

# Combined treatment with a gastric inhibitory polypeptide receptor antagonist and a peptidyl peptidase-4 inhibitor improves metabolic abnormalities in diabetic mice

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## Abstract

**Objectives:** Dipeptidyl peptidase-4 inhibition and gastric inhibitory polypeptide (GIP) receptor antagonism have therapeutic effects in type 2 diabetes mellitus. We assessed the effects of sitagliptin and Pro<sup>3</sup>(GIP) in a mouse model of diabetes.

**Methods:** Diabetes was induced in C57BL/6J mice by a high-fat diet and intraperitoneal injection of streptozocin. Blood glucose was assessed weekly. Six weeks later, serum triglycerides, total cholesterol and glucose tolerance were assessed and pancreatic and adipose tissues were collected.

**Results:** Combination therapy with sitagliptin and Pro<sup>3</sup>(GIP) resulted in significantly greater reductions of blood glucose and triglycerides than either monotherapy. Combination therapy also improved insulin sensitivity and glucose tolerance.  $\beta$ -cell mass and insulin-positive cell percentage in the pancreas was higher in mice receiving combination therapy compared with either monotherapy. Crown-like structures, inflammatory markers in adipose tissue, and serum leptin concentrations were decreased in mice receiving combination therapy compared with either monotherapy.

**Conclusions:** Combination therapy with Pro<sup>3</sup>(GIP) and sitagliptin improved metabolic abnormalities in diabetic mice. Changes in serum leptins and reduced inflammatory cell infiltration in adipose tissue might account for the observed effects.

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## Keywords

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## Introduction

Dipeptidyl peptidase-4 (DPP4) is a trans-membrane glycoprotein expressed by many types of epithelial cells that can break down endogenous glucagon-like peptide 1 (GLP1) and glucose-dependent insulin tropic polypeptide (GIP).<sup>1</sup> DPP4 inhibitors are a class of anti-diabetes agents that suppress degradation of GLP1 and GIP. Sitagliptin is the first commercialized DPP4 inhibitor and remains the most widely used. Sitagliptin lowers glucose and glycosylated hemoglobin levels,<sup>2,3</sup> thereby improving  $\beta$ -cell function by reducing the fasting proinsulin-to-insulin ratio, fasting insulin secretion, and the insulinogenic index.<sup>4</sup> A meta-analysis showed that sitagliptin significantly decreased serum triglycerides (TGs) and increased high-density lipoprotein cholesterol levels.<sup>5</sup> Sitagliptin was also reported to lower total cholesterol (TC) and non-high-density lipoprotein cholesterol, although it remains unclear whether improvements in serum lipids are caused directly by sitagliptin or indirectly via improved glucose control.<sup>6</sup> There is evidence demonstrating that sitagliptin reduces inflammation in adipose tissue<sup>7-9</sup> as shown by decreased levels of interleukin (IL)-6, IL-12, IL-18, tumor necrosis factor (TNF)- $\alpha$ , secretory phospholipase A<sub>2</sub>, and cell adhesion molecules. In addition, sitagliptin exerts anti-inflammatory activity in the adipose tissue of obese mice by decreasing expression of adipocyte inflammatory factors.<sup>10</sup>

GIP is a nutrient-dependent insulinotropic hormone released by jejunal mucosal

K cells that possesses metabolic activity in type 2 diabetes and obesity.<sup>1</sup> GIP is an incretin that stimulates insulin release to improve diabetes control. Fasting levels of GIP are high in type 2 diabetes mellitus, and are considered a risk factor for this disease.<sup>11</sup> In previous studies, GIP was demonstrated to increase the absorption of peptides, amino acids and blood glucose by activating peptide transporter-1 in the apical membranes of intestinal mucosal cells.<sup>12</sup> GIP-induced obesity and insulin resistance are attributed to excessive absorption of nutrients. Furthermore, GIP single nucleotide polymorphisms have been linked to elevated visceral fat, plasma TGs, and hemoglobin A1c levels in Japanese adults.<sup>13</sup> Shibue et al.<sup>14</sup> reported that GIP contributes to diet-induced obesity by promoting fatty acid binding protein 5 activity. Additionally, GIP may promote inflammatory cell infiltration into adipose tissue and impair insulin sensitivity via the hypoxia-inducible factor-1 $\alpha$  pathway.<sup>15</sup> Previous studies have indicated that human Pro<sup>3</sup>(GIP) is a GIP receptor antagonist in obese diabetic mice.<sup>15</sup> Daily administration of Pro<sup>3</sup>(GIP) can improve glucose homeostasis, insulin resistance and blood parameters in mouse models of diet-induced diabetes.<sup>15</sup> However, the effectiveness of Pro<sup>3</sup>(GIP) in mouse models of diabetes remains controversial because it is regarded as a partial agonist.<sup>15</sup> Discrepancies among studies may relate to differences in cell types or species.

