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Administration of pioglitazone alone or with alogliptin delays diabetes onset in UCD-T2DM rats

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Abstract

There is a need to identify strategies for type 2 diabetes prevention. Therefore, we investigated the efficacy of pioglitazone and alogliptin alone and in combination to prevent type 2 diabetes onset in UCD-T2DM rats, a model of polygenic obese type 2 diabetes. At 2 months of age, rats were divided into four groups: control, alogliptin (20 mg/kg per day), pioglitazone (2.5 mg/kg per day), and alogliptin + pioglitazone. Non-fasting blood glucose was measured weekly to determine diabetes onset. Pioglitazone alone and in combination with alogliptin lead to a 5-month delay in diabetes onset despite promoting increased food intake and body weight (BW). Alogliptin alone did not delay diabetes onset or affect food intake or BW relative to controls. Fasting plasma glucose, insulin, and lipid concentrations were lower and adiponectin concentrations were threefold higher in groups treated with pioglitazone. All treatment groups demonstrated improvements in glucose tolerance and insulin secretion during an oral glucose tolerance test with an additive improvement observed with alogliptin + pioglitazone. Islet histology revealed an improvement of islet morphology in all treatment groups compared with control. Pioglitazone treatment also resulted in increased expression of markers of mitochondrial biogenesis in brown adipose tissue and white adipose tissue, with mild elevations observed in animals treated with alogliptin alone. Pioglitazone markedly delays the onset of type 2 diabetes in UCD-T2DM rats through improvements of glucose tolerance, insulin sensitivity, islet function, and markers of adipose mitochondrial biogenesis; however, addition of alogliptin at a dose of 20 mg/kg per day to pioglitazone treatment does not enhance the prevention/delay of diabetes onset.

Key Words

- ▶ pioglitazone
- ▶ type 2 diabetes
- ▶ alogliptin
- ▶ islet

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Introduction

The prevalence of type 2 diabetes is steadily rising with approximately one in ten adults affected in industrialized countries (Danaei *et al.* 2011), necessitating the advent of more effective therapeutic strategies for the prevention

and treatment of type 2 diabetes (DeFronzo & Abdul-Ghani 2011). While the pharmacotherapeutic options for the treatment of type 2 diabetes are expanding and improving, patients suffering from type 2 diabetes

continue to experience a higher risk of developing comorbidities such as cardiovascular disease, cancer, and renal failure (Ritz *et al.* 1999, Czyzyk & Szczepanik 2000, Mazzone *et al.* 2008). Delaying the onset of frank type 2 diabetes will likely delay the development of these long-term complications. The development of multimodal therapies may be more effective in the treatment and prevention of type 2 diabetes and its co-morbidities than monotherapy. Two common drug targets for the treatment of type 2 diabetes are peroxisome proliferator receptor γ (PPAR γ) and glucagon-like peptide 1 (GLP1).

PPAR γ is a nuclear receptor that is highly expressed in adipose tissue and macrophages and acts to upregulate the expression of factors involved in adipocyte differentiation and triglyceride storage (Ferre 2004). PPAR γ is targeted by the thiazolidinedione (TZD) class of drugs (Nolte *et al.* 1998, Rosen & Spiegelman 2001, Semple *et al.* 2006). Agonism of PPAR γ by TZDs leads to decreases in circulating glucose and lipid concentrations and marked improvements of insulin sensitivity in type 2 diabetic patients, making TZDs commonly prescribed for the treatment of type 2 diabetes (Olefsky 2000, Sharma & Staels 2007, Nissen *et al.* 2008). Pioglitazone is a member of the TZD family and is commonly used in the treatment of type 2 diabetes due to its potent glucose-lowering and insulin-sensitizing effects (Olefsky 2000, Semple *et al.* 2006). Furthermore, a recent clinical study demonstrated that pioglitazone treatment of patients with impaired glucose tolerance reduced the risk of conversion to type 2 diabetes by 72%, demonstrating that pioglitazone alone is an effective strategy for delaying type 2 diabetes onset (DeFronzo *et al.* 2011).

GLP1 has become a well-established drug target for the treatment of type 2 diabetes (Drucker & Nauck 2006). We have previously reported that the GLP1 receptor agonist, liraglutide, substantially delays the onset of diabetes in the UCD-T2DM rat model (Cummings *et al.* 2010). Endogenous GLP1 has a short half-life in the circulation of <2 min due to rapid degradation by DPP-IV (Baggio & Drucker 2007). Therefore, DPP-IV inhibitors, such as alogliptin, have been developed in order to prolong the activity of endogenous GLP1 (Feng *et al.* 2007, Thomas *et al.* 2008). As an incretin hormone, GLP1 potentiates glucose-stimulated insulin secretion (Baggio & Drucker 2007). Furthermore, GLP1 has been shown to lower glucagon secretion, preserve β -cell mass, and improve insulin sensitivity, independent of its effects on insulin secretion (Brubaker & Drucker 2004, Baggio & Drucker 2007). Alogliptin has proven effective for improving glucose and lipid homeostasis in rodent models of type 2 diabetes

and in type 2 diabetic patients (DeFronzo *et al.* 2008, Moritoh *et al.* 2008, Pratley *et al.* 2009a, Zhang *et al.* 2011).

These complementary mechanisms of action suggest that alogliptin and pioglitazone given in combination may act synergistically to improve glucose and lipid homeostasis. Indeed, studies on rodents and clinical studies on type 2 diabetic patients report that addition of alogliptin to pioglitazone monotherapy results in additive effects to lower blood glucose concentrations (Moritoh *et al.* 2009, Pratley *et al.* 2009b, Rosenstock *et al.* 2010, DeFronzo *et al.* 2012). However, the efficacy of alogliptin alone and in combination with pioglitazone to delay the onset of type 2 diabetes has not been previously investigated. Therefore, we used the UCD-T2DM rat model to test the hypothesis that pioglitazone and alogliptin in combination would be more effective in delaying type 2 diabetes onset in prediabetic UCD-T2DM rats than either treatment alone. The UCD-T2DM rat model develops adult-onset polygenic obesity, insulin resistance, and subsequent type 2 diabetes (Cummings *et al.* 2008).

Materials and methods

Diets and animals

Male UCD-T2DM rats were individually housed in wire cages in the Department of Nutrition animal facility at the University of California, Davis, and maintained on a 14 h light:10 h darkness cycle. Starting at 2 months of age, male siblings were divided into four groups: control, alogliptin (20 mg/kg per day), pioglitazone (2.5 mg/kg per day), and alogliptin + pioglitazone ($n=32$ per group). Groups were matched for weight at the initiation of treatment and all animals received ground chow (no. 5012, Ralston Purina, Belmont, CA, USA). Drug compounds were mixed into the ground chow such that animals received the appropriate daily dose throughout the study. Food intake and body weight (BW) were measured three times a week. Non-fasting blood glucose was monitored every week with a glucose meter (One-Touch Ultra, LifeScan, Milpitas, CA, USA) at 1300–1400 h using a lancet to collect a drop of blood from the tail. Diabetes onset was defined as a non-fasted blood glucose value above 11.1 mmol/l on 2 consecutive weeks (Cummings *et al.* 2008). One half of the animals in each group ($n=16$) were killed for tissue collection at 6.5 months of age (short-term study), and the remaining half ($n=16$) continued treatment up to 1 year of age (long-term study). Monthly blood samples were collected up to 8 months of age. An oral glucose tolerance test (OGTT; 1 g/kg BW gavage with dextrose) was conducted after

3.5 months of treatment. The experimental protocols were approved by the UC Davis Institutional Animal Care and Use Committee. Standard methods for plasma and tissue analyses are described in detail in the [Supplemental Materials and Methods](#), see section on supplementary data given at the end of this article.

