Endocrine (2019) 63:513–519
https://doi.org/10.1007/s12020-018-1766-2

ORIGINAL ARTICLE

Spexin in the physiology of pancreatic islets—mutual interactions with insulin

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Received: 20 June 2018 / Accepted: 15 September 2018 / Published online: 28 September 2018

Abstract

Spexin is an interesting peptide, which may play an important role in the regulation of the metabolic homeostasis of an organism. Current knowledge on spexin expression, secretion, and influence on tissues and endocrine glands is very limited. We investigated spexin localization in the endocrine pancreas and measured its in vitro secretion from isolated pancreatic islets at various glucose concentrations, simultaneously monitoring insulin release. Also, gene expression for spexin and insulin was estimated. We found the presence of spexin inside beta cells and an increase in its release from islets after a short term and decrease after a long term following glucose administration. Finally, negative feedback loops between spexin and insulin were found, indicating the presence of multilateral relationships between glucose, insulin, and spexin inside pancreatic islets.

Keywords Spexin · Pancreatic islets · Insulin · Pancreas

Introduction

Many aspects of the functions of the pancreatic islets still remain unknown and need clarification. This is especially important because of the role of pancreatic islets in chronic disorders of the present day, such as metabolic syndrome and diabetes mellitus types 1 and 2. The endocrine part of the pancreas producing hormones plays a crucial role in the control of metabolic homeostasis. So, the pancreas may be a target for some therapies, which can improve physiological functions of the organism. Recently, many novel proteins playing important roles in metabolism control have been discovered. Some of these may affect the pancreas and may even be expressed in this organ.

One such peptide is spexin. This was discovered in 2007 [1]. A mature peptide consists of 14 amino acids (aa) and is formed from a 116 aa long preprospexin. Spexin is a peptide, which undergoes secretion to the circulation and which can be measured in the blood [2]. Some authors suggest that spexin may be classified as a member of a common family together with galanin and kisspeptin [3]. This thesis may be supported by the fact that spexin seems to be a specific ligand of two galanin receptors: Gal2R and Gal3R [3, 4]. To date, no separate receptor for spexin has been identified. Spexin was discovered as a neuropeptide, but there is compelling evidence that it is present in peripheral tissues and may even show the ability to regulate endocrine gland functions [5, 6]. Additionally, the presence of spexin in a large number of tissues suggests that this peptide may be involved in the regulation of the metabolism and homeostasis. Among various spexin effects, the following have been described: stomach muscle contraction [1], reduction of food intake in fish [7], increased arterial pressure, decreased heart rate, and increased urinary sodium excretion [8].

The relatively small amount of knowledge on spexin led us to investigate its effect on endocrine pancreas activity. The aim of this study was to identify the potential role of spexin in the physiology of the endocrine pancreas. As a model, we chose porcine pancreas and porcine pancreatic islets, because they show the largest similarity to human islets [9].

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Materials and methods

Tissue section preparation and immunofluorescence analyses

A pancreas obtained from a pig was used partially for islet isolation and the rest of the organ was fixed in Bouin's reagent. The immunofluorescence procedure was performed using primary anti-spexin (cat. no. H-023-81, Phoenix Pharmaceuticals, USA) and anti-insulin antibodies (cat. no. A0564, Dako, USA) and secondary anti-guinea pig (cat. no. A11073, Life Technologies, USA) and anti-rabbit antibodies (cat. no. A10520, Life Technologies, USA). All antibodies were used at a dilution of 1:400. Control slices were incubated with anti-spexin antibody and 10 µg of spexin mixed 2 h before and used as a blocking peptide. The detailed procedure has been described previously [10].

Isolation of pig pancreatic islets

The basic animal materials were pancreatic islets obtained from 5- to 7-day-old piglets of the Zlotnicka White Pig subspecies. All experiments were conducted in accordance with the rules of the Local Ethical Commission for Investigations on Animals. The detailed isolation procedure has been described previously [11].

Secretion experiments

Islets obtained from the pig pancreas were used in secretory experiments in which spexin and insulin concentrations in incubation medium (Krebs–Ringer buffer, KRB) were measured. Experimental factors were: spexin (10, 100, and 1000 nM), insulin (1, 10, and 100 nM), and glucose (1, 6, and 16 nM). When glucose was not treated as an experimental factor, its concentration in KRB was 6 or 16 mM. The experiments lasted 1.5 or 24 h and were conducted in an incubator at 37 °C and in a 5% CO_2 atmosphere. Additionally, diazoxide at a concentration of 250 µM, a strong inhibitor of insulin secretion, was used. Each time, five islets per well were incubated in 0.5 ml of KRB, and the concentrations of secreted spexin and insulin were measured with RIA kit Phoenix Pharmaceuticals, USA, and ELISA kit DRG Diagnostic, Germany, respectively.

