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Chronic effects of neuroendocrine regulatory peptide (NERP-1 and -2) on insulin secretion and gene expression in pancreatic β-cells

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ABSTRACT

Neuroendocrine regulatory peptides (NERP-1 and -2) are novel amidated peptides derived from VGF, a polypeptide secreted from neurons and endocrine cells through a regulated pathway. Dr. Nakazato Masamitsu reported that NERP-1 and -2 may have a local modulator function on the human endocrine system, and clearly showed expression of NERP-1 and -2 in human pancreas islets. Based on these data, we investigated the alteration of insulin secretion, insulin granule-related protein, and pancreas-specific transcription factors in response to NERPs expression. We confirmed the expression of NERP-1 and -2 in the pancreas of a human diabetes patient, in addition to diabetic animal models. When INS1 cells and primary rat islets were incubated with 10 nM NERPs for 3 days, glucose-stimulated insulin secretion levels were blunted by NERP-1 and -2. The number of insulin granules released from the readily releasable pool, which is associated with the first phase of glucose-stimulated insulin release, was decreased by NERP-1 and -2. Insulin granule-related proteins and mRNAs were down-regulated by NERP-2 treatment. NERP-2 decreased the expression of BETA2/NeuroD and insulin and controlled the nucleo-cytoplasmic translocation of FOXO1 and Pdx-1. We observed that NERP-2 levels were dramatically increased in diabetic pancreas. In conclusion, NERP-2 may play an important role in insulin secretion through the regulation of insulin secretory granules and β-cell transcription factors. In addition, NERP-2 expression is increased in diabetic conditions. Therefore, we suggest that NERPs may be potent endogenous suppressors of glucose-dependent insulin secretion.

1. Introduction

Neuroendocrine regulatory peptides (NERP-1 and NERP-2) are novel amidated peptides derived from VGF, a polypeptide secreted from neurons and endocrine cells through a regulated pathway [1]. NERP-1 and NERP-2 are secreted from human medullary thyroid carcinoma TT cells in a strategy for discovering endogenous bioactive peptides [2] and are 25- and 38-amino acid peptides, respectively, with C-terminal amidation, and are abundant in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) in the rat hypothalamus. In the PVN and SON, NERPs frequently co-localize with vasopressin (AVP), but rarely with oxytocin. NERPs dose-dependently suppress AVP release induced by intracerebroventricular (icv) injection of high levels of salt or AngII *in vivo*. NERPs also suppress basal and AngII-induced AVP secretion from hypothalamic explants *in vitro*. C-terminal amidation of NERPs is essential for their biological activity [2].

NERPs are derived from distinct regions of a neurosecretory protein, originally identified as a product of a nerve growth factor-responsive gene in PC12 cells. Dr. Nakazato Masamitsu demonstrated NERP expression in the peripheral tissues. NERP-1 and NERP-2 were detected in the islets of the human pancreas, thyroid C-cells, and gastrin-producing G cells in the stomach antrum, where they co-localized with insulin/glucagon, calcitonin, and gastrin, respectively. In the case of islets, NERPs colocalized extensively with insulin and partially with glucagon, but not with somatostatin [3]. These results suggest that the processing and amidation of NERPs occurred intracellularly before secretion in the tissues, as is the case for amidated bioactive peptides secreted

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from endocrine cells. VGF-derived peptides have been detected in insulinoma cell lines such as the rat pancreatic β-cell line INS-1, as well as in human insulinoma [4,5]. NERP immunoreactivity is present in human hypothalamus, islets of pancreas, thyroid, gastric antrum, and insulinoma. Mature forms of NERP-1 and -2 are present in the pancreas, thyroid, and gastric antrum.

However, there has been no report of the role of NERP in the expression of pancreas-specific genes in β-cells, or whether NERP affects insulin synthesis. Here, we confirmed that the alteration of insulin secretion and insulin granule-related protein is affected by NERPs-induced expression of the gene encoding insulin and pancreas specific-transcription factors.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, CA). RPMI-1640 medium was purchased from Welgene (Daegu, Korea). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). The antibody for insulin was purchased from Zymed (South San Francisco, CA, USA), the antibodies for NERP-1 and -2 were obtained from Phoenix Pharmaceuticals (Burlingame, CA). Anti-Pdx-1 was a gift from Dr. Kaneto Hideaki (Osaka University Graduate School of Medicine, Osaka, Japan). Trizol reagent and SuperScript®II RT were purchased from Invitrogen (Carlsbad, CA, USA). The cell culture media, 0.025% trypsin, and 0.001% DNase I were purchased from GIBCO (Grand Island, NY, USA), and collagenase P was obtained from Boehringer-Mannheim (Indianapolis, IN, USA). The insulin radioimmunoassay (RIA) kit was purchased from Linco (Charles, MO, USA). All other chemicals were acquired from Sigma–Aldrich (Louis, MO, USA).

