Role of β-Adrenergic Receptors in the Oral Activity of Zinc-α2-Glycoprotein (ZAG)

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Zinc-α2-glycoprotein (ZAG) is an adipokine with the potential as a therapeutic agent in the treatment of obesity and type 2 diabetes. In this study we show that human ZAG, which is a 41-kDa protein, when administered to *ob/ob* mice at 50 $\mu g/d^{-1}$ orally in the drinking water produced a progressive loss of body weight (5 g after 8 d treatment), together with a 0.5 C increase in rectal temperature and a 40% reduction in urinary excretion of glucose. There was also a 33% reduction in the area under the curve during an oral glucose tolerance test and an increased sensitivity to insulin. These results were similar to those after iv administration of ZAG. However, tryptic digestion was shown to inactivate ZAG. There was no evidence of human ZAG in the serum but a 2-fold elevation of murine ZAG, which was also observed in target tissues such as white adipose tissue. To determine whether the effect was due to interaction of the human ZAG with the β-adrenergic (β-AR) in the gastrointestinal tract before digestion, ZAG was coadministered to *ob/ob* mice together with propanolol (40 mg/kg^{-1}), a nonspecific β-AR antagonist. The effect of ZAG on body weight, rectal temperature, urinary glucose excretion, improvement in glucose disposal, and increased insulin sensitivity were attenuated by propanolol, as was the increase in murine ZAG in the serum. These results suggest that oral administration of ZAG increases serum levels through interaction with a β-AR in the upper gastrointestinal tract, and gene expression studies showed this to be in the esophagus. **(*Endocrinology* 153: 4696–4704, 2012)**

The increasing prevalence of obesity and the associated type 2 diabetes are a major health problem for the twenty first century. Current therapeutic agents are limited in efficacy, whereas treatment-associated adverse events have meant many agents being withdrawn.

We have previously investigated zinc-α2-glycoprotein (ZAG) as a treatment for obesity and type 2 diabetes (1). ZAG is a soluble protein of 41 kDa, which resembles a class 1 major histocompatability complex heavy chain and has a major groove capable of binding hydrophobic molecules, which could be important in its action (2). ZAG was first identified as the lipid mobilizing factor in cancer cachexia following its isolation from the cachexia-inducing MAC16 tumor, and from the urine of cachectic patients (3). Treatment of either aged (4), or obese (1), mice with ZAG produced a time-dependent decrease in body weight through specific loss of carcass lipid, whereas there was an expansion of the nonfat carcass mass. ZAG is produced by a range of tissues including white (WAT) and brown (BAT) adipose tissue, liver, heart, lung, and skeletal muscle as well as certain tumors that induce cachexia (5). Expression of ZAG mRNA in adipose tissue is high in cancer cachexia, in which lipid stores are low (5), and low in obesity, in which lipid stores are high (6). Thus, ZAG expression is negatively correlated with body mass index and fat mass (6). ZAG expression is negatively regulated by TNF-α and positively regulated by the peroxisomal proliferator-activated receptor-γ agonist rosiglitazone (7), β3-adrenergic receptor (β3-AR) agonists (5), and glucocorticoids (8). ZAG also induces its own expression in adipose tissue through interaction with a β3-AR (8). In this way extracellular ZAG can induce expression of intracellular ZAG in target tissues, which has been suggested to be more important locally than circulating ZAG (6).

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Abbreviations: β1-AR, β2-AR, β3-AR, β-Adrenergic; BAT, brown adipose tissue; KRBB, Krebs-Ringer bicarbonate; WAT, white adipose tissue; ZAG, zinc-α2-glycoprotein.

Previously studies have administration ZAG by either the ip (4) or iv (1) routes. However, neither route is convenient for clinical use. Here our study investigates the effect of ZAG on obesity and diabetes in the ob/ob mouse when administered by the oral route. This would not normally be considered to be ineffective for a protein molecule. However, the ability of ZAG to induce its own expression through a β3-AR (8) enables the message to be transmitted from gastrointestinal tract to the rest of the body before digestion occurs.

Materials and Methods

Materials

Fetal calf serum was from Biosera (Sussex, UK), whereas DMEM was from PAA (Somerset, UK) and freestyle medium was purchased from Invitrogen (Paisley, UK). Hybond A nitrocellulose membranes and peroxidase-conjugated rabbit anti-mouse antibody were from GE Healthcare (Bucks, UK), whereas enhanced chemiluminescence development kits were purchased from Thermo Scientific (Northumberland, UK). Mouse monoclonal antibodies to full-length human and mouse ZAG were from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse insulin ELISA kit was purchased from DRG (Marburg, Germany), and glucose measurements in both urine and plasma were made using a Boots (Nottingham, UK) glucose kit. L-[U-^{14}C] tyrosine (sp.act 16.7 GBq/mmol^{-1}) was purchased from PerkinElmer Ltd. (Cambridge, UK), whereas 2-[1-^{14}C] deoxy-D-glucose (specific activity 1.85GBq/mmol^{-1}) was from American Radiolabeled Chemicals (Cardiff, UK).

Production and purification of ZAG

Recombinant human ZAG was produced by human embryonic kidney 293F cells transfected with pcDNA3.1 containing human ZAG (1). Cells were grown for 2 wk in freestyle medium containing neomycin (50 μg/ml^{-1}) under an atmosphere of 5% CO_2 in air. The cells were then removed by centrifugation (700 g for 15 min), 1 liter of medium was concentrated to 1 ml, and the ZAG was extracted by binding to activated diethylaminoethyl cellulose, followed by elution with 0.3 M NaCl before washing and concentrating with sterile PBS. The ZAG produced was greater than 95% pure mainly due to ZAG's negative charge, as determined by SDS-PAGE, and was free of endotoxin (1). For [^{14}C] ZAG, L-[U-^{14}C]tyrosine was added to the media (1 μCi/ml^{-1}), the cells were allowed to grow for 2 wk, and ZAG was purified as above. The specific activity of the ZAG was 221 μCi/μmol^{-1}, and the purity of the product is shown elsewhere (see Fig. 3A).

cAMP determination

CHOK1 cells transfected with human β1-adrenergic receptor (β1-AR), β2-adrenergic receptor (β2-AR), and β3-AR were maintained in DMEM supplemented with 2 mM glutamine, hygromycin B (50μg/ml^{-1}), G418 (200 mg/ml^{-1}), and 10% fetal calf serum, under an atmosphere of 10% CO_2 in air. For cAMP production, cells were grown in 24-well plates in 1 ml nutrient medium, and ZAG, after tryptic digestion as described in the legend to Fig. 3C, was incubated for 30 min. The medium was then removed and 0.5 ml of 20 mM HEPES (pH 7.5), 5 mM EDTA, and 0.1 mM isobutylmethylxanthine was added, followed by heating on a water bath for 5 min and cooling on ice for 10 min. The concentration of cAMP was determined using a Parameter cAMP assay kit (New England Biolabs, Hitchin, Herts, UK).

