Neuronal activation of brain vagal-regulatory pathways and upper gut enteric plexuses by insulin hypoglycemia

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Yuan, Pu-Qing, and Hong Yang. Neuronal activation of brain vagal-regulatory pathways and upper gut enteric plexuses by insulin hypoglycemia. Am J Physiol Endocrinol Metab 283: E436–E448, 2002;10.1152/ajpendo.00538.2001.—Neuronal activation of brain vagal-regulatory nuclei and gastric/duodenal enteric plexuses in response to insulin (2 U/kg, 2 h) hypoglycemia was studied in rats. Insulin hypoglycemia significantly induced Fos expression in the paraventricular nucleus of the hypothalamus, locus coeruleus, dorsal motor nucleus of the vagus (DMN), and nucleus tractus solitarii (NTS), as well as in the gastric/duodenal myenteric/submucosal plexuses. A substantial number of insulin hypoglycemia-activated DMN and NTS neurons were choline acetyltransferase and tyrosine hydroxylase positive, respectively, whereas the activated enteric neurons included NADPH- and vasoactive intestinal peptide neurons. The numbers of Fos-positive cells in each above-named brain nucleus or in the gastric/duodenal myenteric plexus of insulin-treated rats were negatively correlated with serum glucose levels and significantly increased when glucose levels were lower than 80 mg/dl. Acute bilateral cervical vagotomy did not influence insulin hypoglycemia-induced Fos induction in the brain vagal-regulatory nuclei but completely and partially prevented this response in the gastric and duodenal enteric plexuses, respectively. These results revealed that brain-gut neurons regulating vagal outflow to the stomach/duodenum are sensitively responsive to insulin hypoglycemia.

dorsal motor nucleus of the vagus; nucleus tractus solitarii; glucose; vagus; stomach

CONVERGING EVIDENCE SUGGESTS that hyper- or hypoglycemia affects gastrointestinal (GI) functions by influencing vagal-cholinergic outflow to the viscera. The involvement of upper GI tract organs in the hyperglycemia-induced delay of gastric emptying (5) corresponds to the distribution of the vagus in the GI tract (52). GI functions that are well established to be stimulated by vagal efferent activation, such as sham feeding-induced gastric acid secretion and pancreatic polypeptide release from the pancreas, were remarkably reduced during hyperglycemia (34). In contrast to hyperglycemia, insulin hypoglycemia is well established as a central vagal stimulus on upper GI functions (63, 64, 67). Visceral response to insulin hypoglycemia has been widely used to test vagus nerve integrity (67), especially used postoperatively to test the result of vagotomy (45). These findings established a role of the vagus nerve in mediating the regulation of GI functions by altered glucose metabolism. However, how the neurons in the brain vagal-regulatory nuclei and GI enteric plexuses respond to hypo- or hyperglycemia is still poorly understood.

The medullary dorsal vagal complex (DVC) is composed of the dorsal motor nucleus of the vagus (DMN) and the nucleus tractus solitarii (NTS), which respectively contain somata of parasympathetic efferents that project to the GI tract (4, 48, 61) and neurons receiving vagal afferent input from the viscera (29). The nearby area postrema (AP) and portions of the NTS, where the blood-brain barrier is incomplete, can be the portal of entry for circulating hormones entering the brain (15). The DMN and NTS receive powerful influence from higher brain levels. Stimulation of the neurons in the paraventricular nucleus of the hypothalamus (PVN) activates DMN neurons projecting to the gut (30, 76). In addition, the DMN also receives descending connections from the locus coeruleus (LC), which is the origin of the noradrenergic innervation of the preganglionic autonomic nuclei in the medulla oblongata (66). The LC-DMN pathway may participate in the regulation of vagal preganglionic neurons in the DMN by the PVN (66). At the medullary level, it has been well established in recent years that the DVC receives thyrotropin-releasing hormone (TRH)-containing afferent projections from the raphe pallidus (Rpa) and raphe obscurus (Rob) (36). This caudal raphe-DVC pathway plays an important role in vagal-mediated stimulation of gastric functions by cold stress (6, 70, 71). Peripherally, the gastric and duodenal enteric plexuses, which innervate smooth muscle/mucosal layers and play important roles in regulating gastric and duodenal secretion and motility (14, 50), receive a dense and intricate network of vagal effferent axons (2, 19, 77). Studies on the neuronal responses to abnormal blood glucose levels in these central vagal-
regulatory pathways and the vagal-innervated GI enteric plexuses will provide important information regarding the pathophysiological mechanisms of diabetes-induced GI disorders as well as the hypoglycemia-induced activation of gastric/duodenal functions. Previous studies have revealed that certain brain areas, including the hypothalamus and the brain stem, contain glucose-sensing neurons (47, 62) and are responsive to insulin hypoglycemia (8, 21, 44). However, data showing neuronal activation induced by changed glucose levels simultaneously in the central and peripheral pathways involved in the vagal regulation of GI functions are still lacking.