Statistics and data analysis

Data are presented as mean \pm s.e.m. Statistical analyses were performed using GraphPad Prism 4.00 for Windows, GraphPad Software (San Diego, CA, USA). All time course data were compared by two-factor repeated measures ANOVA followed by *post hoc* analysis with Bonferroni's multiple comparison test. Incidence data were analyzed by log-rank testing of Kaplan–Meier survival curves. Age of onset, OGTT incremental area under the curve (AUC), tissue weights, and tissue measurements were analyzed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Pioglitazone delays type 2 diabetes onset alone and in combination with alogliptin

Compared with control animals, pioglitazone administration alone and in combination with alogliptin delayed type 2 diabetes onset by ~ 5 months (Fig. 1A).

In the long-term study, the average age of diabetes onset was 6.0 ± 0.6 , 5.8 ± 0.5 , 11.0 ± 0.6 , and 11.1 ± 0.6 months in control, alogliptin-, pioglitazone-, and alogliptin + pioglitazone-treated animals respectively ($P < 0.001$). In the long-term study, all animals in the control and alogliptin-treated groups developed diabetes whereas only 43.8% (7/16) of animals in the pioglitazone and 37.5% (6/16) of animals in the alogliptin + pioglitazone-treated groups developed diabetes. When data from animals up to 6 months of age in the long- and short-term studies were combined, the incidence of diabetes up to 6 months of age was 0% for both the pioglitazone- and alogliptin + pioglitazone-treated groups, whereas diabetes incidence was 53.1% (17/32) and 59.4% (19/32) in the control and alogliptin-treated groups respectively (Fig. 1B). The delay of diabetes onset in animals treated with pioglitazone alone and in combination with alogliptin was reflected in lower fasting and fed circulating glucose concentrations and lower HbA1c concentrations at 5 and 6 months of age compared with the control group ($P < 0.001$; Fig. 1C, D and E).

Pioglitazone alone and in combination with alogliptin led to a marked delay in diabetes onset despite its well-known effects to increase food intake, BW, and adiposity. Pioglitazone alone and in combination with alogliptin led to a $\sim 10\%$ increase in food intake between 2 and 4.5 months of age and increased BW throughout the first 6 months of study ($P < 0.01$; Fig. 2A, B and C).

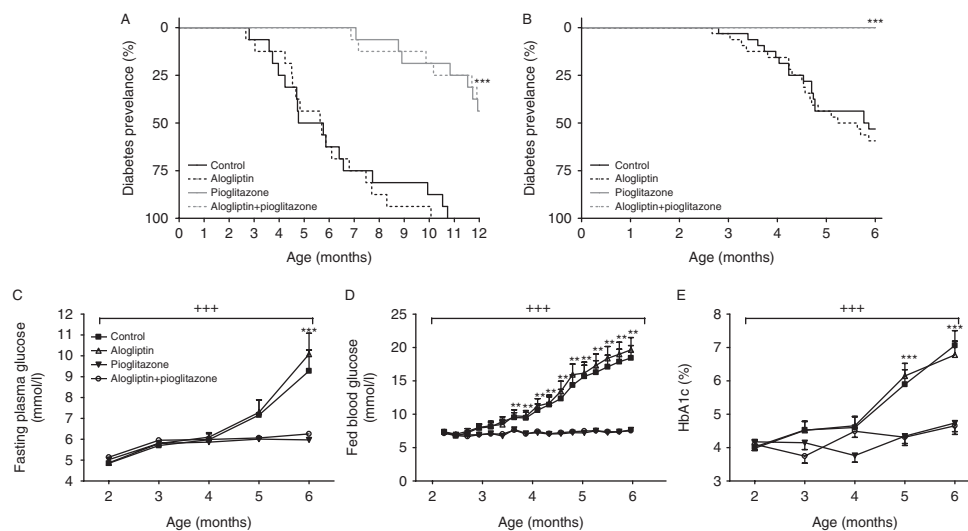


Figure 1

Kaplan–Meier analysis of diabetes incidence in control, alogliptin-, pioglitazone-, and alogliptin + pioglitazone-treated animals up to 12 months ($n = 16$ per group) (A) and 6 months ($n = 32$ per group) (B) of age. $***P < 0.0001$ compared with control and alogliptin by log-rank test.

Fasting plasma glucose (C), non-fasting blood glucose (D), and fasting HbA1c (E). $+++P < 0.001$ by two-factor repeated measures ANOVA, $***P < 0.001$, and $**P < 0.01$ compared with control and alogliptin by Bonferroni's posttest.

