Peripheral gastric leptin modulates brain stem neuronal activity in neonates

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Peripheral gastric leptin modulates brain stem neuronal activity in neonates. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G626–G630, 1999.—Afferent sensory fibers are the primary neuroanatomic link between nutrient-related events in the gastrointestinal tract and the central neural substrates that modulate ingestion. In this study, we evaluated the peripheral gastric effects of leptin (OB protein) on brain stem neuronal activities using an in vitro neonatal rat preparation. We also tested gastric leptin effects as a function of age in neonates. For ~33% of the nucleus tractus solitarius units observed, gastric leptin (10 nM) produced a significant activation of 188.2 ± 8.6% (mean ± SE) compared with the control level of 100% (P < 0.01). Concentration-dependent leptin effects have also been shown. The remaining neurons (67%) had no significant response to gastric leptin application. Next, we evaluated the peripheral gastric effects of leptin (10 nM) on brain stem unitary activity in three different age groups (1–2 days old, 3–5 days old, and 7–8 days old) of neonatal rats. In the 1- to 2-day-old and the 3- to 5-day-old groups, we observed that response ratios and activity levels were similar. However, there was a significant difference between the 7- to 8-day-old group and the two younger age groups in both the response ratios and the activation levels. The percentage of activation responses increased from ~26% in the 1- to 2-day-old and the 4- to 5-day-old age groups to 70% in the 7- to 8-day-old group (P < 0.05). The level of activation increased from 168.3 ± 2.7% (compared with the control level) in the 1- to 2-day-old and the 4- to 5-day-old age groups to 231.4 ± 11.9% in the 7- to 8-day-old group (P < 0.01). Our data demonstrate that peripheral gastric leptin modulates brain stem neuronal activity and suggest that gastric leptin has a significantly stronger effect in the 7- to 8-day-old animals than in the younger neonates.

gastric vagal branch; nucleus tractus solitarius; OB protein; neonatal rat

IT HAS BEEN ESTABLISHED that both central and peripheral signals comprise the complicated circuitry that regulates feeding and energy homeostasis (18). In the past few years, leptin (OB protein), an adipose tissue-derived circulating hormone, has had a considerable impact on hyperphagia and obesity research, and numerous investigations have been performed to study leptin’s effects on the central nervous system (8, 9). Although widespread peripheral leptin receptors have been located (22, 24), to date, only a few investigations have focused on leptin’s peripheral actions. Barrachina et al. (5) reported a synergistic interaction between leptin and CCK, which caused suppression of food intake, involving CCK-A receptors and capsaicin-sensitive afferents. An electrophysiological study by Wang et al. (26) showed the existence of two types of gastric vagal afferents based on whether leptin was able to stimulate the afferents alone or after pretreatment with CCK. However, due to a lack of appropriate methodologies in the past, there are no reports of leptin’s gastric effects on the region of the brain that regulates the digestive process.

Afferent sensory fibers are the primary neuroanatomic link between nutrient-related events in the gastrointestinal tract and the central neural substrates that mediate the control of food intake (1, 6). This study aimed to address the peripheral gastric aspects of leptin on the central nervous system. In this study, to evaluate the peripheral gastric effects of leptin on nucleus tractus solitarius (NTS) neuronal activity, we employed an in vitro neonatal rat preparation in which the functional circuitry of the brain stem-vagal neuronal link with the gastric system was kept intact. We also examined effects of gastric leptin as a function of age in neonates.

METHODS

Animal and surgical preparations. Experiments were performed on 1- to 8-day-old Sprague-Dawley neonatal rats. After the animal was deeply anesthetized with halothane, a craniotomy was performed and the forebrain was ablated at the caudal border of the pons by transection. The caudal brain stem and cervical spinal cord were isolated by dissection in modified Krebs solution that contained (in mM) 128.0 NaCl, 3.0 KCl, 0.5 NaH2PO4, 1.5 CaCl2, 1.0 MgSO4, 21 NaHCO3, 1.0 mannitol, 30.0 glucose, and 10.0 HEPES. The stomach, connected to the esophagus, with the vagus nerves linking it to the brain stem, was kept, and all the other internal organs were removed. The preparation was then isolated and pinned, with the dorsal surface up, on a layer of Sylgard resin (Dow Corning) in a recording chamber. The preparation was isolated and superfused with Krebs solution at 23 ± 1°C. The bathing solution was aerated continuously with a mixture of 95% O2 and 5% CO2 and adjusted to pH 7.35–7.45 (4, 16, 21, 29, 30).

