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Chronic administration of alogliptin, a novel, potent, and highly selective dipeptidyl peptidase-4 inhibitor, improves glycemic control and beta-cell function in obese diabetic *ob/ob* mice

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ABSTRACT

Dipeptidyl peptidase-4 (DPP-4) inhibitors improve glycemic control in patients with type 2 diabetes by increasing plasma active glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide levels. However, the effects of chronic DPP-4 inhibition on *in vivo* beta-cell function are poorly characterized. We thus evaluated the chronic effects of the DPP-4 inhibitor alogliptin benzoate (formerly SYR-322) on metabolic control and beta-cell function in obese diabetic *ob/ob* mice. Alogliptin (0.002%, 0.01%, or 0.03%) was administered in the diet to *ob/ob* mice for 2 days to determine effects on plasma DPP-4 activity and active GLP-1 levels and for 4 weeks to determine chronic effects on metabolic control and beta-cell function. After 2 days, alogliptin dose-dependently inhibited DPP-4 activity by 28–82% and increased active GLP-1 by 3.2–6.4-fold. After 4 weeks, alogliptin dose-dependently decreased glycosylated hemoglobin by 0.4–0.9%, plasma glucose by 7–28% and plasma triglycerides by 24–51%, increased plasma insulin by 1.5–2.0-fold, and decreased plasma glucagon by 23–26%, with neutral effects on body weight and food consumption. In addition, after drug washout, alogliptin (0.03% dose) increased early-phase insulin secretion by 2.4-fold and improved oral meal tolerance (25% decrease in glucose area under the concentration–time curve), despite the lack of measurable plasma DPP-4 inhibition. Importantly, alogliptin also increased pancreatic insulin content up to 2.5-fold, and induced intense insulin staining of islets, suggestive of improved beta-cell function. In conclusion, chronic treatment with alogliptin improved glycemic control, decreased triglycerides, and improved beta-cell function in *ob/ob* mice, and may exhibit similar effects in patients with type 2 diabetes.
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1. Introduction

In animals and humans, oral glucose elicits a higher insulin response than intravenous glucose, even at identical plasma glucose profiles. This phenomenon is termed the "incretin effect" and is mediated primarily by glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), peptide hormones secreted by gut endocrine cells. Both GLP-1 and GIP stimulate glucose-dependent insulin secretion after nutrient ingestion and play an important role in glucose homeostasis (Meier and Nauck, 2006; Drucker, 2007). In addition, GLP-1, but not GIP, inhibits glucose-dependent glucagon secretion and has been reported to delay gastric emptying, enhance satiety, and reduce food intake. Both incretins also exert direct trophic effects on beta-cell proliferation, differentiation, growth, and survival *in vitro* and in animal models of diabetes (Meier and Nauck, 2006; Drucker, 2007).

In patients with type 2 diabetes, the incretin effect is thought to be partially impaired due to a reduction in GLP-1 secretion (Toft-Nielsen et al., 2001; Vilsboll et al., 2001) and a reduction in the pancreatic response to GIP (Nauck et al., 1993; Meier et al., 2001). However, the therapeutic potentials of GLP-1 and GIP are limited by their rapid degradation and inactivation *in vivo* by dipeptidyl peptidase-4 (DPP-4), a serine protease that is present in soluble form in plasma and is also expressed on a variety of cell types, including lymphocytes and endothelial cells, and in most tissues (Deacon, 2004). Thus, degradation-resistant GLP-1 receptor agonists and DPP-4 inhibitors have emerged as new classes of pharmacological agents to enhance incretin action and thereby improve glycemic control in patients with type 2 diabetes (Mest and Mentlein, 2005; Drucker and Nauck, 2006; Pratley and Salsali, 2007). Studies have demonstrated the ability of these agents to improve glycemic control in animal models of diabetes and in patients with type 2 diabetes (Gallwitz, 2006; Henness and Keam, 2006; Deacon, 2007; Vilsboll, 2007).

The progressive deterioration of glycemic control that is characteristic of type 2 diabetes is thought to be largely due to a

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progressive decline in pancreatic beta-cell function and mass coupled with a progressive increase in alpha-cell mass (Del Prato and Marchetti, 2004). Hence, preservation or restoration of both beta- and alpha-cell function may alter or reverse the course of the disease. Accumulating evidence suggests that exogenously administered GLP-1 receptor agonists exert trophic effects on pancreatic islets in animal models of diabetes, increasing islet neogenesis and differentiation as well as increasing beta-cell mass (Xu et al., 1999; Farilla et al., 2002; Rolin et al., 2002; Tourrel et al., 2002; Sturis et al., 2003; Perfetti and Hui, 2004). In contrast, considerably less data are available on the *in vivo* effects of chronic DPP-4 inhibition on pancreatic islets. Chronic studies with the DPP-4 inhibitor P32/98 revealed that this agent improved glycemic control without increasing beta-cell mass in VDF (*fa/fa*) Zucker rats (Pospisilik et al., 2002) but significantly increased beta-cell mass in streptozotocin-injected diabetic rats (Pospisilik et al., 2003). In a more recent study, chronic treatment with an analogue of the DPP-4 inhibitor sitagliptin increased islet insulin content and improved responsiveness of islets to glucose-stimulated insulin secretion in high-fat-diet fed streptozotocin mice (Mu et al., 2006).

