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Elevated glucagon-like peptide-1 plasma levels, as a possible adaptive response, in diabetic NOD mice

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ABSTRACT

The incretin glucagon-like peptide-1 (GLP-1) and other GLP-1 receptor agonists have been shown to cause both antiapoptotic as well as regenerative effects on beta-cells in different animal models for diabetes. Our aim of this study was to test the hypothesis that spontaneously diabetic non obese diabetic (NOD) mice show an altered expression of GLP-1 compared to normoglycemic age-matched controls as a consequence of a diabetic state. To do this we used an ELISA prototype for mouse GLP-1 to measure plasma total GLP-1 from recently diabetic NOD mice as well as from age-matched normoglycemic NOD mice (controls). We also stained sections of pancreatic glands for GLP-1 from diabetic NOD mice and controls. We found increased levels of plasma total GLP-1 in diabetic NOD mice, when compared to control mice, both from non-fasted mice and from mice fasted for 2 h. Furthermore, diabetic NOD mice displayed a higher GLP-1 response to an oral glucose tolerance test, compared to control mice. We also found that sections of pancreatic glands from diabetic NOD mice had an increased GLP-1 positive islet area in regard to relative islet area (i.e. total islet area / total pancreas area of the sections) compared to control mice. To our knowledge, this study is the first to show increased levels of GLP-1 in plasma in spontaneously diabetic NOD mice. We suggest that these results might represent a compensatory mechanism of the diabetic NOD mice to counteract beta-cell loss and hyperglycemia.

1. Introduction

Type 1 diabetes, classified as a chronic autoimmune disease, and type 2 diabetes, classified as a metabolic disorder, both share the inability to meet the demand of produced insulin required to keep normal blood glucose levels. In type 2 diabetes this could be both due to a too low insulin production as well as peripheral resistance to insulin. In type 1 diabetes, however, this is due to the autoimmune destruction of insulin producing beta-cells that follows the infiltration of T-cells and macrophages in the islets of Langerhans in the pancreas [1,2].

Glucagon-like peptide-1 (GLP-1) is one of several different peptides that originate from the processing of the pre-proglucagon gene. In the pancreas, prohormone convertase 2 (PC2) expressed in the alpha-cells in pancreatic islets cleaves proglucagon to the main products glucagon, glicentin-related pancreatic polypeptide and major proglucagon fragment. In the gut, main products are glicentin, GLP-1 and GLP-2 derived from PC1 cleavage of pro-glucagon in intestinal L-cells [3–9]. GLP-1 can be further processed to form GLP-1 (7–37) and GLP-1 (7–36) amide, both of which are strong agonists of the GLP-1 receptor (GLP-1R). These are both rapidly degraded ($t_{1/2}$ = 1–2 min.) by the enzyme dipeptidyl peptidase-4 (DPP-IV) to form GLP-1 (9–37) and (9–36) amide, weak agonists of the GLP-1 receptor (GLP-1R), which make up the majority of the total circulating forms of GLP-1 [10–13]. The complex expression pattern of the pre-proglucagon gene and further processing of GLP-1 gives intriguing possibilities for modulating tissue-specific regulation.

Circulating levels of GLP-1 are low in the fasted state and rise within minutes following food ingestion, lowering gastrointestinal motility, gastric emptying, hepatic glucose output and increasing insulin secretion trough GLP-1R (the incretin effect) [9].

Several studies on GLP-1, and other agonists of the GLP-1R, in the development and/or treatment of diabetes have been performed in many different animal models as reviewed by Doyle and Egan [14]. One such study investigating the expression of GLP-1 in the spontaneous type 1 diabetic NOD-model, compared GLP-1 mRNA and "immunoreactive GLP-1" levels in homogenates of different gut segments from diabetic and non-diabetic mice, but found no differences between normoglycemic and diabetic mice [15]. Furthermore, it was recently shown that prediabetic NOD mice express prohormone convertase 1/3 which results in GLP-1 production in alpha-cells in the islets of Langerhans [7].

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In the present study we tested the hypothesis that spontaneously diabetic NOD mice would have an altered GLP-1 production compared to age-matched non-diabetic controls as a consequence of the diabetic state influencing the GLP-1 expression. This was performed by measurements of total GLP-1 plasma levels, and by GLP-1 staining of pancreas from both recently diabetic NOD mice and age-matched normoglycemic NOD mice.