Stimulation and recording methods. A suction microelectrode was placed on the gastric vagal branch from the subdiaphragmatic vagi for electrical stimulation, since only those neurons in the medial NTS receiving gastric vagal inputs were evaluated in this study. The gastric vagal fibers were stimulated with single or paired pulses of 200 μA for 0.2 ms at a frequency of 0.5 Hz by a Grass stimulator (model S8800) coupled to a stimulus isolation unit (SIU 5B, Grass Instruments, Quincy, MA). This current provided a
stimulus intensity 1.5–2.0 times that required to produce maximal amplitude of the C wave in the vagal nerve action potential (28).

Single tonic unitary discharges were recorded extracellularly in the medial NTS by glass microelectrodes filled with 3 M NaCl, with an impedance of 10–20 MΩ [see Barber et al. (4) for unitary discharge recordings]. A collision test was applied to identify orthodromic inputs (11) to ensure that only second- or higher-order NTS neurons in the gastric vagal afferent system were used in this study.

The NTS unitary discharges were amplified with high-gain AC-coupled amplifiers (Axoprobe-1A, Axon Instruments, Burlingame, CA), displayed on a Hitachi digital storage oscilloscope (model VC-6525, Hitachi Denshi) and recorded on a Vetter PCM tape recorder (model 200, A. R. Vetter, Rebersburg, PA).

For histological identification purposes, some glass microelectrodes were filled with 2% Pontamine sky blue in 0.5 M sodium acetate solution. After each unitary recording, current was applied at 5 μA in 5-s on/10-s off cycles for ~5 min, with the negative lead connected to the microelectrode.

Experimental protocols. OB protein may have both peripheral and central actions. To investigate the peripheral gastric effects of leptin on brain stem neurons without interfering with central nervous system functions, a partition was made at the midthoracic level of the preparation. An agar seal separated the recording bath chamber into a brain stem compartment and a gastric compartment. Leptin was applied only to the gastric compartment, and its effect on the NTS neuronal activity was evaluated.

The test compound, leptin, was dissolved in the vehicle solution. The concentrated solution was applied to the Krebs solution in the gastric compartment. The final drug concentration in the gastric compartment was calculated on the basis of the amount of concentrated solution and the total Krebs volume. Drug solution was applied for 5 min before any pharmacological observation to provide sufficient time for drug delivery to reach a steady-state level. After each observation, leptin was washed out from the compartment. For example, in Fig. 1, which shows sequential spike frequency histograms, a 30-s recording (10 3-s consecutive samples) before leptin application was used as a control. Five minutes after leptin application, another 30-s recording was used as trial data. Five minutes after leptin washout, an additional 30-s recording was used as a posttrial result. The NTS neuronal responses observed during pretreatment or pretreatment (control) was compared with posttrial (washout) to confirm that brain stem neural activity returned to the control level after washout.

Tachyphylaxis was tested by reapplying the test compound to the gastric compartment and observing whether the response to a given concentration of the compound varied by <5%.

At the end of each experiment, colored solution was applied to one compartment to make sure that there was no leakage to the other compartment.

Drugs. Murine leptin was received as a gift from Amgen (Thousand Oaks, CA).

Data and statistical analysis. The data from the NTS unitary activity experiments were analyzed on the basis of action potential discharge rate and drug concentration-related effects. The number of action potentials in a given duration was measured under pretreatment, trial, and posttreatment conditions. The control data (pretreatment) was normalized to 100%, and the NTS neuronal activities during and after trials were compared with the control data. Data were analyzed using ANOVA for repeated measures, Student’s t-test, and χ² test with P < 0.05 considered statistically significant.

RESULTS

Peripheral gastric effects of leptin. Thirty tonic NTS units receiving gastric vagal inputs were tested with leptin from neonatal rats ages 2–6 days old. The mean basal firing rate of the NTS neurons tested was 0.8 ± 0.5 Hz.

As shown in the sequential spike density histogram of Fig. 1, the discharge rate of an NTS unit increased after leptin application to the gastric compartment. In 10 of 30 units observed, leptin (10 nM) produced an activation of 188.2 ± 8.6% (mean ± SE) of the control level (normalized to 100%) of the brain stem neuronal activity (P < 0.01 using Student’s t-test). Concentration-dependent effects were also observed (Fig. 2). The differences in NTS neuronal discharge frequencies between the control recording and the recordings after leptin (1.0–30.0 nM) applications were significant (F = 54.3, P < 0.01 using ANOVA for repeated measures). The remaining 20 NTS units had no significant response to peripheral gastric leptin application.

