

Relationship Between Acyl and Desacyl Ghrelin Levels with Insulin Resistance and Body Fat Mass in Type 2 Diabetes Mellitus

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Purpose: Although strong evidence suggests that ghrelin plays an important role in regulating energy balance, the effects of acylated ghrelin (AG) and deacylated ghrelin (DAG) on fat mass are largely undefined. This study aimed to investigate the differential associations of both forms of ghrelin with insulin resistance and body fat mass in patients with type 2 diabetes mellitus (T2DM).

Patients and Methods: A total of 162 patients with type 2 diabetes were recruited and classified based on BMI and visceral fat area (VFA) as VFA normal group (n = 78), normal-BMI VFA obesity group (n = 20) and high-BMI VFA obesity group (n = 64). VFA and subcutaneous fat area (SFA) were detected by bioelectrical impedance analysis. Blood samples were collected to measure fasting glucose, insulin, lipids, AG and DAG levels after clinical examination.

Results: Compared with VFA normal group, DAG levels were significantly lower (421.7 ± 106.0 and 388.7 ± 96.5 pg/mL vs 524.4 ± 141.5 pg/mL, $P < 0.01$) in the two VFA obesity groups. No significant difference was found in AG levels within three groups. Among all subjects, BMI, VFA, SFA, fasting insulin and HOMA-IR were negatively correlated with DAG but positively with AG/DAG ratio ($P < 0.01$). In contrast, AG was positively correlated with HOMA-IR and fasting glucose ($P < 0.01$). Multiple stepwise regression analysis showed that fasting glucose was the independent factor of AG, VFA and HOMA-IR were the independent factors related to DAG.

Conclusion: DAG levels have a strong negative association with excess body fat mass and insulin resistance, whereas AG levels are closely related to elevated blood glucose levels in T2DM patients.

Keywords: acylated ghrelin, deacylated ghrelin, body fat mass, insulin resistance, type 2 diabetes mellitus

Introduction

The pathogenesis of type 2 diabetes mellitus (T2DM) is known to involve impaired insulin secretion and insulin resistance (IR). IR is closely related to metabolic syndrome, which is mainly characterized by obesity and metabolic dysfunction leading to increased cardiovascular mortality and morbidity.¹ With a significant increase in the global obesity population, IR is prevalent and plays a key pathophysiological role in patients with metabolic syndrome. Thus, it is necessary to explore effective methods to improve insulin resistance and obesity in diabetic individuals.

Ghrelin is a 28-amino acid hormone that is mainly secreted from stomach A-like cells.² It is divided into acylated ghrelin (AG) and deacylated ghrelin (DAG) according to whether its N-terminal serine residue is acylated.³ Ghrelin circulates in vivo mainly as deacylated ghrelin corresponding to more than 80% of the hormone.⁴ A growing body of evidence suggests that ghrelin has a role in insulin resistance and development of type 2 diabetes.^{5,6} Considering that total ghrelin (T-Ghr) is mainly composed of DAG form, T-Ghr and DAG values likely lead to the same conclusions in terms of differences among groups and associations with insulin resistance and glycometabolism in previous studies.⁷⁻⁹ Insulin resistance and obesity are closely related to low circulating levels of total ghrelin (T-Ghr) or DAG.⁷⁻¹⁰ However, the potential differential effects

of two circulating ghrelin forms on energy balance in humans are largely undefined. Ghrelin circulating in acylated form has been reported to have a negative influence on pancreatic insulin secretion and lipid metabolism.^{11,12} On the other hand, DAG also has important metabolic activities, which presumably are GHSR-independent, including the ability to counteract the effect of AG in certain circumstances.^{13–15} Studies have shown different results in the circulating levels of AG and DAG in obese individuals, reflected not only at baseline but also at postprandial levels.^{6,14,15} Since acyl and des-acyl ghrelin appear to have opposite actions and interact with different receptors, it is important to note that assessing total ghrelin levels alone could not reflect the real metabolic status in obesity and diabetes.^{5,16} Therefore, more research should be conducted to elucidate the differential effects of AG and DAG on metabolic function.

