Effects of sitagliptin on circulating zinc- α_2 -glycoprotein levels in newly diagnosed type 2 diabetes patients: a randomized trial

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Abstract

Objective: Zinc- α_2 -glycoprotein (ZAG) has recently been characterized as a potent metabolic regulator. However, the effects of anti-diabetic agents on circulating ZAG levels in humans remain largely unknown. To explore the possible mechanisms by which the dipeptidyl peptidase-IV (DPP-IV) inhibitor improves insulin resistance, we investigated the effect of sitagliptin, a DPP-IV inhibitor, on circulating cytokine levels in newly diagnosed type 2 diabetes (nT2DM) patients.

Design and methods: A subset of 141 subjects with nT2DM were assigned to receive placebo ($n=47$) or sitagliptin ($n=94$) for 3 months. Before and after treatment, subjects received a 75 g oral glucose tolerance test, euglycemic-hyperinsulinemic clamp (EHC), and measurement of ZAG and adiponectin (ADI) concentrations.

Results: Circulating ZAG levels were lower in nT2DM than in control individuals ($P<0.01$). After 3 months of sitagliptin treatment, HbA1c, fasting plasma glucose, postprandial glucose, 2-h insulin after glucose overload, triglycerides, and homeostasis model assessment of insulin resistance (HOMA-IR) were decreased significantly compared with pre-treatment ($P<0.05$ or $P<0.01$), whereas the glucose infusion rate during the stable period of the clamp (M values) during EHC were significantly increased ($P<0.01$). In addition, circulating ZAG and ADI concentrations were significantly increased along with improved glucose metabolism and insulin sensitivity compared with pre-treatment (both $P<0.01$) and the change of ZAG (Δ ZAG) was positively associated with Δ ADI, Δ HOMA-IR, Δ BMI, Δ fasting insulin and negatively associated with Δ tumor necrosis factor- α (TNF- α). Furthermore, sitagliptin treatment resulted in significantly lowered plasma TNF- α level ($P<0.05$).

Conclusion: A low level of circulating ZAG is associated with insulin resistance and sitagliptin treatment significantly increases circulating ZAG levels. These observations have implications in relation to the mode of action of the DPP-IV inhibitor as an insulin sensitizing agent.

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Introduction

Adipose tissue produces several adipocytokines such as leptin, adiponectin (ADI), tumor necrosis factor alpha (TNF- α), and interleukin 6 (IL6). These adipocytokines modulate insulin sensitivity and play an important role in

the pathogenesis of insulin resistance, diabetes, dyslipidemia, inflammation, and atherosclerosis (1, 2, 3). Zinc- α_2 -glycoprotein (also known as ZAG, ZA2G, and ZNGP1) is a 40-kD soluble glycoprotein first isolated from human

plasma (4) and later found in other secretions, including saliva, milk, seminal plasma, sweat, urine, and cerebrospinal fluids (5). Cumulating evidence suggests that ZAG is also an adipokine because it is expressed and secreted by human adipocytes (6) and mouse and human adipose tissues (7). ZAG has been shown to act as a lipid-mobilizing factor (6). Treatment with ZAG stimulates lipolysis in isolated mouse and human adipocytes, and it selectively reduces body fat in both with adiposity, triglycerides (TG), and the key components of metabolic syndrome (8). Additionally, ZAG expression and protein levels have been reported to be down-regulated in subcutaneous and visceral adipose tissue of obese men and women in comparison with the lean controls (9). Very recently, we have demonstrated that circulating ZAG levels are lower in the patients with impaired glucose tolerance (IGT) and patients with newly diagnosed type 2 diabetes mellitus (nT2DM) than in control subjects. Circulating ZAG levels are also positively correlated with ADI, but inversely with BMI, waist-to-hip ratio (WHR), TG, fasting plasma glucose (FPG), fasting insulin (FIns), HbA1c, and homeostasis model assessment of insulin resistance (HOMA-IR) (10). Since the biological actions of ZAG have not been fully understood, it is important to characterize the effect of anti-diabetic treatment, especially new anti-diabetic agents, on the plasma levels of ZAG in nT2DM patients. To date, however, no study has examined the effects of dipeptidyl peptidase-IV (DPP-IV) inhibitors on circulating ZAG levels in nT2DM patients. In the present study, we investigate circulating ZAG levels in a Chinese population, including nT2DM and non-diabetic control subjects in order to explore the possible mechanism of sitagliptin therapy on circulating levels of ZAG in nT2DM subjects.