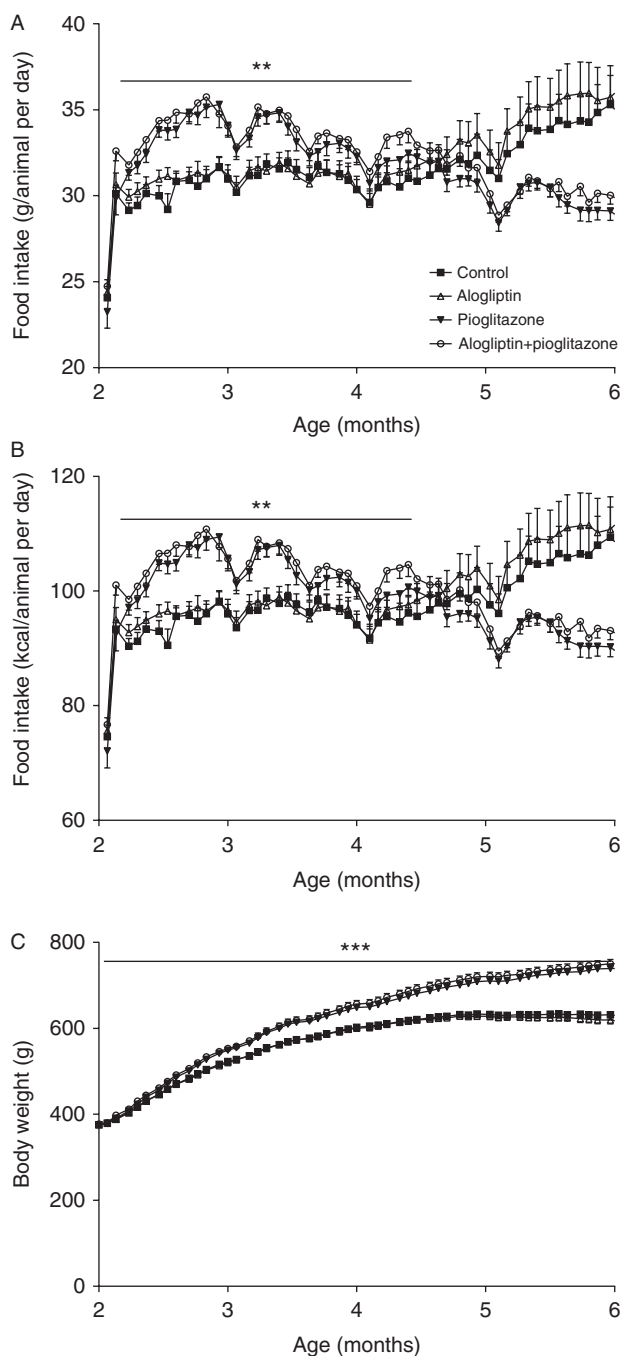


Figure 2 Food intake (g/animal per day) (A), food intake (kcal/animal per day) (B), and body weight (C). *** $P < 0.001$ and ** $P < 0.01$ by two-factor repeated measures ANOVA and Bonferroni's posttest compared with control and alogliptin, $n = 32$ per group.

Alogliptin treatment did not affect food intake or BW compared with the control group. Food intake increased in the control and alogliptin-treated animals starting at 5 months of age because diabetes prevalence in these groups had increased resulting in diabetic hyperphagia,

as previously reported (Cummings *et al.* 2008). The epididymal, retroperitoneal, subcutaneous, and total white adipose tissue (WAT) weights were significantly elevated in pioglitazone and alogliptin+pioglitazone groups compared with the control group ($P < 0.001$); however, mesenteric adipose depot weight did not differ between groups (Table 1).

Pioglitazone alone and in combination with alogliptin reduces circulating insulin and lipid concentrations

Similar to previous studies demonstrating that pioglitazone markedly improves insulin sensitivity (Ferre 2004, Kim & Ahn 2004), pioglitazone alone and in combination with alogliptin significantly reduced fasting plasma insulin concentrations (Fig. 3A). Insulin concentrations began to fall in control and alogliptin-treated groups at 5 and 6 months of age because a large proportion of these animals had developed diabetes and were undergoing pancreatic β -cell decompensation with the progression of their diabetes, as previously described (Cummings *et al.* 2008). Furthermore, pioglitazone alone and in combination with alogliptin resulted in a threefold increase in circulating adiponectin concentrations (Fig. 3B) with corresponding increases in AMPK phosphorylation (Thr¹⁷²) in subcutaneous WAT and brown adipose tissue (BAT) (Fig. 3C). Interestingly, animals treated with alogliptin alone exhibited a significant increase in AMPK phosphorylation in both subcutaneous BAT and WAT compared with controls; however, this enhancement of AMPK signaling was significantly lower than that seen in pioglitazone-treated animals ($P < 0.01$). Pioglitazone treatment resulted in a twofold increase in fasting plasma leptin concentrations compared with controls, which was likely due to the expansion of adiposity (Fig. 3D). Interestingly, pioglitazone and alogliptin in combination significantly reduced fasting plasma glucagon concentrations after 2 and 3 months of treatment compared with controls and compared with pioglitazone and alogliptin alone (Fig. 3E).

Pioglitazone treatment alone and in combination with alogliptin resulted in improvements of lipid metabolism including marked reductions of fasting plasma free fatty acid (FFA), cholesterol, and triglyceride (TG) concentrations ($P < 0.001$; Fig. 4A, B and C). Enzymes involved in the regulation of fatty acid oxidation and lipogenesis were measured in subcutaneous WAT and BAT by immunoblotting in order to perform an initial investigation of the molecular basis for the observed improvement of circulating lipid concentrations with pioglitazone treatment. Phosphorylation and inactivation of acetyl-CoA carboxylase

Table 1 Tissue weights, tissue TG content, and pancreatic insulin and glucagon content. Values are mean \pm s.e.m. ($n = 16$)

	Control	Alogliptin	Pioglitazone	Alogliptin + Pioglitazone
Epididymal fat depot (g)	8.1 \pm 0.4	7.0 \pm 0.4*	10.9 \pm 0.6 [‡]	12.0 \pm 0.7 [‡]
Retroperitoneal fat depot (g)	12.9 \pm 0.6	10.9 \pm 0.8*	22.8 \pm 1.4 [‡]	23.3 \pm 1.2 [‡]
Subcutaneous depot (g)	45.8 \pm 2.8	40.1 \pm 3.9	85.1 \pm 6.5 [‡]	94.7 \pm 5.0 [‡]
Mesenteric depot (g)	7.2 \pm 0.5	5.7 \pm 0.6	7.8 \pm 0.4	8.1 \pm 0.4
Total white adipose tissue (g)	74.8 \pm 4.0	64.5 \pm 5.6	129.4 \pm 8.6 [‡]	141.1 \pm 7.2 [‡]
Heart (g)	1.6 \pm 0.1	1.5 \pm 0.1	1.7 \pm 0.1*	1.7 \pm 0.1*
Kidney (g)	2.0 \pm 0.1	2.0 \pm 0.1	1.9 \pm 0.1*	1.9 \pm 0.1
Liver (g)	21.1 \pm 0.5	20.5 \pm 0.4	17.8 \pm 0.3 [‡]	18.8 \pm 0.4 [‡]
Liver TG (μ mol/g tissue)	25.2 \pm 3.2	19.6 \pm 4.0	21.0 \pm 2.0	19.8 \pm 1.9
Skeletal muscle TG (μ mol/g tissue)	4.2 \pm 0.7	3.0 \pm 0.5	7.6 \pm 0.9 [†]	7.4 \pm 0.7 [†]
Pancreas insulin (μ mol/g tissue)	3.4 \pm 0.9	2.7 \pm 0.8	4.9 \pm 0.5	5.3 \pm 0.5*
Pancreas glucagon (pmol/g tissue)	95 \pm 17	62 \pm 8*	72 \pm 11	60 \pm 11*

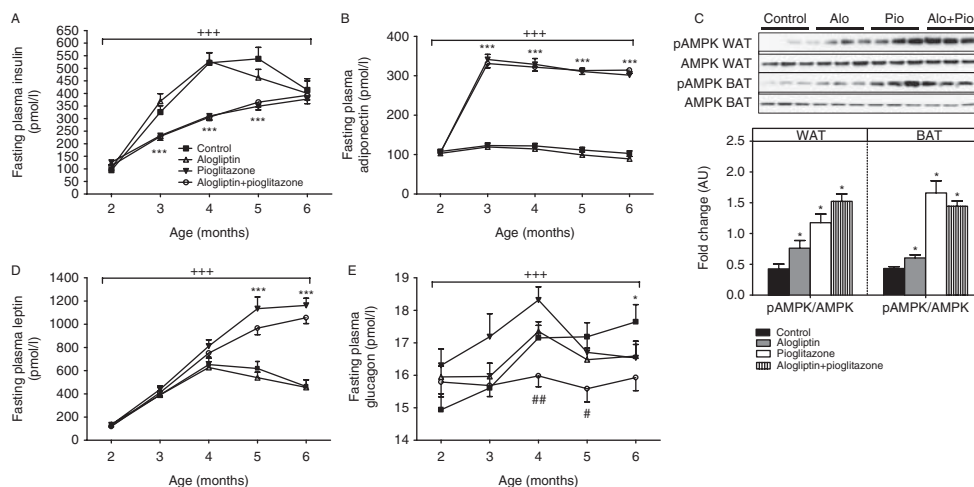
* $P < 0.05$, [†] $P < 0.01$, and [‡] $P < 0.001$ compared with control by one-factor ANOVA.