Gene expression experiments

To investigate spexin and insulin expression, pancreatic islets were incubated for 24 h (37 °C; 5% CO_2) in 1 ml of KRB enriched with spexin, insulin, and glucose at the concentrations described above. Then, islets were transferred to Eppendorf tubes and Tripure Reagent (Roche Diagnostic, Germany) was added. Subsequently, the isolation of RNA according to the attached manual was performed. RNA quantity and purity were measured using a Nano Drop 1000 (Thermo Scientific, USA). In the next step, 1 µg of RNA was rewritten to complementary DNA (cDNA) using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and following the manufacturer's instructions. cDNA obtained after 10-fold dilution was used for gene expression measurements by real-time PCR. Fast SYBR Green Master Mix reagents and QuantStudio 12K Flex thermomixer (Applied Biosystems, USA) were used for the analyses. Expression analyses were conducted in relation to the tata-binding protein—TBP reference gene. Primers were designed using the NCBI/Primer-BLAST tool as follows: for insulin—F: 5′–GTGGCAT CGTGGAGCATG–3′; R: 5′–CGGCCTAGTTGCAGTA GTTCTC–3′, for spexin—F: 5′–TGTGCTATTGGCT TCCTTGA–3′ and R: 5′–GGAACCTGGTTTGATCGAA GT–3′, and for the tata-binding protein—F: 5′–TTGAG AACATCTACCCTATCCT–3′ and R: 5′–CGTCCACAAC ACCACCATT–3′.

Statistical analysis

Statistical analyses was performed using the Student's *t*-test with alpha set to 0.05 and 0.01. Statistical significance was marked by lowercase $P < 0.05$ and capital letter $P < 0.01$, respectively.

Results

Spexin localization in pancreatic islets

Immunofluorescence staining of pig pancreatic islets showed the presence of spexin inside the endocrine structures of the pancreas (Fig. 1a). Simultaneously, insulin staining was performed to detect if the obtained signal came from insulin-positive cells (Fig. 1b). The imposition of images taken from spexin and insulin staining confirmed the presence of both these hormones in the same cells (Fig. 1c). Moreover co-localization of spexin and insulin was also observed in single or in pairs of endocrine cells, which were widely scattered across the whole organ. Simultaneously, the signal for spexin was absent when it was used as a blocking peptide (Fig. 1e).

Spexin secretion after glucose treatment

Three concentrations of glucose (1, 6, and 16 mM) and two periods of incubation were applied (90 min and 24 h). Additionally, diazoxide (250 µM), as a strong inhibitor of insulin secretion, was used. During the short-term experiment (90 min incubation), increasing concentrations of

