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Glucagon-like peptide-1 and insulin synergistically activate vagal afferent neurons

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ABSTRACT

Intestinal glucagon-like peptide-1 (GLP-1) and pancreatic insulin, released postprandially, commonly regulate glucose metabolism. Recent clinical experience indicates that the GLP-1R agonist and insulin in combination, compared to insulin alone, results in better glycemic and weight controls in type 2 diabetic patients. These observations suggest possible interactive effect of these hormones. These hormones, in addition to peripherally controlling glycemia, exert central regulation of food intake and glucose metabolism, the effect at least partly mediated by signaling to the brain via the vagal afferents. However, whether the vagal afferents are involved in the interactive effects of GLP-1 and insulin remains unknown. The present study explored possible cooperative effect of GLP-1 and insulin on vagal afferent neurons isolated from nodose ganglion (NG) of mice, while monitoring the neuronal activity by measuring cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura-2. GLP-1 at 10^{-8} M increased $[Ca^{2+}]_i$ in 8–11% of single NG neurons. GLP-1-induced $[Ca^{2+}]_i$ increases were inhibited by GLP-1 receptor antagonist exendin (9–39). Majority (92%) of GLP-1-responseive NG neurons also responded to 10^{-7} M insulin with $[Ca^{2+}]_i$ increases. Both GLP-1 and insulin at lower concentration of 10^{-9} M induced $[Ca^{2+}]_i$ increases with smaller amplitude in lesser NG neuron population (4–7%). These hormones at 10^{-9} M in combination recruited the unresponsive neurons to $[Ca^{2+}]_i$ increases, and induced $[Ca^{2+}]_i$ increases with greater amplitude in the responsive neurons. The results demonstrate that GLP-1 and insulin synergistically and additively activate vagal afferent neurons. This interaction may be linked to the postprandial functions mediated commonly by GLP-1 and insulin and in the beneficial outcome of the therapy with GLP-1 receptor agonist and insulin in combination.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is produced and released from the intestinal L-cells in response to meal (Diakogiannaki et al., 2012; Holst, 2007; Tian and Jin, 2016), and enhances glucose-stimulated insulin release from pancreatic islets and regulates glycemia, being recognized as the incretin effect, and also contributes to induction of satiety, the functions involving the brain. Peripheral molecules can inform the brain principally through two routes: penetrating the blood-brain barrier (BBB) to directly act on the brain and/or interacting with the vagal afferent nerves that signal to the brain (Iwasaki and Yada, 2012). GLP-1 receptor (GLP-1R) is expressed in the hypothalamic neurons and the vagal afferent nodose ganglion (NG) neurons (Nakagawa et al., 2004; Richards et al., 2014), key regions for regulating feeding and glucose metabolism.

GLP-1 is rapidly degraded to inactive form by dipeptidyl peptidase-4 (DPP-4), resulting in a short half-life of 1–2 min. Consequently, < 25% of the portal GLP-1 reaches the liver, and only 10–15% reaches the circulation (Tian and Jin, 2016). Furthermore, entry of GLP-1 into the brain is strictly limited by BBB (Hassan et al., 1999; Kastin et al., 2002). It was shown that peripheral, but not central, injection of GLP-1R antagonist exendin 9–39 (Ex(9–39)) increases food intake and counteracts the inhibition of food intake by peripheral injection of GLP-1 (Williams et al., 2009). These results suggest that the effect of circulating GLP-1 on feeding is mediated primarily by peripheral GLP-1R. The vagal afferents terminate in the lamina propria of intestinal mucosa and the portal (Berthoud and Neuhuber, 2000; Bohorquez et al., 2015), the areas of immediate access to GLP-1 released from L-cells (Bohorquez et al., 2015). We first reported that GLP-1 directly interacts with the vagal afferent NG neurons to evoke action potentials and increase cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Kakei et al., 2002). Studies using surgical or chemical ablation of vagal afferents have demonstrated that GLP-1-induced insulin secretion (Nishizawa et al., 2013), anorexigenic effect (Abbott et al., 2005; Talsania et al., 2005), and suppression of

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gastric empting (Imeryuz et al., 1997) are mediated at least partly by vagal afferents. The rats whose GLP-1R is knocked down selectively in vagal afferent neurons exhibit increased meal size, reduced insulin and enhanced glycemic responses in meal tolerance tests, and increased gastric emptying (Krieger et al., 2016). Furthermore, the insulin-releasing and glucose-lowering effects of DPP-4 inhibitors, which extend the life of GLP-1, are also mediated largely by vagal afferents and efferents (Ahlkvist et al., 2016; Fujiwara et al., 2012; Smith et al., 2014). These findings have led to the current consensus that the vagal afferent GLP-1R largely contributes to the physiological effects of endogenous GLP-1 including suppression of food intake, promotion of insulin release, attenuation of hyperglycemia, and inhibition of gastric emptying after meal. Thus, accumulating evidences indicate that the vagal afferents play a key role in mediating the effects of GLP-1 and DPP-4 inhibitors on insulin release, glycemia and feeding. However, the property of the GLP-1-responsive vagal afferents is less defined.

The first aim of the present study was to determine the precise concentration dependency of the GLP-1 action to increase $[Ca^{2+}]_i$ in vagal afferent neurons, and to characterize the identity of the subpopulation of vagal afferent neurons that respond to GLP-1 in comparison to the subpopulation responding to cholecystokinin (CCK), another postprandial hormone with anorexigenic ability.