2. Materials and methods

2.1. Animals

The NOD mice used were originally obtained from the Clea Company (Aobadi, Japan), and subsequently inbred under pathogen-free conditions at the Animal Department, Biomedical center, Uppsala, Sweden. The incidence of spontaneous diabetes is 60–80% in females, and much lower in males. The onset of diabetes is often at 12–14 weeks of age, but can occur at 25 weeks or even later [16,17]. We used female NOD mice, aged 13–50 weeks, which had been screened weekly for diabetes by measuring urine (Clinistix) glucose levels. With the use of a glucose meter (FreeStyle Mini; Abbot Scandinavia AB, Solna, Sweden) diabetes was then confirmed after obtaining non-fasted blood glucose measurements above 11.1 mM on two consecutive days. NOD mice with a non-fasted blood glucose level between 4–8 mM were considered normoglycemic and used as age-matched controls. Animal care, use and experimental protocols were approved by the local animal ethics committee in Uppsala and performed in accordance with EU Directive 2010/63/EU.

2.2. Sample collection

Blood samples were collected from the tail vein of diabetic female NOD mice and age matched normoglycemic female NOD mice for analysis of GLP-1, insulin or glucose. For GLP-1 the blood samples were collected in EDTA tubes and liquid DPP-IV inhibitor was added immediately after blood collection [18]. Plasma was then prepared and stored at −20 °C. For insulin measurements serum samples was prepared and stored at −20 °C.

2.3. Gastric glucose tolerance test

Diabetic and normoglycemic female NOD mice were fasted for 2 h after which a blood sample was taken from the tail vein. D-glucose (75 mg/mouse) dissolved in 250 μl saline was then administered through a gavage tube. Blood glucose was monitored at 0, 10, 30, 60 and 120 min. Blood samples were collected after 0, 10, 30, 60 and 120 min and prepared for analysis of GLP-1 or insulin.

2.4. Measurements of insulin

Serum insulin samples in duplicates were analyzed using a rat insulin ELISA according to the manufacturers instructions (Mercodia, Uppsala, Sweden).

2.5. Measurement of GLP-1

Plasma samples in duplicates were analyzed for total GLP-1 in a non-commercial ELISA prototype for mouse GLP-1 (Mercodia AB, Sweden). The GLP-1 total ELISA is specific for GLP-1 (1–36) amide, (7–36) amide and (9–36) amide but do not detect non-amidated forms of GLP-1 [18]. There is no cross reaction (<0.0005%) to GRPP (glicentin-related pancreatic polypeptide), GLP-2, oxyntomodulin, glucagon or mini-glucagon in the mouse GLP-1 ELISA (each peptide was tested at a concentration range of 16–200,000 pM).

2.6. GLP-1 staining of pancreas

NOD mice were sacrificed by cervical dislocation, and their pancreatic glands were removed, fixed in 10% formalin for 24 h and embedded in paraffin. Five μm thin sections were cut and deparaffination and antigen retrieval were performed (Diva Decloaker, #DV2004MX and Hot Rinse Buffer #HTR1001 M, Biocare Medical, Concord, CA, USA). After a 30 min incubation with protein block (#X0909, Dako AB, Stockholm, Sweden) tissue sections were incubated for 2 h with anti-GLP-1 antibody (rabbit polyclonal to epitope GLP-1 (1–19) (enabling immunoreactivity with N terminal truncated and C terminally extended forms of GLP 1), 1:1000, #ab22625, Abcam, Cambridge, UK). Immunoreactivity was amplified using the Mach 3 Rabbit AP polymer detection kit (#M3R533H, Biocare Medical) and visualized with the Vulcan Fast Red chromogen kit (#FR805S, Biocare medical). Finally, sections were counterstained with Mayer's haematoxylin (Histolab, Gothenburg, Sweden).

2.7. Determination of GLP-1 positive islet area, total islet area and total pancreas area

Sections were photographed with a Leica DF420c, with a resolution of 3888 × 2916 pixels. The GLP-1 positive islet area, the total islet area and the total pancreas area of the sections was then determined with a computerized system for morphometry (Image J, NIH, Bethesda, MD). Briefly, for the total islet area and the total pancreas area, the areas were circumscribed and measured by Image J. Next, the sections were analyzed for GLP-1 (red color) positive islet areas inside the circumscribed islet areas, using Image J. In all these measurements the examiner was unaware of the origin of the specimens.

