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PROGRESSIVE CHANGE OF INTRA-ISLET GLP-1 PRODUCTION DURING DIABETES DEVELOPMENT

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Abstract

Background—Glucagon-like Peptide 1 (GLP-1) and glucagon shares the same precursor molecule proglucagon, but each arises from a distinct posttranslational process in a tissue-specific manner. Recently it has been shown that GLP-1 is co-expressed with glucagon in pancreatic islet cells. This study was aimed to investigate the progressive changes of GLP-1 vs glucagon production in pancreatic islets during the course of diabetes development.

Methods—Both type 1 (NOD mice) and type 2 (db/db mice) diabetes models were employed in this study. The mice were monitored closely for their diabetes progression, and were sacrificed at different stages according to their blood glucose levels. GLP-1 and glucagon expression in the pancreatic islets was examined using immunohistochemistry assays. Quantitative analysis was performed to evaluate the significance of the changes.

Results—The ratio of GLP-1-expressing cells to glucagon expressing cells in the islets showed significant, progressive increase with the development of diabetes in db/db mice. The increase of GLP-1 expression was in accordance with the upregulation of PC1/3 expression in these cells. Interestingly, intra-islet GLP-1 expression was not significantly changed during the development of type 1 diabetes in NOD mice.

Conclusions—The study demonstrated GLP-1 was progressively up-regulated in pancreatic islets during type 2 diabetes development. In addition, the data suggest clear differences in intra-islet GLP-1 production between type 1 and type 2 diabetes developments. These differences may have an impact on the clinical and pathophysiological processes of these diseases and may be a target for therapeutic approaches.

Keywords

GLP-1; alpha cells; islets; diabetes

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INTRODUCTION

Glucagon-like Peptide 1 (GLP-1) was originally discovered as a gut hormone that is secreted by the endocrine L-cells in the small intestine and colon following exposure to ingested nutrients [1–3]. In the gastrointestinal system, GLP-1 inhibits gastrointestinal motility, reduces gastric acid secretion, and slows gastric emptying [4–7]. In addition, it has been well established that, in the pancreas, GLP-1 stimulates insulin secretion from insulin-producing β -cells in a blood-glucose dependent manner, and inhibits glucagon secretion from the glucagon-producing α -cells, thus plays an essential role in blood glucose regulation [2, 3, 8].

GLP-1 and glucagon share the same precursor molecule—proglucagon. The diversification takes place at the post-translational stage in a tissue-specific manner. In pancreatic α -cells, proglucagon is cleaved by prohormone convertase 2 (PC2), giving rise to glucagon, glicentin-related polypeptide (GRPP), and the major proglucagon fragment (MPGF) [9–11]. In contrast, in enteroendocrine L-cells, proglucagon is processed by prohormone convertase 1/3 (PC1/3), resulting in the production of GLP-1, GLP-2 and several other small peptides [12, 13]. Interestingly, early studies suggested the production of GLP-1 within the pancreas in addition to that produced by intestinal L-cells, first by reporting the presence of GLP-1 in pancreas [14, 15], followed by immunohistochemical localization of pancreatic GLP-1 production within α -cells [16]. More recently, with the advancement of fluorescence microscopy, other researchers have confirmed these original studies, reporting that GLP-1 can, indeed, be produced in the glucagon-producing α -cells in pancreas [17, 18], which is in agreement with our own observations.

Recent studies have also demonstrated factors such as interleukin 6 (IL-6) and stromal cell-derived factor (SDF-1) can promote GLP-1 production in islet α -cells, probably through upregulation of PC1/3 [17, 19, 20]. It has been reported that signals in the external environment, including high glucose concentration, general β -cell destruction, and high circulating levels of the cytokine IL-6, can up-regulate α -cell expression of PC1/3 with either no change or concurrent downregulation in the expression of PC2. The upregulation of PC1/3 in these α -cells in turn may be responsible for the increased expression of GLP-1, possibly as a defense mechanism against hyperglycemia and further β -cell stress [19, 21, 22].