Despite differences in the modes of action of sitagliptin and Pro<sup>3</sup>(GIP),

Pro<sup>3</sup>(GIP) inhibits and offsets defective glucoregulation following augmentation of endogenous GIP by sitagliptin.<sup>3</sup> This effect results from insulin sensitization following degradation of lipid profile-induced Pro<sup>3</sup>(GIP). However, the effects of combination therapy with sitagliptin and Pro<sup>3</sup>(GIP) in models of type 2 diabetes remain unclear. Therefore, this study was designed to assess the metabolic effects of administering sitagliptin and Pro<sup>3</sup>(GIP) alone or in combination in a mouse model of streptozocin-induced diabetes.

## Material and methods

Protocols for animal experiments were approved by the Animal Care Committee of Xi'an Jiaotong University. Male C57BL/6J mice were purchased from the Animal Care Committee of Xi'an Jiaotong University at 6 to 7 weeks of age. Mice were housed in nonspecific pathogen-free rooms at 22 ± 2°C under a 12-hour/12-hour light/dark cycle with free access to food and water. The mice were fed either a high-fat diet consisting of 45% fat, 20% protein, and 35% carbohydrates or a standard rodent diet consisting of 10% fat, 30% protein, and 60% carbohydrate for 4 weeks. Mice were then injected intraperitoneally with streptozocin (40 mg/kg) dissolved in sodium citrate buffer (pH 4.5) or a similar volume of citrate buffer alone once a day for 3 days. Mice with blood glucose levels ≥ 16.9 mmol/L were considered diabetic.

## Experimental procedures

Sixty mice were randomly allocated in equal numbers to the following groups: normal control, diabetes model, diabetes model + sitagliptin, diabetes model + Pro<sup>3</sup>(GIP), or diabetes model + sitagliptin + Pro<sup>3</sup>(GIP). Pro<sup>3</sup>(GIP) (25 nmol/kg) or a 0.9% saline vehicle were administered intraperitoneally once daily. Sitagliptin (10 mg/kg) or a 0.5%

sodium carboxymethyl cellulose vehicle were administered once daily by gavage for 6 weeks.<sup>16</sup> In the control and diabetes model group, 0.9% saline and 0.5% sodium carboxymethyl cellulose were administered according to the same protocol. Animals had free access to food and water. Blood glucose levels were recorded weekly. Handlers weighed the total mass of all feed placed in each cage as well as leftovers at regular intervals to calculate daily food consumption. All experiments began at 10:00 AM. After the final administration, mice were anesthetized with 100 mg/kg ketamine and 16 mg/kg xylazine. Pancreatic tissue was collected and subcutaneous adipose tissue, perirenal adipose tissue, and epididymal adipose tissue were stripped and weighed. Blood samples were collected from the tail vein. The mice were sacrificed by cervical dislocation.

## Pro<sup>3</sup>(GIP) and sitagliptin

Pro<sup>3</sup>(GIP) was synthesized on an Applied Biosystems (Foster City, CA, USA) Model 432 automated peptide synthesizer. GIP was dried *in vacuo* and purified by reverse-phase high pressure liquid chromatography using a Millennium 2010 chromatography system version 2.1.5 (Waters, Milford, MA, USA). Sitagliptin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

## Metabolic assays

After 6 weeks of treatment, plasma TG and TC levels were determined using a lipid profile automatic analyzer (Boehringer Mannheim, Mannheim, Germany). An intravenous glucose tolerance test (IVGTT) was performed at the same time. Mice fasted for 18 hours and were then injected intraperitoneally with glucose (18 mmol/kg). Blood samples were taken via the tail vein 0, 30, 60, and 120 minutes after glucose administration. Plasma

glucose was measured using a glucometer. Serum insulin and leptin levels were measured using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