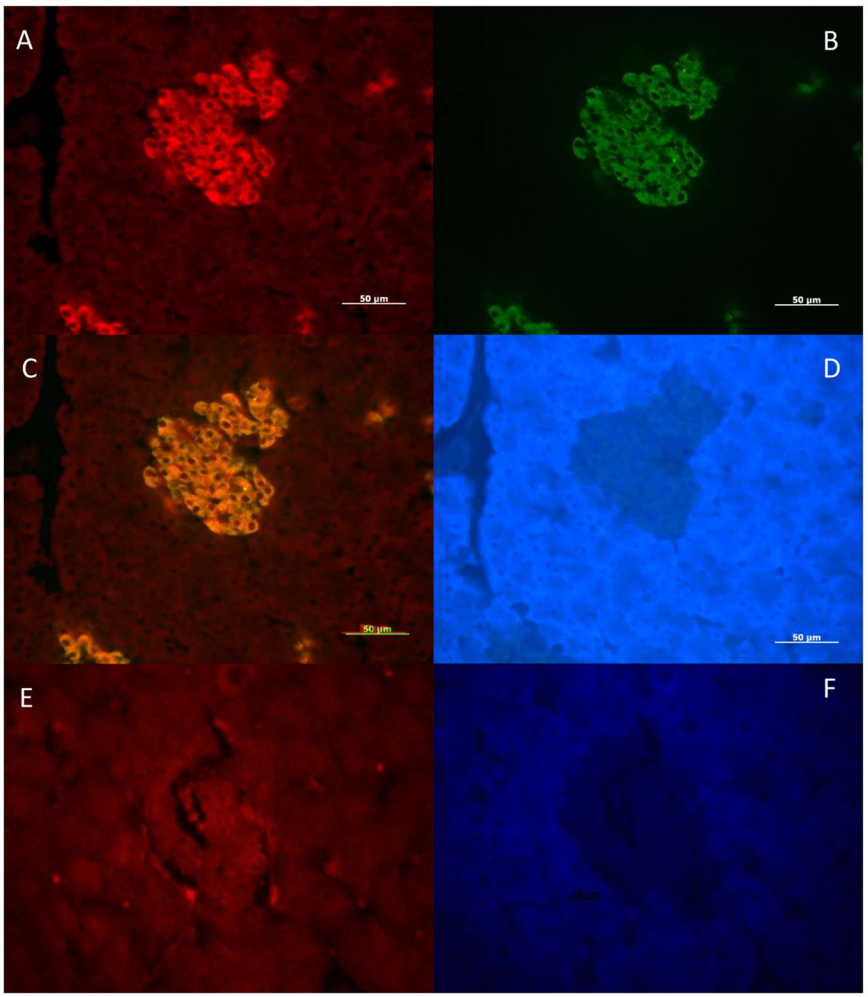

Fig. 1 **a** Detection of spexin (red) in the center of pig pancreatic islets. **b** Detection of insulin (green) in the center of pig pancreatic islets. **c** The imposition of images taken from spexin staining and insulin staining. **d** DAPI staining. **e** Control with anti-spexin antibody and spexin as a blocking peptide. **f** DAPI staining

glucose caused spexin secretion in addition to stimulation of insulin (Fig. 2). An over 1.4-fold enhancement of spexin concentration in KRB was detected for 6 mM glucose in comparison to a 1 mM concentration. A comparable difference was observed between 6 and 16 mM glucose, and a twofold increase in spexin secretion was noticed between 1 and 16 mM glucose concentrations. Similar observations were found in variants with an addition of diazoxide: a 1.3-fold increase in spexin secretion between 1 and 6 mM glucose concentrations, a 1.25-fold increase between 6 and 16 mM glucose levels, and a 1.6-fold increase between 1 and 16 mM glucose levels. Finally, spexin secretion was compared at the same glucose concentrations, but with or without the addition of diazoxide. Always in the presence of diazoxide, spexin was secreted in greater amounts and these changes were statistically significant. For 1, 6, and 16 mM glucose concentrations, simultaneous addition of diazoxide caused 1.6-, 1.45-, and 1.3-fold increases, respectively (Fig. 2a). After prolonged (24 h) incubation with glucose, an inverse regulation of spexin secretion was detected in the variant without diazoxide. In comparison to the 1 mM glucose concentration, a significant decrease in spexin secretion was observed for 6 mM (over 1.7-fold) and for the 16 mM concentration (over 2.25-fold). On the other hand, in variants with diazoxide, an increase in spexin secretion was found. For 6 mM glucose, an almost 1.7-fold increase in spexin in KRB was detected in comparison to the 1 mM glucose group. Also, a 1.25-fold increase in spexin secretion was found between 16 and 6 mM glucose concentrations, and an over twofold increase was observed between 16 and 1 mM glucose. Eventually, spexin secretion was again measured at the same glucose levels, but with or without diazoxide. An over 2.7-fold increase was observed for 6 mM glucose and an over 4.5-fold enhancement for 16 mM glucose (Fig. 2b). To complete the above described data, the concentrations of insulin in those samples were measured after 90 min and 24 h incubation with glucose. For both periods of time, an increase in insulin secretion at rising glucose concentrations and an unchanged insulin level after additional treatment with diazoxide were found. Moreover, insulin concentrations at physiological and supraphysiological