2.2. Cell culture and NERP treatment

Rat pancreatic islets were isolated from Sprague–Dawley rats (200–230 g) by digesting the pancreatic duct with collagenase P (1 mg/mL in Medium 199), as previously described [6]. After digestion, the islets were separated with Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The islets were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 μg/mL antibiotic–antimycotic (GIBCO BRL, Carlsbad, CA). The rat insulinoma cell line INS1 was maintained in RPMI1640 medium supplemented with 11.1 mM glucose, 10 mM HEPES, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 100 μg/mL antibiotic–antimycotic (GIBCO BRL). NERP-1 and -2, and a form of NERP-1 and -2 bearing a COOH-terminal glycine (Gly) extension (NERP-1 and 2-Gly, kindly provided by Dr. Masamitsu Nakazato, University of Miyazaki, Miyazaki, Japan) [7] were diluted in distilled water to a final concentration of 10 μM. Cells were treated with 10 nM NERP-1, -2, NEPR-1-Gly, and NEPR-2-Gly.

2.3. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was obtained from cells using a Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using superscript II RT (Invitrogen) and 1 μg of total RNA according to the manufacturer's instructions. PCR was conducted with 3 μl of first-strand cDNA as follows: 98 °C for 1 min followed by 27 cycles for all genes at 94 °C for 1 min, 55.5 °C for 30 s, 72 °C for 1 min and 72 °C for 7 min. The PCR primers for FOXO1 5′-CGTGCCCTACTTCAAGGATA-3′ (forward) and 5′-GGTGAGTCCTTCCACGGTTT-3′ (reverse); for Pdx-1 5′-GGCCACACAGCTCTACAAGGA-3′ (forward) and 5′-CATCACTGCCAGCTCCACCC-3′ (reverse); for BETA2/NeuroD 5′-CTCCGGGGTTATGAGATCGTCAC-3′ (forward) and 5′-GCCTTCATG-CGCCTTAATTT-3′ (reverse); for insulin 5′-TCCTGCCCCTGCTGG CCCTGCT-3′ (forward) and 5′-CTAGTTGCAGTAGTTCTCCAG-3′ (reverse); for SUR1 5′-ATCCACGCTGGAGTGTCTGA-3′ (forward) and 5′-GGTGAGTCCTTCCACGGTTT-3′ (reverse); for Kir6.2 5′-GGCTCCTAGTGACCTGCACCA-3′ (forward) and 5′-CCACAGCCACACT GCGCTTGCG-3′ (reverse); for SCG2 5′-CCCTACCAAGGTGTCTCTGT-3′ (forward) and 5′-GGTGCAGACTGAGGCTCATTT-3′ (reverse); for SCG5 5′-GGCTGCTTCATGGTGTTATGG-3′ (forward) and 5′-CAGTCA ACTCTGCCACGATGT-3′ (reverse); for β-actin 5′-CATGTTTGAGAC CTTCAACACCCC-3′ (forward) and 5′-GCCATCTCCTGCTCGAAGTC-TAG-3′ (reverse); were designed to recognize the separate exons to eliminate possible DNA contamination. The PCR products were electrophoresed through 1.5% agarose gel. Band intensity was calculated densitometrically using a densitometer VDS analyzer (Pharmacia Biotech AB).

2.4. Glucose-stimulated insulin secretion (GSIS)

Cultured cells were washed in Krebs–Ringer-bicarbonate (KRB) washing buffer (130 mM NaCl, 3.6 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 2.0 mM $NaHCO_3$, and 10 mM HEPES) and incubated in KRB buffer containing 5.5 mM glucose for 1 h. The cells were then stimulated for 1 h in KRB buffer containing 25.5 mM glucose. Insulin concentrations were measured with a RIA kit (Millipore Corporation, Bedford, MA).

