ROLE OF β-ADRENERGIC RECEPTORS IN THE
ORAL ACTIVITY OF ZINC-α2-GLYCOPROTEIN (ZAG)

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Running Title: Role of β-AR in the oral activity of ZAG

Abbreviations: ZAG, Zinc-α2-glycoprotein; β1-AR, β1-adrenergic receptor; β2-AR, β2-adrenergic receptor; β3-AR, β3-adrenergic receptor; TNF-α, Tumour necrosis Factor-α; UCP-1, uncoupling protein-1; UCP-3, uncoupling protein-3; WAT, white adipose tissue; BAT, brown adipose tissue.
Abstract

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 41kDa protein, when administered to ob/ob mice at 50μg day⁻¹ po in the drinking water produced a progressive loss of body weight (5g after 8 days 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-adrenoceptor (β-AR) in the gastrointestinal tract before digestion, ZAG was co-administered to ob/ob mice together with propanolol (40mgkg⁻¹), a non-specific 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 oesophagus.

Keywords: Zinc-α2-glycoprotein; oral administration; diabetes; obesity; β-adrenoceptors
Introduction

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therapeutic agents are limited in efficacy, while treatment-associated adverse events have meant many agents being withdrawn.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 Mr41kDa, which resembles a class 1 major histocompatibility complex (MHC) heavy chain, and has a major groove capable of binding hydrophobic molecules, that could be important in its action (2). ZAG was first identified as the lipid mobilising factor in cancer cachexia following its isolation from the cachexia-inducing MAC16 tumour, 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, while there was an expansion of the non-fat 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 tumours that induce cachexia (5). Expression of ZAG mRNA in adipose tissue is high in cancer cachexia, where lipid stores are low (5), and low in obesity, where lipid stores are high (6). Thus ZAG expression is negatively correlated with BMI and fat mass (6). ZAG expression is negatively regulated by tumour necrosis factor-α (TNF-α), and positively regulated by the PPARγ 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).

Previously studies have administration ZAG by either the i.p. (4), or i.v. (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

FCS (foetal calf serum) was from Biosera (Sussex, UK), while DMEM (Dulbecco’s modified Eagles Medium) was from PAA (Somerset, UK) and Feestyle medium was purchased from Invitrogen (Paisley, UK). Hybond A nitrocellulose membranes and peroxidise – conjugated rabbit anti-mouse antibody were from GE Healthcare (Bucks, UK), while enhanced chemiluminescene (ECL) 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-14C] tyrosine (sp.act 16.7 GBqmmol⁻¹) was purchased from Perkin Elmer Ltd, (Cambridge, UK), while 2-[1-14C] deoxy-D-glucose (sp.act 1.85GBqmmol⁻¹) was from American Radiolabeled Chemicals (Cardiff, UK).

Production and purification of ZAG

Recombinant human ZAG was produced by HEK293F cells transfected with pcDNA3.1 containing human ZAG (1). Cells were grown for 2 weeks in Freestyle medium containing neomycin (50μgml⁻¹) under an atmosphere of 5% CO₂ in air. The cells were then removed by centrifugation (700g for 15min), 1litre of medium was concentrated to 1ml, and the ZAG was extracted by binding to activated DEAE cellulose, following by elution with 0.3M 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 sodium dodecylsulphate polyacrylamide electrophoresis (SDS PAGE), and was free of endotoxin (1). For [14C] ZAG L-[U-14C] tyrosine was added to the media (1μCi ml⁻¹), the cells were
allowed to grow for 2 weeks 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 in Fig 3A.

Cyclic AMP determination

CHOK1 cells transfected with human β1-, β2- and β3-AR were maintained in DMEM supplemented with 2mM glutamine, hygromycin B (50μgml⁻¹), G418 (200mgml⁻¹) and 10% FCS, under an atmosphere of 10% CO₂ in air. For cyclic AMP production cells were grown in 24-well plates in 1ml nutrient medium, and ZAG, after tryptic digestion as described in the legend to Fig. 3C, was incubated for 30min. The medium was then removed and 0.5ml of 20mM HEPES, pH7.5, 5mM EDTA and 0.1mM isobutylmethylxanthine was added, followed by heating on a water bath for 5min, and cooling on ice for 10min. The concentration of cyclic AMP was determined using a Parameter cyclic AMP assay kit (New England Biolabs, Hitchin, Herts, UK).

