RAPID COMMUNICATION

Nesfatin-1 exerts a direct, glucose-dependent insulinotropic action on mouse islet β- and MIN6 cells

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Abstract

Nesfatin-1 is a recently discovered multifunctional metabolic hormone abundantly expressed in the pancreatic islets. The main objective of this study is to characterize the direct effects of nesfatin-1 on insulin secretion in vitro using MIN6 cells and islets isolated from C57BL/6 mice. We also examined the expression of the nesfatin-1 precursor protein, nucleobindin 2 (NUCB2) mRNA, and nesfatin-1 immunoreactivity (ir) in the islets of normal mice and in the islets from mice with streptozotocin-induced type 1 diabetes and diet-induced obese (DIO) mice with type 2 diabetes. Nesfatin-1 stimulated glucose-induced insulin release in vitro from mouse islets and MIN6 cells in a dose-dependent manner. No such stimulation in insulin secretion was found when MIN6 cells/islets were incubated with nesfatin-1 in low glucose. In addition, a fourfold increase in nesfatin-1 release from MIN6 cells was observed following incubation in high glucose (16.7 mM) compared to low glucose (2 mM). Furthermore, we observed a significant reduction in both NUCB2 mRNA expression and nesfatin-1-ir in the pancreatic islets of mice with type 1 diabetes, while a significant increase was observed in the islets of DIO mice. Together, our findings indicate that nesfatin-1 is a novel insulinotropic peptide and that the endogenous pancreatic islet NUCB2/nesfatin is altered in diabetes and diet-induced obesity.

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Introduction

Nesfatin-1 is an anorectic hormone encoded in the N-terminal region of the precursor peptide nucleobindin 2 (NUCB2; Oh-I et al. 2006). Nesfatin-1 is proposed to be processed from NUCB2 by prohormone convertases, the enzymes that process proinsulin (Oh-I et al. 2006). Nesfatin-1 immunoreactivity (ir) has been reported in the rat pancreas (Stengel et al. 2009b), and we first reported the exclusive co-localization of nesfatin-1 and insulin-ir in the islet β-cells of both rats and mice (Gonzalez et al. 2009). Similarly, co-localization of nesfatin-1 in the β-cells, but not in other endocrine cell types of rat and human pancreata, was also reported (Foo et al. 2010). Since our initial histochemical observations, i.e., but not central administration of nesfatin-1 was shown to cause anti-hyperglycemic effects in db/db mice (Su et al. 2010). However, this reduction in blood glucose levels was not observed in non-hyperglycemic animals (Su et al. 2010). Furthermore, oral glucose administration in healthy adults significantly elevated basal nesfatin-1 levels compared to saline controls (Li et al. 2010). Significantly lower levels of nesfatin-1 in isolated islets of Goto-Kakizaki (GK) rats, a model of type 2 diabetes (Foo et al. 2010), and reduced circulating levels of nesfatin-1 in fasted humans with type 2 diabetes have been observed (Li et al. 2010). Based on these early reports, it is clear that nesfatin-1 is a regulator of blood glucose levels, and endogenous nesfatin-1 is altered in diabetes.

Other studies in this emerging area primarily focused on the appetite regulatory effects of nesfatin-1. Both brain and peripheral injections of synthetic nesfatin-1 cause a significant reduction in food intake of both rats and mice (Oh-I et al. 2006, Shimizu et al. 2009, Stengel et al. 2009a, Su et al. 2010, Atsuchi et al. 2010, Goebel et al. 2010, Yosten & Samson 2010). Chronic i.c.v. administration of nesfatin-1 caused a significant reduction in food intake, body weight, and subcutaneous, mesenteric and epididymal fat mass (Oh-I et al. 2006). Together, these studies provide support for nesfatin-1 as an appetite regulatory peptide with weight-reducing effects.