Subjects and methods

Cross-sectional studies

The trial was conducted from February 2013 to May 2014. A total of 513 subjects including 413 patients with nT2DM (nT2DM group) and 100 normal subjects participated in this study. The diagnosis of nT2DM was based on World Health Organization 1998 diagnostic criteria (11). Subjects with nT2DM had not been treated with hypoglycemic agents, insulin, diet control, physical exercise, or other pharmacological agents (anti-hypertensive drugs, statins, etc.) prior to the present study. Inclusion criteria were age 40–75 years with a BMI of 20–40 kg/m², and HbA1c level between 7 and 9.0%. All patients were in good general health without evidence of cardiac, hepatic, renal, or other

chronic diseases as determined by history, examination, and screening blood tests. Exclusions included patients with type 1 diabetes or ketoacidosis, symptomatic heart failure, malignant disease in the previous 10 years, liver cirrhosis, hypertension, hepatic and renal failure, or other known major diseases. One hundred age-matched healthy subjects without clinical evidence of major diseases were selected from an unselected population that underwent routine medical check-ups and were used as the controls. These subjects had normal glucose tolerance (NGT), an FPG level <6.1 mmol/l, a 2 h oral glucose tolerance test (OGTT) level <7.8 mmol/l, no family history of T2DM, and were not using medications. This study was carried out in accordance with the recommendations of the Declaration of Helsinki and was approved by the Human Research Ethics Committee of Chongqing Medical University. An informed consent was obtained from all participants in this study. The study was also registered at chictr.org (CHICTR-OCC-13003185).

Oral glucose tolerance test

After an overnight fasting of 10–12 h, all subjects underwent a standard 75-g, 2-h OGTT between 0800 and 0900 h. Blood samples were collected at 0, 30, 60, and 120 min for the measurements of glucose, insulin, free fatty acids (FFA), blood lipid, ZAG, and ADI.

Euglycemic-hyperinsulinemic clamp

A 2-h euglycemic-hyperinsulinemic clamp (EHC) was performed as previously described (12). Briefly, after an overnight fast, an intravenous catheter was placed in an antecubital vein to infuse insulin and glucose. Another catheter was placed retrograde in the dorsal vein of the contralateral hand for blood withdrawal. Regular human insulin (1 mU/kg per min, Novolin; Novo Nordisk, Denmark) was infused for 2 h and a variable rate infusion of 20% glucose was administered to maintain euglycemia. The rate of glucose disposal (GRd) was defined as the glucose infusion rate (GIR) during the stable period of the clamp (13).

Interventional studies

A subset of 141 patients from the nT2DM group were selected for sitagliptin therapy. All patients were weight stable (<2.5 kg change) in the last 3 months before the study. Inclusion criteria included age from 40 to 60 years, BMI 22–30 kg/m², and HbA1c 7.2–8.6%. Patients were

excluded if they adhered poorly to diabetes management recommendations, had recurrent hypoglycemia within the last 3 months, or had a history of hypoglycemia unawareness. In addition, the subjects were advised to continue their usual eating and exercise habits during the study. The patients with nT2DM were randomized (2:1) to 12 weeks of double-blind treatment with 100 mg sitagliptin (sitagliptin group, $n=94$) or placebo (placebo group, $n=47$) taken once daily before the first meal. To prevent acute complication, the subjects with three consecutive FPG values >13.7 mmol/l were withdrawn from the study. Fasting blood samples for biochemical parameters, TNF- α , ZAG, and ADI measurements were obtained at 0800 h (pre-treatment) on day 2 of the last admission.

