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Glucose and insulin induce Ca^{2+} signaling in nesfatin-1 neurons in the hypothalamic paraventricular nucleus

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ABSTRACT

Nucleobindin-2 derived nesfatin-1 in the hypothalamic paraventricular nucleus (PVN) plays a role in inhibition of feeding. The neural pathways downstream of PVN nesfatin-1 have been extensively investigated. However, regulation of the PVN nesfatin-1 neurons remains unclear. Since starvation decreases and refeeding stimulates nesfatin-1 expression specifically in the PVN, this study aimed to clarify direct effects of meal-evoked metabolic factors, glucose and insulin, on PVN nesfatin-1 neurons. High glucose (10 mM) and insulin (10^{-13} M) increased cytosolic calcium concentration ($[Ca^{2+}]_i$) in 55 of 331 (16.6%) and 32 of 249 (12.9%) PVN neurons, respectively. Post $[Ca^{2+}]_i$ measurement immunocytochemistry identified that 58.2% of glucose-responsive and 62.5% of insulin-responsive neurons were immunoreactive to nesfatin-1. Furthermore, a fraction of the glucose-responsive nesfatin-1 neurons also responded to insulin, and *vice versa*. Some of the neurons that responded to neither glucose nor insulin were recruited to $[Ca^{2+}]_i$ increases by glucose and insulin in combination. Our data demonstrate that glucose and insulin directly interact with and increase $[Ca^{2+}]_i$ in nesfatin-1 neurons in the PVN, and that the nesfatin-1 neuron is the primary target for them in the PVN. The results suggest that high glucose- and insulin-induced activation of PVN nesfatin-1 neurons serves as a mechanism through which meal ingestion stimulates nesfatin-1 neurons in the PVN and thereby produces satiety.

1. Introduction

Food intake is regulated by the feeding center in the hypothalamus, whose activity is altered by nutritional and hormonal status of the body. Hypothalamic paraventricular nucleus (PVN) receives afferent inputs via the first order feeding centers, the arcuate nucleus (ARC) and nucleus tractus solitarius (NTS), communicates with other brain areas related to various functions including stress, circadian rhythm and reward, and integrates the peripheral and central signals to finally control feeding. Therefore, PVN is considered the second order or integrative feeding center [1–5]. The PVN is located bilaterally bordering the third ventricle and equipped with neurons synthesizing anorexigenic neuropeptides including corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) oxytocin, and newly discovered nesfatin-1 [6–8].

Nesfatin-1, a 82-amino-acid peptide derived from the nucleobindin2 (NUCB2) is an emerging new player in regulation of food intake [8,9] and is expressed in several nuclei including the hypothalamic PVN, ARC, and the brain stem NTS [8,10]. Acute intracerebroventricular (i.c.v) injection of nesfatin-1 decreases food intake in rats and mice [8,11,12]. Chronic infusion of nesfatin-1 into the third ventricle significantly decreases food intake and body weight gain in rats, while i.c.v injection of NUCB2 antisense oligonucleotide increases food intake and body weight [8]. Regarding the neural mechanisms downstream of the PVN nesfatin-1, central injection of nesfatin-1 reportedly evokes anorexia by activating the oxytocin neurons in the PVN which project to pro-opiomelanocortin (POMC) neurons in the NTS, thereby inducing melanocortin-dependent anorexia [13]. It has also been reported that CRH mediates the effects of central nesfatin-1 [11]. A physiologic role of central nesfatin-1 in food intake regulation is further supported by the finding that the expression of NUCB2/nesfatin-1 in the PVN is altered by nutritional states [8,14]. Altogether, these data have evidenced that nesfatin-1 is an anorectic molecule implicated in the physiologic regulation of food intake.

Although the neural pathways downstream of PVN nesfatin-1 have been extensively studied, the factors that regulate nesfatin-1

Abbreviations: PVN, paraventricular nucleus; ARC, arcuate nucleus; NTS, nucleus tractus solitarius; CRH, corticotropin-releasing hormone; TRH, thyrotropin releasing hormone; NUCB2, nucleobindin2; $[Ca^{2+}]_i$, cytosolic calcium concentration; POMC, pro-opiomelanocortin; BBB, blood-brain barrier; HKRB, Hepes-buffered Krebs-Ringer bicarbonate buffer; IR, immunoreactive; i.c.v, intracerebroventricular.