Animals

Obese (*ob/ob*) mice (average weight 65 g) were bred in our own colony and were kept in an air conditioned room at 22 ± 2 C with *ad libitum* feeding of a rat and mouse breeding diet (Special Diet Services, Witham, UK) and tap water. These animals exhibit a more severe form of diabetes then C57BL/6J *ob/ob* mice, and the origins and characteristics of the Aston *ob/ob* mouse has been previously described (9). Animals were grouped (n = 5) to receive either ZAG/PBS (50 μg/d^{-1}) or PBS in their drinking water, and the experiment was repeated three times after a power analysis was performed. Each mouse consumed 5 ml/d^{-1} water, and this did not change on ZAG administration (1). The ZAG was replaced every 48 h. One group of mice receiving ZAG were also administered propanolol (40 mg/kg^{-1}, orally) daily. The dose of ZAG was chosen to be the same as that previously administered iv (1) so that a direct comparison could be made between the two routes.

Body weight, food and water intake, urinary glucose excretion, and body temperature, determined by the use of a rectal thermometer (RS Components, Northants, UK), were measured daily. A glucose tolerance test was performed on d 3. Animals were fasted for 12 h, followed by oral administration of glucose (1 g/kg^{-1} in a volume of 100 μl by gavage). Blood samples were removed from the tail vein at 15, 30, 60, and 120 min and used for the measurement of glucose. Urinary glucose was measured by collecting 0.5 ml urine and testing glucose concentration using a Boots glucose monitor. After 8 d of treatment, the animals were killed by cervical dislocation, and tissues were removed and rapidly frozen in liquid nitrogen and maintained at −80 C. Future work would be to repeat this work in diet-induced animals as an alternative to a model with gene alteration, although previous studies have shown the *ob/ob* mouse to be a good indicator of potential human treatments (9). Animal studies were conducted under Home Office License according to the U.K. Coordinating Committee on Cancer Research Guidelines for the Care and Use of Laboratory Animals.

Glucose uptake into adipocytes

Single-cell suspensions of white and brown adipocytes were obtained by incubation of minced epididymal sc and visceral WAT and BAT for 2 and 2.5 h, respectively, with Krebs-Ringer bicarbonate (KRBB) containing 1.5 mg/ml^{-1} collagenase and 4% BSA under 95% oxygen-5% CO_2 at 37 C. Adipocytes were washed twice in 1 ml KRBB (pH 7.2) and then incubated for 10 min at room temperature in 0.5 ml KRBB, containing 18.5 M Bq 2-[1-^{14}C] deoxy-D-glucose, together with nonradioactive 2-[1-^{14}C] deoxy-D-glucose, to give a final concentration of 0.1 mM, in the absence or presence of insulin (10 nM). Uptake was terminated by the addition of 1 ml ice-cold KRBB without glucose. Adipocytes were washed three times with 1 ml KRBB and lysed by the addition of 0.5 ml 1 M NaOH. The uptake of 2-[1-^{14}C] DG was determined by liquid scintillation counting.

Glucose uptake into soleus muscle

The uptake of 2-[1-14C] DG into freshly isolated soleus muscles in the absence and presence of insulin (10 nM) was determined as previously described (1).

Western blotting analysis

Tissues were thawed, washed in PBS, and lysed in Phosphosafe extraction reagent (Merck, Darmstadt, Germany) for 5 min at room temperature, followed by sonication at 4 C. Cytosolic protein (5–20 μg) formed by centrifugation at 18,000 × *g* for 5 min at 4 C, was resolved on 12% SDS-PAGE by electrophoresis at 180 V for about 1 h. To determine ZAG in serum, 30-μl samples containing 20 μg total protein were electrophoresed as above. Protein was transferred to 0.45 μm nitrocellulose membranes, which had been blocked with 5% (wt/vol) nonfat dried milk (Marvel Premier Foods, St. Albans, UK) in Tris-buffered saline (pH 7.5) at 4 C overnight. Membranes were washed for 15 min in 0.1% Tween 20-buffered saline before adding the primary antibodies. Both the primary and secondary antibodies were used at a dilution of 1:1000. Incubation was for 1 h at room temperature, and development was by enhanced chemiluminescence. Blots were scanned by a densitometer to quantify differences.

Polymerase chain reaction

Total RNA was extracted from tissues (50–120 mg) and adipocytes with Trizol. RNA samples used for real-time PCR were treated with a DNA-free kit (Invitrogen, Paisley, UK) to remove any genomic DNA. The RNA concentration was determined from the absorbance at 260 nM. One microgram of total RNA of each sample was reverse transcribed to cDNA in a final volume of 20 μl by using a Reverse-iT first strand kit (Fisher, Loughbourgh, UK). One microliter of each cDNA sample was then amplified in a PCR mixture containing 0.02 mM of each primer and 1.1 · Reddy Mix PCR master mix (Fisher) in a final volume of 25 μl. Human β-actin was used as a housekeeping gene. The primer pair used and the PCR cycling conditions were as follows: mouse ZAG, 5′-GCCTTCTTCCAC TACAACAG-3′ (forward), 5′-TTCAGGACACTCCTCCTCTA-3′ (reverse), annealing temperature 54 C, 33 cycles; and mouse β-actin, 5′-GTGGCATCCACGAAACTACCTT-3′ (forward), 5′-GGACTCGTCATACTCCTGCTTG-3′ (reverse) (antisense), annealing temperature 57 C, 23 cycles. PCR was performed on a thermal cycler (Hybaid, Basingstoke, UK) with an initial denaturation at 94 C for 2 min followed by cycles consisting of denaturation at 94 C for 20 sec, annealing at the specified temperature for 25 sec, and extension at 72 C for 59 sec; the final step was an extension at 72 C for 5 min. PCR products were separated on a 1% agarose gel stained with ethidium bromide. The PCR products were sequenced commercially to confirm their identity (MWG Biotech, Warrington, UK) (7).

Reverse transcription-polymerase chain reaction

Relative ZAG mRNA levels were quantified using real-time PCR with an ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, UK). Mouse β-actin mRNA levels were similarly measured and served as the reference gene. Primers and Taqman probes were designed using PrimerExpress software (Applied Biosystems). The sequences of primers and Taqman probes were as follows: mouse

