Ghrelin inhibits sympathetic nervous activity in sepsis

Rongqian Wu, Mian Zhou, Padmalaya Das, Weifeng Dong, Youxin Ji, Derek Yang, Michael Miksa, Fangming Zhang, Thanjavur S. Ravikumar, and Ping Wang

Center for Immunology and Inflammation, The Feinstein Institute for Medical Research and Department of Surgery, North Shore University Hospital and Long Island Jewish Medical Center, Manhasset, New York

Submitted 13 February 2007; accepted in final form 24 September 2007

Wu R, Zhou M, Das P, Dong W, Ji Y, Yang D, Miksa M, Zhang F, Ravikumar TS, Wang P. Ghrelin inhibits sympathetic nervous activity in sepsis. Am J Physiol Endocrinol Metab 293: E1697–E1702, 2007. First published October 2, 2007; doi:10.1152/ajpendo.00098.2007.—Our previous studies have shown that norepinephrine (NE) upregulates proinflammatory cytokines by activating \( \alpha_2 \)-adrenoceptor. Therefore, modulation of the sympathetic nervous system represents a novel treatment for sepsis. We have also shown that a novel stomach-derived peptide, ghrelin, is downregulated in sepsis and that its intravenous administration decreases proinflammatory cytokines and mitigates organ injury. However, it remains unknown whether ghrelin inhibits sympathetic activity through central ghrelin receptors [i.e., growth hormone secretagogue receptor 1a (GHSR-1a)] in sepsis. To study this, sepsis was induced in male rats by cecal ligation and puncture (CLP). Ghrelin was administered through intravenous or intracerebroventricular injection 30 min before CLP. Our results showed that intravenous administration of ghrelin significantly reduced the elevated NE and TNF-\( \alpha \) levels at 2 h after CLP. NE administration partially blocked the inhibitory effect on TNF-\( \alpha \) in sepsis. GHSR-1a inhibition by the administration of a GHSR-1a antagonist, [\(-\text{Arg}^1,\text{D-Phe}^6,\text{D-Trp}^7,\text{9-Leu}^{11}\) substance P], significantly increased both NE and TNF-\( \alpha \) levels even in normal animals. Markedly elevated circulating levels of NE 2 h after CLP were also significantly decreased by intracerebroventricular administration of ghrelin. Ghrelin’s inhibitory effect on NE release was completely blocked by intracerebroventricular injection of the GHSR-1a antagonist or a neuropeptide Y (NPY)/\( Y_1 \) receptor antagonist. However, ghrelin’s downregulatory effect on TNF-\( \alpha \) release was only partially diminished by these agents. Thus ghrelin has sympathoinhibitory properties that are mediated by central ghrelin receptors involving a NPY/\( Y_1 \) receptor-dependent pathway. Ghrelin’s inhibitory effect on TNF-\( \alpha \) production in sepsis is partially because of its modulation of the overstimulated sympathetic nerve activation.

ghrelin; sepsis; sympathetic nervous system; norepinephrine; cytokines

THE INTERACTION BETWEEN the central nervous system and immune system under various inflammatory diseases has found considerable interest in the past several decades. Sympathetic influence on immune function was initially demonstrated by showing that epinephrine and norepinephrine (NE) inhibited histamine secretion from mast cells (18). Along with the discovery of cytokines in the late 1970s, greater research efforts were undertaken to investigate neuro-immune interaction. Our recent studies have indicated that intraperitoneal injection of NE, at concentrations found under septic conditions (~20 nM), produced an increase of circulating levels of tumor necrosis factor (TNF)-\( \alpha \), interleukin (IL)-1\( \beta \) and IL-6, similar to that found in sepsis (38, 39, 42). Moreover, NE upregulates TNF-\( \alpha \) and IL-1\( \beta \) production in Kupffer cells through an \( \alpha_2 \)-adrenergic pathway (39, 41, 42). This appears to be, in part, responsible for the increased proinflammatory cytokines in the circulation under such conditions. Therefore, modulation of the sympathetic nervous system represents a novel strategy for sepsis treatment.

