Neural regulation of hindlimb muscle contraction-induced glucagon-like peptide-1 and peptide YY secretion in rats

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Introduction

The role of gut hormones in the treatment of obesity has been widely recognized1-4). Glucagon-like peptide-1 (GLP-1) is a satiety factor5-7) that is released into the circulation after a meal in proportion to the amount of food consumed, with the major source of postprandial GLP-1 release being L-cells in the intestine5-7). GLP-1 is the most powerful known incretin in humans, and manipulating the GLP-1 system has formed the basis of several major new treatments for type 2 diabetes5-7). Peptide YY (PYY) is also recognized as a satiety factor that is secreted from L-cells in the intestine after a meal and suppressed by fasting8,9).

Previous studies have shown that increases in the plasma concentrations of GLP-110-14) and PYY15,16) were mediated by a neural pathway. The vagus nerve efferent pathway, in particular, was examined for its global effects on basal GLP-114,17,18) and PYY15,16) secretion and represents the main parasympathetic nerve innervating the proximal portion of the gastrointestinal tract.

On the other hand, previous studies demonstrated the inhibitory effects of acute exercise on the hunger induced by these hormones in healthy subjects19-21). We also showed that a single bout of aerobic exercise caused significant increases in GLP-1 and PYY plasma levels, and decreases in subsequent energy intake in obese and non-obese subjects22-24). A considerable discrepancy has been identified between vagus nerve-mediated and exercise-induced mechanisms because exercise evokes a decrease in vagus nerve activity25,26). Therefore, the vagus nerve may not play an important role in the increases observed in GLP-1 and PYY plasma levels with exercise. A clearer understanding of this conflicting mechanism may assist in the development of new exercise programs to prevent and treat obesity.

As described above, exercise is accompanied by an increase in GLP-1 and PYY plasma levels; however, the physiological mechanisms for this phenomenon have yet to be elucidated in detail. To the best of our knowledge, only one previous study has investigated the physiological mechanisms underlying this increase in GLP-1 plasma levels27), the findings of which demonstrated that this increase was mediated by skeletal muscle-derived interleukin-6 (IL-6) via a humoral pathway. However, GLP-1, but not PYY, was examined in this study. If these increases are mediated by skeletal muscle-derived IL-6 via a humoral pathway, as previously suggested27), the activation...
of skeletal muscle-derived afferent neurons may also be involved in these increases.

Therefore, we investigated the neural regulation of GLP-1 and PYY secretion during exercise in rats using a hindlimb muscle contraction model. We hypothesized that the increases observed in GLP-1 and PYY plasma levels with exercise may be mediated by the activation of skeletal muscle-derived afferent neurons, and not by mechanisms through the neural pathway of the vagus nerve.

Materials and methods

Animal preparation. Animal care was conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences approved by the Physiological Society Japan. All protocols were reviewed and approved by the Animal Subjects Committee of the Ethics Committee of Morinomiya University of Medical Sciences (Admitting No. 2012-A003).

Twenty-seven male Sprague-Dawley rats (12-14 weeks old, 340-480 g body weight) were fasted overnight, but allowed free access to drinking water and housed two per cage in a temperature-controlled room with a 12:12-h dark-light cycle. Rats were anesthetized by a mixture of urethane (250 mg/ml) and α-chloralose (40 mg/ml), initiated with an intraperitoneal bolus injection of 2 ml/kg. The right carotid artery was cannulated for blood sampling.

Protocol 1. In order to determine whether the increases observed in GLP-1 and PYY plasma levels during exercise were mediated by the activation of skeletal muscle-derived afferent neurons or the vagus nerve efferent pathway, we compared hindlimb muscle contraction-induced GLP-1 and PYY plasma levels in sciatic nerve deafferentation (SND, n = 7), vagotomy (VAG, n = 7), and sham (n = 7) trials (Fig. 1). With the rat in a prone position, the bilateral sciatic nerve was exposed by a skin incision at the proximal thigh region of the hamstring, followed by a blunt incision in the biceps femoris. A pair of bipolar stainless steel electrodes was attached to the bilateral sciatic nerve. In the SND trials, the sciatic nerve afferent, which was 1 cm away from the sciatic nerve and in contact with the pair of bipolar stainless steel electrodes, was cut. The effectiveness of SND was verified by the absence of a muscle contraction with electrical stimulation. In the VAG trials, a midline incision of 2 cm was made in the anterior neck to localize the bilateral vagus trunk. The vagus nerve was separated carefully from the carotid artery and cut, and the skin was sutured. VAG was confirmed to be successful by the presence of an increased heart rate. Heart rate was measured from electrocardiograms obtained using a cardiotachometer. Sham rats were operated on to expose the vagal trunk, but neither the vagus nerve nor sciatic nerve afferent was cut. Hindlimb muscle contraction was induced by electrically stimulating the sciatic nerve for 20 min (5 V, 5 Hz). A fasting arterial blood sample (Baseline) was taken (0.7 ml). Rats were subjected to 20 min of hindlimb muscle contraction in vivo, and blood samples were collected at the end of the hindlimb muscle contraction protocol (0.7 ml).

