Endogenous ghrelin increases in adriamycin-induced heart failure rats

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ABSTRACT. Evidence has indicated that plasma ghrelin was elevated in chronic heart failure (CHF) patients with cachexia. The present report studied whether pathophysiologic increment of endogenous ghrelin levels existed in the progression of adriamycin (ADR)-induced CHF, then the possible compensatory mechanism by which the changes were induced and the relationship between active ghrelin, cardiac function and energy reserve in heart failure (HF) rats were explored. Cardiac function, high energy phosphates (HEP) content, and ghrelin levels in plasma and myocardium were measured at 4 days, 1, 2 and 3 weeks after the last injection of ADR, after which correlation analysis was performed between these markers in HF rats. Results showed that cardiac function decreased early, then was significantly restored and worsened at 3 weeks accompanied by the decrease of myocardial ATP content. Plasma ghrelin level increased significantly at each time point while myocardial ghrelin level increased transiently, then was restored followed by increased oxidative stress status and apoptosis in the weakening heart. Moreover, correlation analysis indicated that the markers of cardiac function and HEP were positively correlated to the endogenous ghrelin levels at the HF stage. This study indicated that the increase of endogenous ghrelin levels during the progression of the HF induced by ADR represent a compensatory self-protective effect by improving cardiac function and retaining myocardial energy reserve; this may be closely linked to anti-oxidative and anti-apoptosis mechanisms through regulating myocardial mitochondria function by ghrelin; but further investigations are necessary.

INTRODUCTION
Cardiovascular disease and malignant tumor have become two main problems endangering health in the 20th century. Though the application of anti-neoplastic agents with a very broad anti-tumor spectrum has already markedly improved cancer survival, the use of anthracyclines is limited by a dose-dependent cardiotoxicity (1), so it is urgent to prevent and cure adriamycin (ADR)-induced chronic heart failure (CHF). Ghrelin is a new kind of brain-gut peptide. Plasma ghrelin was significantly higher in CHF patients with cachexia than those without cachexia (2), and chronic sc administration of ghrelin improved left-ventricular (LV) dysfunction and attenuated the development of LV remodeling and cardiac cachexia in rats with CHF (3), but the detailed mechanism is still not clear. Considering ghrelin-induced positive energy effects, increased ghrelin may represent a compensatory effect under catabolic-anabolic imbalance in cachectic patients with CHF. In this study, the changes of endogenous ghrelin level in plasma and myocardium during the progression of CHF induced by ADR were observed and the possible involved mechanisms were explored.

MATERIALS AND METHODS
Experimental protocol
Male Wistar rats (200-250 g) were obtained from the Experimental Animal Center of Sun Yat-sen University (Clean grade, Certificate No SCXK2004-0011) and randomly assigned for all of the experiments. Animal use was in accordance with the Chinese council on animal care guidelines. All rats were randomly divided into control group and model group [heart failure (HF) group]. Following the ADR-induced CHF procedure previously described by Zhao M. et al. (4), 4 mg/kg of ADR from Wanle
Pharmaceuticals Co. (Shenzhen, China) were given to the model group every 5 days; three injections were performed through the tail vein. An equal volume of normal saline was given to the control group. Cardiac catheterization was performed after the last injection, and rats were then sacrificed at 4 days, 1, 2 and 3 weeks, respectively, during the next observation period in the HF group. The control group was sacrificed to establish the initial values in HF group. Subsequently, blood was collected and the hearts were immediately removed, rinsed in saline, and tissues were quickly cut into very small pieces. Ventricles pieces were then separated into two parts, one part for the assay of antioxidant enzyme activity and lipid peroxidation, the rest for the determination of ghrelin, ATP, phosphocreatine (PCr) and DNA ladder. In the sub-HF group, rats were observed for 3 weeks for general appearance, behavior and mortality. Body weight (BW) was recorded before killing only at the end of the next 3 weeks, then heart, liver, spleen and lung were removed quickly and weighed, with calculation of viscera: body weight (the ratio between organ weight and body weight, g·g⁻¹). Last hearts were stored in liquid nitrogen, formalin or glutaraldehyde till analysis, and the same protocols were applied to control rats.