Anthropometry and blood samples

Anthropometric measurement was performed in the morning, before breakfast, with the subjects wearing light clothing, but without shoes. Body weight and height were measured in all subjects using a scale and a wall mounted stadiometer to the nearest 0.5 kg and 0.5 cm respectively. Waist and hip circumferences were measured, and the WHR was calculated. BMI was calculated as weight divided by height squared. The percentage of fat *in vivo* (FAT %) was measured by bioelectrical impedance (BIA-101; RJL Systems, Shenzhen, China). $\text{HOMA-IR (14)} = \text{FIns (microunits/milliliter)} \times \text{FPG (millimoles/l)} / 22.5$, and homeostasis model assessment of insulin secretion ($\text{HOMA-}\beta$) = $20 \times \text{fasting insulin (mU/ml)} / \text{FPG (mmol/l)} - 3.5$. Plasma glucose and HbA1c were measured immediately by the glucose oxidase method and anion exchange high-performance liquid chromatography, respectively. Plasma samples were frozen and stored at -80°C for the measurements of ZAG, insulin, ADI, TNF- α , FFA, and blood fat levels.

Measurements of biochemical parameters and cytokines

Circulating ZAG levels were determined with an ELISA obtained from RayBiotech, Inc. (Norcross GA, USA) following the manufacturer's protocol. The limit of detection was 21 pg/ml, and intra- and inter-assay coefficient of variations (CV) were <10 and $<15\%$, respectively. Circulating ADI level was also measured by ELISA (Cusabio Co., Wuhan Hubei, China) as previously described (10). Detection range was 1.562–100 ng/ml, and intra- and inter-assay variations were <8 and 10% , respectively. In addition, circulating TNF- α level was examined

using a commercially available ELISA Kit (4A Biotech Co. Ltd, Beijing, China). The linear range of the assay was 1.56–100 pg/ml. The intra- and inter-assay CV were <10 and $<12\%$, respectively. Insulin was measured by RIA using human insulin as standard. FFAs were measured with a commercial kit (Randox Laboratories Ltd, Antrim, UK). Total cholesterol, HDL-cholesterol, LDL-cholesterol, and TG were determined enzymatically using an autoanalyzer (Hitachi 747; Hitachi).

Statistical analysis

Statistical analysis was carried out using SPSS 19.0 Software (SPSS, Inc.). Data are expressed as mean $\pm$ s.d. unless stated otherwise. Variables with a non-normal distribution were transformed logarithmically before analysis. Comparisons between groups were performed by unpaired *t*-test, or paired *t*-test as appropriate. All *t*-tests were two-tailed and $P<0.05$ was considered statistically significant. Pearson's correlation coefficient was used to determine correlations and regression analysis. All statistical analyses were performed by a single operator who was blinded to treatment group.

Results

Circulating ZAG and ADI levels and anthropometric and biochemical parameters in healthy and nT2DM subjects

A total of 513 middle-aged subjects were enrolled in this study. The main clinical features, biochemical parameters, and circulating ZAG and ADI levels are represented in Table 1. Fasting plasma concentrations of ADI were significantly lower in nT2DM patients compared with healthy subjects ($P<0.01$). Importantly, significantly lower circulating ZAG was also observed in nT2DM patients compared with the controls ($P<0.01$). These differences remained significant after the adjustment of age, BMI, and sex. In the nT2DM group, Pearson's correlation analysis showed that ZAG levels correlated with ADI, HOMA-IR, HbA1c, HDL-C, LDL-C, FFA, FAT (%) (the percentage of fat *in vivo*), TG, and total cholesterol (TC), whereas no correlation with BMI, FIns, FPG, diastolic blood pressure (DBP), systolic blood pressure (SBP), or WHR was found (Table 2). Lastly, we performed multiple stepwise regressions analyses to determine variables that had independent associations with circulating ZAG levels. The results showed that only HOMA-IR, ADI, TG, FFA, and LDL-C were independently related to

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Table 1 Main clinical features and circulating ZAG levels in NGT and nT2DM subjects.

