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ORIGINAL ARTICLE

Fatty Acid Synthase and Hormone-sensitive Lipase Expression in Liver Are Involved in Zinc- α 2-glycoprotein-induced Body Fat Loss in Obese Mice[△]

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Key words: zinc- α 2-glycoprotein; adipose tissue; fatty acid synthase; hormone-sensitive lipase; obesity

Objective To explore the effects of zinc- α 2-glycoprotein (ZAG) on body weight and body fat in high-fat-diet (HFD)-induced obesity in mice and the possible mechanism.

Methods Thirty-six male mice were fed with standard food (SF) ($n=9$) and HFD ($n=27$), respectively. Five weeks later, 9 mice fed with HFD were subjected to ZAG expression plasmid DNA transfection by liposome transfection method, and another 9 mice to negative control plasmid transfection. Two weeks later, serum ZAG level in the mice was assayed by Western blot, and the effects of ZAG over-expression on body weight, body fat, serum biochemical indexes, and adipose tissue of obese mice were evaluated. The mRNA expressions of fatty acid synthase (FAS) and hormone-sensitive lipase (HSL) in liver tissue were determined by reverse transcription-polymerase chain reaction.

Results Serum ZAG level significantly lowered in simple HFD-fed mice in comparison to SF-fed mice (0.51 ± 0.10 AU vs. 0.75 ± 0.07 AU, $P < 0.01$). Further statistical analysis demonstrated that ZAG level was negatively correlated with body weight ($r = -0.56$, $P < 0.001$), epididymal fat mass ($r = -0.67$, $P < 0.001$), percentage of epididymal fat ($r = -0.65$, $P < 0.001$), and increased weight ($r = -0.57$, $P < 0.001$) in simple SF- and HFD-fed mice. ZAG over-expression in obese mice reduced body weight and the percentage of epididymal fat. Furthermore, FAS mRNA expression decreased ($P < 0.01$) and HSL mRNA expression increased ($P < 0.001$) in the liver in ZAG over-expressing mice.

Conclusions ZAG is closely related to obesity. Serum ZAG level is inversely correlated with body weight and percentage of body fat. The action of ZAG is associated with reduced FAS expression and increased HSL expression in the liver of obese mice.

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EVIDENCE accumulated over the past decades demonstrates that adipose tissue is not only an energy storage organ, but also plays an important role in monitoring and controlling whole body metabolism by secreting a variety of bioactive molecules, known as adipokines or adipocytokines, in an autocrine, paracrine, and/or endocrine manner.¹

Zinc- α 2-glycoprotein (ZAG) is a 43 kD glycoprotein, first isolated from human plasma.² The concentration of ZAG in normal human plasma or serum has been reported as between 3.65 μ g/mL and 140 μ g/mL in different populations using various analytical techniques.³ ZAG was initially reported to be correlated with body weight loss in cancer patients with cachexia, a severe life-threatening wasting syndrome defined in 1998 as massive depletion of both adipose and skeletal muscle tissues.⁴ The concentration of serum ZAG in cancer patients with cachexia was 20-fold higher than that in age- and sex-matched normal people.⁵ *In vivo*, administration of ZAG in mice caused a highly significant reduction in body weight. Further body composition analysis showed that loss of body weight could be attributed entirely to the loss of body fat.⁶ Loss of adipose tissue may be due to the lipolytic effect of ZAG, since incubation of adipocytes isolated from murine adipose tissue with ZAG has been shown to stimulate great glycerol release in a dose-dependent manner.⁴ Interestingly, recent studies performed by Bing et al⁷ and Bao et al⁸ identified that ZAG was not only expressed in adult mouse white adipose tissue and brown adipose tissue, but also could be secreted in human Simpson-Golabi-Behmel syndrome and by mouse 3T3-L1 adipocytes. All these findings suggest that ZAG may be a new adipokine produced by adipocytes, and it is associated with body fat loss that may be involved in stimulatory action of ZAG in lipolysis of adipose tissue. However, it still remains unclear whether ZAG has the similar stimulating action on lipolysis in obesity and whether ZAG has any influence in lipogenesis of adipose tissue.