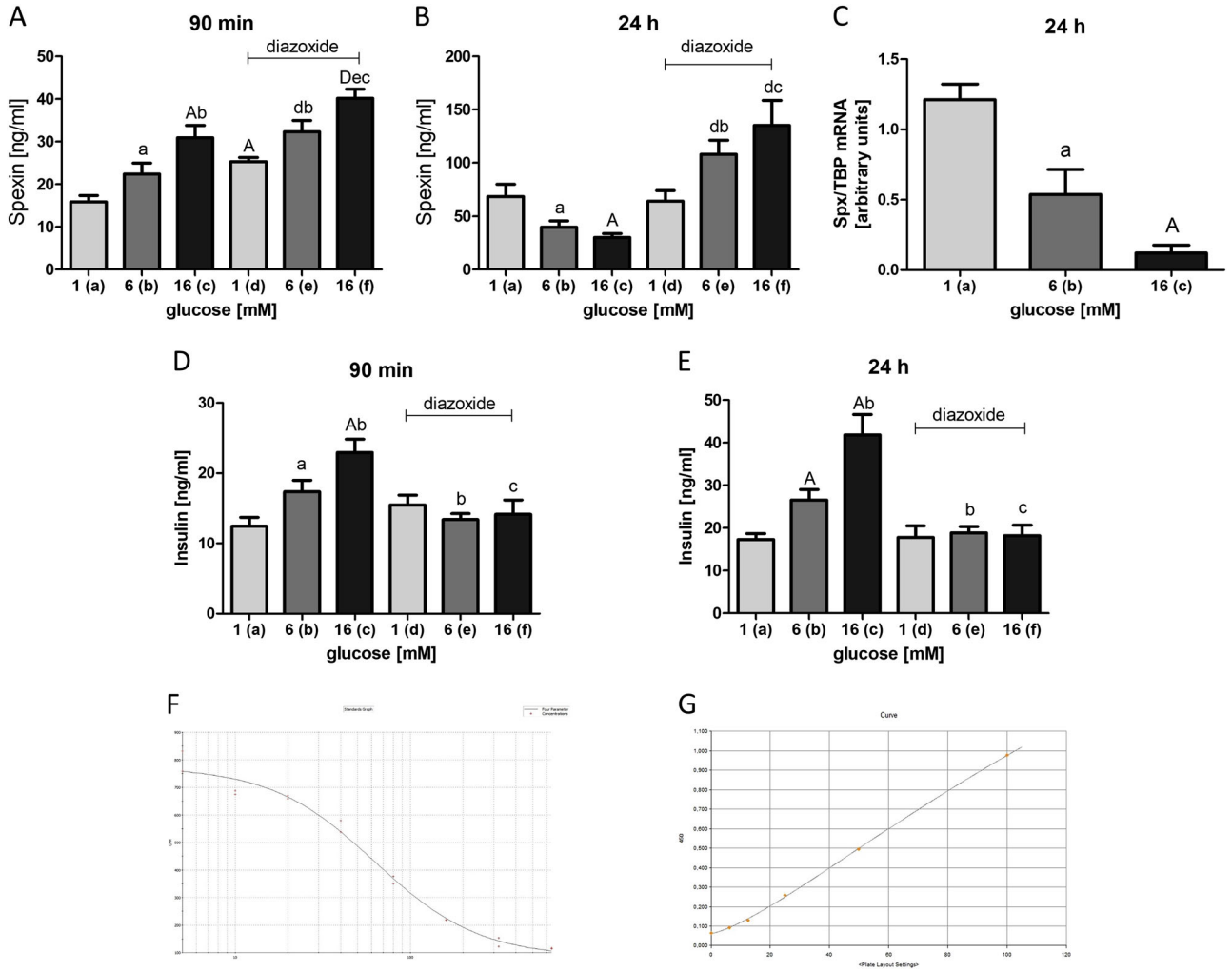

Fig. 2 **a**, **b**, **d**, **e** Spexin and insulin secretion from pig pancreatic islets exposed for 90 min and 24 h to various concentrations of glucose and diazoxide. Data are presented as means ± SEM (three experiments, $n = 6$ in each, total $n = 18$). **c** Real-time PCR analysis of a spexin gene after glucose treatment. Data are normalized against TBP and presented as means ± SEM (three experiments, $n = 3$ in each, total $n = 9$). Each column is marked by a letter. Statistical significance was denoted by lowercase $p < 0.05$ and capital letter $P < 0.01$. Each letter above the column indicates the column where the statistically significant difference is observed. **f**, **g** Representative standard curves for **f** spexin and **g** insulin assays

glucose levels (6 and 16 mM, respectively) were strongly reduced, to the level measured for 1 mM glucose. No differences were observed between 1 mM glucose and 1 mM glucose plus diazoxide. Simultaneously, statistically significant differences in comparison to 1 mM glucose were found after 90 min of incubation for 6 and 16 mM glucose (over 1.8- and over 1.3-fold increases, respectively). An increase in insulin secretion was also observed between 6 and 16 mM glucose concentrations (over 1.3-fold) (Fig. 2d). When incubation lasted much longer (24 h), similar statistically significant differences were observed—over 1.5-fold and 2.4-fold higher secretions of insulin for 6 and 16 mM glucose concentrations, respectively, in comparison to medium with 1 mM glucose. Moreover, an almost 1.6-fold increase was found in insulin secretion between 6 and 16 mM glucose concentrations (Fig. 2e).