Although BMI was a valuable anthropometric indicator to identify insulin resistance, it is affected by various factors such as age and muscle mass and thus does not accurately measure fat mass and reflect fat distribution.¹⁷ Additionally, obese individuals may differ in their fat distribution and disease risk. Regional adipose tissue distribution is suggested to play a crucial role in understanding the relationship between adiposity and metabolism.^{18,19} Visceral and subcutaneous fat mass is considered as good indicators to assess fat mass and fat distribution; moreover, the accumulation of visceral fat is closely related to adverse cardiovascular events.^{18,20} Few studies have explored the correlation of ghrelin concentration with fat mass, including visceral and subcutaneous fat. The potential influence of fat mass on different ghrelin forms remains largely undetermined. The goal of the present study was to illustrate the possible relationships among both forms of ghrelin and body fat mass, insulin resistance, and related markers of metabolic abnormalities in T2DM patients.

Materials and Methods

Study Individuals and Experimental Protocol

In a retrospective study, a total of 162 patients with T2DM (109 males and 53 females) were enrolled randomly from the Department of Endocrinology in Jinling Hospital. Their age ranged from 18 to 70 years. The inclusion criteria were diagnosis of T2DM according to the WHO 1999 criteria. Most patients were administered oral hypoglycemic agents (metformin and acarbose), combined with moderate diet and exercise intervention. Twenty-five patients were controlled by diet and exercise, and no subject was using insulin. The numbers of unmedicated patients were comparable among the three groups. The three patient groups were also matched for age, sex, duration of illness, medication type and dosage. The exclusion criteria were diagnosis of T1DM, special types of diabetes mellitus, acute and severe chronic illnesses, liver failure, renal failure, cancer, autoimmune disease and pregnancy. No subject had been treated with weight-reduction surgeries for obesity.

In order to investigate the influence of fat mass on different parameters in the presence of metabolic abnormalities, visceral fat area (VFA) and subcutaneous fat area (SFA) were assessed by dual bioelectrical impedance analysis. According to the Japanese visceral obesity criteria,²¹ visceral obesity was defined as a VFA ≥ 100 cm². Using BMI and VFA value, patients with VFA < 100 cm² were classified as VFA normal group (N = 78), VFA ≥ 100 cm² BMI ≥ 25 kg/m² were classified as high-BMI VFA obesity group (N = 64) and patients with VFA ≥ 100 cm² and BMI < 25 kg/m² were classified as normal-BMI VFA obesity group (N = 20). The study protocol was approved by the local ethics committee. All patients were informed of the aims of the study.

Anthropometric and Biochemical Data

Medical history including age, gender and other baseline characteristics were gained through medical records. We measured the height and weight of all subjects. Venous blood samples were collected from all subjects in the morning after overnight fasting for at least 8 hours. Fasting blood samples were collected using standard EDTA-aprotinin tubes, then chilled on ice and centrifuged at 4°C as soon as possible. The supernatants were acidified with 1 mol/L HCl (10% of sample volume) and stored at -80°C until assayed. VFA and SFA were measured by the dual bioelectrical impedance analysis method (HDS-2000; Omron Healthcare Co., Ltd.).

Serum Measurements

Insulin concentration was examined by electrochemiluminescence immunoassay (IMMULITE2000 XPI, Siemens, Germany). Other biochemical indices were detected by an automatic biochemical analyzer (Model 7600 Series Automatic Analyzer,

Hitachi, Japan). Insulin resistance was quantified using HOMA-IR [fasting insulin (mU/L) \times fasting glucose (mmol/L)]/22.5. Plasma acylated ghrelin (AG) and desacyl ghrelin (DAG) levels were determined using ELISA kits (Human acylated and deacylated Ghrelin EIA Kits, Bertin Corporation, reference range: 2–250 pg/mL; intraassay coefficient of variation <5%; interassay coefficient of variation <15%). These tests were done following the manufacturer's instruction manual. AG/DAG ratio was calculated as AG divided by DAG.

Statistical Analysis

One-way ANOVA was applied to compare the groups for quantitative and chi square test was used for qualitative variables. Correlation analysis was performed using Pearson's or Spearman's rank correlation test. The effect of several variables on AG and DAG concentrations was considered with multiple linear regression analysis. P values <0.05 were considered statistically significant. All statistical analyses were conducted using SPSS software version 22.0.