The immunohistochemical and limited functional studies available to date point towards a functional role for nesfatin-1...
in rodent pancreatic islets. Does nesfatin-1 influence insulin secretion? The focus of this work is to unravel the direct effects of nesfatin-1 on insulin secretion in mice. In our studies, nesfatin-1 induced glucose-stimulated insulin release in vitro from pancreatic islets isolated from C57BL/6 mice, and from MIN6 cells. We also observed a significant reduction in both NUCB2 mRNA expression and nesfatin-1-ir in the pancreas of mice with type 1 diabetes, while a significant increase was observed in the pancreas of diet-induced obese (DIO) mice with type 2 diabetes. Our new findings indicate that nesfatin-1 is a novel glucose-dependent insulinotropic peptide and that alterations in endogenous nesfatin-1 in the pancreatic islets could contribute to diabetes and DIO.

Materials and Methods

Animals

Age- and weight-matched male C57BL/6 mice were purchased from Charles River Canada (St-Constant, Quebec, Canada) and housed in a 12 h light:12 h darkness cycle (lights off at 1900 h and on at 0700 h) in a temperature- and humidity-controlled vivarium. Animals had ad libitum access to standard mouse chow (SMC) from TestDiet (Catalog # 5012, Richmond, IN, USA) (60, 27, and 13% of calories from carbohydrate, protein, and fat respectively, energy density = 3.43 kcal/g, LabDiets, Inc., St Louis, MO, USA) and water. The DIO mice with type 2 diabetes were developed by 8 weeks of feeding a high-fat diet (20, 20, and 50% of calories from carbohydrate, protein, and fat (lard/soybean oil) respectively, energy density = 5.24 kcal/g from Research Diets, Inc., Catalog # D12492, Richmond, IN, USA). Only hyperglycemic and obese mice were used for collecting the pancreas used in immunohistochemical studies. Type 1 diabetes was induced in C57BL/6 mice by a single injection of 50 mg/kg streptozotocin (STZ) using previously reported protocols (Unniappan et al. 2009). Three and six days post-injection, pancreata were collected from STZ-treated mice, which had severe hyperglycemia and decreased body weight compared to controls. All research protocols used in this study adhered to the guidelines of the Canadian Council for Animal Care and were approved by the York University Animal Care Committee.

Table 1 PCR primers used for quantitative real-time PCR of NUCB2, insulin, glucagon, β-actin, and 19S cDNA from MIN6 cells and pancreatic mouse tissue

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse NUCB2</td>
<td>CAGTTGAAAAATGCAAGGAT</td>
<td>GCTCATCCAGTCTCGTCTCT</td>
<td>94°C 30 s</td>
<td>61°C 30 s</td>
<td>72°C 30 s</td>
<td>35</td>
</tr>
<tr>
<td>Mouse preproinsulin</td>
<td>GCCCTTCTCACCACACCA</td>
<td>CAGATGTTCCTCACGCTGGA</td>
<td>94°C 30 s</td>
<td>59°C 30 s</td>
<td>72°C 30 s</td>
<td>35</td>
</tr>
<tr>
<td>Mouse preproglucagon</td>
<td>TGGCAGT GTTGCGAAGTGTG</td>
<td>CAGAGGAGAAACCCACGATCA</td>
<td>94°C 30 s</td>
<td>59°C 30 s</td>
<td>72°C 30 s</td>
<td>35</td>
</tr>
<tr>
<td>19S</td>
<td>CCCATATGATGAAACTGCTT</td>
<td>CAGATCTCTGTGTCCTGGAG</td>
<td>94°C 30 s</td>
<td>55°C 30 s</td>
<td>72°C 30 s</td>
<td>35</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CCATCGCCGATCCTCTCTT</td>
<td>CTCGTTGGCAAATGAGTACG</td>
<td>94°C 30 s</td>
<td>60°C 30 s</td>
<td>72°C 30 s</td>
<td>35</td>
</tr>
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</table>