In the present study, we assessed neuronal activation by insulin hypoglycemia in components of the brain vagal-regulatory pathways, including the PVN, LC, DMN, NTS, AP, Rpa, and Rob, as well as in the peripheral GI myenteric and submucosal plexuses. Immunohistochemical detection of the nuclear protein Fos was used as a marker of cellular activation (40) in the brain sections and GI longitudinal muscle/myenteric plexus (LMMP) (37) or submucosal whole-mount preparations. Double staining of Fos and cuprolinic blue, an established marker for enteric neurons (20), was used to assess the neuronal identity of Fos-immunoreactive (IR) cells in the myenteric plexus. The sensitivity of neuronal responses to hypoglycemia was studied by observing the correlations between serum glucose levels and the numbers of Fos-positive cells in the brain vagal-regulatory nuclei and the upper GI myenteric plexus. The role of vagal afferent signaling in the Fos induction in the brain, and the role of the extrinsic vagal efferent innervation in Fos expression in the gastric/duodenal myenteric plexus in response to insulin hypoglycemia were investigated by vagotomy. We also partly examined the biochemical coding of the brain and myenteric Fos-IR cells in insulin-hypoglycemic rats by use of double labeling of Fos with choline acetyltransferase (ChAT) (39), tyrosine hydroxylase (TH) (27), or vasoactive intestinal peptide (VIP) or with dopamine beta-hydroxylase (NADPH-d), an established marker for nitric oxide (NO)-synthesizing neurons (12, 69). Part of this study has been published in abstract form (74).

MATERIALS AND METHODS

 Animals

Male Sprague-Dawley rats (Harlan Laboratory, San Diego, CA) weighing 280–320 g were housed under controlled conditions (22–24°C, light on from 6:00 AM to 6:00 PM). Animals had free access to rat chow (Ralston Purina, St. Louis, MO) and tap water. All rats were deprived of food, but not water, overnight before experiments. Studies were approved by the Animal Research Committee of the West Los Angeles Veterans Affairs Medical Center.

Drugs

The following drugs were used: crystalline porcine insulin (Eli Lilly, Indianapolis, IN) and atropine sulfate (Sigma Chemical, St. Louis, MO). Both were dissolved in sterile saline immediately before use.

Treatments

Insulin hypoglycemia. Hypoglycemia was induced by insulin (2.0 U/kg) injected subcutaneously. Two hours later, the rats were euthanized and tissues and blood samples collected. A group of untreated rats was used as the euglycemic control. Dose-related response to insulin hypoglycemia was studied in six groups of rats, which received saline (1 ml/kg) or insulin (0.1, 0.25, 0.5, 1.0, or 2.0 U/kg sc) injection 2 h before the tissue and blood collections.

Acute bilateral cervical vagotomy. In one group of overnight-fasted rats, acute bilateral cervical vagotomy was performed as in our previous studies (73) under short enflurane anesthesia (Éthérane; 3–5-min inhalation, 5% vapor concentration in oxygen; Ohmeda Pharmaceutical Products Division, Liberty Corner, NJ). After a cervical midline incision, bilateral cervical vagal trunks were cut. Atropine (8 mg/kg) was injected intraperitoneally 10 min before surgery to alleviate acute respiratory complications associated with bilateral cervical vagotomy (11). Another group of sham-operated rats underwent the same procedure without sectioning of the vagus nerves; the atropine treatment was replaced with saline (1 ml/kg) injection. The rats were awake within 2 min after the surgery. Insulin (2 U/kg sc) was injected immediately after the rats became awake.

Tissues and Blood Collections

The rat was deeply anesthetized with pentobarbital sodium (70 mg/kg ip, Abbott Laboratories, North Chicago, IL). The abdominal cavity was opened, and the whole stomach, whole duodenum, and ∼1.5 cm of the jejunum, ileum, and colon, respectively, were quickly collected. Then the thoracic cavity was opened, and the rat was transcardially perfused with 100 ml of isotonic saline followed by 500 ml of fixative [0.1 M sodium phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde and 14% picric acid]. Blood samples (1.0 ml/rat) were collected from the left heart ventricle immediately before the perfusion. The sera were kept at −75°C until the glucose levels were measured using a Beckman Glucose Analyzer 2 (Beckman Instrument, Diagnostic System Group, Brea, California). The brain was collected after the perfusion.