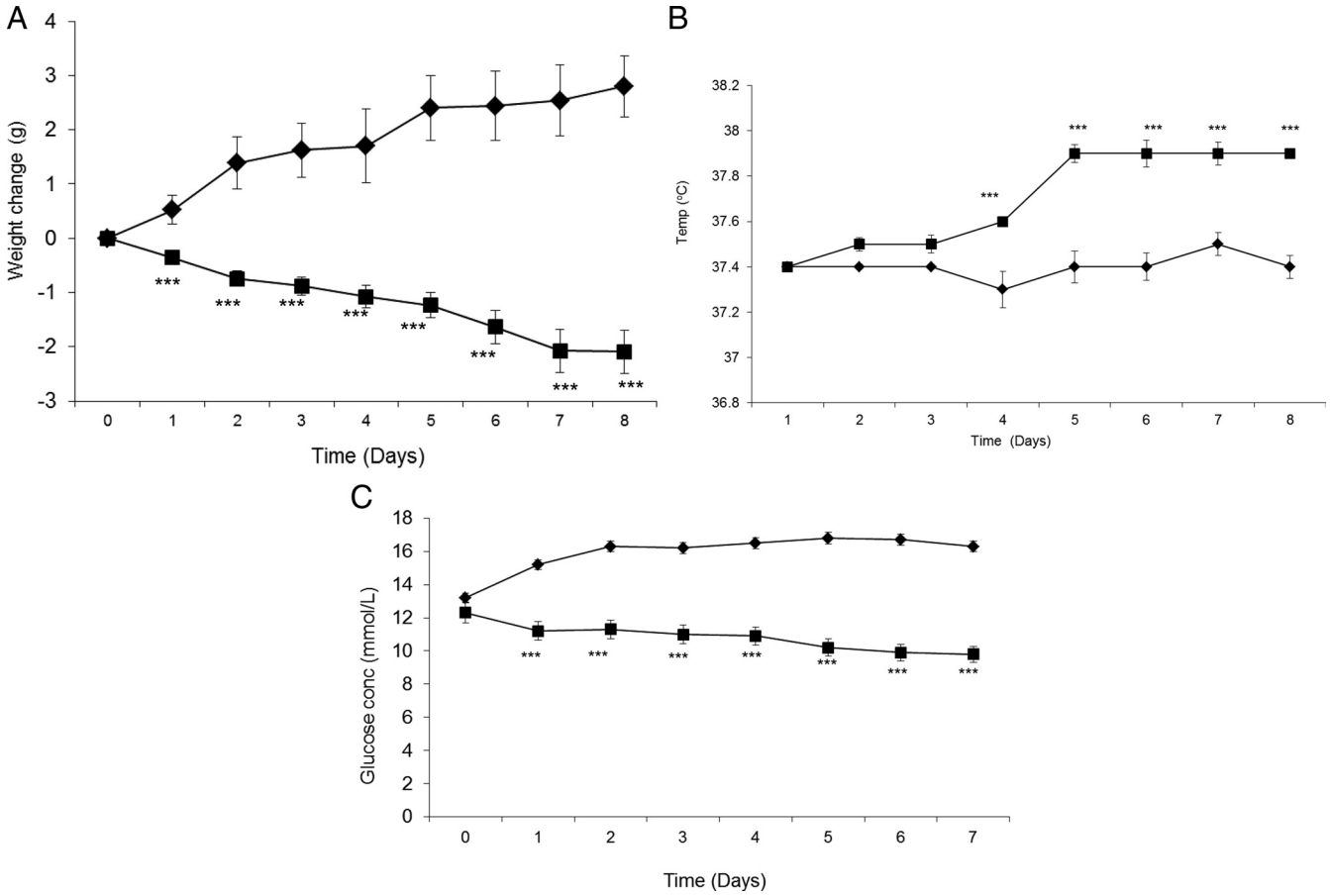

FIG. 1. Effect of human ZAG on body weight (A), rectal temperature (B), and urinary glucose excretion (C) in *ob/ob* mice. ZAG was dissolved in the drinking water so that animals consumed 50 $\mu g/d^{-1}$ (■), whereas a control group received an equal volume of PBS (◆) (0.5 ml in 5 ml water). ***, Differences from PBS controls, $P < 0.001$.

ZAG, 5′-GAGCCTGTGGGACCTTGGA-3′ (forward), 5′-CCTC CCTGGCCCTCTGAA-3′ (reverse), and 5′-FAM-AATGGAGGA CTGGGAGAAGGAAAGCCAG-TAMRA-3′; and mouse β-actin, 5′-ACGGCCAGGTCATCACTATTG-3′ (forward), 5′-CAAG AAGGAAGGCTGGAAAAGA-3′ (reverse), and 5′-FAM-AC GAGCGGTTCCGATGCCCTG-TAMRA-3′. Amplification was performed in a 96-well plate using a master mix made from a quantitative PCR core kit (Eurogentec, Herstal, Belgium), with 300 nM forward (900 nM in the case of β-actin) and 900 nM reverse primers, 225 nM probe, and 1 μl of cDNA in a final volume of 25 μl. Each sample was run in triplicate for ZAG and in duplicate for β-actin. The PCR parameters were as follows: initial 2 min at 50 C, denaturation at 95 C for 10 min followed by 40 cycles of denaturation at 95 C for 15 sec, and combined annealing and extension at 60 C for 1 min. Data were recorded and analyzed with Sequence Detector software (Applied Biosystems). ZAG mRNA levels were normalized to the values of β-actin and the results expressed as relative fold changes using the 2-DDCt method, (7).

Statistical analysis

Results are shown as mean ± SEM for at least three replicate experiments. Differences in means between groups were determined by one-way ANOVA followed by Tukey-Kramer multiple comparison tests, and $P < 0.05$ was considered significant.

Results

Previous studies (1) have shown that animals treated with iv ZAG consume the same amount of food and water as PBS controls. It was therefore convenient for oral admin-

