Nucleobindin-2/nesfatin in the endocrine pancreas: distribution and relationship to glycaemic state

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Abstract

The protein nucleobindin-2 (NUCB2, also known as nesfatin) was recently implicated as a mediator of anorexia and catabolism in the central nervous system, and has been suggested to act as a cleaved and secreted messenger. Given the overlap of signalling molecules between the brain and pancreas, we have explored the presence of NUCB2 in the islets of Langerhans. We also performed an investigation of the dynamic regulation of pancreatic NUCB2 in different metabolic states. NUCB2-like immunoreactivity was detected by immunofluorescence in all human and rat islet β-cells (as detected by co-localization with insulin), but not in other islet cells or in the exocrine pancreas. Islet NUCB2 content, as measured by enzyme immunoassay, did not change significantly following an overnight fast, but was substantially lower in islets isolated from an animal model of type 2 diabetes, the Goto-Kakizaki (GK) rats (48% of non-diabetic Wistar rat control). Serum levels, however, were not different between Wistar and GK rats. The release of NUCB2 from isolated rat islets was significantly elevated following glucose challenge (123%), but this effect was substantially lower than that observed for insulin (816%). In contrast, serum levels of NUCB2 showed a reversible decrease in an i.p. glucose tolerance test. These data suggest a role for NUCB2 in β-cell function and a potential involvement in diabetic pathology. However, our findings, together with previous reports, appear more compatible with intracellular actions rather than with endocrine/paracrine communication, and suggest that NUCB2 in serum derives primarily from non-islet sources.

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Introduction

The central nervous system (CNS) and the endocrine pancreas overlap significantly in their signalling molecule repertoires. Thus, a number of neuropeptides expressed in the brain are also found in the islets of Langerhans (Langerhans 1869, Laguesse 1893), e.g. somatostatin in δ-cells (Luft *et al.* 1974) and thyrotrophin-releasing hormone (TRH) in β-cells (Kawano *et al.* 1983). In some cases, the functional output of signalling via these peptides converges to a common effect: the anorexigenic CNS actions of TRH (Vijayan & McCann 1977) have a correlate in paracrine hypoglycaemic effects in the pancreas (Kulkarni *et al.* 1995, Yamada *et al.* 1997). A recent addition to the group of centrally acting catabolic signalling molecules is the protein nucleobindin-2 (NUCB2, also known as NEFA; Barnikol-Watanabe *et al.* 1994, Oh-I *et al.* 2006, Stengel *et al.* 2009*a*). Intracerebral administration of an N-terminal 82-amino acid residue (a.a.r.) fragment of NUCB2, named nesfatin-1, results in the inhibition of feeding and concomitant reduction in body weight. Conversely, administration of nesfatin-1 antiserum or anti-sense oligonucleotides stimulates food intake. In contrast, no effect was observed with the C-terminal nesfatin-2 and -3 fragments, leading Oh-I *et al.* (2006) to propose that nesfatin-1 is cleaved from NUCB2 and secreted, acting as an anorexigenic mediator. This interpretation is, however, complicated by previous research that identified helix-turn-helix and EF-hand motifs within the NUCB2 sequence that suggest a direct interaction with DNA and Ca^{2+} (Barnikol-Watanabe *et al.* 1994), and by the demonstration that nesfatin is virtually absent in axon terminals in neurons (Foo *et al.* 2008).

We (Foo *et al.* 2008) and others (Oh-I *et al.* 2006, Brailoiu *et al.* 2007) have recently described a broad, but discrete, distribution of NUCB2 in the CNS, including almost all neuroendocrine neurons. In addition, we also found a wide expression of NUCB2 in the anterior pituitary gland (Foo *et al.* 2008), which may indicate the involvement of this protein in endocrine secretion. A recent report suggests the presence of nesfatin-1 immunoreactivity in the pancreas (Gonzalez *et al.* 2009, Stengel *et al.* 2009*b*). Because of the endocrine expression and the proposed involvement of NUCB2 and related fragments in metabolic processes, we have examined the distribution and co-expression of NUCB2 with four major pancreatic hormones in the rat and human

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pancreata. We have also performed the first investigation of the relationship between pancreatic NUCB2 and metabolic state. Thus, we investigated the levels of NUCB2 in serum and pancreatic islets under different metabolic conditions, including food restriction, and in the Goto-Kakizaki (GK) rats, model of type 2 diabetes mellitus (T2DM; Goto *et al.* 1975, Östenson & Efendic 2007, Portha *et al.* 2009).