2.8. Statistics

The results are expressed as means ± S.E.M or as medians with 25th and 75th percentile as vertical boxes. Statistical analysis was performed with Student's t-test or Wilcoxon's rank sum test and a probability (P) for a chance difference < 0.05 was regarded as significant. Statistical analysis was performed using SigmaStat (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Basal GLP-1, glucose and insulin in NOD mice

Blood samples were collected from non-fasted diabetic NOD mice (diabetic) and from non-fasted age-matched normoglycemic NOD mice (control). The body weight of these mice varied between 22 and 29 g. Mean blood glucose concentrations in diabetic mice were 26.3 ± 0.7 mM ($n = 24$) compared to 7.2 ± 0.3 mM ($n = 18$) in control mice ($P < 0.001$). Furthermore, the mean serum insulin levels were lower in diabetic mice (0.49 ± 0.1 μg/l, $n = 18$) compared to corresponding controls (0.90 ± 0.1 μg/l, $n = 19$) ($P < 0.05$). Plasma GLP-1 measurements from non-fasted mice revealed considerably higher levels in diabetic mice compared to the corresponding control animals (Fig. 1). This was true both if we subdivided the mice into different age groups (13–25 weeks and 26–50 weeks) and when we pooled animals of different ages (Fig. 1).

3.2. GLP-1, glucose and insulin responses to gastric glucose tolerance test in NOD mice

After a fasting period of 2 h, administration of 75 mg glucose by a gavage tube led to an increase in blood glucose after 10 min in

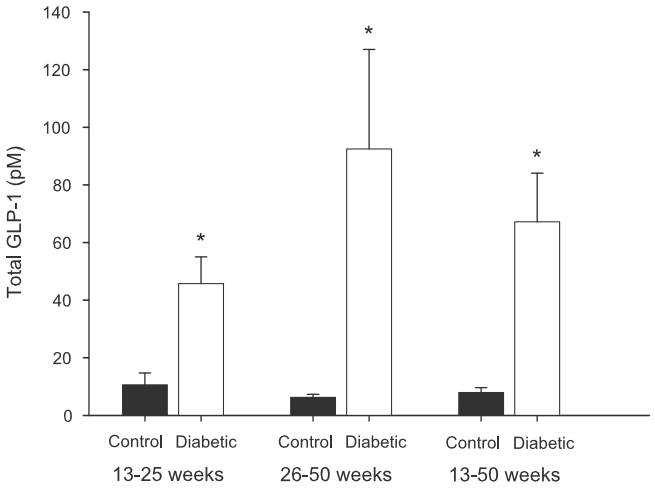

Fig. 1. Plasma measurements of total GLP-1 from non-fasted diabetic (white bars), and from non-fasted age-matched normoglycemic (control (black bars)), NOD mice. Results are shown both for the animals subdivided into different age groups (13–25 weeks, $n = 7–13$ and 26–50 weeks, $n = 11$) and for all the animals pooled into one group (13–50 weeks, $n = 18–24$). Values are means ± S.E.M. and * denotes $P < 0.05$ vs control, using Student's t-test.

both diabetic and control mice (Fig. 2A). After 120 min the mean blood glucose levels returned below 11.1 mM in control mice (9.3 ± 1.9 mM), but still remained hyperglycemic in diabetic mice (23.5 ± 2.1 mM) (Fig. 2A). At all time points measured, diabetic mice had significantly higher mean blood glucose levels compared to control mice ($P < 0.001$).

In diabetic mice a lower serum insulin response to the gastric glucose challenge was observed after 10 min compared to control mice. At the other time points no significant differences were found between diabetic and control mice (Fig. 2B).

We found a significant difference in plasma GLP-1 between diabetic and control mice after a 2 h fasting period (Fig. 2C). The diabetic animals also showed an increased GLP-1 response to the gastric glucose challenge in the form of an increased area under the curve (AUC) (1260 ± 210, $n = 6$), compared to control mice (630 ± 72, $n = 5$) ($P < 0.05$) (Fig. 2C).

