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Therapeutic effects of ghrelin on endotoxic shock in rats

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Abstract

We investigated the effects of ghrelin in a rat endotoxic shock model, and also observed the direct role of endotoxin on ghrelin generation in gastric mucosa. About 55% (11/20) of rats treated with lipopolysaccharide (5 mg/kg i.v.) alone died within 24 h of endotoxin injection. However, administration of ghrelin either at the same time as lipopolysaccharide injection (early treatment) or 12 h after lipopolysaccharide injection (late treatment) significantly decreased the mortality rate and ameliorated the hypotension seen in rats with endotoxic shock. Early and late treatment with ghrelin increased markedly the plasma glucose concentration and decreased the plasma lactate concentration. Early treatment with ghrelin attenuated significantly the deficiency in myocardial ATP content, but late treatment with ghrelin had no effect on myocardial ATP content. The plasma ghrelin level was significantly increased in the rats with endotoxin shock, and it increased further after ghrelin administration. Exposure of rat gastric mucosa in vitro to lipopolysaccharide (1.0 to 100 μg/ml) triggered the release of ghrelin from mucosa tissue in a dose- and time-dependent manner, meaning that lipopolysaccharide stimulated directly gastric mucosa to synthesize and secrete ghrelin. The results suggest that ghrelin could have therapeutic value for endotoxic shock.

Keywords: Endotoxic shock; Hemodynamics; Ghrelin

1. Introduction

Ghrelin, a novel growth hormone-releasing peptide, was recently isolated from rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (Kojima et al., 1999). The discovery of ghrelin ends one mystery and hopefully initiates many new intrigues, intrigues about how the gastrointestinal tract and nutritional intake exert an influence on the hypothalamic-pituitary unit in the regulation of growth hormone secretion and appetite. Ghrelin specifically stimulates the release of growth hormone from the anterior pituitary gland in rats and humans. Ghrelin regulates neuropeptide Y expression in the arcuate nucleus of the hypothalamus and stimulates the hypophysis to secrete growth hormone, which plays an important role in energy balance regulation (Asakawa et al., 2001; Nakazato et al., 2001; Shintani et al., 2001). Recently, ghrelin receptors were detected in cardiovascular tissues (Papotti et al., 2000), indicating that ghrelin may possess important regulatory roles in peripheral tissues as well as in the central nervous system. Endotoxin shock is accompanied by marked cardiovascular dysfunction (Tang and Liu, 1996). There are no reports of changes in plasma ghrelin level following endotoxin administration or of the effects of exogenous ghrelin on cardiovascular function in endotoxin shock. The purpose of the present study was to examine the effects of ghrelin on the hemodynamic and metabolic consequences of endotoxin shock in the rats. We also examined the effects of endotoxin on plasma concentrations of ghrelin and on the release of ghrelin from gastric mucosa in vitro.

2. Materials and methods

2.1. Reagents and experimental instruments

Endotoxin (lipopolysaccharide *w. E. coli*, lot number 3123.25) was purchased from Difco Laboratories Detroit

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MI, USA. Glucose oxidase kit, lactate kit, and ATP assay kit were all purchased from Sigma (MO, USA). Synthetic rat ghrelin and ghrelin radioimmunoassay kit (lot no. 416753) were produced by Phoenix Pharmaceuticals, (CA, USA). The other reagents were of analytical purity. Sep-Pak C18 cartridge was the product of Milford (MA, USA). The PE-50 cannula was produced by Intramedic (NY, USA). The pressure transducer was produced by Gould P231D, USA. The physiological polygraph was a product of San ei 2G66, Janpen.

2.2. Preparation of endotoxic shock model

All animal experiments in this study were performed with the approval of the Animal Care Committee of Peking University. Fifty-four male Sprague–Dawley rats, weighing about 260 ± 10 g, were obtained from the Experimental Animal Center of Peking University (Clean grade, Certificate No SCXK 11-00-0008). Animals were randomly divided into an endotoxic shock group ($n = 20$), an early ghrelin-treated group ($n = 12$), a late ghrelin-treated group ($n = 15$) and a normal group ($n = 7$). Except for rats in the normal group, all rats were injected with lipopolysaccharide (5 mg/kg, in 2 ml of 0.9% NaCl) through a tail vein. All rats were given 0.9% NaCl at a dose of 5 ml/kg by intravenous injection after lipopolysaccharide injection, and intraperitoneal injection of 0.9% NaCl at a dose of 5 ml/kg again 12 h after lipopolysaccharide injection. However, 0.9% NaCl containing ghrelin 10 nmol/kg was given by intravenous injection in the early ghrelin-treated group, and by intraperitoneal injection in the late ghrelin-treated group, respectively. At 24 h after the first injection, hemodynamic parameters including heart rate, mean arterial blood pressure, $+LVdp/dt_{max}$, $-LVdp/dt_{max}$ and left ventricular end-diastolic pressure were measured in all surviving rats. Arterial blood was collected and anticoagulated with heparin to measure concentrations of plasma glucose, lactate and ghrelin. The heart was then removed for myocardial-ATP content assay. The mortality rate in all rats was also recorded.