Materials and Methods

Animal and human tissue sources

For histochemistry, 6–8-week-old male Sprague–Dawley ($n=5$) and Wistar ($n=4$) rats (Scanlab, Sollentuna, Sweden) weighing *ca.* 300 g were used. For rat islet measurements and serum samples, 8–12-week-old male Wistar rats (Scanlab) weighing 295 ± 25 g and age- and sex-matched GK rats weighing 258 ± 21 g from our own breeding were used. The GK rats are derived after repeated breeding of non-diabetic Wistar rats with high-normal serum glucose levels as breeding selection criterion (Goto *et al.* 1975). Glucose tolerance and other characteristics of GK rats of the Stockholm colony have been described in detail elsewhere (see Östenson 2001). All animals were maintained under 12 h light:12 h darkness conditions (lights on at 0600 h) in a temperature-controlled environment and were allowed free access to standard rat chow and tap water. For measurement of serum NUCB2/nesfatin concentrations, blood samples were drawn from the tail vein of animals first during *ad lib* feeding conditions (Wistar, $n=20$; GK, $n=19$), and next following an overnight fast (chow removed immediately prior to the lights were put out) and an i.p. glucose tolerance test (ipGTT) in these fasted animals (Wistar, $n=20$; GK, $n=19$), 30 min (Wistar, $n=12$; GK, $n=12$), 90 min (Wistar, $n=5$; GK, $n=5$), 120 min (Wistar, $n=7$; GK, $n=7$) and 240 min (Wistar, $n=7$; GK, $n=7$) after i.p. glucose injection (2 g/kg body weight of a 2·8 M glucose solution). All animal experiments had been approved by the local ethics committee, Stockholm Norra Djurförsöksetiska Nämnd.

Human pancreatic islets were isolated from pancreata retrieved from three non-diabetic patients. The patients were brain-dead, heart-beating, multiorgan donors, and the use of pancreata for scientific purpose was approved in all cases. The study had been approved by the Human Research Ethics Committee of the Karolinska Institutet. The cold ischaemia time period ranged from 8 to 12 h. The procedure of islet isolation was a refinement of the automated method described previously (Ricordi *et al.* 1988). The isolated islets were suspended in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 5·5 mM glucose, 10% human serum, 10 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin, 0·25 μg/ml fungizone (Gibco), 20 μg/ml ciprofloxacin (Bayer Healthcare) and 10 mM nicotinamide, and were cultured in a culture bag system Fenwal (Baxter Medical, Eskilstuna, Sweden) at 37 °C in 5% CO_2 and humidified air for 2–4 days. The culture medium was changed on day 1 and then every other day. The purity of islets was checked by microscopic sizing on a grid after staining with dithizone, and it exceeded 90% in all cases. The islets were stored frozen at −80 °C until processing for immunofluorescence.

Immunofluorescence

Perfusions were performed between 1400 and 1700 h in the afternoon. Rats were anaesthetized with an i.p. injection of sodium pentobarbital and perfused via the ascending aorta with 50 ml of Tyrode's Ca^{2+}-free solution (37 °C) followed by 50 ml of a mixture of formalin and picric acid (4% paraformaldehyde and 0·4% picric acid in 0·16 M phosphate buffer, pH 6·9, 37 °C) according to Zamboni & De Martino (1967), and then followed by 200–300 ml of the same but ice-cold fixative. The pancreata were rapidly dissected out, immersed in the same fixative for 90 min and rinsed for at least 24 h in 0·1 M phosphate buffer (pH 7·4) containing 10% sucrose, 0·02% bacitracin and 0·01% sodium azide. The pancreata were frozen and sectioned on a cryostat (Microm, Walldorf, Germany) at 35-μm thickness and thaw-mounted onto gelatin-coated glass slides. In addition, frozen human pancreatic islets isolated as described above ($n=100$) were sectioned at 14-μm thickness and were fixed for 5 min in formalin/picric acid as described above. Immunofluorescence on both rat and human tissues was performed using the Tyramide Signal Amplification (TSA) protocol (Perkin Elmer, Waltham, MA, USA). Following incubation in PBS (0·01 M), sections were treated with 0·03% hydrogen peroxide in PBS for 30 min and were subsequently rinsed with PBS for another 30 min before incubation with primary anti-NUCB2 antisera (as described below), diluted in PBS containing 0·3% Triton X-100, at 4 °C for 72 h. Sections were processed for conventional indirect immunofluorescence as described (Broberger *et al.* 1999); all reactions were performed in room temperature unless stated otherwise. Sections were rinsed in TNT buffer (0·1 M Tris, 0·15 M NaCl and 0·05% Tween 20), then pre-incubated with TNB blocking reagent supplied with TSA kit (Perkin Elmer) for 30 min and incubated for another 30 min with HRP-conjugated swine anti-rabbit immunoglobulin (1:200 in TNB buffer; Dako, Glostrup, Denmark). The sections were then rinsed and incubated for 10 min with tyramide-conjugated fluorescein (1:100 in Amplification Diluent supplied with the TSA kit; Perkin Elmer). For double staining, the sections were washed for 30 min in PBS, then incubated with Alexa 594-conjugated secondary antisera (as described below) in 37 °C for 30 min and subsequently mounted with anti-fade agent (2·5% DABCO; Sigma) diluted in glycerol. Some sections were incubated with the nuclear marker TOTO-3 (1:3000; Invitrogen) diluted in 0·3% Triton for 20 min after detection of secondary antisera. For confocal microscopy, a Zeiss LSM 510 META confocal scanning laser microscope was used (Carl Zeiss, Jena, Germany).