Spexin secretion after insulin treatment

Experiments were performed for two periods of time (90 min and 24 h incubation), three concentrations of insulin (1, 10, and 100 nM), and 6 mM glucose concentration. At 6 mM glucose level and after 90 min of incubation with insulin, a significant decrease in spexin secretion was observed for all three concentrations of insulin. The mean concentration in the variant without any insulin addition was over 1.35-fold of its concentration at 1 nM insulin, and over 1.4-fold at 10 and 100 mM insulin (Fig. 3a). After prolongation of the incubation to 24 h, a statistically significant decrease in spexin secretion was observed at 10 and 100 nM insulin concentrations. Spexin concentration in the variant without any insulin addition was, respectively, 1.55- and 1.5-fold higher (Fig. 3b).

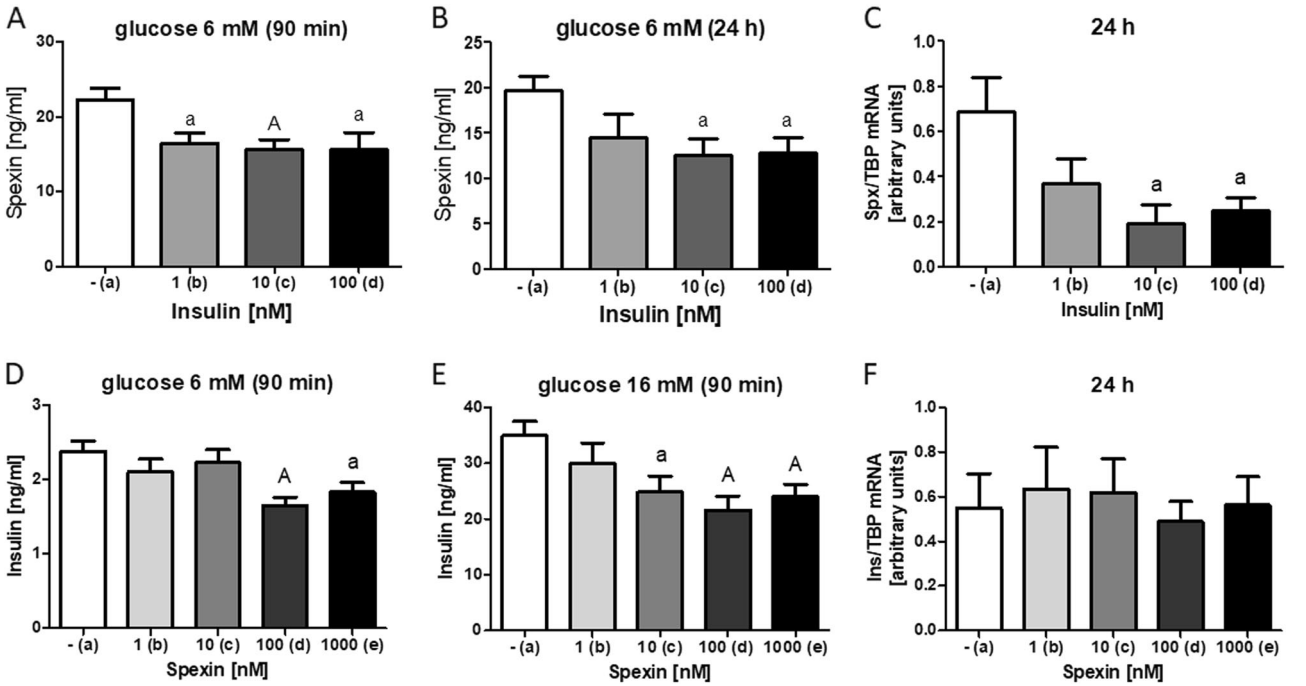

Fig. 3 **a**, **b** Spexin secretion from pig pancreatic islets exposed for 90 min and 24 h to various concentrations of insulin and glucose. Data are presented as means ± SEM (three experiments, $n = 6$ in each, total $n = 18$). **c** Real-time PCR analysis of a spexin gene after insulin treatment. Data are normalized against TBP and presented as means ± SEM (three experiments, $n = 3$ in each, total $n = 9$). **d**, **e** Insulin secretion from pig pancreatic islets exposed for 90 min and 24 h to various concentrations of spexin at 6 and 16 mM glucose. Data are presented as means ± SEM (three experiments, $n = 6$ in each, total $n = 18$). **f** Real-time PCR analysis of an insulin gene after spexin treatment. Data are normalized against TBP and presented as means ± SEM (three experiments, $n = 3$ in each, total $n = 9$). Each column is marked by a letter. Statistical significance was denoted by lowercase $p < 0.05$ and capital letter $P < 0.01$. Each letter above the column indicates the column where the statistically significant difference is observed

Insulin secretion after spexin treatment

Incubations of pancreatic islets with four concentrations of spexin (1, 10, 100, and 1000 nM) lasted 90 min and were conducted in the presence of 6 or 16 mM glucose. When incubations were performed using 6 mM glucose, a statistically significant decrease in insulin secretion for 100 and 1000 nM of spexin was observed. Insulin concentrations in medium without spexin were, respectively, 1.4- and 1.3-fold higher (Fig. 3d). A more visible reduction of insulin secretion was found when experiments were performed at 16 mM glucose level. A decrease in insulin secretion was observed for three concentrations of spexin (10, 100, and 1000 nM) and insulin concentrations in medium without spexin were 1.4-fold, over 1.6-fold and 1.45-fold higher, respectively (Fig. 3e).

Spexin and insulin gene expression in porcine islets

All experiments lasted 24 h. Firstly, expression of spexin gene after glucose treatment (1, 6, and 16 mM) was measured. We found a distinct decrease in spexin gene expression for 6 and 16 mM glucose levels in comparison to 1 mM glucose concentration, about a twofold and 10-fold drop, respectively (Fig. 2c). Secondly, according to the main schedule of experiments, spexin expression after insulin treatment (1, 10, and 100 nM) was studied and statistically significant about threefold, decreases were observed for 10 and 100 nM insulin (Fig. 3c). Thirdly, insulin gene expression after spexin treatment (1, 10, 100, and 1000 nM) was measured and no statistically significant changes were observed (Fig. 3f).

