GH-independent mechanism [7,8]. The ghrelin receptor is widely distributed in cardiovascular tissues, such as the myocardium, aorta, coronary arteries, and veins [10], which suggest that ghrelin could directly exert effects on cardiovascular tissue through GH-independent mechanism. Ghrelin has various effects on cardiovascular system, including amelioration of hemodynamic dysfunction, activation of the GH/IGF-1 axis, prevention of the formation of lipid peroxidation, and inhibition of endothelin 1, maintaining cardiovascular homeostasis [13,14,16,32–35]. However, the cardioprotective mechanisms of ghrelin, in particular, how ghrelin attenuates the development of cardiac cachexia with end-stage CHF are still unclear.

The model of heart injury induced by ISO has been used to show the cardioprotective role of ghrelin. ISO is a β-adrenergic receptor agonist that functions in the heart predominantly by stimulating β_1-adrenergic receptors. ISO of high dosage is commonly applied to induce myocardial injury through catecholamine intoxication and oxidative stress [25]. In this study, subcutaneous injections of ISO for 10 days resulted in significant myocardial injury and cardiac dysfunction, as was found previously [25], and the level of ghrelin in plasma and myocardium was increased, with a negative correlation between ghrelin content and cardiac function in rats, as similar reports in patients [36]. Exogenous administration

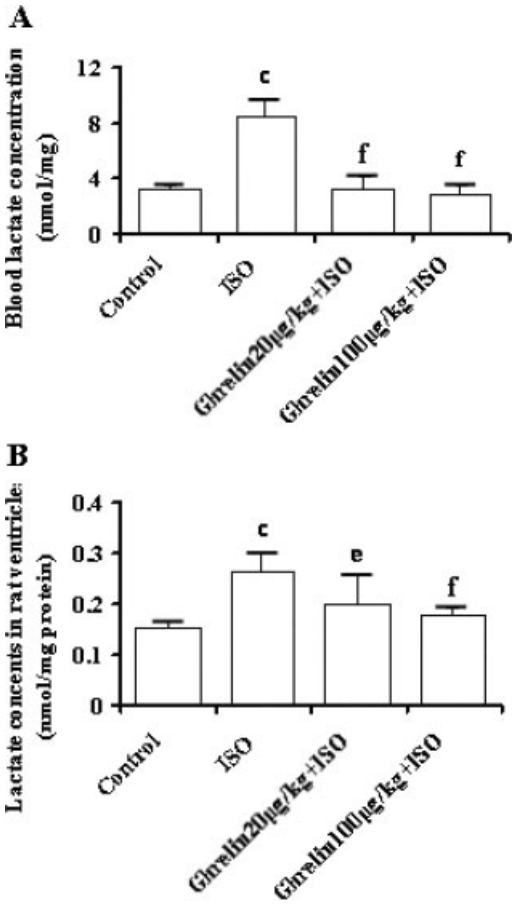

Figure 7. Effects of ghrelin on lactate levels in blood and myocardium in rats with CHFinduced by ISO. (A) Blood lactate concentrations. (B) Myocardial lactate contents. $n = 6$. Data are mean $\pm$ SD and compared by Student–Newman–Keuls test. $^{c}P < 0.01$ *versus* control group, $^{e}P < 0.05$, and $^{f}P < 0.01$ *versus* ISO group.