### Adipose tissue inflammation

Abdominal adipose tissues were isolated from sacrificed mice, fixed in 10% formalin overnight, and embedded in paraffin. Tissues were sectioned (thickness 20  $\mu$ m) and stained with hematoxylin and eosin for observation of inflammatory cells. The number of crown-like structures (CLSs) in each field of 100 adipocytes was recorded as previously described.<sup>17</sup> Tissue sections were observed and photographed using a BZ-9000 microscope (Keyence, Shanghai, China).

### Histological evaluation

Pancreatic tissues were fixed in 10% formalin and embedded in paraffin. Sections were cut (thickness 5  $\mu$ m) and stained with anti-insulin antibody. Images were captured with a BX-51 microscope (Olympus, Tokyo, Japan). The percentage of  $\beta$ -cells

in each pancreas tissue sample was determined using ImagePro plus software (Media Cybernetics, Rockville, MD, USA).

### Statistical analysis

Data were expressed as means  $\pm$  standard deviations. Differences among multiple groups were assessed using analysis of variance followed by the Student–Newman–Keuls post hoc test. Values of  $P < 0.05$  were considered statistically significant. Statistical analysis was conducted using SPSS 22.0 software (IBM, Armonk, NY, USA).

## Results

### Plasma glucose changes

Plasma glucose levels in diabetic mice treated for 1 to 4 weeks with sitagliptin or Pro<sup>3</sup>(GIP), alone or combination, were similar and lower than those of untreated diabetic mice (Table 1). After 5 weeks of treatment, plasma glucose was lower in the combination therapy group than in the sitagliptin and Pro<sup>3</sup>(GIP) groups; however, only the difference between the combination therapy and sitagliptin groups reached statistical significance ( $P < 0.05$ ). After 6 weeks of

**Table 1.** Plasma glucose levels in normal mice, untreated diabetic mice and sitagliptin/Pro<sup>3</sup>(GIP)-treated diabetic mice.

Weeks of treatment	Number of mice	Plasma glucose (mmol/L)				
		Normal	Diabetes (untreated)	Diabetes (sitagliptin)	Diabetes (combination therapy)	Diabetes (Pro <sup>3</sup> (GIP))
0	9	8.53 $\pm$ 1.03***	22.23 $\pm$ 1.61	22.80 $\pm$ 1.02	22.61 $\pm$ 1.55	22.18 $\pm$ 1.73
1	9	8.60 $\pm$ 0.88***	23.53 $\pm$ 0.94*	20.68 $\pm$ 0.89#	20.40 $\pm$ 1.48#	18.86 $\pm$ 1.39#
2	9	8.60 $\pm$ 0.84***	24.58 $\pm$ 1.02*	21.70 $\pm$ 1.77#	20.70 $\pm$ 1.59#	19.42 $\pm$ 1.36#
3	9	8.60 $\pm$ 0.85***	25.63 $\pm$ 1.18**	20.70 $\pm$ 1.39###	18.23 $\pm$ 1.40###	19.08 $\pm$ 0.96###
4	9	8.65 $\pm$ 0.79***	28.9 $\pm$ 1.56**	20.16 $\pm$ 1.33###	17.07 $\pm$ 1.67###	18.45 $\pm$ 1.19##
5	9	8.57 $\pm$ 0.87***	29.06 $\pm$ 1.27**	19.08 $\pm$ 1.00***	15.24 $\pm$ 1.22###	17.83 $\pm$ 1.26###
6	9	8.72 $\pm$ 0.88***	29.35 $\pm$ 1.36**	18.10 $\pm$ 1.90***	13.11 $\pm$ 0.96###	17.06 $\pm$ 1.15***

Values represent means  $\pm$  standard deviations. \* $P < 0.05$  and \*\* $P < 0.01$  vs. combination therapy. # $P < 0.05$  and ### $P < 0.01$  vs. untreated diabetic mice.

treatment, plasma glucose levels in the combination therapy group were significantly lower than those in both the sitagliptin and Pro<sup>3</sup>(GIP) groups.