Immunohistochemistry

Brain tissue preparation. The brains were postfixed for 4 h at 4°C in the same fixative and subsequently cryoprotected overnight in 20% sucrose–0.1 M PB. Frozen coronal sections (30 μm) were cryostat cut (Microtome) at the interaural levels of 7.30 to 6.88 mm (PVN), −0.5 to −1.04 mm (LC), −2.60 to −4.24 mm (Rpa and Rob), and −4.24 to −5.08 mm (DMN, NTS, and AP) according to the atlas of Paxinos and Watson (49). The brain sections were collected in 0.01 M phosphate-buffered saline (PBS).

GI tissue preparation. Immediately after collection, the stomach was opened along the greater curvature, and the intestinal samples were cut along the mesenteric border. Each GI tissue was pinned flat and fixed overnight in the fixative. After being rinsed in PBS, the corpus and the antrum were separated. Samples were dissected under a surgical microscope to obtain the LMMP whole-mount preparations. Briefly, the mucosa was scraped off, and the submucosa and the circle muscle were carefully removed using fine forceps. The LMMP whole-mount preparation includes the myenteric plexus adhering to the longitudinal muscle. To avoid regional differences, in each rat, ∼0.5 cm² of preparation dissected from the middle portion of the ventral corpus (−0.4 mm from the boundary between the corpus and
the antrum, 0.7 mm from the lesser curvature, and 1.0 mm from the greater curvature) and the entire antrum preparation were collected for morphological staining. In some rats, the corpus and duodenal submucosa were collected.

Fos immunohistochemistry. Fos immunostaining was performed as previously described (37, 73, 75). Briefly, the brain sections or the LMMP whole-mount preparations were rinsed in PBS, and the LMMP preparations were incubated with 0.3% H₂O₂ for 30 min to remove endogenous peroxidase activity. All of the sections and preparations were incubated for 24 h at 4°C with a polyclonal rabbit anti-Fos serum (Fos Ab-5, 1:10,000; Oncogene Research Products, Cambridge, MA) diluted in PBS containing 0.1% sodium azide and 0.3% Triton X-100 (PBS-T, pH 7.4) and then rinsed in PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit secondary antibody (1:1,000; Jackson ImmunoResearch, West Grove, PA). Finally, brain sections or GI preparations were processed using the standard biotin-avidin-horseradish peroxidase method (22). Fos immunoreactivity was detected as a dark brown nuclear staining. Immunohistochemical controls were routinely performed following the same procedure except that the primary antibody was replaced by PBS-T.

Double immunostaining for Fos and ChAT or TH in the brain stem sections or Fos and VIP in the LMMP whole-mount preparations. After Fos immunohistochemistry, as described in Fos immunohistochemistry, except that dianisobenzidine enhanced with nickel ammonium sulfate was used as the first chromogen in the biotin-avidin-horseradish peroxidase method, the sections and GI preparations were rinsed with 0.01 M PBS for 3 h at room temperature. The brainstem sections were then incubated with a rabbit polyclonal antibody raised against ChAT (Chemicon, AB143, dilution 1:1,000 in PBS containing 0.1% Triton and 3% normal goat serum) overnight at 4°C followed by biotinylated goat anti-rabbit secondary antibody (1:1,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature or a mouse monoclonal antibody raised against TH (1:2,000; Boehringer Mannheim, 1 017 381) followed by biotinylated goat anti-mouse IgG (1:1,000; Jackson ImmunoResearch). The LMMP whole-mount preparations were incubated with a polyclonal rabbit anti-VIP serum (1:1,000; CURE Antibody Core no. 7913) overnight at 4°C followed by biotinylated goat anti-rabbit IgG (1:1,000; Jackson ImmunoResearch) for 1 h at room temperature. The sections or GI preparations were finally processed by an avidin-biotin-peroxidase procedure with diaminobenzidine as the second chromogen. Fos immunoreactivity was detected as a dark blue reaction product in the nuclei, and ChAT, TH, or VIP immunoreactivity appeared as a brown reaction product in the cytoplasm.

Fos immunohistochemistry combined with neuronal staining using cuprolinic blue in the LMMP whole-mount preparations. Slightly modified cuprolinic blue counterstaining (20) was used to assess the neuronal identity of myenteric cells expressing Fos. LMMP whole-mount preparations were incubated in PB containing 0.3% H₂O₂ followed by washing with PBS and 0.05 M sodium acetate buffer (pH 5.6) and were then stained for 2 h at 42°C in cuprolinic blue [0.3% quininoline phthalocyanine in 0.05 M sodium acetate-1.0 M magnesium chloride buffer, pH 4.9 (Electron Microscopy Sciences, Fort Washington, PA)]. After being washed in PBS, tissues were processed for Fos immunohistochemistry as described. Neurons were recognized by the turquoise cuprolinic blue staining in cytoplasm, whereas nuclear Fos immunoreactivity was revealed as brown staining in cell nuclei.