GLP-1, GLP-1R agonists and DPP-4 inhibitors all enhance insulin release, resulting in the state in which both GLP-1 and insulin increase in circulation. The two hormones regulate common functions such as insulin and glucagon secretion, β-cell growth and/or survival, glucose metabolism, feeding, neural protection and memory. Moreover, recent clinical experience has indicated that the switching from insulin therapy to combination of GLP-1R agonist and insulin results in better control of both glycated hemoglobin (HbA1c) and body weight in patients with type 2 diabetic (Ahren, 2015; Eng et al., 2014; Li et al., 2012; Nauck and Meier, 2011; Seino et al., 2016) and after partial pancreatectomy (Kitazawa et al., 2016), suggesting a beneficial cooperative effect of GLP-1 and insulin. However, how these two hormones commonly regulate these diverse functions and exert cooperative effects is little known. Many of these functions involve the central nervous system (Niswender et al., 2004). Insulin can enter the brain across BBB but in a limited rate (Banks and Kastin, 1998). We have previously demonstrated that insulin directly interacts with the vagal afferent NG neurons to inform the brain (Iwasaki et al., 2013). These reports by us and others prompted us to hypothesize that GLP-1 and insulin might cooperatively regulate the NG neurons.

The second aim of the present study was to explore cooperative effects of GLP-1 and insulin on single vagal afferent neurons isolated from NG by measuring $[Ca^{2+}]_i$, a neuronal activation marker (Kohno et al., 2003). We examined whether co-administration of GLP-1 and insulin at their threshold concentrations could effectively induce $[Ca^{2+}]_i$ increases. We found additive and synergistic effects of GLP-1 and insulin on $[Ca^{2+}]_i$ in vagal afferent neurons.

2. Materials and methods

2.1. Materials

GLP-1 (Human, 7–36 Amide) and cholecystokinin-8 (CCK-8, 26–33, sulfated form) were purchased from Peptide Institute (Osaka, Japan). Porcine insulin and capsaicin were obtained from Sigma (MO, USA) and Ex(9–39) from GenScript (NJ, USA). These peptides were dissolved in ultrapure water or 1 N acetic acid (for insulin) in 10^{-3} M and stored at − 80 °C.

2.2. Animals

Male ICR mice aged 1–3 months (Japan SLC, Shizuoka, Japan) were housed for at least 1 week under conditions of controlled temperature (23 ± 1 °C), humidity (55% ± 5%), and lighting (light on at 7:30 and off at 19:30). Food and water were available ad libitum. Animal experiments were carried out after receiving approval from the Institutional Animal Experiment Committee of the Jichi Medical University, and in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

2.3. Preparation of single neurons from nodose ganglia

Single neurons were isolated from mouse NGs as previously reported (Iwasaki et al., 2009). The NGs of both right and left sides from 2 mice were used in a preparation of single NG neurons. The NGs were treated for 20 min at 37 °C with 0.1–0.5 mg/ml collagenase Ia (Sigma), 0.4–0.6 mg/ml dispase II (Roche, Basel, Swiss), 15 μg/ml DNase II type IV (Sigma), and 0.75 mg/ml bovine serum albumin (Sigma) in HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) composed of (in mM) 4.7 KCl, 1.2 KH_2PO_4, 129 NaCl, 5 $KaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 10 HEPES with pH adjusted at 7.4 using NaOH supplemented with 5.6 glucose. Cells were gently triturated by using fire-polished pasteur pipettes and tapping the tube, and centrifugated 2 time in Eagle's minimal essential medium (MEM) containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were resuspended in MEM and plated onto 6 coverslips coated with poly-L-lysine. Cells were cultured in MEM for 12–24 h at 37 °C under 5% CO_2/air.

2.4. Measurements of $[Ca^{2+}]_i$ in single nodose ganglion neurons

Measurements of $[Ca^{2+}]_i$ in primary cultured NG neurons were carried out as described previously (Iwasaki et al., 2009). Briefly, following incubation with 2 μM fura-2 AM (DOJINDO, Kumamoto, Japan) for 30 min at 37 °C, the cells on coverslips were mounted in a chamber and superfused using peristaltic pump (Gilson Inc., WI) at 1.3 ml/min at 30 °C with HKRB containing 5.6 mM glucose. Fluorescence ratio images at 510 nm due to excitation at 340 and 380 nm were produced by an Aquacosmos ver. 2.5 (Hamamatsu Photonics, Shizuoka, Japan). The responsiveness of NG neurons to hormones was comparable at 30 °C and 37 °C, but kept for a longer period at 30 °C than 37 °C suggesting a better maintenance of healthy states during measurements. Accordingly, we performed the experiments at 30 °C. NG neurons were selected by their round shape, while non-neuronal cells had spindle or filamentous shape. In one $[Ca^{2+}]_i$ imaging experiment, 30 to 50 neurons on a coverslip were monitored. Test reagents were administered for 4–5 min and, in repetitive administration, washing periods of 8 min or longer were given for cells to recover from possible desensitization. When $[Ca^{2+}]_i$ changed within 5 min after addition of agents and their amplitudes were at least twice larger than fluctuations of baseline, they were considered responses. We analyzed the neurons with $[Ca^{2+}]_i$ response to 55 mM KCl, a sign of healthy state. In each category of study, neurons from at least 3 separate preparations from at least 6 mice were used to ensure that the observed responses were representative.

2.5. Statistical analysis

All data were shown as means ± SEM. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test or Mann Whitney test using the Prism 5 (GraphPad Software, CA). $P < 0.05$ was considered significant. More specific and detailed statistics were indicated in corresponding figure legends.