2.3. Measurement of hemodynamic parameters

Hemodynamic parameters were determined with a polygraph via the femoral artery and an intraventricular cannula (Zimmer, 1983). The rats were anesthetized by intraperitoneal injection of urethane (1 g/kg). Two PE-50 tubings were inserted into the left femoral artery and the left carotid artery, respectively. The latter was further inserted into the left ventricle. All catheters were filled with 0.9% NaCl containing 10 kU/l of heparin. The ventricular and arterial catheters were separately connected to pressure transducers. Heart rate, mean arterial blood pressure, $+LVdp/dt_{max}$, $-LVdp/dt_{max}$ and left ventricular end-diastolic pressure were recorded on a microcomputer-controlled physiological polygraph 20 min after insertion of the catheters.

2.4. Assay for plasma lactate, plasma glucose and myocardial ATP content

Plasma was prepared by centrifugation of whole blood at $2900 \times g$ for 10 min at 4 °C. Plasma glucose was determined by glucose oxidase method, and plasma lactate was assayed by colorimetric method. The heart was promptly frozen by freeze-clamping with aluminum clamps precooled in liquid nitrogen, and the tissue was pulverized with a pestle and mortar precooled in liquid nitrogen. Myocardial ATP content was assayed spectrophotometrically using glucose-6-phosphate dehydrogenase and hexokinase methods.

2.5. Gastric mucosa culture in vitro

Eight male Sprague–Dawley rats weighing 260 ± 10 g were fasted for 12 h with free access to tap water. The stomach was obtained by laparotomy after anesthesia was induced with an intraperitoneal injection of urethane (1 g/kg). The stomach was opened along the delimitation of the gastric fundus and the corpus. The gastric fundus was washed in ice-cold Krebs–Henseleit buffer (in mM: 118.0 NaCl, 4.74 KCl, 0.93 KH_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.3 $CaCl_2$, 10.0 glucose, pH 7.4), and the gastric fundus mucosa was then dissected out and cut into strips (1–3 mm^3) using a sterile razor blade in a sterile tissue culture dish. Tissue strips were incubated in vitro in 1-ml Krebs–Henseleit buffer under 95% O_2 and 5% CO_2 for 2, 4 and 6 h at 37 °C with 1, 10 and 100 μg/ml of lipopolysaccharide, respectively. After the end of incubation, medium and gastric mucosa were collected and boiled in 5 volumes of 1 M acetic acid for 10 min to inactivate intrinsic proteases. Boiled tissue was extracted with 1-ml normal saline with a Polytron mixer for 2 min, and the tissue protein content was determined with the Coomassie brilliant blue method. The homogenate was centrifuged at $2500 \times g$ for 20 min and the supernatant was collected for radioinnunoassay (RIA) assay.

2.6. Radioimmunoassay for ghrelin level

Blood samples were collected into tubes containing 1 g/l of ethylene diamine tetraacetate-2 Na and 500 MIU/l of aprotinin. Plasma was prepared by centrifugation at $2900 \times g$ for 10 min at 4 °C and stored at −80 °C until use. Prepared plasma, incubation medium and homogenate of gastric mucosa were acidified with 60 μl of 1 M acetic acid, diluted with 5 ml of saline solution, and then loaded onto a Sep-Pak C18 cartridge equilibrated with saline. After the cartridge was washed with 2.5 ml of saline and 10% acetonitrile in 0.1% trifluoroacetic acid, the absorbed material was eluted with 2 ml of 50% acetonitrile in 0.1% trifluoroacetic acid. The extracted material was lyophlized and subjected to RIA for ghrelin. The sensitivity IC_{50} was 6.95 pmol/tube, and binding was 35% in this assay. There was 100% cross-reactivity with rat and human ghrelin. All

