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Ghrelin counteracts insulin-induced activation of vagal afferent neurons via growth hormone secretagogue receptor

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ABSTRACT

Vagal afferent nerves sense meal-related gastrointestinal and pancreatic hormones and convey their information to the brain, thereby regulating brain functions including feeding. We have recently demonstrated that postprandial insulin directly acts on the vagal afferent neurons. Plasma concentrations of orexigenic ghrelin and anorexigenic insulin show reciprocal dynamics before and after meals. The present study examined interactive effects of ghrelin and insulin on vagal afferent nerves. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated nodose ganglion (NG) neurons was measured to monitor their activity. Insulin at 10^{-7} M increased $[Ca^{2+}]_i$ in NG neurons, and the insulin-induced $[Ca^{2+}]_i$ increase was inhibited by treatment with ghrelin at 10^{-8} M. This inhibitory effect of ghrelin was attenuated by [D-Lys^3]-GHRP-6, an antagonist of growth hormone-secretagogue receptor (GHSR). Des-acyl ghrelin had little effect on insulin-induced $[Ca^{2+}]_i$ increases in NG neurons. Ghrelin did not affect $[Ca^{2+}]_i$ increases in response to cholecystokinin (CCK), a hormone that inhibits feeding via vagal afferent neurons, indicating that ghrelin selectively counteracts the insulin action. These results demonstrate that ghrelin via GHSR suppresses insulin-induced activation of NG neurons. The action of ghrelin to counteract insulin effects on NG might serve to efficiently inform the brain of the systemic change between fasting-associated ghrelin-dominant and fed-associated insulin-dominant states for the homeostatic central regulation of feeding and metabolism.

1. Introduction

Insulin, released from the pancreas, regulates peripheral glucose and lipid metabolism via acting on the peripheral organs such as the liver, skeletal muscle and adipose tissue. Insulin is also known to influence the brain, thereby regulating glucose and lipid metabolisms (Obici et al., 2002; Scherer et al., 2011) and inhibiting feeding (Filippi et al., 2013; Niswender et al., 2004; Woods et al., 1979). Ghrelin, released primarily from the stomach (Date et al., 2000; Kojima et al., 1999) and to a lesser extent from the pancreas (Dezaki et al., 2004, 2006), stimulates feeding (Nakazato et al., 2001), promotes growth hormone release (Kojima et al., 1999), inhibits insulin release (Dezaki et al., 2004), and regulates glucose and lipid metabolism (Dezaki et al., 2006; Gahete et al., 2014). Plasma concentrations of orexigenic ghrelin and anorexigenic insulin change in a reciprocal manner both before and after meals (Cummings et al., 2001), which is thought to contribute to dynamic regulation of feeding, glucose and energy metabolism. Regarding possible mechanisms for the diurnal reciprocal changes of these hormones, it has been demonstrated that ghrelin interacts with the pancreatic β-cells to suppress insulin release (Dezaki et al., 2004), and that insulin interacts with gastric X/A-like cells to suppress ghrelin release (Sakata et al., 2012). Prior to meal intake, plasma ghrelin level is high and plasma insulin level is low, and this ghrelin-dominant state stimulates appetite and increases food intake. Following food intake, plasma ghrelin and insulin levels change in a reciprocal manner (Cummings et al., 2001), and the resultant insulin-dominant state could induce satiety and decrease food intake.

The mechanisms by which the peripheral insulin and ghrelin inform the brain are still obscure. It has been reported that both insulin and ghrelin can cross the blood–brain barrier (BBB) and enter the brain (Banks and Kastin, 1998; Banks et al., 2002), and their direct actions on the brain have been suggested. Furthermore, these hormones are thought to regulate feeding via acting on the first order neurons in the hypothalamic arcuate nucleus (ARC) that senses systemic metabolic signals (Niswender et al., 2004; Schwartz et al., 2000). In the ARC, the neurons co-expressing orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) play a pivotal role in regulation of feeding. Ghrelin directly activates ARC NPY neurons by increasing cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Kohno et al., 2003), and the ghrelin-induced $[Ca^{2+}]_i$ increase is suppressed by insulin (Iwasaki et al., 2013; Maejima et al., 2011).

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Insulin and ghrelin enter the brain across BBB in a limited manner (Banks and Kastin, 1998; Banks et al., 2002). The vagal afferent nerves, one of the primary visceral sensory nerves, sense peripheral factors including gastrointestinal and pancreatic hormones and transmit their signals to the brain, thereby regulating food intake (Iwasaki and Yada, 2012). Peripheral administration of ghrelin reportedly increases food intake via the vagal afferents (Date et al., 2002) that express GHRS (Burdyga et al., 2006; Date et al., 2002; Sakata et al., 2003). Moreover, peripheral administration of ghrelin increases dopamine β hydroxylase mRNA expression in the nucleus tractus solitaries (NTS) to which vagal afferents project, elevates noradrenaline release in the ARC where the NTS noradrenergic neurons innervate, and activates the ARC NPY neurons through adrenergic receptors, thereby increasing food intake (Date et al., 2006). We have recently demonstrated that insulin directly activates the vagal afferents including those innervating the pancreas, via signaling cascade of insulin receptor (IR) – insulin receptor substrate 2 (IRS2) – phosphatidylinositol 3 kinase (PI3 kinase) (Iwasaki et al., 2013). In IRS2 knockout mice that exhibit hyperphagic obesity, insulin action in the vagal afferent neurons was impaired while it was intact in the ARC NPY neurons, suggesting that the impaired insulin sensing by the vagal afferents is linked to hyperphagic obesity in IRS2 knockout mice (Iwasaki et al., 2013).