Results

Baseline Characterization and Hormonal Profile Comparison

The clinical characteristics and hormonal profiles of each group are reported in Table 1. No statistically significant difference was observed within the three groups in terms of their age, sex, fasting glucose, TG and LDL. Compared with the visceral normal group, VFA obesity patients with high or normal BMI had significantly higher insulin levels and HOMA-IR ($P < 0.01$). Besides, TC in high-BMI VFA obesity group was higher than that in VFA normal group, whereas HDL was lower ($P < 0.05$).

As shown in Figure 1, compared with VFA normal group, DAG concentrations were significantly lower (421.7 ± 106.0 and 388.7 ± 96.5 pg/mL vs 524.4 ± 141.5 pg/mL, $P < 0.01$) in the two VFA obesity groups, whereas AG/DAG ratio was significantly higher ($P < 0.01$). In contrast, no significant difference was observed in AG concentrations within three groups (Figure 2). There were no differences with respect to baseline characteristics of the patients between the two VFA obesity groups.

Table 1 Comparisons of Baseline Characteristics and Hormonal Profile Among Three Groups of T2DM Subjects

Parameters	VFA Normal	VFA Obesity	
		BMI <25kg/m ²	BMI \geq 25kg/m ²
N	78	20	64
Sex (male/female)	53/25	13/7	43/21
Age (years)	54.8 \pm 6.6	55.5 \pm 6.0	53.4 \pm 7.5
BMI (kg/m ²)	23.1 \pm 2.3	23.4 \pm 1.0	27.5 \pm 1.8**
TC (mmol/L)	4.9 \pm 0.9	5.1 \pm 1.0	5.2 \pm 0.9*
TG (mmol/L)	2.3 \pm 1.7	2.2 \pm 1.2	2.5 \pm 1.4
HDL (mmol/L)	1.1 \pm 0.3	1.1 \pm 0.2	0.9 \pm 0.2*
LDL (mmol/L)	3.2 \pm 0.8	3.1 \pm 0.9	3.4 \pm 0.7
Fasting glucose (mmol/L)	7.3 \pm 1.7	7.8 \pm 2.0	7.8 \pm 2.0
Fasting insulin (mU/L)	3.9 \pm 1.2	7.7 \pm 1.4 ^{###}	9.1 \pm 2.7**
HOMA-IR	1.3 \pm 0.4	2.8 \pm 0.8 ^{###}	3.2 \pm 1.1**
AG (pg/mL)	59.0 \pm 12.8	60.5 \pm 14.2	63.8 \pm 17.4
DAG (pg/mL)	524.4 \pm 141.5	421.7 \pm 106.0 ^{###}	388.7 \pm 96.5**
AG/DAG	0.12 \pm 0.04	0.15 \pm 0.04 [#]	0.17 \pm 0.05**

Notes: Values are expressed as mean \pm SD.* $P < 0.05$, ** $P < 0.01$ visceral normal vs visceral obesity with BMI \geq 25; [#] $P < 0.05$, ^{###} $P < 0.01$ visceral normal vs visceral obesity with BMI <25.

Abbreviations: BMI, body mass index; TC, total cholesterol; TG, triglycerid; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AG, acylated ghrelin; DAG, deacylated ghrelin; VFA, visceral fat area; SFA, subcutaneous fat area; HOMA-IR, homeostatic model assessment of insulin resistance.