Variable	nT2DM (n=413)	NGT (n=100)
Sex, male/female (%)	49.4	50
Age (years)	54.0±8.1	53.1±11.3
BMI (kg/m ²)	25.6±3.9*	22.7±3.2
WHR	0.91±0.06*	0.87±0.06
FAT (%)	30.0±6.0*	27.2±8.4
SBP (mmHg)	127±13*	117±16
DBP (mmHg)	77±8	76±10
TC (mmol/l)	5.00±0.66*	3.78±1.21
TG (mmol/l)	2.11±0.53*	1.09±0.71
FFA (mmol/l)	0.73±0.34**	0.41±0.21
HDL-C (mmol/l)	1.31±0.31*	1.46±0.39
LDL-C (mmol/l)	2.68±0.74	2.77±0.87
HbA1c (%)	8.19±1.08**	5.37±0.33
FPG (mmol/l)	9.38±1.01**	5.33±0.63
2h-BG (mmol/l)	17.78±5.05**	5.75±0.65
Fins (pmol/l)	96.4±35.5*	62.3±27.0
2h-Ins (pmol/l)	331.5±129.6*	280.0±152.6
HOMA-IR	5.79±2.39**	2.12±0.95
HOMA-β	49.26±21.02**	107.64±60.88
ADI (μg/l)	31.5±9.4**	41.8±13.6
ZAG (mg/l)	35.2±11.3**	59.3±16.2
ZAG (adjusted)*	35.2±1.31**	59.3±1.59

Data are mean±s.d. *Mean±s.e.m. by general linear model with adjustment of age, gender, and BMI. **P*<0.05 and ***P*<0.01 compared with controls.

circulating ZAG levels. The multiple regression equation was: $Y_{ZAG} = 13.138 - 0.640X_{HOMA-IR} + 0.246 X_{ADI} + 4.572 X_{TG} + 5.608X_{FFA} + 2.014 X_{LDL-C}$ (Table 2).

The effects of sitagliptin on clinical characteristics and biochemical parameters in nT2DM patients

Baseline demographics and laboratory characteristics of nT2DM patients in the interventional study are given in Table 3. All nT2DM patients had not been treated with oral anti-hyperglycemic agents or insulin. Anthropometric and clinical data at baseline between two study groups were not significantly different. Two patients in the placebo group discontinued this study due to lack of glycemic control. Six patients in sitagliptin group were withdrawn from the study for diarrhea, headache, or peripheral edema with unknown causes. As shown in Table 3, after 12 weeks treatment with sitagliptin, HbA1c, FPG, post-prandial glucose (2h-PG), 2-h insulin after glucose overload (2h-Ins), and TG declined significantly (*P*<0.05 or *P*<0.01) compared with pre-treatment, whereas these metabolic parameters were no significantly altered in the placebo treated group. In sitagliptin groups, HOMA-IR significantly decreased and HOMA-β significantly increased after treatment compared with pre-treatment (*P*<0.05).

The effects of sitagliptin on cytokine levels in nT2DM patients

Basal circulating ZAG and ADI levels were similar in nT2DM and control groups before treatment (ZAG: 34.2±12.6 and 33.4±10.9 mg/l; ADI: 32.2±7.0, and 32.6±8.3 μg/l; Fig. 1A and B). After therapy, circulating ZAG levels in the sitagliptin group increased significantly (from 34.2±12.6 to 40.1±12.3, *P*<0.01; Fig. 1A), whereas circulating ZAG showed no change in the placebo group. Similar to the changes of circulating ZAG, ADI levels were also increased significantly in therapy groups at the end of the therapy (from 32.2±7.0 to 34.6±6.3 μg/l, *P*<0.01; Fig. 1B), whereas in the placebo group, ADI levels did not change at pre- and post-treatment (32.6±10.9 vs 31.8±8.5 μg/l, Fig. 1B). Furthermore, compared with baseline, sitagliptin treatment resulted in significantly lowered plasma TNF-α levels (*P*<0.01; Table 3).

Changes of circulating ZAG and ADI levels during OGTT pre- and post-treatment with sitagliptin

After sitagliptin treatment, the ZAG concentration at each time point during OGTT increased significantly compared with pre-treatment (0 min: 42.2±9.16 vs 3.0±12.5 mg/l; 30 min: 56.5±11.6 vs 30.9±9.1 mg/l; 60 min: 48.9±12.8 vs 28.6±9.1 mg/l; 120 min: 45.5±15.9 vs 23.6±8.19 mg/l; all *P*<0.01; Fig. 2A). Notably, after sitagliptin treatment, ZAG levels showed an initial increase when subjected to a glucose challenge and then decreased gradually to baseline at 60 min when compared with the changes during pre-treatment, which were characterized

Table 2 Linear and multiple regression analyses of variables associated with circulating ZAG levels in nT2DM subjects.