assays included 12 plasma control samples from common stock solutions, which were frozen in aliquots at the beginning of the study, in order to normalize each test for inter-assay variability. Based on these controls, the intra-assay coefficient of variation was 8.7% and the interassay coefficient of variation was 14.6% ($n=10$). No cross-reactivity was seen with leptin, orexin A and B, neuropeptide Y, galanin or vasoactive intestinal polypeptide, assessed with doubling dilutions from 100 to 1 ng/ml.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. One-factor analysis of variance (ANOVA) was performed when more than two groups were compared, and when significant ($P<0.05$) Student–Newman–Keuls test was applied to test for differences between individual groups. X^2 test was used for comparisons of mortality rate among groups. P values <0.05 were considered statistically significant.

3. Results

3.1. Effects of ghrelin on the mortality rate in the rats with endotoxic shock

Endotoxic shock may cause death. To investigate whether ghrelin affects mortality due to endotoxic shock, rats were injected with endotoxin with or without ghrelin, and 24 h later mortality was assessed. All seven rats in the normal control group remained alive during the experiment period. About 55% (11/20) of rats in the endotoxic shock group died within 24 h of lipopolysaccharide injection, whereas the mortality rate was only 25% (3/12) in the early ghrelin-treated group ($P<0.05$) and 47% (7/15) in the late ghrelin-treated group ($P>0.05$) compared with the endotoxic shock group. These results suggest that early ghrelin treatment decreases mortality in rats with endotoxic shock.

3.2. Effects of ghrelin on hemodynamic parameters in rats with endotoxic shock

Hemodynamic change is key factor in death due to endotoxic shock. To assess if ghrelin improves the hemodynamic parameters, blood pressure and cardiac function were determined 24 h after injection of lipopolysaccharide. As shown in Table 1, rats with endotoxic shock had severe hypotension, bradycardia, decreased cardiac systolic and diastolic function (decreased $+\mathrm{LV d}p/\mathrm{d}t_{max}$ and $-\mathrm{LV d}p/\mathrm{d}t_{max}$) and increased left ventricular end-diastolic pressure compared with those of normal controls. Rats with early ghrelin treatment demonstrated a significant improvement in mean arterial blood pressure, heart rate, and decreased cardiac systolic and diastolic function compared with those of the endotoxic shock rats. Although the mean arterial blood pressure and heart rate were not significantly different between the late ghrelin-treated group and the endotoxic shock group ($P>0.05$), the values of $+\mathrm{LV d}p/\mathrm{d}t_{max}$ and $-\mathrm{LV d}p/\mathrm{d}t_{max}$ in the late ghrelin-treated group increased by 13% ($P<0.05$) and 16% ($P<0.01$), respectively, and left ventricular end-diastolic pressure decreased by 33% ($P<0.01$) compared to those of the endotoxic shock group. These results suggest that ghrelin improves hemodynamic changes in endotoxic shock.

3.3. Effects of ghrelin on the concentrations of plasma glucose and lactate and myocardial ATP content in rats with endotoxic shock

Metabolic abnormality has been shown to play an important role in the development and progression of endotoxic shock. To investigate whether ghrelin improves the metabolic abnormality in rats with endotoxic shock, plasma glucose, lactate and myocardial ATP content were

Table 2
Effects of ghrelin on the concentrations of plasma glucose (mmol l^{-1}), plasma lactate (mmol l^{-1}) and myocardial-ATP content (μmol g^{-1} wet wt) in rats with endotoxic shock

	Plasma glucose	Plasma lactate	Myocardial ATP
Normal group ($n=7$)	6.5 ± 0.6	1.1 ± 0.2	4.2 ± 0.5
Endotoxic shock group ($n=9$)	2.5 ± 0.5[c]	7.5 ± 0.7[c]	2.3 ± 0.4[c]
Early ghrelin-treated group ($n=9$)	4.4 ± 0.5[c,f]	4.1 ± 0.7[c,f]	3.1 ± 0.3[c,f]
Late ghrelin-treated group ($n=8$)	3.4 ± 0.4[c,f]	5.9 ± 0.8[c,f]	2.5 ± 0.3[c,d]

Data are means ± S.E.M. [c]$P<0.01$ compared with normal group. [d]$P>0.05$, [f]$P<0.01$ compared with endotoxic shock group.