3.3. GLP-1 staining of pancreas

Morphological examination of sections of pancreatic glands revealed that GLP-1 positive islet area in regard to relative islet area (i.e. total islet area / total pancreas area of the sections) was increased in diabetic mice compared to control mice (Fig. 3). Fig. 4 shows examples of sections of pancreatic glands stained for GLP-1 from age-matched normoglycemic NOD mouse (A) and recently diabetic NOD mouse (B). In Fig. 4A, a noticeable periinsulitis surrounds several islets that are stained for GLP-1 to various degrees, while (B) shows no signs of insulitis but a pronounced GLP-1 staining. There was no significant difference in relative islet area between the groups (control: 0.33 ± 0.15% vs diabetic: 0.19 ± 0.06%, $p = 0.349$).

4. Discussion

To our knowledge, this study is the first to show increased levels of GLP-1 in plasma in spontaneously diabetic NOD mice compared to age-matched normoglycemic NOD mice.

This was true for both non-fasted mice and for mice subjected to a 2 h long fast and appears to be independent of age. We also found that fasted diabetic mice had a higher GLP-1 secretion after a glucose challenge compared to normoglycemic mice.

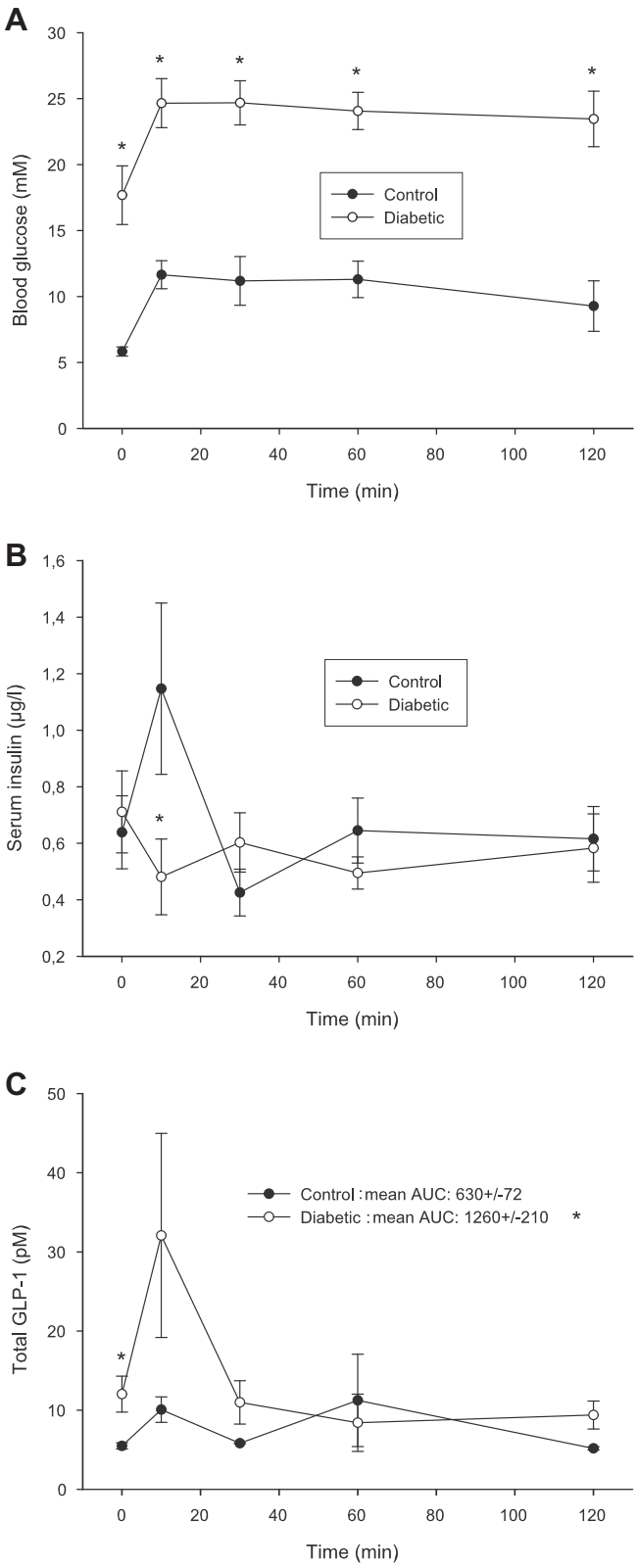

Fig. 2. Blood glucose (A), serum insulin (B) and plasma total GLP-1 (C) measurements performed just before (0 min), and at different time points after (10, 30, 60 and 120 min), an oral glucose tolerance test (75 mg glucose/mouse administered by a gavage tube). White circles denote diabetic NOD mice and black circles denote age-matched normoglycemic (control) NOD mice. The mice were fasted for 2 h before the experiments. In (C) we also show the calculated area under the curve (AUC) for the different groups. Values are means ± S.E.M, * denotes $P < 0.05$ vs control, using Student's t-test and (A) shows the results from 5–14 experiments, (B) the results from 2–9 experiments and (C) the results from 2–6 experiments.

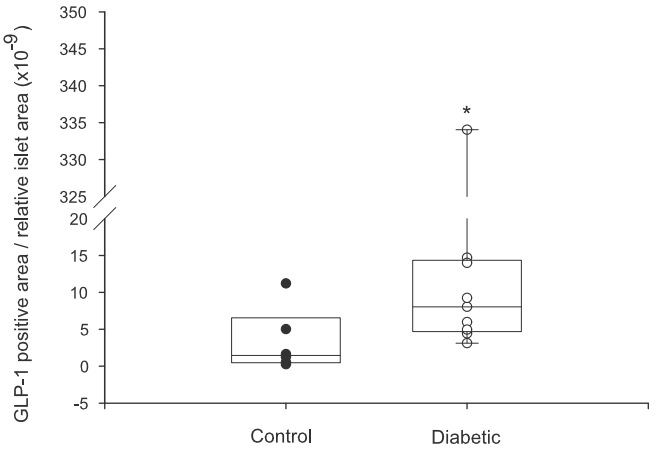