Peptide

A previous study has shown that i.p. injections of rat nesfatin-1 suppress food intake and in mice with similar potency to the synthetic native mouse nesfatin-1 (Shimizu et al. 2009). Furthermore, peripheral injection of rat nesfatin-1 produces an anti-hyperglycemic effect in mice which is insulin dependent (Su et al. 2010). In addition, Shimizu et al. (2009) showed that the active core of the peptide that causes physiological effects lies within the mid-segment of nesfatin-1 (24–53). Although rat and mouse nesfatin-1 differ in two amino acids (aa 13 and 18), this proposed bioactive core is completely conserved between rat and mouse sequences. Based on these, rat nesfatin-1 was used in our studies. Rat full length (82 amino acids) nesfatin-1 (VPIDVDKTKHVNPVESARIIEPPIDTGLYYDEYDIKQVIEVLETDPPHFRKQLQADIEIRSGRLSQELDIVSHKVRTRLDEL) was synthesized by Abgent Technologies (San Diego, CA, USA). Synthetic nesfatin-1 was HPLC purified to ≥95% purity. The mass and purity of the peptide were confirmed by mass spectrometry.

In vitro studies

To explore whether nesfatin-1 has direct effects on islets to regulate insulin secretion, we performed in vitro secretion experiments using MIN6 cells and isolated islets. MIN6 cells were seeded at 1×10^5 cells per well density in 1 ml DMEM in 12-well plates 24 h prior to glucose-stimulated insulin secretion (GSIS). Briefly, on the day of the study, cells were pre-treated with low glucose (2.0 mM) DMEM for 2 h and then replaced with buffer consisting of Krebs-Ringer bicarbonate buffer (KRB) with 1% BSA. Prior to GSIS, isolated islets were washed with low glucose (2.0 mM) KRB. The cells were washed once after 30 min and replaced with the same buffer mixed with 0, 0.1, or 1 nM nesfatin-1 and incubated for 1 h. Subsequently, low glucose KRB media were collected and replaced with high glucose (16-7 mM) KRB media containing 0, 0.1, or 1 nM nesfatin-1 for 1 h prior to collection of media and storage at −20°C. Insulin levels in media samples were determined using mouse ultrasensitive insulin ELISA kit (ALPCO, Salem, NH, USA). MIN6 cells were lysed in acid ethanol for determination of total insulin content as described previously (Zhang et al. 2009). Results are presented as insulin secreted normalized to the total insulin content. Duplicated plates
for mRNA extraction were conducted to determine preproinsulin and NUCB2 mRNA expression.

The experiments with pancreatic islets were conducted as previously described (Zhang et al. 2009). Isolated islets were evenly divided in 12-well plates (25 islets/well) and pretreated with low glucose (2.0 mM) DMEM. On the day of the study, the DMEM media were replaced with low glucose KRB for 2 h. Subsequently, the islets were washed once and then replaced with low glucose KRB buffer mixed with 0, 0.001, 0.01, 0.1, or 1.0 nM nesfatin-1 and incubated for 1 h. Subsequently, low glucose KRB media were collected and replaced with high glucose (16·7 mM) KRB media containing 0, 0.001, 0.01, 0.1, or 1·0 nM nesfatin-1 for 1 h prior to collection of media and storage at −20 °C. Insulin levels in media samples were determined using mouse ultrasensitive insulin ELISA kit (ALPCO). cDNAs were synthesized using the iScript cDNA synthesis kit (Bio-Rad). Primer sequences and qRT-PCR conditions are listed in Table 1. Amplification and detection of NUCB2 and housekeeping genes were performed in triplicate on four samples in each experimental group using an iQ SYBR Green Supermix on a Chromo4 Multicolor Real-Time PCR Detection System (Bio-Rad). Relative gene expression data were obtained after normalizing the data using the Pfaffl method (Pfaffl 2001).

Quantitative real-time PCR

Total RNA was extracted from rodent tissues using the TRIzol RNA isolation reagent (Invitrogen) or Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). cDNAs were synthesized using the iScript cDNA synthesis kit (Bio-Rad). β-Actin and 19S RNA were used as internal controls. Primer sequences and qRT-PCR conditions are listed in Table 1. Amplification and detection of NUCB2 and housekeeping genes were performed in triplicate on four samples in each experimental group using an iQ SYBR Green Supermix on a Chromo4 Multicolor Real-Time PCR Detection System (Bio-Rad). Relative gene expression data were obtained after normalizing the data using the Pfaffl method (Pfaffl 2001).