Variable	Simple		Multiple	
	Estimate	P value	Estimate	P value
BMI	−0.013	0.782		
WHR	−0.044	0.355		
FAT (%)	−0.263	<0.001		
SBP	0.032	0.503		
DBP	0.014	0.772		
TG	0.317	<0.001	4.572	<0.001
TC	0.094	0.049		
HDL-C	0.106	0.027		
LDL-C	0.116	0.015	2.014	0.002
FFA	0.321	<0.001	5.608	0.001
FPG	0.020	0.684		
Fins	−0.078	0.102		
HbA1c (%)	−0.124	0.010		
HOMA-IR	−0.185	<0.001	−0.640	<0.001
ADI	0.324	<0.001	0.246	<0.001

Table 3 Clinical characteristics and biochemical parameters before and 12 weeks after treatment in nT2DM patients.

Variable	Sitagliptin (n = 88)		Placebo (n = 45)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Weight (kg)	64.0 ± 8.1	63.6 ± 8.0	61.1 ± 6.6	60.7 ± 6.2
BMI (kg/m ²)	25.9 ± 4.1	25.8 ± 4.1	25.5 ± 3.1	25.3 ± 2.9
WHR	0.90 ± 0.05	0.89 ± 0.05	0.90 ± 0.05	0.90 ± 0.04
FAT (%)	29.7 ± 4.9	29.6 ± 4.8	28.1 ± 5.1	29.0 ± 4.1
SBP (mmHg)	129 ± 11	127 ± 10	127 ± 9	127 ± 8
DBP (mmHg)	76 ± 6	76 ± 7	74 ± 5	75 ± 5
TC (mmol/l)	5.06 ± 0.58	4.99 ± 0.61	4.66 ± 0.78	4.67 ± 0.60
TG (mmol/l)	2.27 ± 0.60	1.91 ± 0.46*	2.12 ± 0.79	2.17 ± 0.61
FFA (μmol/l)	0.78 ± 0.33	0.77 ± 0.38	0.83 ± 0.20	0.83 ± 0.45
HDL-C (mmol/l)	1.31 ± 0.24	1.29 ± 0.31	1.35 ± 0.27	1.32 ± 0.24
LDL-C (mmol/l)	2.62 ± 0.73	2.61 ± 0.82	2.59 ± 0.49	2.58 ± 0.52
HbA1c (%)	8.08 ± 0.60	7.72 ± 0.58*	8.16 ± 0.48	8.18 ± 0.83
FPG (mmol/l)	9.00 ± 1.11	7.91 ± 0.92*	9.10 ± 1.28	8.92 ± 0.83
2hPG (mmol/l)	17.5 ± 3.6	12.1 ± 3.1*	17.1 ± 4.5	17.4 ± 3.2
Flns (pmol/l)	103.2 ± 45.9	109.2 ± 18.4	110.0 ± 29.7	103.5 ± 26.9
2hIns (pmol/l)	291.2 ± 66.2	404.7 ± 81.5*	300.9 ± 62.0	286.3 ± 56.4
HOMA-IR	6.18 ± 3.29	5.13 ± 1.05**	6.24 ± 1.26	5.84 ± 1.40
HOMA-β	52.0 ± 16.4	73.6 ± 20.4**	62.2 ± 32.5	57.0 ± 20.8
TNF-α (μg/l)	12.01 ± 0.49	7.83 ± 0.51*	11.70 ± 0.31	12.03 ± 0.61

Data are mean ± s.d. * $P < 0.01$, ** $P < 0.05$ compared with pre-treatment.

by a gradual decrease in ZAG levels. Circulating ZAG levels decreased significantly at 120 min of OGTT compared with 0 min at pre-treatment ($P < 0.01$; Fig. 2A). In response to OGTT-induced high glucose and insulin levels, circulating ADI at post-treatment significantly and rapidly elevated from 28.8 ± 8.9 to 45.8 ± 9.6 μg/l at 30 min, then to 40.2 ± 11.4 μg/l at 60 min, and finally to 37.1 ± 9.9 μg/l at 120 min (all $P < 0.01$ vs 0 min; Fig. 2B). Although during OGTT, the ADI changes at post-treatment at each time point were similar to that at pre-treatment, the increase in amplitude at 0, 30, 60, and 120 min was significantly higher than that of at pre-treatment ($P < 0.05$ or $P < 0.01$; Fig. 2B), suggesting that in response to the glucose challenge, ADI and ZAG release is increased following insulin sensitivity improvement.