Alogliptin benzoate (formerly SYR-322) is a novel, oral, potent, and highly selective DPP-4 inhibitor with once-daily dosing potential that is being developed to improve glycemic control in patients with type 2 diabetes (Feng et al., 2007). Alogliptin is >10,000 fold more selective for DPP-4 over other related serine peptidases *in vitro* (IC_{50} of 6.9 nM vs DPP-4; >100,000 nM vs DPP-2, DPP-8, DPP-9, fibroblast activation protein/seprase, and prolyl endopeptidase; >390,000 nM vs tryptase (Feng et al., 2007; Lee et al., 2008). A single dose of alogliptin has been demonstrated to improve glucose tolerance and increase early-phase insulin secretion after an oral glucose challenge in Wistar fatty rats (Feng et al., 2007) and neonatally streptozotocin-injected diabetic (N-STZ-1.5) rats (Takeuchi et al., 2006), an obese and non-obese rat model of type 2 diabetes, respectively (Ikeda et al., 1981; Matsuo et al., 1991). In addition, chronic treatment with alogliptin for 4 weeks has been demonstrated to decrease glycosylated hemoglobin levels and increase pancreatic insulin content in N-STZ-1.5 rats (Takeuchi et al., 2006).

The objectives of the present research were to characterize the *in vivo* effects of chronic administration of the DPP-4 inhibitor alogliptin on metabolic control and pancreatic islet function using *ob/ob* mice, an obese rodent model of type 2 diabetes that exhibits hyperglycemia, glucose intolerance, hyperinsulinemia, and hyperglucagonemia (Herberg and Leiter, 2001).

2. Materials and methods

2.1. Chemicals

Alogliptin benzoate (2-[[6-[(3*R*)-3-amino-1-piperidinyl]-3,4-dihydro-3-methyl-2,4-dioxo-1(2*H*)-pyrimidinyl]methyl]benzonitrile monobenzoate) was synthesized by Albany Molecular Research Institute (Albany, NY). The dose of alogliptin is expressed as the free base form. All reagents were purchased from Wako Pure Chemical Industries or Sigma-Aldrich.

2.2. Animals

Male Lep^{ob}/Lep^{ob} (*ob/ob*) mice and their non-diabetic, untyped (*?/+*) male littermates were obtained from Charles River Japan (B6.V-Lep^{ob}/J). All mice were housed in individual metal cages in a room with controlled temperature (23 °C), humidity (55%), and lighting (lights on from 7:30 am to 7:30 pm) and were maintained on a laboratory chow diet (CE-2, Clea). The care and use of the animals and the experimental protocols used in this research were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company, Ltd (Osaka, Japan).

2.3. Short-term study in ob/ob mice

After a 6-day acclimation period, 7-week old *ob/ob* mice were divided into 4 groups (8 mice per group) based on body weight and food consumption and fed a CE-2 diet containing 0.002%, 0.01%, or 0.03% alogliptin for 2 days. Control diabetic *ob/ob* and non-diabetic *?/+* mice (8 and 5 mice, respectively) were fed a drug-free CE-2 diet (vehicle). Blood samples were collected after the 2-day treatment, and plasma DPP-4 activity and active GLP-1 levels were analyzed.

2.4. Dose-dependent chronic study in ob/ob mice

After a 6-day acclimation period, 7-week old *ob/ob* mice were divided into 3 groups (8 mice per group) based on glycosylated hemoglobin, plasma glucose, plasma insulin, and body weight, and fed a CE-2 diet containing 0.002% (2.8 mg/kg/day) or 0.01% (14.1 mg/kg/day) alogliptin during the experimental period. Control diabetic *ob/ob* and non-diabetic *?/+* mice (8 and 4 mice, respectively) were fed a drug-free CE-2 diet (vehicle). After 27 days of treatment, blood samples were collected at 8:00 am (0.5 h after dark period; fed state), and plasma metabolic parameters were determined. After 28 days of treatment, plasma glucose levels were determined at 4:00 pm (3.5 h before dark period; state of reduced food intake). After 29 days of treatment, blood samples were collected at 8:00 am (fed state) and plasma glucagon, DPP-4 activity, and active GLP-1 levels were determined; the pancreas was then isolated for measurement of insulin and glucagon content (fed state). Body weight and food consumption were measured at regular intervals.

2.5. High-dose chronic study in ob/ob mice

After a 6-day acclimation period, 7-week old *ob/ob* mice were divided into 3 groups (7 mice per group) based on glycosylated hemoglobin, plasma glucose, plasma insulin, and body weight, and fed a CE-2 diet containing 0.03% (42.2 mg/kg/day) alogliptin during the experimental period. Control diabetic *ob/ob* and non-diabetic *?/+* mice (7 and 4 mice, respectively) were fed a drug-free CE-2 diet (vehicle). After 28 days of treatment, blood samples were collected at 8:00 am (fed state), and plasma metabolic parameters and DPP-4 activity were determined. After this blood draw, all mice then underwent an oral meal tolerance test, after which the pancreas was isolated for measurement of insulin and glucagon content and immunohistochemical analysis (dietary status considered fasting for pancreatic analyses). Body weight and food consumption were measured at regular intervals.

2.6. Oral meal tolerance test in ob/ob mice

In the high-dose chronic study, after 28 days of treatment, all mice were fed with drug-free diet for 24 h, fasted for 18 h, and then given an oral meal load using the Lieber–DeCarli liquid diet with some modifications (20 kcal/15 ml/kg, Oriental Yeast Co., Ltd). The composition of the liquid diet was as follows (1000 kcal/750 ml/215.33 g): sodium-casein 41.4 g; L-cystine 0.5 g; DL-methionine 0.3 g; corn oil 8.5 g; olive oil 28.5 g; vitamin mixture 5 g; mineral mixture 10 g; ethyl linoleate 2.7 g; sucrose 115.9 g; carrageenan 2.5 g; and DL-α-tocopherol acetate 0.03 g. Blood samples were collected before the meal load (time=0), and 10, 30 and 60 min after the meal load, and plasma glucose and insulin levels were analyzed. The area under the concentration–time curve (AUC) from 0 to 60 min after meal administration was calculated for plasma glucose. Plasma DPP-4 activity before the meal load (time=0; fasting state) was also measured.

