Effects of Linagliptin on Pancreatic \( \alpha \) Cells of Type 1 Diabetic Mice

Yanqing Zhang,1 Genevieve E. Fava,1 Meifen Wu,1 Wynn Htun,1 Thomas Klein,2 Vivian A. Fonseca,1 and Hongju Wu1

1Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70112; and
2Boehringer-Ingelheim Pharma GmbH & Co. KG, Biberach 88597, Germany

The dipeptidyl peptidase-4 inhibitor linagliptin promotes \( \beta \)-cell survival and insulin secretion by prolonging endogenous glucagon-like peptide 1 (GLP-1) action and therefore helps to maintain normoglycemia in diabetic patients. The effect of linagliptin on glucagon-producing \( \alpha \) cells, however, was not clear. In this study, we investigated whether linagliptin had any effects on \( \alpha \) cells with regard to their proliferation and hormonal production using type 1 diabetes mouse models, including streptozotocin-induced and nonobese diabetes mice. After diabetes development, the mice were either untreated or treated with linagliptin or insulin for up to 6 weeks. Our results showed that linagliptin significantly increased circulating GLP-1 levels in both type 1 diabetes models, but therapeutic benefit was detected in nonobese diabetes mice only. Circulating C-peptide and glucagon levels (nonfasting) were not significantly altered by linagliptin treatment in either model. In addition, we found that linagliptin did not increase \( \alpha \)-cell proliferation compared with the untreated or insulin-treated controls as assessed by in vivo 5-bromo-2'-deoxyuridine labeling assay. Finally, we examined whether linagliptin treatment altered GLP-1 vs glucagon expression in pancreatic \( \alpha \) cells. Immunohistochemistry assays showed that linagliptin treatment resulted in detection of GLP-1 in more \( \alpha \) cells than in control groups, suggesting linagliptin was able to increase intraislet GLP-1 presence, presumably by inhibiting GLP-1 degradation. In summary, this study indicates that linagliptin would not confer adverse effect on \( \alpha \) cells, such as causing \( \alpha \) cell hyperplasia, and instead may facilitate a blood glucose–lowering effect by increasing GLP-1 presence in \( \alpha \) cells.

Glucagon-like peptide 1 (GLP-1) plays essential roles in maintaining blood glucose homeostasis by acting on multiple organs involved in food intake and glucose regulation [1–3]. Most notably, GLP-1 stimulates insulin secretion and promotes \( \beta \)-cell survival and proliferation, whereas it inhibits glucagon secretion in pancreas [1–3]. Therefore, GLP-1 is a highly attractive target for diabetes treatment. Because native GLP-1 is prone to dipeptidyl peptidase-4 (DPP4)-mediated degradation, two types of drugs (collectively called incretin drugs) have been developed: GLP-1 receptor (GLP-1R) agonists that mimic GLP-1 action but are resistant to DPP4 degradation and DPP4 inhibitors that prolong the activity of endogenous GLP-1, which include linagliptin [3–5]. Studies have shown linagliptin improves \( \beta \)-cell survival and function in animal models [6, 7] and protects \( \beta \) cells from gluco-, lipo-, and cytokine-induced toxicity in human islets [8].

Although diabetes mellitus is mainly caused by the lack of insulin-producing \( \beta \) cells [type 1 diabetes (T1D)] or deficiency in insulin signaling/secretion pathways (type 2 diabetes), the glucagon-producing \( \alpha \) cells, which coreside with \( \beta \) cells in pancreatic islets, play important counterpart and regulatory roles to \( \beta \) cells and thus are also crucial in the regulation of blood

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glucose. Although many studies have been performed to examine the effects of GLP-1–based therapies on \( \beta \) cells, little has been done for \( \alpha \) cells. Recently, in an autopsy study, the investigators found that several patients under incretin treatment showed \( \alpha \)-cell hyperplasia and some even had glucagon-expressing neuroendocrine tumors [9, 10]. Although the study was questionable with regard to the comparability of the patient groups and the methodological assays [11–13], it raised an important safety issue that needed to be investigated. Because GLP-1 has survival- and growth-promoting effects of GLP-1 on \( \beta \) cells, is it possible that it has similar effects on \( \alpha \) cells?