The effects of sitagliptin on insulin sensitivity and adipocytokine levels during EHCs

To directly evaluate the quantitative effect of sitagliptin treatment on insulin sensitivity *in vivo*, EHCs were performed in 25 patients treated with sitagliptin at pre- and post-treatment. During insulin clamp, the steady-state (80–120 min) plasma glucose and insulin concentrations were similar at pre- and post-treatment (4.9 ± 0.2 vs 5.1 ± 0.1 mmol/l and 100.7 ± 11.1 , and 96.2 ± 7.1 mU/l, respectively). After 3 months, sitagliptin treatment resulted in a significant increase in GIR ($P < 0.05$; Fig. 2C)

and GRd shown as the GIR during the stable period of the clamp (M values) (from 5.02 ± 1.70 to 6.19 ± 1.63 mg/kg per min, $P < 0.01$; Fig. 2D) during the steady-state of clamp. Importantly, after 3 months of sitagliptin treatment in nT2DM patients, circulating ZAG during EHC was significantly and rapidly elevated from 44.9 ± 12.9 to 73.3 ± 17.8 mg/l at 80 min ($P < 0.01$ vs 0 min), then fell to 55.8 ± 14.5 mg/l at 100 min ($P < 0.05$ vs 0 min), and finally to 53.0 ± 13.6 mg/l at 120 min, whereas in pre-treatment, ZAG levels had only a slight change without significant difference at each time point (Fig. 2E). As shown in Fig. 2F, before sitagliptin treatment, ADI had no significant changes during the steady-state (80–120 min)

Figure 1

Effects of sitagliptin on circulating ZAG and ADI levels.

(A) Circulating ZAG levels in two groups of pre- and post-treatment. (B) Circulating ADI levels in two groups of pre- and post-treatment. Values were given as means ± s.d. * $P < 0.01$ vs pre-treatment.

Figure 2

Effects of sitagliptin administration on circulating ZAG and ADI levels during OGTT and EHCs. (A) Circulating ZAG levels pre- and post-treatment with sitagliptin in nT2DM subjects during an OGTT with 75 g glucose. (B) Circulating ADI levels pre- and post-treatment with sitagliptin in nT2DM subjects during an OGTT with 75 g glucose. (C) Time course of GIR changes pre- and post-treatment with sitagliptin in nT2DM subjects during the EHCs. (D) M values pre- and post-treatment with sitagliptin in nT2DM subjects. (E) Circulating ZAG levels pre- and post-treatment with sitagliptin in nT2DM subjects during the EHCs. (F) Circulating ADI levels pre- and post-treatment with sitagliptin in nT2DM subjects during the EHCs. $n=25$, values were given as means $\pm$ s.d., $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ vs 0 min; $\Delta P < 0.05$, $*P < 0.01$ vs pre-treatment.

of EHC when compare with 0 min. After 3 months of treatment, ADI showed a significant decrease during the steady-state (80 and 100 min) when compared with 0 min (both $P < 0.01$; Fig. 2F), whereas ADI levels has a slight

increase without significant difference at 80, 100, and 120 min of EHC compared with that of pre-treatment (Fig. 2F). These results suggested that sitagliptin treatment leads to an increase of insulin-stimulated ZAG release during EHCs. Finally, we assessed the associations between the changes of circulating ZAG (Δ ZAG) and Δ ADI as well as several parameters related to IR at pre- and post-treatment. Interestingly, Δ ZAG was positively associated with Δ ADI ($r=0.32$, $P < 0.01$), Δ HOMA-IR ($r=0.49$, $P < 0.01$), Δ BMI ($r=0.34$, $P < 0.01$), Δ FIns ($r=0.53$, $P < 0.01$), but negatively correlated with Δ TNF- α ($r = -0.385$, $P < 0.01$; Fig. 3 A, B, C, D, and E).