Double labeling of Fos and NADPH-d in the LMMP whole-mount preparations. LMMP whole-mount preparations were rinsed in PB and incubated for 30–60 min at 37°C in PB containing 0.3% Triton X-100, 1 mg/ml β-NADPH, and 0.1 mg/ml nitroblue tetrazolium. After a further rinsing, tissues were processed for Fos immunohistochemistry as described. Fos immunoreactivity was detected as a brown nuclear staining, and NADPH-d appeared as a dark blue reaction product in the cytoplasm.

Quantiative Analysis and Statistics

The number of Fos-IR cells was counted under microscopy. In each rat, the mean Fos-positive cells in a number of brain sections of each observed brain nucleus, or the mean in a number of ganglia of each GI location, was calculated [intersural levels of brain sections were according to the atlas of Paxinos and Watson (49): PVN, 10 sections (7.30 to 6.88 mm); LC, 15 sections (−0.50 to −1.04 mm); DMN/NTS/AP, 20 sections (interaural −4.24 to −5.08 mm); myenteric plexus of the corpus, antrum, and duodenum, 25 ganglia. Myenteric ganglia were recognized separately as clearly delineated groups of neurons by well defined internodal fiber tracts (37). The mean from each animal was used to calculate the group mean. Data are expressed as means ± SE of the number of cells or neurons per brain nucleus or per myenteric ganglion. Pictures of brain and gastric or duodenal myenteric plexus illustrated from rats in different groups were taken under identical conditions. Comparisons between group mean values were performed using one-way analysis of variance (ANOVA) followed by Duncan’s contrast. P < 0.05 was considered statistically significant.

RESULTS

Serum Glucose Levels in Control and Insulin-Treated Rats

Serum glucose levels in the control rats were 129.8 ± 7.21 mg/dl (n = 6). In rats receiving insulin treatment (2 U/kg sc, 2 h), the serum glucose levels were 29.8 ± 0.9 mg/dl (n = 4) 2 h after the insulin injection, which was significantly different from the control levels.

Induction of Fos Expression in PVN, LC, DMN, NTS, AP, Rpa, and Rob and in Myenteric Ganglia of the GI Tract by Insulin Hypoglycemia

In the control rats, the numbers of Fos-like IR-positive neurons in each of the observed brain nucleus and the GI myenteric plexus were low (Fig. 1). Insulin hypoglycemia markedly induced Fos expression in the PVN, LC, DMN, and NTS (Figs. 1 and 2) 54-, 1.9-, 42-, and 4.8-fold, respectively, and in the myenteric ganglia of the corpus, antrum, and duodenum 43-, 16-, and 15-fold, respectively (Figs. 1 and 3), compared with the controls. In contrast, Fos-IR cells in the AP were not significantly increased (Table 1). Fos expression was also induced in the submucosal plexus of the corpus, antrum, and duodenum (Figs. 9 and 10). The gastric submucosal ganglia are scarce (16); therefore, the Fos induction was not quantitatively calculated. Noticeable Fos induction was observed neither in the Rpa and Rob in the brain medulla nor in the myenteric plexus of...
Fig. 1. Fos expression in the paraventricular nucleus of the hypothalamus (PVN; A), dorsal motor nucleus of the vagus (DMN; B), and nucleus tractus solitarii (NTS; C) and in the myenteric plexus of the corpus (D), antrum (E), and duodenum (F) in control rats and in rats treated with insulin (2 U/kg sc, 2 h). Each column represents the mean ± SE of no. of rats indicated. *P < 0.05 compared with the corresponding values of the control rats.

Fig. 2. Photomicrographs of the hypothalamic, pontine, and medullary sections showing Fos immunoreactivity in the PVN (A), locus coeruleus (LC; B), and DMN/NTS (C) in control rats and in rats treated with insulin (2 U/kg sc, 2 h). The anatomic locations of the photomicrographs are indicated in the coronal sections (top), adapted from the atlas of Paxinos and Watson (49). Fos-positive nuclei are presented as dark brown staining in the cell nuclei. Scale bars, 100 µm. AP, area postrema; III, third ventricle; cc, central canal.
the jejunum, ileum, and colon, in either the control or insulin-hypoglycemic rats (data not shown).