Ghrelin, a novel endogenous ligand for the growth hormone secretagogue receptor 1a (GHSR-1a), is a small peptide (28 amino acids) that was discovered in 1999 by Kojima et al. (14). In addition to its somatotropin properties (1), ghrelin has now been proved to possess other endocrine and nonendocrine activities reflecting central and peripheral GHSR-1a distribution (7, 34). Recent studies have shown a decrease in the sympathetic nerve activity in brown adipose tissues and kidneys after intravenous or intracerebroventricular injection of ghrelin (19, 22, 40). Therefore, this novel peptide may be used to directly modulate the sympathetic nervous activity.

Our recent studies demonstrated that plasma levels of ghrelin decrease significantly in a rat model of polymicrobial sepsis induced by cecal ligation and puncture (CLP; see Ref. 36), and ghrelin administration decreases proinflammatory cytokines and mitigates organ injury under such conditions (33, 35). In addition, recent studies have shown that treatment with ghrelin significantly downregulates circulating levels of cytokines in endotoxemia (6, 17). However, it remains to be determined whether ghrelin inhibits sympathetic activity in sepsis and, if so, whether this inhibition plays any role in ghrelin’s anti-inflammatory effect. The present study was conducted to test the hypothesis that administration of exogenous ghrelin inhibits sympathetic activity, which contributes to its anti-inflammatory effect in sepsis.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (275–325 g), purchased from Charles River Laboratories (Wilmington, MA), were used in this study. The rats were housed in a temperature-controlled room on a 12:12-h light-dark cycle and fed on a standard Purina rat chow diet. The rats were fasted for 6 h before the procedure. Animal experimentation was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources). This project was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Animal model of sepsis. Sepsis was induced by CLP, as we previously described (35). Briefly, the rats were anesthetized, and a 2-cm ventral midline abdominal incision was performed. The cecum was then exposed, ligated just distal to the ileocecal valve to avoid

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
intestinal obstruction, punctured two times with an 18-gauge needle, and returned to the abdominal cavity. The incision was then closed in layers. Sham-operated animals (i.e., control animals) underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with 3 ml/100 g body wt normal saline subcutaneously immediately after surgery.

**Intravenous injection of ghrelin.** Before the onset of sepsis (30 min), rats were anesthetized with pentobarbital sodium (ip injection, 40 mg/kg body wt). A catheter (PE-50 tubing) was placed in the femoral vein after carefully separating the femoral nerve and blood vessels. Ghrelin (4 nmol/rat; Phoenix Pharmaceuticals, Belmont, CA) or vehicle (1 ml normal saline) was administered intravenously over a period of 2.5 h through a pump (Harvard Apparatus, Holliston, MA).

In an additional group of animals, NE (20 nmol; Sigma, St. Louis, MO) was administered together with ghrelin. The rats were then subjected to CLP or sham operation as described above. Blood samples were collected at the end of infusion (i.e., 2 h after CLP or sham operation). Blood samples were centrifuged at 3,000 g for 10 min at 4°C, and the plasma samples were stored at −80°C until assayed.

**Determination of plasma levels of NE and TNF-α.** The concentrations of NE and TNF-α in the plasma were quantified by the use of commercially obtained enzyme-linked immunosorbent assay (ELISA) kits specific for NE (IBI-America, Minneapolis, MN) and rat TNF-α (BD Biosciences, San Diego, CA). The assay was carried out according to the instructions provided by the manufacturers.

**Intravenous administration of ghrelin receptor antagonist.** Intravenous administration of ghrelin receptor antagonist [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P (700 nmol/kg body wt in 1 ml normal saline) or vehicle (1 ml normal saline) was infused in the plasma samples were stored at −80°C until assayed. The concentrations of NE and TNF-α in the plasma were quantified by the use of commercially obtained enzyme-linked immunosorbent assay (ELISA) kits specific for NE (IBI-America, Minneapolis, MN) and rat TNF-α (BD Biosciences, San Diego, CA). The assay was carried out according to the instructions provided by the manufacturers.

**Intravenous administration of ghrelin receptor antagonist.** Intravenous administration of ghrelin receptor antagonist [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P (700 nmol/kg body wt in 1 ml normal saline) or vehicle (1 ml normal saline) was infused in the plasma samples were stored at −80°C until assayed.