GLP-1 and glucagon are coencoded by the proglucagon gene but derived from different posttranslational processes [14]. The classical view is that in intestinal \( L \)-cells, proglucagon is cleaved by prohormone convertase 1/3 to produce GLP-1, whereas in pancreatic \( \alpha \) cells, proglucagon is cleaved by prohormone convertase 2 to generate glucagon [14]. Recent studies have shown GLP-1 is also produced in pancreatic \( \alpha \) cells and that its expression is upregulated under certain circumstances such as diabetes development and exercise [15–19]. More interestingly, emerging evidence has suggested that intraislet-expressed GLP-1 could be largely responsible for its role in glucose regulation [20]. Therefore, it would be interesting to examine whether linagliptin affected the relative production of GLP-1 vs glucagon in pancreatic \( \alpha \) cells.

The main objective of this study was to investigate whether linagliptin treatment affected \( \alpha \)-cell proliferation and its hormone production. To accomplish this, we used T1D mouse models because these mice contain abundant numbers of \( \alpha \) cells and few \( \beta \) cells.

1. Materials and Methods

A. Antibodies

The guinea pig polyclonal anti-insulin antibody (RRID: AB_306130) and mouse monoclonal antibody (RRID: AB_470838) specific for the amidated C-terminus of active form GLP-1-7-36 were purchased from Abcam (Cambridge, MA). Rabbit antiglucagon (RRID: AB_10698611) antibody was purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-5-bromo-2′-deoxyuridine (BrdU) monoclonal antibody (RRID: AB_94897) was purchased from EMD Millipore (Billerica, MA). All of the secondary antibodies, including antimouse, antirabbit, and anti-guinea pig antibodies, which were conjugated with either tetramethyl rhodamine (red), fluorescein isothiocyanate (green), or coumarin (blue), were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA).

B. Animals

Nonobese diabetic (NOD) mice and streptozotocin (STZ)-induced diabetic C57BL/6 mice were used in this study. NOD mice (8 to 10 weeks old, female) were purchased from Jackson Laboratory (Bar Harbor, ME), and their random blood glucose levels were monitored twice a week with AlphaTRAK small animal blood glucose monitoring system (Abbott Animal Health, Abbott Park, IL). Mice with blood glucose levels >250 mg/dL were remeasured the next day, and diabetes was defined as a random (nonfasting) blood glucose level >250 mg/dL for 2 consecutive days. The diabetic mice were randomly assigned into three groups: untreated, insulin-treated, or linagliptin-treated. Efforts were taken to ensure similar-age animals were used among different treatment groups (for example, if three mice were found diabetic in the same week, one would be treated with insulin, one with linagliptin, and one would be untreated). The physiological data on the NOD mice that did not develop hyperglycemia by 25 weeks old of age were used as nondiabetic controls. For treatment, insulin (Humulin 70/30; Eli Lilly and Company, Indianapolis, IN) was given subcutaneously, 0.05 to 0.2 units every 12 hours depending on blood glucose levels; linagliptin (Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT) was administered orally at 3 mg/kg body weight, once daily, at 1 hour after lights off.
For STZ-induced T1D models, C57BL/6 mice (8 to 10 weeks old, female) were purchased from Charles River Laboratories (Wilmington, MA), and rendered diabetic with five consecutive daily injections of 50 mg STZ per kilogram of body weight. Their blood glucose levels were monitored three times a week as described previously. Similarly, they were randomly assigned into three groups: untreated, insulin-treated, and linagliptin-treated. Normal mice were included as controls. The treatment followed the same schedule as described for NOD mice. In addition, BrdU (1 mg/mL)-containing drink water was provided to all mice from when treatment started until the end of the experiment. All animal experiments followed the protocol that was approved by Tulane University Institutional Animal Care & Use Committee.