2.5. TIRFM analysis

Rat pancreatic islets were isolated by the collagenase digestion method as described above and transferred onto a high-refractive-index glass coverslip (refractive index, n = 1.8 at 488 nm; Olympus) which was coated with 10 mg/cm^2 laminin (Sigma) at 37 °C for 3 h. The cells were then infected with adenovirus carrying insulin-Venus, at a multiplicity of infection (MOI) of 10, and cultured in RPMI medium 1640 supplemented with 10% FBS under a humidified condition in 95% air and 5% CO_2. After culturing of infected primary β-cells for 48 h, the cells on the glass coverslips were pre-incubated on a thermostat-controlled stage at 37 °C in HEPES-KRB containing 2.8 mM glucose for 60 min with 10 nM NERP-1 or NERP-2. Analysis of the dynamics of insulin granule exocytosis was performed using the Olympus total internal reflection fluorescence microscope (TIRFM) system. Briefly, light from an argon laser (488 nm, 3 mW) was introduced to an inverted microscope (IX71; Olympus) through a single-mode fiber and 2 illumination lenses, and the light was focused at the back focal plane of a high aperture objective lens (Apo 100′ OHR; NA 1.65; Olympus). The focal point was moved off-axis to the most-peripheral position in the objective lens by shifting the position of the fiber. Di-iodomethane sulfur immersion oil (n = 1.81 at 488 nm) (Cargille Laboratories) was used to make contact between the objective lens and the coverslip. At a measured incidence angle of 69.0° with nglass = 1.8 and ncytosol = 1.38, a 488-nm beam had a calculated penetration depth of 40.7 nm. Images were collected with a cooled charge-coupled-device camera (ORCA-R2; Hamamatsu) operated with Metamorph 6.1 (Universal Imaging). Images were acquired every 300 ms. In total, 4000 frame time lapses were collected. The collected data were analyzed according to a method described [7].

2.6. Western blot analysis

Cells were lysed in PBS containing 1 mM Na_3VO_4, 1 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.0), and 1 mM PMSF. Protein concentration was determined by the

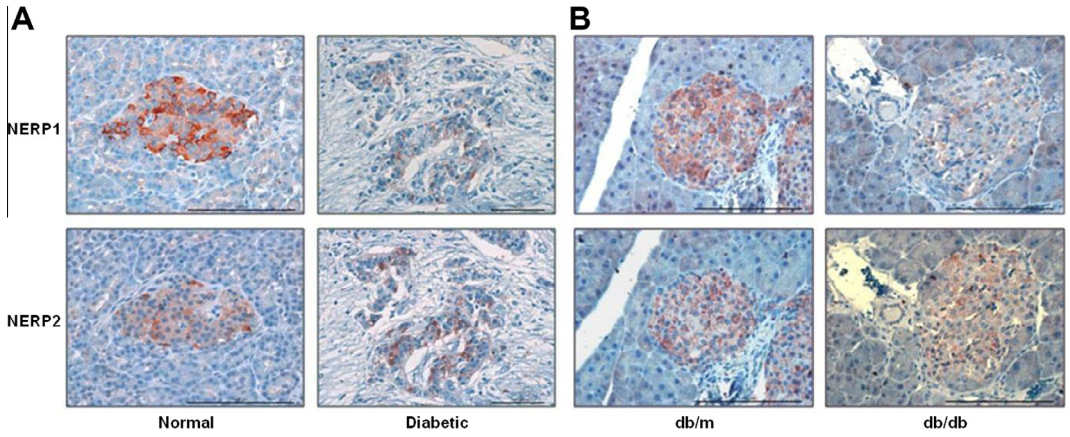

Fig. 1. The expression of NERP-1 and -2 in normal or diabetic pancreatic islets. Normal and diabetic human pancreas were immunostained with anti-NERP-1 and -2 antibodies. The potency of NERP-2 was weaker than NERP-1 in the normal pancreas, whereas in diabetic pancreas, the expression level of NERP-2 was higher compared to normal pancreas (A). Similar results were also obtained from immunochemistry with pancreas that was from well-established mouse model, db/db and db/m for the control (B).