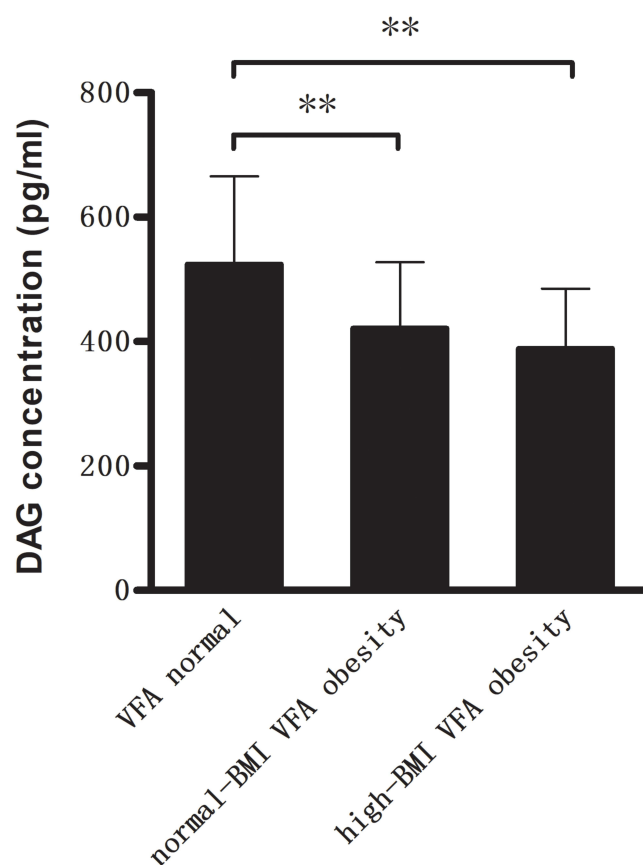


Figure 1 Compared with VFA normal group, plasma levels of deacylated ghrelin (DAG) were significantly lower in two VFA obesity groups (** $P < 0.01$).

The Association Between Clinical Characteristics and AG, DAG and AG/DAG Ratio

In all T2DM patients ($n = 162$), fasting insulin levels and HOMA-IR were negatively associated with DAG but positively with AG/DAG ratio ($P < 0.001$). In contrast, AG was positively correlated with HOMA-IR ($P = 0.006$). Fasting glucose levels showed a positive correlation with AG ($P < 0.001$), whereas they had no correlations with DAG. A strong inverse correlation was observed between DAG and BMI, VFA, and SFA in the whole studied groups ($P < 0.001$). In contrast, no difference was found between these obesity parameters and AG. The associations between AG, DAG and AG/DAG ratio with other variables within all individuals are summarized in [Table 2](#).

Multiple Stepwise Regression Analysis of Influencing Factors of AG and DAG

After multiple stepwise regression analysis, among the parameters only fasting glucose was independently associated with AG concentration ($\beta = 0.390$, $P < 0.001$). In addition, VFA and HOMA-IR were the independent factors related to DAG concentration ($\beta = -0.422$, -0.196 , respectively; $P < 0.05$). The independent factors associated with AG and DAG are shown in [Tables 3](#) and [4](#).

Discussion

The present study assessed the relationship of different circulating forms of ghrelin and their ratio with fat content and insulin resistance in T2DM subjects. AG can stimulate food intake, growth hormone secretion and adipogenesis by activating the growth hormone secretagogue receptor 1a (GHSR).^{5,22} DAG is originally considered as an inactive form of ghrelin since it cannot activate GHSR. However, further research suggests that DAG seems to have a positive impact on energy metabolism, including inhibiting glucose production and improving insulin resistance.^{14,15,23} Absolute or relative increase of AG potentially has a negative impact on insulin action,^{12,24} which is contrary to the effect of DAG.^{23,25} We found that circulating AG is positively correlated with fasting glucose levels; this is in agreement with the previous