Discussion

Information about spexin is very limited and that is why this peptide seems to be very intriguing. Interestingly, the presence of spexin within pancreatic islets has been confirmed in humans but not in rats [12, 13]. This fact encourages us to investigate the possible localization of spexin and its influence on pig pancreas function. Our experiments are the first to show the presence of spexin in porcine pancreatic islets, the effect of glucose on spexin release and its mutual interactions with insulin.

Our main aim was to examine the localization of spexin in isolated pig pancreatic islets. The potential presence of this peptide within the islets opens new possibilities for the

impact of spexin on this tissue because, in addition to endocrine effects, autocrine and paracrine interactions are also possible. We confirmed co-localization of this peptide with insulin in beta cells. These findings enrich understanding of spexin and indicate a new species and its endocrine pancreas where this peptide is detected. So, spexin is present in pig pancreatic islets as in human islets but not in rat ones. Moreover, these results suggest that pig islets are a better model to investigate the potential functions of spexin in the pancreas than rat islets.

The second aim was to investigate the potential influence of glucose, insulin, and spexin on pancreatic islet secretory abilities. Firstly, we examined and documented the influence of glucose on spexin release from islets. Three divergent concentrations of glucose were chosen to check the whole spectrum of its possible effects. The lowest concentration (1 mM glucose) imitates a state of deep hypoglycemia when stimulation of insulin secretion is minimalized. The second concentration (6 mM glucose) mimics the typical level of glucose in the circulation of a healthy organism. The third concentration (16 mM glucose) imitates diabetes with the high glucose level strongly stimulating insulin secretion. Simultaneously, an analogical experiment was performed with the addition of 250 μM diazoxide, which is a potent inhibitor of insulin secretion. This compound affects potassium channels in beta-cell membranes and through their hyperpolarization prevents the secretion of insulin. The results show that elevating the concentration of glucose over a short period of time causes an increase in spexin release. These facts may suggest that spexin secretion is sensitive to glucose level, but it is also dependent on insulin simultaneously secreted in response to glucose. Therefore, this experiment was also conducted with diazoxide. This provides evidence that spexin release is positively regulated by glucose and negatively so by secreted insulin. Surprisingly, positive regulation of spexin secretion by glucose was inverted when incubation time was prolonged to 24 h. We presumed that this effect may be due to downregulation of the potential receptors of spexin. Lower secretion after long incubation may also indicate that glucose regulates the expression and production of spexin in pancreatic islet cells. Interestingly, the treatment with diazoxide once again eliminated the combined effect of glucose and insulin and showed a strong increase in spexin secretion. This result again confirms that insulin shows an inhibitory effect on spexin release.