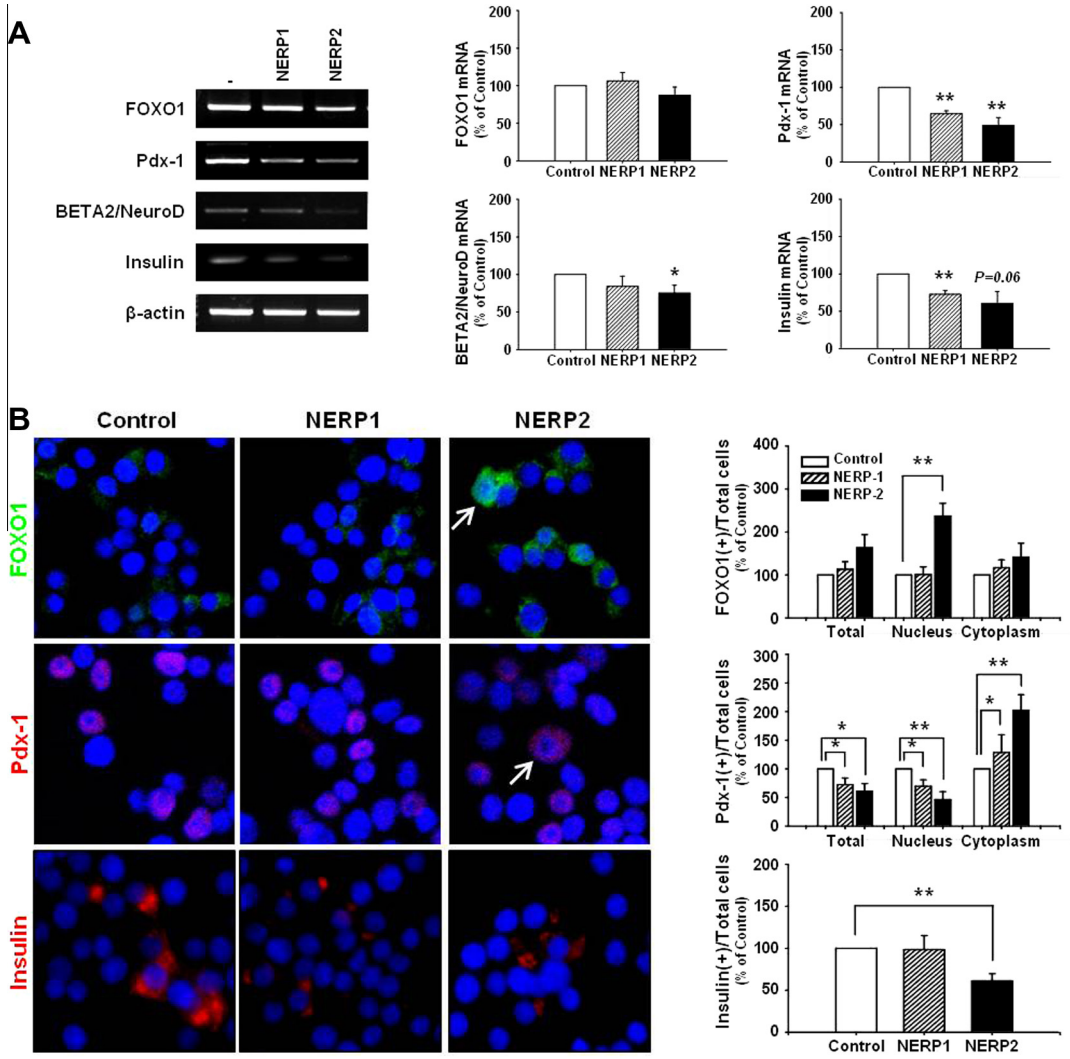

Fig. 2. The alteration of β-cells transcription factors by NERP-1 and -2. To determine the effect of chronic NERP-1 or -2 treatments, INS1 cells at 70% confluence were treated with NERP-1 or -2 (10 nM, each) for 3 days. At day 3 post-NERPs treatment, Pdx-1, BETA2/NeuroD and insulin mRNA levels were down-regulated by NERP-1 and -2, but FOXO1 mRNA had little change (A). The mRNA levels of these genes were normalized to β-actin mRNA and the RT-PCR data from four independent experiments are summarized and expressed as the mean ± standard errors relative to those of the untreated islets ($^{*}P < 0.05$, $^{**}P < 0.01$). Increased nucleic-FOXO1 and cytosolic-Pdx-1 levels were observed in NERP-2 treatment group by immunocytochemistry. Decreased insulin levels were also observed on equal terms (B).