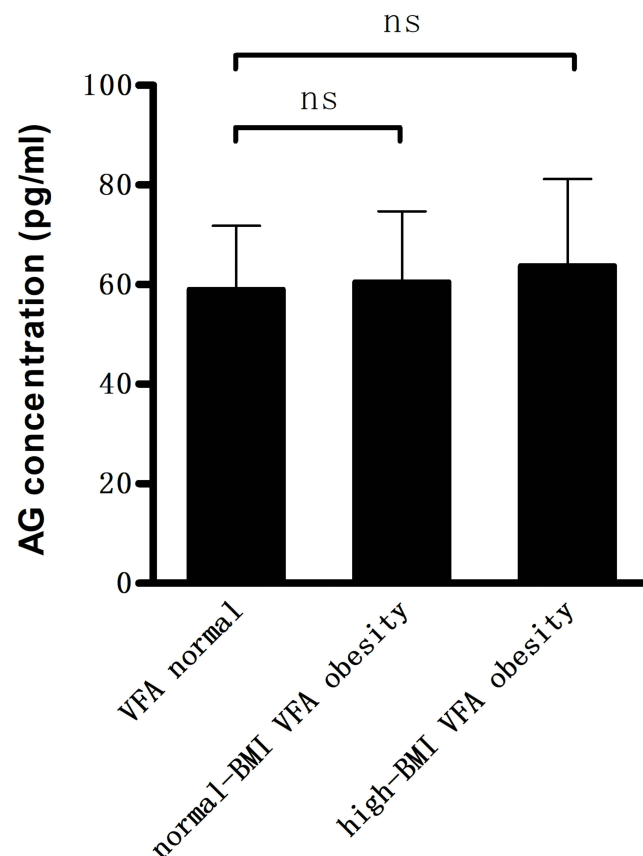


Figure 2 Compared with VFA normal group, the two VFA obesity groups showed no significant difference in the plasma levels of acylated ghrelin (AG) (ns.P>0.05).

reports.^{6,24} Continuous infusion of AG worsens glucose metabolism and acutely reduced oral glucose disposal in humans and rodents.^{12,24} AG is expressed in pancreatic islets and released into the pancreatic microcirculation, where it suppresses insulin secretion, which may partially explain its physiological role in glucose metabolism.²⁶ In contrast, no difference was observed between DAG and fasting glucose levels, suggesting that DAG levels are not affected by glucose status in T2DM. This is supported by the report that DAG levels are unrelated to glucose homeostasis parameters.²⁷

Table 2 Correlations Between AG, DAG and AG/DAG Ratio and Other Variables in All Subjects

	AG		DAG		AG/DAG Ratio	
	r	P-value	r	P-value	r	P-value
Sex (male/female)	-0.084	0.288	-0.069	0.383	0.173	0.028
Age (years)	0.002	0.978	-0.017	0.826	0.013	0.873
BMI (kg/m ²)	0.085	0.282	-0.444	<0.001	0.399	<0.001
TC (mmol/L)	-0.087	0.272	-0.055	0.491	-0.070	0.375
TG (mmol/L)	0.109	0.166	-0.062	0.433	-0.098	0.214
HDL (mmol/L)	-0.058	0.463	0.107	0.176	0.004	0.263
LDL (mmol/L)	-0.079	0.317	0.028	0.724	-0.089	0.263
Fasting glucose (mmol/L)	0.388	<0.001	-0.109	0.169	0.392	<0.001
Fasting insulin (mU/L)	0.105	0.182	-0.509	<0.001	0.459	<0.001
HOMA-IR	0.214	0.006	-0.483	<0.001	0.527	<0.001
VFA (cm ²)	0.005	0.953	-0.547	<0.001	0.359	<0.001
SFA (cm ²)	0.101	0.886	-0.403	<0.001	0.319	<0.001

Notes: Pearson correlation when both variables have normal distribution and Spearman correlation otherwise.

Table 3 Multiple Stepwise Regression Analysis of AG Concentration and Other Variables in All Subjects

Variable	β	SE	β'	t	P
Constant	36.911	4.661		7.919	<0.001
FPG	3.201	0.600	0.390	5.338	<0.001

Table 4 Multiple Stepwise Regression Analysis of DAG Concentration and Other Variables in All Subjects

Variable	β	SE	β'	t	P
Constant	668.8	27.016		24.755	0
VFA	-1.644	0.342	-0.422	-4.806	<0.001
HOMA-IR	-20.786	9.3	-0.196	-2.235	0.027

Although the exact mechanisms of physiological effect of DAG are still unclear, emerging evidence suggests that DAG can counteract the adverse effects of AG on glycometabolism and insulin action.^{28,29} DAG seems to have inherent effects on energy metabolism that are independent of AG, probably because DAG acts on its own receptor. Those actions of DAG may support or antagonize the effects of AG.^{15,29} Our results showed that DAG was inversely associated with IR markers such as BMI, fasting insulin levels and HOMA-IR, whereas AG was positively associated with IR. This is in line with the main studies.⁶⁻¹⁰ DAG can improve insulin sensitivity and metabolic profile independent of GHSR,²⁸⁻³⁰ but has no effect on basal or stimulated insulin secretion.³¹ On the other hand, moderate hyperinsulinemia could inhibit ghrelin secretion, and therefore ensue to worsen insulin resistance through progressive reduction of total and deacylated ghrelin in obese individuals³². The potential role of these alterations in the development of obesity and IR should be further tested in future experimental and clinical prospective studies.