Intra-islet production of GLP-1 may provide means by which to further explain the regulatory roles of GLP-1 on insulin and glucagon secretion in pancreas. It is known GLP-1 is highly susceptible to degradation by Dipeptidyl-peptidase IV (DPP-IV), which is enriched in the endothelium of capillaries adjacent to the L-cells [2, 23]. Therefore, the majority of gut-produced GLP-1 is inactivated before it reaches pancreas. Thus, local production of GLP-1 within α -cells of the endocrine pancreas may contribute more efficiently to the insulinotropic and glucagonostatic effects of this incretin within the pancreas.

Given the essential regulatory role GLP-1 has on insulin and glucagon secretion, and because GLP-1 and glucagon share the same prohormone but have opposite effect on blood glucose homeostasis, we hypothesized that intra-islet production of GLP-1 (relative to glucagon production) could change during the course of diabetes development to reflect

their physiological response to high blood glucose. To test the hypothesis, we employed two widely employed diabetic mouse models and examined the progressive change of GLP-1 production in islets at different stages of diabetes development. In addition, we investigated whether the expression of GLP-1 processing enzyme, PC1/3, changed during the progression of diabetes.

MATERIALS AND METHODS

Animals

Non-obese diabetes (NOD) mice (female, 8-10 weeks old), db/db mice (male, 3-4 weeks old), and C57BLKS/J mice (male, 3-4 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Of note, either male or female mice were used, and sex of the mice was not considered a factor affecting the study. All of the mice were maintained in sterile, autoclaved cages, 5 per cage, on a standard chow diet. Body weight and random blood glucose measurements were recorded for each mouse on a weekly basis unless specified. For db/db mice (n=20), their weight and random blood glucose were monitored twice weekly. The mice were sacrificed by random selection on weekly basis (n=2 for 6-week group, and n=4 for other groups listed). Their pancreases were processed for paraffin embedding and subsequent immunohistochemistry assays. C57BLKS/J mice (n=10) were used as normal control for db/db mice because they have the same genetic background. NOD mice (n=10) were monitored weekly until they developed diabetes, which was marked by two consecutive random blood glucose measurements of ≥ 250 mg/dL. The mice were then monitored twice a week until sacrificing. The physiological parameters of NOD mice at 12-weeks of age were used as baseline (n=2). The mice that developed diabetes within 29 weeks of age were classified as diabetic NOD (n=5), and those failed to develop diabetes by the age of 29 weeks were classified as pre-diabetic NOD (n=3). All animal experiments followed the guidelines established by Tulane University Institutional Animal Care and Use Committee and proper approvals were obtained prior to the study.

Antibodies

The mouse anti-GLP-1 monoclonal antibody specific for the amidated C-terminus of the active form GLP-1₇₋₃₆ was purchased from AbCam (Cambridge, MA). The rabbit anti-PC1/3 antibody was obtained from Millipore (Billerica, MA). Mouse anti-glucagon monoclonal antibody was obtained from Sigma-Aldrich Corp (St. Louis, MO), and a rabbit anti-glucagon polyclonal antibody was from Cell Signaling Technology (Danvers, MA). The Texas-Red (TR) and FITC-conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA).

Blood glucose monitoring and glucose tolerance test

Random blood glucose concentration was measured using the AlphaTrak blood glucose monitoring system (Abbott laboratories, Chicago, IL). Mice were considered diabetic once tail vein blood glucose readings of ≥ 250 mg/dL were obtained on two consecutive days. Glucose tolerance test was performed on mice every two weeks, as described previously[24]. Briefly, following overnight fasting, each mouse received 2g/ Kg body

weight of glucose via intraperitoneal injection, and their blood glucose was monitored at 15, 30, 45, 60, 90, and 120 minutes post-injection.