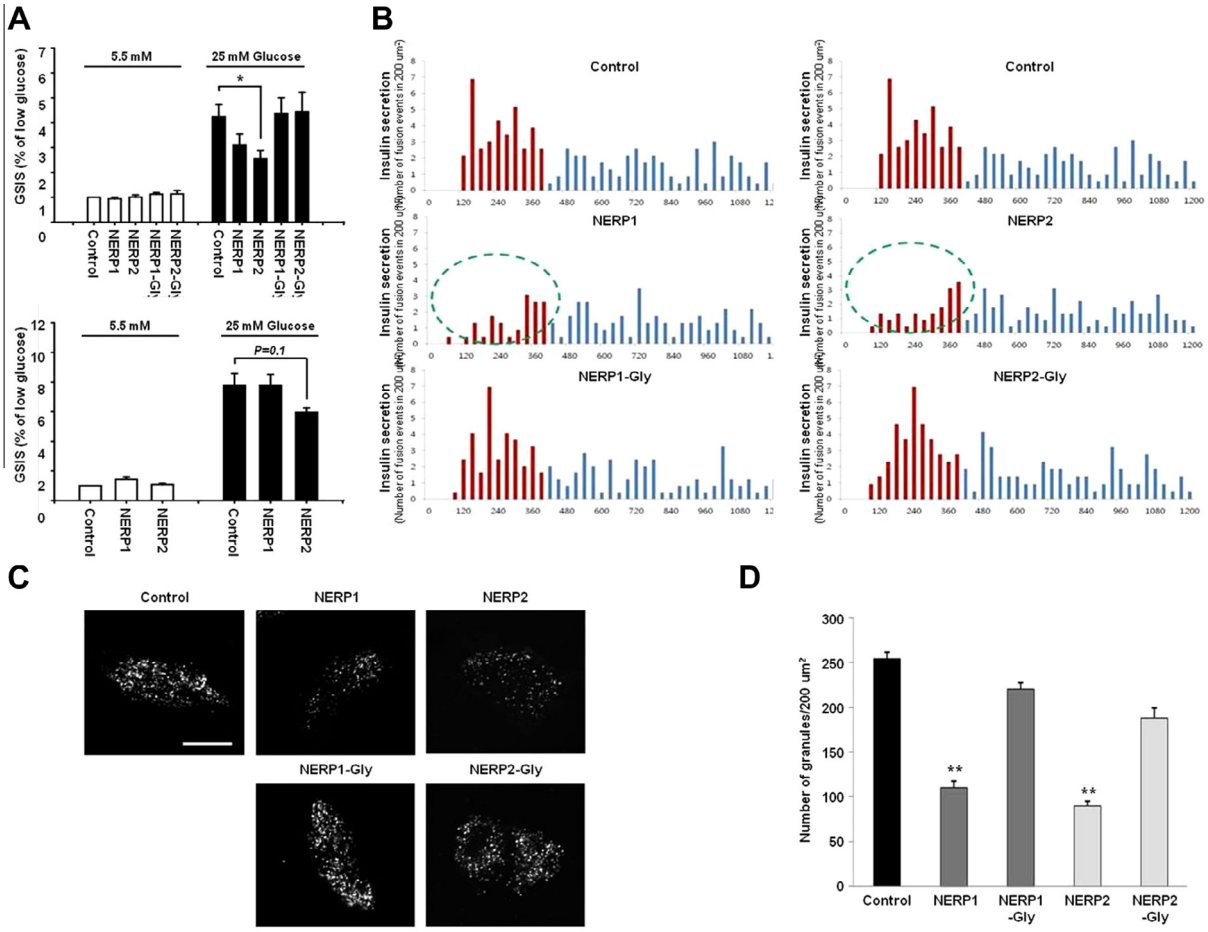

Fig. 3. Chronic NERP-2 treatment decrease insulin secretion and first phase insulin secretion in β-cells. INS1 cells or isolated rat islets were treated with NERP-1 or -2 (10 nM, each) for 3 days. Cells and islets were then incubated in KRB buffer containing 5.5 mM glucose for 1 h, stimulated for 1 h in KRB buffer containing 25 mM glucose. Glucose stimulated insulin secretion (under static condition) were blunted by NERP-2 in NERPs treated INS1 (upper panel) and isolated rat islets (lower panel) (A). Similar results were also obtained from dynamic glucose-stimulated insulin secretion in NERPs treated rat islets (B). The real-time motion of insulin granules was imaged close to the plasma membrane. Isolated rat islets were treated with NERP-1 or -2 (10 nM, each) for 3 days, then in turn incubated in 5.5 and 25 mM glucose. In islets, the first-phase insulin response arose from the readily releasable pool of insulin granules at the plasma membrane (scale bar; 10 μm). Impaired first-phase insulin release was shown in NERP-1 and -2 treatment groups (C). The number of docked insulin granules to the plasma membrane in NERP-1 and -2 treated islets. Reduced number of granules was shown in the β-cells by NERP-1 and -2 treatment groups (D). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group (mean ± SE).