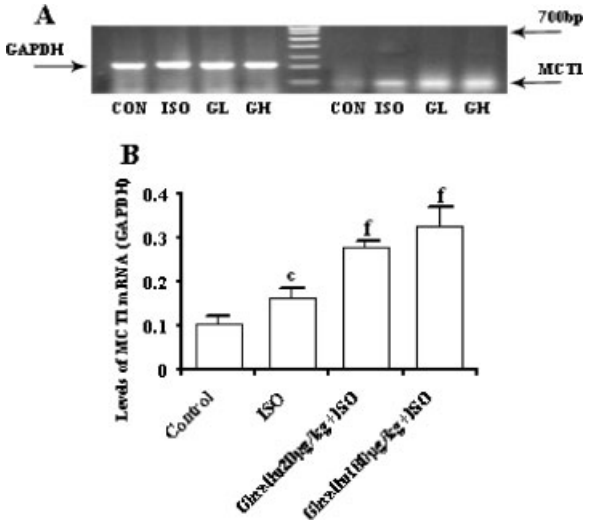

Figure 8. Effects of ghrelin on myocardial MCT1 mRNA expressions in left ventricles of rats with CHF induced by ISO. (A) Lanes 1–4, myocardial GAPDH mRNA expressions; lanes 5–8, myocardial MCT1 mRNA expressions. (B) Relative amount of myocardial MCT1 mRNA in rats with CHF given ghrelin. $n = 6$. Data (as ratio of level of target gene to that of GAPDH gene) are mean $\pm$ SD and compared by Student–Newman–Keuls test. $^{c}P < 0.05$ *versus* control group and $^{f}P < 0.01$ *versus* ISO group.

of ghrelin attenuated the disturbance of hemodynamics and improved myocardial injury in rats treated with ISO. Myocardial apomorphosis, necrosis, and massive fibrosis were effectively ameliorated, LVEF was increased, and the levels of LVEDV and myocardial BNP mRNA were decreased after ghrelin treatment. These findings suggested that ghrelin could be an endogenous protective factor against myocardial damage.

The mechanisms by which ghrelin improves myocardial function and metabolism following ISO damage are still unclear. Various agents that affect the endocrine system and metabolism participate in the regulation of the expression, synthesis, and secretion of ghrelin. The levels of ghrelin mRNA in blood and stomach are increased by fasting, hypoglycemia or weight loss, and decreased by food intake, obesity, or hyperglycemia [37]. As a marker of augmented catabolism, ghrelin is negatively correlated with body weight, and high-ghrelin levels were found in cachectic patients with ischemic cardiac disease [38], cancer [39], urinemia [40] or obstructive lung disease [41]. Cardiac cachexia, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage CHF and is a strong independent risk factor of mortality in patients with CHF [42,43]. Nagaya *et al*. [38,44,45] demonstrated the increase in plasma ghrelin level in patients with cardiac cachexia, which has some relations with the decrease in body mass index and the increase in the levels of plasma tumor necrosis factor-α (TNF-α) and GH. These results suggest that increased plasma ghrelin level may represent a compensatory mechanism under conditions of anabolic/catabolic imbalance in cachectic patients with CHF. In this study, rats with CHF induced by ISO showed significant decreases in body weight and food intake, which suggests the presence of cardiac cachexia. Exogenous administration of ghrelin stimulated feeding and increased weight, near the levels of the control group, which suggests that ghrelin could ameliorate the development of cardiac cachexia in CHF rats.

However, the mechanisms by which ghrelin ameliorated cardiac cachexia in rats with CHF remain to be elucidated. Recent studies showed that ghrelin administration in both rodents and humans stimulated appetite and increased food intake, thus inducing a positive energy balance and obesity by decreasing fat oxidation through a GH-independent mechanism [7,8,46]. As the ultimate anabolic hormone [5], ghrelin directly regulates the metabolism of adipocytes. In rat adipocytes, ISO-stimulated lipolysis was significantly reduced by ghrelin treatment in a dose-dependent manner [47]. Ghrelin stimulated the activation of glycerol-3-phosphate dehydrogenase and the differentiation of rat preadipocytes by upregulating the mRNA level of peroxisome proliferator-activated receptor-γ_2 [47]. These findings in rats indicate that the administration of ghrelin may be beneficial for cachexia with CHF via regulation of systemic and cellular metabolisms.