Protocol 2. As a supplemental protocol, we investigated the effects of electrical vagal stimulation on GLP-1 and PYY concentrations in rats. With the rat in a supine position, a pair of bipolar stainless steel electrodes was attached to the vagus nerves in the neck. Six rats were subjected to a 20-min electrical stimulation protocol (5 V, 5 Hz). A fasting arterial blood sample (Baseline) was taken (0.7 ml). The vagus nerves of rats were stimulated for 20 min in vivo, and blood samples were collected at the end of this protocol (0.7 ml). The successful stimulation of the vagus nerves in the neck was confirmed by the presence of a decreased heart rate. Heart rate was measured from the electrocardiogram obtained using a cardiotachometer.

Blood sampling. Blood samples were immediately transferred into disodium EDTA-treated tubes to measure...
plasma GLP-1 and PYY. Test tubes were centrifuged at 3000 rpm. for 15 min at 4 °C immediately after collection, and plasma samples were then stored at -80 °C until their later use in hormone assays. Plasma GLP-1 (GLP-1 (7-36) amide) and PYY levels were determined by EIA (GLP-1/ PYY EIA kit, Yanaihara Institute Inc., Shizuoka, Japan). The PYY ELISA quantified the total amount of both PYY$_{1–36}$ and PYY$_{3–36}$. The between-run reproducibilities for GLP-1 and PYY were 5.51-18.87% and 4.2-14.2%, respectively. The within-run reproducibilities for GLP-1 and PYY were 5.36-6.60% and 3.1-9.8%, respectively. The within-run reproducibilities for GLP-1 and PYY were 5.36-6.60% and 3.1-9.8%, respectively. All sample measurements were performed in duplicate according to the manufacturer’s instructions. pH and Paco2 were measured using a blood gas analysis apparatus (GEM premier 4000, Instrumentation Laboratory, Tokyo, Japan). Blood lactate was analyzed using a simplified blood lactate test meter (Lactate Pro2, Arkray Inc., Kyoto, Japan).

Statistical analyses. All data are presented as the mean and SD (standard deviation) values. A two-way analysis of variance (ANOVA) was performed in protocol 1 to examine the effects of hindlimb muscle contraction and the trial on GLP-1, PYY, lactate, pH, and Paco2 levels. Changes in (delta) GLP-1 and PYY levels were also compared by a one-way ANOVA. If significance was detected, post-hoc multiple comparisons (Dunnett’s test) were used. In protocol 2, electrical vagal stimulation effects on heart rate, GLP-1, and PYY were tested by the Student’s paired t-test. P-values less than 0.05 were considered significant.

Results

Protocol 1. The hindlimb muscle contraction protocols performed in the sham, SND, and VAG trials were well-matched, with no significant differences being observed in lactate, arterial pH, or Paco2 levels between either session ($P > 0.05$; Table 1). The baseline heart rate was significantly higher in the VAG trial than in the sham trial ($P < 0.05$), which confirmed that VAG was performed appropriately (Table 1). The significant main effects of hindlimb muscle contraction ($P < 0.001$), trial ($P < 0.01$), and interaction ($P < 0.01$) were observed on GLP-1 and PYY levels (Table 1). No significant differences were observed in baseline GLP-1 and PYY levels between the sham and SND or VAG trials (Table 1). Although GLP-1 and PYY levels were significantly increased after hindlimb muscle contraction (Sham: $P < 0.001$, SND: $P < 0.05$, VAG: $P < 0.001$), they were significantly lower in the SND trial than in the sham trial ($P < 0.01$ respectively) (Table 1). Furthermore, changes in (delta) GLP-1 and PYY levels were significantly lower in the SND trial than in the sham trial ($P < 0.001$, respectively) (Fig. 2). On the other hand, no significant differences were observed in GLP-1 or PYY levels between the sham and VAG trials (Table 1, Fig. 2).