Table 1
Effects of ghrelin on hemodynamic parameters in rats with endotoxic shock

	MABP	HR	LVEDP	$+\mathrm{LV d}p/\mathrm{d}t_{max}$	$-\mathrm{LV d}p/\mathrm{d}t_{max}$
Normal group ($n=7$)	90 ± 7	407 ± 27	5 ± 2	4794 ± 303	4366 ± 308
Endotoxic shock group ($n=9$)	47 ± 8[c]	239 ± 41[c]	18 ± 4[c]	2399 ± 414[c]	1983 ± 272[c]
Early ghrelin-treated group ($n=9$)	67 ± 11[c,f]	316 ± 39[c,f]	10 ± 3[c,f]	3812 ± 490[c,f]	3411 ± 555[c,f]
Late ghrelin-treated group ($n=8$)	52 ± 6[c]	234 ± 36[c]	12 ± 2[c,f]	2744 ± 272[c,e]	2353 ± 300[c,f]

MABP: mean artery blood pressure (mm Hg); HR: heart rate (beats per min); LVEDP: left ventricular end-diastolic pressure (mm Hg); $\mathrm{LV d}p/\mathrm{d}t_{max}$: left ventricular $\mathrm{d}p/\mathrm{d}t$ maximum (mm Hg s^{-1}). Data are means ± S.E.M. [c]$P<0.01$ compared with normal group. [e]$P<0.05$, [f]$P<0.01$ compared with endotoxic shock group.

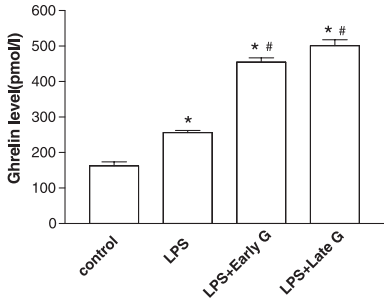

Fig. 1. Plasma ghrelin level in rats with endotoxic shock. Rat blood samples were collected and ghrelin was determined by radioimmunoassay as described in Section 2. Ghrelin levels increased significantly in the endotoxic shock group compared with the control group. Early or late administration of ghrelin further increased ghrelin level. Control: normal group ($n=7$); LPS: endotoxic shock group($n=9$); LPS + Early G: early ghrelin-treated group ($n=9$); LPS + Late G: late ghrelin-treated group($n=8$). *$P<0.01$ compared with normal group. #$P<0.01$ compared with endotoxic shock group.

measured. The rats with endotoxic shock displayed serious hypoglycemia and hyperlactacidemia (Table 2). The concentration of plasma glucose significantly increased by 76% and 36% in the early and late ghrelin-treated groups, respectively, and the concentration of plasma lactate in both groups decreased by 45% and 21% respectively, compared with levels in the endotoxic shock group ($P<0.01$). The myocardial ATP content significantly increased by 26% in the early ghrelin-treated group compared to endotoxic shock group ($P<0.01$); there was no significant difference in myocardial ATP content between the late ghrelin-treated group and the endotoxic shock group ($P>0.05$). These results suggest that both early and late treatment with ghrelin improves the glucose and lactate metabolic abnormalities, but only early treatment with ghrelin increases myocardial ATP content.

3.4. Alteration of plasma ghrelin level

As shown in Fig. 1, the plasma ghrelin level at the end of the experiment increased by 36% in the endotoxic shock group compared with the normal group ($P<0.01$). The plasma ghrelin levels in early and late ghrelin-treated groups were further increased by 77.5% ($P<0.01$) and by 95.7% ($P<0.01$), respectively, compared with those of the endotoxic shock group.

3.5. Effects of lipopolysaccharide on ghrelin synthesis and release from gastric mucosa in vitro

The source of the elevated plasma ghrelin levels in endotoxic shock is unclear. Ghrelin-producing cells are most abundant in the oxyntic glands of the stomach. To investigate if endotoxin induces ghrelin synthesis and