Inverse relationship between ghrelin and insulin has been demonstrated; ghrelin and insulin are released in a reverse diurnal pattern, ghrelin suppresses insulin secretion from pancreatic β-cells, and ghrelin and insulin reciprocally regulate NPY neurons in the hypothalamus. Based on these findings, we propose the insulin-counteracting nature of ghrelin (Yada et al., 2008, 2014). To further substantiate it, it is of particular importance to verify whether ghrelin counteracts the insulin action on the vagal afferents, the essential pathway for information flow from the periphery to the brain. In this study, we investigated the effect of ghrelin on insulin-induced $[Ca^{2+}]_i$ increases in the vagal afferents neurons isolated from the nodose ganglion (NG) in mice. We also investigated the involvement of GHSR in the effects of ghrelin.

2. Materials and methods

2.1. Materials

Rat ghrelin, rat des-acyl ghrelin and cholecystokinin-8 (CCK-8, 26-33, sulfated form) were purchased from Peptide Institute (Osaka, Japan). Porcin insulin and [D-Lys^3]-GHRP-6 were obtained from Sigma (MO).

2.2. Animals

Male ICR mice aged 1–3 months were purchased from Japan SLC (Shizuoka, Japan). The animals were housed at least for 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). Briefly, 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 4.7 mM KCl, 1.2 mM KH_2PO_4, 129 mM NaCl, 5 mM $KaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 10 mM HEPES with pH adjusted at 7.4 using NaOH supplemented with 5.6 mM glucose. Single neurons were cultured for 12–24 h in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

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 were mounted in a chamber and superfused 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). 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. Regarding the suppression of $[Ca^{2+}]_i$ increases, when ghrelin decreased the amplitude of insulin-induced $[Ca^{2+}]_i$ increases by 50% or greater in a single NG neuron, it was considered the suppression. Only the neurons that responded to 55 mM KCl were analyzed.

2.5. Statistical analysis

All data were shown as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison tests or unpaired t-test using the Prism 5 (GraphPad Software, CA). $P < 0.05$ was considered significant.

3. Results

3.1. Ghrelin inhibits insulin-induced $[Ca^{2+}]_i$ increases but not CCK-8-induced $[Ca^{2+}]_i$ increases in NG neurons.

To determine the direct effects of insulin and ghrelin on vagal afferent neurons, we measured $[Ca^{2+}]_i$ in single neurons isolated from NG of mice. We previously showed that insulin increases $[Ca^{2+}]_i$ in NG neurons with its maximal effect obtained at 10^{-7} M, and that the incidence of $[Ca^{2+}]_i$ response to insulin (10^{-7} M) is approximately 10% (Iwasaki et al., 2013). As shown in Fig. 1A, pulsatile administration of 10^{-7} M insulin twice induced repeated $[Ca^{2+}]_i$ increases in 59 of 409 NG neurons (14.4%). This incidence of $[Ca^{2+}]_i$ responses to insulin well fits with the that of NG neurons expressing insulin receptor (13.4%) (Iwasaki et al., 2013). In these NG neurons that responded to 10^{-7} M insulin with $[Ca^{2+}]_i$ increases, ghrelin at 10^{-8} M had no effect on the basal $[Ca^{2+}]_i$ ($n = 10$, Fig. 1B). We next examined whether ghrelin could influence the insulin-induced $[Ca^{2+}]_i$ increases on NG neurons. In the presence of 10^{-8} M ghrelin, administration of 10^{-7} M insulin induced little increases in $[Ca^{2+}]_i$, and after washing out ghrelin, second stimulation with insulin induced robust increases in $[Ca^{2+}]_i$ (Fig. 1C). Insulin increased $[Ca^{2+}]_i$ in 31 of 331 (9.4%) NG neurons, and ghrelin at 10^{-8} M inhibited insulin-induced $[Ca^{2+}]_i$ increases in 19 of 31 (61.3%) insulin-responsive NG neurons. The average amplitude of $[Ca^{2+}]_i$ responses to 10^{-7} M insulin was significantly smaller in the presence of ghrelin ($n = 31$, Fig. 1D). Ghrelin at 10^{-9} M suppressed the insulin-induced $[Ca^{2+}]_i$ increases only in 7 of 25 NG neurons (28%), and the average amplitude of $[Ca^{2+}]_i$ responses to 10^{-7} M insulin was not significantly altered (Fig. 1D). These results show that ghrelin concentration-dependently inhibits insulin-induced $[Ca^{2+}]_i$ increases in NG neurons.

CCK-8 is well established as the gastrointestinal hormone that inhibits feeding via directly interacting with vagal afferents (Iwasaki and Yada, 2012; Lankisch et al., 2002; Simasko et al., 2002; Smith et al., 1981), and as large as 40% of NG neurons respond to CCK-8 (Iwasaki and Yada, 2012; Simasko et al., 2002). Hence, the response to CCK may be so far the most representative anorexigenic response of