Additionally, we decided to investigate directly the effect of insulin on spexin secretion and islets were incubated with increasing concentrations of insulin. Our results confirm our previous assumption about the role of insulin in spexin release. Interestingly, a reduction of spexin secretion was observed only at the 6 mM glucose level. This also confirms that glucose seems to be a stronger upregulator of spexin release than insulin is a downregulator of it.

To complete the interactions between spexin and insulin, we performed a third set of experiments where insulin secretion was measured after spexin treatment. This shows that bilateral interactions between spexin and insulin are based on negative feedback. Insulin inhibits spexin secretion and simultaneously spexin inhibits secretion of insulin. At higher glucose levels, the reduction of insulin secretion is more easily visible because of the generally higher insulin secretion in these conditions. It is understandable that a potential decrease in insulin secretion can be better noticed when insulin is stimulated to release.

Finally, we investigated the expression of messenger RNA (mRNA) for spexin and insulin in pancreatic islets to reveal potential changes at a molecular level. The experiments lasted 24 h, which is sufficient time to demonstrate differences in expression. Once more, these results fit with data obtained from secretion experiments where spexin release was also reduced. So, we can conclude that in both cases, reduction of spexin secretion is also connected with attenuation of its production. Finally, no changes in insulin mRNA expression in pancreatic islets after spexin treatment was observed. This suggests that spexin affects the secretion rather than expression of insulin.

Literature about the regulation of spexin expression and secretion is very limited. In particular, there are no data about the role of spexin in the pancreas, but some literature report on the connection between spexin and glucose homeostasis in fish [14], mice [15], and humans [12]. To date, there are no similar data for other organisms. Ma et al. [14] investigated the effects of feeding and glucose and insulin administration on spexin production. They found that food intake stimulates spexin as well as glucose and insulin level in circulation. Glucose also increases mRNA level for spexin in the liver. Insulin exerts a similar effect on spexin concentration in blood and mRNA level in the liver and brain [14]. These data are partially consistent with our observations, except for the effect of insulin on spexin production. However, it must be mentioned that the role of insulin in fish is much more complicated than in mammals; for example, its expression has been found in the liver, heart, gut, spleen, brain, muscle, and gonads. This suggest that interactions between spexin and insulin in fish can be completely different than in mammals.

However, spexin seems to be a regulator of glucose homeostasis in mammals as well as in fish. Ge et al. [15] performed oral glucose tolerance tests (OGTTs) and observed a diminution effect of spexin on glucose level in the circulation in mice C57BL/6J, which were maintained on a high-fat diet. Also, OGTT were performed on humans and provided more information about spexin regulation after glucose administration [12, 16]. Gu et al. [12] and

Hodges et al. [16] also measured spexin concentrations in blood during OGTTs. Gu et al. [12] observed a negative correlation between glucose as well as glycated hemoglobin (HbA1c) and spexin level in the circulation after a glucose bolus and significantly lower spexin concentrations in type 2 diabetes mellitus than in healthy volunteers. Hodges et al. [16] found that there were no changes in blood spexin level in response to glucose administration during OGTT in normal weight adolescents. Our data clearly show upregulation of spexin release from pancreatic islets by glucose for 90 min and downregulation for 24 h of incubation. Simultaneously, after use of diazoxide, which mimics diabetic state, the spexin level was univocally positively correlated with glucose concentration for 90 min as well as for 24 h incubation. The explanation of these observed discrepancies may be that our research was performed in a different way, in vitro, and on porcine material in contrast to in vivo OGTT in humans.

Funding This study was supported by the National Science Centre, Poland 2017/01/X/NZ4/00466 MINIATURA grant and 2015/19/N/NZ4/00572 PRELUDIUM grant.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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