### Lipid profiles

As shown in Table 2, there were no significant differences in body weight, food intake, or fat weight among the different groups. After 6 weeks of treatment, TG levels in diabetic mice treated with either sitagliptin or Pro<sup>3</sup>(GIP) were higher than those of normal mice ( $P < 0.01$ , Table 3). Mice treated with either sitagliptin, Pro<sup>3</sup>(GIP), or the combination thereof had significantly lower TG levels compared with untreated diabetic mice. However, only combination therapy decreased the mean concentration of TGs to a normal level ( $P < 0.01$ , Table 3). Changes in TC levels following treatment with sitagliptin and

Pro<sup>3</sup>(GIP), alone or in combination, were not significant compared with diabetic or control mice (Table 3).

### IVGTT

IVGTTs were performed to evaluate insulin sensitivity and glucose metabolism. Glucose tolerance in untreated diabetic mice and diabetic mice receiving sitagliptin, Pro<sup>3</sup>(GIP), or combination therapy was similar (Figure 1a). The area under the curve from 0 to 120 minutes in the combination therapy group was smaller compared with the sitagliptin monotherapy and Pro<sup>3</sup>(GIP) monotherapy groups (Figure 1b). There was a consistently significant decrease in the insulin values of diabetic mice treated with sitagliptin or combination therapy compared with untreated diabetic mice. Moreover, decreases in insulin values were greater

**Table 2.** Food intake and adipose tissue weight in normal mice, untreated diabetic mice and sitagliptin/Pro<sup>3</sup>(GIP)-treated diabetic mice.

Group	Body weight	Food intake (g/day)	Perirenal fat (g)	Epididymal fat (g)	Subcutaneous fat (g)	White fat (g)
Normal	30 ± 1.77	3.55 ± 0.08	0.21 ± 0.05	0.47 ± 0.12	0.12 ± 0.03	0.76 ± 0.07
Diabetes (untreated)	32.80 ± 1.23	3.82 ± 0.25	0.29 ± 0.19	0.58 ± 0.38	0.15 ± 0.12	1.01 ± 0.14
Diabetes (sitagliptin)	29.50 ± 2.39	3.70 ± 0.31	0.25 ± 0.04	0.47 ± 0.21	0.13 ± 0.09	0.78 ± 0.12
Diabetes (Pro <sup>3</sup> (GIP))	28.96 ± 1.13	3.64 ± 0.44	0.23 ± 0.09	0.49 ± 0.07	0.12 ± 0.05	0.69 ± 0.16
Diabetes (combination therapy)	29.90 ± 2.77	3.61 ± 0.28	0.27 ± 0.08	0.51 ± 0.10	0.13 ± 0.08	0.74 ± 0.27

Values represent means ± standard deviations. <sup>#</sup> $P < 0.05$  vs. untreated diabetic mice.

**Table 3.** Triglyceride and cholesterol levels in normal mice, untreated diabetic mice and sitagliptin/Pro<sup>3</sup>(GIP)-treated diabetic mice.