Figure 1 (A) Nesfatin-1 at 0·01 and 0·1 nM stimulates glucose-dependent (16·7 mM) insulin release in vitro from MIN6 cells. Incubation media with low glucose (2 mM) did not significantly alter insulin release between nesfatin-1-treated and control conditions. Values presented in the figure are expressed as fractional release (% of total insulin content) in 60 min. (B) Preproinsulin mRNA expression was up-regulated in MIN6 cells treated with nesfatin-1 at 0·01 and 0·1 nM. (C) Glucose stimulates nesfatin-1 release from MIN6 cells in vitro during 1 h incubation. Data are presented as mean±s.e.m. N=4 wells/group. *P<0·5, **P<0·01.

Figure 2 (A) Nesfatin-1 at 0·01, 0·1, and 1·0 nM stimulates glucose-dependent (16·7 mM) insulin release in vitro from isolated mouse islets. Incubation media with low glucose (2 mM) did not significantly alter insulin release between nesfatin-1-treated and control conditions. Values presented in the figure are expressed as insulin release per 25 islets. Data are presented as mean±s.e.m. N=4 wells/group. *P<0·5, **P<0·01.
Immunohistochemistry

Pancreata from control, DIO, and STZ-treated mice were processed as previously described (Gonzalez et al. 2009) for detecting nesfatin-1, insulin, and glucagon immunoreactivity. Pixel intensity and morphometric analyses were done on pancreas sections of experimental and control mice (n=5/group). Three randomly chosen fields of 0.27 mm² from representative pancreas from each mouse were taken and analyzed for islet density, size, and area. In addition, the pixel intensity of nesfatin-stained islets was determined using NIS-elements basic research imaging software (Nikon Canada, Mississauga, Canada).

Statistical analysis

All values were reported as mean±S.E.M. Statistical analyses were performed by Student’s t-tests or one-way ANOVA followed by Student–Newman–Keuls post-hoc test.

Results

Nesfatin-1 stimulates glucose-induced insulin release from cultured MIN6 cells

Nesfatin-1 (0.1 and 1 nM) significantly augmented GSIS from cultured MIN6 cells (Fig. 1A) during 1 h incubation in the presence of high glucose (16.7 mM). No significant difference in insulin secretion was observed in nesfatin-1-treated MIN6 cells in the presence of low glucose (2 mM). Nesfatin-1 also increased preproinsulin mRNA expression in the same MIN6 cells (Fig. 1B) from which insulin release was stimulated.

Glucose stimulates nesfatin-1 release

A fourfold increase in nesfatin-1 release from MIN6 cells was observed following incubation in high glucose (16.7 mM) compared to low glucose (2.0 mM) (Fig. 1C).

Nesfatin-1 stimulates glucose-induced insulin release from islets isolated from mice

As observed with MIN6 cells, nesfatin-1 induced dose-dependent increase in insulin secretion in isolated mouse islets. In the presence of high glucose (16.7 mM), nesfatin-1 (0.01, 0.1, and 1.0 nM) significantly elevated GSIS from isolated islets during a 1 h incubation period (Fig. 2). No increase in GSIS was observed when isolated islets were exposed to lower (0.001 nM) concentrations of nesfatin-1. In the presence of low glucose (2.0 mM), none of the tested concentrations of nesfatin-1 caused any significant change in basal insulin secretion during the 1 h incubation (Fig. 2).