Antisera

Three antisera raised in a rabbit against separate portions of the NUCB2 protein were used for immunofluorescence staining: against the putative nesfatin-1 fragment (corresponding to rat NUCB2 a.a.r. 1–82; 1:8000; Phoenix Pharmaceuticals, Burlingame, CA, USA), against the putative nesfatin-2 fragment (corresponding to rat NUCB2 a.a.r. 117–182; 1:20 000; a generous gift from Dr M Mori, Gunma University Graduate School of Medicine, Maebashi, Japan; Oh-I *et al.* 2006) and against the putative nesfatin-3 fragment (corresponding to rat NUCB2 a.a.r. 401–420; 1:1000; Phoenix Pharmaceuticals). Specificity of the nesfatin-1 antiserum was determined by preabsorbing the antiserum with the corresponding immunogenic peptide (10^{-5} M; Phoenix Pharmaceuticals) prior to immunostaining. As this antiserum detects only the full-length 420-a.a. NUCB2 protein (Oh-I *et al.* 2006, Foo *et al.* 2008) in the western blot analysis, the signal detected with this reagent is subsequently referred to as 'NUCB2-like immunoreactivity'. Characteristics of the nesfatin-2 and -3 antisera have been published previously (Foo *et al.* 2008). For double labelling, the following antisera were used: antiserum targeted against insulin (1:2000) and glucagon (1:500; a generous gift from Dr S Efendic, Karolinska Institute, Stockholm, Sweden) raised in a guinea pig and detected using Alexa 594-conjugated goat anti-guinea pig secondary antiserum (1:500; Invitrogen). Antisera against somatostatin (1:2000; a generous gift from Dr R Elde, University of Minnesota, Minneapolis, MN, USA) and pancreatic polypeptide (1:2000; a generous gift from Dr J R Kimmel, VA Medical Center, Kansas City, MO, USA) were raised in a rabbit and detected with Alexa 594-conjugated donkey anti-rabbit secondary antiserum (1:500; Invitrogen). The increased sensitivity of immunodetection that can be achieved with TSA staining (and tenfold-decreased antiserum concentrations) allows for double labelling with antisera raised in the same species as demonstrated previously (Broberger *et al.* 1999).

Studies in isolated rat islets

Rats were killed by decapitation when they were unconscious after inhalation of CO_2. Islets were isolated by injecting collagenase A in Hanks' solution (9 mg/10 ml for Wistar rats and 24 mg/10 ml for GK rats) into the pancreas through the pancreatic duct. Then, the gland was removed, incubated for 24 min at 37 °C and washed with Hanks' solution, and islets were picked up after separation on Histopaque gradient (Sigma). Isolated islets were cultured overnight free floating in Petri dishes in RPMI 1640 medium (Flow Laboratory, Irvine, UK) with 11 mM glucose, 2 mM glutamine, 10% heat-inactivated FCS, 100 IU/ml penicillin and 0·1 mg/ml streptomycin at 37 °C in an atmosphere of 95% O_2 and 5% CO_2. To yield homogenates, batches of 100 isolated islets were homogenized by ultrasound in 200 µl acid ethanol (1 M HCl in 70% ethanol) and extracted for 24 h at +4 °C before freezing at −20 °C. To investigate the effect of ambient glucose on the release of nesfatin and insulin, cultured islets were pre-incubated for 30–45 min at 37 °C in 5 ml of Krebs–Ringer bicarbonate (KRB) buffer containing 118·4 mM NaCl, 4·7 mM KCl, 1·9 mM $CaCl_2$, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4$, 25 mM $NaHCO_3$ (equilibrated with 5% CO_2 and 95% O_2), 10 mM HEPES, 300 units/ml aprotinin (Trasylol, Bayer) and 0·2% bovine albumin with 3·3 mM glucose, pH 7·4. Batches of 20 islets were then incubated for 60 min at 37 °C in 0·4 ml KRB supplemented with HEPES and aprotinin as described above and with 3·3 or 16·7 mM glucose. Aliquots of the incubation medium were stored at −20 °C for the determination of NUCB2 (see below) and insulin by RIA later (Herbert *et al.* 1965).

Enzyme immunoassay

Immunoreactive nesfatin levels in rat samples were measured using a nesfatin-1 (1–82) rat enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals) according to the manufacturer's instructions. The assay had a detection limit of 0·1 ng/ml. All samples were analyzed in duplicates. Total islet protein content in islet homogenates was measured using the Bio-Rad protein assay kit according to the manufacturer's instructions. The assay had a detection range of 200–1400 µg/ml. All samples were analyzed in duplicates.

Statistical analysis

Data obtained from experiments on islets and sera were analyzed using GraphPad Prism 4.0 software (La Jolla, CA, USA). Mann–Whitney test was used for non-parametric two-way comparison between groups. $P<0·05$ was regarded as statistically significant. All data are expressed as mean ± S.E.M.

Results

Immunofluorescence

Sections of formalin-fixed rat pancreas stained with antiserum raised against the NUCB2 fragment displayed prominent labelling in the islets of Langerhans (Fig. 1A). NUCB2-immunoreactive (-ir) cells were observed primarily in the central parts of islets, while several of the outermost peripherally located cell bodies were void of staining. No NUCB2-like immunoreactivity (-LI) was observed in the exocrine acini or in the blood vessels. To control for specificity of the immunolabelling, we also stained adjacent sections with NUCB2 antiserum that had been pre-incubated with the cognate peptide. This pre-treatment abolished staining in pancreatic islets (Fig. 1B). We also used three different antisera raised against segments corresponding to the postulated nesfatin-1, -2 and -3 portions of the NUCB2 protein (Fig. 1C–E). All the three antisera yielded identical staining patterns in the pancreas as shown previously in the CNS (Foo *et al.* 2008).