Fatty acid synthase (FAS) plays a central role in *de novo* lipogenesis by converting acetyl-CoA and malonyl-CoA into the final end product, palmitate, which can subsequently be esterified into triacylglycerols and then stored in adipose tissue. It has been reported that FAS gene expression in human adipose tissue is associated with obesity and type 2 diabetes.⁹ FAS activities were found significantly higher in obese patients and genetically obese rats than those in normal weight subjects.^{9,10} FAS gene promoter activities were 4- to 6-fold higher in adipocytes of obese rats than in those of non-obese rats. As a crucial factor in the opposite process, hormone-sensitive lipase (HSL) is a

rate-limiting enzyme in lipolysis. Its physiological role is to hydrolyze the triglyceride stored in adipose tissue into fatty acids and glycerol. It has been shown that over-expression of HSL prevents triglyceride accumulation in adipocytes. Adipose triglyceride lipase and HSL protein expression decreased in the obese insulin-resistant state,¹¹ and the HSL C-60G promoter polymorphism was associated with increased waist circumference in normal weight subjects.¹² These findings demonstrated that both FAS and HSL are related with obesity. Interestingly, our recent study found FAS and HSL expression in epididymal adipose tissue involved in ZAG-induced body fat loss in obese mice.¹³

In this study, we investigated the association of the serum ZAG level with body weight and percentage of body fat in normal weight and high-fat-diet (HFD)-induced obese mice. In addition, the mRNA expressions of FAS and HSL in liver tissue of HFD-induced obese mice with or without ZAG gene transfection were also determined by reverse transcription-polymerase chain reaction (RT-PCR) to explore the possible involvement in the action of ZAG.

MATERIALS AND METHODS

Animals

Male KM mice (purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College) were housed individually in the animal center of Peking Union Medical College at an ambient temperature of 22°C $\pm$ 1°C under a 12 : 12-hour light-dark cycle with free access to food and drinking water. At the age of six weeks, the mice weighing 25-27 g were divided into two groups and were submitted to standard food (SF, $n=9$) and HFD ($n=27$), respectively. The composition of SF was protein 24%, fat 11%, and carbohydrates 65%; and HFD was protein 22%, fat 55%, and carbohydrates 23%. The quantity of food provided to each mouse was monitored everyday, and the body weight was measured twice a week. We assure that all applicable institutional and governmental regulations concerning the ethical use of laboratory animals were followed during this research.

Construction and identification of pcDNA3.1(-)-mZAG expression plasmid

pcDNA3.1(-)-mZAG expression plasmid contained coding sequences of murine ZAG (mZAG) full-length cDNA (+1-+924 bp). The construction process was similar to what has been previously described.¹⁴ In brief, total RNA from the liver of BALB/c mice was extracted using EZNA total RNA

kit (OmegaBio-Tek, Doraville, GA, USA), then reverse transcribed with SuperScript first-strand synthesis system kit (Invitrogen, Carlsbad, CA, USA). And 2 μ L cDNA was amplified with mZAG primers as follows: forward primer 5'-ATGGTGCCTGTCTGCTGTC-3' (20 bp), reverse primer 5'-TTACTGAGGCTGAGCTACAAC-3' (21 bp). The process consisted of 35 cycles at 95°C for 40 seconds, at 57°C for 40 seconds, and at 72°C for 90 seconds. The product length was 924 bp. The second PCR amplification was performed in order to get products with *EcoR* V and *Hind*III enzyme sites in both ends. The second group of PCR products and pcDNA3.1(-) vector both digested by *EcoR* V and *Hind*III were ligated by T4 DNA ligase to yield a pcDNA3.1(-)-mZAG expression plasmid, which was then sequenced.