### Negative Correlations Between Serum Glucose Levels and Numbers of Fos-IR-Positive Neurons in PVN, LC, DMN, and NTS and in Gastric and Duodenal Myenteric Ganglia in Insulin-Treated Rats

The correlation of blood glucose levels with Fos induction in the brain nuclei or the myenteric plexus was obtained in rats treated with saline or different doses of insulin (0.1, 0.25, 0.5, 1.0, and 2.0 U/kg sc). The number of Fos-IR-positive cells in the PVN, LC, DMN and NTS of the rats injected subcutaneously with saline (Fig. 4 and Table 1) was as low as that in rats that received no treatment (Fig. 1). Fos expression gradually increased along with the dose-dependent decrease of serum glucose levels in insulin-treated rats (Fig. 4 and Table 1). In the same animals, the Fos expression in the corpus, antral, and duodenal myenteric ganglia was also increased in an insulin dose-related manner, with an abrupt increase to the peak levels when insulin was injected at the dose of 0.5 U/kg (Fig. 4 and Table 1), which was different from the pattern of the Fos induction in the brain (Fig. 4 and Table 1). Significant negative correlations between serum glucose levels and the numbers of Fos-IR-positive cells in the PVN, LC, DMN, and NTS, as well as in the myenteric ganglia of the corpus, antrum, and duodenum, were observed (Fig. 5 and Table 1). It is obvious that the numbers of Fos-IR cells were increased as soon as the serum glucose levels dropped below 80 mg/dl.

### Effect of Acute Bilateral Cervical Vagotomy on Insulin Hypoglycemia-Induced Fos Expression in PVN, LC, DMN, and NTS and in Corpus, Antral, and Duodenal Myenteric Ganglia

In rats undergoing sham operation, the hypoglycemia-induced Fos expressions in the observed brain nuclei and in the corpus, antral and duodenal myenteric ganglia (Fig. 6 and Tables 2 and 3) were similar to those observed in rats that did not receive any surgery (Fig. 1 and Table 1). Acute bilateral cervical vagotomy did not significantly influence the Fos expression in the brain PVN, LC, DMN, and NTS induced by insulin (2 U/kg) hypoglycemia (Table 2). In contrast, vagotomy completely abolished the insulin-induced Fos expression.

### Table 1. Increase of Fos-like IR positive neurons in the PVN, NTS, AP, and LC and the myenteric plexus of the antrum and duodenum after different doses of subcutaneous insulin injection

<table>
<thead>
<tr>
<th>Location</th>
<th>Dose of Insulin Treatment, U/kg (n = 4/group)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PVN</td>
<td>60.8 ± 7.4</td>
</tr>
<tr>
<td>NTS</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td>AP</td>
<td>17.6 ± 0.21</td>
</tr>
<tr>
<td>LC</td>
<td>62.6 ± 6.3</td>
</tr>
<tr>
<td>Antrum</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>Duodenum</td>
<td>3.7 ± 0.3</td>
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</table>

Each value represents the mean ± SE of 10, 15, or 20 sections in the paraventricular nucleus (PVN), locus coeruleus (LC), or nucleus tractus solitarii (NTS)/area postrema (AP), respectively, or 25 ganglia in the antral or duodenal myenteric plexus per rat and 4 rats per group. IR, immunoreactive. The values of serum glucose levels with each dose of insulin treatment are shown in Fig. 4. *P < 0.05 compared with corresponding value of saline-treated rats (dose 0).
sion in the corpus/antral myenteric plexus and significantly reduced it by 53% in the duodenum (Fig. 6 and Table 3). Fos expression in the submucosal plexus of the corpus and antrum in insulin-treated rats was also abolished by vagotomy (data not shown).

**Colocalization of Fos-IR/ChAT-IR in DMN and Fos-IR/TH-IR in NTS in Insulin-Hypoglycemic Rats**

Double immunostaining of Fos-IR/ChAT-IR or Fos-IR/TH-IR cells in the brainstem sections revealed that...
most, if not all, of insulin hypoglycemia-induced Fos expressions in the DMN were in the ChAT-containing neurons, whereas most of the Fos expressed in the NTS, especially in the medial subnucleus of the NTS (28), were in TH-containing neurons (Fig. 7).

Colocalization of Fos-IR/NADPH-d and Fos-IR/VIP-IR in Gastric and Duodenal Enteric Plexuses in Insulin-Hypoglycemic Rats

Cuprolinic blue staining revealed individual myenteric neurons with clearly outlined cytoplasm (Fig. 6). About 90–93% of the cuprolinic blue-positive neurons expressed Fos after insulin treatment (2 U/kg; Fig. 6), indicating that the majority of Fos-IR myenteric cells in the corpus, antrum, and duodenum in insulin-hypoglycemic rats were neurons. The Fos/NADPH-d double staining in the myenteric ganglia yielded low background and revealed well defined NADPH-d-positive cells (Fig. 8) similar to those described for nitric oxide synthase (NOS)-containing neurons (26). Fibers displaying NADPH-d activity were most prominent in the internodal fiber tracts (Fig. 8). About 8–10 NADPH-d-positive neurons per ganglion and rare Fos-IR cells were observed in the corpus, antral, and duodenal myenteric plexus in rats receiving saline injection (data not shown). Insulin hypoglycemia did not change the number of NADPH-d-positive neurons while inducing a considerable amount of Fos expression in myenteric cells, including 90–100% of NADPH-d-positive neurons in the stomach and 50% of that in the duode-