(ACC Ser⁷⁹) was elevated in pioglitazone-treated animals in both BAT and WAT compared with controls ($P < 0.05$), suggesting an enhancement of fatty acid oxidation (Fig. 4D and E). Inactive phosphorylated ACC (Ser⁷⁹) was also significantly elevated in alogliptin-treated animals in BAT only ($P < 0.05$; Fig. 4D). Similar to a previous report in humans (Ranganathan *et al.* 2006), fatty acid synthase (FAS) protein expression was elevated with pioglitazone treatment in both BAT and WAT, suggesting an enhancement of lipogenesis in adipose tissue. Liver TG content did not differ significantly between groups (Table 1); however, when diabetic animals were excluded from the control group, liver TG concentrations were significantly elevated in control

animals (control = 29.7 \pm 3.8 μ mol TG/g tissue) compared with pioglitazone and alogliptin + pioglitazone ($P < 0.05$). By contrast, skeletal muscle TG concentrations were significantly elevated in pioglitazone- and alogliptin + pioglitazone-treated animals (Table 1). This difference persisted when diabetic animals were excluded from the analysis.

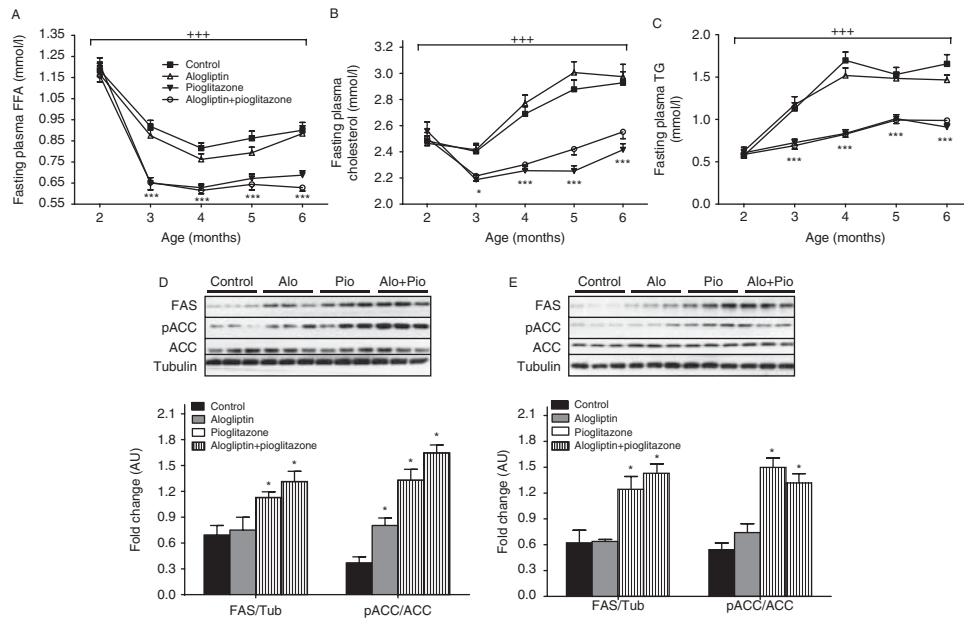
Pioglitazone and alogliptin improve glucose tolerance and insulin secretion

Based on the significant improvements of fasting plasma glucose concentrations with pioglitazone treatment, we investigated potential improvements of glucose tolerance

**Figure 3**

Fasting plasma insulin (A), adiponectin (B), leptin (D), and glucagon (E) concentrations. ⁺⁺⁺ $P < 0.001$ by two-factor repeated measures ANOVA, ^{***} $P < 0.001$ compared with control and alogliptin, ^{##} $P < 0.01$, and [#] $P < 0.05$ for alogliptin + pioglitazone compared with control, alogliptin, and pioglitazone by Bonferroni's posttest, $n = 32$ per group. Representative

immunoblots for AMPK and pAMPK (Thr¹⁷²) in subcutaneous white adipose tissue (WAT) and brown adipose tissue (BAT). All blots were scanned and quantified using FluorChem 9900. Results were quantified in densitometric units and expressed relative to AMPK (C). * $P < 0.05$ compared with control by Student's *t*-test, $n = 16$ per group.

**Figure 4**

Fasting plasma FFA (A), cholesterol (B), and TG (C) concentrations.

+++ $P < 0.001$ by two-factor repeated measures ANOVA, *** $P < 0.001$, and * $P < 0.05$ compared with control and alogliptin by Bonferroni's posttest, $n = 32$ per group. Representative immunoblots for FAS, pACC (Ser⁷⁹), ACC

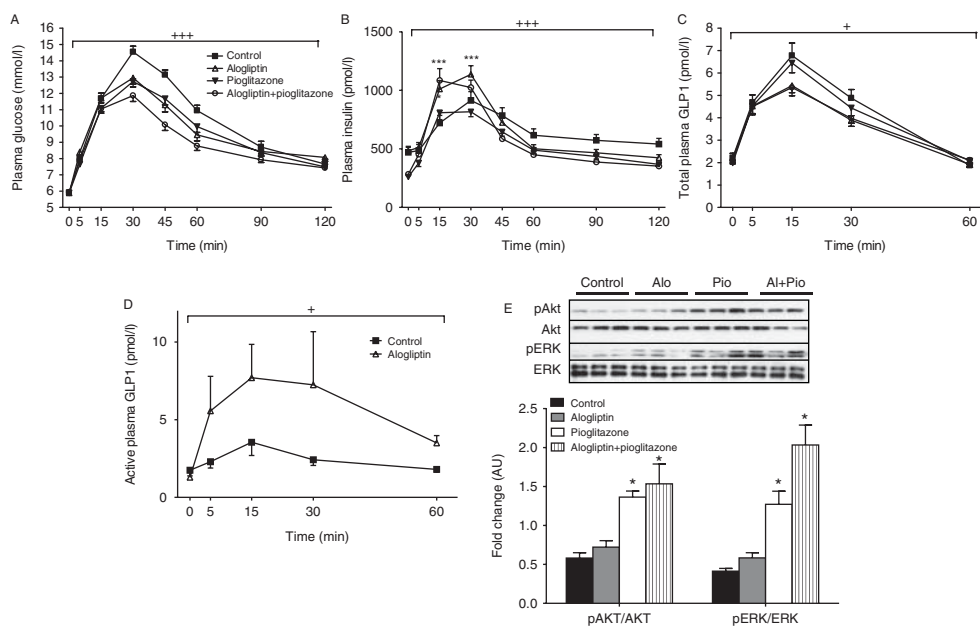
and tubulin. All blots were scanned and quantified using FluorChem 9900. Results were quantified in densitometric units and expressed relative to the protein of interest or to tubulin for FAS in subcutaneous BAT (D) and WAT (E). * $P < 0.05$ compared with control by Student's *t*-test, $n = 16$ per group.