The constructed pcDNA3.1(-)-mZAG expression plasmid was confirmed by DNA sequencing. Further *in vitro* experiments confirmed that ZAG mRNA and protein could be expressed better in 3T3-L1 preadipocytes and cell medium following transfection of pcDNA3.1(-)-mZAG expression plasmid into these cells, demonstrated by real-time quantitative RT-PCR and Western blot (data not shown).

***In vivo* plasmid DNA transfection**

After five weeks, the HFD-induced obese mice were divided into three groups ($n=9$ in each group): the simple HFD group, ZAG over-expression group (HFD+ZAG), and negative control plasmid group (HFD+NC). Plasmid transfection was performed as previously described.¹⁵ Briefly, ZAG expression plasmid (25 μ g) or pcDNA3.1(+) negative control plasmid (25 μ g) in 150 μ L Opti-MEM medium (a kind of serum-free medium often used in transfection, produced by Invitrogen) was mixed thoroughly with Lipofectamine 2000 (40 μ L) in 150 μ L Opti-MEM medium, and the total 300 μ L mixture was incubated at room temperature for 30 minutes, then injected into the mice by tail vein. Mice in the simple HFD group were injected with the same volume of Opti-MEM medium as control. This injection was performed at 9:00 am once every two days for seven times.

After two weeks, all the mice were killed following overnight fasting. Blood samples were obtained for biochemical indexes and ZAG assays, and epididymal adipose tissue was dissected and weighed. The percentage of epididymal fat (epididymal fat%) in the mice was calculated as body weight divided by epididymal fat mass. A piece of liver tissue was dissected from each mouse and frozen immediately in liquid nitrogen for FAS and HSL mRNA expression analysis.

Western blot for serum ZAG level in mice

Western blot analysis was performed as previously described.¹⁶ In brief, the protein concentration of murine serum was determined using BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Samples containing 10 μ g of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was then transferred to a nitrocellulose membrane (Immobilon-P blotting membrane, Millipore, Billerica, MA, USA) and immunodetection was performed using a mouse anti-human monoclonal antibody (SC-21720, Santa Cruz Biotechnology, CA, USA) at 4°C overnight at a 1:1 000 dilution. Blots were then incubated with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at a 1:2 000 dilution. Signals were detected by Western blotting luminol reagents (Santa Cruz Biotechnology). A similar process was also performed to assay serum β -actin in mice as an internal reference. The signal intensity of bands was analyzed using BandsScan software, and the intensity of each ZAG band was normalized to the corresponding β -actin band.

Semi-quantitative RT-PCR for FAS and HSL mRNA expressions in liver tissue

The process of total RNA extraction from liver tissue and reverse transcription were the same as described above for the construction of pcDNA3.1(-)-mZAG expression plasmid. The primers used for the PCR amplification of FAS were as follows: forward primer 5'-CCAGCCCCGACCCACAACA-3' and reverse primer 5'-GCCATAGGTGCCGCTGTCTT-3'. The process consisted of 30 cycles at 95°C for 40 seconds, at 52°C for 40 seconds, and at 72°C for 30 seconds. The product length was 387 bp. The primers used for HSL were: forward primer 5'-GCTGGTGCAGAGACAC-3' and reverse primer 5'-GAAAGCAGCGCGCACGCG-3'. The process consisted of 30 cycles at 95°C for 40 seconds, at 59°C for 40 seconds, and at 72°C for 30 seconds. The product length was 409 bp. The primers for β -actin were: forward primer 5'-ATGGATGACGATATCGCT-3' and reverse primer 5'-ATGAGGTAGTCTGTCAGGT-3'. The process consisted of 29 cycles at 95°C for 40 seconds, at 52°C for 40 seconds, and at 72°C for 30 seconds. The product length was 569 bp. The PCR products were separated on 1.5% agarose gel and were scanned using AlphaImager M2200 system (Alpha Innotech, San Leandro, CA, USA), and the signal intensity of bands was counted. The intensity of each cDNA band was normalized to the corresponding β -actin band as was done in the above Western blot. Sequence analysis of the amplicons of FAS and HSL revealed 100% homology with the corresponding regions of murine FAS and HSL cDNA.