<table>
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<tr>
<th>Surgery</th>
<th>n</th>
<th>PVN</th>
<th>LC</th>
<th>DMN</th>
<th>NTS</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>4</td>
<td>391.0 ± 28.3</td>
<td>103.8 ± 7.4</td>
<td>48.1 ± 6.7</td>
<td>81.6 ± 4.9</td>
<td>12.4 ± 1.3</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>5</td>
<td>415.1 ± 7.2</td>
<td>93.3 ± 9.9</td>
<td>42.5 ± 3.0</td>
<td>89.0 ± 10.6</td>
<td>11.6 ± 0.9</td>
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</table>

Each value represents the mean ± SE of 10, 15, or 20 sections in each rat in the PVN, LC, dorsal motor nucleus (DMN), NTS, or AP, respectively, and the no. of rats indicated in the Table. Acute bilateral cervical vagotomy or sham operation was performed immediately before the insulin injection (2 U/kg sc).
The ganglionic NADPH-d/Fos-IR neurons comprised nearly 35–40% of the total Fos-IR cells in the corpus and antral myenteric plexus. NADPH-d-positive neurons in the gastric submucosal plexus of insulin-treated rats also expressed Fos (Fig. 9). Few NADPH-d-positive neurons were observed in the duodenal submucosal plexus (data not shown). VIP-IR neurons are rare in the gastric (data not shown) but are abundant in the duodenal myenteric and submucosal ganglia. Nearly all of the VIP-IR neurons in the duodenal enteric plexuses were Fos-IR positive (Fig. 10).

DISCUSSION

Data obtained from the present study show that insulin hypoglycemia strongly and simultaneously induces neuronal activation in the brain vagal-regulatory nuclei, including the PVN, LC, DMN, and NTS, as well as in the vagal-innervated gastric and duodenal enteric plexuses. These data are in agreement with the well-established concept that insulin hypoglycemia is a central vagal stimulus on GI functions (45, 63, 64) and provide morphological evidence for the central and peripheral pathways mediating insulin hypoglycemia-induced visceral function changes.

Insulin can affect brain neurons directly (60). It was reported that microinjection of insulin into the ventromedial hypothalamic nucleus (VMH) (65) or DVC (33) altered GI functions by activating the efferent vagus. However, several lines of evidence suggest that the Fos induction observed in the present study results from the decrease of blood glucose concentrations, rather than from a direct action of insulin. First, it is generally accepted that peripheral gut hormones influence medullary DVC neurons, either through the entrance in the AP and portions of the NTS, where the blood-
brain barrier is incomplete, such as peptide YY, (18) or through the mediation of capsaicin-sensitive vagal afferents such as cholecystokinin (53, 55). In the present study, however, the Fos expression in the AP was not influenced by insulin treatment, a fact that confirms the observations of Horn et al. (21) but does not support the idea that peripheral insulin acts in the DVC through this entrance. Current literature does not suggest that vagal afferent fibers are sensitive to insulin but rather supports its sensitivity to glucose levels (43, 68). Second, in the present study, we observed strong negative correlations between serum glucose levels and Fos expression in the PVN, DMN, and NTS in insulin-treated rats. It is interesting to note that, when serum glucose levels dropped below 80 mg/dl, Fos expression began to increase in these vagal-regulatory nuclei and further increased along with the decrease of glucose levels. Confirmation that the brain Fos induction by insulin hypoglycemia was related to hypoglycemia-induced cellular energy deficiency comes from the fact that acute glucose deprivation induced by 2-deoxyglucose (2-DG) induced similar vagal-mediated stimulation on visceral functions (32) and Fos expression in the PVN, DMN, and NTS (7, 59). Finally, the observation that glucose microinjected into the DVC or injected into the portal vein inhibited insulin hypoglycemia-induced gastric acid secretion and motility (57, 58) provides additional support for this view. Because hypoglycemia induces complicated changes in the release of central and peripheral counterregulatory hormones (25), the possibility that these reactions contribute to the brain Fos induction could not be excluded. Fos expression was not changed in the AP, an entrance for peripheral hormones into the brain (18). Although this observation does not support a role of peripheral hormone action, the participation of neural and hormonal interactions in the Fos induction of brain vagal-regulatory pathways during hypoglycemia is still to be investigated.