At the end of seven experiments, after the NTS neuronal responses to gastric leptin were observed, the vagus nerve was severed at the low thoracic level. For all seven units that responded to gastric leptin before vagal discontinuation, gastric leptin effects were abolished after the vagus was cut off.
Age factor in gastric leptin modulation on NTS unitary activity. The peripheral gastric effect of leptin (10 nM) on NTS unitary activity was evaluated in the neonatal rat in three different age groups: 1–2 days old, 3–5 days old, and 7–8 days old. Two categories of gastric leptin effects on brain stem were examined. The ratio of activation and the no effect responses in the NTS units recorded in the three different age groups were first compared. The second comparison involved the activity level of the NTS neurons in each age group with activation responses to leptin application.

In both categories, the ratios of response and the activity levels in the 1–2 day-old and the 4–5 day-old age groups were similar. However, there was a significant difference between the 7–8 day-old group and the two younger age groups in both the response ratios and the activation levels. As shown in Table 1, the percentage of activation responses increased from ~26% in the 1–2 day-old and the 4–5 day-old age groups to 70% in the 7–8 day-old group (χ² = 6.0, P < 0.05). We also observed that the level of activation increased from 168.3 ± 2.7% (compared with the control level) in the 1–2 day-old and the 4–5 day-old age groups to 231.4 ± 11.9% in the 7–8 day-old group (P < 0.01 using Student’s t-test). These results suggest that leptin had a significantly stronger activation effect on NTS unitary activity in the 7–8-day-old age group than it did on the 1–2 day and 4–5 day-old age groups.

**DISCUSSION**

The in vitro neonatal rat preparation of the brain stem-spinal cord was initially developed for the study of the respiratory and locomotion systems (21). Anatomically, the stomach is linked to the caudal part of the brain stem by the vagal fibers. To investigate the gastric neurochemical effects on gastric vagally evoked brain stem responses, we modified the brain stem-spinal cord preparation to a brain stem-gastric preparation (4, 28). This in vitro preparation mimics an in vivo preparation in which the gastric vagal inputs to the recorded brain stem neurons can be identified. With the use of our bicompartamental preparation, the local environment in the gastric compartment can be changed by the addition of test compound without interference to any brain stem function. The development of obesity in rodent models has been shown to be concomitant with effects from hormonal and metabolic changes on leptin homeostasis (19). Because our experiments were performed on nonobese, preweaned animals, the complicating effects of metabolic patterns on leptin activity in adults were avoided.

Leptin achieves its metabolic effects by interacting with specific receptors (OB-R) located in the brain and peripheral tissues (22). The different OB-R isoforms are widely distributed throughout the choroid plexus and hypothalamus, as well as in the lung, kidney, gut, and adipose tissue (22, 24). Leptin activity in the hypothalamus results in inhibition of food intake and an increase in energy expenditure (31). Although the hypothalamus is a critical target for the satiety effects of leptin (14, 20), several observations suggest that leptin may have other functions through direct effects on peripheral tissues (3, 7).

In this study, we examined the peripheral gastric effects of leptin on NTS units processing gastric vagal inputs, an issue that has not been previously addressed. We used a 30-s data recording time for each observation (i.e., before or after drug application), the time epoch successfully utilized in our previous studies (4, 28–30). Our results indicate that peripheral gastric application of leptin increased the activity of NTS units in a concentration-dependent manner. There is evidence that, in its role of maintaining optimum body

**Table 1. Percentage of activation and no effect responses of NTS neurons to peripheral gastric effects of leptin (10 nM) in different neonatal rat age groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Activation result (activation level)</th>
<th>1–2 day old</th>
<th>4–5 day old</th>
<th>7–8 day old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation effect</td>
<td>25% (170.0 ± 2.5%)</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>No effect</td>
<td>75% (167.0 ± 4.6%)</td>
<td>9</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>12</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate level of activation compared with control (100%) and are means ± SE; n = no. of units recorded. NTS, nucleus tractus solitarius. There is a significant difference in the response ratios (P < 0.05) and level of activation (P < 0.01) between the 7- to 8-day-old group and the 2 younger age groups.
weight, leptin may signal the brain via gastric vagal afferents. Wang et al. (26) observed that intra-arterial injections of leptin significantly increased the poststimulus spike count of some gastric vagal terminals. With the use of our experimental model, which included the brain stem, our results show that second- or higher-order neurons in the vagal afferent system are sensitive to stimulation by gastric leptin. A recent investigation showed that leptin mRNA and leptin protein are present in the rat gastric epithelium and that glands of the gastric fundic mucosa are immunoreactive for leptin (2), indicating that the stomach is a source of leptin. Our results suggest that gastric leptin, by influencing vagal afferents, may potentially be involved in early events activated by food intake, such as CCK-induced satiety (5).