Bradford method using BSA as a standard (Bio-Rad, Hercules, CA). Whole cell lysates were fractionated by SDS–PAGE (12% gels) and transferred to immobilon polyvinylidene difluoride membrane (Millipore Corporation) in transfer buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. After blocking with milk, the membranes were incubated with 1 of the following antibodies: anti-phospho-FOXO1, anti-FOXO1 (Cell signaling, Danvers, MA), anti-Pdx-1 (Millipore Corporation), anti-Rab43 (Abcam, Cambridge, UK), anti-Chromogranin, anti-SCG2, (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-Actin (Sigma Aldrich, St. Louis, MO) antibodies. The membranes were then treated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad). Signals were visualized by Enhanced Peroxidase Detection Western Blot Detection Kit (GenDEPOT, Barker, TX).

2.7. Immunochemistry

Whole pancreases were immersion-fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4). Sections were prepared for immunostaining using anti-NERP-1 (1:200, Phoenix Pharmaceuticals, Burlingame, CA) and anti-NERP-2 (1:200, kindly provided by Dr. Naoto Minamino, National Cardiovascular Center Research Institute, Osaka, Japan) antibodies and developed with diaminobenzidine tetrahydrochloride. For immunostaining of cultured cells, cells were fixed with 4% paraformaldehyde for 10 min and incubated at 4 °C overnight with 1 of the following antibodies: anti-FOXO1 (1:100), anti-Pdx-1 (1:500, Developmental Studies Hybridoma Bank, Iowa City, IA), and anti-insulin (1:200, Invitrogen) antibodies.

2.8. Image acquisition

Digital images at 2 or 3 fluorescent emission wavelengths were acquired using a Bio-Rad confocal microscope. The light sources were an Argon/He and multi-photon laser giving excitation wavelengths of 368–647 nm. FITC and Texas Red were excited at 488 and 568 nm using an Argon/He laser. FITC and Texas Red images were scanned separately at different laser wavelengths and captured as overlapped images.

3. Results and discussion

3.1. NERP-2 expression was increased in diabetic conditions

In a previous study, NERPs were highly abundant in the pancreas; the tissue contents of NERP-1 and NERP-2 were 4.5 ± 2.2