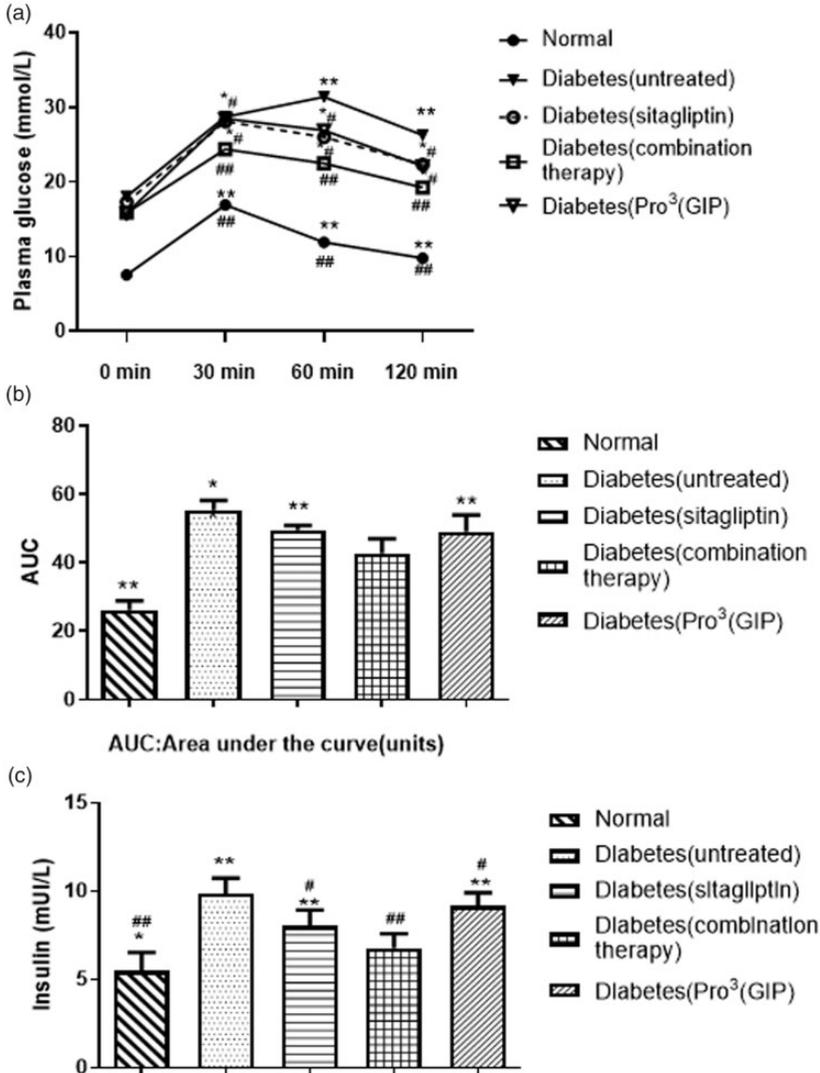
Group	Number of mice	Triglycerides (mmol/L)	Cholesterol (mmol/L)
Normal	9	1.12 ± 0.30 <sup>**</sup> <sup>###</sup>	2.60 ± 0.33
Diabetes (untreated)	7	1.55 ± 0.23 <sup>**</sup>	2.82 ± 0.48
Diabetes (sitagliptin)	6	1.37 ± 0.40 <sup>*</sup>	2.81 ± 0.21
Diabetes (combination therapy)	6	0.88 ± 0.20 <sup>###</sup>	2.48 ± 0.37
Diabetes (Pro <sup>3</sup> (GIP))	6	1.27 ± 0.09 <sup>*</sup>	2.80 ± 0.26

Values represent means ± standard deviations. <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  vs. combination therapy. <sup>###</sup> $P < 0.01$  vs. untreated diabetic mice.

when sitagliptin and Pro<sup>3</sup>(GIP) were combined (Figure 1c). Combined administration of sitagliptin and Pro<sup>3</sup>(GIP) resulted in better improvements of glucose tolerance and insulin resistance compared with either agent alone.

### Adipose tissue morphometry and inflammation

Adipose tissue inflammation is characterized by formation of CLSs. Significantly fewer CLSs were observed in the adipose tissues of mice treated with sitagliptin,