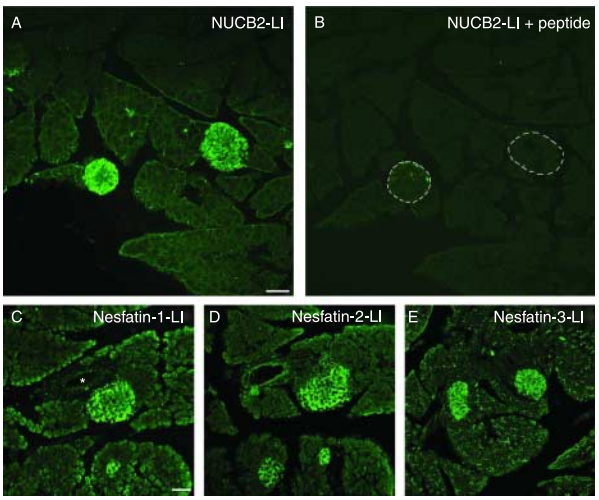

Figure 1 NUCB2 in rat pancreas. Confocal micrographs of sections of rat pancreas processed for immunofluorescence staining with antisera raised against nesfatin fragments. (A) NUCB2-LI is observed only in islets, while exocrine acini are void of labelling. (B) Following pre-incubation of the antiserum with cognate peptide, staining of islets (indicated by stippled line) in an adjacent section is abolished. (C–E) Similar immunofluorescence patterns are observed following staining with antisera raised against the nesfatin-1 (C), nesfatin-2 (D) and nesfatin-3 (E) fragments. Scale bar in A and C = 100 μm for A, B, and C–E respectively. Asterisk indicates blood vessel. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-09-0254.

To determine the phenotype of the NUCB2-ir islet cells, we performed immunofluorescence double staining for four peptides (Fig. 2): glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells) and pancreatic polypeptide (PP cells). NUCB2-LI was not observed in cells that were glucagon-, somatostatin- or PP-ir. However, all insulin-ir cells also contained NUCB2-LI. When double-stained sections were analyzed with high-resolution confocal microscopy, a partly non-overlapping subcellular distribution was observed (Fig. 3A–D). NUCB2-LI was distributed in cytoplasmic clusters; insulin-LI partly aggregated in puncta lining the nuclear envelope in the β-cells that were relatively void of NUCB2-LI. Staining of isolated human islets with the NUCB2 antiserum revealed a similar distribution and coexistence with insulin-LI as in rats (Fig. 3E–H).

Islet and serum immunoreactive nesfatin levels

The levels of ir NUCB2 were measured in homogenates of isolated rat pancreatic islets by EIA (Fig. 4). In islets isolated from control (Wistar) rats, the concentration of ir NUCB2 was 50·2 ± 9·5 pg/islet ($n=8$). In islet homogenates obtained from GK rats ($n=7$), ir NUCB2 levels were significantly lower (23·9 ± 3·9 pg/islet; 48% of control; $P<0·05$). Islet levels of ir NUCB2 did not change significantly after an overnight fast in Wistar control rats (41·0 ± 9·2 pg/islet; $n=6$; $P=0·51$). In GK rats, however, overnight fasting resulted in a significant increase in NUCB2-LI (48·7 ± 10·4 pg/islet; $n=7$; $P<0·05$) towards the same level as in *ad lib* fed Wistar rats ($P=0·92$).

To assess whether NUCB2 is released from the endocrine pancreas, isolated rat islets were incubated in 3·3 and 16·7 mM glucose (Fig. 5). Elevation of glucose from 3·3 to 16·7 mM resulted in a slightly higher release of NUCB2-LI from Wistar islets (20 ± 1 pg/islet per h and 24 ± 1 pg/islet per h respectively; 123% of control; $n=11$; $P<0·01$). While this change was significant, it was notably smaller than the glucose challenge-induced increase in secreted ir insulin measured from the same islet preparations (7·4 ± 1·9 and 60·4 ± 5·1 μU/islet per h respectively; 816% of control; $P<0·001$; $n=11$; not shown). The ir NUCB2 release at 3·3 mM of glucose from GK rat islets (23 ± 1 pg/islet per h; $n=6$) was not significantly different from that from Wistar rat islets ($P=0·116$). In GK rat islets, there was a tendency towards an increase in ir NUCB2 (31 ± 4 pg/islet per h; $n=5$) in 16·7 mM glucose compared with 3·3 mM glucose, but this change failed to reach statistical significance ($P=0·061$). Insulin release from GK rat islets was 5·6 ± 0·6 μU/islet per h ($n=6$) and 16·0 ± 2·3 μU/islet per h ($n=5$) at 3·3 and 16·7 mM glucose respectively (286% of control; $P<0·05$), which is in agreement with previous reports of impaired insulin release from this diabetic model (Portha *et al.* 1991, Östenson *et al.* 1993).

