Ghrelin Acts at the Nucleus of the Solitary Tract to Decrease Arterial Pressure in Rats

Yingzi Lin, Kiyoshi Matsumura, Masayo Fukuhara, Shuntaro Kagiyama, Koji Fujii, Mitsuo Iida

Abstract—Ghrelin is an orexigenic peptide originally isolated from the stomach. Intracerebroventricular administration of ghrelin has been shown to elicit decreases in arterial pressure and renal sympathetic nerve activity in conscious rabbits. The aim of the present study was to determine the role of ghrelin in the brain stem in cardiovascular responses in rats. Unilateral microinjection of ghrelin into the nucleus of the solitary tract significantly decreased the mean arterial pressure and heart rate ($-17.3\pm0.8$ mm Hg and $-13.6\pm3.5$ bpm by 20 pmol). The microinjection of ghrelin into the nucleus of the solitary tract also suppressed the renal sympathetic nerve activity ($-29.5\pm3.4\%$; $P<0.0001$). Pretreatment with intravenous injection of pentolinium (5 mg/kg), a ganglion-blocking agent, eliminated these cardiovascular responses induced by the microinjection of ghrelin (20 pmol) into the nucleus of the solitary tract; however, pretreatment with intravenous injection of atropine sulfate (0.1 mg/kg), an antagonist of muscarinic acetylcholine receptors, failed to prevent them. In contrast, unilateral microinjection of ghrelin into the area postrema, rostral, and caudal ventrolateral medulla caused no significant changes in the mean arterial pressure and heart rate.

It has been suggested that ghrelin may participate not only in feeding behavior but also in cardiovascular and sympathetic regulation. From the stomach, is an endogenous ligand for the growth hormone secretagogue (GHS) receptor. Although ghrelin is likely to regulate pituitary growth hormone (GH) secretion, along with GH-releasing hormone and somatostatin, intracerebroventricular (ICV) administration of ghrelin has been shown to generate a dose-dependent increase in food intake and body weight, suggesting that ghrelin participates in the regulation of both food intake and GH secretion.

The nucleus of the solitary tract (NTS), where baroreceptor and chemoreceptor afferents terminate, is known to play an important role in central cardiovascular regulation. On the other hand, the area postrema, known as a circumventricular organ, has a diminished blood-brain barrier and thus can monitor blood-borne peptides. Eff erent fibers from the area postrema project to a number of central nervous system structures involved in the regulation of sympathetic outflow and cardiovascular function. Anterograde and retrograde anatomic tracing studies have demonstrated projections from the area postrema to the NTS. In addition, GHS receptors have been identified in hypothalamic neurons and in the brain stem, suggesting that these brain regions may be involved in central cardiovascular regulation of ghrelin, although the precise distribution of GHS receptors in the medulla oblongata has not been determined. We hypothesized that ghrelin acts at the NTS and the area postrema to decrease sympathetic nerve activity and blood pressure. Accordingly, the aim of the present study was to determine the role of the brain stem on cardiovascular responses to ghrelin. To determine the distribution of GHS receptors in the medulla oblongata, an immunohistochemical procedure was also performed.

Key Words: blood pressure ■ immunohistochemistry ■ rats

Received October 13, 2003; first decision October 28, 2003; revision accepted February 3, 2004.
From the Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan.
Correspondence to Kiyoshi Matsumura, MD, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. E-mail matsumk@intmed2.med.kyushu-u.ac.jp
© 2004 American Heart Association, Inc.
Methods

Microinjection Procedures

All experiments were performed using adult male Wistar rats (10 to 12 weeks old; Kyudo, Saga, Japan) according to the institutional guidelines for animal experimentation at Kyushu University. Rats were anesthetized with urethane (1.2 g/kg IP). Surgical procedures and recording of RSNA were performed as described previously.16–18 Microinjections were made from multibarrel micropipettes with tip diameters of 20 to 50 μm. Injections (50 nL or 100 nL) were made over a 30-second period with a hand-held syringe. The injection volume was measured by observing the movement of the fluid meniscus along a reticle in a microscope.16–18 The appropriate placement of the pipette tip within the medial NTS was established by microinjection of L-glutamate (0.2 nmol/50 nL) and observing a depressor response of at least 15 mm Hg. On this basis, injections into the medial NTS had coordinates of 0.4 to 0.5 mm rostral and 0.5 to 0.6 mm lateral to the calamus scriptorius and 0.4 mm below the dorsal surface of the medulla. Injections into the area postrema had coordinates of 0.2 mm rostral to the calamus scriptorius and 0.2 mm below the surface of the medulla. Moreover, the appropriate placement of the pipette tip within the rostral ventrolateral medulla (RVLM) and the caudal ventrolateral medulla (CVLM) were established by microinjection of L-glutamate (2 nmol/50 nL) and observing a pressor or a depressor response of at least 20 mm Hg, respectively. Injections into the RVLM had coordinates of 1.5 to 1.7 mm rostral and 1.8 to 2.0 mm lateral to the calamus scriptorius and 3.0 to 3.6 mm below the dorsal surface of the medulla, and injections into the CVLM had coordinates of 0.3 to 0.5 mm rostral and 1.8 to 2.0 mm lateral to the calamus scriptorius and 3.0 to 3.6 mm below the dorsal surface of the medulla. All drugs for microinjection were dissolved in artificial cerebrospinal fluid (aCSF).16–18 Alcian blue dye (50 nL) was injected to mark the site of injection at the end of the experiment.