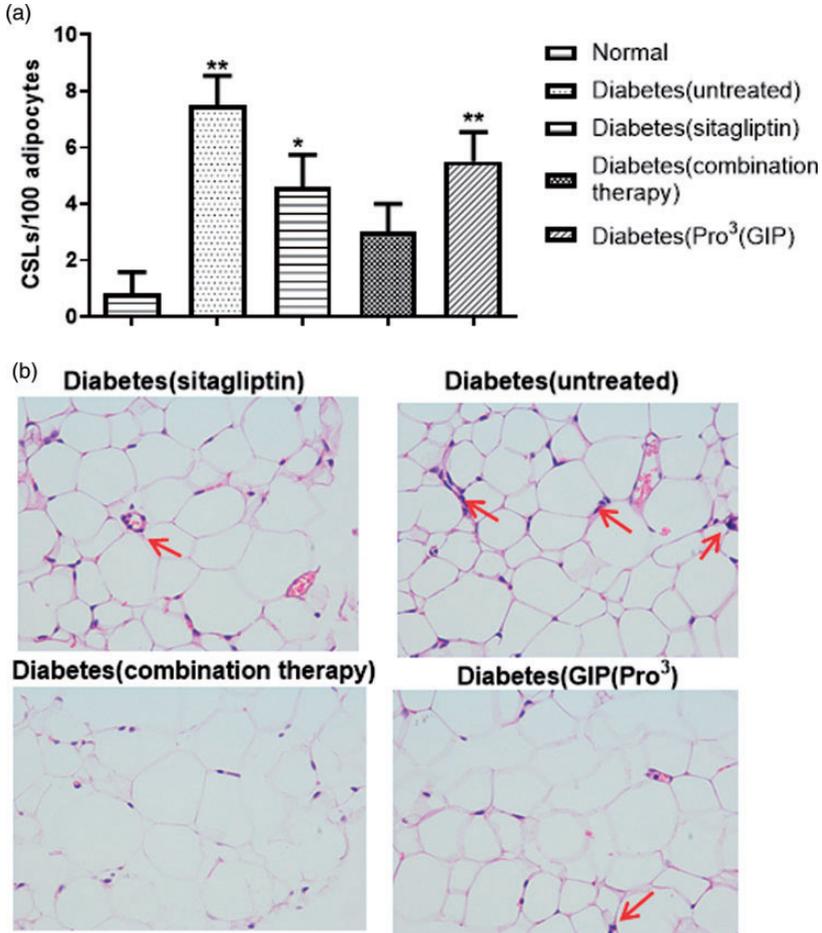


**Figure 1.** Effects of sitagliptin and Pro<sup>3</sup>(GIP), alone or in combination, on glucose tolerance. a: Serum glucose concentrations. b: Area under the curve from 0 to 120 minutes in intravenous glucose tolerance tests. c: Fasting insulin concentrations. Values represent means of five to seven mice and error bars indicate standard errors of the means. \**P* < 0.05 vs. combination therapy, \*\**P* < 0.01 vs. combination therapy.

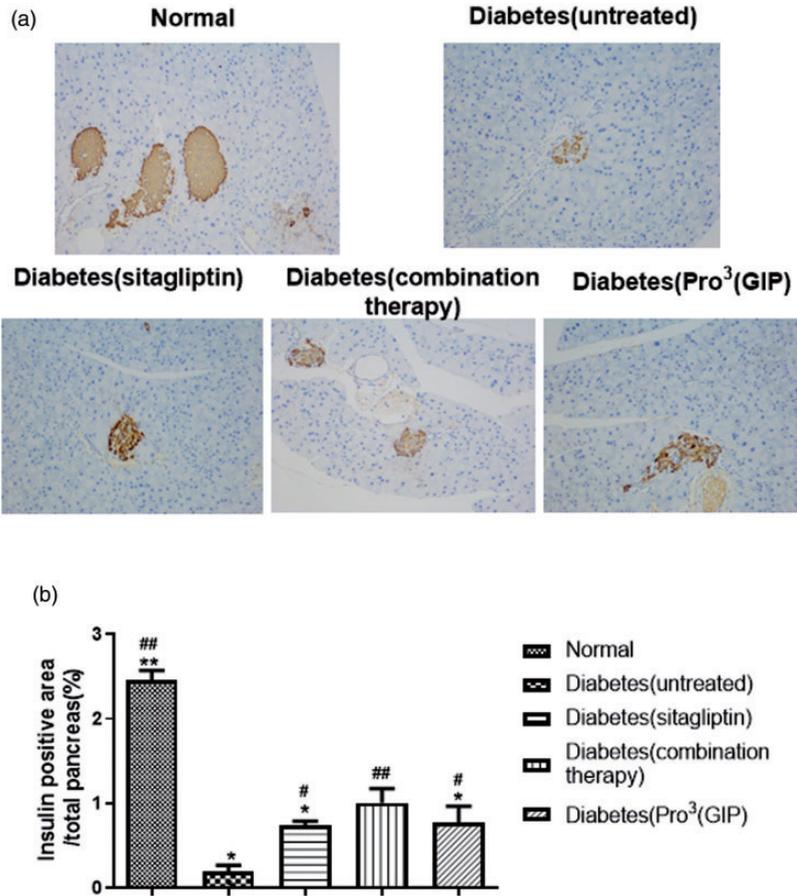
Pro<sup>3</sup>(GIP), or the combination thereof relative to untreated diabetic mice (Figure 2a). Inflammatory cell infiltration was also less extensive in the combination therapy group than in the untreated diabetic mice (Figure 2b). However, the amount of subcutaneous adipose tissues was unchanged after treatment with Pro<sup>3</sup>(GIP) or sitagliptin, alone or in combination (data not shown).