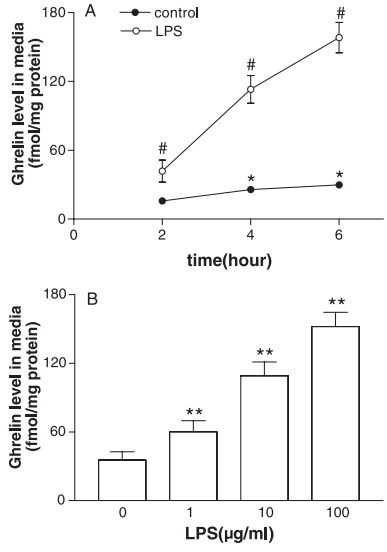

Fig. 2. Lipopolysaccharide (LPS) induces ghrelin release from gastric mucosa in a dose- and time-dependent manner. Cultured gastric mucosa was treated with or without LPS (10 μg/ml) for various times (2–6 h) (A), or incubated with LPS at 0–100 μg/ml for 4 h (B); and the media were then collected for ghrelin assay. The ghrelin levels in media are shown as fmol per mg gastric mucosa tissue protein. Tissue protein content was determined with Coomassie brilliant blue method. (A) shows that both basal and LPS-stimulated ghrelin release from gastric tissue were increased in a time-dependent manner; however, LPS induced greater ghrelin release than in controls at the same time. (B) shows dose-dependent increased release of ghrelin from gastric mucosa. *$P<0.05$ compared with 2 h value, #$P<0.05$ compared with the corresponding time values of control. **$P<0.05$ compared with LPS at 0 μg/ml. $N=6$.

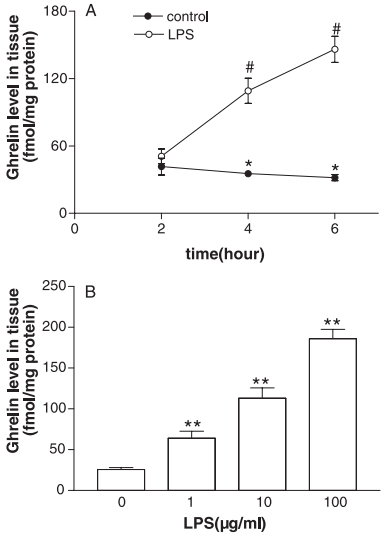

Fig. 3. Lipopolysaccharide (LPS) induces ghrelin production in gastric mucosa. Cultured gastric mucosa was treated with or without LPS (10 μg/ml) for various times (2–6 h) (A), or incubated with LPS at 0–100 μg/ml for 4 h (B), and gastric mucosa were collected and homogenized for ghrelin assay, and tissue protein content was determined with Coomassie brilliant blue method. (A) shows that the level of ghrelin in gastric mucosa was decreased in the controls without LPS. LPS significantly increased the level of ghrelin in gastric mucosa in a time-dependent manner. (B) shows a dose-dependent increase in the level of ghrelin in gastric mucosa. *$P<0.05$ compared with 2 h value, #$P<0.05$ compared with the corresponding time values of control. **$P<0.05$ compared with LPS at 0 μg/ml. $N=6$.

release, the gastric fundus mucosa was dissected, cultured and incubated with lipopolysaccharide at various times and concentrations, and the media and incubated tissues were collected for measurement of ghrelin. There were time- and concentration-dependent increases in ghrelin level in the media of tissues incubated with lipopolysaccharide (Fig. 2A and B). Furthermore, the content of ghrelin in tissues also increased in a time- and dose-dependent way stimulated by lipopolysaccharide (Fig. 3A and B), suggesting that lipopolysaccharide increases ghrelin synthesis and release in gastric fundus mucosa.

4. Discussion

Ghrelin, a 28-amino-acid peptide predominantly produced by the stomach, displays strong growth hormone-releasing activity mediated by activation of the ghrelin receptor, a G-protein coupled receptor of the growth hormone secretagogue receptor. The ghrelin receptor has a widespread distribution in the body: in addition to pituitary gland, cardiovascular tissues such as myocardium, aorta, coronary artery and vein are rich in ghrelin receptors (Papotti et al., 2000), suggesting that ghrelin might directly exert cardiovascular effects by growth hormone-independent mechanisms. Here, we demonstrated that the ghrelin level was increased in rats with endotoxic shock, that endotoxin induced the production and release of ghrelin from cultured gastric mucosa, and that administration of ghrelin decreased mortality and improved metabolic and hemodynamic disturbance in rats with endotoxic shock. These findings suggest that ghrelin could be a very important factor in the development and progression of endotoxic shock.

It has been shown that ghrelin elicits potent, long-lasting growth hormone release and has beneficial hemodynamic effects by reducing cardiac afterload and increasing cardiac output without increasing heart rate in humans (Nagaya et al., 2001a,b,c). Chronic administration of ghrelin improved left ventricular dysfunction and attenuated the development of cardiac cachexia in rats with heart failure (Nagaya et al., 2001a,b,c).