2.7. Assays for plasma metabolic parameters

Glycosylated hemoglobin levels were analyzed by a high-pressure liquid chromatography-based method using an automated analyzer

HLC-723 G7 (Tosoh). Plasma glucose and triglyceride levels were determined using an autoanalyzer 7080 (Hitachi). Plasma insulin and glucagon levels were determined by radioimmunoassay (Rat Insulin RIA Kit; Linco and Glucagon Kit Daiichi; TFB, respectively). Plasma active GLP-1 levels were determined by enzyme-linked immunosorbent assay (Glucagon Like Peptide-1 [Active] ELISA Kit; Linco). Plasma insulin levels during the oral meal tolerance test were determined by enzyme-linked immunosorbent assay (Mouse Insulin ELISA Kit; Shibayagi).

2.8. Plasma DPP-4 enzymatic assay

After the blood sampling, 10 μl of plasma was mixed with 40 μl of assay buffer containing 250 mM Tris–HCl (pH 7.5), 0.25% (wt/vol) bovine serum albumin, and 0.125% (wt/vol) 3-[(3-Cholamidopropyl) dimethylammonio]propanesulfonic acid (CHAPS, Dojindo) in 96-well plates. The plates were then shaken and samples were mixed with 50 μl of 1 mM Gly-Pro-pNA·Tos (Peptide Institute) to initiate the reactions. The samples were incubated at 30 °C on a plate shaker, and the increase in absorbance at 405 nm was monitored at both 20 and 60 min after the reaction initiation using a microtiter plate reader (Dainippon Sumitomo Pharma). The plasma DPP-4 activity was assessed by the increase in absorbance at 405 nm between 20 and 60 min. Plasma DPP-4 activity of the alogliptin-treated mice was compared with that of the vehicle-treated *ob/ob* mice, which was defined as 100%.

2.9. Pancreas isolation and measurement of insulin and glucagon content

At the end of the dose-dependent and high-dose chronic studies, the mice were euthanized with carbon dioxide, and the pancreas was isolated and cut into two sections. In both studies, one section was homogenized in acid-ethanol containing 74% ethanol with 0.15 M HCl for the determination of insulin and glucagon concentrations. In the high-dose chronic study only, the other section was placed in Bouin fixative solution (Polysciences) for immunohistochemical analysis. The homogenized tissues were extracted overnight at 4 °C and centrifuged at 12,000×g for 10 min. The resultant supernatants were then diluted with phosphate-buffered saline containing 0.1% bovine serum albumin, and the insulin and glucagon levels in the supernatants were determined by radioimmunoassay.

2.10. Immunohistochemical analysis

In the high-dose chronic study, after the overnight fixation, tissue samples were placed in 70% ethanol for 2 days, and subsequently embedded in paraffin. The tissue sections were dried on slides overnight at 37 °C. Paraffin sections (6 μm for insulin and 4 μm for glucagon) were cut, deparaffinized, and rehydrated at room temperature. The sections were then heated for 15 min at 90 °C in a microwave oven for glucagon staining only, and the endogenous peroxidase was blocked with 80% methanol containing 0.6% hydrogen peroxide for 15 min. The sections were rinsed with distilled water and placed in 3% hydrogen peroxide for 15 min. Next, the sections were rinsed with distilled water and washed in Tris-buffered saline with Tween (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 5 min. The sections were then reacted with the primary antibodies, ready-to-use guinea pig anti-insulin antibody (Dako) overnight at 4 °C or ready-to-use rabbit anti-glucagon antibody (Dako) for 60 min at room temperature. The sections were washed with Tris-buffered saline with Tween, and bound antibody was detected using a ready-to-use polymer-labeled Envision+ system (Dako) for 30 min. The sections were rinsed with Tris-buffered saline with Tween and developed for 1 min using 3,3'-diaminobenzidine tetrahydrochloride substrate. Finally, slides were washed with distilled water, counterstained with hematoxylin, and mounted.

2.11. Statistical analysis

Statistical analysis was performed using the SAS system Version 8.2 (SAS institute Inc.). To evaluate the effect of alogliptin in the short-term and dose-dependent chronic studies, statistical significances were analyzed using the one-tailed Williams' or one-tailed Shirley–Williams test. To evaluate the effect of alogliptin in the high-dose chronic study, statistical significances between vehicle-treated *ob/ob* mice and alogliptin-treated *ob/ob* mice were analyzed using the Student's *t*-test or Aspin–Welch test. To evaluate differences in DPP-4 activity between the *ob/ob* mice and *?/+* control mice, statistical significances were analyzed using the Aspin–Welch test. All data were presented as mean and S.D.

3. Results

3.1. Effects of short-term alogliptin administration on plasma DPP-4 activity and active GLP-1 levels in ob/ob mice

The dose-dependent effects of short-term treatment with alogliptin (0.002%, 0.01%, and 0.03%) on plasma DPP-4 activity and active GLP-1 levels in *ob/ob* mice were examined. Interestingly, at 7 weeks of age, plasma DPP-4 activity was significantly ($P \leq 0.01$) higher (1.9-fold) in the vehicle-treated diabetic *ob/ob* mice than in the vehicle-treated non-diabetic *?/+* mice (Fig. 1). After 2 days of treatment, alogliptin at doses of 0.002%, 0.01%, and 0.03% dose-dependently and significantly ($P \leq 0.025$) decreased plasma DPP-4 activity by 28%, 71%, and 82%, respectively, and increased active GLP-1 levels by 3.2-, 5.0-, and 6.4-fold, respectively, in *ob/ob* mice compared with vehicle alone (Fig. 1).