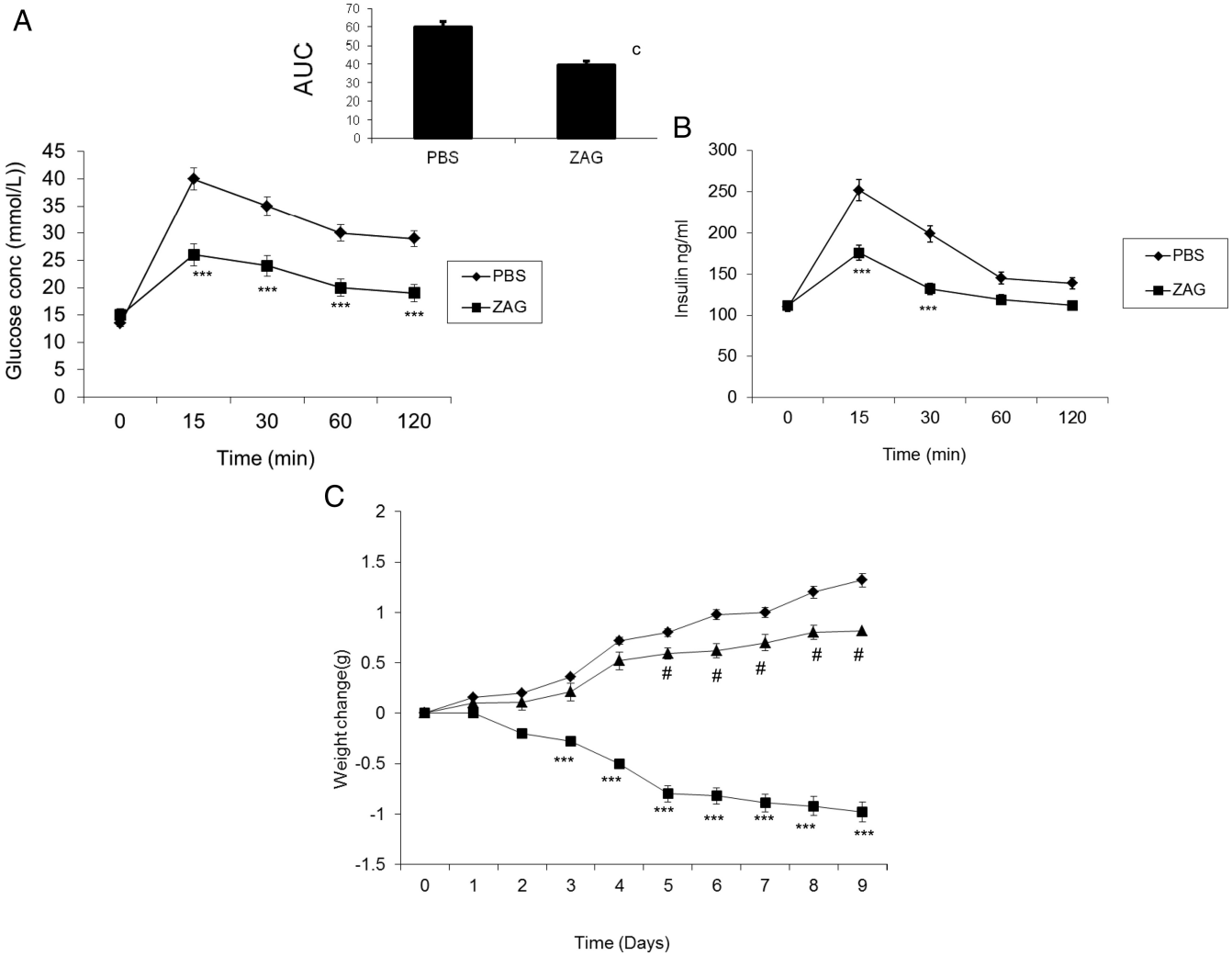

FIG. 2. Effect of oral human ZAG (■) compared with PBS (♦) on glucose and insulin tolerance of *ob/ob* mice after 3 d of treatment. Animals were fasted for 12 h before oral administration of glucose (1g/kg^{-1} in a volume of 100 μl). Blood samples were removed from the tail vein at the time intervals shown and used for the measurement of serum glucose (A) and insulin (B). The *inset* in A shows the total area under the glucose curves (AUC) in arbitrary units. ***, Differences from PBS controls, $P < 0.001$. The effect of propranolol (40 mg/kg^{-1}, orally, daily) on ZAG-induced reductions in obesity and diabetes in *ob/ob* mice are shown. Animals received ZAG (50 μg daily) in their drinking water as described in the legend to Fig. 1, either alone (■) or in the presence of propranolol (▲), whereas a control group received PBS (♦). Changes in body weight (C), rectal temperature (D), and urinary glucose excretion (E) were monitored over an 8-d period. A glucose tolerance test (F), with measurement of serum insulin levels (G), was made 3 d after starting the oral ZAG. ***, Differences from PBS controls, $P < 0.001$; #, differences from ZAG alone, $P < 0.001$.

istration to dissolve the ZAG in drinking water because this would avoid the stress associated with dosing by gavage. The concentration of ZAG in the drinking water was such that the animals would consume 50 μg/d so that a direct comparison could be made with the iv route (1). The effect of oral ZAG on the body weight of *ob/ob* mice is shown in Fig. 1A. After 5 d of treatment, the difference in body weight between the ZAG and PBS groups was 3.5 g, which was the same as that found after iv administration (1), whereas after 8 d of treatment, there was 5 g weight difference between the groups. As with iv administration of ZAG (1), there was an increase in rectal temperature, which became significant after 4 d of treatment (Fig. 1B), whereas there was a 40% reduction in urinary glucose excretion, which became significant after 1 d of treatment (Fig. 1C). This suggests that the oral ZAG also reduced the severity of diabetes in the *ob/ob* mouse. A glucose tolerance test, performed on animals after 3 d of oral ZAG, showed a reduced peak blood glucose concentration and a 33% reduction in the total area under the curve during the entire glucose tolerance test in ZAG treated animals (Fig. 2A). ZAG also decreased the insulin response to the glucose challenge (Fig. 2B), although a direct comparison has not been made. These results suggest that oral administration of ZAG is as effective in inducing weight loss and reducing the severity of diabetes in *ob/ob* mice as when given by the iv route (1).

To determine whether this effect was due to interaction with a β-adrenergic receptor (β-AR), ZAG was administered orally to *ob/ob* mice that were coadministered the nonspecific β-AR antagonist propanolol (40 mg/kg). As shown in Fig. 2C, whereas mice administered ZAG orally lost weight, this was blocked with propanolol, which had no effect on weight gain of mice, administered PBS. Initially propanolol was administered at 20 mg/kg^{-1}, but this did not prevent the weight loss with ZAG so the dose was increased to 40 mg/kg^{-1}. Antagonists of β1-AR and β2-AR are known to be less effective against β3-AR responses. Propanolol also completely attenuated the ZAG induced increase in rectal temperature (Fig. 2D) and the reduction in urinary excretion of glucose (Fig. 2E). Propanolol also blocked the reduced peak blood glucose concentration in the glucose tolerance test (Fig. 2F) and the increase in insulin sensitivity (Fig. 2G). Propanolol also completely attenuated the decrease in serum glucose and insulin levels after ZAG administration to ob/ob mice. The elevation of serum glycerol level suggested that it blocked lipolysis-induced by ZAG along with the decrease in serum triglycerides and nonesterified fatty acids (Table 1).

One possibility by which this could occur is that ZAG escapes digestion by proteolytic enzymes and is absorbed directly into the blood stream. To investigate this possibility, ZAG was biosynthetically labeled with L-[U-^{14}C] tyrosine. SDS-PAGE showed that the purified product