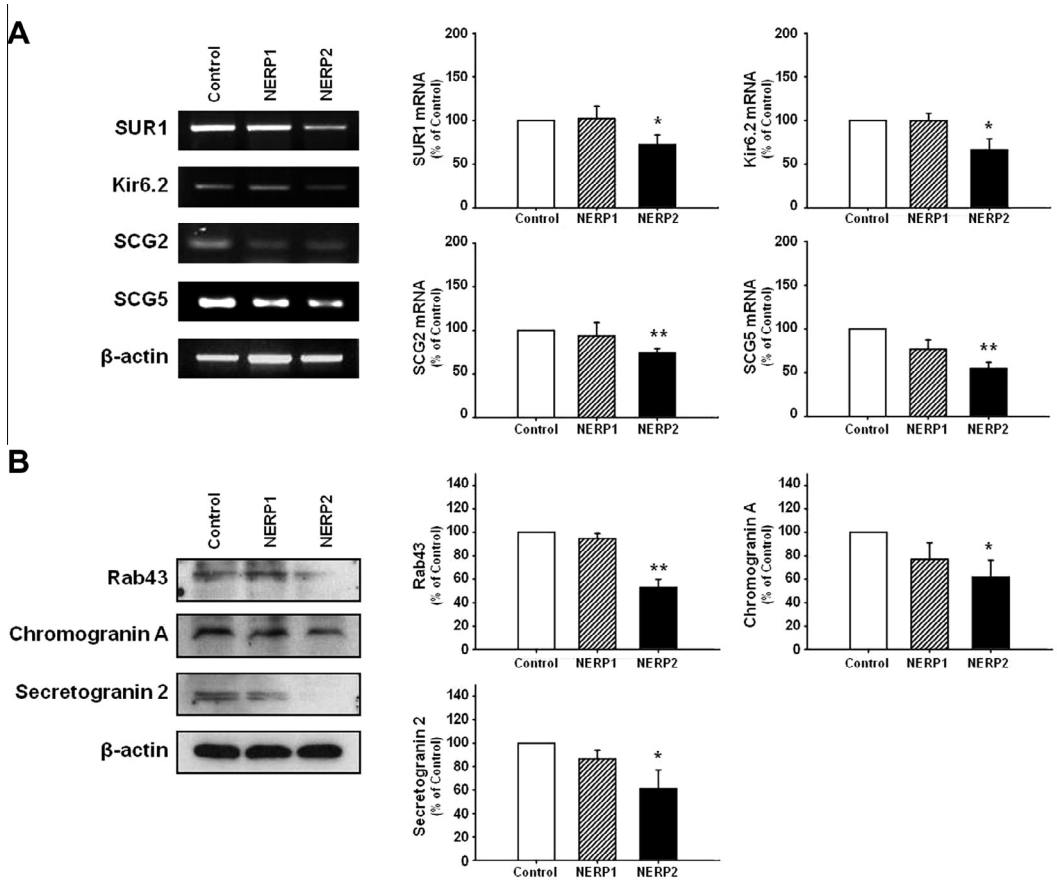

Fig. 4. The alteration of secretory granule-related genes by chronic NERP-1 and -2 treatments. INS1 cells at 70% confluence were treated with NERP-1 or -2 (10 nM, each) for 3 days. SUR1, Kir6.2, SCG2 and SCG5 were estimated by semiquantitative RT-PCR. All secretory granule-related genes were decreased by NERP-2 at day 3 after NERPs treatment (A). The mRNA levels of these genes were normalized to GAPDH mRNA and the RT-PCR data from the four independent experiments have been summarized as the mean ± standard error relative to those of untreated islets. After 3 days NERP-1 or -2 (10 nM, each) treatment in INS cells, western blot was performed using anti-Rab43, chromogranin A and secretogranin 2 antibody to verify vesicular protein levels in INS1 cells. Various vesicular proteins were blunted by NERPs treatment especially NERP-2 (B). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group (mean ± SE).

and 1.0 ± 0.3 pmol/g wet tissue, respectively. In the islets of the pancreas, NERP immunoreactivity colocalized extensively with insulin, and partially with glucagon, but not with somatostatin [3]. To extend this study, we investigated NERP expression in normal and diabetic pancreatic islets. By immunohistochemistry, NERP-1 and NERP-2 were found to be mainly localized in pancreatic islets, with NERP-2 expression weaker than that of NERP-1 in the normal pancreas. By contrast, in diabetic pancreas, the expression level of NERP-2 was higher than that in normal pancreas (Fig. 1A). Subsequently, we performed immunohistochemical staining of mouse pancreas from db/db, a well-known obese model of type 2 diabetes, and db/dm, a control mouse model. Similar results were obtained using immunochemistry to examine NERP-1 and NERP-2 expression in each group. The expression of NERP-2 was weaker than that of NERP-1 in normal pancreatic islets, whereas its expression was increased in pancreatic islets of db/db mice (Fig. 1B). These results indicate that NERP-2 expression, which is lower than NERP-1 expression under normal condition, increased in diabetic conditions in both human and mouse pancreatic islets.

3.2. β-Cell transcription factors were decreased by chronic treatment of NERP-2

In type 2 diabetes, chronic hyperglycemia has long been thought to have negative consequences on β-cell function [8,9]. The reduction of insulin-gene transcription is thought to be secondary to reductions in the transcription or activity of β-cell transcription factors such as Pdx-1 and BETA2/NeuroD [10,11]. Thus, to confirm the relationship between NERP-2, which was increased in the diabetic condition, and β-cell transcription factors, INS1 cells were treated with NERP-1 or NERP-2, chronically. Cells were incubated with 10 nM NERP-1 or NERP-2 for 3 days, and expression of these NERPs was confirmed by RT-PCR amplification. At day 3 post-NERP treatment, Pdx-1, BETA2/NeuroD, and insulin mRNA levels were down-regulated by NERP-1 and NERP-2, but little change was observed in FOXO1 mRNA levels (Fig. 2A). It was reported that oxidative stress resulted in nuclear-to-cytoplasmic translocation of Pdx-1, and that this was blocked by dominant-negative FOXO1 [12]. Therefore, to examine alterations of Pdx-1 and FOXO1 proteins in response to NERP-2 treatment, we next performed immunocytochemistry of Pdx-1, FOXO1, and insulin (Fig. 2B). Interestingly, increased nucleic FOXO1 and cytosolic Pdx-1 levels were observed in the NERP-2 treatment group. Consequently, decreased insulin levels were decreased by NERP treatment. These results suggest that chronic treatment with NERP-2 decreased β-cell transcription factors, controlled the nucleo-cytoplasmic translocation of FOXO1 and Pdx-1, and decreased expression of insulin mRNA and protein.