3.2. Dose-dependent effects of chronic alogliptin administration on plasma DPP-4 activity, active GLP-1 levels, and metabolic control in ob/ob mice

To evaluate the dose-dependent effects of chronic treatment with alogliptin, 7-week old *ob/ob* mice were treated with 0.002% (2.8 mg/kg/day) or 0.01% (14.1 mg/kg/day) alogliptin or vehicle alone for 4 weeks, and plasma DPP-4 activity, active GLP-1 levels, and metabolic parameters were analyzed. At the end of the 4-week treatment period (11 weeks of age), the vehicle-treated diabetic *ob/ob* mice had significantly elevated levels of DPP-4 activity compared with the vehicle-treated non-diabetic *?/+* mice (Table 1), as was observed for the 7-week old mice in the short-term study (Fig. 1). However, alogliptin at doses of 0.002% and 0.01% dose-dependently inhibited plasma DPP-4 activity by 24% (not significant) and 62% ($P \leq 0.025$), respectively, and significantly ($P \leq 0.025$) increased active GLP-1 levels by 2.3- and 5.3-fold, respectively, in the *ob/ob* mice compared with vehicle alone (Table 1).

After 4 weeks, the vehicle-treated diabetic *ob/ob* mice exhibited hyperglycemia, having elevated glycosylated hemoglobin and plasma glucose levels compared with the vehicle-treated non-diabetic *?/+* mice (Table 1). Chronic treatment with 0.002% and 0.01% alogliptin dose-dependently decreased non-fasting (8:00 am; 0.5 h after dark period) glycosylated hemoglobin levels by 0.4% (not significant) and 0.7% ($P \leq 0.025$), respectively, compared with vehicle alone in the *ob/ob* mice. Although decreases in non-fasting plasma glucose levels (7% and 13%, respectively) were also observed in alogliptin-treated *ob/ob* mice, these changes were not statistically significant when compared with the vehicle-treated *ob/ob* mice. However, plasma glucose levels measured at 4:00 pm (3.5 h before dark period; state of reduced food intake) were significantly ($P \leq 0.025$) decreased by 26% and 28%, respectively, in alogliptin-treated *ob/ob* mice compared with vehicle-treated *ob/ob* mice.

In addition to hyperglycemia, the vehicle-treated diabetic *ob/ob* mice also exhibited hyperinsulinemia and hyperglucagonemia after 4 weeks (11 weeks of age), having elevated plasma insulin and

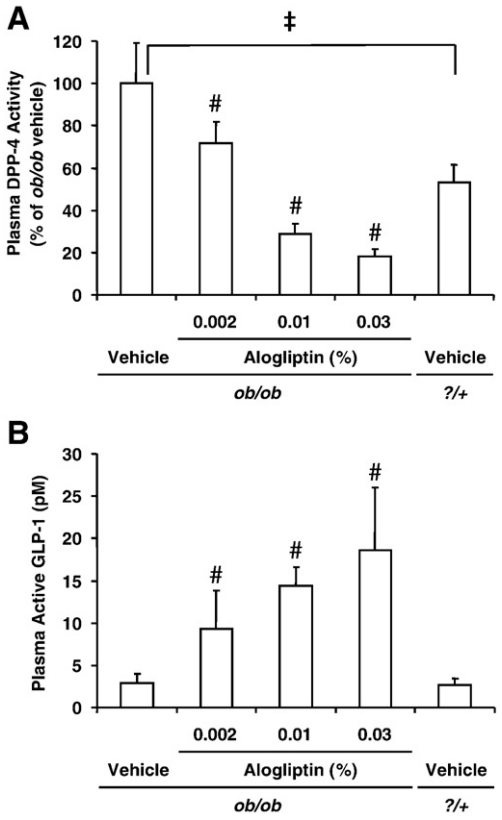

Fig. 1. Effects of short-term alogliptin treatment on plasma DPP-4 activity and active GLP-1 levels in *ob/ob* mice. Mice were fed a CE-2 diet containing 0.002%, 0.01%, or 0.03% alogliptin or a drug-free diet (vehicle) for 2 days. (A) Plasma DPP-4 activity. (B) Plasma active GLP-1 levels. #$P \leq 0.025$ vs vehicle-treated *ob/ob* mice by one-tailed Shirley–Williams test; ‡$P \leq 0.01$ vs vehicle-treated *?/+* mice by Aspin–Welch test. Values are mean±S.D. ($n=8$ for *ob/ob* mice, $n=5$ for *?/+* mice).

glucagon levels compared with the vehicle-treated non-diabetic *?/+* mice (Table 1). Treatment with 0.002% and 0.01% alogliptin increased non-fasting plasma insulin levels by 1.5- (not significant) and 1.8-fold ($P \leq 0.025$) and reduced non-fasting plasma glucagon levels by 26% (not significant) and 23% (not significant), respectively, in the *ob/ob* mice compared with vehicle alone. Furthermore, non-fasting pancreatic insulin content in *ob/ob* mice treated with 0.002% and 0.01% alogliptin was dose-dependently and significantly ($P \leq 0.025$) increased by 1.5- and 1.9-fold, respectively, compared with vehicle-treated *ob/ob* mice. In contrast, alogliptin had no effect on non-fasting pancreatic glucagon content, which was elevated in the diabetic *ob/ob* mice compared with the vehicle-treated non-diabetic *?/+* mice.