Fig. 3. Morphological examinations of sections of pancreatic glands stained for GLP-1. Results are presented as GLP-1 positive area per relative islet area (relative islet area is defined as total islet area per total pancreas area). Data are presented as a combination of vertical box plot and vertical point plot in which white circles denotes pancreatic glands from diabetic NOD mice ($n = 9$) and black circles denotes pancreatic glands from age-matched normoglycemic (control) NOD mice ($n = 6$). Values are medians with 25th and 75th percentile as vertical boxes, error bars highlights the outliers and * denotes $P < 0.05$ vs control, using Wilcoxon's rank sum test.

We expected no significant difference in relative islet area between the groups, since it is well known that also normoglycemic NOD mice display signs of insulitis already at 4 weeks of age [19]. Thus, even though the control mice in our study are normoglycemic, it is likely that they have ongoing beta-cell destruction and therefore an already decreased relative islet mass.

Furthermore, we observed an increase in GLP-1 staining in sections of pancreatic glands from diabetic mice compared to corresponding normoglycemic control mice. One possible explanation may be that NOD mice after diabetes onset show a diminished beta-cell mass, which might be consistent to a relative increase of alpha-cells in these islets, combined with a PC1 driven shift in pro-glucagon processing governing GLP-1 production. Since the intestinal L-cells are the primary source of GLP-1, an increased secretion from these cells would be the most likely explanation for the increased plasma total GLP-1 in diabetic NOD mice that we found in our study. However, the increased GLP-1 staining in pancreatic islets does not exclude that an increased expression of GLP-1 in the islets of Langerhans might also contribute to the increased plasma total GLP-1 observed herein. It could be noted that in the study from Berghöfer et al. [15], no difference in GLP-1 mRNA and "immunoreactive GLP-1" levels in homogenates of different gut segments from diabetic and normoglycemic NOD mice, was found.