Statistical analysis

Data were presented as means±SD. Inter-group differences were analyzed by one-way analysis of variance (ANOVA). Pearson and partial correlation coefficients were used to determine linear association between serum ZAG level and other obesity-related parameters in mice. All statistical computations were run on SPSS 12.0. $P<0.05$ was considered statistically significant.

RESULTS

Serum ZAG level and other laboratory results in SF- and HFD-fed mice

After 7-week feeding, a marked increase in body weight ($P<0.01$), epididymal fat mass ($P<0.05$), epididymal fat% ($P<0.05$) of HFD-fed mice was first observed compared with SF-fed mice. Meanwhile, the serum fasting glucose ($P<0.01$), total cholesterol ($P<0.01$), and low density lipoprotein (LDL)-cholesterol ($P<0.05$) of HFD-fed mice also became higher than those of SF-fed mice. In contrast, serum ZAG level in HFD-fed mice was 32% lower than that in SF-fed mice (0.51 ± 0.10 AU vs. 0.75 ± 0.07 AU, $P<0.01$) (Table 1).

Effects of ZAG on body weight, epididymal fat% and biochemical parameters in HFD-induced mice

Serum ZAG level in mice receiving ZAG gene transfection (1.01 ± 0.16 AU) increased by 98% and 87% respectively when compared with two controls: Opti-MEM medium treated mice (HFD group, 0.51 ± 0.10 AU, $P<0.01$) and pcDNA3.1(+) negative control plasmid treated mice (HFD+NC group, 0.54 ± 0.08 AU, $P<0.01$), as detected by Western blot. The mean serum ZAG level in SF-fed mice was 0.75 ± 0.07 AU (Table 1, Fig.1).

Over-expression of ZAG in HFD mice produced a 12.7% decrease in final body weight (39.96 ± 2.62 g vs. $45.70\pm$

4.04 g, $P<0.01$) and a 31.7% decrease in increased weight (in comparison to initial weight) with HFD group as the reference (12.77 ± 2.35 g vs. 18.71 ± 3.92 g, $P<0.01$), without a significant change in food intake. Over-expression of ZAG in HFD mice also caused a great reduction in epididymal fat mass (54.9%, $P<0.01$) and epididymal fat% (47.5%, $P<0.01$) (Table 1). Among serum glucose and lipid profiles parameters, only fasting glucose decreased significantly after transfecting ZAG gene ($P<0.01$). Finally, further analysis showed that a negative correlation existed between serum ZAG and body weight ($r=-0.56$, $P<0.001$), epididymal fat mass ($r=-0.67$, $P<0.001$), epididymal fat% ($r=-0.65$, $P<0.001$), and increased weight ($r=-0.57$, $P<0.001$) in SF- and HFD-fed mice (Fig. 2). There was no relationship between ZAG and fasting glucose, total cholesterol, triglycerides, high density lipoprotein (HDL)-cholesterol, and LDL-cholesterol in mice.

FAS and HSL mRNA expression in liver tissue of HFD+ZAG mice

As shown in Figure 3, FAS and HSL mRNAs were detected in all of the 36 murine liver tissue samples. FAS mRNA in the liver tissue of HFD-fed mice increased by 46.7% compared with that in SF-fed mice (2.67 ± 0.46 vs. 1.82 ± 0.32 , $P<0.05$). After transfection of ZAG gene, FAS mRNA decreased by 58.1% compared with that in HFD group (1.12 ± 0.32 vs. 2.67 ± 0.46 , $P<0.01$). In contrast to FAS mRNA, HSL mRNA in the liver tissue of HFD-fed mice reduced by 53.5% compared with that in SF-fed mice (1.20 ± 0.20 vs. 2.58 ± 0.30 , $P<0.01$). Transfection of ZAG gene to HFD-fed mice resulted in a significant increase in HSL mRNA expression (3.17 ± 0.75 vs. 1.20 ± 0.20 , $P<0.001$), 1.6-fold higher than that of HFD group.