3.3. Chronic treatment with NERPs results in depleted insulin secretion

NERPs are derived from distinct regions of VGF, a neurosecretory protein [13]. The mature forms of VGF and insulin may be

stored in the same type of vesicles [14], and the involvement of VGF in the control of circulating glucose and insulin levels was confirmed by previous studies [15,16]. Thus, we confirmed the effect of NERPs in insulin secretion and secretory granule-related genes. To verify the role of NERPs in insulin secretion, we performed glucose stimulated insulin secretion under real-time and static conditions. For measurement of glucose-stimulated insulin secretion by static incubation, INS1 cells or isolated rat islets were incubated in KRB buffer containing 5.5 mM glucose for 1 h, and stimulated for 1 h in KRB buffer containing 25 mM glucose, after treatment with NERP-1 or NERP-2 (10 nM each) for 3 days. After chronic NERP-2 treatment, decreased insulin secretion was observed in INS1 cells and rat islets (Fig. 3A). Similar results were also obtained from dynamic glucose-stimulated insulin secretion in NERP-treated rat islets (Fig. 3B). Glucose-stimulated insulin secretions, especially first-phase insulin secretion, were blunted by NERP-1 and NERP-2 in treated rat islets. Next, the docking and fusion processes of insulin granules were imaged in isolated rat islets after 3 days of treatment with NERPs. TIRF images showed the real-time motion of insulin granules close to the plasma membrane and the fusion of granules after stimulation with high levels of glucose, during first-phase insulin secretion. In islets, the first-phase insulin response arose from the readily releasable pool of insulin granules at the plasma membrane (scale bar: 10 μm). The number of insulin granules docked to the plasma membrane was decreased in NERP-1 and NERP-2-treated islets (Fig. 3C). Moreover, a reduced number of granules was observed in the β-cells of NERP-1 and NERP-2 treatment groups (Fig. 3D). These results indicate that NERPs inhibit fusion events by decreasing the number of readily releasable pools.

3.4. Chronic treatment with NERPs results in depleted insulin secretion via inhibition of insulin granule components

Finally, we examined how secretory granule-related genes were altered by NERP-1 and NERP-2. INS1 cells were treated with NERP-1 or NERP-2 (10 nM, each) for 3 days, and expression levels of *SUR1*, *Kir6.2*, *SCG2* (*secretogranin2*) and *SCG5* (*secretogranin5*) were estimated by semi-quantitative RT-PCR. The expression levels of all secretory granule-related genes were decreased by chronic NERP-2 treatment (Fig. 4A). To evaluate the alteration of vesicular protein levels in INS1 cells at 3 days after NERP-1 or NERP-2 treatment, western blots were performed using anti-Rab43, chromogranin A, and secretogranin 2 antibodies. The expressions of various vesicular proteins were repressed by treatment with NERPs, particularly NERP-2 (Fig. 4B). Taken together, chronic treatment with NERPs depleted insulin secretion via inhibition of granule docking and fusion in the first phase of insulin secretion and insulin granule. In summary, the present data indicate that NERP-1 and NERP-2 have different patterns of expression between postnatal and adult stages of pancreatic islets. NERP-2 expression disappears in pancreatic islets of human diabetes patients, and NERPs inhibit fusion events by decreasing the number of readily releasable insulin pools. Therefore, we suggest that NERPs are potent endogenous suppressors of glucose-dependent insulin secretion. Further study will attempt to confirm the expression of pancreatic β-cell specific genes and GSIS in response to NERP-1 and NERP-2 treatments in *in vitro* diabetic conditions, such as gluco- or lipotoxicity, and we will investigate NERP levels in human diabetes patients.

Acknowledgments

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