Protocol 2. Heart rate decreased significantly from 328.0 ± 23.0 bpm at baseline to 261.3 ± 22.3 bpm after vagus nerve stimulation in the neck ($P < 0.001$), which confirmed that this stimulation was performed appropriately. GLP-1 and PYY levels did not change with the vagus nerve stimulation in the neck (Fig. 3).

Discussion

To the best of our knowledge, this is the first study to investigate the neural regulation of exercise-induced increases in anorectic gut hormones in rats. The main result of the present study is that the increases observed in

| Table 1. Effects of the hindlimb muscle contraction protocol on blood parameters. |
|-----------------|-----------------|-----------------|
|                  | Sham ($n = 7$)  | SND ($n = 7$)   | VAG ($n = 7$)   |
| GLP-1 (pmol/ml) | 1.3 ± 0.3       | 2.8 ± 0.3***    | 1.4 ± 0.4       | 2.2 ± 0.7†       | 1.2 ± 0.3       | 2.8 ± 0.4***    |
| PYY (pmol/ml)   | 0.6 ± 0.4       | 1.5 ± 0.3***    | 0.6 ± 0.2       | 1.2 ± 0.3**      | 0.7 ± 0.2       | 1.5 ± 0.4***    |
| Lactate (mmol/l)| 1.5 ± 0.3       | 3.2 ± 0.2***    | 1.5 ± 0.4       | 3.4 ± 0.9***     | 1.7 ± 0.9       | 4.3 ± 1.1***    |
| Arterial pH     | 7.36 ± 0.07     | 7.32 ± 0.08*    | 7.35 ± 0.04     | 7.25 ± 0.09**    | 7.33 ± 0.06     | 7.22 ± 0.06**   |
| Paco2 (mmHg)    | 44.9 ± 6.9      | 42.7 ± 6.9      | 50.8 ± 6.3      | 47.3 ± 6.7       | 54.1 ± 6.7      | 51.5 ± 9.2      |
| Heart rate (bpm)| 298.2 ± 38.0    | 410.8 ± 16.8**  | 356.8 ± 35.9    | 397.2 ± 24.3*    | 383.8 ± 27.1†   | 408.2 ± 24.5*   |

All values are shown as the mean ± S.D. Data were analyzed using a two-way ANOVA and Dunnett’s post hoc test.

$***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ significantly different from baseline. $†P < 0.05$ significantly different from the Sham trial.

SND sciatic nerve deafferentation trial; VAG vagotomy trial
GLP-1 and PYY plasma levels with exercise required the activation of skeletal muscle-derived afferent neurons. Direct stimulation of the celiac branch of the vagus nerve was previously shown to result in a significant increase in gut glucagon-like secretion. Furthermore, infusions of muscarinic cholinergic agonists into an isolated perfused rat ileum and colon were reported to result in an increase in GLP-1 secretion. On the other hand, the electrical stimulation of vagus nerves was shown to have no effect in anesthetized pigs. No significant differences were observed in plasma GLP-1 levels between sham and VAG trials in the present study (Table 1, Fig. 2A). Although the increase observed in GLP-1 plasma levels may have been mediated by specific exercise-induced pathways, we confirmed that the vagus nerve had no effect. The results of the present study showed that although hindlimb muscle contraction induced increases in GLP-1 plasma levels, sciatic nerve deafferentation attenuated

**Fig. 2** Plasma level responses of GLP-1 (A) and PYY (B) to a hindlimb muscle contraction protocol. The mean values ± SD of each parameter are presented. Data were analyzed using a one-way ANOVA and Dunnett’s post hoc tests. SND sciatic nerve deafferentation trial; VAG vagotomy trial

**Fig. 3** Plasma level responses of GLP-1 (A) and PYY (B) to vagus nerve stimulation in the neck. The mean values ± SD of each parameter are presented. Data were analyzed using the Student’s paired t-test.
this increase (Table 1, Fig. 2A), which indicated that the increase observed in GLP-1 plasma levels during exercise was mediated by the activation of skeletal muscle-derived afferent neurons. A previous study demonstrated that this increase was mediated by skeletal muscle-derived IL-6 via a humoral pathway\(^27\). In order to determine whether IL-6 was required for this exercise-induced increase in GLP-1, Ellingsgaard et al. (2011)\(^27\) subjected IL-6 knock-out mice to a bout of exercise and found they were unable to increase plasma GLP-1 levels. IL-6 increases in response to muscle contractions. Serum IL-6 exponentially increases with the duration of exercise up to 100-fold, is rapidly released into circulation, and precedes the appearance of other cytokines\(^28\). Although this exponential increase is related to exercise intensity, we speculated that serum IL-6 levels increased with muscle contractions in this study. Lactate levels were significantly higher after the muscle contraction protocol than baseline in all trials, and arterial pH was significantly lower than baseline (Table 1). Therefore, exercise-induced increases in IL-6 levels influenced the present results. Further studies are needed to elucidate the interplay between the two mechanistic pathways (neural and humoral) underlying the increase in GLP-1 plasma levels during hindlimb muscle contraction.