Table 1. Body weight, body composition, food intake, and laboratory measurements in normal weight and obese mice[§] ($n=9$)

Group	Initial weight (g)	Final weight (g)	Increased weight (g)	Food intake (g/d)	Epididymal fat mass (g)	Epididymal fat%
SF	27.16 ± 0.54	40.39 ± 1.90	13.23 ± 2.11	7.51 ± 0.38	0.60 ± 0.14	1.49 ± 0.34
HFD	27.00 ± 0.47	$45.70\pm4.04^{**}$	$18.71\pm3.92^{**}$	$9.62\pm0.51^{**}$	$1.02\pm0.29^{*}$	$2.21\pm0.48^{*}$
HFD+ZAG	27.19 ± 0.58	$39.96\pm2.62^{\#}$	$12.77\pm2.35^{\#}$	$9.57\pm0.34^{**}$	$0.46\pm0.06^{\#}$	$1.16\pm0.10^{\#}$
HFD+NC	26.72 ± 0.77	$46.44\pm4.33^{**}$	$19.69\pm3.76^{**}$	$9.80\pm0.52^{**}$	$1.02\pm0.57^{*}$	$2.15\pm0.98^{*}$
Group	Fasting glucose (mmol/L)	Total cholesterol (mmol/L)	Triglycerides (mmol/L)	HDL-cholesterol (mmol/L)	LDL-cholesterol (mmol/L)	ZAG (AU)
SF	8.74 ± 1.60	3.66 ± 0.81	1.03 ± 0.10	2.67 ± 0.40	0.59 ± 0.44	0.75 ± 0.07
HFD	$10.87\pm1.77^{**}$	$5.20\pm1.24^{**}$	1.24 ± 0.16	$4.04\pm1.05^{**}$	$1.10\pm0.24^{*}$	$0.51\pm0.10^{**}$
HFD+ZAG	$9.13\pm1.00^{\#}$	$5.28\pm1.10^{**}$	1.07 ± 0.23	$3.91\pm0.69^{**}$	$0.73\pm0.15^{**}$	$1.01\pm0.16^{\#}$
HFD+NC	10.08 ± 1.36	$5.49\pm0.66^{**}$	1.17 ± 0.38	$4.13\pm0.72^{**}$	$1.55\pm0.81^{**}$	$0.54\pm0.08^{**}$

§: Plus-minus values are means±SD.

SF: standard food; HFD: high-fat-diet; ZAG: zinc- α 2-glycoprotein; HFD+ZAG: ZAG over-expression group; HFD+NC: negative control plasmid group; epididymal fat%: percentage of epididymal fat; HDL: high density lipoprotein; LDL: low density lipoprotein; AU: arbitrary unit.

* $P < 0.05$, ** $P < 0.01$ compared with SF group; # $P < 0.01$ compared with HFD group.

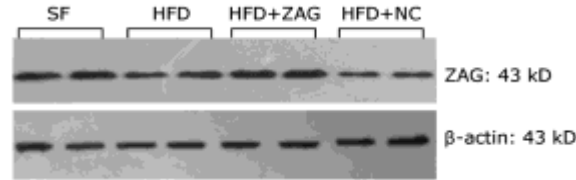


Figure 1. Serum ZAG level determined by Western blot in SF- and HFD-fed mice with or without ZAG transfection.