**Pancreatic  $\beta$ -cell mass**

The effects of sitagliptin and Pro<sup>3</sup>(GIP) therapy on insulin release were evaluated via assessment of  $\beta$ -cell mass and islet function. The proportion of islet cells and  $\beta$ -cell mass were both reduced in untreated diabetic mice compared with normal mice (Figure 3a). Following sitagliptin or Pro<sup>3</sup>(GIP) treatment, islet cell area,  $\beta$ -cell mass, and insulin release were significantly



**Figure 2.** Combination therapy with sitagliptin and Pro<sup>3</sup>(GIP) resulted in decreased inflammation infiltration into subcutaneous adipose. a: Number of crown-like structures (CLSs, normalized to 100 adipocytes). b: Markers of inflammation infiltration in crown-like structures. \*P < 0.05 vs. combination therapy, \*\*P < 0.01 vs. combination therapy.

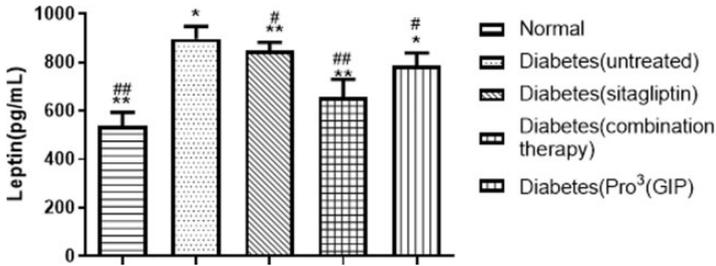


**Figure 3.** Sitagliptin and Pro<sup>3</sup>(GIP) increased  $\beta$ -cell mass in diabetic mice. a:  $\beta$ -cells are indicated by brown areas of positive insulin staining. b: Insulin-positive percentage of the total area of pancreas tissue. Error bars show standard deviations. # $P < 0.05$  and ## $P < 0.01$  vs. untreated diabetic mice; \* $P < 0.05$  and \*\* $P < 0.01$  vs. combination therapy.

increased compared with untreated diabetic mice. Furthermore, combination therapy was more effective than either onotherapy in improving islet cell area,  $\beta$ -cell mass, and insulin release. The percentages of insulin-positive areas within the pancreas were greater in all three treatment groups compared with those of untreated diabetic mice, but smaller than those of normal mice (Figure 3b). The largest responses were observed in diabetic mice treated with both sitagliptin and Pro<sup>3</sup>(GIP).

### Serum adipocytokine levels

The effects of sitagliptin and Pro<sup>3</sup>(GIP) in adipose tissue were investigated by quantitating secretion of the adipocytokines leptin and adiponectin. Serum leptin was 1.8-fold higher in diabetic mice compared with normal mice (Figure 4). Mice treated with sitagliptin and Pro<sup>3</sup>(GIP), alone or in combination, had reduced leptin secretion compared with untreated diabetic mice. However, the effect of combination therapy



**Figure 4.** Levels of plasma leptin in normal mice, untreated diabetic mice, and sitagliptin- and/or Pro<sup>3</sup>(GIP)-treated diabetic mice. Values represent means of five to seven mice and error bars indicate standard errors of the means. \* $P < 0.05$  and \*\* $P < 0.01$  vs. combination therapy; # $P < 0.05$  and ## $P < 0.01$  vs. untreated diabetic mice

was smaller than either sitagliptin or Pro<sup>3</sup>(GIP) alone. Serum adiponectin was significantly lower in diabetic mice compared with normal mice. No significant differences in serum adiponectin levels were observed in untreated diabetic mice or diabetic mice in any treatment group (data not shown).