To further investigate the possibility that NUCB2 is secreted and regulated by metabolic state, we measured ir protein levels in serum obtained from Wistar control and GK rats (Fig. 6A and B) under *ad lib* feeding conditions after an overnight fast and in an ipGTT. Circulating ir NUCB2 levels in *ad lib* fed Wistar control rats (4·73 ± 0·32 ng/ml) were similar to those in *ad lib* fed GK rats (4·54 ± 0·31 ng/ml; Fig. 6A). The ipGTT caused a typical transient elevation in blood glucose that was characteristically delayed in GK rats (Fig. 6B; Abdel-Halim *et al.* 1994). While the ir NUCB2 levels were not affected by an overnight fast in Wistar rats (4·56 ± 0·28 ng/ml; Fig. 6A), i.p. injection of glucose resulted in a transient and significant decrease within 30 min (2·89 ± 0·14 ng/ml; $P<0·01$). Plasma ir NUCB2 levels in GK rats displayed a similar response, with no change following fasting (4·66 ± 0·34 ng/ml), but a significant transient decrease was observed following i.p. glucose injection (3·11 ± 0·13 ng/ml, $P<0·001$). In both Wistar and GK rats, the plasma ir NUCB2 levels appeared to follow an inverse relationship with plasma glucose concentration in the ipGTT (Fig. 6A and B).

Discussion

We have investigated the distribution and regulation of pancreatic NUCB2. Our data confirm and extend recent reports (Gonzalez *et al.* 2009, Stengel *et al.* 2009*b*) by showing a wide expression of NUCB2 in pancreatic islets confined to β-cells. The present data provide the first demonstration of NUCB2 in human islets. We have also shown that islet

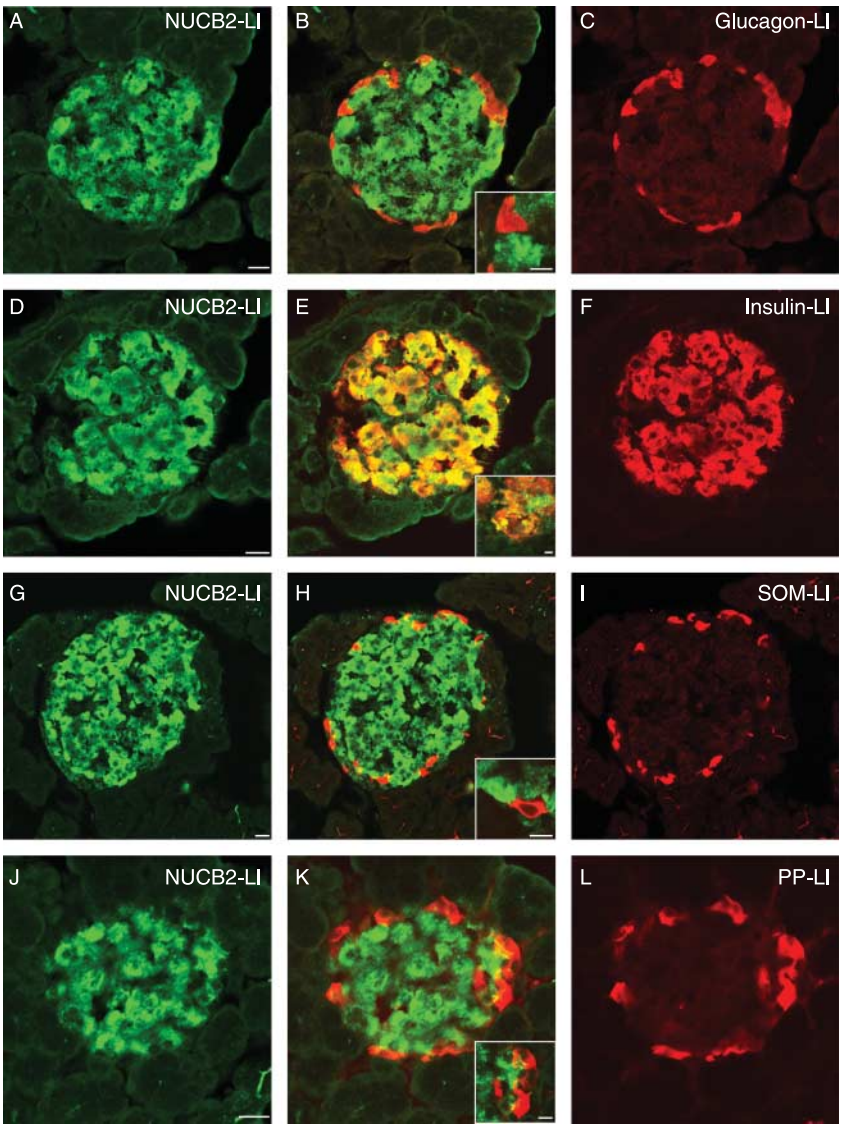

Figure 2 NUCB2-LI is co-localized with insulin. Confocal micrographs of sections of rat pancreata processed for immunofluorescence double staining for NUCB2 (A, D, G and J; green) and glucagon (C), insulin (F), somatostatin (SOM; I) and pancreatic polypeptide (PP; L) respectively (all in red). Merged images with high-resolution images in insets are shown in B, E, H, and K. Note an almost complete coexistence between NUCB2-LI and insulin-LI (E). In contrast, NUCB2 and glucagon, and somatostatin and pancreatic polypeptide form mutually exclusive populations in the islet (B, H and K). Scale bar in A, D, G and J = 20 μm for A–C, D–F, G–I and J–L respectively. Scale bar = 10 μm for insets in B, E, H and K.

