Biochemical and Biophysical Research Communications 462 (2015) 124–130

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Nesfatin-1 stimulates glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide secretion from STC-1 cells *in vitro*

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ARTICLE INFO

Article history:
Received 11 April 2015
Available online 28 April 2015

Keywords:
Nucleobindin 2
Nesfatin-1
Glucagon-like peptide-1
Glucose-dependent insulinotropic polypeptide
Insulin secretion

ABSTRACT

Nesfatin-1 is an 82 amino acid peptide encoded in a secreted precursor, nucleobindin 2. It is an anorexigenic and insulinotropic peptide found abundantly in the hypothalamus, pancreas and gastric oxyntic mucosa. NUCB2 mRNA expression is 10 fold higher in the gastric mucosa than in brain, suggesting gastrointestinal tract as a main source of nesfatin-1. Meal responsive insulin secretion is regulated by incretins glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP). Since both nesfatin-1 and incretins modulate insulin secretion, we hypothesized that nesfatin-1 is present in the enteroendocrine cells, and that it regulates incretin secretion. RT-PCR analysis found NUCB2 mRNA expression, and immunofluorescence microscopy determined nesfatin-1 immunoreactivity in STC-1, an enteroendocrine cell line. NUCB2/nesfatin-1 is co-localized with GLP-1 and GIP in mouse small intestinal cells. Static incubation of STC-1 cells with nesfatin-1 upregulated preproglucagon (GLP-1 precursor) mRNA (0.01, 0.1, 1 and 10 nM) and GLP-1 secretion (0.1, 1 and 10 nM). Nesfatin-1 also enhanced GIP mRNA (0.1, 1 and 10 nM) and GIP secretion (1 and 10 nM). Together, our data support the hypothesis that nesfatin-1 is present in enteroendocrine cells and that it stimulates incretin secretion. Future studies should aim for nesfatin-1 and incretin interactions *in vivo*.

1. Introduction

Gastrointestinal (GI) hormones contribute to the regulation of insulin secretion and glucose homeostasis. Intestinal hormones glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted from L and K cells of the distal ileum and upper small intestine, respectively. Both GLP-1 and GIP, collectively named as incretins, stimulate insulin secretion from rat pancreas and human islets [1–3]. Under atypical physiological conditions including type 2 diabetes (T2D), the secretion of GLP-1 is impaired. However its insulinotropic and glucagonostatic activity are preserved [4]. Unlike GLP-1, GIP secretion is intact in patients with T2D, however its insulinotropic action is impaired [4]. GLP-1 analogue based drugs, for example, Byetta™ are already available as therapeutic agents for T2D [5]. The crucial role of GIP and its receptor in T2D were demonstrated in a variety of studies [2,6–8]. In addition, both GLP-1 and GIP have been shown to attenuate glucagon stimulated hepatic glucose production in both humans and rodents [9,10]. Together, incretins are important insulinotropic and glucoregulatory hormones with significant roles in energy homeostasis. In 2006, another peptide, nesfatin-1, with metabolic actions was reported [11].

Nesfatin-1 (NEFA/nucleobindin-2-Encoded Satiety and Fat-Influencing proteiN-1) is an 82 amino acid anorexigenic peptide encoded in the N-terminal region of its precursor, nucelobindin-2 (NUCB2) [11]. Administration of full length nesfatin-1 (1-82 amino acids) or its mid-segment (23-53, 30 amino acids), considered to be its bioactive core reduces food intake and fat mass [11,12] in rodents and fish [13]. Plasma nesfatin-1 concentrations are inversely correlated with glucose levels in rats and diabetic humans [14,15]. Our research group showed for the first time that nesfatin-1 and insulin are co-localized in the β cells of pancreatic islets [16], and that nesfatin-1 is insulinotropic [17]. Nesfatin-1 increases glucose stimulated insulin secretion (GSIS) by direct action involving Ca^{2+} influx through L-type calcium channels [18]. Studies conducted thereon have confirmed NUCB2 expression in gastric mucosa [19]. The expression of NUCB2 mRNA is 10-fold higher in gastric mucosa of rats than in brain, suggesting stomach as the

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http://dx.doi.org/10.1016/j.bbrc.2015.04.100
0006-291X/

major source of circulating nesfatin-1. In addition, it crosses the blood-brain barrier via a non-saturable mechanism, providing the possibility that nesfatin-1 secreted peripherally may act centrally [20]. Overall, nesfatin-1 is now emerging as a multifunctional peptide [21] with reproductive [22], cardiac [23], and endocrine functions [24]. What tissues contribute to the post-meal increase in NUCB2/nesfatin-1 release? It has been shown that the stomach and pancreas are two major sources of endogenous NUCB2/nesfatin-1 [16,25]. We recently reported that NUCB2/nesfatin-1 mRNA and protein expression in the small and large intestines of C57BL/6 mice [26]. These results are in agreement with previous reports [27,28] that showed NUCB2/nesfatin-1 immunoreactivity (IR) in the duodenal submucosal layer of Sprague Dawley (SD) rats and in Institute of Cancer Research (ICR) mice.

While the above studies determined nesfatin-1 in tissues, the identity of NUCB2/nesfatin-1 expressing cells within the intestine is not known. Considering that nesfatin-1 is insulinotropic, and incretins, which are predominant insulinotropins are primarily secreted from the intestine, it warrants further research to determine whether nesfatin-1 is present in enteroendocrine cells. We hypothesized that nesfatin-1 is present in intestinal endocrine cells, especially those producing incretins, and that nesfatin-1 stimulates incretin secretion. This article presents our novel results on nesfatin-1 colocalization of GLP-1 and GIP in mice intestine. We also report the discovery that nesfatin-1 stimulates both the expression of mRNAs encoding GLP-1 (proglucagon) and GIP, and incretin secretion from STC-1 cells.

