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Nesfatin-1 stimulates cholecystokinin and suppresses peptide YY expression and secretion in mice

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ABSTRACT

Nesfatin-1 is an 82 amino acid secreted peptide encoded in the precursor, nucleobindin-2 (*NUCB2*). It is an insulinotropic anorexigen abundantly expressed in the stomach and hypothalamus. Post-prandial insulin secretion is predominantly regulated by incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Nesfatin-1 was previously reported to modulate GLP-1 and GIP secretion *in vitro* in an enteroendocrine (STC-1) cell line. Intestine is a source of additional hormones including cholecystokinin (CCK) and peptide YY (PYY) that regulate metabolism. We hypothesized that nesfatin-1 modulates CCK and PYY secretion. Immunofluorescence histochemistry showed NUCB2/nesfatin-1 co-localizing CCK and PYY in the intestinal mucosa of mice. Static incubation of STC-1 cells with nesfatin-1 upregulated both CCK mRNA expression (1 and 10 nM) and secretion (0.1, 1 and 10 nM) at 1 h post-incubation. In contrast, nesfatin-1 treatment for 1 h downregulated PYY mRNA expression (all doses tested) and secretion (0.01 and 0.1 nM) in STC-1 cells. Continuous infusion of nesfatin-1 using osmotic mini-pumps for 12 h upregulated CCK mRNA expression in large intestine, and downregulated PYY mRNA expression in both large and small intestines of male C57BL/6J mice. In these tissues, Western blot analysis found a corresponding increase in CCK and a decrease in PYY content. Collectively, we provide new information on the cell specific localization of NUCB2/nesfatin-1 in the intestinal mucosa, and a novel function for nesfatin-1 in modulating intestinal CCK and PYY expression and secretion in mice.

1. Introduction

Gut hormones play a major role in regulating insulin secretion, glucose levels and energy homeostasis [1]. Cholecystokinin (CCK) and peptide YY (PYY) are secreted from intestinal I and L cells, respectively [2,3]. CCK secreted in response to meal ingestion is a potent hunger suppressant [2] that inhibits gastric emptying [4]. There are several known bioactive forms of CCK, of which CCK-8 and -33 are found in plasma and small intestine [5]. CCK stimulates insulin granule exocytosis [6] and promotes growth of pancreatic β cells in islets of *ob/ob* mice [7]. Administration of an N-terminally stable CCK analogue "(pGlu-Gln)-CCK-8" protects against diabetes and obesity in animal models [8]. PYY is secreted in response to a protein rich meal and exists endogenously in two forms: PYY_{1-36} and PYY_{3-36} [9–12]. PYY_{3-36} reduces food intake and weight gain in rodents [11,13,14]. Besides colon, PYY immunopositive cells are also localized in the pancreatic α cells, and PYY is co-stored in glucagon-positive secretory granules [15]. PYY inhibits insulin secretion *in vivo* in rats, dogs and mice [16–18] and could mediate its action by decreasing pancreatic flow rate and stimulating glucagon secretion [19]. Together, CCK and PYY are important glucoregulatory hormones that modulate insulin secretion and energy homeostasis. In 2006, another peptide, nesfatin-1, with metabolic actions was reported [20].

Nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-influencing protein-1) is an 82 amino acid anorexigenic peptide encoded in the N-terminal of its secreted precursor, nucleobindin-2 (NUCB2) [20–23]. The mid-segment of the peptide (23–53, 30 amino acids) is considered to be its bioactive core, known to reduce food intake and fat mass [20,24] in rodents and goldfish [25]. Nesfatin-1 levels in plasma and blood glucose concentrations are inversely correlated in rats and diabetic humans [26,27]. We showed for the first time that nesfatin-1 co-localizes insulin in pancreatic islet β cells [22,28] and nesfatin-1 is insulinotropic [29].