Previous and current findings indicate that the medullary DVC is a critical center mediating central and GI responses to insulin hypoglycemia. In dogs, decerebration and midbrain or pontine section could not prevent the insulin hypoglycemia-induced gastric acid secretion that was profoundly reduced after destruction of the DMN (31). The NTS neurons transmit information on local glucose availability (72) as well as peripheral glucose metabolic signals received from the vagal...
afferents (43) to hypothalamic structures, including the PVN, via ascending adrenergic and noradrenergic pathways (10). A number of NTS neurons directly, or indirectly via interneurons, connect with vagal motor neurons in the DMN, forming a vago-vagal reflex (56). On the other hand, although the PVN receives ascending signals from the NTS, it provides direct control over neuronal activities in the DMN and the NTS (76) and is well established to be the principal source of descending projections to the DVC related to glucose metabolism in rats (51). We observed strong simultaneous Fos induction in these nuclei by insulin hypoglycemia, indicating that central vagal regulatory pathways are strongly activated. In addition, we characterized the Fos-expressing neurons in the DMN as ChAT-containing neurons, a marker of vagal preganglionic motor neurons, and those in the NTS as TH-containing neurons, a marker of catecholaminergic neurons. Other hypothalamic areas, such as the VMH, also contain glucose-sensitive neurons (1) and have direct connections with the DMN and NTS (46). Therefore, the possibility cannot be excluded that the Fos induction in the DVC and PVN may be partly attributable to signals from the glucose-sensitive neurons located in other brain nuclei. Compared with the PVN, NTS, and DMN, the Fos induction in the pontine LC in response to hypoglycemia was relatively modest. Our findings indicate that the LC may also participate in the central vagal response to hypoglycemia. In support of this indication, it has been reported that the LC sends direct projections to the medullary DMN (66), and intracerebroventricular injection of 2-DG induces Fos expression in the TH neurons of the NTS and LC (7).

Glucose-sensitive neurons and glucose sensors are located both centrally (47) and peripherally (35). Electrophysiological studies revealed that glucose had no direct excitatory effect on DMN neurons, which appear to be affected by an action of glucose on cell bodies of NTS neurons (13). Besides sensing local glucose availability (72), the NTS relays vagal afferent-mediated information from glucoreceptors in the gut, portal vein, or liver (43). However, our data suggest that the central neuronal activation induced by insulin hypoglycemia is independent of the vagal afferents. This was evidenced by the finding that bilateral cervical vagotomy did not influence insulin-induced Fos expression in the brain. In support of this view, a cholecystokinin A receptor antagonist, which blocked duodenal glucose loading-induced and vagal afferent-mediated Fos expression in the NTS (68), has no effect on insulin hypoglycemia-induced stimulation of gastric and pancreatic secretions (32). Similarly, by use of vagal cooling approaches, it was shown that the vagal afferents do not play an important role in the physiological neural and hormonal counterregulatory response to insulin hypoglycemia (9, 25). Taken together, these findings indicate that neuronal activation in the brain vagal-regulatory nuclei was mediated through glucose sensors and signals responding to hypoglycemia in the brain.

The GI smooth muscle and mucosal layers are directly innervated by enteric plexuses (14, 50). Consistent with the strong gastric acid- and motor-stimulatory effects of insulin hypoglycemia, as expected, we observed remarkable Fos expression in the myenteric and submucosal plexuses of the corpus, antrum, and duodenum in rats treated with insulin. Glucose-responsive neurons have been identified and characterized in the gut myenteric plexus of guinea pigs by electrophysiological studies (35), indicating that the GI enteric nervous system may respond independently to blood glucose changes. However, converging evidence suggests that central vagal stimulation is involved in the insulin hypoglycemia-induced Fos expression in the gastric and duodenal enteric neurons. First, we simultaneously observed Fos expression in brain vagal-regulatory nuclei, especially the medullary DMN, which is indicative of vagal preganglionic neuronal activation, in the same animals. Second, the Fos expression was induced in the enteric plexuses of the stomach and duodenum but not in the jejunum, ileum, and colon, a fact that accords with the denser distribution of the vagal innervation in the upper GI tract (52). Anterograde tracing studies showed that vagal efferent terminals form a dense network encircling or making putative contact with nearly all myenteric neurons in the rat corpus and antrum (19). The diffuse, widespread, and similar magnitude of Fos expression in the corpus, antral, and duodenal myenteric ganglia in response to insulin hypoglycemia is consistent with the vagal efferent innervation in these areas (19). Third, bilateral cervical vagotomy completely abolished the insulin hypoglycemia-induced Fos expression in the gastric myenteric ganglia and significantly reduced it in the duodenum. The abolition of Fos expression by vagotomy did not result from a nonspecific stressful effect of acute surgical or atropine treatment, as the Fos induced by central vagal stimulation was not altered in acute sham-operated rats that underwent the same anesthesia, surgical procedures (present study and Ref. 73), and atropine treatment (73) except for the sectioning of the vagus. Fourth, recent studies have shown that electrical stimulation of vagal efferents (77) or central vagal activation by intracerebral TRH analog (38) or cold stress (73) induces Fos expression in the gastric myenteric plexus. Unlike the pattern of the dose-related increase of Fos expression in the brain vagal-regulatory nuclei that was gradually changed along with decreasing blood glucose levels, the Fos expression in the gastric and duodenal myenteric plexus reached its maximal expression before glucose reached its lowest levels. This indicates that activating only a portion of the DMN neurons by hypoglycemia was sufficient to mobilize nearly all the myenteric-responsive neurons. The incomplete abolishment of the Fos expression in the duodenal myenteric plexus by vagotomy is consistent with relatively less vagal innervation in this location compared with the stomach (52) and suggests that other mechanisms besides the vagal mediation may contribute to the neuronal activation in duodenal myenteric plexus in response to insulin hy-
poglycemia. Both the gluoresponsive neurons, which were excited by increases in extracellular glucose, and the glucosensitive neurons, which were excited by decreases in extracellular glucose, were found in the intestinal myenteric plexus of guinea pigs (35). It is not clear whether the sensitivity of myenteric neurons for directly sensing changes of glucose levels is identical or not in different portions of the GI tract. One explanation for the failure of Fos induction in the lower GI tract in response to insulin hypoglycemia could be that the Fos expression is a much less sensitive parameter for indicating cellular excitation compared with electrophysiological recordings (35).