_Determination of cardiac function_

Rats were anesthetized with sodium pentobarbital (45 mg/kg, ip), and then underwent tracheotomy, endotracheal intubation and mechanical ventilation with room air (the respiratory rate was 60-70 breaths/min and the tidal volume was 5 ml). A transverse incision was then performed on the right side of the chest wall in the second intercostal space just below the axillary area (5). The pectoral musculature was carefully opened by following the fiber orientation to avoid damage. An ultrasonic perivascular flow probe (2 mm; MF-1200, Japan) was placed around the ascending aorta just above the coronary arteries and connecting wires emerged through the back of the rat. After the measurement of cardiac output (CO), the right carotid artery was intubated with PE-50 tubing and connected with a blood pressure analyzer (AP-611G, RM-6000 System, Japan). After mean arterial pressure and heart rate were recorded, this PE-50 tubing was advanced into the left ventricle and connected to a heart performance analyzer (AT-601G) to monitor and record maximum LV developed pressure increase rate (dp/dtmax, mmHg/s).

_Radioimmunoassay (RIA) for endogenous active ghrelin_

_Prepaiation of plasma samples_

Blood samples were collected with EDTA·Na₂ (2 mg/ml) and aprotinin (Amresco, U.S.A., 500 IU/ml). Plasma was diluted with an equal volume of 0.9% saline after centrifugation at 4 °C, then stored at −80 °C until use. According to the manufacturer’s specifications, the sample did not require extraction.

_Preparation of tissue samples_

Fresh tissue samples from rats were quickly frozen and stored at −80 °C until use. Each tissue was diced and boiled for 7 min in 5-fold volume of water to inactivate intrinsic proteases. The solution was adjusted to 1 mol/l acetic acid after cooling, and the tissue was homogenized with a Polytron mixer. The supernatant was obtained after centrifugation at 10,000 rpm for 30 min, and then lyophilized.

**RIA**

RIA kit of Phoenix Pharmaceuticals in USA (sensitivity: IC₅₀, 11.45 pg/tube, the intra-assay and the inter-assay coefficient of variation were both no >9%. The minimum and maximum sensitivities of these kits were 1 and 128 pg/ml, respectively, and 100% cross-reactivity with ghrelin, Des-Octanoyl-Ser² ghrelin and ghrelin (17-28) in rat. Based on the above controls, the plasma eluate was diluted by RIA buffer (1: 3-5) and subjected to ghrelin RIA. The lyophilized material was dissolved in RIA buffer and ghrelin RIA were performed.

**High performance liquid chromatography (HPLC) for ATP and phosphocreatine (PCr)**

High-energy phosphates (HEP) assay was performed according to Hallstrom S. et al. (6) with some changes. The hearts were removed and frozen immediately in liquid nitrogen. One hundred mg tissue was homogenized with 1.2 ml of 0.5 mol/l perchloric acid in a cooled mill. After thawing and centrifugation (10,000 rpm) at 4 °C, the supernatant was neutralized with 0.5 mol/l potassium hydroxide. HPLC (Agilent chemstation for LC, Germany) was carried out using a BDS C-18 (2) 5 μm column (300 x 4.6 mm; Hypersil, USA) with a stainless-steel frit filter (2 μm). Detector signals (absorbance at 254 nm for PCr and 214 nm for ATP) were recorded automatically. LC/MS System (Agilent) was used as controller for data requisition and analysis. A flow rate of 0.5 ml/min was used and ATP as well as PCr were identified by comparing their retention times relative to the retention time. The peak identity was confirmed by mixing tissue extracts and ATP (Boehringer GmbH, Germany) and PCr (Amresco, U.S.A.) standards, before HPLC separation. For each, the peak area was calculated by integrating the area under the curve and the mass was calculated using a standard curve derived from the standards, respectively.

**Separation of mitochondria and cytosol**

The heart tissue was placed in ice-cold buffer. The mitochondria fraction was separated according to Murfit et al. (7). The heart was sliced into several pieces and immersed in ice-cold isolation media consisting of 0.8% NaCl, 0.01 mol/l sucrose and 0.01 mol/l Tris-HCL (PH 7.4). Two hundreds mg minced tissue was homogenized with 2 ml isolation media, then 10% tissue homogenate was centrifuged at 2,000 rpm for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 10,000 rpm for 10 min, the mitochondria pellet was isolated and washed by suspending in the media, centrifuged again and the mitochondria pellets were re-suspended and stored at −80 °C for analysis.

**Biochemical analysis of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content**

The heart tissue homogenate, mitochondria suspension and plasma were prepared as described before (4). Briefly, the enzyme activity was assayed by inhibition of pyrogallol auto-oxidation (8, 9). We measured the absorbance at 420 nm at 1-min intervals for 5 min to monitor the auto-oxidation of pyrogallic acid. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol auto-oxidation with a known SOD activity. MnSOD and CuZnSOD activities were differentiated by measuring the enzyme activity in the presence of potassium cyanide (KCN). Measurement of lipid peroxidation by determination of thiobarbituric acid-reactive substances (TBARS) was performed as described previously (10).