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Nesfatin-1 modulates insulin secretion by increasing Ca^{2+} influx through L-type calcium channels [30]. It is a multi-functional peptide [22,23,31] with reproductive [32], cardiac [33] and endocrine functions [34] in mammals. It has also been characterized in non-mammals including goldfish and zebrafish [21,35]. Nesfatin-1 is expressed in the gastric mucosa with ghrelin in X/A like cells [36]. Further *NUCB2* mRNA is 10-fold higher in gastric mucosa of rats than in brain, suggesting stomach and/or gastrointestinal (GI) tract as the major source for nesfatin-1 in circulation. We recently confirmed NUCB2/nesfatin-1 mRNA and protein expression in small and large intestines of C57BL/6J mice [37] confirming previous findings of NUCB2/nesfatin-1 like immunoreactivity (IR) in the duodenal submucosal layer of Sprague Dawley (SD) rats and Institute of Cancer Research (ICR) mice. It was also observed that enteroendocrine L and K cells that secrete incretins (glucagon-like peptide 1; GLP-1 and glucose dependent insulinotropic polypeptide; GIP) co-localize NUCB2/nesfatin-1. Nesfatin-1 stimulates incretin secretion *in vitro* in STC-1 cells [38]. These findings suggest a possible incretin mediated insulinotropic action for nesfatin-1 in mice.

The objectives of this research were to determine if nesfatin-1 is expressed in other types of enteroendocrine cells, and whether nesfatin-1 regulate CCK and PYY, two important enteric metabolic hormones. We hypothesized that nesfatin-1 is present in the intestinal I (CCK) cells and L (PYY) cells, and that nesfatin-1 modulates CCK and/or PYY synthesis and/or secretion. Here we report that both I and L cells express NUCB2/nesfatin-1, and that nesfatin-1 stimulates CCK expression and secretion and that it inhibits PYY expression and secretion from STC-1 cells.

2. Materials and methods

2.1. Cell culture and immunohistochemistry

NUCB2/nesfatin-1 like immunoreactivity was recently confirmed in mice intestinal L and K cells (co-localization with GLP-1 and GIP) [38]. Is nesfatin-1 expressed in other enteroendocrine cells, especially I and L cells? Cell culture and immunohistochemistry were carried out to address this. STC-1 cells derived from intestinal enteroendocrine tumors were a kind gift from Dr. Timothy Kieffer (University of British Columbia, Vancouver, Canada). The cells were previously shown to secrete both CCK [39] and PYY [40] besides incretins [38,41,42]. Cells were cultured as previously described [40]. Total RNA was extracted using TRIzol RNA isolation reagent (Invitrogen, Catalog# 15596-026) and cDNAs were synthesized using iScript™ reverse transcription supermix (Bio-Rad, Catalog# 170-8840) for further quantification of mRNA expression by RT-qPCR, as described later.