At the completion of the experiment, the rats were prepared for transcardial perfusion and then euthanized with a lethal dose of intravenous sodium pentobarbital (75 mg/kg). Animals were then perfused transcardially, and the brain was removed as described previously.17,18 The medulla oblongata was cut into 50-μm serial coronal frozen sections stained with neutral red. The locations of the microinjection sites in the medial NTS, area postrema, RVLM, and CVLM were confirmed microscopically according to the atlas of Paxinos and Watson.19

Experimental Protocols

Cardiovascular Effects of Ghrelin in the NTS and the Area Postrema

To determine the cardiovascular effect of ghrelin (Peptide Institute, Osaka, Japan) in the NTS and the area postrema, ghrelin (10 pmol/50 nL and 20 pmol/100 nL) was injected into the NTS or the area postrema. As the vehicle control, we administered a microinjection of aCSF (100 nL).

Effects of Pentolinium or Atropine on Cardiovascular Responses Induced by Microinjection of Ghrelin in the NTS

To determine the role of the sympathetic nervous system, rats were injected with pentolinium (5 mg/kg IV, Sigma Chemical Co), a ganglion blocking agent (n=6). Five minutes later, 20 pmol of ghrelin was injected into the NTS. To determine the role of the parasympathetic nervous system, another group of rats was injected with atropine sulfate (0.1 mg/kg IV, Tanabe Seiyaku, Osaka, Japan), an antagonist of muscarinic acetylcholine receptors (n=8). Five minutes later, 20 pmol of ghrelin was injected into the NTS.

Cardiovascular Effects of Ghrelin in the RVLM and the CVLM

To determine the cardiovascular effect of ghrelin in the RVLM and the CVLM, ghrelin (20 pmol/100 nL and 80 pmol/100 nL) was injected into the RVLM (n=6 and 8, respectively) or the CVLM (n=6 and 8, respectively). As the vehicle control, we administered a microinjection of aCSF (100 nL).

Immunohistochemistry

Five Wistar rats were used for the immunohistochemistry experiments. Rats were anesthetized with sodium pentobarbital (100 mg/kg, IP) and perfused transcardially with 150 mL of 0.1-mol/L phosphate-buffered saline (PBS), followed by 200 mL of 10% formaldehyde solution. The fixed brains were embedded in paraffin and processed for immunohistochemistry.

Serial sections were cut to 3-μm thick. They were deparaffinized and rehydrated in graded alcohols. The slides were treated in an antigen retrieval, and then incubated with 0.3% H2O2 in methyl alcohol for 30 minutes to suppress endogenous peroxidase activity. The rabbit antibody specific to rat ghrelin receptor (GHS receptor, 1:2000 dilution, Alfa Diagnostic International, San Antonio, Tex) was applied at 4°C overnight. After being washed with PBS, the slides were incubated with biotinylated goat antirabbit IgG (Histofine SAB-PO(R) Kit, Nichirei, Osaka, Japan) for 30 minutes at room temperature. The immunoreactivity for the GHS receptor was visualized by the streptavidine peroxidase staining method. Hematoxylin was used for counterstaining. Control slides were incubated with PBS.

Statistics

All values are expressed as mean±SE. A 1-way ANOVA with repeated measurements was used to evaluate the effects of the microinjection of ghrelin into the NTS, area postrema, RVLM, or CVLM, followed by Duncan multiple range test to determine the significance of the mean differences in response to the microinjection of vehicle. A value of P<0.05 was considered significant.