Previous investigators have shown that intraperitoneal leptin injections in mice at a high dose (27) or low dose (24) did not increase Fos expression in several observed nuclei in the central nervous system. However, it is difficult to compare leptin doses that were used in these in vivo studies to the leptin concentrations used in our in vitro organ-specific neonatal rat preparation. Understanding the pharmacological effects of leptin is further complicated by differences among species, animal age, route of administration, and region of anatomic site examined, as well as the precise experimental conditions. Nonetheless, it is interesting to investigate whether peripherally acting leptin shows a synergistic effect with CCK, as has been previously reported (5) in our experimental model.

Experimental evidence suggests that leptin affects fat accumulation and metabolism, which are independent of its central inhibitory action on food intake (10). Supportive evidence for a peripheral inhibitory role in fat absorption is also provided by a recent study showing that OB-Rb isoform expression in the jejunal epithelium of the mouse is associated with a decrease in nutrient absorption and lipid intake (15), suggestive of a feedback loop involving fat stores and the gastrointestinal tract. Results from our study suggest that gastric leptin, by modulating vagal afferents in the NTS, provides rapid information to central processing stations while food is in transit. One of the axonal projections of the NTS neurons receiving gastric vagal afferent inputs is the dorsal motor nucleus of the vagus, an area of the preganglionic parasympathetic motoneurons that provides vagal outflow to the viscera (23). Although it is possible that the leptin-initiated vagal stimuli to the NTS that we observed may modify gastric motility and gastric emptying, these stimuli could also potentially provide advance signals to sites of lipid handling in the small intestine.

It is plausible that leptin increased the discharge frequency of vagal afferent fibers in our experimental model by activating gastric OB-Rs. Several alternatively spliced forms of OB-Rs have been found in tissues of different organs, including the stomach (25), suggesting multiple sites of leptin's actions. The OB-Rb, whose COOH-terminal tail appears to be required for transducing the leptin-induced signals that regulate food intake, thermogenesis, and body weight, is also expressed in the stomach and other organs (22, 25). In addition, short isoforms OB-Ra and OB-Rf, whose signaling functions are not known, are relatively abundant in the stomach (25). Although the signaling-competent OB-Rb receptor is expressed in the stomach (22), whether OB-Rbs are bound to vagal afferent fiber membranes and are sufficient to mediate responses to leptin is unknown. Alternatively, leptin may activate vagal afferent fibers secondary to leptin-induced changes in local muscle tension and/or intragastric pressure. In our experimental paradigm, we were unable to differentiate whether the NTS neuronal responses to gastric leptin were mediated through the activation of leptin receptors directly on nerve fibers or were secondary to activation of receptors on gastric smooth muscle cells or both.

It has been shown that leptin is also produced in the placenta (13), suggesting it has an important role in fetomaternal signaling. The leptin system, with respect to ob gene expression and leptin production, is operational one day after birth in the rat (17). As further support for this observation, plasma leptin levels in infants highly correlated to the size of adipose tissue mass (12). Thus it seems that our preparation, which utilized neonatal animals, is appropriate for investigating whether age is a factor in leptin’s gastric actions that influence brain stem neuronal activity.

In the present study, the effect of gastric leptin and its modulation of brain stem neuronal activity were observed in 1-day-old neonatal rats. From day 1 after birth to day 8, both the percentage of activation responses and the level of activity of NTS neurons receiving leptin-sensitive gastric vagal inputs increased. These changes may reflect changes in leptin receptor expression and/or gastric binding during the development of neonatal rats. Rats are born with little white adipose tissue in which the ob gene is highly expressed (31), and they rapidly lay down fat stores during the suckling period. The subcutaneous site is the major fat storage site during this period (17). Rayner and co-workers (17) recently reported that, in lean Zucker rats (+/+), subcutaneous white adipose tissue, as a percentage of body weight, increased rapidly, reaching its maximum at 12 days of age (17). Our results indicate that leptin’s effect system becomes functional immediately after birth, and age, even within the first 8 days after the birth, may affect leptin’s gastric activities. Thus leptin may potentially exert biological effects on the neonate at a time in which both the adipose tissue and the appetite regulatory systems are immature. In summary, our data demonstrate the effects of gastric leptin on brain stem neuronal activity and suggest that leptin may play a role in regulating the ingestive process in neonates.

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