Figure 3 In islets from normal mice, no glucagon (green) and nesfatin-1 (red) co-staining was found (A), but insulin and nesfatin-1 co-localization (yellow) was seen (D). Three days post-STZ injection, no glucagon (green) and nesfatin-1 (red) co-localization was found (B), while nesfatin-1 (red) and insulin (green) co-staining (yellow) (E) completely disappeared in diabetic mice. On day 6 post-STZ injection, some nesfatin-1 alone (C and F) or nesfatin-1 and glucagon (C) staining alone was found in the islets, but no insulin was present (C). Scale bar = 50 μm. Both NUCB2 (G) and preproinsulin (H) mRNA expression were significantly reduced in the pancreas of streptozotocin (STZ)-injected mice with type 1 diabetes, while a significant increase in proglucagon mRNA (I) expression was found. Data are presented as mean±S.E.M. n=3 mice/group, *P<0.05.
NUCB2 mRNA and nesfatin-1-ir are reduced in type 1 diabetes

As we previously reported (Gonzalez et al. 2009), glucagon-ir does not co-localize with nesfatin-1-ir in mice (Fig. 3A and B), while insulin-ir does (Fig. 3D). As expected, we found a complete loss of detectable insulin-ir in mice with type 1 diabetes 3 days post-STZ injection (Fig. 3E). Nesfatin-ir was also significantly reduced on day 3 post-STZ injection (Fig. 3B and E). Furthermore, 6 days post-STZ injection, we observed nesfatin-1-immunoreactive cells (Fig. 3C and F) in the islets, and some of these cells co-localize glucagon (Fig. 3C). We found significantly lower levels of NUCB2 (Fig. 3G) and preproinsulin (Fig. 3H) mRNA expression 6 days post-STZ injection, while preproglucagon mRNA expression was significantly higher (Fig. 3I).

Increased nesfatin-ir and NUCB2 mRNA expression was found in type 2 diabetes

We observed nesfatin-ir co-localized with insulin-ir (Fig. 4C) but did not co-localize with glucagon-ir (Fig. 5C) in the islets of DIO mice. No staining was found when the primary antibody was preabsorbed with rat nesfatin-1 peptide or stained with secondary antibodies alone (D). All nuclei are stained with DAPI (blue; A–D). Scale bar=50 μm.

Discussion

Our studies indicate that nesfatin-1 is an insulinotropic peptide and that endogenous pancreatic nesfatin-1 is altered in diabetes and obesity. We found that short-term exposure of nesfatin-1 enhances GSIS from isolated islets and cultured MIN6 cells. These findings provide clear evidence that nesfatin-1 directly acts on the β-cells to stimulate insulin secretion. In addition, nesfatin-1 also stimulated preproinsulin mRNA expression in MIN6 cells, suggesting that this novel insulinotropic peptide could also influence insulin synthesis. Other regulatory peptides, including glucose-dependent insulinotropic peptide, glucagon-like peptide-1, vasoactive intestinal polypeptide, and pituitary adenylate cyclase-activating polypeptide, have been shown to exert glucose-dependent stimulatory effects on insulin release in a meal-responsive manner (Winzell & Ahren 2007, Kim & Egan 2008). Our results show for the first time that nesfatin-1 exerts a stimulatory effect on GSIS from mice islets in vitro. The mechanisms that mediate the insulinotropic actions of nesfatin-1 require further investigations.

We have also observed an approximately fourfold increase in the level of nesfatin-1 release from MIN6 cells in high glucose, indicating that endogenous nesfatin-1 is released in a glucose-responsive manner. Our results are in agreement
with recent findings, which provide strong evidence that nesfatin-1 is a meal-responsive factor that may regulate insulin release in both humans and rodents. Foo et al. (2010) reported that NUCB2 release from islets of normal Wistar rats and diabetic GK rats is stimulated by high glucose. They also reported that fasting decreases and i.p. injections of glucose induce serum NUCB2 in rats ( Foo et al. 2010). In a recent study by Stengel et al. (2009b), NUCB2 mRNA in rat gastric endocrine cells was down-regulated after a 24 h fast. In addition, fasting in rats has been shown to decrease nesfatin-1 levels in circulation, while re-feeding normalizes nesfatin-1 levels (Goebel et al. 2009 ). Administration of nesfatin-1 reduced blood glucose levels in hyperglycemic db/db mice, and this effect was insulin dependent (Su et al. 2010). In humans, healthy subjects given an oral glucose drink had higher levels of nesfatin-1 in circulation relative to saline-treated controls (Li et al. 2010), whereas nesfatin-1 levels in restricting-type anorexia nervosa were lower in the anorexic group compared to those in the control group (Ogiso et al. 2010). These reports suggest that nesfatin-1 in the circulation is regulated by nutritional status and response to starvation. Our results suggest that pancreatic islets are possibly one of the tissues that contribute to nesfatin-1 in circulation.