C. Hormone Measurements

Insulin, glucagon, and active GLP-1 concentrations in circulation (plasma) were measured using corresponding enzyme-linked immunosorbent assay kits from ALPCO Diagnostics (Salem, NH) according to the manufacturer’s protocols. To determine the hormone expression (production) in the pancreas, the pancreatic tissues (100 mg/mL) were first homogenized in RIPA buffer containing a cocktail of protease inhibitors and phosphatase inhibitors, then centrifuged at 4°C, 13,000 rpm for 10 minutes. The supernatants (cell lysates) were processed for hormone measurements as described previously. Total protein concentrations of the pancreatic lysates were determined using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA).

D. Immunofluorescence Staining

The paraffin-embedded mouse pancreatic slices were deparaffinized, permeabilized, and blocked in blocking solution (2% glycine, 2% bovine serum albumin, 5% fetal bovine serum, 50 M NH4Cl in phosphate-buffered saline) for 1 hour. The slices were then incubated with primary antibodies and fluorescence-conjugated secondary antibodies following standard protocols [19].

E. Quantification of GLP-1+ and Glucagon+ Cells

Following immunofluorescence staining and microscopy, the numbers of GLP-1–expressing cells (GLP-1+) and glucagon-expressing cells (glucagon+) were counted from 15 to 25 islets in each mouse. The ratio of GLP-1+ cells (including GLP-1+ only and GLP-1+glucagon+ double-positive cells) to glucagon+ cells (including glucagon+ only and GLP-1+glucagon+ double-positive cells) was calculated for each mouse and then averaged for each treatment group. The data were expressed as mean ± standard error of the mean.

F. Statistical Analysis

The statistical analysis was performed with SAS software. All data were expressed as mean ± standard error of the mean. One-way analysis of variance was used to compare the differences among different treatment groups. Student t test was used to compare the differences between two designated groups. \( P < 0.05 \) was considered statistically significant.

2. Results

A. Effect of Linagliptin on \( \alpha \)-Cell Proliferation in STZ-Induced Diabetic Mice

As terminally differentiated cells, the pancreatic islet cells that include \( \alpha \) cells have limited proliferation capacity. Nonetheless, studies have shown spontaneous \( \alpha \)-cell proliferation occurs and the rate of proliferation increases in response to STZ-induced \( \beta \)-cell injury that results in T1D [21, 22]. To examine whether linagliptin treatment had any effect on \( \alpha \)-cell
proliferation, we thus used STZ-induced T1D mice. In the study, C57BL/6 mice were first rendered diabetic by STZ injection and then randomly allocated into three treatment groups: untreated (n = 6), insulin-treated (n = 9), or linagliptin-treated (n = 9) for up to 4 weeks, as described in the Materials and Methods section. Normal mice (n = 6) were included as controls. As shown in Fig. 1, insulin treatment, as expected, fully normalized the blood glucose of the diabetic mice, whereas linagliptin treatment did not show any therapeutic benefits.

Figure 1. Linagliptin did not affect β-cell proliferation in STZ-induced diabetic mice. C57BL/6 mice were first rendered diabetic by STZ injection and then treated with either insulin (n = 9) or linagliptin (n = 9) for 2 or 4 weeks as described in the Materials and Methods section. Untreated diabetic mice (n = 6) and normal mice (n = 6) were included as control. BrdU-containing drink water was provided to the mice during the experimental period. (A) Blood glucose (nonfasting) of the mice. (B) AUC for each group in part A. *P < 0.05 and n.s. (P > 0.05) compared with the normal group. (C) GLP-1 concentration in blood. *P < 0.05 and **P < 0.01 compared with all controls (normal, untreated, and insulin-treated). Mouse C-peptide in pancreatic lysates (D) and (E) blood. *P < 0.05 and **P < 0.01 between normal and all diabetic groups; n.s. (P > 0.05) among the treatment groups. (F) Glucagon concentrations in blood. *P < 0.05 between normal and all diabetic groups; n.s. (P > 0.05) among the treatment groups. (G) Immunofluorescence staining for BrdU (red), glucagon (green), and insulin (blue). Representative images are shown. Arrows mark examples of BrdU+ cells. (H) Percentage of BrdU+ a cells (as total a cells) in each treatment group. Although STZ-induced diabetic mice showed a significant increase in α-cell proliferation compared with normal mice, linagliptin-treated mice did not show a significant difference from untreated or insulin-treated mice. *P < 0.05 compared with normal mice. n.s. (P > 0.05) among the treatment groups. AUC, area under the curve; C-pep, C peptide; N, normal; n.s., not significant. Insulin treated, black fill; N, filled strip; linagliptin treated, gray fill; untreated, white fill.
despite the circulating GLP-1 concentration being significantly increased (Fig. 1A–1C). C-peptide measurements in pancreatic lysates confirmed that the STZ-induced diabetic mice had significantly fewer β cells (Fig. 1D). Interestingly, C-peptide and glucagon concentrations in blood (nonfasting) were not significantly different among the treatment groups (Fig. 1E and 1F). Of note, we measured C-peptide instead of insulin for β-cell function to avoid interference from exogenous insulin; the half-life of C-peptide is three to four times longer than insulin; thus, its readings in blood is often much higher than insulin.