**Intracerebroventricular injection of ghrelin.** Before the onset of sepsis (30 min), rats were anesthetized with pentobarbital sodium (ip injection, 40 mg/kg body wt) and placed in a stereotactic head frame (Stoelting, Wood Dale, IL). The incisor bar was adjusted until the plane defined by the lambda and bregma was parallel to the base plate. The musculature on the skull was removed, and the skull was exposed. A hole (1–2 mm diameter) was drilled through the skull with a hand-operated drill (Dremel Robert Bosch Tool, Mount Prospect, IL). The needle of a Hamilton syringe (25 μl) was stereotactically guided in the lateral ventricle (0.8 mm posterior to bregma, 1.5 mm lateral to midline, 3.0 mm below the dura). Ghrelin (1 nmol) was dissolved in sterile endotoxin-free normal saline (10 μl) and administered in 1 min. The hole was sealed by the use of cyanoacrylic glue. The rats were then removed from the stereotactic head frame and placed in a supine position on an acrylic board. The rats were then subjected to CLP or sham operation as described above. Blood samples were collected 2 h after CLP or sham operation. Blood samples were centrifuged at 3,000 g for 10 min at 4°C, and the plasma samples were stored at −80°C until assayed. The location of intracerebroventricular injections was confirmed by histological examination of the brain after the experiment.

**Intracerebroventricular injection of ghrelin.** Before the onset of sepsis (30 min), rats were anesthetized with pentobarbital sodium (ip injection, 40 mg/kg body wt). [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P (0.5 nmol/rat, 10 μl), BIBP-3226 (a specific NPY/Y₁ receptor antagonist, Peninsula Laboratories, Belmont, CA; 40 nmol/rat, 10 μl), or vehicle (10 μl normal saline) was administered through intracerebroventricular injection. Immediately after [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P or BIBP-3226 administration, ghrelin (4 nmol/rat) was administered intravenously over a period of 2.5 h through a pump. The blood samples were collected at the end of infusion. Plasma levels of NE and TNF-α were measured by ELISA as described above.

**Statistical analysis.** All data are expressed as means ± SD and compared by two-way or one-way ANOVA and the Student-Newman-Keuls method for multiple-group analysis or Student’s t-test for two group analysis. Differences in values were considered significant if P < 0.05.

**RESULTS**

**Effects of intravenous ghrelin administration on plasma levels of NE and TNF-α.** Consistent with our previous observations, plasma levels of NE and TNF-α increased significantly at 2 h after CLP compared with those in sham-operated animals (P < 0.05, Fig. 1, A and B). Intravenous administration of ghrelin decreased plasma levels of NE and TNF-α by 33 and 79%, respectively, in septic animals (P < 0.05). However, intravenous administration of ghrelin did not have any significant effects on either NE or TNF-α levels in the plasma in sham-operated animals (Fig. 1, A and B).

**Effects of NE administration on ghrelin’s inhibition of TNF-α releases after CLP.** To determine whether ghrelin’s downregulatory effect on TNF-α in sepsis is mediated at least in part via inhibition of the sympathetic activity, NE was
administered together with ghrelin. As indicated in Fig. 2, NE administration partially blocked the inhibitory effect of ghrelin on the circulating levels of TNF-α.

**Effects of intravenous ghrelin receptor antagonist administration on plasma levels of NE and TNF-α in normal animals.** As indicated in Fig. 3A, plasma levels of NE were increased by 51% after intravenous infusion of ghrelin receptor antagonist [d-Arg¹,d-Phe⁵,d-Trp⁷,⁹,Leu¹¹]substance P, for 1 h in normal animals (P < 0.05). This increase is associated with a 188% increase in plasma levels of TNF-α in normal animals (P < 0.05, Fig. 3B).

**Effects of intracerebroventricular ghrelin administration on plasma levels of NE.** To determine whether the inhibitory effect of ghrelin on NE production is mediated through the central nervous system, ghrelin was administered through intracerebroventricular injection. As shown in Fig. 4, markedly increased circulating levels of NE at 2 h after CLP were also significantly decreased by intracerebroventricular administration of ghrelin (P < 0.05). However, intracerebroventricular administration of ghrelin did not have any significant effects on plasma levels of NE in sham-operated animals (Fig. 4).