However, it has not been reported whether ghrelin can improve cardiac cachexia by regulating myocardial metabolism. In this study, we demonstrated that ghrelin increased plasma glucose concentrations and myocardial contents, thus attenuating hypoglycemia and myocardial depletion in cachectic rats with CHF. Ghrelin may have a metabolism-regulating effect on the whole body and the heart as well. The mechanisms by which ghrelin regulates glucose homeostasis may involve insulin secretion and peripheral insulin sensitivity. Sun *et al*. [48] demonstrated that ablation of ghrelin in mice(ghrelin-/-) augmented insulin secretion in response to glucose challenge and increased peripheral insulin

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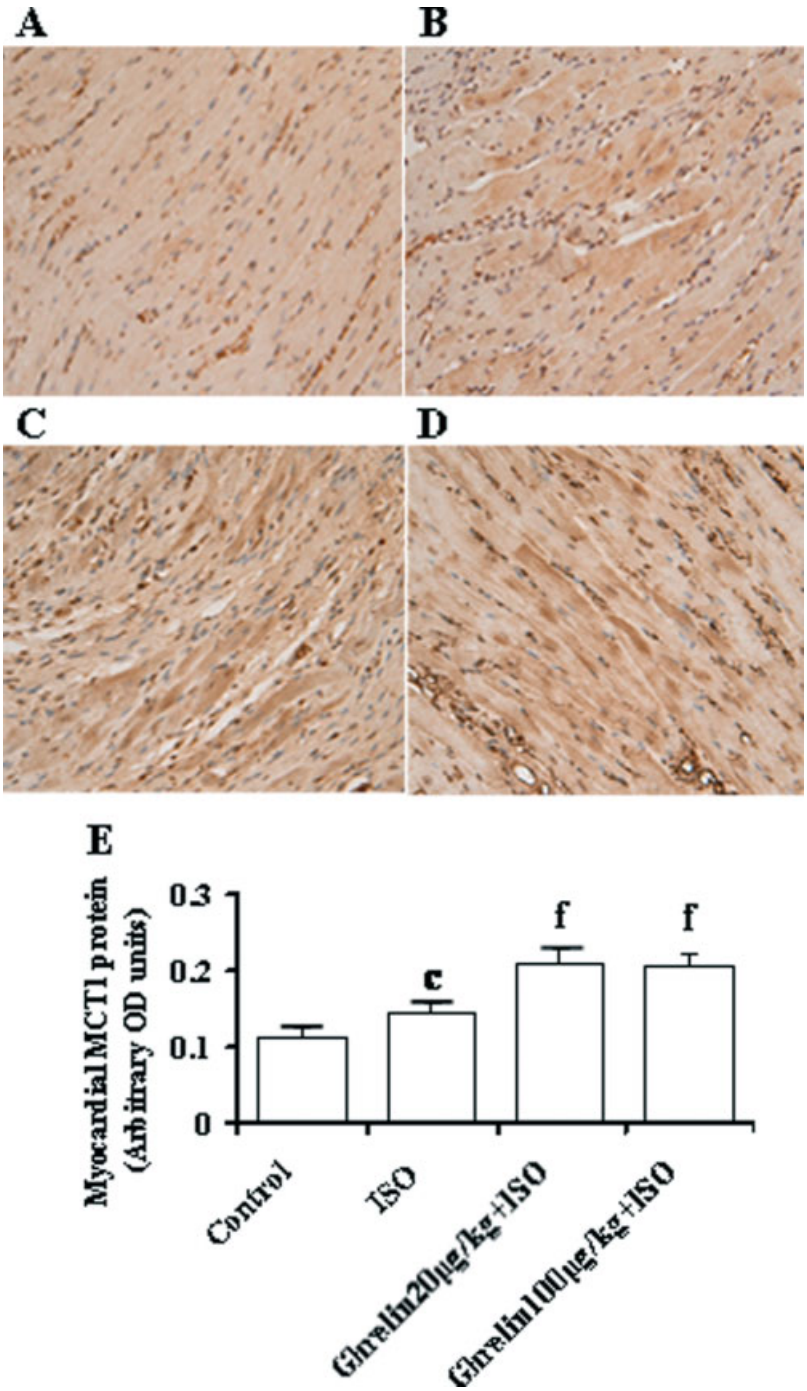