Compared with vehicle-treated *ob/ob* mice, non-fasting plasma triglyceride levels in mice treated with 0.002% and 0.01% alogliptin were significantly ($P \leq 0.025$) decreased by 51% and 42%, respectively (Table 1). Alogliptin also had a neutral effect on body weight and food consumption.

Table 2
Metabolic parameters in mice treated with alogliptin for 4 weeks (high-dose study)

Parameter (units)[a]	*ob/ob* mice		*?/+* mice
	Vehicle	0.03% alogliptin	Vehicle
Plasma DPP-4 activity (% of vehicle-treated *ob/ob* mice)	100±27[b]	20±3[c]	45±10
Glycosylated hemoglobin (%)	6.9±0.3	6.0±0.6[c]	3.1±0.1
Plasma glucose (mM)	20.5±1.6	17.3±3.4[d]	7.8±0.5
Plasma triglyceride (mM)	1.06±0.24	0.80±0.25[e]	1.44±0.49
Plasma insulin (pM)	8135±3772	16,656±5041[f]	154±50
Fasting pancreatic insulin content (pmol/mg pancreas)	53.9±9.4	133.3±36.9[c]	36.7±6.5
Fasting pancreatic glucagon content (ng/mg pancreas)	14.5±1.6	15.0±3.1	5.1±1.0
Food consumption (g/day)	6.2±0.5	6.1±0.7	5.3±0.5
Body weight (g)	47.3±2.7	47.8±1.6	25.0±1.2

Values are mean±S.D. ($n=7$ for *ob/ob* mice, $n=4$ for *?/+* mice).
[a] All parameters were determined in the fed state, unless otherwise indicated.
[b] $P \leq 0.01$ vs vehicle-treated *?/+* mice by Aspin–Welch test.
[c] $P \leq 0.01$ and [d] $P=0.057$ vs vehicle-treated *ob/ob* mice by Aspin–Welch test.
[e] $P=0.075$ and [f] $P \leq 0.01$ vs vehicle-treated *ob/ob* by Student's *t*-test.

3.3. High-dose effects of chronic alogliptin administration on DPP-4 activity and metabolic control in ob/ob mice

In a second chronic study, the effects of a higher dose of 0.03% alogliptin (corresponding to 42.2 mg/kg/day) on plasma DPP-4 activity, metabolic parameters, glucose tolerance, and beta-cell function were investigated in *ob/ob* mice. As observed in the dose-dependent chronic study, after 4 weeks of treatment (11 weeks of age), the vehicle-treated diabetic *ob/ob* mice exhibited elevated plasma DPP-4 activity, hyperglycemia, and hyperinsulinemia when compared with the vehicle-treated non-diabetic *?/+* mice (Table 2). After the 4-week treatment period, alogliptin significantly ($P \leq 0.01$) inhibited plasma DPP-4 activity by 80%, significantly ($P \leq 0.01$) decreased non-fasting glycosylated hemoglobin by 0.9%, and signifi-

Table 1
Metabolic parameters in mice treated with alogliptin for 4 weeks (dose-dependent study)

Parameter (units)[a]	*ob/ob* mice			*?/+* mice
	Vehicle	0.002% alogliptin	0.01% alogliptin	Vehicle
Plasma DPP-4 activity (% of vehicle-treated *ob/ob* mice)	100±24[b]	76±20	38±8[c]	34±4
Plasma active GLP-1 (pM)	2.4±0.8	5.7±0.9[c]	12.9±2.9[c]	2.0±0.0
Glycosylated hemoglobin (%)	7.2±0.7	6.9±0.5	6.5±0.6[d]	3.1±0.1
Plasma glucose (mM)	22.8±3.9	21.1±4.6	19.8±2.6	8.4±0.6
Plasma glucose (mM) (4:00 pm; 3.5 h before the dark period; state of reduce food intake)	23.5±6.6	17.5±3.6[d]	17.0±4.8[d]	9.3±1.6
Plasma triglyceride (mM)	1.60±0.89	0.78±0.22[c]	0.92±0.21[c]	1.55±0.19
Plasma insulin (pM)	8018±4055	12,344±5490	14,545±7822[d]	176±62
Plasma glucagon (ng/l)	361±177	267±91	276±82	141±49
Pancreatic insulin content (pmol/mg pancreas)	14.8±3.9	21.7±6.4[d]	28.5±6.7[d]	26.6±4.7
Pancreatic glucagon content (ng/mg pancreas)	11.6±1.5	11.1±2.6	11.7±1.7	3.8±1.0
Food consumption (g/day)	6.3±0.5	6.3±0.7	6.3±0.3	5.4±0.9
Body weight (g)	48.5±1.9	49.8±2.1	49.3±1.7	26.2±0.6

Values are mean±S.D. ($n=8$ for *ob/ob* mice, $n=4$ for *?/+* mice).
[a] All parameters were determined in the fed state.
[b] $P \leq 0.01$ vs vehicle-treated *?/+* mice by Aspin–Welch test.
[c] $P \leq 0.025$ vs vehicle-treated *ob/ob* mice by one-tailed Shirley–Williams test.
[d] $P \leq 0.025$ vs vehicle-treated *ob/ob* mice by one-tailed Williams' test.

cantly ($P \leq 0.01$) increased non-fasting plasma insulin and fasting pancreatic insulin content by 2- and 2.5-fold, respectively, compared with vehicle alone in *ob/ob* mice. Alogliptin also decreased non-fasting plasma glucose and triglyceride levels by 15% ($P=0.057$) and 24% ($P=0.075$), respectively, compared with vehicle alone. Consistent with the dose-dependent study, no significant changes in body weight, food consumption, and fasting pancreatic glucagon content were observed in the alogliptin-treated mice.