and glucose-stimulated insulin secretion by OGTT (at 5.5 months of age). Glucose excursions during the OGTT were significantly reduced in all treatment groups compared with controls. However, the glucose AUC was significantly lower in animals treated with pioglitazone and alogliptin in combination compared with either pioglitazone or alogliptin alone, suggesting that pioglitazone and alogliptin act in an additive manner to improve glucose tolerance (Fig. 5A; glucose AUC: control = 549 ± 30 , alogliptin = 468 ± 34 , pioglitazone = 458 ± 28 , and alogliptin + pioglitazone = 376 ± 24 mmol/l \times 120 min; $P < 0.05$).

While fasting plasma insulin concentrations at baseline were significantly lower in animals treated with pioglitazone alone and in combination with alogliptin, the insulin AUC was significantly higher in animals treated with pioglitazone alone and in combination with alogliptin compared with control animals (insulin AUC: control = $22\,039 \pm 3506$, alogliptin = $19\,268 \pm 3075$, pioglitazone = $33\,946 \pm 2484$, and alogliptin + pioglitazone = $34\,857 \pm 2510$ pmol/l \times 120 min; $P < 0.05$). However, insulin concentrations were significantly lower in pioglitazone- and pioglitazone + alogliptin-treated animals at multiple time points and all three treatment groups demonstrated a significantly greater percent increase in circulating insulin concentrations from baseline to peak

values (% change from baseline: control = $118 \pm 14\%$, alogliptin = $206 \pm 35\%$, pioglitazone = $319 \pm 49\%$, and alogliptin + pioglitazone = $458 \pm 91\%$; $P < 0.05$; Fig. 5B).

GLP1 concentrations were measured during the OGTT in order to identify potential influences of chronic alogliptin and pioglitazone treatment on endogenous postprandial GLP1 secretion. Surprisingly, the GLP1 AUC was significantly lower in animals treated with alogliptin alone and in combination with pioglitazone (GLP1 AUC: control = 130 ± 13 , alogliptin = 93 ± 10 , pioglitazone = 131 ± 13 , and alogliptin + pioglitazone = 101 ± 11 pmol/l \times 120 min; $P < 0.05$; Fig. 5C). In order to ensure that alogliptin treatment was prolonging the half-life of endogenously secreted active GLP1, the acute effects of alogliptin administration on plasma active GLP1 concentrations was measured in a separate group of male UCD-T2DM rats. Rats were fasted overnight and the next morning they received a gavage of dextrose (1 g/kg dextrose) with or without alogliptin (1 mg/kg BW) and active GLP1 was measured. Animals receiving alogliptin exhibited a twofold higher active GLP1 AUC than control animals, demonstrating that UCD-T2DM rats exhibit appropriate postprandial increases in active GLP1 (active GLP1 AUC: control = 148 ± 19 pmol/l \times 60 min and alogliptin = 317 ± 90 pmol/l \times 60 min; $P < 0.05$; Fig. 5D).

**Figure 5**

Circulating glucose (A), insulin (B), and total GLP1 (C) concentrations during an OGTT at 3.5 months after the initiation of treatment. Animals were fasted overnight and received a 1 g/kg gavage of a 50% dextrose solution. $^{+++}P < 0.001$ for all groups compared with control, and $^{+}P < 0.05$ for alogliptin and alogliptin + pioglitazone compared with control by one-factor ANOVA of the AUC. $^{***}P < 0.05$ by one-factor ANOVA of the percent increase in insulin from baseline to peak values compared with the control group, $n = 32$ per group. Plasma active GLP1 in a separate group of male UCD-T2DM rats ($n = 4$). Rats were fasted overnight and then received a

gavage of dextrose (1 g/kg) with or without alogliptin mixed in with the dextrose to provide a dose of 1 mg alogliptin/kg body weight. $^{+}P < 0.05$ compared with control by Student's t -test of the AUC (D). Representative immunoblots for pAkt (Ser⁴⁷³), Akt, pERK (Thr²⁰²/Tyr²⁰⁴), and ERK in white adipose tissue. All blots were scanned and quantified using FluorChem 9900. Results were quantified in densitometric units and expressed relative to the protein of interest (E). $^{*}P < 0.05$ compared with control by Student's t -test, $n = 16$ per group.

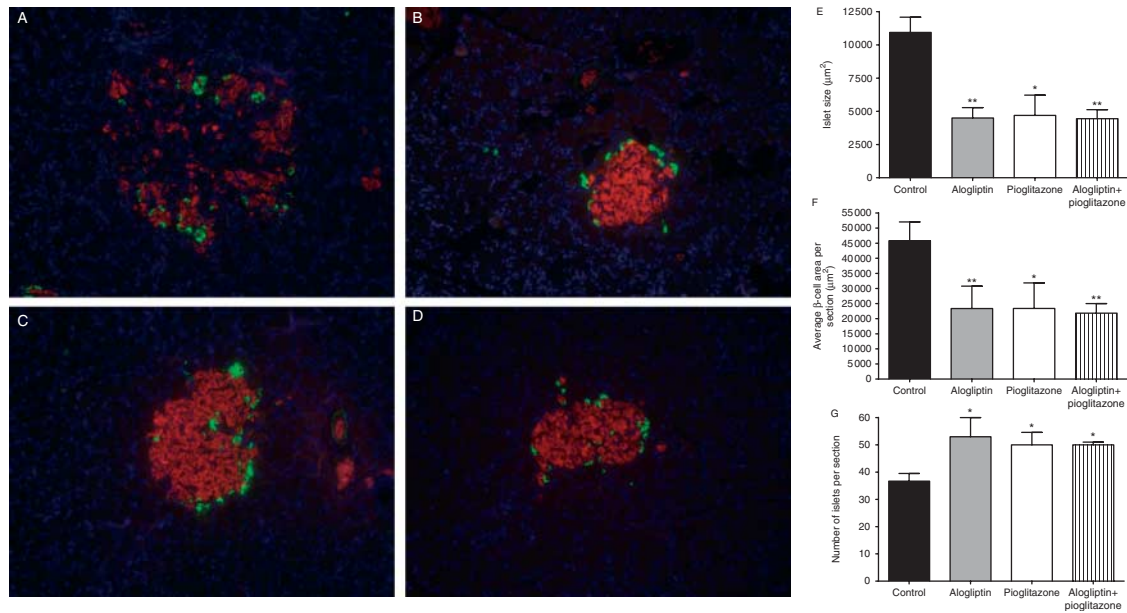
In order to investigate the molecular basis for enhanced insulin sensitivity in pioglitazone-treated animals, we analyzed downstream components of insulin signaling pathways in subcutaneous WAT of fasted animals at study termination (6.5 months of age). Akt (Ser⁴⁷³) and ERK1/2 (Thr²⁰²/Tyr²⁰⁴) phosphorylation, normalized to their protein expression, were two- to threefold higher in WAT in animals treated with pioglitazone compared with controls ($P < 0.05$; Fig. 5E). Alogliptin did not significantly affect Akt or ERK1/2 phosphorylation.