To evaluate whether linagliptin had any effects on α-cell proliferation, BrdU-containing drink water was provided to the mice during the 4 weeks of treatment. BrdU, a thymidine analog, can be incorporated into DNA during cell division and thus is often used as a marker for cell proliferation. After the mice were euthanized, their pancreases were processed for immunohistochemistry staining of BrdU, insulin, and glucagon. BrdU+ α cells (BrdU+ glucagon+) were readily detected in each treatment group, and occasionally in normal mice (Fig. 1G). Further quantification showed that the percentage of BrdU+ α cells in STZ-induced diabetic mice were significantly higher than that of normal mice (Fig. 1H), which is in agreement with previous observations [22]. Nonetheless, there was no substantial difference between linagliptin group and the untreated or insulin-treated groups. These results indicated that linagliptin did not significantly affect α-cell proliferation in STZ-induced T1D mice.

B. Effect of Linagliptin on GLP-1 vs Glucagon Expression in Pancreatic α Cells of STZ-Induced Diabetic Mice

Emerging evidence suggests pancreatic α cells produce not only glucagon, but also GLP-1, through distinct posttranslational processing of their shared precursor, proglucagon [14, 20]. We thus examined whether linagliptin treatment had any effects on GLP-1 vs glucagon production in α cells using the STZ-induced T1D model. Following linagliptin and control treatments as described previously, mice pancreatic tissues were either fixed for immunohistochemistry or lysed for biochemical hormone measurements. Immunofluorescence staining showed expression of both GLP-1 and glucagon in pancreatic islets (Fig. 2A; Supplemental Fig. S). In general, more glucagon positive cells (green) were detected than GLP-1+ cells (red), and many cells expressed both GLP-1 and glucagon (yellow or orange) (Fig. 2A; Supplemental Fig. S). Of note, the anti–GLP-1 and antiglucagon antibodies used were generated against the cleaved peptides so that they would not recognize the proglucagon precursor or intermediate peptides. Indeed, the detection of GLP-1 only (red) or glucagon only (green) cells confirmed the specificity of these antibodies (Fig. 2A). Quantification of the GLP-1+ and glucagon+ cells showed that linagliptin increased the ratio of GLP-1+ cells to glucagon+ cells because GLP-1 expression became detectable in more α cells, and the increase became statistically significant after 4 weeks’ treatment (Fig. 2B; Supplemental Fig. S).

The number of GLP-1+ or glucagon+ cells did not reflect the amount of GLP-1 or glucagon produced in these cells because of the nature of immunohistochemistry assay: a cell would become GLP-1+ as long as its expression reached the detectable level. To further determine whether linagliptin treatment increased intraislet GLP-1 vs glucagon production, we lysed the pancreases and measured the hormones in the lysates. Our data showed no significant differences between linagliptin-treated mice and untreated or insulin-treated mice for either GLP-1 or glucagon (Fig. 2C and 2D). This suggested that, although GLP-1 expression became detectable in more α cells after linagliptin treatment, the increase was too slight to cause substantial changes in the total amount of GLP-1 expressed in the pancreas.