Results

Cardiovascular Effects of Ghrelin in the NTS

Baseline values for mean arterial pressure (MAP) and HR were 90.9±1.6 mm Hg and 399.1±11.2 bpm in 20 pmol of ghrelin-microinjected rats, and 90.7±2.1 mm Hg and 388.3±13.4 bpm in aCSF-microinjected rats, respectively. The microinjection of ghrelin (10 and 20 pmol) into the NTS caused dose-related decreases in MAP and HR (Figure 1). To determine the contribution of sympathetic nervous system, 20 pmol of ghrelin or aCSF was injected into the NTS in another group of rats (n=10 and 8, respectively) with the recording of the RSNA. Figure 2 shows the typical responses of MAP, HR, and RSNA elicited by the microinjection of ghrelin (20 pmol) into the NTS. The microinjection of ghrelin (20 pmol) into the NTS significantly suppressed the RSNA (−29.5±3.4%; P=0.0001 versus aCSF-microinjected rats).

Cardiovascular Effects of Ghrelin in the Area Postrema

Baseline values for MAP and HR were 85.8±3.4 mm Hg and 390.0±11.5 bpm in 20 pmol of ghrelin-microinjected rats (n=8), and 84.9±2.9 mm Hg and 390.7±15.6 bpm in aCSF-microinjected rats (n=7), respectively. The microinjection of ghrelin (10 and 20 pmol) into the area postrema failed to cause significant changes in MAP and HR (−1.8±1.4 mm Hg and −1.3±2.1 bpm in 20 pmol ghrelin).

Effects of Pentolinium or Atropine on Cardiovascular Responses Induced by Microinjection of Ghrelin in the NTS

Baseline values for MAP and HR were 88.0±3.1 mm Hg and 399.2±11.4 bpm, respectively, in pentolinium-injected rats.
After pentolinium administration, MAP decreased to 58.3 ± 2.2 mm Hg without a significant change in HR. However, microinjection of ghrelin (20 pmol) failed to cause any further responses in MAP in pentolinium-pretreated rats. In contrast, baseline values for MAP and HR were 90.3 ± 1.0 mm Hg and 379.4 ± 14.7 bpm, respectively, in atropine sulfate-injected rats (n=8). After atropine sulfate administration, HR increased to 434.4 ± 11.1 bpm without a significant change in MAP. Microinjection of ghrelin (20 pmol) caused a significant decrease in MAP (−7.8 ± 1.3 mm Hg, P<0.05) without a significant change in HR in atropine sulfate-pretreated rats (Figure 3).

**Cardiovascular Effects of Ghrelin in the RVLM and the CVLM**

The microinjection of ghrelin (20 and 80 pmol) into the RVLM or the CVLM failed to cause significant changes in MAP and HR (+3.0 ± 1.1 mm Hg and +8.1 ± 4.1 bpm, respectively, in 80-pmol ghrelin in the RVLM; −0.3 ± 1.3 mm Hg and −5.0 ± 3.3 bpm, respectively, in 80-pmol ghrelin in the CVLM).

**Immunohistochemistry**

The GHS receptor was expressed in the neuronal cells of the NTS, the dorsal motor nucleus of the vagus, RVLM, and CVLM (Figure 4C-4F). However, expression of the GHS receptor was not found in the cells of the area postrema (Figure 4C). The negative controls for PBS did not exhibit any specific staining patterns (Figure 4A and 4B).
Discussion

The results of the present study indicate that microinjection of ghrelin into the NTS, but not into the area postrema, RVLM, and CVLM, elicits dose-related decreases in MAP and HR. The depressor response induced by microinjection of ghrelin into the NTS was prevented by pretreatment with intravenous pentolinium, but not with intravenous atropine sulfate, suggesting that suppression of the sympathetic nervous system is the primary mechanism of a decrease in arterial pressure. Furthermore, the GHS receptor was found to be expressed in the neuronal cells of the NTS and the dorsal motor nucleus of the vagus, whereas the expression of GHS receptor was not found in the cells of the area postrema. These physiological and immunohistochemical findings in the present study suggest that ghrelin acts at the neurons of the NTS to decrease arterial pressure in rats. To the best of our knowledge, this is the first study to demonstrate the potential role of ghrelin in the medulla oblongata on cardiovascular and sympathetic regulation.