NUCB2 is decreased in a model of T2DM. Parallel expression of signalling molecules in brain and endocrine pancreas appears to be more a rule than an exception, and applies to neuropeptides (e.g. Luft *et al.* 1974), transcription factors (e.g. Naya *et al.* 1995) and intracellular signalling mediators (see Mountjoy & Rutter 2007), as well as to proteins involved in the secretory process (e.g. Jacobsson *et al.* 1994). Our data, along with recent papers by Gonzalez *et al.* (2009) and Stengel *et al.* (2009*b*), suggest that this is also true for NUCB2. Indeed, our immunofluorescence data suggest that all, rather than just a subpopulation, of rat β-cells possess NUCB2-LI. We also demonstrate that other islet cells, as revealed by immunofluorescence for somatostatin (δ-cells) and PP – in addition to glucagon-containing α-cells – lack NUCB2, further underlining its specific co-expression with insulin. The controls included in our study indicate that the signal detected reflects true NUCB2: NUCB2-LI is abolished following pre-incubation of the antiserum with the

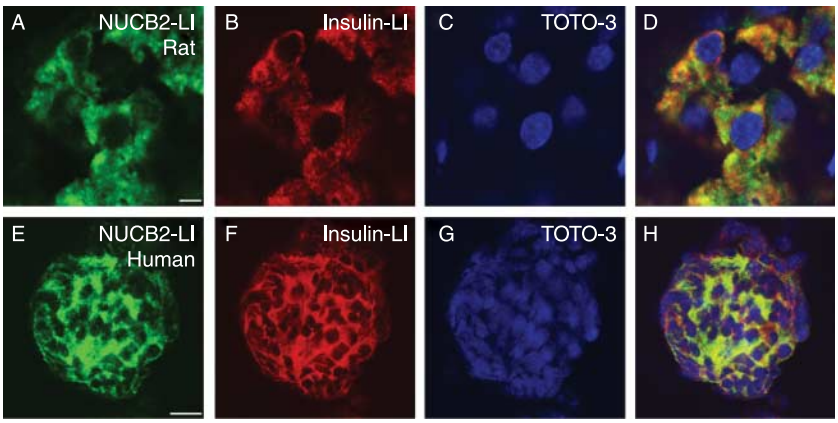

Figure 3 Subcellular distribution of NUCB2 and insulin in rat and human islets. (A–D) High-resolution confocal micrographs of sections of rat islet processed for immunofluorescence triple staining for NUCB2 (A), insulin (B) and the nuclear marker TOTO-3 (C). Merged image shown in D. Note partly non-overlapping subcellular distribution of NUCB2-LI and insulin-LI (D). However, neither protein is observed in the nucleus. (E–H) Confocal micrographs of human islet processed for immunofluorescence triple staining for NUCB2 (E), insulin (F) and the nuclear marker TOTO-3 (G). Merged image shown in H. Note coexistence of NUCB2-LI (E) with insulin-LI (F). Scale bar in A indicates 5 μm for A–D; 20 μm in E for E–H.

immunogenic peptide, and the same staining pattern was observed using antisera raised against the three different putative nesfatin fragments.

Given the blood–brain barrier permeability of nesfatin-1 fragments (Pan *et al.* 2007, Price *et al.* 2007) and the presence of NUCB2-LI in serum (present results), it is tempting to speculate that the anorexigenic CNS effects (Oh-I *et al.* 2006, Stengel *et al.* 2009*a*) may result from peripheral NUCB2 entering the brain equally well as local, neuronally produced protein. Previous demonstrations of NUCB2 in the pituitary (Foo *et al.* 2008) and in enteroendocrine cells (Stengel *et al.* 2009*b*) indicate that these can act as additional potential sources of this protein in serum. Examples of direct effects of nesfatin fragments on membrane excitability have been described (Price *et al.* 2008*a*,*b*). Importantly, however, several data from the present study and earlier literature are difficult to reconcile with the concept of NUCB2 or nesfatin protein(s) as (a) secreted neurohormonal messenger(s). First, though extracellular levels from *in vitro* islet preparations increased slightly following glucose challenge, this increase was notably smaller than that observed for insulin (23 and 716% increase respectively), suggesting that this standard protocol for stimulating degranulation of β-cells does not lead to substantial release of NUCB2. Our observation that GK rats display serum ir NUCB2 levels that are similar to those displayed by Wistar control rats even though islet content is dramatically decreased also argues against the pancreas as a major contributor of blood NUCB2.