3.4. High-dose effects of chronic alogliptin administration on oral meal tolerance and beta-cell function in ob/ob mice

After 4 weeks of treatment in the high-dose chronic study, the mice were fed a drug-free diet for 24 h, fasted for a further 18 h, and then given an oral meal load. As shown in Fig. 2, despite the lack of measurable plasma DPP-4 inhibition at time 0 (after the 42-h washout but before oral meal challenge), early-phase insulin secretion was significantly ($P \leq 0.05$) increased by 2.4-fold, and plasma glucose AUC was significantly ($P \leq 0.05$) decreased by 25% in the alogliptin-treated *ob/ob* mice compared with vehicle-treated *ob/ob* mice, indicative of improved oral meal tolerance with chronic alogliptin treatment.

After the oral meal tolerance test, the pancreas was isolated from each animal and analyzed by immunohistochemistry using anti-insulin and anti-glucagon antibodies. This analysis revealed decreased insulin staining in the beta-cells of the vehicle-treated diabetic *ob/ob* mice compared with the vehicle-treated non-diabetic *?/+* mice after 4 weeks (11 weeks of age), indicative of impaired beta-cell function in

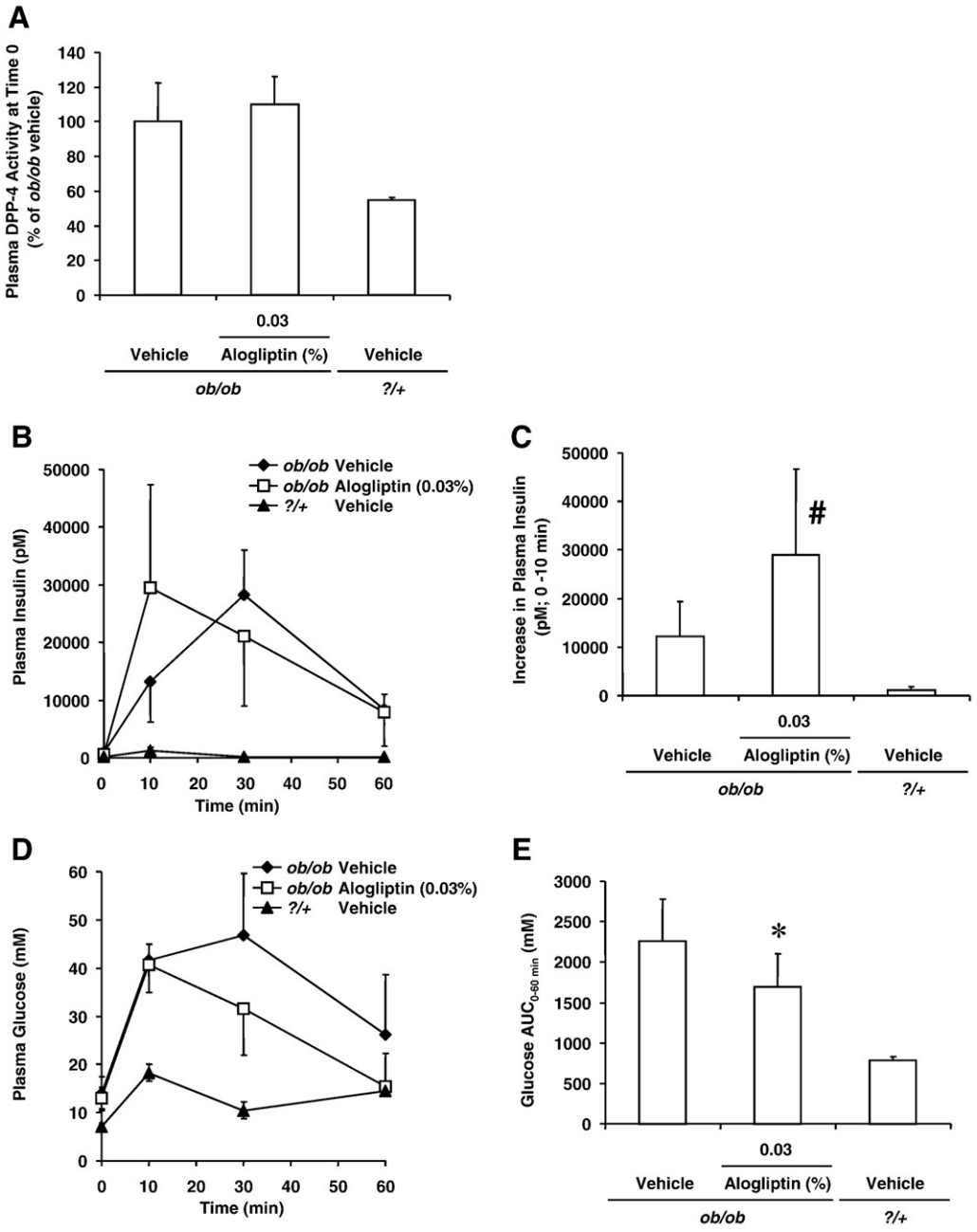

Fig. 2. Effects of chronic alogliptin treatment on oral meal tolerance and insulin secretion in *ob/ob* mice. Mice were fed a CE-2 diet containing 0.03% alogliptin (42.2 mg/kg/day) or a drug-free diet (vehicle) for 4 weeks. Mice were then fed a drug-free diet for 24 h, fasted for 18 h, and given an oral meal load. (A) Plasma DPP-4 activity at time 0 (before oral meal load). (B) Plasma insulin levels. (C) Increase in plasma insulin levels from time 0 to 10 min. (D) Plasma glucose levels. (E) Plasma glucose $AUC_{0-60\ min}$. $^*P \leq 0.05$ vs vehicle-treated *ob/ob* mice by Student's *t*-test. $\#P \leq 0.05$ vs vehicle-treated *ob/ob* mice by Aspin–Welch test. Values are mean ± S.D. ($n=7$ for *ob/ob* mice, $n=4$ for *?/+* mice).