Pioglitazone and alogliptin treatment preserve islet morphology

In order to investigate the potential effects of pioglitazone and alogliptin on β -cell mass and islet morphology, immunostaining for pancreatic insulin and glucagon was performed in a subset of animals killed at 6.5 months of age. Cohorts in which none of the animals had developed diabetes were specifically selected for immunohistochemistry in order to avoid confounding by the presence

of diabetes. In general, islets from all the three treatment groups appeared smaller with better preservation of islet architecture than islets from control animals (Fig. 6A, B, C and D). Quantification of β -cell mass, average islet size, and average number of islets per section revealed that control animals exhibited greater β -cell mass with larger islets and fewer islets per section compared with all the three treatment groups ($P < 0.05$; Fig. 6E, F and G).

As a confirmation of the immunohistochemistry data set, insulin and glucagon were extracted from whole pancreas samples as an index of β -cell and α -cell mass. When all animals were included in the analysis, pancreatic insulin content was significantly higher in pioglitazone- and pioglitazone + alogliptin-treated groups compared with the control and alogliptin treatment groups (Table 1). However, consistent with the islet immunohistochemistry results, when diabetic animals were excluded from the analysis, pancreatic insulin content was significantly higher in the control animals compared with pioglitazone- and pioglitazone + alogliptin-treated groups (excluding diabetic animals: control = $7.3 \pm 0.9 \mu\text{mol/g}$ pancreas; $P < 0.05$).

**Figure 6**

Representative images from pancreas sections from prediabetic control, alogliptin-, pioglitazone-, and alogliptin + pioglitazone-treated animals. Insulin immunostaining is in red, glucagon is in green, and nuclei are stained blue with DAPI. Merged insulin, glucagon, and DAPI staining in control (A), alogliptin (B), pioglitazone (C), and alogliptin + pioglitazone (D).

Interestingly, pancreatic glucagon content was significantly lower in animals treated with alogliptin alone and in combination with pioglitazone compared with controls ($P < 0.05$).

Pioglitazone increases markers of mitochondrial biogenesis

In order to determine whether changes in adipose tissue mitochondrial biogenesis may be contributing to the improvements of glucose tolerance with pioglitazone and alogliptin treatment, markers of energy uncoupling and mitochondrial biogenesis were measured in subcutaneous WAT and BAT by RT-PCR. All the three treatment groups exhibited significant increases in uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1 α), and cytochrome *c* oxidase subunit 8b (*Cox8b*) mRNA in BAT and WAT (Fig. 7A and B). UCP1 is an inner mitochondrial membrane transporter of FFAs that allows for the dissipation of energy as heat (Cardoso *et al.* 2010). PGC1 α is a key transcriptional regulator of oxidative metabolism and *Cox8b* is a gene involved in mitochondrial oxidative phosphorylation that is upregulated by PGC1 α . Increases in markers of mitochondrial biogenesis were more pronounced in pioglitazone-treated animals than in

Quantification of average islet size (E), average β -cell area per section (F), and average number of islets per section (G); ** $P < 0.01$ and * $P < 0.05$ compared with control by Student's *t*-test, $n = 3$ per group. Ten sections per pancreas used for quantification.

animals receiving alogliptin alone ($P < 0.05$). Addition of alogliptin to pioglitazone treatment did not produce an additional elevation in UCP1 or PGC1 α expression. However, alogliptin and pioglitazone in combination resulted in higher *Cox8b* mRNA levels than either alogliptin or pioglitazone alone ($P < 0.05$).

Discussion

Results from previous studies have suggested that pioglitazone and alogliptin in combination may act synergistically to provide greater glucose-lowering effects than either agent alone (DeFronzo *et al.* 2008, Pratley *et al.* 2009a, Rosenstock *et al.* 2009, Kaku *et al.* 2011, Eliasson *et al.* 2012). However, the efficacy of alogliptin alone and in combination with pioglitazone to delay the onset of type 2 diabetes has not been previously assessed. In this study, we report that while pioglitazone and alogliptin both produced metabolic benefits, the addition of alogliptin at a dose of 20 mg/kg per day to pioglitazone treatment did not produce a greater delay in diabetes onset than pioglitazone alone. Furthermore, alogliptin did not produce a delay in diabetes onset at this dose on its own. Of note, only one dose of alogliptin was tested in this study. While this dose is similar to or higher than doses of

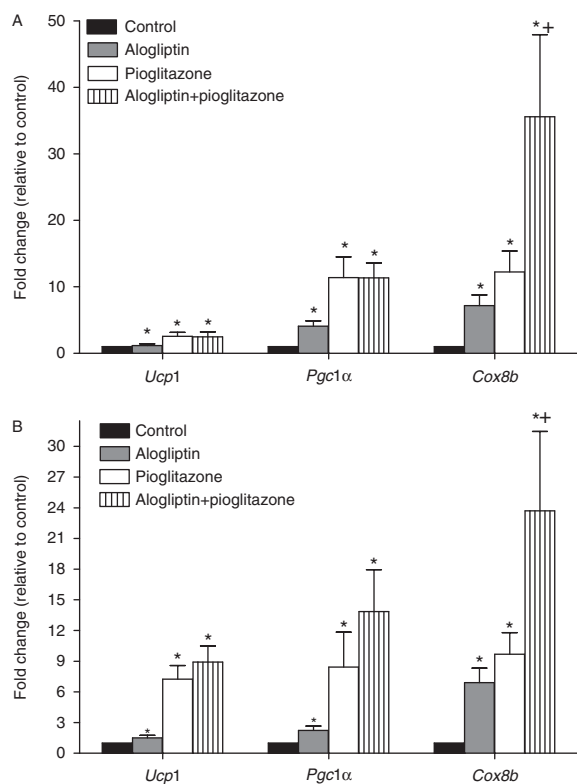


Figure 7 mRNA of *Ucp1*, *Pgc1α*, and *Cox8b* in subcutaneous BAT (A) and WAT (B), normalized to β -actin mRNA. * $P < 0.05$ compared with control and + $P < 0.05$ compared with pioglitazone alone by Student's *t*-test, $n = 16$ per group.

alogliptin employed in most previously published rodent studies (Feng *et al.* 2007, Lee *et al.* 2008, Asakawa *et al.* 2009), there is a possibility that the efficacy of alogliptin would have been enhanced at a higher dose. By contrast, pioglitazone alone and in combination with alogliptin delayed type 2 diabetes onset by ~ 5 months compared with control UCD-T2DM rats.