Further studies show that the negative correlation between ghrelin and insulin resistance does not remain significant after removing the effects of obesity, which indicates obesity may play a crucial role in ghrelin reduction and insulin resistance.^{6,8} Excessive fat accumulation is a vital feature of obesity and is largely involved in the development of insulin resistance.³³ Additionally, excess visceral fat mass, characterized by abdominal fat accumulation, is strongly related to various metabolic syndrome features and adverse cardiovascular events.¹⁸⁻²⁰ Recently, bioelectrical impedance analysis (BIA) is considered as a fast, simple, noninvasive and readily reliable body composition assessment technique with a good association with dual-energy X-ray absorptiometry (DXA) results.³⁴ Thus, this study assessed VFA and SFA of T2DM patients by bioelectrical impedance analysis.

Previous observations have shown that obese mice and humans have lower DAG levels than their normal-weight counterparts where AG levels are similar, suggesting that obesity may reflect a relative DAG deficiency.^{8,35,36} Our study found that compared with VFA normal group, VFA obese individuals with high or normal BMI have lower DAG levels and higher HOMA-IR. VFA was significantly negatively correlated with DAG levels. Further analyses showed that VFA and IR were the only two definite independent correlation factors for DAG. These results provide evidence that excess visceral fat mass is involved in the reduction of DAG concentrations, even in the normal-BMI VFA obese individuals. In addition, lower DAG levels have been identified as potential contributors to obesity-related reduction of insulin sensitivity,^{9,10,35} this is probably because obese subjects have lower levels of insulin receptors, which increase insulin levels in consequence.³⁷ Besides, DAG can inhibit inflammation of adipose tissue caused by HFD and decrease macrophage infiltration of adipose tissue, thus alleviating obesity and improving insulin sensitivity in rodents.³⁶ In contrast, administering AG reduces energy expenditure, enhances gene expression of fat storage-promoting enzymes in adipose tissue, and consequently promotes adipose tissue deposition in animal models.³⁸ No correlation between AG concentrations and body fat mass was found in the study. Perhaps, the influencing factors and metabolic mechanism of AG are more complicated in the human body. Further research is needed to observe this issue in humans.

The balance of two different forms of ghrelin was assessed by AG/DAG ratio in our study. The high AG/DAG ratio in obese individuals indicates that excess visceral fat mass is likely to be related to attenuated ghrelin acylation.¹⁶ Furthermore, AG/DAG ratio was significantly positively correlated with insulin resistance and body fat mass, suggesting that the modulation of AG/DAG ratio may be a new treatment strategy for obese-related individuals.^{16,39}

The present study is limited by its retrospective design and a relatively small sample size. The link between both forms of ghrelin and body fat mass can suggest but not prove causality. Thus, prospective cohort studies with larger sample size are needed to validate these findings. In addition, we did not investigate the ghrelin values after a standardized meal in T2DM patients, and this data may provide additional useful information. Nevertheless, the results provide more evidence of the correlation between different forms of ghrelin and body fat mass.

Conclusion

This study demonstrates that AG and DAG differentially affect body fat mass and glycometabolism in T2DM patients. DAG was negatively correlated with insulin resistance and body fat mass, whereas it did not associate with glucose homeostasis parameters. In contrast, AG was closely related to elevated blood glucose levels but unrelated to body fat mass. Further research is warranted to better understand the different roles and mechanisms of AG and DAG in human obesity and energy homeostasis.

Ethical Statement

This study was approved by the Ethics Committee of Jinling Hospital, Nanjing University, and was performed according to the Declaration of Helsinki. As our study was retrospective design, the individual Institutional Review Boards or the Data Protection and Research committees waived the requirement of informed consent due to appropriate handling of patient data and maintenance of patient data confidentiality.

Acknowledgments

This work was supported by Jiangsu Provincial Natural Science Foundation (BK20150558), Postdoctoral Foundation of China (2018M633693), and Jinling Hospital Foundation (YYBJ 2021046).

Disclosure

The authors report no conflicts of interest in this work.

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