Figure 9. Effects of ghrelin on myocardial MCT1 protein expressions in rats with CHF induced by ISO (immunohistochemical staining, 200×). (A and B) Myocardial MCT1 protein expressions in control group and ISO-treated group. (C and D) Myocardial MCT1 protein expressions in groups treated with ISO and ghrelin of low and high dosages. (E) Relative amount of myocardial MCT1 protein expression in CHF rats given ghrelin. $n = 6$. Data (optical density, OD) are mean ± SD and compared by Student–Newman–Keuls test. $^{c}P < 0.01$ *versus* control group, $^{f}P < 0.01$ *versus* ISO group.

sensitivity. In addition, more and more evidence has shown that ghrelin can directly regulate glucose metabolism in tissues and cells. Barazzoni *et al*. [49] reported that ghrelin induced the glycogenic gene expression via inhibiting the activation of AMP-activated protein kinase in liver. Murata *et al*. [50] showed that ghrelin stimulated phosphoenolpyruvate carboxykinase mRNA expression to upregulate gluconeogenesis through suppressing Akt kinase activity and stimulating the IRS-1–GRB2–MAPK pathway in hepatoma cells.

Normal cardiac energy metabolism is characterized by various metabolic fuels, especially lactate, as energy substrate [1,2,51]. Jennings *et al*. [52] demonstrated that ATP depleted and lactate accumulated in the ischemic myocardium, which, in turn, was responsible for the expansion of myocardial tissue necrosis. Here, we found that ghrelin significantly decreased hyperlactacidemia and ischemic myocardial lactate accumulation induced by large doses of ISO. Myocardial lactate accumulation was mainly transported by MCTs into or out of the cardiocytes and oxidated in

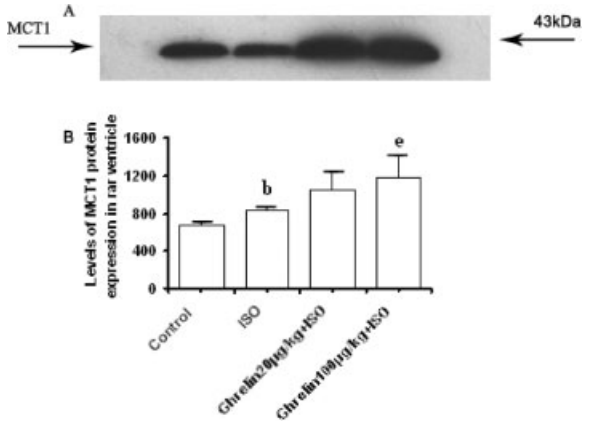

Figure 10. Effects of ghrelin on myocardial MCT1 protein expression in rats with CHF given ISO (Western blot analysis). (A) MCT1 protein expressions ventricular tissues. (B) Relative amount of myocardial MCT1 protein expressions in rats with CHF given ghrelin. CON, control group; ISO, ISO-treated group; GL, group treated with ISO + ghrelin of low dosage; GH, group treated with ISO + ghrelin of high dosage. $^{b}P < 0.05$ *versus* control group, $^{e}P < 0.05$ *versus* ISO group.

the mitochondria. Jóhannsson *et al*. [53] expressed that the lactate transport capacity into cardiocytes was greatly increased through a higher rate of MCT1 synthesis and more transit of MCT1 molecules to the cell surface in CHF rats with post-myocardial infarction. Murry *et al*. [54] reported that higher myocardial MCT1 expression and activity enhanced the lactate-proton cotransport system (H^+/L^-) of lactate and H^+ across the plasma membrane under hypoxic or ischemic conditions. In our study, ghrelin further upregulated MCT1 expression levels, as indicated by increased myocardial MCT1 protein expressions in both damaged and non-damaged parts of the left ventricles in rats with CHF. Our results suggest that upregulated MCT1 protein expression could be closely associated with increased oxidative clearance of myocardial lactate.