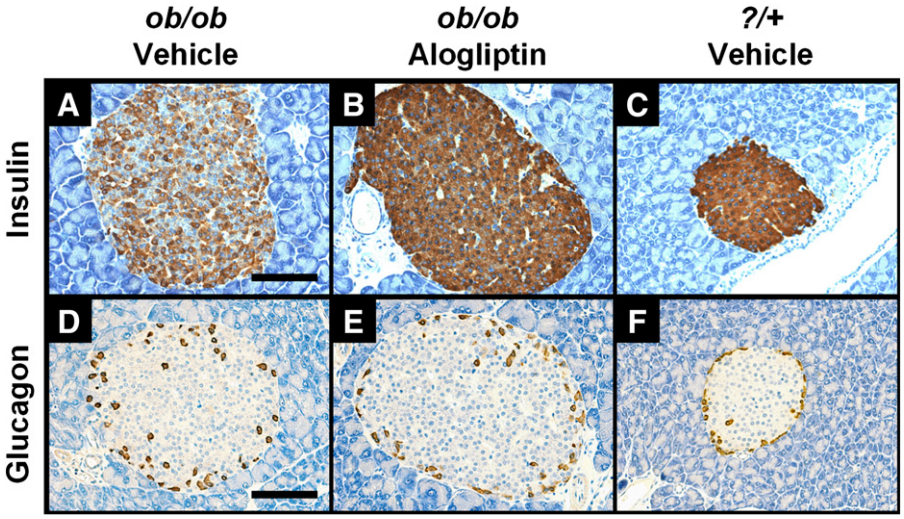

Fig. 3. Effects of chronic alogliptin treatment on pancreatic insulin and glucagon staining in *ob/ob* mice. Mice were fed a CE-2 diet containing 0.03% alogliptin (42.2 mg/kg/day) or a drug-free diet (vehicle) for 4 weeks. Mice were then fed a drug-free diet for 24 h, fasted for 18 h, and given an oral meal load. After the oral meal tolerance test, pancreata were isolated and pancreatic sections were stained with anti-insulin or anti-glucagon antibodies. (A, D) Vehicle-treated *ob/ob* mice. (B, E) 0.03% alogliptin-treated *ob/ob* mice. (C, F) Vehicle-treated *?/+* mice. Scale bar = 100 μm.

the *ob/ob* mice (Fig. 3). In contrast, increased insulin staining in the beta-cells was observed in the alogliptin-treated *ob/ob* mice compared with the vehicle-treated *ob/ob* mice after 4 weeks of treatment (Fig. 3).

Glucagon staining revealed an abnormal distribution of alpha cells in islets in the vehicle-treated diabetic *ob/ob* mice compared with the vehicle-treated non-diabetic *?/+* mice after 4 weeks, consistent with the elevated pancreatic glucagon content and plasma glucagon levels in the *ob/ob* mice. However, the distributions of glucagon-positive cells in islets were similar in the alogliptin-treated and vehicle-treated *ob/ob* mice after 4 weeks of treatment (Fig. 3).

4. Discussion

In the present studies, the effects of chronic treatment with alogliptin on metabolic control and beta-cell function were investigated in *ob/ob* mice, an obese rodent model of type 2 diabetes that exhibits hyperglycemia, glucose intolerance, hyperinsulinemia, and hyperglucagonemia. After 4 weeks of treatment, alogliptin potently and dose-dependently inhibited plasma DPP-4 activity and increased plasma active GLP-1 levels, resulting in improved glycemic control, decreased plasma triglyceride levels, improved glucose tolerance, and improved beta-cell function in the *ob/ob* mice. The degree of improvement in glycemic control observed in the alogliptin-treated *ob/ob* mice was dependent on the extent of plasma DPP-4 inhibition.

The improved glycemic control observed in the alogliptin-treated *ob/ob* mice was associated with not only increased plasma insulin levels but also decreased plasma glucagon levels, both of which were abnormally regulated in the *ob/ob* mice. Studies have revealed that glucagon-induced hepatic glucose production makes an important contribution to the hyperglycemia observed in *ob/ob* mice and other animal models of diabetes (Sloop et al., 2004). Furthermore, accumulating evidence suggests that hyperglucagonemia is a contributing factor for hyperglycemia in patients with type 2 diabetes (Shah et al., 2000; Ahren and Larsson, 2001), and DPP-4 inhibitors have been shown to decrease plasma glucagon levels in these patients (Ahren et al., 2004). Due to the key role of insulin and glucagon in regulating hepatic glucose production, the increased plasma insulin to glucagon ratio induced by alogliptin in the present studies may have contributed to the observed improved glycemic control. In fact, 3.5 h before the dark period, in which both reduced food intake and increased glucose production are thought to be occurring in rodents (Armstrong, 1980), plasma glucose levels in alogliptin-treated *ob/ob* mice were significantly decreased compared with vehicle-treated *ob/ob* mice, indicating that alogliptin may have ameliorated the abnormal regulation of glucose production. Interestingly, chronic alogliptin treatment did not affect pancreatic glucagon content and alpha-cell distribution, both of which were abnormally regulated in the *ob/ob* mice. These observations suggest that the decreased plasma glucagon levels observed with alogliptin treatment are not due to decreased pancreatic glucagon content or to normalization of islet beta/alpha-cell distribution, which is thought to be important for the function of these cells, but are rather due to the increased plasma insulin and active GLP-1 levels, both of which inhibit glucagon secretion (Gromada et al., 2007).