**Effects of intracerebroventricular ghrelin receptor antagonist or NPY/Y₁ receptor antagonist administration on ghrelin’s inhibition of NE and TNF-α releases after CLP.** To determine whether the effect of ghrelin is mediated via the central nervous system, a very low dose of [d-Arg¹,d-Phe⁵,d-Trp⁷,⁹,Leu¹¹] substance P was administered through intracerebroventricular injection before intravenous injection of ghrelin in CLP animals. Our result showed that ghrelin’s inhibitory effect on NE release was completely blocked by intracerebroventricular injection of the ghrelin receptor antagonist (Fig. 5A). However, ghrelin’s downregulatory effect on TNF-α release was only partially diminished by intracerebroventricular injection of the ghrelin receptor antagonist (Fig. 5B). To determine whether the NPY/Y₁ receptor pathway is involved in ghrelin’s sympathetic nerve inhibitory effect, a specific NPY receptor antagonist, BIBP-3226, was administered through intracerebroventricular injection before intravenous injection of ghrelin. Similar to the intracerebroventricular ghrelin receptor antagonist data, ghrelin’s inhibitory effect on NE release was completed blocked by intracerebroventricular injection of NPY/Y₁ receptor antagonist (Fig. 5A). However, ghrelin’s downregulatory effect on TNF-α release was only partially diminished by intracerebroventricular injection of NPY/Y₁ receptor antagonist (Fig. 5B).

**DISCUSSION**

The present study clearly demonstrates that ghrelin possesses sympathoinhibitory properties. The results that intracerebroventricular injection of ghrelin decreased NE levels in the circulation and intracerebroventricular injection of ghrelin re-
receptor antagonist completely blocked the inhibitory effect of intravenous ghrelin administration on NE release suggest that ghrelin’s sympathoinhibitory effect appears to be mainly mediated by ghrelin receptors in the central nervous system. Since the discovery of ghrelin as an endogenous ligand of the GHSR-1a, numerous publications on its orexigenic activity (i.e., the enhancing effect on hunger and food intake) have been found. Ghrelin is now thought to be the most important mediator of food intake and a functional antagonist of leptin receptors (21, 23). Recent studies have shown that plasma levels of ghrelin decrease by 64–72% in addition to a significant decrease in gastric ghrelin gene expression and content in the early and late stages of sepsis (36). In this regard, there is a close correlation of the increased NE and reduced ghrelin levels in sepsis (11, 36, 38). Therefore, downregulation of the novel peptide ghrelin in sepsis appears to play a role in activating sympathostimulatory nuclei in the brain and increasing NE release from the sympathetic nerve fibers.

The adrenergic effects of the sympathetic nervous system are mediated through nine different adrenoceptors: three α1 receptors (α1A, α1B, α1C), three α2 receptors (α2A, α2B, α2C), and three β receptors (β1, β2, β3). Catecholamines possess both anti-inflammatory and proinflammatory activities. The adrenergic anti-inflammatory effects are mostly mediated by β2-adrenoceptors, which are expressed on lymphocytes and monocytes (10, 31). Only supraphysiological levels of NE can inhibit cytokine release from monocytes/macrophages (20). Many studies focusing on the immunomodulation by NE used high concentrations of NE (i.e., 10⁻⁴ M) and thus were more likely to activate β2-receptors that override α2-receptor-mediated proinflammatory responses (9, 12, 26). Our previous study showed that intraperitoneal infusion of NE at a concentration of 20 nM did not alter cardiac output (39), suggesting that the NE concentration in the circulation was low enough not to influence β-receptor-mediated cardiac performance. This further implies that α2-adrenoceptors on Kupffer cells/macrophages become selectively activated (31). Stimulation of Kupffer cell

**Fig. 5.** Effects of intracerebroventricular (icv) administration of the ghrelin receptor antagonist ([D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P) or neuropeptide Y (NPY)/Y₁ receptor antagonist (BIBP-3226) on ghrelin’s inhibition of NE (A) and TNF-α (B) releases at 2 h after CLP. Data are presented as means ± SE (n = 5–6) and compared by 2-way ANOVA and the Student-Newman-Keuls method: P < 0.05 vs. Sham group (†), vs. Vehicle group, and vs. Ghrelin alone group (*)