Homeostasis of myocardial energy metabolism is of importance in maintaining the normal heart performance. However, in advanced end-stage CHF, the metabolic substrate preference has already switched to reduced fatty acid oxidation and accelerated glucose oxidation [3,4]. A recently emerged concept of metabolic remodeling is that cardiac substrate metabolism shift gives rise to alterations in the metabolic pathway following cardiac structural abnormalities and contractile dysfunction [5].

These findings raise the possibility that improving of metabolic remodeling partly by directly modulating myocardial energy

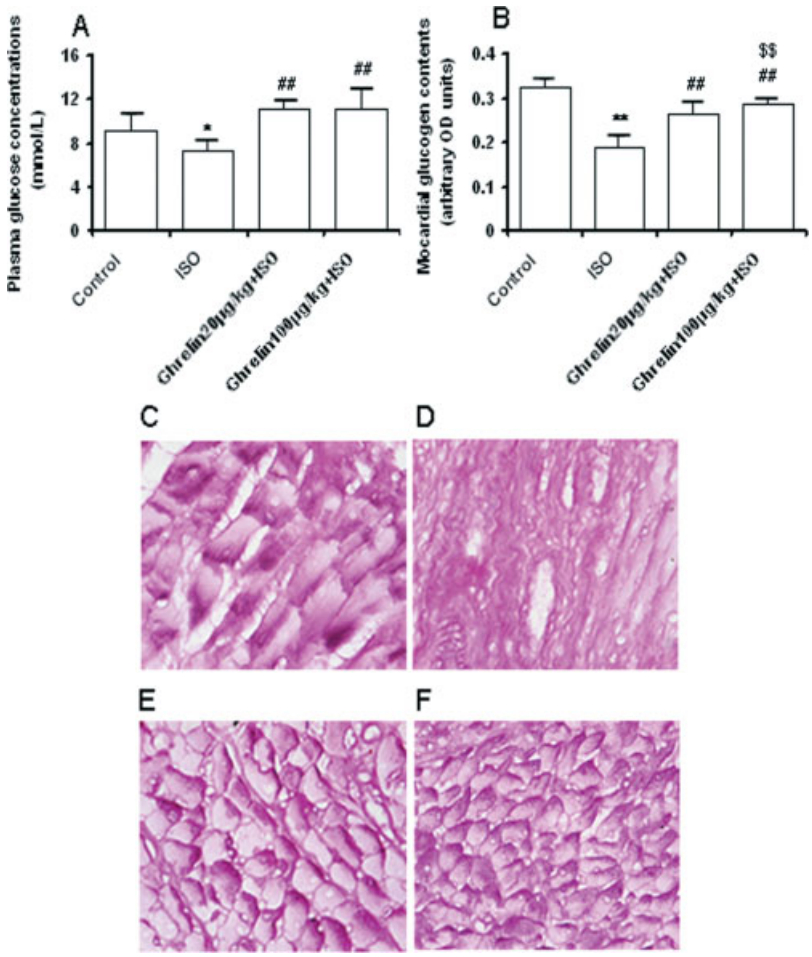

Figure 11. Effects of ghrelin on levels of plasma glucose and myocardial glycogen in rats with CHFinduced by ISOand treated with ghrelin. (A) Plasma glucose concentration. (B) Relative content of myocardial glycogen in rats with CHF given ghrelin (PAS staining, ×400). (C and D) Myocardial glycogen expressions in control group and ISO-treated group. (E and F) Myocardial glycogen expressions in groups treated with ISO and ghrelin of low and high dosages. $n = 6$. Data (optical density, OD) are mean ± SD and compared by Student–Newman–Keuls test. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* control, $^{\#\#}P < 0.01$ *versus* ISO, $^{\$\$}P < 0.01$ *versus* ISO + ghrelin of low dosage.

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metabolism may be an effective therapeutic strategy in cachectic patients with end-stage CHF. Therefore, exogenous administration of ghrelin could be a new therapeutic approach to the treatment of severe CHF because it can attenuate the myocardial metabolic disorders.

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