The effect of pioglitazone to delay diabetes onset was independent of food intake and BW as both were higher in pioglitazone-treated animals compared with controls. Numerous studies on rodents and humans have reported that TZDs increase BW by increasing adipogenesis, fluid retention, and food intake (Shimizu *et al.* 1998, Lehrke *et al.* 2005). Similar to previous studies, alogliptin did not affect food intake or BW (Kawashima *et al.* 2011, Shah *et al.* 2011). Increases of adiposity in pioglitazone-treated animals primarily involved the subcutaneous adipose depot and not the mesenteric adipose depot. Previous studies have shown that the effect of TZDs to increase adiposity is primarily restricted to the subcutaneous adipose depot, sparing the more metabolically detrimental

visceral depot, allowing for improved insulin sensitivity in the face of increased adiposity (Miyazaki *et al.* 2002).

Pioglitazone and alogliptin appear to act in an additive manner to improve glucose tolerance and insulin secretion during the OGTT. Furthermore, fasting plasma insulin concentrations were lower and activation of downstream insulin signaling proteins were higher in pioglitazone-treated animals, suggesting that improvements of insulin sensitivity likely contributed to the improvement of glucose tolerance with pioglitazone treatment. The effect of pioglitazone treatment to improve insulin sensitivity has been previously described in both human clinical studies and studies on rodents (Olefsky 2000, Ferre 2004). We hypothesize that the decrease in GLP1 secretion during the OGTT in alogliptin-treated animals is likely due to downregulation of GLP1 secretion over time following repeated increases in postprandial GLP1 secretion.

Islet immunohistochemistry revealed marked improvements of islet morphology in all treatment groups compared with the control group. We hypothesize that β -cell mass was lower in the groups treated with pioglitazone because these groups were more insulin sensitive resulting in less islet hypertrophy. The improvement of islet morphology in alogliptin-treated animals was surprising in light of alogliptin's lack of effect on diabetes onset. This suggests that the development of type 2 diabetes in the UCD-T2DM rat is, at least initially, more heavily reliant on development of insulin resistance. The effect of alogliptin to reduce pancreatic glucagon content is in line with previous studies reporting that GLP1 signaling results in decreased glucagon secretion from α -cells, partially indirectly due to GLP1-induced pancreatic somatostatin secretion (Fehmann & Habener 1991). The effect of alogliptin to reduce pancreatic glucagon likely contributed to the effect of alogliptin in combination with pioglitazone to reduce circulating glucagon concentrations and represents a metabolic benefit of addition of alogliptin to pioglitazone therapy.

Another potential mechanism by which pioglitazone treatment delayed diabetes onset is by increasing circulating adiponectin concentrations leading to subsequent increases in AMPK activation. Activation of AMPK promotes glucose uptake and lipid oxidation and inhibits glucose and lipid production (Long & Zierath 2006). We confirmed that pioglitazone treatment increases AMPK activation with corresponding increases in phosphorylation and inactivation of ACC in BAT and subcutaneous WAT. Previous studies have demonstrated that AMPK can inactivate ACC resulting in reduced malonyl-CoA concentrations, which enhances the movement of long-chain acyl-CoA into the

mitochondria for β -oxidation (Winder & Hardie 1996, Winder *et al.* 1997). These findings suggest that pioglitazone treatment promotes an increase in fatty acid oxidation in adipose tissue, which likely contributed to the decreases in circulating and liver lipid concentrations; however, further studies are needed to confirm these findings. This reduction of hepatic lipid deposition likely contributed to the improvement of insulin sensitivity with pioglitazone treatment as lipid deposition in liver has been shown to inhibit insulin signaling through promotion of serine phosphorylation of insulin receptor substrate proteins (Samuel & Shulman 2012). Surprisingly, TG deposition was increased in skeletal muscle in pioglitazone-treated animals compared with controls despite improvements of fasting plasma insulin concentrations and glucose tolerance observed with pioglitazone treatment. Interestingly, a previous study on human type 2 diabetic patients reports that treatment with pioglitazone improves insulin sensitivity without altering skeletal muscle TG deposition (Rabøl *et al.* 2010). Furthermore, a previous study in sucrose-fed rats reports an increase in muscle TG deposition with pioglitazone treatment (Markova *et al.* 2010). By contrast, pioglitazone treatment also appears to enhance fatty acid synthesis as indicated by increased protein expression of FAS in BAT and WAT. This is similar to a previous report on humans and suggests that pioglitazone also reduces hepatic ectopic lipid deposition by diverting lipid into adipose stores (Ranganathan *et al.* 2006). In line with this finding, studies have shown that increases in *de novo* lipogenesis in WAT improve insulin sensitivity (Cao *et al.* 2008, Roberts *et al.* 2009, Eissing *et al.* 2013).

Interestingly, alogliptin treatment also resulted in a small but significant elevation of AMPK activation in BAT and subcutaneous WAT and inactivation of ACC in BAT. However, alogliptin treatment did not produce decreases in circulating lipid concentrations or ectopic lipid deposition, similar to what has been reported in previous clinical studies (Bosi *et al.* 2011). This suggests that these modest elevations of AMPK activity are not sufficient to lower circulating lipids or ectopic lipid deposition.

Increases in markers of mitochondrial biogenesis in WAT and BAT may have also contributed to the delay in diabetes onset in pioglitazone-treated animals. BAT is highly thermogenic leading to the loss of energy as heat and subsequent BW loss. Recent reports have revealed that WAT has the capacity to express a BAT-like phenotype under certain conditions such as cold exposure, β -adrenergic stimulation, and PPAR γ agonism (Kajimura *et al.* 2010). Results from previous studies indicate that PPAR γ agonists can enhance the thermogenic activity of

BAT and can induce a WAT-to-BAT conversion by activating PPAR γ and PPAR response elements on the promoter and/or enhancer region of brown adipose genes (Sears *et al.* 1996, Ohno *et al.* 2012). Increased markers of mitochondrial biogenesis in BAT and WAT with pioglitazone treatment corresponded with increased markers of β -oxidation. Previous studies have reported that pioglitazone does not increase energy expenditure despite its effects on mitochondrial biogenesis but that it does increase the capacity for sympathetically mediated energy expenditure in rodents (Sell *et al.* 2004). However, increases in mitochondrial biogenesis may have also contributed to the improvements of lipid metabolism with pioglitazone treatment by favoring the use of lipids for energy through β -oxidation.

Interestingly, alogliptin-treated animals also exhibited increases in markers of mitochondrial biogenesis compared with control animals, although these increases were less dramatic than those observed in pioglitazone-treated animals. These results are similar to those from a previous report that another DDP-IV inhibitor, des-fluorositagliptin, increases mRNA expression of UCP1 and PGC1 α in BAT (Shimasaki *et al.* 2013).

