Ghrelin inhibits sympathetic nervous activity in sepsis

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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 α2-adrenergic receptor. 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-α levels at 2 h after CLP. NE administration partially blocked the inhibitory effect of ghrelin on TNF-α in sepsis. GHSR-1a inhibition by the administration of a GHSR-1a antagonist, [d-Arg1,d-Phe5, d-Trp7,9-Leu11]substance P, significantly increased both NE and TNF-α 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)/Y1 receptor antagonist. However, ghrelin’s downregulatory effect on TNF-α release was only partially diminished by these agents. Thus ghrelin has sympathoinhibitory properties that are mediated by central ghrelin receptors involving a NPY/Y1 receptor-dependent pathway. Ghrelin’s inhibitory effect on TNF-α 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 intraportal injection of NE, at concentrations found under septic conditions (~20 nM), produced an increase of circulating levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, similar to that found in sepsis (38, 39, 42). Moreover, NE upregulates TNF-α and IL-1β production in Kupffer cells through an α2-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 somatotrope 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

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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 (IBL-America, Minneapois, 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 [d-Arg^1,d-Phe^3]substance P.** To further define the role of acute ghrelin deficiency in the sympathetic activity and proinflammatory response, a specific and potent ghrelin receptor antagonist, [d-Arg^1,d-Phe^3]substance P (Bachem, Torrance, CA; see Ref. 2), was administered to normal animals. Briefly, the animals were anesthetized by isoflurane inhalation. The femoral vein was carefully separated from the artery and cannulated with PE-50 tubing. [d-Arg^1,d-Phe^3]substance P (700 nmol/kg body wt in 1 ml normal saline) or normal saline (vehicle, 1 ml) was infused in normal animals over a period of 1 h through a pump (Harvard Apparatus). The blood samples were collected at the end of infusion. Plasma levels of NE and TNF-α were measured by ELISA as described above.

**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 cyanoacryl 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 the ghrelin receptor antagonist or neuropeptide Y (NPY)/Y₁ receptor antagonist.** Before the onset of sepsis (30 min), rats were anesthetized with pentobarbital sodium (ip injection, 40 mg/kg body wt). [d-Arg^1,d-Phe^3,d-Trp^7,9,Leu^11]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-Arg^1,d-Phe^3,d-Trp^7,9,Leu^11]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 ± SE and compared by two-way or one-way ANOVA and the Student-Newman-Keuls’s 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 $[^{[\text{D-Arg}_1,\text{D-Phe}_5,\text{D-Trp}_{7,9},\text{Leu}_{11}]}\text{substance P}$, 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 $[^{[\text{D-Arg}_1,\text{D-Phe}_5,\text{D-Trp}_{7,9},\text{Leu}_{11}]}\text{substance P}$ 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 completed 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 completely 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 sympatohibinceptive properties. The results that intracerebroventricular injection of ghrelin decreased NE levels in the circulation and intracerebroventricular injection of ghrelin re-
release suggests that a NPY/Y\textsubscript{1} receptor-dependent pathway is involved ghrelin’s sympathoinhibitory effect in sepsis. Neurons expressing the GHSR-1a are localized within the brain sites rich in the melanocortin-3/4 (MC3/4) receptors, which suggests that ghrelin and MC3/4 receptor ligands may share some of their target cells (21). A synthetic MC3/4 receptor agonist, melanotan II (MTII) injected in the hypothalamic paraventricular nucleus, decreases feeding generated by ghrelin (23, 28). A recent study has shown that centrally administered MTII increases the sympathetic outflow (5). Therefore, ghrelin’s effect on the sympathetic nervous system may also involve the balance between ghrelin and the melanocortin system. Nevertheless, the precise mechanism of ghrelin’s sympathoinhibitory effect warrants further investigation.

The sympathetic division of the autonomic nervous system originates in nuclei within the brain stem and gives rise to preganglionic efferent fibers that leave the central nervous system through the thoracic and lumbar spinal nerves. Most of the sympathetic preganglionic fibers terminate in ganglia located in the paravertebral chains that lie on either side of the spinal column. The postganglionic noradrenergic fibers innervate a wide variety of target organs, including the gastrointestinal tract, blood vessels, heart, and lymphoid organs. These fibers give rise to widespread arborizations and release NE as the principal neurotransmitter (10, 12). To support ghrelin’s role in the sympathoinhibition, our current study has demonstrated that ghrelin can inhibit NE release from the sympathetic nervous system in septic animals, and intravenous administration of a specific ghrelin receptor antagonist increases the release of NE in normal rats. Hahn et al. (11) reported that sustained elevation in circulating catecholamine levels was present during polymicrobial sepsis. Despite the fact that ghrelin clearance is reduced in sepsis (37), our 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 \( \alpha \) receptors (\( \alpha_{1A} \), \( \alpha_{1B} \), \( \alpha_{1C} \)), three \( \alpha \) receptors (\( \alpha_{2A} \), \( \alpha_{2B} \), \( \alpha_{2C} \)), and three \( \beta \) receptors (\( \beta_{1} \), \( \beta_{2} \), \( \beta_{3} \)). Catecholamines possess both anti-inflammatory and proinflammatory activities. The adrenergic anti-inflammatory effects are mostly mediated by \( \beta_{2} \)-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 \( \beta_{2} \)-receptors that override \( \alpha_{2} \)-receptor-mediated proinflammatory responses (9, 12, 26). Our previous study showed that intraportal 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 \( \beta \)-receptor-mediated cardiac performance. This further implies that \( \alpha_{2} \)-adrenoceptors on Kupffer cells/macrophages become selectively activated (31). Stimulation of Kupffer cell

![Graph A](http://ajpendo.physiology.org/)

- **Fig. 5.** Effects of intracerebroventricular (icv) administration of the ghrelin receptor antagonist (L-Arg\textsuperscript{1}-D-Phe\textsuperscript{5}-D-Trp\textsuperscript{7,9}-Leu\textsuperscript{11}\textsubscript{substance P}) or neuropeptide Y (NPY)/Y\textsubscript{1} receptor antagonist (BIBP-3226) on ghrelin’s inhibition of NE (A) and TNF-\( \alpha \) (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: \( * \) vs. Sham group (*), vs. Vehicle group, and vs. Ghrelin alone group (†).

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 (\( \alpha \textsubscript{3} \), \( \alpha \textsubscript{4} \)), three \( \alpha \) receptors (\( \alpha \textsubscript{2A} \), \( \alpha \textsubscript{2B} \), \( \alpha \textsubscript{2C} \)), and three \( \beta \) receptors (\( \beta \textsubscript{1} \), \( \beta \textsubscript{2} \), \( \beta \textsubscript{3} \)). Catecholamines possess both anti-inflammatory and proinflammatory activities. The adrenergic anti-inflammatory effects are mostly mediated by \( \beta \textsubscript{2} \)-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 \( \beta \textsubscript{2} \)-receptors that override \( \alpha \textsubscript{2} \)-receptor-mediated proinflammatory responses (9, 12, 26).
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 hepatocellular 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 the 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 a 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.

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