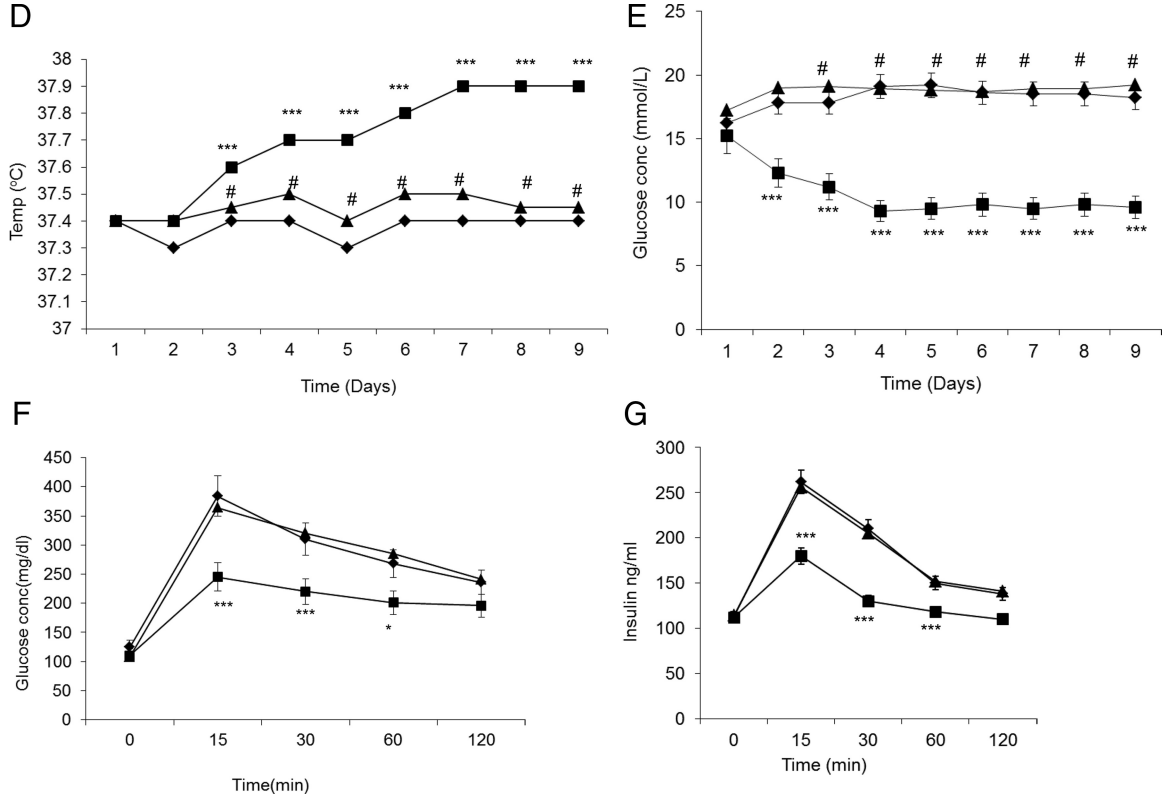

FIG. 2. Continued.

TABLE 1. Serum metabolites in ob/ob mice treated with ZAG in the absence or presence of Propanolol for 8 d

	PBS	ZAG	ZAG+ Prop
Glucose mmol^{-1}	24.1 ± 0.6	19.5 ± 0.5^{b}	25.5 ± 0.6^{d}
Insulin ng mL^{-1}	42.1 ± 0.1	28.3 ± 0.3^{c}	38.9 ± 0.7^{f}
Triglycerides mmol L^{-1}	1.3 ± 0.13	0.8 ± 0.1^{a}	1.18 ± 0.1^{d}
Glycerol μmol^{-1}L^{-1}	365 ± 15	430 ± 12^{c}	362 ± 14^{f}
NEFA mEq L^{-1}	0.62 ± 0.12	0.28 ± 0.08^{b}	0.59 ± 0.13^{d}

Differences from PBS treated animals are shown as: ^{a}, $P < <0.05$; ^{b}, $P < 0.01$; ^{c}, $P < 0.001$. Differences from ZAG treated animals are shown as: ^{d}, $P < 0.05$; ^{e}, $P < 0.01$; ^{f}, $P < 0.001$.

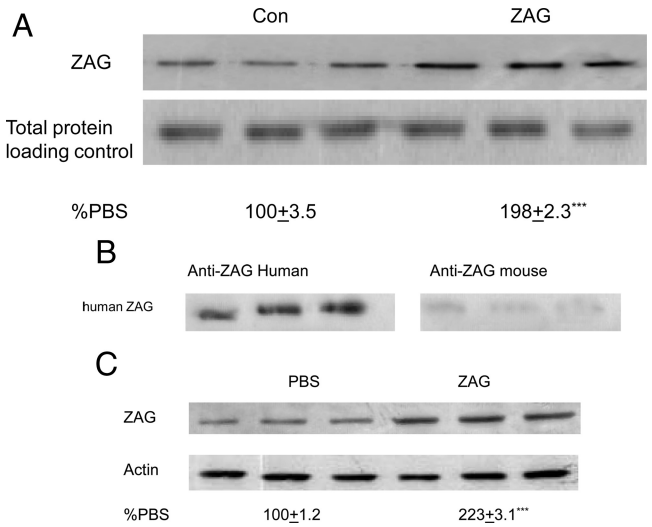

FIG. 4. Western blots of ZAG. A, Expression of murine ZAG in serum of ob/ob mice administered human ZAG or PBS orally for 8 days as shown in Fig. 1. Each lane is a sample from an individual mouse. The blot was probed with antimouse ZAG antibody. (B, Human ZAG was electrophoretically blotted, and probed with antibodies specific to human and mouse ZAG. (C, Expression of ZAG in WAT quantitated using an antimouse ZAG antibody after 8 days treatment with human ZAG Differences from PBS treated animals are shown as ***, $P < 0.001$.

contained a single band of radioactivity of Mr43 kDa (Fig. 3A). The [^{14}C]ZAG was then administered to ob/ob mice by the oral route. SDS-PAGE of serum proteins provided no evidence for intact ZAG (Fig. 3A). Western blotting of serum, using mouse monoclonal antibody to full-length human ZAG, confirmed the absence of human ZAG (Fig. 3B). Another possibility is that a tryptic digest of ZAG could mediate the effect, but there is no evidence for absorption of peptides into the blood stream (Fig. 3A). Alternatively, a peptide could act within the gastrointestinal tract. The effect of ZAG has been shown to be manifested through interaction with a β3-AR (10). However, treatment of Chinese hamster ovary cells transfected with human β1-AR, β2AR, or β3-AR with a tryptic digest of ZAG had no effect on cAMP production (Fig. 3C), whereas intact ZAG stimulated cAMP production in cells with β2-AR and β3-AR. This suggests that interaction with trypsin in the stomach would inactivate ZAG. Therefore, ZAG must act before it reaches the stomach.

Previous studies (8) have shown that ZAG can induce its own expression through interaction with a β3-AR and may be able to induce its own expression through interaction with β3-AR in the esophagus before being digested in the stomach and other parts of the gastrointestinal tract. Because there was an absence of human ZAG in the serum of orally dosed mice (Fig. 3B). Western blotting of serum from mice dosed orally with ZAG for 8 d showed a 2-fold ($P < 0.001$) in-