Endotoxic shock is a syndrome characterized by hypotension, diminished response to vasoconstrictor agents, and myocardial dysfunction. The pathogenesis of endotoxic shock is not fully understood yet. Although great progress has been made by using antibiotics and therapeutics regulating vascular function, the mortality rate is still high in endotoxemia, particularly when there is multiple system organ failure (Tang and Liu, 1996). Therefore, it is important theoretically and clinically to clarify the regulatory mechanism and to explore new therapeutics for endotoxic shock. In this study, we used a rat endotoxic shock model induced by intravenous injection of lipopolysaccharide. In this study, the endotoxic shock rats had severe hypotension, heart failure, critical metabolic disturbances and a high mortality rate, mimicking the clinical situation in septic shock. Administration of ghrelin either early or late significantly decreased the mortality rate and improved cardiac function and metabolic abnormalities. The mechanisms by which ghrelin improves cardiovascular function and metabolic disturbance are still unclear. Several papers indicated that the plasma ghrelin level was positively correlated with plasma growth hormone concentration both in animals and in humans (Nagaya et al., 2001a,b,c). Growth hormone has some beneficial effects on serious heart failure and ischemic heart disease (Giustina et al., 1999; Osterziel et al., 2000). Further studies need to be performed to identify whether ghrelin improves cardiovascular function by stimulating endogenous growth hormone release in endotoxic shock. Since ghrelin receptors have been detected in cardiovascular tissues, ghrelin seems likely to have direct effects on the cardiovascular system. Recent reports showed that hexarelin, another ligand of growth hormone secretagogue receptors, protected the heart directly, in a growth hormone-independent manner, in both isolated myocardial cells and a myocardial ischemia-reperfusion model (Locatelli et al., 1999; Rossoni et al., 2000; Ivesten et al., 2000). Besides stimulated growth hormone secretion, ghrelin administration induced a prompt increase in serum glucose level in healthy volunteers, and the hyperglycemia continued up to 165 min. There was a secondary decrease in serum insulin level after ghrelin administration. The absolute insulin level was significantly reduced from 30 min, reached a nadir at 45 min and then persisted at a low level up to 105 min (Broglio et al., 2001).

Exposure of hepatoma cells to ghrelin caused up-regulation of several insulin-induced activities, including tyrosine phosphorylation of insulin receptor substrate-1, association of the adapter molecule growth factor receptor-bound protein 2 with insulin receptor substrate-1, mitogen-activated protein kinase activity and cell proliferation. Unlike insulin, ghrelin inhibited Akt kinase activity and up-regulated gluconeogenesis. These findings raise the possibility that ghrelin modulates insulin activity in humans (Murata et al., 2002). The regulatory action of ghrelin on glucose metabolism may have the important pathophysiological function of correcting metabolic disturbances in endotoxic shock.

In this study, we also observed that the plasma ghrelin level was elevated in rats with endotoxic shock, suggesting that the increased secretion of endogenous ghrelin could have implications for compensation in endotoxic shock. The source of elevated plasma ghrelin in endotoxic shock is unclear. Ghrelin-producing cells are most abundant in the oxyntic glands of the stomach (Date et al., 2000; Dornonville de la Cour et al., 2001; Lee et al., 2002). It is known that during shock, gastroenteric tract tissues release many bioactive substances into the circulation, such as prostaglandins, adremomedullin, catecholamine, etc., which play an important role in the pathogenesis of shock. In this study lipopolysaccharide stimulated ghrelin secretion from rat

gastric mucosa in vitro in a time- and concentration-dependent manner, indicating that lipopolysaccharide directly stimulated gastric mucosa to secret ghrelin and thus increased the plasma ghrelin level, which could play a protective role during endotoxic shock. Nagaya et al. (2001a,b,c) reported that in cachexia of chronic heart failure, the plasma ghrelin level was increased, inhibiting catabolism and enhancing anabolism, leading to an energy balance. Therefore, an elevation of plasma ghrelin level could be considered as an adaptive protective response to endotoxic shock. The fact that exogenous administration of ghrelin partially corrected the heart dysfunction and metabolic disturbance in endotoxic shock indicates that ghrelin might be an endogenous anti-shock factor and play an important regulatory role in cardiovascular function in endotoxic shock.

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