Immunohistochemistry

Immediately following sacrifice, the pancreas was removed from each mouse, formalin-fixed, embedded, and sectioned for immunohistochemistry (IHC) as described previously [24, 25]. The tissue sections were co-stained with anti-GLP-1 monoclonal antibody and anti-glucagon or anti-PC1/3 antibodies, followed by incubation with Texas Red or FITC-conjugated secondary antibodies. In addition, the slices were incubated with the nuclear dye Hoechst 33342 at a final concentration of 2 µg/ml for 10 minutes. The stained pancreas sections were imaged using a Nikon Eclipse Ti-S inverted microscope that was equipped with NIS-BR Microscope Imaging software (Nikon Instruments, Inc., Melville, NY). The final images were processed in Adobe Photoshop software (San Jose, CA).

Quantification and statistical analysis

Following immunofluorescence staining of the pancreatic slices, each islet was assessed for the number of GLP-1⁺Glucagon⁺ and double positive (GLP-1⁺/Glucagon⁺) cells based on associated nuclei. 20–30 islets with a minimum of 5 α-cells per islet were analyzed for each group. The number of GLP-1-expressing cells (GLP-1⁺) and glucagon-expressing cells (Glucagon⁺) in each islet were manually counted. For each islet, the ratio of total GLP-1⁺ cells (including GLP-1⁺ only and GLP-1⁺Glucagon⁺ double positive) to total Glucagon⁺ cells (including Glucagon⁺ only and GLP-1⁺Glucagon⁺ double positive) was determined and averaged within age group. The statistical analysis was performed using SAS software. The data were expressed as Mean ± SEM (standard error of the mean). $P < 0.05$ was considered statistically significant.

RESULTS

GLP-1 progression in type 2 diabetes model db/db mice

In order to investigate the progression of GLP-1 production in islets during the course of diabetes development, we first employed the widely used type 2 diabetes mouse model, db/db mice, and analyzed their pancreatic sections by immunofluorescence assays at various ages (Fig. 1). The db/db mice became obese by the age of 4 weeks (data not shown), and developed diabetes (defined as random blood glucose >250 mg/dL for 2 consecutive days) shortly after (Fig. 1A). Without any treatment, the disease progressed quickly, and in fact, it became so severe that the mice needed to be sacrificed by the age of 12 weeks.

As shown in Fig. 1B, in normal islets, glucagon expression was predominant. GLP-1 was detectable but at lower levels, and mostly co-expressed with glucagon. However, in db/db mice, we observed a clear shift from glucagon predominant to GLP-1 predominant expression as evidenced by more GLP-1 signals than glucagon signals with increasing diabetes severity/ages of the mice (Fig. 1B). In addition, the cells with only GLP-1 expression (that is, GLP-1⁺/Glucagon⁻) became readily detectable with diabetes development, suggesting proglucagon processing in these cells was completely switched to

support GLP-1 production. To further understand the progressive change of GLP-1 expression during diabetes development, we quantified the total numbers of GLP-1⁺ cells and Glucagon⁺ cells in each islet. The GLP-1⁺: Glucagon⁺ ratio showed significant increase between diabetic mice and normal control ($p<0.005$), and between 6-week and 10-week db/db mice ($p<0.005$) (Fig. 1C). Taken together, these data suggest intra-islet GLP-1 production in db/db mice was progressively up-regulated during the course of diabetes development.

PC1/3 expression in GLP-1⁺ cells during diabetes development

We examined whether PC1/3 expression changed in accordance with GLP-1 expression by co-staining the pancreatic slices with anti-PC1/3 and anti-GLP-1 antibodies. As shown in Fig. 2, PC1/3 was not detected in GLP-1⁺ cells of normal islets, suggesting PC1/3 expression level was below detectable concentration in normal condition. High level of PC1/3 expression was detected in insulin-producing β -cells that are typically localized in the core of mouse islets, which is not surprising because it has been well established that PC1/3 plays essential roles in proinsulin processing that gives rise to insulin and C-peptide, and is abundant in pancreatic β -cells[26, 27]. Interestingly, with diabetes development, PC1/3 became readily detectable in GLP-1⁺ cells in the islets of db/db mice (Fig. 2), suggesting PC1/3 was up-regulated to support GLP-1 production in these cells.