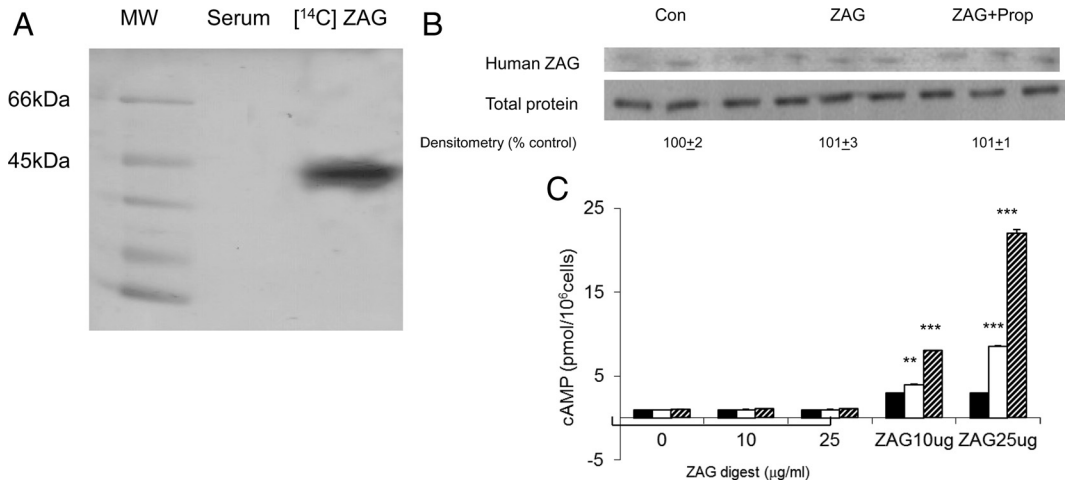

FIG. 3. A, SDS-PAGE of purified biosynthetically labeled [^{14}C]ZAG (15 μg) and serum from *ob/ob* mice administered [^{14}C] ZAG (50 μg; 212 μCi/μmol^{-1}) orally for 24 h. Con, Control. B, Western blot of serum from *ob/ob* mice administered nonradioactive ZAG for 8 d in the absence or presence of propanolol (40 mg/kg^{-1}) using antihuman ZAG monoclonal antibody. C, Effect of a tryptic digest of ZAG in comparison with intact ZAG on cAMP production by Chinese hamster ovary cells transfected with human β1-AR (*black columns*), β2-AR (*white columns*), and β3-AR (*hatched columns*) after 30 min incubation. ZAG (1 mg) was incubated with trypsin (200 μg) in 1 ml 10 mM Tris-HCl (pH 8) for 4 h at 37 C, and proteolysis was terminated by addition of the trypsin inhibitor (200 μg). High-molecular-weight material was removed by a Sephadex G25 column followed by dialysis using an Amicon filtration cell (Millipore, Watford, UK) containing a 10-kDa cutoff membrane filter.

creased level of murine ZAG (Fig. 4A). The specificity of the antibodies against human ZAG is shown in Fig. 4B. Thus, the antimouse ZAG antibody did not detect human ZAG. Therefore, the human ZAG administered orally has resulted in an increase in mouse ZAG in the serum, and this has also caused a 2-fold rise of mouse ZAG in WAT ($P < 0.001$) (Fig. 4C). Administration of propanolol also attenuated the oral route ZAG-induced stimulation of glucose uptake *ex vivo* into epididymal, sc, and visceral adipocytes in the absence and presence of insulin (Fig. 5A), It also attenuated glucose uptake into BAT in the absence and presence of insulin (Fig. 5B) and glucose uptake *ex vivo* into gastrocnemius muscle in the presence of insulin (Fig. 5C). In addition, there was no increase in murine ZAG in the serum (Fig. 5D) and no evidence of human ZAG (Fig. 3B) in animals coadministered propanolol. These results suggest that oral administration of ZAG increases circulatory levels by interaction with a β-AR, probably in the esophagus because ZAG mRNA appears to be dramatically increased in esophageal tissue compared with that of the stomach, small intestine, or colon and is on par with that seen in the liver in mice treated with ZAG orally (Fig. 6, A and B). Gene expression for ZAG in the various sections of the gastrointestinal tract is shown in Fig. 6C.

Discussion

Previous studies (11) have shown ZAG to bind to a high-affinity binding site on the β3-AR, with an affinity constant value of 78 ± 45 nM and a maximal binding of 282 ± 1 fmol/mg protein^{-1}. Many of the effects of ZAG are also found with β3-AR agonists, including an increased lipid