Previous studies revealed that electrical vagal stimulation induced the release of PYY in a dog and pig\(^29\). In the dog, bethanechol i.v. and feeding induced the release of PYY, while atropine and hexamethonium blocked the food-induced release of PYY\(^10\). Vagal stimulation-induced PYY release was also blocked by atropine in the pig\(^15\). These findings indicated that vagal cholinergic nerves mediated the postprandial release of PYY\(^29\). However, no significant differences were observed in plasma PYY levels between the sham and VAG trials in rats in the present study (Table 1, Fig. 2B). Although we cannot provide a definite explanation for this difference, the following factors may have affected this outcome: 1) possible variations between species. In the present study, rats were anesthetized and subjected to a 20-min electrical stimulation protocol to the vagus nerves in the neck in vivo, at the end of which PYY and GLP-1 levels were measured. However, no significant differences were observed in PYY levels between pre and post stimulation, which was consistent with the results obtained for GLP-1 (Fig. 3). 2) The increase observed in PYY plasma levels with exercise may have been mediated by the same specific exercise-induced pathways for GLP-1. Although hindlimb muscle contraction induced increases in PYY plasma levels, sciatic nerve deafferentation attenuated this increase (Table 1, Fig. 2B).

A previous in vitro study demonstrated that PYY and GLP-1 were co-localized in endocrine L-cells and both were synchronically secreted from these cells in the rat isolated distal ileum after the direct administration of intestinal regulatory peptides\(^11\). Of particular interest in the present study is that similar results were obtained for both GLP-1 and PYY. In other words, the neural regulation of GLP-1 and PYY secretion during exercise occurred via a similar mechanism. Collectively, the present results may provide a new insight into the mechanisms underlying the exercise-induced changes in GLP-1 and PYY levels.

There were some potential limitations to the present study. We could not measure other appetite-related hormones such as ghrelin. GLP-1 and PYY originate from the same secretory cells and have anorexigenic functions\(^5\,\text{-}\,9\). In addition, previous studies reported a correlation between the magnitude of the exercise-induced increase in plasma GLP-1 and PYY levels and concurrent decrease in energy intake\(^9\). Therefore, we focused on GLP-1 and PYY. However, a recent meta-analysis revealed that exercise may influence appetite by suppressing acylated ghrelin levels\(^3\). Additionally, we could not measure other hormone and cytokine levels, including IL-6. The major limitation inherent to the EIA of GLP-1 and PYY is the large plasma volume that is needed to accurately measure GLP-1 and PYY levels in rats. The involvement of hormonal mechanisms to explain the rapid onset of the secretion of these anorectic gut hormones has been considered\(^18\,\text{-}\,29\). Glucose-dependent insulinotropic peptide (GIP) and catecholamines have been shown to stimulate the secretion of GLP-1\(^10\,\text{-}\,12\,\text{and}\,32\), while cholecystokinin (CCK)\(^3\,\text{and}\,34\) and cortisol\(^3\) were found to stimulate that of PYY. We intend to measure other hormone (e.g., ghrelin, CCK, GIP) and cytokine (e.g., IL-6) levels in future studies using anesthetized pigs or rabbits because larger amounts of blood can be collected from these animals than from rats. Total GLP-1 was measured rather than active GLP-1 in this study. As we did not add a dipeptidyl peptidase 4 (DPP-4) inhibitor, we were unable to measure active GLP-1 levels. A previous study demonstrated that active GLP-1 and total GLP-1 levels showed the same pattern during the glucose tolerance test\(^3\). Therefore, these findings prompted us to speculate that total GLP-1 levels may reflect active GLP-1 responses to exercise. Future studies incorporating a DPP-4 inhibitor need to be conducted in order to enable the assessment of active GLP-1 levels because previous findings have suggested that total GLP-1 levels reflect changes in active GLP-1 levels.

In conclusion, the increases observed in GLP-1 and PYY plasma levels with exercise were mediated by the activation of skeletal muscle-derived afferent neurons, and not by mechanisms through the neural pathway of the vagus nerve.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.
References


ization, acclimatization, and sample processing affect gut hormone levels and appetite in humans. *Gastroenterology* 136: 2115-2126.