Ghrelin is predominantly produced by the stomach, whereas substantially lower amounts are derived from the bowel, pituitary, kidney, placenta, and hypothalamus. In contrast, GHS receptors have also been identified on hypothalamic neurons and in the brain stem, although the precise distribution of GHS receptors in the brain stem has not been determined. ICV administration of ghrelin has been shown to induce c-fos expression in the neurons of the NTS and the dorsal motor nucleus of the vagus, suggesting that centrally administered ghrelin activates the neurons of these regions. The present study has directly demonstrated that GHS receptors are predominantly present in the NTS, the dorsal motor nucleus of the vagus, RVLM, and CVLM, but not in the area postrema. The NTS, where baroreceptor and chemoreceptor afferents terminate, is one of the most important brain regions to regulate blood pressure and the sympathetic nervous system. We have previously demonstrated that ICV administration of ghrelin suppresses the RSNA and decreased blood pressure in conscious rabbits. The present

Figure 4. Photographs showing immunohistochemical localization of growth hormone secretagogue (GHS) receptor in the brain stem of a Wistar rat. A, Control staining for PBS in the brain stem. B, Control staining for PBS in the nuclei of the solitary tract (NTS). C, GHS receptor immunoreactivity in the brain stem. D, GHS receptor immunoreactivity in the NTS. E, GHS receptor immunoreactivity in the rostral ventrolateral medulla. F, GHS receptor immunoreactivity in the caudal ventrolateral medulla. AP indicates area postrema; X, dorsal motor nucleus of the vagus.
study, along with previous findings, suggest that ICV-injected ghrelin may act at the NTS to suppress sympathetic activity, resulting in a decrease in arterial pressure.

The present study alone is not sufficient to evaluate the mechanisms involved in the depressor response induced by microinjection of ghrelin into the NTS, because the antagonist of ghrelin was not used to prevent the cardiovascular responses to ghrelin. However, the present study indicates that microinjection of ghrelin into the NTS decreases arterial pressure and that the GHS receptor immunoreactivity-positive neurons are present in the NTS. These results strongly indicate that ghrelin acts at the NTS to decrease arterial pressure. Furthermore, to evaluate the contribution of the sympathetic and parasympathetic nervous systems on cardiovascular responses to the microinjection of ghrelin in the NTS, pentolinium or atropine was injected intravenously before ghrelin administration. Pretreatment with intravenous injection of pentolinium prevented the depressor response induced by the microinjection of ghrelin. In contrast, microinjection of ghrelin in the NTS caused a smaller but significant decrease in arterial pressure, even after the pretreatment with intravenous injection of atropine. In addition, the microinjection of ghrelin into the NTS suppressed the RSNA. These findings suggest that suppression of the sympathetic nervous system is primarily responsible for the depressor response induced by the microinjection of ghrelin in the NTS.

Before conducting the present study, we hypothesized that ghrelin in the blood stream might reach to the area postrema, which has a diminished blood-brain barrier, and that it might then elicit the cardiovascular responses. However, GHS receptor immunoreactivity was not found in cells of the area postrema, and microinjection of ghrelin in the area postrema failed to cause any cardiovascular responses. These findings indicate that the area postrema is not the primary brain region where ghrelin acts. On the other hand, ghrelin is predominantly produced by the stomach.3 It has been shown that ghrelin produced in the stomach reaches the NTS through the gastric vagal afferent nerve and influences neuronal activity.21 resulting in an increase in feeding behavior, although the role of the vagal afferent nerve on cardiovascular responses to ghrelin has not been elucidated. Based on the present and previous findings, ghrelin may primarily act at the NTS to regulate feeding, sympathetic activity, and blood pressure. Further studies will be necessary to determine the role of the vagal afferent nerve on cardiovascular regulation of ghrelin.

Brain regions other than the NTS, such as the hypothalamus, may be involved in the central cardiovascular action of ghrelin. Chronic central administration of ghrelin has been shown to increase both neuropeptide Y and agouti-related protein (AGRP) mRNA levels in the arcuate nucleus.22 On the other hand, AGRP is an endogenous melanocortin-3 and -4 receptor (MC3-R and MC4-R) antagonist, and central cardiovascular and sympathetic responses to ICV leptin are reportedly mediated through activation of hypothalamic MC3-R and MC4-R.23–25 Furthermore, ICV administration of neuropeptide Y decreases arterial pressure and renal sympathetic nerve activity in conscious rabbits.26 The interactions of ghrelin and these peptides on cardiovascular and sympathetic regulations in the brain have not been clearly understood.9 ghrelin, however, may also act at the hypothalamus to activate neuropeptide Y and AGRP, resulting in a decrease in arterial pressure and sympathetic activity.