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neurons in PVN remain unknown. By now, it has been shown that starvation for 24 h decreases the NUCB2 mRNA level and nesfatin-1 protein content selectively in the PVN [8]. Conversely, refeeding activates nesfatin-1 neurons in the PVN, as measured by c-Fos expression [14]. Hence, it is possible that the nesfatin-1 neuron in the PVN responds to the meal-evoked signals. Plasma glucose and insulin surge postprandially [15,16], and penetrate into the cerebrospinal fluid through the blood–brain barrier (BBB) [17,18]. The present study aimed to clarify whether high glucose and insulin could directly activate nesfatin-1 neurons in the PVN. We investigated the effects of glucose and insulin on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the neurons isolated from PVN of mice, followed by immunocytochemical identification of nesfatin-1 neurons. We found that high glucose and insulin directly interact with and increase $[Ca^{2+}]_i$ in PVN nesfatin-1 neurons, and that they cooperate to recruit some of unresponsive nesfatin-1 neurons to $[Ca^{2+}]_i$ increases.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) aged 5–6 weeks were used in the experiments. Animals were maintained on a 12 h light/dark cycle (19:30 lights off) and given conventional food (CE-2; Clea, Osaka, Japan) and water *ad libitum*. Experimental procedures and care of animals were carried out according to the Jichi Medical University Institute of Animal Care and Use Committee.

2.2. Preparation of single neurons from PVN

Single neurons were prepared according to procedures reported previously [19] with slight modifications. Briefly, brain slices containing the entire PVN were prepared and the PVN was excised from the left and right sides. The dissected tissues were washed with 10 mM HEPES-buffered Krebs–Ringer bicarbonate buffer (HKRB) containing 1 mM glucose. They were then incubated in the HKRB supplemented with 20 U/ml papain (Sigma Chemical Co., St. Louis, MO), 0.015 mg/ml deoxyribonuclease, 0.75 mg/ml BSA, and 1 mM cysteine for 15 min at 36 °C in a shaking water bath, followed by gentle mechanical trituration for 5–10 min. After trituration, the cell suspension was centrifuged at 100 g for 5 min. The pellet was resuspended in HKRB. The single neurons obtained were distributed onto coverslips and incubated in the humidified chamber at 30 °C for 30 min and then at 25 °C for up to 6 h until use.

2.3. Measurement of $[Ca^{2+}]_i$ in single PVN neurons

$[Ca^{2+}]_i$ was measured by ratiometric fura-2 fluorescence imaging as previously reported [13,19]. Briefly, single neurons on coverslips were incubated with 2 μmol/l fura-2/AM (Dojin chemical,