Animals

Obese (ob/ob) mice (average weight 65g) 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 (50μg day⁻¹), or PBS in their drinking water, the experiment was repeated three times after a power analysis was performed. Each mouse consumed 5ml day⁻¹ water, and this did not change on ZAG administration (1). The ZAG was replaced every 48h. One group of mice receiving ZAG were also administered propanolol (40mgkg⁻¹, p.o.) daily. The dose of ZAG was chosen to be the same as that previously administered i.v. (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 day 3. Animals were fasted for 12h, followed by oral administration of glucose (1gkg⁻¹ in a volume of 100µl 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 0.5ml urine and testing glucose concentration using a Boots glucose monitor. After 8 days of treatment the animals were terminated by cervical dislocation, 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 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 Licence according to the UKCCCR 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 subcutaneous and visceral WAT and BAT for 2 and 2.5h, respectively, with Krebs-Ringer bicarbonate (KRBB) containing 1.5mgml⁻¹ collagenase and 4% BSA under 95% oxygen-5% CO₂ at 37°C. Adipocytes were washed twice in 1ml KRBB, pH7.2, and then incubated for 10min at room temperature in 0.5ml KRBB, containing 18.5MBq 2-[1⁴C] deoxy-D-glucose (2-DG), together with non-radioactive 2-DG, to give a final concentration of 0.1mM, in the absence or presence of insulin (10nM). Uptake was terminated by addition of 1ml ice-cold KRBB without glucose. Adipocytes were washed three times with 1ml KRBB and lysed by the addition of 0.5ml 1M NaOH. The uptake of 2-[1⁴C] 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 (10nM) was determined as previously described (1).
Western blotting analysis

Tissues were thawed, washed in PBS and lysed in Phosphosafe™ Extraction reagent for 5 min at room temperature, followed by sonication at 4°C. Cytosolic protein (5-20μg) formed by centrifugation at 18,000g for 5 min at 4°C, was resolved on 12% SDS PAGE by electrophoresis at 180V for about 1 h. To determine ZAG in serum 30μl samples containing 20μg total protein were electrophoresed as above. Protein was transferred to 0.45μm nitrocellulose membranes, which had been blocked with 5% (w/v) non-fat dried milk (Marvel) in Tris-buffered saline, pH 7.5, at 4°C overnight. Membranes were washed for 15 min in 0.1% Tween 20 buffer saline prior to adding the primary antibodies. Both primary and secondary antibodies were used at a dilution of 1:1000. Incubation was for 1 h at room temperature, and development was by ECL. Blots were scanned by a densitometer to quantify differences.

PCR

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 (Ambion) to remove any genomic DNA. The RNA concentration was determined from the absorbance at 260 nM. 1 μg 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 (ABgene). 1μl 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 (ABgene) 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′-GCCCTTCTCCACTACAACAG-3′ (forward), 5′-TTCAGGACACTCCTCCTCTA-3′ (reverse); annealing temperature (TA) 54 °C; 33 cycles. Mouse β-actin: 5′-GTGGCATCCACGAAACTACCTT-3′ (forward), 5′-GGACTCGTCATACTCCTGCTTG-3′ (reverse) (antisense); TA 57 °C; 23 cycles. PCR was performed on a thermal cycler (Hybaid) with an initial denaturation at 94 °C for 2 min followed by cycles consisting of denaturation at 94 °C for 20 s, annealing at the specified temperature for 25 s, and extension at 72 °C for 59 s; 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). (7)

**RT-PCR**

Relative ZAG mRNA levels were quantified using real-time PCR with an ABI Prism 7700 Sequence Detector (Applied Biosystems). 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 ZAG**: 5’-GAGGCTGTGGGACCTTGGA-3’ (forward), 5’-CCTCCCTGGGCCCTCTGAA-3’ (reverse), and 5’-FAM-AATGGAGGACTGGGAGAAAGCCAG-TAMRA-3’;  **Mouse β-actin**: 5’-ACGCCAGTGTCATCATATTG-3’ (forward), 5’-CAAGAAGGAAGGCTGGAAAAGA-3’ (reverse), and 5’-FAM-ACGAGGGGTTCCGATGCCCTG-TAMRA-3’. Amplification was performed in a 96-well plate using a master mix made from qPCR core kit (Eurogentec), 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 s and combined annealing and extension at 60 °C for 1 min. Data were recorded and analysed 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 analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests, p values <0.05 were 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 administration to dissolve the ZAG in drinking water, since 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 50μg per day, 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 days of treatment the difference in body weight between the ZAG and PBS groups was 3.5g, which was the same as that found after i.v. administration (1), while after 8 days of treatment there was 5g weight difference between the groups. As with i.v. administration of ZAG (1) there was an increase in rectal temperature, which became significant after 4 days of treatment (Fig. 1B), while there was a 40% reduction in urinary glucose excretion, which became significant after 1 day 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 days of oral ZAG, showed a reduced peak blood glucose concentration, and a 33% reduction in the total area under the curve (AUC) 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 i.v. route (1). To determine whether this effect was due to interaction with a β-AR, ZAG was administered orally to ob/ob mice that were co-administered the non-specific β-AR antagonist propanolol (40mg/kg). As shown in Fig. 2C while 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 20mgkg⁻¹, but this did not prevent the weight loss with ZAG so the dose was increased to 40mgkg⁻¹. Antagonists of β1- 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, suggesting that it blocked lipolysis induced by ZAG, along with the decrease in serum triglycerides and non-esterified 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 ZAG was biosynthetically labelled with L-[U-14C] tyrosine. SDS/PAGE showed that the purified product contained a single band of radioactivity of Mr43kDa (Fig. 3A). The [14C] 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 CHO cells transfected with human β1-, β2 or β3-AR with a tryptic digest of ZAG had no effect on cyclic AMP production (Fig. 3C), while intact ZAG stimulated cyclic AMP production in cells with β2- 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 oesophagus before being digested in the stomach and other parts of the gastrointestinal tract. Since 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 days showed a two-fold (P<0.001) increased level of
murine ZAG (Fig. 4A). The specificity of the antibodies against human ZAG is shown in Fig. 4B. Thus the anti-mouse 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 two 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, subcutaneous 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 co-administered propanolol. These results suggest that oral administration of ZAG increases circulatory levels by interaction with a β-AR, probably in the oesophagus, since ZAG mRNA appears to be dramatically increased in oesophageal tissue compared to that of the stomach, small intestine or the colon and is on par with that seen in the liver in mice treated with ZAG orally (Fig 6A and B). Gene expression for ZAG in the various sections of the GI Track are shown in (Fig 6C).
DISCUSSION