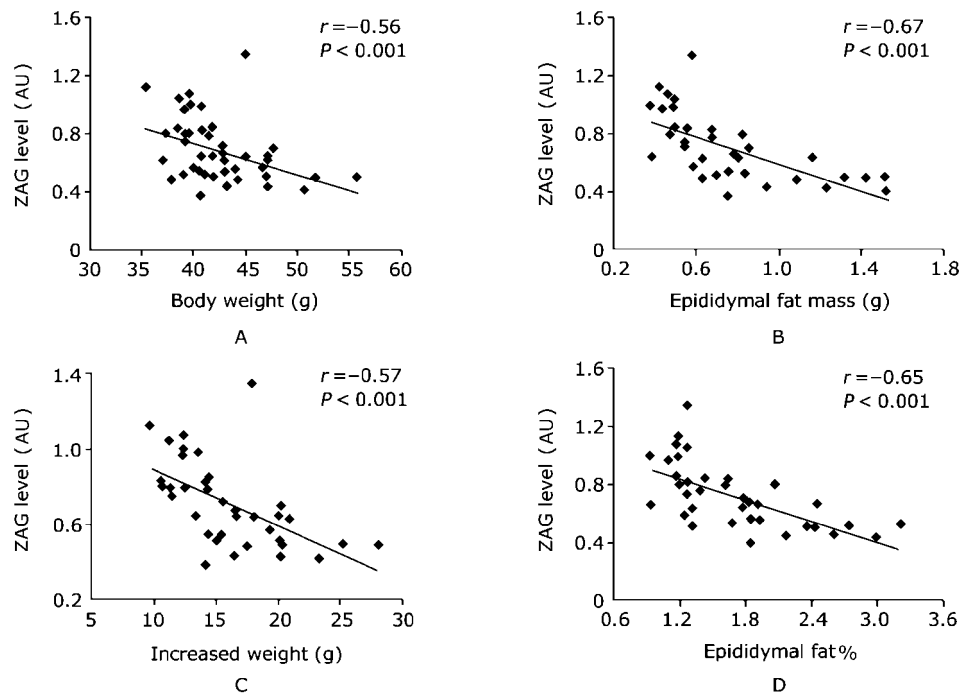


Figure 2. Correlation of serum ZAG level with body weight (A), epididymal fat mass (B), increased weight (C) and epididymal fat% (D) in SF- and HFD-fed mice.

All blood samples are taken after an overnight fasting. Serum ZAG level is detected by Western blot.

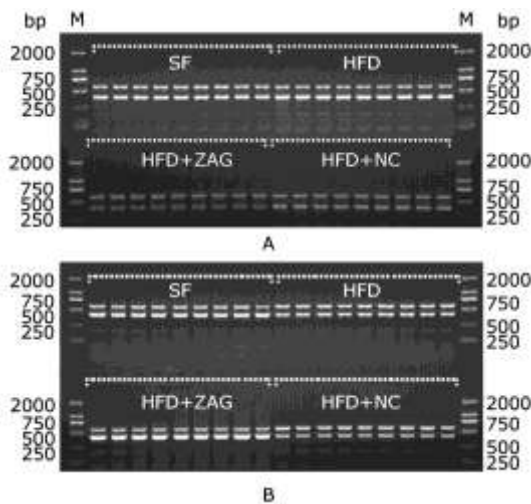


Figure 3. mRNA expression levels of fatty acid synthase (FAS, A) and hormone-sensitive lipase (HSL, B) in liver tissue of mice as demonstrated by semi-quantitative reverse transcription-polymerase chain reaction. M: marker.