Immunohistochemical study revealed that GHS receptor was also expressed in the neuronal cells of the RVLM and the CVLM. However, 80 pmol of ghrelin, 4 times larger a dose than microinjected into the NTS, failed to cause cardiovascular responses. This dose was the highest concentration we could prepare in the present study, because the injected volume was limited to 100 nL. It might be difficult to account for the dissociated results of the immunohistochemical study and the microinjection study in the VLM; however, the neurons in the NTS may be more sensitive than those in the VLM. In fact, the dose of L-glutamate needed to elicit the similar magnitude responses of arterial pressure in the VLM is about 10 times larger than that in the NTS. In addition, in our preliminary analysis, GHS receptor immunoreactivity-positive cells in the NTS were more abundant than those in the RVLM or CVLM. The lack of cardiovascular responses induced by microinjection of ghrelin into the VLM might be explained by the small number of GHS receptor immunoreactivity-positive cells in these brain regions. Ghrelin in the VLM may only participate in some pathological conditions, such as congestive heart failure.

The present study was limited by fact that the experiments were conducted in anesthetized animals, because the sympathetic nervous system and baroreceptor reflex are greatly affected by anesthesia.27,28 Therefore, it seems difficult to infer the role of ghrelin in the NTS on cardiovascular and sympathetic regulation in conscious animals. However, because ICV administration of ghrelin suppressed the RSNA and decreased blood pressure in conscious rabbits,8 ghrelin may act at the NTS to suppress sympathetic nerve activity and decrease arterial pressure even in conscious animals. In addition, the present study has only evaluated the acute cardiovascular effect of ghrelin in the brain stem. It is also difficult to infer the chronic central cardiovascular effect of it. Although we do not have any evidence, it may be possible that chronic central administration of ghrelin suppresses the sympathetic nerve activity and augments the baroreceptor reflex, as shown in the acute experiments. Further studies will be necessary to determine the chronic central effect of ghrelin on blood pressure and sympathetic nerve activity in conscious animals.

In the present study, to determine the dose of ghrelin which elicits cardiovascular responses, 10 or 20 pmol of ghrelin was injected into the NTS and the area postrema. Our preliminary experiments had shown that <10 pmol of ghrelin failed to cause any cardiovascular responses; therefore, we elected to inject 10 and 20 pmol of ghrelin into the NTS and the area postrema to determine the dose response relationships. Furthermore, in our previous study in conscious rabbits, ICV administration of 1 nmol of ghrelin suppressed the RSNA and decreased arterial pressure. Accordingly, we considered that 20 pmol of ghrelin might be the reasonable dose for microinjection into the NTS. In addition, ghrelin produced in the stomach has been shown to reach the NTS through the gastric vagal afferent nerve, not through CSF stream.22 Although ghrelin microinjected into the NTS might be beyond the physiological range of CSF concentration of ghrelin, local brain tissue concentration of ghrelin may be independent of CSF concentration of ghrelin, and ghrelin in
the NTS may modulate sympathetic nerve activity even in physiological condition. Plasma ghrelin concentration is increased in patients with cachexia associated with chronic heart failure. Intravenous administration of ghrelin, however, has been shown to increase the cardiac index not only in healthy men but also in patients with congestive heart failure. Furthermore, chronic administration of ghrelin decreases systemic vascular resistance, resulting in an improvement in left ventricular function in rats suffering from congestive heart failure. Extreme activation of the sympathetic nervous system is harmful with regard to congestive heart failure. Accordingly, suppression of sympathetic nerve activity is one way to treat congestive heart failure. In fact, clonidine, a central sympathetic suppressant, has been shown to improve cardiac function in patients with congestive heart failure. Therefore, sympathoinhibitory effects of ghrelin in the NTS may be one of the mechanisms improving cardiac function. Further studies will be necessary to evaluate the clinical application of ghrelin in patients with congestive heart failure.

Perspectives
Ghrelin is primarily found as an endogenous ligand for the GHS receptor and stimulates food intake. Moreover, intravenous injection of ghrelin decreases arterial pressure and increases the cardiac index and stroke volume in healthy men, suggesting that ghrelin may be applicable to the treatment of congestive heart failure. We have previously demonstrated that ICV administration of ghrelin suppresses the RSNA and decreases arterial pressure in conscious rabbits, and it augments the baroreceptor reflex. The results of the present study indicating that ghrelin acts at the NTS to decrease arterial pressure support the previous finding. The present and previous findings indicate that ghrelin may be applicable to the treatment of congestive heart failure by suppressing sympathetic activity and by improving baroreflex control. Ghrelin may be able to be developed as a medicine for congestive heart failure in the near future.

References
Ghrelin Acts at the Nucleus of the Solitary Tract to Decrease Arterial Pressure in Rats
Yingzi Lin, Kiyoshi Matsumura, Masayo Fukuhara, Shuntaro Kagiyama, Koji Fujii and Mitsuo Iida

Hypertension. 2004;43:977-982; originally published online March 1, 2004; doi: 10.1161/01.HYP.0000122803.91559.55
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/43/5/977