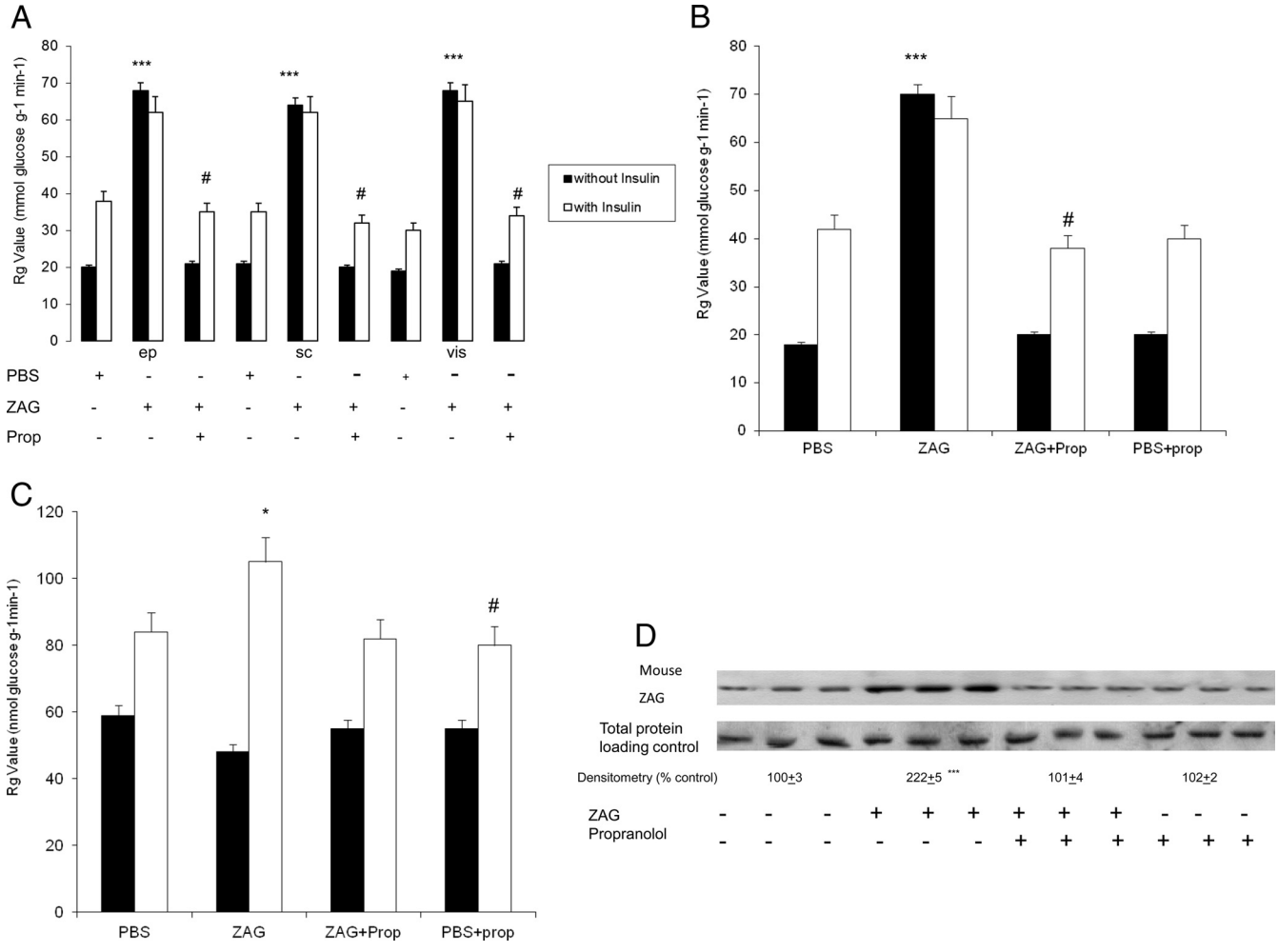

FIG. 5. Effect of propanolol on the stimulation of glucose uptake into WAT, BAT and skeletal muscle of *ob/ob* mice *ex vivo* after administration of ZAG. A, Glucose uptake into epididymal (ep), sc, and visceral (vis) adipocytes from animals treated with PBS and ZAG with or without propanolol (Prop) for 8 d in the absence (*closed bars*), or presence (*open bars*) of insulin (10 nM). B, Glucose uptake into brown adipocytes from mice treated with PBS, ZAG, or ZAG+propanolol for 8 d with or without insulin (10 nM). C, Glucose uptake into isolated gastrocnemius muscle of ob/ob mice administered either PBS or ZAG with or without propanolol for 8 d. D, Quantitation of serum ZAG in mice treated with PBS, ZAG, or ZAG+propanolol for 3 d by immunoblotting using an antimouse ZAG monoclonal antibody. Each lane represents serum form an individual mouse. *, Differences from PBS-treated animals, $P < 0.05$; ***, differences from PBS-treated animals, $P < 0.001$; ##, differences from ZAG-treated animals, $P < 0.001$.

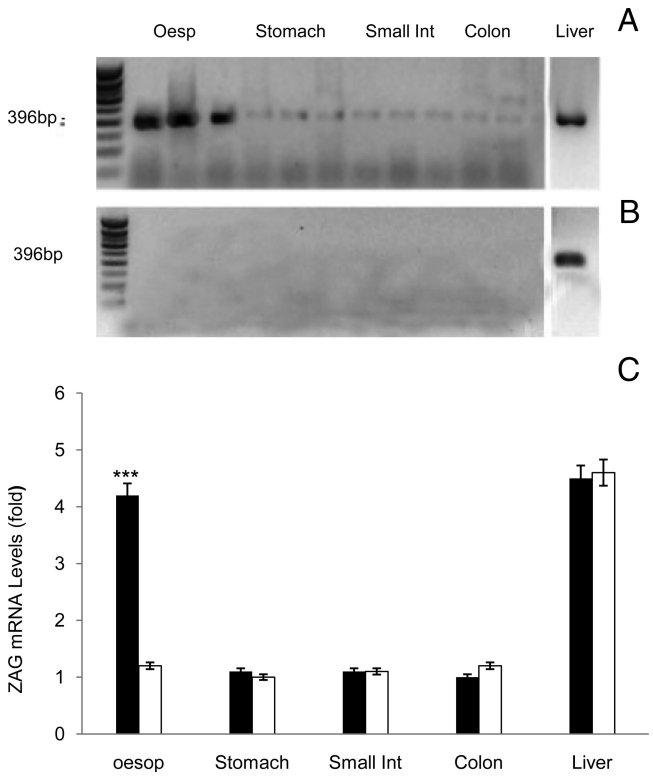

FIG. 6. ZAG gene expression in mouse tissues examined by RT-PCR. A, Tissue specificity of expression from mice treated with ZAG orally. B, ZAG expression in control mice. C, ZAG mRNA expression in mouse tissue after either oral administration of ZAG (*black bars*) or PBS (*white bars*). ***, Differences from PBS treated animals, $P < 0.001$.

mobilization and reduction of body fat (11), an increase of rectal temperature and induction of uncoupling protein 1capacity in BAT (11), normalization of hyperglycemia and hyperinsulinemia, improvement in glucose tolerance and reduction of the insulin response during a glucose tolerance test (12), and also attenuation of muscle wasting (13). The β3-AR is found predominantly on adipocytes (14), but has also been reported on BAT and prostate (15) as well as in the smooth muscle of the gastrointestinal tract in a variety of species and mediates relaxation in the ileum, gastric fundus, jejunum, colon, and esophagus (16–18). This study has shown that the previously described presence of a β-AR in the gastrointestinal tract, coupled with the ability of ZAG to induce its own expression through a β-AR (8), enables ZAG to be administered orally and this stimulus to be converted into circulating ZAG. The β-AR responsive to oral ZAG must be in the mouth or esophagus because tryptic digestion of ZAG produced a product with no stimulation of the β-AR. Using RT-PCR analysis of ZAG mRNA, this study shows a large increase in the esophagus of animals receiving ZAG orally. The lack of expression of ZAG in the lower part of the gastrointestinal tract, despite the reported presence of β-AR (16–18), would support the contention that ZAG is digested in the stomach. Previous studies have suggested that a tryptic digest of a cancer lipolytic factor called toxohormone L still retains biological activity (19). The mechanism by which the ZAG signal is transmitted from the gastrointestinal tract to the general circulation has been elucidated by administration of human ZAG to a mouse and depends on the specificities of the antibodies to human and murine ZAG. As expected, human ZAG is digested, but murine ZAG appears in the serum and responsive tissue such as WAT. This effect is mediated through a β-AR because mice treated with the nonspecific β-AR antagonist, propanolol, showed no murine ZAG in their serum, and the effects of ZAG on body weight, lipolysis, and glucose disposal were completely attenuated.

Previous studies (20) have shown that the lipolytic effect of ZAG *in vitro* was also completely attenuated by propanolol. We did not use agents that have been reported to be specific for β3-AR, such as SR59230A (21), because our previous studies have indicated that this antagonist also attenuates activation through both the β1-AR and β2-AR, whereas other investigators (22, 23) have shown it to be an antagonist of the α1-AR. SR59230A has also been seen to bind to albumin when used *in vivo* (21). The specific β-AR involved can be determined only using specific β-AR knockout animals. The ability of propanolol to attenuate the reduction in body weight, increase in temperature, reduction in blood glucose, insulin, nonesterified fatty acid, and triglycerides, increase in serum glucose, disposal of glucose, and increased insulin sensitivity induced by ZAG in *ob/ob* mice suggests that these effects are mediated through a β-AR.

The effects of orally administered human ZAG at a dose of 50 $\mu g/d^{-1}$ are almost identical with those found when human ZAG was administered by the iv route (1), suggesting a quantitative transfer of the message from human ZAG into the serum as mouse ZAG. ZAG is unusual in inducing its own expression, and the mechanism is unknown apart from a requirement of the β3-AR (8). The β3-AR agonist BRL37344 has also been shown to increase levels of ZAG mRNA in 3T3-L1 adipocytes, suggesting a common mechanism. The cAMP formed from interaction with a β3-AR would lead to activation of protein kinase A, the C-subunits of which are capable of passively diffusing into nucleus, in which they can regulate gene expression through direct phosphorylation of cAMP response element binding protein (24).