In conclusion, we have demonstrated that pioglitazone markedly delays the onset of type 2 diabetes in the UCD-T2DM rat and that this delay in onset is not enhanced by co-administration with alogliptin. Alogliptin treatment at a dose of 20 mg/kg per day resulted in modest improvements of glucose tolerance, glucose-stimulated insulin secretion, islet morphology, and markers of mitochondrial biogenesis. While alogliptin is effective in the management of type 2 diabetic patients (Bosi *et al.* 2011, DeFronzo *et al.* 2012), the metabolic improvements observed with alogliptin treatment in prediabetic UCD-T2DM rats do not appear to be sufficiently robust to result in a delay in diabetes onset alone or in combination with pioglitazone.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0601>.

Declaration of interest

The authors have no conflicts to disclose except that P J H received funding from Takeda Pharmaceuticals USA, Inc. to perform this project.

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Supplementary Material

Materials and Methods:

Monthly Hormone and Metabolic Profiles

Blood samples were collected from rats in both the short term and long term treatment groups once a month after an overnight (13 hour) fast and placed into EDTA treated tubes. The plasma was separated by centrifugation and assayed for glucose, insulin, glucagon, free fatty acids (FFA), triglycerides (TG), cholesterol, leptin and adiponectin. Whole blood samples were collected for the measurement of HbA_{1c}. Plasma glucose, cholesterol, FFA and TG concentrations were measured using enzymatic colorimetric assays (Thermo DMA Louisville, CO). Leptin, glucagon and adiponectin were measured with rodent/rat specific RIAs (Millipore, St. Charles, MO). HbA_{1c} was measured using an enzymatic colorimetric assay (Diazyme; Poway, CA).

Oral Glucose Tolerance Testing

An OGTT was performed after 3.5 months of treatment on animals from the short term and long term groups. Animals were fasted overnight and then received a 50% dextrose solution (1 g/kg BW) by oral gavage. Blood was collected from the tail for measurement of glucose and insulin concentrations. A second aliquot of blood was placed in tubes containing EDTA, aprotinin and a DPP-IV inhibitor and analyzed for total GLP-1. Serum glucose was measured using an enzymatic colorimetric assay for glucose (Thermo DMA Louisville, CO). Serum insulin and plasma GLP-1 were measured by sandwich electrochemiluminescence immunoassay (Meso Scale Discovery; Gaithersburg, MA).

The same procedure was followed for the performance of an acute study of the effects of alogliptin administration on circulating concentrations of active GLP-1. A separate set of male UCD-T2DM rats were fasted overnight and received an oral gavage of dextrose (1 g/kg) with or without alogliptin added to the gavage (1 mg alogliptin/kg body weight).

Body Composition and Liver and Muscle Triglyceride Content

After 4.5 months of treatment (6.5 months of age) animals in the short-term groups were euthanized with an overdose of pentobarbital (200 mg/kg i.p.) after an overnight fast. Subcutaneous, mesenteric, retroperitoneal and epididymal adipose depots and liver, heart, gastrocnemius muscle and kidney were dissected, weighed and flash frozen in liquid nitrogen and stored at -80 °C. Liver and skeletal muscle TG content were measured using the Folch method [33] for lipid extraction followed by spectrophotometric measurement of TG content (Thermo Electron, Louisville, CO).

Islet Immunohistochemistry and Pancreatic Insulin Content

Pancreas samples were collected and insulin and glucagon were extracted and analyzed as previously described [34]. Pancreas samples were also collected from a subset of animals for immunohistochemistry, as previously described [35]. Briefly, samples were fixed in 4% paraformaldehyde and embedded in paraffin. Ten sections (1 µm) per pancreas were obtained. Sections were deparaffinized in a xylene ethanol series and placed in Tris-EDTA buffer for antigen retrieval (10mM Tris, 1mM EDTA, 0.05% Tween, pH=9.0) and then blocked in 5% BSA. Sections were immunostained for insulin using a monoclonal anti-mouse antibody (1:100) and for glucagon using monoclonal anti-rabbit antibody (1:50) (Santa Cruz Biotechnology;

Dallas, TX). Detection of the primary antibodies was performed using Alexa Fluor 488 anti-goat and Alexa Fluor 633 anti-mouse secondary antibodies (1:200) (Invitrogen; Foster City, CA). Nuclei were detected using 4',6'-diamino-2-phenyl indole (DAPI), included in the mounting solution (Invitrogen; Foster City, CA). Ten sections per pancreas, taken throughout the pancreas, were imaged for quantification. Pancreatic β -cell area was analyzed using Image J software.

rtPCR

RNA was extracted from brown and subcutaneous white adipose tissue using TRIzol reagent (Invitrogen, CA). cDNA was generated using high-capacity cDNA Archive Kit (SuperScript™ III Reverse Transcriptase, Invitrogen). mRNA of UCP1, Cox8b and PGC1 α was assessed by reverse transcription PCR (iCycler, BioRad) and normalized to β -actin. For RT-PCR, *Absolute blue* qPCR premix (Fisher Scientific) was mixed with each primer. UCP1 primers: 5'- A T A C T G G C A G A T G A C G T C C C -3' (For.), 5'-A T C C G A G T C G C A G A A A G A A -3'(Rev.); Cox8b primers: 5'- C C G A G A A T C A T G C C A A G G C T -3' (For.), 5'- T C C T G C T G G A A C C A T G A A G C -3' (Rev.); PGC1 α primers: 5'- T A G C G G T C C T C A C A G A G A C A -3' (For.), 5'- A G T G C T A A G A C C G C T G C A T T -3' (Rev.); β -actin primers: 5'- C A C G G C A T T G T C A C C A A C T G -3' (For.), 5'- G G G G T G T T G A A G G T C T C A A A -3' (Rev.).

Immunoblotting

Tissues were ground in the presence of liquid nitrogen and lysed using radio-immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 mM

NaF, 1 mM sodium orthovanadate and protease inhibitors). Lysates were clarified by centrifugation at 13,000 rpm for 10 min and protein concentrations were determined using bicinchoninic acid protein assay kit (Pierce Chemical, IL). Proteins (20-50ug) were resolved by SDS-PAGE (10-12%) and transferred to PVDF membranes. Immunoblots were performed with the relevant antibodies and proteins were visualized using Luminata™ Forte (Millipore; Billerica, MA). For quantitation purposes, pixel intensities of immuno-reactive bands from blots that were in the linear range of loading and exposure were quantified using FluorChem 9900 (Alpha Innotech, CA). Antibodies for FAS, PGC1 α and Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for acetyl-CoA carboxylase (ACC), pACC (Ser⁷⁹), AMP-activated protein kinase (AMPK), pAMPK (Thr¹⁷²), protein kinase B (AKT), pAKT (Ser⁴⁷³), extracellular-signal-regulated kinase1/2 (ERK1/2) and pERK1/2 (Thr²⁰²/Tyr²⁰⁴) were from Cell Signaling (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from BioResources International (Carlsbad, CA).