GLP-1 and PC1/3 expression in type 1 diabetes model NOD mice

We next investigated whether intra-islet GLP-1 production changed progressively in type 1 diabetes mouse models. NOD mice were used because these mice spontaneously develop diabetes that is attributable to autoimmune-mediated destruction of insulin-producing β -cells, resembling the pathogenesis of human type 1 diabetes. Immunofluorescence staining of the pancreatic slices from baseline, pre-diabetic and diabetic NOD mice was performed similarly as described above using anti-GLP-1 and anti-glucagon antibodies. As shown in the representative images, GLP-1 was detected in the islets of all three NOD groups, and mostly co-expressed with glucagon in α -cells (Fig. 3A). There were more Glucagon⁺ cells than GLP-1⁺ cells in all groups. Further quantitative analysis of the ratio of GLP-1⁺: Glucagon⁺ cells showed no significant differences among the three groups ($p>0.05$), although the ratio was slightly higher in diabetic NOD mice (Fig. 3B).

In addition, we examined PC1/3 expression in the islets of these NOD mice. PC1/3 was not detectable in GLP-1-expressing cells of baseline NOD mice, while abundant expression was found in the insulin-producing β -cells (Fig. 4), similar to what was found in normal mice (Fig. 2). In pre-diabetic and diabetic mice, PC1/3 expression diminished with the loss of β -cells, but was detected in some GLP-1⁺ cells (Fig. 4), suggesting PC1/3 was up-regulated in cells with increased GLP-1 expression during the development of diabetes in NOD mice.

DISCUSSION

This study characterized the progressive changes of intra-islet GLP-1 production during the course of diabetes development. Our data demonstrated GLP-1 production in islets was progressively and significantly up-regulated during the development of type 2 diabetes in

db/db mice, which may represent a self-protective physiological response against the disease. Interestingly, the up-regulation of GLP-1 in pancreatic islet cells was not as prominent during the course of type 1 diabetes development in NOD mice, suggesting intra-islet GLP-1 production has different impact on the development of type 1 and type 2 diabetes.

The mechanism for GLP-1 up-regulation in pancreatic islets is not clear. Previous studies on the GLP-1 production in intestinal L-cells have shown, in addition to nutrients, GLP-1 is modulated by many other factors such as neurotransmitters, cytokines and insulin [2, 19, 20, 28, 29]. Intra-islet GLP-1 production may be modulated by similar means. For example, high blood glucose may play an important role in inducing GLP-1 production in pancreatic islets since this GLP-1 progression was related to diabetes development. Indeed, elevated glucose concentration has been shown to be a potent stimulus for GLP-1 production in both L-cells and islet cultures [2, 21, 28, 30]. On the other hand, high blood glucose did not appear to be the only factor since intra-islet GLP-1 did not increase significantly in type 1 diabetes development. A critical difference between type 1 (NOD mice) and type 2 (db/db mice) diabetes is their insulin content: type 2 diabetics often present high insulin concentrations in blood to compensate for insulin resistance, while type 1 diabetics lack sufficient insulin. Since insulin has been shown to stimulate GLP-1 secretion in L-cells [29], it is highly possible that insulin also plays a role in stimulating intra-islet GLP-1 production.

Furthermore, there are additional factors that may influence the expression, secretion, and action of intra-islet GLP-1 on the endocrine pancreas. In fact, an intra-islet inactivation system may be responsible for regulation of islet-produced peptide hormones, including GLP-1. For example, Poulsen et al. suggested the presence of soluble DPP-IV within α -cells [31], a finding that has more recently been confirmed in a study conducted by Bugliani et al [32]. Inhibition of this islet-derived DPP-IV promoted improved glucose-stimulated insulin release in isolated human pancreatic islets cultured in moderate and high glucose conditions. These findings suggest that there may be an intra-islet regulatory mechanism in regards to the release of GLP-1 by α -cells, thus limiting its effects within the endocrine pancreas, though the implications of this remain to be further investigated.