Although *ob/ob* mice exhibit elevated plasma insulin levels, their beta-cells were clearly impaired as evidenced by abnormal insulin staining and decreased membrane localization of glucose-transporter 2 (data not shown). Alogliptin increased non-fasting plasma insulin levels in *ob/ob* mice. Furthermore, alogliptin-treated *ob/ob* mice showed enhanced early-phase insulin secretion and improved glucose tolerance following the oral meal challenge, despite the complete lack of measurable plasma DPP-4 inhibition after drug washout and before challenge. Alogliptin also increased pancreatic insulin content and the staining of insulin in islets with no apparent proliferation and hypertrophy of beta-cells. In addition, alogliptin treatment resulted in a slight but not significant increase in membrane localization of the glucose-transporter 2 (data not shown). Taken together, these results suggest that chronic treatment with alogliptin improved beta-cell function, most likely by preserving or restoring the function of pre-existing pancreatic beta-cells in the *ob/ob* mice.

Evidence suggests that hyperglycemia is a critical contributor to beta-cell deterioration in diabetes, and improved glycemic control has indirectly resulted in improved beta-cell function in *db/db* mice (Kjorholt et al., 2005), Zucker diabetic fatty rats (Harmon et al., 2001), and partially pancreatectomized Sprague–Dawley rats (Jonas et al., 1999). Studies have demonstrated that the beta-cell insulin response to mixed meal and glucose were improved by decreasing glucose levels in patients with type 2 diabetes, regardless of treatment, suggesting that decreasing glucose levels improves beta-cell function (Kosaka et al., 1980; Hidaka et al., 1982; Garvey et al., 1985). In addition, alogliptin was unable to clearly improve glycemic control and beta-cell function in another obese diabetic model, *db/db* mice,

despite the fact that plasma active GLP-1 levels were increased over 4-fold compared with vehicle (manuscript in preparation). Thus, it is possible that the improved beta-cell function observed in the alogliptin-treated *ob/ob* mice may have been mainly an indirect result of improved glycemic control. However, it is also possible that the sustained high levels of GLP-1, which exhibits trophic effects on beta-cells, may have directly contributed to the observed improvement in beta-cell function when glycemia was improved with alogliptin treatment. In fact, GLP-1 receptor expression was restored in animal models of diabetes and beta-cell responsiveness to GLP-1 was induced in patients with type 2 diabetes when glycemic control was improved (Xu et al., 2007; Hojberg et al., 2008). In addition, other substrates of DPP-4 such as GIP and pituitary adenylate cyclase activating polypeptide, both of which have insulinotropic actions and direct trophic effects on beta-cells, may have contributed to both the improved glycemic control and the improved beta-cell function that was observed with alogliptin. Indeed, a DPP-4-resistant GIP analogue has been reported to improve glycemic control in *ob/ob* mice (Irwin et al., 2007).

In addition to improving glycemic control and beta-cell function, alogliptin decreased non-fasting plasma triglyceride levels in *ob/ob* mice. This observation is consistent with the results of recent studies conducted with an analog of sitagliptin in high-fat-diet fed streptozotocin mice (Mu et al., 2006) or with other DPP-4 inhibitors in streptozotocin rats or Zucker diabetic fatty rats (Sudre et al., 2002; Pospisilik et al., 2003), and with a clinical study conducted with the DPP-4 inhibitor vildagliptin where improvements in postprandial lipid profiles were observed in patients with type 2 diabetes after 4 weeks of treatment (Matikainen et al., 2006). There are several possible mechanisms by which alogliptin could have mediated this triglyceride-lowering effect. Alogliptin may have decreased plasma triglyceride levels by an indirect mechanism. Increasing plasma insulin levels (Taskinen, 2003) and improving glycemic control (i.e. reduced availability of glucose for lipogenesis), are two ways in which alogliptin treatment may have resulted in the decreased production of lipoproteins, such as very-low-density lipoprotein, by the liver. In addition, alogliptin treatment may have decreased plasma triglycerides by a direct mechanism. Preclinical studies have shown that GLP-1 reduced intestinal triglyceride absorption and apolipoprotein production in rats (Qin et al., 2005), and that GIP increased chylomicron-associated triglyceride clearance in dogs (Wasada et al., 1981), and reduced post-load triglyceride levels in rats (Ebert et al., 1991). Hence, the triglyceride-lowering effect of alogliptin may have been, at least in part, a direct result of the activation of the incretin axis and an indirect result of overall improved glycemic control.

Interestingly, in the present studies, plasma DPP-4 activity was significantly elevated in the obese diabetic *ob/ob* mice compared with the non-obese non-diabetic *?/+* mice. Studies in patients with type 2 diabetes have yielded equivocal results, revealing elevated plasma DPP-4 activity in those with severe hyperglycemia (Mannucci et al., 2005) and reduced plasma DPP-4 activity in those with mild to moderate hyperglycemia (Meneilly et al., 2000; Korosi et al., 2001). The mechanisms underlying the increased DPP-4 activity in *ob/ob* mice observed in the present studies remain to be determined.

In conclusion, chronic treatment with the DPP-4 inhibitor alogliptin improved glycemic control, decreased plasma triglyceride levels, and improved beta-cell function in *ob/ob* mice, suggesting that alogliptin may also exhibit these beneficial effects when administered to patients with type 2 diabetes.

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