Secondly, although NUCB2-LI and insulin-LI (the latter following the secretory pathway) coexisted in β-cells, our high-resolution analysis revealed that their subcellular distributions were not identical. The exact relative distribution of these molecules to cellular compartments will need to be determined with electron microscopy. However, it is notable that in neurons also, the subcellular distribution of NUCB2-LI is different from what would be expected for a traditional neuropeptide, as only cell bodies and proximal dendrites, but not axon terminals, are stained (Brailoiu *et al.* 2007, Foo *et al.* 2008, Goebel *et al.* 2009). Thirdly, to date, actual processing of NUCB2 into the proposed nesfatin-1, -2

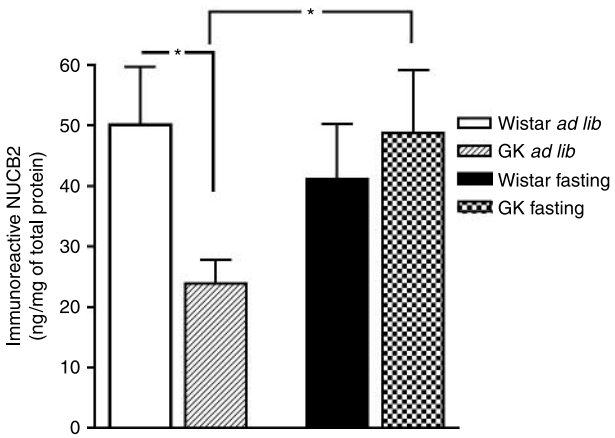

Figure 4 NUCB2 concentration in islet homogenates. Histogram shows levels of ir NUCB2 expressed as ng/mg of total protein in rat islet homogenates obtained from Wistar control and GK rats under *ad lib* feeding and overnight fasting conditions. Level of ir NUCB2 was lower in *ad lib* fed GK rats than in their Wistar control rats (48% of control; $*P<0{\cdot}05$). The difference between *ad lib* fed and fasted Wistar rats was not statistically significant ($P=0{\cdot}52$). Islet ir NUCB2 levels in fasted GK rats, however, were significantly higher than those in *ad lib* fed GK rats ($*P<0{\cdot}05$). Immunoreactive NUCB2 levels are not significantly different between *ad lib* fed Wistar rats and fasted GK rats ($P=0{\cdot}92$). Wistar rats *ad lib* fed $n=8$, and fasting $n=6$; GK rats *ad lib* fed $n=7$, and fasting $n=7$.

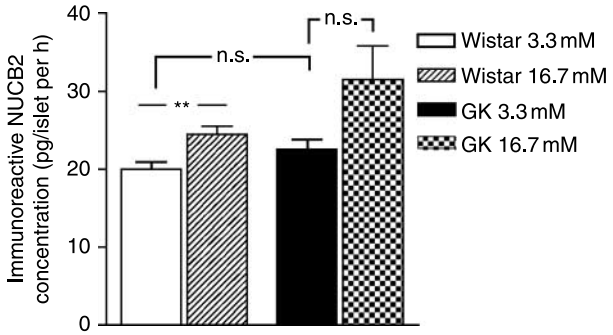

Figure 5 NUCB2 release from glucose-challenged islets. Histogram shows levels of ir NUCB2 in perifusate of rat islets isolated from Wistar control and GK rats exposed to 3·3 or 16·7 mM glucose. Note a significant increase in perifusate ir NUCB2 following glucose challenge of Wistar rat-originated islets. $^{**}P<0{\cdot}01$; NS, not statistically significant. Wistar islets 3·3 mM, $n=11$; Wistar islets 16·7 mM, $n=12$; GK islets 3·3 mM, $n=6$; GK islets 16·7 mM, $n=5$.

and -3 fragments has not been shown. Using western blot analysis, neither we (Foo *et al.* 2008) nor others (Oh-I *et al.* 2006, Stengel *et al.* 2009*b*) have been able to recognize nesfatin-1 from brain, pancreas or gastrointestinal mucosa extracts with antibodies raised against the nesfatin-1 sequence (and which react with synthetic nesfatin-1 peptide), although a protein corresponding to the size of the full-length NUCB2 is readily detectable. While the NUCB2 structure contains a signal peptide and putative cleavage sites (Oh-I *et al.* 2006), several motifs involved in intracellular signalling, including a Ca^{2+}-binding EF hand and a nuclear-binding basic helix-loop-helix sequence (for which the protein is named), have been recognized (Barnikol-Watanabe *et al.* 1994). Taken together, these observations suggest that the potential role of NUCB2 within the β-cell merits further study.