AJP-Endocrinol Metab • VOL 293 • DECEMBER 2007 • www.ajpendo.org
α2-adrenoceptors by α2-adrenergic agonists NE or clonidine upregulates TNF-α production (42). In addition, urethane (an α2-adrenergic antagonist) and rauwolscine improved the survival rate following a lethal bolus injection of endotoxin (15). Similarly, we have shown that administration of α2-adrenergic antagonists rauwolscine or yohimbine protects hepatoceleular function and attenuates TNF-α upregulation during early sepsis or after NE administration (38, 39). As demonstrated in the current study, intravenous administration of ghrelin decreased both NE and TNF-α levels in the circulation in sepsis. NE administration partially blocked the inhibitory effect of ghrelin on the circulating levels of TNF-α. Moreover, intravenous administration of a specific ghrelin receptor antagonist increases the release or NE and TNF-α in normal rats. Therefore, the beneficial effects of ghrelin in sepsis partially are the result of its modulation of the overstimulated sympathetic nerve activation.

It should be pointed out that anti-inflammatory properties of ghrelin may also be partially mediated through stimulation of the vagus nerve (25, 29, 33). The vagus nerve provides another endogenous mechanism to regulate the magnitude of innate immune responses and attenuate inflammation. Activation of parasympathetic efferent nerves during systemic stress confers an additional protective advantage to the host by restraining a potentially adverse peripheral immune response. Borovikova et al. (3, 4) and Wang et al. (32) recently described the novel concept of a cholinergic anti-inflammatory pathway mediated by the activation of the vagus nerve and nicotinic α7-cholinergic receptor on macrophages (29). It has also been shown that electrical stimulation of the vagus nerve during endotoxemia can inhibit synthesis of TNF-α in the liver, spleen, and heart (32). In studies involving surgical dissection of the nerve (vagotomy) before CLP, we found that the anti-inflammatory effects induced by intravenous injection of ghrelin could be abrogated by vagotomy (33). This indicates that ghrelin has not only the ability to inhibit sympathetic excitotoxicity observed in sepsis but also can activate the cholinergic anti-inflammatory pathway. We observed that ghrelin’s inhibitory effect on NE release was completed blocked by intracerebroventricular injection of the ghrelin receptor antagonist, whereas its downregulatory effect on TNF-α release was only partially diminished by intracerebroventricular injection of the ghrelin receptor antagonist. This would suggest that ghrelin’s effect on TNF-α release was only partially mediated through the sympathetic nervous system. Thus ghrelin appears to be a potential modulator to rebalance the dysregulated sympathetic/parasympathetic nervous system during sepsis.

In summary, intravenous administration of ghrelin significantly reduced the elevated NE and TNF-α levels at 2 h after CLP. NE administration partially blocked the inhibitory effect of ghrelin on the circulating levels of TNF-α in sepsis. GHSR-la inhibition by the administration of GHSR-la antagonist, [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P, significantly elevated both NE and TNF-α levels even in normal animals. Markedly increased circulating levels of NE at 2 h after CLP were also significantly decreased by intracerebroventricular administration of ghrelin. Ghrelin’s inhibitory effect on NE release was completed blocked by intracerebroventricular injection of the GHSR-la antagonist or a NPY/Y1 receptor antagonist. However, ghrelin’s downregulatory effect on TNF-α release was only partially diminished by those agents.

These results suggest that ghrelin has sympathoinhibitory properties that are mediated by central ghrelin receptors involving a NPY/Y1 receptor-dependent pathway. The inhibitory effect of ghrelin on TNF-α production in sepsis is partially the result of its modulation of the overstimulated sympathetic nerve activation.

GRANTS

This study was supported by National Institutes of Health grants R01 GM-053008, R01 AG-028352, and R01 GM-057468 (P. Wang). R. Wu was supported by a Postdoctoral Fellowship No. 0325802T from the American Heart Association (the Heritage Affiliate).

REFERENCES