Plasma ZAG protein has been shown to be decreased in *ob/ob* mice (25), and a similar decrease has been reported in high-fat diet-fed mice (26). Serum ZAG levels have also been found to be low in obese human subjects (26, 27). Most of the serum ZAG is thought to come from adipose tissue and liver, and expression levels of ZAG mRNA in these tissues in *ob/ob* mice have been shown to be significantly reduced (25). This is at least partly due to the proinflammatory cytokine TNF-α

(25), which is elevated in the adipose tissue of obese subjects (28). Many of the effects of obesity may be due to this low expression of ZAG because of its function in regulating lipid metabolism (1) and ZAG's ability to increase expression of β3-AR in gastrocnemius muscle, BAT, and WAT (our unpublished results), which are low in obesity (29). The ability of ZAG to increase serum levels when administered by the oral route provides a mechanism for countering some of the effects of obesity. It also raises the possibility of some uncooked foods such as broccoli, rich in ZAG functioning to control obesity and type 2 diabetes, through conversion of vegetable ZAG to human ZAG.

Acknowledgments

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References

1. **Russell ST, Tisdale MJ** 2010 Antidiabetic properties of zinc-α2-glycoprotein in *ob/ob* mice. Endocrinology 151:948–957
2. **Delker SL, West Jr AP, McDermott L, Kennedy MW, Bjorkman PJ** 2004 Crystallographic studies of ligand binding by zinc-α2-glycoprotein. J Struct Biol 148:205–213
3. **Todorov PT, McDevitt TM, Meyer DJ, Ueyama H, Ohkubo I, Tisdale MJ** 1998 Purification and characterisation of a tumor lipid-mobilizing factor. Cancer Res 58:2353–2358
4. **Russell ST, Zimmerman TP, Domin BA, Tisdale MJ** 2004 Induction of lipolysis in vitro and loss of body fat *in vivo* by zinc-α2-glycoprotein. Biochim Biophys Acta 1636:59–68
5. **Bing C, Bao Y, Jenkins J, Sanders P, Manieri M, Cinti S, Tisdale MJ, Trayhurn P** 2004 Zinc-α2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. Proc Natl Acad Sci USA 101:2500–2505
6. **Mracek T, Ding Q, Tzanavari T, Kos K, Pinkney J, Wilding J, Trayhurn P, Bing C** 2010 The adipokine zinc-α2-glycoprotein is downregulated with fat mass expansion in obesity. Clin Endocrinol (Oxf) 72:334–341
7. **Bao Y, Bing C, Hunter L, Jenkins JR, Wabitsch M, Trayhurn P** 2005 Zinc-α2-glycoprotein, a lipid mobilizing factor, is expressed and secreted by human (SGBS) adipocytes. FEBS Lett 579:41–47
8. **Russell ST, Tisdale MJ** 2005 The role of glucocorticoids in the induction of zinc-α2-glycoprotein expression in adipose tissue in cancer cachexia. Br J Cancer 92:876–881
9. **Bailey CJ, Flatt PR, Atkins TW** 1982 Influence of genetic background and age on the expression of the obsese hyperglycaemic syndrome in Aston ob/ob mice. Int J Obes 6:11–21
10. **Russell ST, Hirai K, Tisdale MJ** 2002 Role of β3-adrenergic receptors in the action of a tumour lipid mobilizing factor. Br J Cancer 86:424–428
11. **Inokuma K, Okamatsu-Ogura Y, Omachi A, Matsushita Y, Kimura K, Yamashita H, Saito M** 2006 Indispensable role of mitochondrial UCP1 for antiobesity effect of β3-adrenergic stimulation. Am J Physiol Endocrinol Metab 290:E1014–E1021
12. **Liu X, Perusse F, Bukowiecki LJ** 1998 Mechanism of the antidiabetic effects of the β3-adrenergic agonist CL-316243 in obese Zucker-ZDF rats. Am J Physiol Regul Integr Comp Physiol 43: R1212–R1219
13. **Lynch GS, Ryall JG** 2008 Role of β-adrenoceptor signalling in skeletal muscle: Implications for muscle wasting and disease. Physiol Rev 88:729–767
14. **Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD** 1989 Molecular characterisation of the human β3-adrenergic receptor. Science 245:1118–1121
15. **Krief S, Lönnqvist F, Raimbault S, Baude B, Van Spronsen A, Arner P, Strosberg AD, Ricquier D, Emorine LJ** 1993 Tissue distribution of β3-sdrenergic receptor mRNA in man. J Clin Invest 91:344–349
16. **Manara L, Croci T, Landi M** 1995 β(3)-Adrenergic receptors and intestinal motility. Fundam Clin Pharmacol 9:332–342
17. **Fletcher DS, Candelore MR, Grujic D, Lowell BB, Luell S, Susulic VS, Macintyre DE** 1998 Beta-3 adrenergic receptor antagonists cause an increase in gastrointestinal transit time in wild-type mice, but not in mice lacking the beta3-adrenergic receptor. J Pharmacol Exp Ther 287:720–724
18. **Sarma DN, Banwait K, Basak A, DiMarino AJ, Rattan S** 2003 Inhibitory effect of β3-adrenoceptor agonist in lower esophageal sphincter smooth muscle: *in vitro* studies. J Pharmacol Exp Ther 304:48–55
19. **Masuno H, Yamasaki N, Okuda H** 1981 Purification and characterisation of a lipolytic factor (Toxohormone-L) from cell-free fluid of ascites sarcoma 180. Cancer Res 41:284–288
20. **Khan S, Tisdale MJ** 1999 Catabolism of adipose tissue by a tumour-producing lipid-mobilising factor. Int J Cancer 80:444–447
21. **Nisoli E, Tonello C, Landi M, Carruba MO** 1996 Functional studies of the first selective β3-adrenergic receptor antagonist SR59230A in rat brown adipocytes. Mol Pharmacol 49:7–14
22. **Leblais V, Pourgeaud F, Ivorra MD, Guilbert C, Marthan R, Muller B** Role of α-adrenergic receptors in the effect of the β-adrenergic receptor ligands CGP 12177, Bupranolol and SR59230A, on the contraction of rat intrapulmonary artery. J Pharmacol Exp Ther 309:137–145
23. **Bexis S, Docherty JR** 2009 Role of α1- and β3-adrenoceptors in the modulation of SR59230A of the effects of MDMA on body temperature in the mouse. Br J Pharmacol 158:259–266
24. **Mayr B, Montminy M** 2001 Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599–609
25. **Mracek T, Gao D, Tzanavari T, Bao Y, Xiao X, Stocker C, Trayhurn P, Bing C** 2010 Downregulation of zinc-α2-glycoprotein in adipose tissue and liver of ob/ob mice and by tumour necrosis factor-α in adipocytes. J Endocrinol 204:165–172
26. **Gong FY, Zhang SJ, Ding JY, Zhu HJ, Pan H, Li NS, Shi YF** 2009 Zinc-α2-glycoprotein is involved in regulation of body weight through inhibition of lipogenic enzymes in adipose tissue. Int J Obes 33:1023–1030
27. **Selva DM, Lecube A, Hernández C, Baena JA, Fort JM, Simó R** 2009 Lower zinc-α2-glycoprotein production by adipose tissue and liver in obese patients unrelated to insulin resistance. J Clin Endocrinol Metab 94:4499–4507
28. **Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW** 1999 C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol 19: 972–978
29. **Robidoux J, Martin TL, Collins S** 2004 β-Adrenergic receptors and regulation of energy expenditure: a family affair. Annu Rev Physiol Toxicol 44:297–323