Immunoreactive NUCB2 in islet homogenates (as detected by EIA) did not change significantly with overnight food deprivation, suggesting that transcriptional regulation and/or degradation of NUCB2 is not sensitive to starvation, at least not within this time interval. In gastric endocrine cells (Stengel *et al.* 2009*a*,*b*) and in the hypothalamic paraventricular nucleus (Oh-I *et al.* 2006), the same treatment decreases *NUCB2* mRNA expression (while NUCB2 expression in several other hypothalamic nuclei is insensitive to starvation; Oh-I *et al.* 2006). We did, however, find a notable reduction in ir NUCB2 in islet homogenates obtained from the GK rats, which normalized with fasting (present results). The inbred non-obese GK rats recapitulate several of the salient features of β-cell pathology in T2DM, including impaired insulin secretion and glucose intolerance (Östenson & Efendic 2007, Portha *et al.* 2009). (Of note, in the Stockholm GK colony used in this study, no reduction in β-cell mass is detected at three months, which is the maximum age for animals included in the present study; Guenifi *et al.* 1995). Our findings in the GK rats suggest that impaired glucose homeostasis can affect islet NUCB2 expression, and that this measure, like several other parameters in T2DM, is rectified with food restriction. An alternative explanation, i.e. that the reduced NUCB2 contributes to, rather than results from, the pathogenesis in GK diabetes, cannot be excluded. It has been shown that GK rats display defective β-cell secretory machinery (Abdel-Halim *et al.* 1996) and reduced levels of key exocytotic proteins (Nagamatsu *et al.* 1999, Gaisano *et al.* 2002) that can account for the impaired insulin release (Östenson *et al.* 1993). These data are interesting given the wide distribution of NUCB2 in neurosecretory neurons

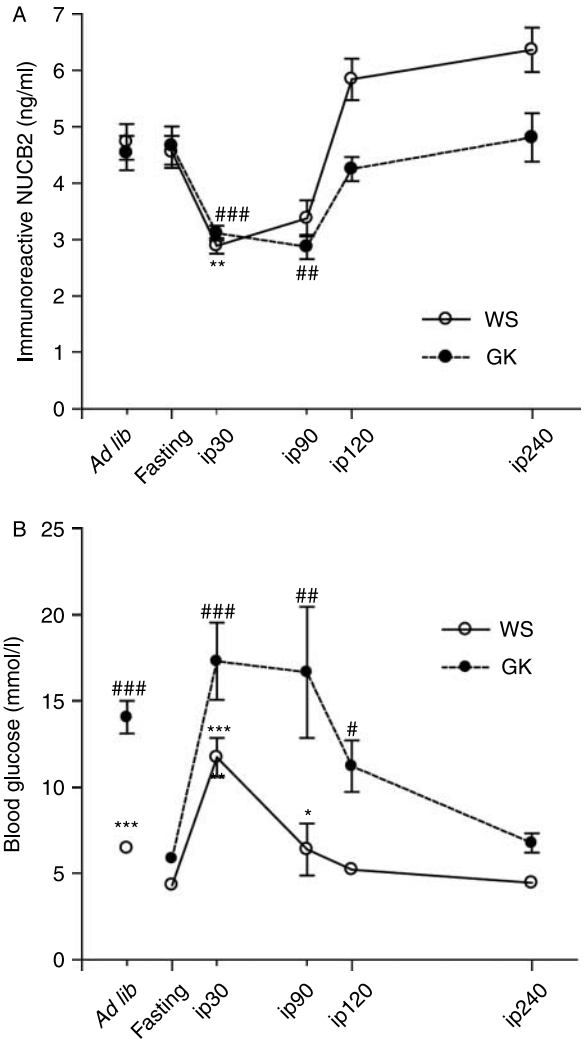

Figure 6 NUCB2 concentration in rat serum following i.p. glucose tolerance test (ipGTT). Graphs displaying plasma NUCB2-LI (A) and glucose (B) concentrations in Wistar (○; WS) and Goto-Kakizaki (●; GK) rats in *ad lib* feeding conditions, following an overnight fast and 30, 90, 120 and 240 min after i.p. glucose injection. Results are displayed as mean ± S.E.M. In Wistar rats as well as in GK rats, fasting did not change ir NUCB2 plasma levels. In both groups of animals, however, a transient decrease was observed in the ipGTT. Indication of significance is relative to '*ad lib*' in A and to 'fasting' in B; $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ for WS rats. $^{\#}P<0{\cdot}05$, $^{\#\#}P<0{\cdot}01$ and $^{\#\#\#}P<0{\cdot}001$ for GK rats.

and endocrine cells (Foo *et al*. 2008) and a potential role in hormone and neurotransmitter turnover. Of relevance to diabetes, it can be noted that in the SQ-5 cell line, *NUCB2* gene expression is induced by the antidiabetic compound, troglitazone (Oh-I *et al*. 2006). Finally, NUCB2 plasma levels, although unaffected by fasting, showed a somewhat surprising transient decrease in the ipGTT that correlated with plasma glucose in both Wistar and GK rats. Although the above described data suggest that the pancreatic islets may not be a dominant source of circulating NUCB2, these findings do indicate a dynamic relationship with glycaemic state. The nature of this relationship as well as the source(s) of plasma NUCB2 remains to be fully determined.

In summary, we demonstrated a β-cell-specific expression of NUCB2 in the rat and human pancreata. The present findings on cellular distribution and extracellular and serum levels, in combination with our previous observations in the nervous system (Foo *et al*. 2008), complicate the interpretation of NUCB2 as a cleaved and secreted protein. Currently available data primarily support an intracellular role. This conclusion and our finding of markedly reduced NUCB2 levels in the diabetic GK rat pancreas warrant further investigation of the role of this protein and its relationship with insulin and glycaemic control.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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