Discussion

In the current study, we examined the effect of 12 weeks of sitagliptin treatment on fasting adipocytokine (ZAG, ADI, and TNF- α) levels in nT2DM subjects and related changes in ZAG and ADI to changes in body composition, glycemic control, and insulin sensitivity. Consistent with previous findings from other laboratories (15, 16, 17, 18), we demonstrated that sitagliptin lowered the HbA1c, FPG concentration, 2hPG, FAT (%), FFA, serum TG, and HOMA-IR in nT2DM patients. As expected, treatment with sitagliptin led to a small decrease from baseline in body weight, with no significant difference compared with the placebo group. Because improvement in glycaemic control can lead to weight gain, the modest reduction in body weight or lack of weight gain with the substantial improvement in glycemic control observed in the sitagliptin groups has a clinically important meaning, particularly given the high prevalence of obesity in patients with T2DM. Noteworthy, the effect of sitagliptin on HbA1c in this study was less than in many studies (19), but similar to some reports (19, 20). This discrepancy in the effect of HbA1c may be due to the difference in

Figure 3

Relationship between the changes in circulating ZAG (Δ ZAG) and the changes in circulating ADI (Δ ADI), HOMA-IR (Δ HOMA-IR), BMI (Δ BMI), FIns (Δ FIns) and TNF- α (Δ TNF- α) after treatment with

sitagliptin. Fold change of ZAG is positively related to fold change of ADI (A), HOMA-IR (B), BMI (C), FIns (D), but negatively related to fold change of TNF- α (E).

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inclusion criteria, such as BMI, age and HbA1c, sample sizes, a history of diabetes, different therapy duration, other medications, and ethnic differences. Importantly, to eliminate the effects of diet control and exercise on ZAG, the patients in the present study were advised to continue their usual eating and exercise habits because these factors could affect the changes of HbA1c during DPP-IV therapy.

A previous study showed that long-term (12 weeks) DPP-IV inhibition improves insulin sensitivity in a diabetes model in rats (21). Furthermore, it has been reported that DPP-IV inhibition improves insulin sensitivity by assessing the oral glucose insulin sensitivity after long-term treatment in patients with T2DM (22). In the present study, HOMA-IR was significantly decreased along with the improvement of glucose metabolism in the DPP-IV group, showing improved insulin sensitivity. However, it cannot be ruled out that this may be an indirect action through improvement of glycemia. Importantly, in a previous study (22), enhanced insulin sensitivity was not judged by the EHCs, a gold standard, in subjects with T2DM. Here, we found that M values during the EHC were significantly increased in the sitagliptin treated patients, further confirming improved insulin sensitivity. However, in the present study, the causal relationship between DPP-IV inhibitor and insulin sensitivity is still unclear, and further investigation is needed to comprehensively understand the roles of DPP-IV in insulin resistance.

It has been demonstrated that the adipocyte is a metabolic factory capable of producing a number of adipocytokines (23). These adipocytokines play important roles in regulating glucose and lipid metabolism, the coagulation cascade, and inflammation (23). A growing body of evidence suggests that ZAG is also an adipokine because it is expressed and secreted by human adipocytes (6) as well as mouse and human adipose tissues (7). In our previous study, we demonstrated that circulating ZAG was lower in patients with IGT and nT2DM, and it correlated positively with ADI, and correlated inversely with BMI, WHR, FAT (%), and HOMA-IR. Therefore, we consider ZAG may be an adipokine associated with insulin sensitivity (10).

In the present study, we focused on the effect of the sitagliptin on ZAG and ADI. Before starting the treatment, the plasma ZAG and ADI concentrations in nT2DM were significantly decreased compared with the values in age and gender matched healthy subjects. After sitagliptin treatment, the circulating ZAG and ADI concentration increased along with the improvement of glucose metabolism and insulin sensitivity in diabetic subjects.