C. Linagliptin Effects on α-Cell Function of NOD Mice

NOD female mice develop T1D resulting from autoimmunity-mediated β-cell destruction, similar to human T1D patients. Previous studies have shown that linagliptin has therapeutic benefits for NOD mouse models [23]. Therefore, we next examined whether linagliptin affected α-cell function, especially on hormone production and secretion, in these mice. Because
NOD mice develop diabetes spontaneously and there are wide variations among individuals regarding when they become diabetic, we monitored their disease progress and started a treatment plan accordingly for each mouse. The diabetic mice were randomly grouped into three treatment groups (untreated, insulin-treated, and linagliptin-treated), and efforts were taken to ensure that each group had mice with comparable ages at the beginning of the treatments (see Materials and Methods section for details). The mice that did not develop hyperglycemia by the 25 weeks of age were used as nondiabetic (ND) controls.

As shown in Fig. 3, linagliptin-treated mice showed lower random blood glucose compared with untreated mice, but much higher than the insulin-treated or ND mice (Fig. 3A and 3B). Further examination confirmed that linagliptin significantly increased circulating GLP-1 concentrations (Fig. 3C), but did not affect C-peptide or glucagon levels in blood (Fig. 3D and 3E). Of note, the ND group of NOD mice had significantly higher C-peptide concentrations than all other groups, which were in line with their ND condition. To see whether linagliptin treatment had any impact on GLP-1 vs glucagon expression in pancreatic α cells, we performed immunofluorescence staining. GLP-1 and glucagon were clearly detected in the islets.
Figure 3. Linagliptin effects in the autoimmune-based T1D mice. NOD mice that developed hyperglycemia were either untreated or treated with insulin or linagliptin (n = 6 to 9 mice) as described in the Materials and Methods section. The NOD mice that did not develop hyperglycemia by the age of 25 weeks were used as ND controls (n = 10). (A) Random (nonfasting) blood glucose levels of the mice. (B) AUC for the blood glucose of each group of mice. *P < 0.05 and **P < 0.01 between the indicated groups. For the ND group, **P < 0.01 compared with all other groups. (C) GLP-1 concentrations in blood following 3 and 6 weeks’ treatment. *P < 0.05 and **P < 0.01 compared with all other groups. (D) C-peptide concentrations in blood. *P < 0.05 compared with all other groups. (E) Glucagon concentrations in the blood. No significant differences were detected among the groups. (F) Immunofluorescence staining showing GLP-1 (red) and glucagon (green) expression in the pancreatic islets. Nuclei staining (blue) was included to mark all cells in the slices. Representative images are show. (F) Ratio of GLP-1+ cells:glucagon+ cells. *P < 0.05 compared with untreated group.
Some cells predominantly expressed glucagon (green), some predominantly GLP-1 (red), and some expressed both GLP-1 and glucagon (yellow or orange) (Fig. 3G). Quantification of the GLP-1+ and glucagon+ cells showed that linagliptin treatment increased the ratio of GLP-1+ cells: glucagon+ cells compared with untreated NOD mice, which became statistically significant after 6 weeks of treatment (Fig. 3F). Nonetheless, similar to what was observed in the STZ models, this increase in GLP-1 detection did not result in significant changes in the GLP-1 or glucagon contents of the pancreases in the mice (data not shown).

3. Discussion

In this study, we evaluated the effects of linagliptin on α-cell function using T1D models. Interestingly, although linagliptin significantly increased circulating GLP-1 levels in both STZ and NOD mice, therapeutic benefits were detected in NOD mice only. This may be explained by the different underlying mechanisms of β-cell destruction in the two models. In NOD mice, β-cell loss is caused by autoimmunity-based cell killing, which occurs through apoptosis [24], a process that can be delayed by GLP-1 because GLP-1 exerts antiapoptotic effects on β cells through various signaling pathways [25–28]. Therefore, an increase in circulating GLP-1 levels by linagliptin was beneficial in delaying the disease progress, which is in agreement with previous studies [23]. In the STZ model, however, the toxin causes rapid β-cell destruction through necrosis that was mainly triggered by DNA alkylation [29, 30], overwhelming the protective effect of GLP-1 on β cells.