DISCUSSION

In the present study, we found that serum ZAG level in HFD-induced obese mice significantly decreased compared with that in normal weight mice. Further statistical analysis demonstrated negative correlation of ZAG level with body weight, epididymal fat mass, epididymal fat%, and increased weight in SF- and HFD-fed mice. Consistent with our findings, Marrades et al¹⁷ also found that ZAG mRNA level was negatively correlated with body mass index (BMI) and fat mass. Studies performed by Marrades et al¹⁷ and Dahlman et al¹⁸ at the tissue level revealed with real-time PCR analysis and microarray technique that ZAG expression was down-regulated by 70% and 69%, respectively, in subcutaneous abdominal adipose tissue of obese subjects

compared with lean subjects. Moreover, a significant positive correlation between ZAG gene expression and serum adiponectin and a negative correlation with the plasma levels of leptin and waist circumference were found in obese subjects,¹⁷ suggesting that ZAG may be a candidate factor in the regulation of body weight at the tissue level, and adiponectin and leptin are also involved in this process.

The relationship between ZAG and body weight was further supported by our experiments in ZAG gene transfected obese mice, where ZAG over-expression was found to result in a reduction of body weight, epididymal fat mass, and epididymal fat% in HFD-fed obese mice without changing food intake. The similar conclusion was also drawn by Hirai et al⁴ who demonstrated that administration of ZAG to both exbreeder mice and *ob/ob* mice induced a rapid and dose-dependent reduction in body weight without a reduction in food and water intake. Loss of body weight could be attributed entirely to the loss of body fat. In contrast to administering ZAG to obese mice, Rolli et al¹⁹ inactivated both ZAG alleles by gene targeting in mice, then subjected these ZAG-deficient mice to standard or lipid rich food regimens for 20 weeks. The results showed that ZAG knock out (ZAG^{-/-}) mice gained significantly more weight than ZAG^{+/+} control mice fed by either standard or lipid rich food. All of these findings, together with the evidence of the reduced serum ZAG level in obesity patients and obese mice, indicate that there is a relationship between ZAG and body weight.

Now that ZAG is associated with body weight in human and mice, and administration of ZAG results in a reduction of body weight and body fat in normal and obese mice, what might be the targets involved in the action of ZAG? To answer this question, mRNA expression of two key enzymes in lipid metabolism, FAS and HSL, were determined by RT-PCR method in liver tissue of HFD-induced obese mice with or without ZAG gene transfection. Our results showed that FAS mRNA decreased and HSL mRNA increased significantly in liver tissue after transfecting ZAG gene into the mice. Since FAS and HSL are key enzymes during lipogenesis and lipolysis, and both are related with obesity, this result suggests that lipid metabolism including lipogenesis and lipolysis are involved in ZAG-induced reduction of body fat in obese mice. It has been reported that *in vivo*, administration of ZAG in mice induced a reduction of body fat together with increased levels of serum free fatty acid and glycerol, and an elevated oxygen uptake in interscapular brown adipose tissue, providing evidence of increased lipolysis, lipid mobilization and utilization.^{4,20} In the present study, we reported that the action of ZAG to reduce body weight and body fat in obese mice involved

inhibiting lipogenesis besides stimulating lipolysis. Experiments performed *in vitro* demonstrated that incubation of adipocytes isolated from murine adipose tissue with ZAG could stimulate lipolysis in a dose-dependent manner,⁴ suggesting that the lipolytic action of ZAG may be direct. However, further investigation is needed to determine whether the action of ZAG in FAS and HSL mRNA expression is direct or indirect.

Finally, in the present study, we amplified coding sequences of mZAG full-length cDNA from the liver of BALB/c mice and constructed mZAG expression plasmid pcDNA3.1 (-)-mZAG. DNA sequence analysis revealed 100% homology with mZAG cDNA. Experiments performed *in vitro* and *in vivo* using real-time RT-PCR and Western blot both confirmed that ZAG mRNA and protein could be better expressed in cell lysis, cell medium, and serum of mice. Thus mZAG expression plasmid provides a good tool for studying biological function of ZAG in future researches.

In conclusion, ZAG is closely linked to obesity. ZAG level is inversely associated with body weight and percentage of body fat. The action of ZAG is correlated with reduced FAS expression and increased HSL expression in liver tissue of mice.

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