## Discussion

In the present study, a mouse model of type 2 diabetes was established by feeding a high-fat diet and streptozocin injection. A previous study showed that a high-fat diet, in conjunction with streptozocin injection, could induce hyperglycemia, insulin resistance, hyperlipidemia, and obesity.<sup>218</sup> We found that sitagliptin or Pro<sup>3</sup>(GIP) treatment significantly reduced blood glucose and increased insulin levels as well as the percentage of insulin-positive areas in the pancreas. These results were consistent with previous studies.<sup>19,20</sup> Surprisingly, combination therapy with Pro<sup>3</sup>(GIP) and sitagliptin showed synergy in decreasing plasma glucose, insulin resistance, and adipose tissue inflammation in a mouse model of type 2 diabetes.<sup>21</sup> Additionally, analysis of IVGTT area under the curve indicated that plasma glucose was more significantly reduced 5 and 6 weeks after combination therapy with sitagliptin and Pro<sup>3</sup>(GIP)

compared with either monotherapy. Furthermore, increased  $\beta$ -cell mass and insulin-positive areas were observed in the pancreas, suggesting that combination therapy increased insulin sensitivity to achieve a hypoglycemic effect. The beneficial effects of combination therapy depended upon the combined functions of sitagliptin and Pro<sup>3</sup>(GIP) on the response of adipose tissue to insulin secretion.<sup>10,22,23</sup>

DPP4 secreted by adipose tissue impairs insulin signaling in adipose cells, leading to insulin resistance. Sitagliptin has been shown to promote insulin activation in adipose cells by increasing protein kinase B (Akt) activity.<sup>24</sup> These data suggest that adipocytes may mediate the effects of sitagliptin and Pro<sup>3</sup>(GIP) on diabetes metabolism. Pro<sup>3</sup>(GIP) and sitagliptin decreased the infiltration of inflammatory cells into the adipose tissue of diabetic mice. The greatest reduction of adipocytes with CLSs was observed in mice receiving combination therapy with sitagliptin and Pro<sup>3</sup>(GIP), suggesting that combination therapy could significantly reduce adipose tissue inflammation. Insulin resistance and inflammatory factors, such as IL-6 and TNF- $\alpha$ , have been linked to chronic inflammation in the adipose tissue of diabetic patients.<sup>25</sup> Some trials have demonstrated benefits of immunomodulatory therapy in diabetic patients.<sup>26</sup>

Both sitagliptin and Pro<sup>3</sup>(GIP) markedly decreased plasma leptin concentrations in diabetic mice. Combination therapy resulted in a larger decrease in leptin levels than either monotherapy. However, leptin concentrations were still higher in treated diabetic mice than in normal mice. The adipocytokines leptin and adiponectin produced by adipose tissue play central roles in insulin resistance.<sup>27</sup> Adiponectin is an insulin-sensitizing cytokine that augments fatty acid oxidation in muscle, increasing glucose uptake in adipose tissue and reducing free fatty acid influx and gluconeogenesis in the liver.<sup>28</sup> Leptin blocks insulin biosynthesis and secretion upon binding to leptin receptors on pancreatic  $\beta$ -cells.<sup>29,30</sup> In obese or diabetic patients, dysregulated leptin function results in upregulated plasma leptin levels, dysfunctional leptin signaling in the hypothalamus and increased insulin levels. Our results suggested that the mechanism underlying combination therapy with sitagliptin and Pro<sup>3</sup>(GIP) involved leptin, but not adipocytokines, because serum adiponectin was unaffected by either monotherapy or combination therapy.

In conclusion, combination therapy with Pro<sup>3</sup>(GIP) and sitagliptin more significantly decreased plasma glucose, insulin resistance, and adipose tissue inflammation than either monotherapy in a mouse model of type 2 diabetes. Combination therapy with sitagliptin and Pro<sup>3</sup>(GIP) had a greater effect on diabetic mice than either monotherapy, providing more metabolic benefits than administration of either single agent. Regulation of inflammatory infiltrates in adipose tissue and plasma leptin concentrations may be responsible for the superiority of combination therapy. Sitagliptin combined with Pro<sup>3</sup>(GIP), or similar combination regimens, may be effective for treating diabetes through modulation of adipose tissue metabolism.

## Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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