Considering the survival and growth-promoting effects of GLP-1 on β cells, we examined whether linagliptin had similar effects on α cells using STZ-induced diabetic mice, a mouse model for α-cell proliferation [21, 22]. Our results showed that linagliptin did not stimulate α-cell proliferation and did not cause α-cell hyperplasia or glucagon-producing tumors. It has been shown that, in pancreatic β cells, GLP-1 acts by binding to its receptor, GLP-1R, and activates several signaling pathways such as cyclic adenosine monophosphate/protein kinase A, mitogen-activated protein kinase/ extracellular signal-regulated kinase 1/2, phosphatidylinositol 3 kinase/Akt, and mTOR pathways, all of which may stimulate β-cell proliferation [25–28]. Expression of GLP-1R in α cells has been a controversial topic: using both antibody- and nonantibody-based methods, some studies reported the presence of GLP-1R in α cells [31], or at least in a portion of α cells [32, 33]; some reported no GLP-1R expression [34]. Taking these studies together, it is reasonable to conclude that GLP-1R is expressed in α cells but at a much lower level than in β cells, or only in a subpopulation of α cells. This might explain why linagliptin, via upregulation of GLP-1, was not sufficient to cause substantial α-cell proliferation. Another reason may be the treatment duration in our study, which was up to 4 weeks, whereas patients could be under treatment for years. Nonetheless, it should be noted that we used in vivo BrdU labeling assay to identify α-cell proliferation, which occurs before the formation of visible α-cell hyperplasia or tumors in pancreas [9, 10], and 4 weeks’ treatment would allow us to detect any substantial change should it be present.

On another note, the impact of GLP-1–based incretin therapies on β-cell proliferation and mass expansion has its own controversies. Many studies have shown GLP-1R agonists and DPP-4 inhibitors have mitogenic effects on β cells in rodents [35–37], but some showed otherwise. For instance, a newly published study using a large cohort of young male mice has shown that incretin (exenatide and sitagliptin) therapies have little impact on β-cell proliferation and mass expansion following long-term (4.5 months) treatment [38], although the authors indeed have observed moderate β-cell proliferation following short-term (2 weeks) treatment. Together, these studies suggest that the mitogenic potential of incretin therapies may be affected by drug dosage, administration route, treatment duration, and the nature of diabetes models [38, 39].

Many studies have now confirmed that α cells produce not only glucagon, but also GLP-1, although the latter was produced at much lower level than the former [14–16, 19, 40, 41]. The effect of linagliptin on α-cell hormone expression was thus investigated in this study. The number of α cells expressing detectable levels of GLP-1 was significantly up-regulated by
linagliptin treatment in both T1D models. This may be attributable to the inhibition of GLP-1 degradation rather than stimulation of production. Indeed, DPP-4, a widely expressed protease, has been shown to be expressed in mouse islets [42]. On the other hand, despite increased detection of GLP-1–expressing cells by immunohistochemistry, it did not result in significant changes in the total amount of the hormones when compared with controls, probably because the hormones constituted such a tiny fraction of pancreatic proteins that any changes became insignificant in the context of whole pancreatic extracts (Fig. 2).

Taken together, this study clarified some important questions regarding whether linagliptin modulated α-cell function using T1D models. Our data showed that linagliptin did not significantly induce α-cell proliferation, but could up-regulate intraislet GLP-1 expression. The results suggest that linagliptin would not confer adverse effects, such as inducing α-cell proliferation; instead, it may facilitate blood glucose–lowering effects by upregulation of GLP-1 expression in α cells.

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Current Affiliation: M. Wu’s current affiliation is the Department of Medicine, Guangdong Medical University, Zhanjiang, Guangdong Province 524023, China.

Correspondence: Hongju Wu, PhD, Department of Medicine, Tulane University School of Medicine, 1430 Tulane Ave., # 8553, New Orleans, Louisiana 70112. E-mail: hwu3@tulane.edu.

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References and Notes