It has previously been shown that administration of GLP-1 and/or exendin-4, a more potent and degradation-resistant GLP-1R agonist (derived from the saliva of Gila monster *Heloderma suspectum*), to different animal models for diabetes can result in antiapoptotic and regenerative effects on beta-cells [20–23]. Also, Zhang et al. [24] showed that a continuous subcutaneous infusion of GLP-1 (for 4 or 8 weeks) to 8 weeks old female NOD mice, via an osmotic pump, could induce beta-cell proliferation and neogenesis, suppress beta-cell apoptosis and delay the onset of type 1 diabetes. Furthermore, in the recent study by Whalley et al. [8] they show that isolated rat pancreatic islets subjected to beta-cell toxicity by treatment with streptozotocin display a significantly increased GLP-1 expression after 48 h. This process was shown to be upregulated by elevated glucose, activation of G protein coupled receptors and β-cell destruction. In light of these studies, we speculate that the results from our study represent GLP-1 upregulation driven by the diabetic state, including elevated glucose and an ongoing autoimmune destruction of β-cells. Despite of what has been seen in different studies where GLP-1 upregulation and/or GLP-1R stimulation have shown promising results in reducing diabetes development [14,24], the increase in GLP-1 observed in this study may represent an attempt, albeit not sufficient, of the diabetic NOD mice to try to compensate for its beta-cell loss. This endogenous attempt to compensate for beta-cell loss might be initiated too late, or at a too low concentration, to be effective.

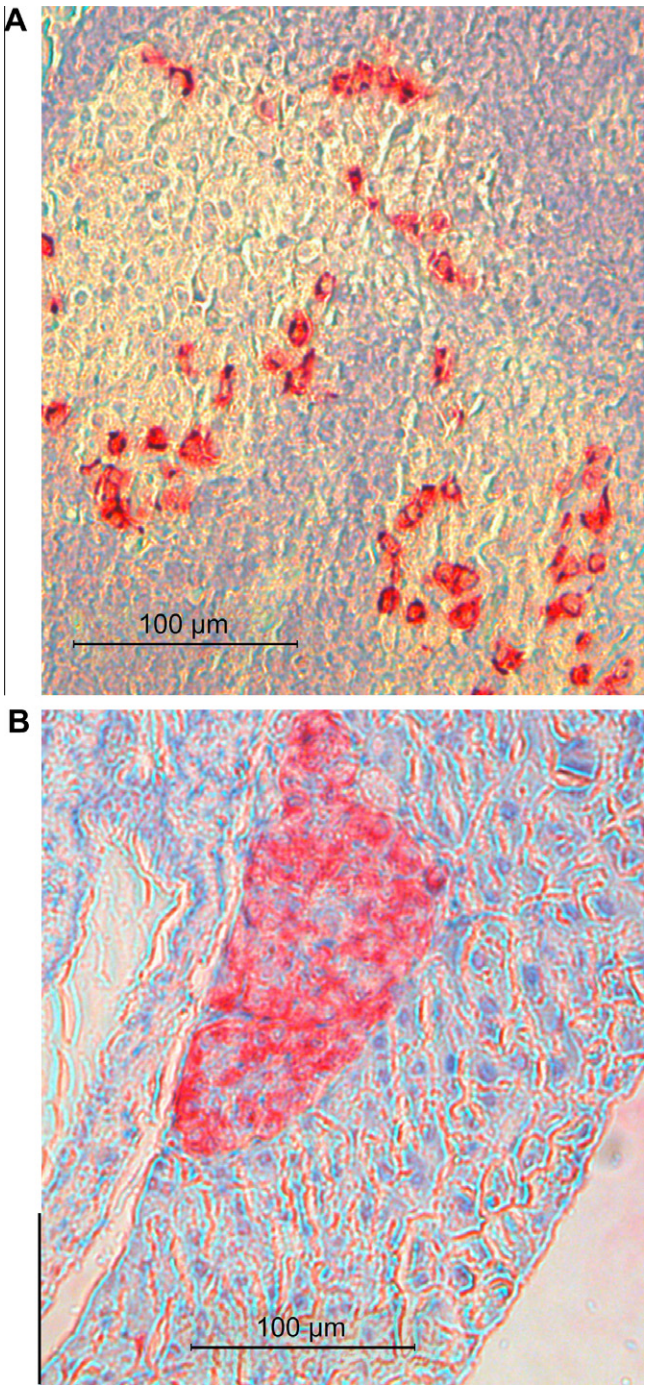

Fig. 4. Examples of sections of pancreatic glands stained for GLP-1 from age-matched normoglycemic NOD mouse (A) and recently diabetic NOD mouse (B) (not necessarily representative for the experiments).

Disclosure statement

TR, AB and SS have nothing to declare. AC is employed by Mercodia and has developed the GLP-1 ELISA used in the study.

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