For immunohistochemical studies small intestinal sections were collected from *ad libitum* fed male C57BL/6J mice (Charles River, Quebec, Canada) cared under the Canadian Council of Animal Care guidelines, as approved by the University of Saskatchewan Animal Care Committee. Mice were euthanized by cervical dislocation. The intestinal sections were collected and fixed in 4% paraformaldehyde overnight at 4 °C and were processed and sectioned (4 μm thickness). These sections were deparaffinized with xylene (incubated twice in 100% xylene; 5 min at 25 °C) and rehydrated in graded ethanol series (incubated twice in 100% ethanol, once in each 95% ethanol, 70% and 50% ethanol, 2 min each at 25 °C). The sections were then incubated with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity (30 min at 25 °C). The sections were then blocked with serum-free protein block reagent (DAKO, Catalog #S0809) for 10 min before being incubated with primary antibodies overnight at 4 °C followed by incubation with secondary antibody for 4 h at room temperature. Primary antibodies used were: Goat anti-nesfatin-1 (Santa Cruz, Catalog# SC65160, 1:500 dilution), rabbit anti-$PYY_{(3\text{-}36)}$ (Phoenix Pharmaceuticals, Catalog# H-59-04, 1:200 dilution) and rabbit anti-CCK (Abcam, Catalog# ab43842, 1:500 dilution). The secondary antibodies used were donkey anti-goat Alexa Flour-488 (Invitrogen, Catalog# A11055, 1:500 dilution, Green) for nesfatin-1 and goat anti-rabbit Texas-Red IgG (Vector Laboratories, Catalog# TI-1000, 1:100 dilution, Red) for CCK and PYY. For the negative control slides, no primary antibodies were added. Slides were washed in 1× PBS and mounted using Vectashield® medium containing the nuclear dye DAPI (Vector Laboratories, Catalog# H-1200, Blue). Tissues were imaged under Nikon Eclipse-Ti inverted fluorescence microscope (Nikon, Canada), images were captured using a Nikon DS-Qi1 MC camera. Images were analyzed using NiS Elements basic research software on a Lenovo ThinkPad workstation. Since the nesfatin-1 antibody used here detects both nesfatin-1 and NUCB2, we used nesfatin-1/NUCB2 like immunoreactivity to refer to the fluorescence detected with this antibody.

2.2. Nesfatin-1 effects on CCK, PYY mRNA expression and secretion

Nesfatin-1 was previously reported to stimulate incretin secretion from STC-1 cells [38]. Does it modulate CCK and PYY secretion from STC-1 cells? Static incubation studies were performed to confirm this rationale. STC-1 cells at 2×10^5 cells/well density were seeded in 1 mL DMEM (25 mM glucose) in 24-well plates. On the day of study, medium was removed and cells were washed twice with PBS. The cells were then treated for 1 h with 1 mL of DMEM containing 0, 0.001, 0.01, 0.1, 10 nM synthetic rat full length nesfatin-1 (Abgent Technologies, USA, >95% purity) [38]. Media samples were then collected and the levels of CCK and PYY secreted into the media were measured using rat/mouse Total CCK ELISA kit (Abnova, Catalog# KA1862) and PYY radioimmunoassay kit (Millipore Inc., Catalog# RMPYY-68HK). Cells were collected and quantitative PCRs (qPCR) were conducted using primers mouse CCK (NM_001284508) [sense primer 5′-TTTCCTGCCCGCATTTGAAC-3′ and antisense primer 5′-AATCCATCCAGCCCATGTAGTC-3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 60 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 153 bp] and mouse PYY (NM_145435.1) [sense primer 5′-TTCAGGCCA-GAAGGTTTGGA-3′ and antisense primer 5′-ACACCGAGA-TATGAAGTGCCC-3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 59 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 122 bp]. Mouse beta actin (internal control) RT-qPCR was conducted employing the primers and conditions described earlier. Amplification and detection of respective genes were performed in duplicates in each experimental group for relative mRNA expression using iQ™ SYBR® Green Supermix (Bio-Rad, Catalog #170-8880) on a CFX-Connect™ Real-Time PCR detection system (Bio-Rad, Canada). A melting curve analysis was carried out at 65 °C to 95 °C and the absence of any dimer formation or artifacts was confirmed. The PCR efficiency was 97% and relative gene expression data were obtained after normalizing the data using the Livak method [43].

2.3. In vivo effect of Nesfatin-1 infusion on CCK and PYY mRNA expression in male C57BL/6J mice

Nesfatin-1 upregulated both GLP-1 and GIP in STC-1 cells. Similar to this, will nesfatin-1 affect CCK and PYY in mouse intestine? *In vivo* studies were conducted to determine the changes in CCK and PYY mRNA expression in small and large intestine in response to subcutaneous infusion of nesfatin-1 using an osmotic mini-pump. Age matched (5 weeks old, average body weight: 20 g) C57BL/6J male mice (The Jackson Laboratory, USA) were housed

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