One caveat to the findings of this study is that the results are based primarily on data obtained via immunohistochemical methods. Therefore, antibody sensitivity and specificity are critical to the validity of the results. Specifically, there have been concerns regarding anti-GLP-1 antibody due to the potential for cross-reactivity with proglucagon or MPGF (the byproduct of glucagon processing), both of which contain GLP-1 sequence in the middle. In an attempt to account for this, we selected a GLP-1 antibody specific for the amidated C-terminus of cleaved, bioactive GLP-1 (7–36) (AbCam, Cambridge, MA), an epitope not present within uncleaved proglucagon or other proglucagon-derived peptides including MPGF. Indeed, the detection of cells that were only positive for GLP-1 or glucagon confirmed the specificity and sensitivity of the antibodies, and thus the validity of our results. Nonetheless, it should be noted that it is not clear whether the increased production of GLP-1 in pancreas is associated with increased release of bioactive GLP-1 from the islets, which requires further studies using purified islets from normal and diabetic subjects.

In summary, our data suggest that there are clear differences in intra-islet GLP-1 production during the development of type 1 and 2 diabetes. These differences may have an impact on the clinical and pathophysiological processes of these diseases and may be a target for therapeutic approaches.

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Figure 1.

Progression of intra-islet GLP-1 expression during diabetes development in db/db mice. (A) Random blood glucose levels of db/db mice at different ages. (B) Immunofluorescence co-staining of pancreatic slices from normal and db/db mice at different ages using anti-glucagon (red) and anti-GLP-1 (green) antibodies. Hoechst 33342 (blue) was used to mark nuclei. Note GLP-1 signals increased with diabetes development (in synergy with the age of the mice). (C) Ratio of GLP-1⁺: Glucagon⁺ cells in pancreatic islets of normal and db/db mice of various ages. The ratio of total GLP-1⁺ cells (including GLP-1⁺ only and

GLP-1⁺Glucagon⁺ double positive) to total Glucagon⁺ cells (including Glucagon⁺ only and GLP-1⁺Glucagon⁺ double positive) from 20–30 islets in each group was quantified as described in the Methods. The error bars represent the standard error of the mean (SEM). The * marks statistical significance when compared to normal islets or between two specified groups (**: $p < 0.005$; ***: $p < 0.0001$).

GLP-1/ PC1/3/ Nuclei

Figure 2.

Expression of PC1/3 in GLP-1-producing cells in the islets of db/db mice. Pancreatic slices of normal and db/db mice of different ages were co-stained with anti-PC1/3 (green) and anti-GLP-1 (red) antibodies. PC1/3 was not detected in the GLP-1⁺ cells of normal islets. In db/db mice, however, PC1/3 became readily detectable in certain GLP-1⁺ cells (arrows). Of note, PC1/3 is known to be essential for insulin production from proinsulin, and abundantly expressed in β -cells (located at the core area of mouse islets).

Figure 3.

GLP-1 expression in pancreatic islets of NOD mice. Pancreas sections from NOD mice at different stages of diabetes development were co-stained with anti-GLP-1 (green) and anti-glucagon (red) antibodies. Nuclei were stained with Hoechst 33342 (blue). (A) Representative images of islets from baseline, prediabetic, and diabetic NOD mice. The arrows mark cells that co-expressed GLP-1 and glucagon. Note the islets in NOD mice were under the attack of immune cells (* marked area). (B) Quantitative assessment of the ratio of GLP-1⁺: Glucagon⁺ cells. Total GLP-1⁺ and total Glucagon⁺ cells in each islet were counted

and the ratios were calculated and averaged. 20–25 islets from each group were used in the quantification assay. The error bars represent SEM. The ratios of GLP-1⁺:Glucagon⁺ cells in the islets of baseline, pre-diabetic, and diabetic NOD mice did not show significant difference although diabetic mice appeared to have slightly more GLP-1⁺ cells. The mark # indicates $p>0.05$ compared to baseline.

Figure 4.

PC1/3 expression in NOD mice. Pancreatic slices of baseline, prediabetic and diabetic NOD mice were processed for immunofluorescence staining with anti-PC1/3 (red) and anti-GLP-1 (green) antibodies. Shown are representative images. The stars (*) mark the infiltrating immune cells, and arrows mark the examples of PC1/3 and GLP-1 co-expressing cells.