The vagus nerve displays major autonomic control over food digestion, which is the main source for blood glucose. In addition, the vagus nerve regulates the release of hormones controlling glucose metabolism such as insulin and glucagon. The tight control of vagal activity by glucose concentration is important for maintaining energy homeostasis. Several energy-unbalanced states, such as hyperthermia and hypothermia, have been well established to elevate vagal effferent outflow and induce vagal-mediated gastric secretary and motor stimulation (17, 71). In these situations, the TRH-containing projections arising from the caudal raphe nuclei and innervating the DVC (36) were activated (6, 75). In contrast, insulin hypoglycemia induces a vagal-mediated gastric functional response (63, 64, 67) and gastric myenteric neuronal activation (present study) similar to that induced by acute cold stress (71, 73) but does not induce Fos expression in the Rpa and Rob. These findings indicate that energy-unbalanced states induced by specific factors may activate separate brain pathways by different mechanisms, with the DVC serving as the common, final, key nuclei for vagal regulation of GI functions.

The vagal-dependent, atropine-sensitive stimulation of gastric functions induced by insulin hypoglycemia (63, 64, 67) is indicative of cholinergic myenteric neuronal activation. ChAT, the enzyme synthesizing acetylcholine, is contained in ~60% of rat myenteric neurons (41). However, ChAT immunoreactivity, which has been used to visualize cholinergic neurons and their processes in the central nervous system, is less successfully applied to the peripheral cholinergic system, especially in rats, without the use of specific antisera against peripheral ChAT (41, 54). The number of ChAT-positive neurons in the gastric myenteric plexus in acute cold-exposed rats observed in our previous study (73) did not match the data obtained using antisera against peripheral ChAT (41). Further studies using this specific antisera will be needed to investigate the activation of ChAT-containing myenteric neurons by insulin hypoglycemia. In rats, the NADPH-dand NO-sensitive gastric myenteric neurons also receive vagal efferent contacts (2, 3) and provide an extensive network of axonal projections running within the circular smooth muscle layer (2). NO in the myenteric nervous system plays an important role in the nonadrenergic, noncholinergic relaxation of smooth muscles (42). VIP is an intestinal neuropeptide that is released in response to vagal stimulation and plays important roles in intestinal secretion, smooth muscle relaxation, and vasodilatation (23, 24). Insulin hypoglycemia-induced and vagally mediated activation of NADPH-d and VIP-positive neurons in the gastric and duodenal enteric plexuses indicates that these neurons are responsive to changed blood glucose levels. These results also provide additional evidence, besides that observed in an acute cold exposure model (73), supporting the central vagal regulation of enteric excitatory and inhibitory systems.

In summary, the present study demonstrates that the neuronal activities in the brain vagal-regulatory pathways, including neurons in the PVN, LC, DMN, and NTS, are sensitively influenced by insulin hypoglycemia. These central reactions are responsible for the altered vagal efferent outflow to the stomach and duodenum. The strong vagally-dependent neuronal activation in the gastric and duodenal enteric plexuses convincingly revealed the role of the vagus nerve in mediating the profound upper GI functional changes that originate from the brain reactions to hypoglycemia. These findings extend our understanding of the central and peripheral mechanisms of the vagal-mediated regulation of upper GI functions by glucose metabolism and the pathophysiology of GI abnormalities observed in diabetes such as delayed gastric emptying and upper GI bleeding in cachexia patients.

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