2. Materials and methods

2.1. Cell culture, RT-PCR and immunohistochemistry

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 GLP-1 [29] and GIP [30,31]. Cells were cultured as previously described [32]. Total RNA was extracted using TRIzol™ RNA isolation regent (Invitrogen, Catalog #15596-026) and cDNAs were synthesized using iScript™ reverse transcription supermix (Bio-Rad, Catalog #170-8840). Mouse NUCB2 (NM_001130479.2) [sense primer, 5′−CCAGTGGAAAATGCAAGGAT−3′ and antisense primer, 5′−GCTCATCCAGTCTCGTCCTC−3'; PCR conditions: denaturation: 95 °C (10 s), annealing: 61 °C (30 s) and elongation: 73 °C (30 s), 35 cycles, amplicon size: 202 bp] and mouse beta actin (NM_007393.3) [sense primer, 5′−CCACTGCCGCATCCTCTTCC−3′ and antisense primer, 5′−CTCGTTGCCAATAGTGATGAC−3'; PCR conditions: denaturation: 95 °C (10 s), annealing: 60 °C (30 s) and elongation: 73 °C (30 s), 35 cycles, amplicon size: 77 bp] mRNAs were detected using RT-PCR. The primers were validated for high primer efficiency and annealing temperatures. Gel electrophoresis (1.5% agarose) was conducted and images were captured using a gel imager (Gel Doc™ EZ system, Bio-Rad).

For immunohistochemistry, STC-1 cells were cultured in a Lab-Tek™ chamber slide system (Nalge Nunc, New York). Cells were washed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Catalog #14190-250). The cells were then fixed in 4% paraformaldehyde for 15 min at 4 °C, permeabilized using 0.3% Triton-X (Bioshop, Catalog #TRX-777) and then washed with DPBS. For immunohistochemical studies small intestinal section 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). Sections were blocked in Antibody blocking buffer (10% protein block; DAKO, Cat#S0809, 3%BSA; Sigma A7906, and 100 uL of Triton-X 20%) for 10 min and permeabilized in 1% PBS-Triton-X for another 10 min. Following this, sections were incubated with primary antibodies overnight at room temperature followed by incubation with secondary antibody for 1 h at 37 °C. Primary antibodies used were: rabbit polyclonal anti-NUCB2 (custom antibody, Pacific Immunology, Catalog #1312-PAC-01, 1:200) for STC-1 cells, intestine (1:500) and mouse monoclonal anti-GLP-1 (Abcam, Catalog #ab26278, 1:500) for GLP-1 sections, mouse polyclonal NUCB2/nesfatin-1 (ENZO Life Sciences, Catalog #ALX-804-854-C100, 1:100) and rabbit polyclonal anti-GIP (Abcam, Catalog #ab22624; 1:500) for GIP sections. The respective secondary antibodies used were goat polyclonal anti-rabbit Texas Red® IgG (Red − NUCB2/nesfatin-1; Vector Laboratories, Catalog #T1-1000, 1:200), goat polyclonal anti-mouse FITC (Green − GLP-1; Abcam, Catalog #A-11034, 1:500) goat polyclonal anti-mouse Alexa Flour-594 (Red − Nesfatin-1; Invitrogen, Catalog # ab150108, 1:500) and goat polyclonal anti-rabbit Alexa Flour 488 (Green − GIP; Invitrogen, Catalog #A-11037, 1:500). Primary antibody pre-absorbed in 10 μg synthetic nesfatin-1 overnight was used as pre-absorption controls for STC-1 cells to confirm the antibody specificity. The slides were washed in 1X PBS and mounted using Vectashield® medium containing the nuclear dye DAPI (Blue; Vector Laboratories). Tissue and cells were analyzed 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, NUCB2/nesfatin-1 like immunoreactivity is used as the term to identify any immunostaining using this reagent.

2.2. Nesfatin-1 effects on preproglucagon/GIP mRNA expression and incretin secretion

For static incubation studies, 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 DPBS. The cells were then treated for 1 h with 1 mL of DMEM containing 0, 0.001, 0.01, 0.1, 1 or 10 nM synthetic rat full length nesfatin-1 (Abgent Technologies, USA, >95% purity; 17). Media samples were then collected and the levels of GLP-1 and GIP secreted into the media were measured using multi-species GLP-1 total ELISA kit (Millipore Inc., Catalog #EZGLP1T-36K) and rat/mouse GIP (total) ELISA kit (Millipore Inc., Catalog #EZRMGIP-55K) respectively. Cells were collected and quantitative PCRs (qPCR) were conducted using primers mouse preproglucagon (AF276754.1) [sense primer 5′−AATCTTGCCACCAGGGACTT−3′ and antisense primer 5′−AGTGACTGGCACGAGATGTT−3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 56.3 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 112 bp] and mouse GIP (NM_008119.2) [sense primer 5′− ACAAAGAGGCACAGGAGAGC−3′ and antisense primer 5′−AGCCAAGCAAGCTAAGGTCA−3′, PCR conditions: denaturation: 95 °C (10 s), annealing: 60 °C (30 s) and elongation: 72 °C (30 s), 35 cycles, amplicon size: 180 bp]. Mouse beta actin (internal control) qPCR was conducted employing the primers and conditions described earlier. Amplification and detection of respective genes were performed in duplicates in each