The NUCB2 mRNA and nesfatin-ir, together with preproinsulin mRNA and insulin-ir, were significantly reduced in type 1 diabetes. However, we found some cells in the endocrine pancreas that stain exclusively for nesfatin-1 on day 6 post-STZ injection. Nesfatin-1 does not co-localize with glucagon ( Gonzalez et al. 2009 , Foo et al. 2010), or somatostatin and PP cells (Foo et al. 2010) in adult rodent pancreas. It is currently not known whether nesfatin-1 is involved in the regulation of islet endocrine cells, especially β-cell neogenesis, survival, and proliferation. Several other insulinogetic endocrine factors including GLP-1 also have important local roles in islet

Figure 6 Larger nesfatin-1-immunoreactive islets were abundant in DIO mice (B) compared to standard mouse chow (SMC) fed controls (A). Scale bar=50 µm. Both the percentage average pixel intensity of nesfatin-1 immunoreactivity (C) and NUCB2 mRNA expression were higher in DIO mice (D). Nesfatin-1-immunoreactive islet density (E), islet area (F), islet size (G), and percentage of larger islets positive for nesfatin-1 (H) are significantly higher in DIO mice. Data are presented as mean±S.E.M. n=minimum five sections from three different mice/group, *P<0.05, **P<0.01. Representative images are shown.

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growth, proliferation, and survival (Perfetti et al. 2000, Farilla et al. 2002). The abundant expression and very strong co-localization of nesfatin-1 with insulin in the islets suggest a local role for nesfatin-1 in the functioning of endocrine pancreas.

In DIO mice islets, nesfatin-ir co-localized with insulin-ir, but not with glucagon-ir as we previously reported in normal mice (Gonzalez et al. 2009). NUCB2 mRNA increased twofold in the pancreas, and nesfatin-1-ir increased 37% above mice fed standard chow. This is in agreement with the fivefold increase in NUCB2 mRNA expression in the pancreatic islets of DIO mice, previously discovered in a microarray study (supplemental data in Iguchi et al. 2005). We found significant increases in the density, size, and distribution of pancreatic islets in DIO mice, similar to previous reports of increases in islet mass in DIO mice (Reimer et al. 2002, Bock et al. 2003). In a recent report, Foo et al. (2010) reported that islets from ad libitum fed, but not from fasted, GK rats had lower nesfatin-1 in islet homogenates compared to control animals. However, the variations observed here are likely due to differences in analysis (nesfatin-1 in islet homogenates versus nesfatin-1 pixel intensity), strain used (GK rats versus DIO mice), and physiology-dependent response differences to nesfatin-1 actions.

Collectively, our results indicate that nesfatin-1 is a glucose-responsive insulinotropic peptide that acts directly on pancreatic islet β-cells. Altered expression of pancreatic nesfatin-1 in both type 1 and type 2 diabetes implicates endogenous nesfatin-1 in the pathophysiology of diabetes and obesity. While the precise mechanisms underlying nesfatin-1 action, particularly within the context of diabetes and obesity, remain elusive, this work illuminates a potential role for nesfatin-1 in regulating glucose homeostasis. Future studies examining the glucoregulatory and insulinotropic effects and mechanisms of actions of nesfatin-1 under normal and aberrant physiological conditions, especially in diabetes, warrant consideration.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
R. G conducted the experiments, collected and analyzed data, and wrote the manuscript. B K R and X G helped with the in vivo MING6 studies. M P G helped with the DIO study. R. G T provided some materials and supplies and feedback on the manuscript. S U helped with the research planning, conducted some experiments, secured funding for the project, and wrote the manuscript.

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