Therefore, we speculated that sitagliptin can increase the release of adipocytokines related to increase insulin sensitivity, indirectly confirming that DPP-IV inhibitor improves insulin resistance. Furthermore, these results are also similar to our previous report that liraglutide, a glucagon-like peptide 1 (GLP 1) analogue, treatment for 16 weeks in nT2DM patients leads to a significant increase in circulating ZAG levels (10). Therefore, it might be speculated that increased ZAG levels can be due to a prolonged activation of the GLP-1 receptor or due to inhibition of DPP-IV and thereby higher availability of endogenous GLP-1. Further studies will be required to address this issue. In addition, these results are also consistent with prior work investigating the insulin sensitivity of DPP-IV inhibitor in db/db mice by means of euglycemic hyperinsulinemic clamp techniques (24). However, one study in patients with T2DM showed that the DPP-IV inhibitor had no effect on HOMA-IR or ADI levels (25). The discrepancy in findings between that and our study might be attributable to differences in study design, patient selection, sample size, and methodological problems. In the present study, it has also been observed that sitagliptin treatment failed to lead to a change in FFA concentration. Clearly, therefore, the effect of the sitagliptin on insulin sensitivity is independent of FFA concentration. Several reports have demonstrated that improved glycemic control and insulin sensitivity are associated with an increase in plasma ADI concentration in T2DM patients (26, 27, 28). Therefore, increasing ZAG levels in parallel with an increase in ADI after sitagliptin treatment may also be due to improved glycemic control and insulin sensitivity.

Chronic subclinical inflammation has emerged as a characteristic feature of T2DM. TNF- α plays a key role in the pathogenesis of chronic inflammation and is also known to affect insulin signaling, lipid metabolism, and adipocyte function. In interventional study, we found that when compared with baseline, sitagliptin treatment significantly lowered plasma TNF- α level. This is consistent with Rizzo *et al.*'s report (29). Therefore, one can speculate that increasing ZAG may be also associated with anti-inflammatory effects of sitagliptin (29). This may be because insulin signal transduction is interfered by inflammatory mediators like TNF α and IL6. On the basis of that concept, an anti-inflammatory effect would have to precede insulin sensitization (29, 30, 31). However, the precise mechanism(s) by which ZAG exerts its actions on glucose metabolism and insulin sensitivity remain unclear.

To further investigate the effects of glucose and insulin on circulating ZAG levels at different metabolism state (pre-

and post-treatment), we preformed EHCs and OGTT to observe the changes of circulating ZAG during glucose and insulin challenges. After treatment with sitagliptin, both glucose and insulin stimulation significantly increased the circulating ZAG level in nT2DM patients, suggesting that glucose and insulin mediated ZAG release is more pronounced under improved insulin resistance conditions. Importantly, increasing ZAG levels were also accompanied by an increase in circulating ADI levels. Therefore, these findings further show that circulating levels of ZAG may be a marker related to insulin resistance, and it may change in response to nutritional status after DPP-IV inhibitor treatment. These findings also support that ZAG may act as a metabolic regulator in humans.

Nevertheless, this study has some limitations. First, it does not allow for causal inference between circulating ZAG concentrations and the development of insulin resistance and diabetes. Additional studies are needed to clarify their precise relationship. Secondly, because of the limited follow-up, we could not evaluate the clinical events following treatment.

In conclusion, we have provided evidence that sitagliptin treatment for 12 weeks decreased the FPG concentration, improved oral glucose tolerance, reduced HbA1c in nT2DM patients. Importantly, sitagliptin enhanced hepatic and peripheral tissue insulin sensitivity assessed by EHCs. Furthermore, we have first time demonstrated that sitagliptin treatment significantly increased the circulating ZAG and ADI levels in nT2DM. These observations enhance our understanding of the mechanism of action for the DPP-IV inhibitor in nT2DM patients, and further suggest that ZAG can be used as a novel biomarker for insulin resistance syndrome and T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M Tian, Z Liang, R Liu, K Li, X Tan, Y Luo, and M Yang conceived and carried out the experiments. L Li analyzed data and drafted the manuscript. H F Gu and H Liu revised and edited the manuscript. G Yang is the guarantor of this work and, as such, had full access to all the data in the study and takes

responsibility for the integrity of the data and the accuracy of the data analysis. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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