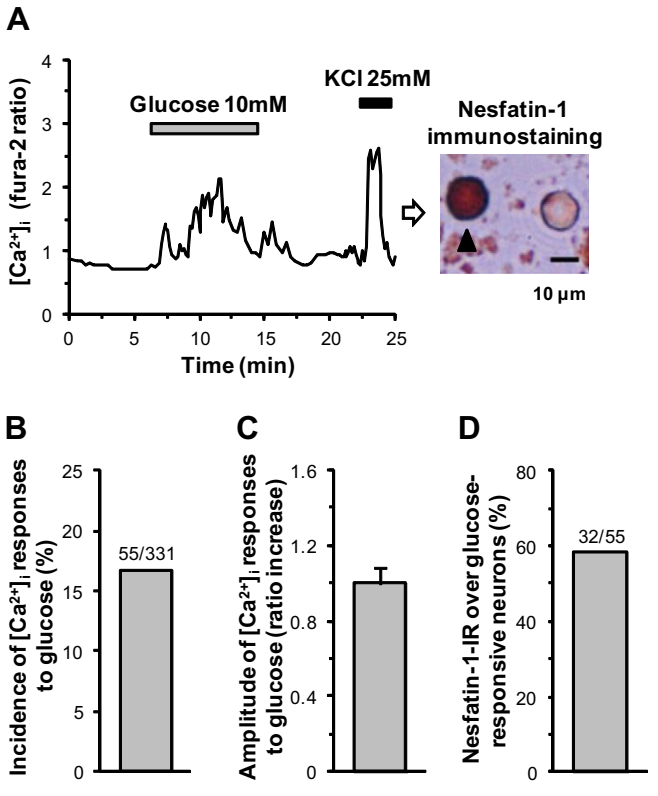

Fig. 1. Glucose increases $[Ca^{2+}]_i$ in PVN nesfatin-1 neurons. (A) Elevation of the glucose concentration to 10 mM from a basal level of 1 mM under superfusion conditions increased $[Ca^{2+}]_i$ in a single neuron isolated from PVN that subsequently responded to 25 mM KCl with an increase in $[Ca^{2+}]_i$ (left panel). Superfusate contained 1 mM glucose unless indicated by the bars. The bars above the tracing indicate the period of treatment specified. This neuron was proven to be nesfatin-1-immunoreactive (IR), as designated by arrow head, (right panel). Scale bar, 10 μm. (B) Percentage of PVN neurons that responded to glucose with $[Ca^{2+}]_i$ increases. The numbers above the bar indicate the number of PVN neurons that responded to 10 mM glucose over the number of PVN neurons examined. (C) Amplitude of glucose-induced $[Ca^{2+}]_i$ increases is expressed as the increment of ratio. Bar represents mean ± SE. (D) Percentage of nesfatin-1-IR over glucose-responsive neurons. The numbers above the bar indicate the number of PVN neurons IR to nesfatin-1 over that responded to 10 mM glucose.

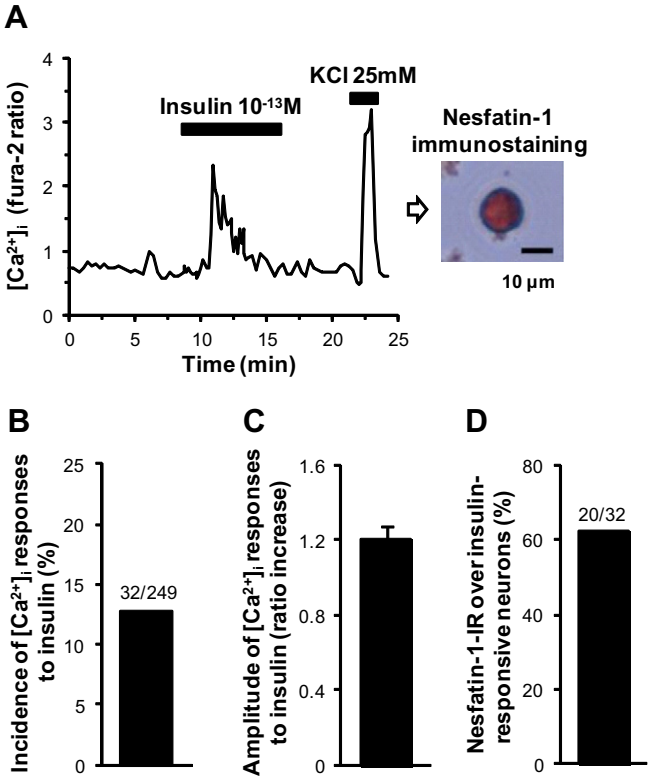

Fig. 2. Insulin increases $[Ca^{2+}]_i$ in PVN nesfatin-1 neurons. (A) Insulin at 10^{-13} M increased $[Ca^{2+}]_i$ (left panel) in a PVN neuron, which was shown to be immunoreactive to nesfatin-1 (right panel). (B) Incidence of $[Ca^{2+}]_i$ responses to insulin in PVN neurons, expressed by percentage. (C) Amplitude of $[Ca^{2+}]_i$ responses to insulin is expressed as delta ratio. Bar represents mean ± SE. (D) Twenty of 32 insulin-responsive neurons (62.5%) were nesfatin-1-immunoreactive.