Previous studies (11) have shown ZAG to bind to a high affinity binding site on the β3-AR, with a Kd value of 78±45nM and Bmax of 282±1 fmole mg protein⁻¹. Many of the effects of ZAG are also found with β3-AR agonists, including an increased lipid mobilization and reduction of body fat (11), an increase of rectal temperature and induction of UCP1 in BAT (11), normalization of hyperglycaemia and hyperinsulinaemia, improvement in glucose tolerance and reduction of the insulin response during a glucose tolerance test (12), and also attenuation of muscle wasting (17).

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 oesophagus (16, 17, 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 oesophagus, since 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 oesophagus 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, 17, 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, since mice treated with the non-specific 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), since our previous studies have indicated that this antagonist also attenuates activation through both the β1 and β2-ARwhile 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 only be determined using specific β-AR “knock-out” animals. The ability of propanolol to attenuate the reduction in body weight, increase in temperature, reduction in blood glucose, insulin, NEFA, 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 50µg day\(^{-1}\) are almost identical to those found when human ZAG was administered by the i.v. 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 cyclic AMP formed from interaction with a β3-AR would lead to activation of protein kinase A (PKA), the C-subunits of which are capable of passively diffusing into nucleus, where they can regulate gene expression through direct phosphorylation of cyclic AMP response element binding protein (CREB) (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 pro-inflammatory cytokine tumour necrosis factor-α (TNF-α), (25) which is elevated in 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 (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.

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REFERENCES


28. Yudkin JS, Stehouwer CD, Emesis JJ, Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for

FIGURE LEGENDS

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 50μg/day (■), while a control group received an equal volume of PBS (▲) (0.5ml in 5ml water). Differences from PBS controls are shown as *** p<0.001.

Fig. 2 Effect of oral human ZAG (■) compared with PBS (▲) on glucose and insulin tolerance of ob/ob mice after 3 days of treatment. Animals were fasted for 12h before oral administration of glucose (1g/kg in a volume of 100μl). 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 are shown as ***, p<0.001. Effect of propranolol (40mg/kg, po, daily) on ZAG-induced reductions in obesity and diabetes in ob/ob mice. Animals received ZAG (50μg daily) in their drinking water as described in the legend to Fig 1, either alone (■), or in the presence of propranolol (▲), while a control group received PBS (▲). Changes in body weight (C), rectal temperature (D), and urinary glucose excretion (E) were monitored over a 8 day period. A glucose tolerance test (F), with measurement of serum insulin levels (G) was made 3 days after starting the oral ZAG. Differences from PBS controls are shown as ***, p<0.001, while differences from ZAG alone are shown as #, p<0.001.

Fig. 3 (A) SDS/PAGE of purified biosynthetically labelled [14C] ZAG (15μg) and serum from ob/ob mice administered [14C] ZAG (50μg; 212μCi/μmol) orally for 24h. (B) Western blot of serum from ob/ob mice administered non-radioactive ZAG for 8 days in the absence or presence of propranolol (40mg kg⁻¹) using anti-human ZAG monoclonal antibody. (C) Effect of a tryptic digest of ZAG in comparison with intact ZAG on cyclic AMP production by CHO cells transfected with human β1-AR (■), β2-AR (▲) and β3-AR (▼) after 30min incubation. ZAG (1mg) was incubated with trypsin (200μg) in 1ml 10mM Tris.HCl, pH8 for 4h 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 containing a 10kDa cut-off membrane filter.

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 anti-mouse 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 anti-mouse ZAG antibody after 8 days treatment with human ZAG Differences from PBS treated animals are shown as ***, p<0.001.

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), subcutaneous (sc) and visceral (vis) adipocytes from animals treated with PBS and ZAG with or without propanolol (Prop) for 8 days in the absence (closed bars), or presence (open bars) of insulin (10nM). (B) Glucose uptake into brown adipocytes from mice treated with PBS, ZAG or ZAG+propanolol for 8 days with or without insulin (10nM). (C) Glucose uptake into isolated gastrocnemius muscle of ob/ob mice administered either PBS or ZAG with or without propanolol for 8 days. (D) Quantitation of serum ZAG in mice treated with PBS, ZAG or ZAG+propanolol for 3 days by immunoblotting using an anti-mouse ZAG monoclonal antibody. Each lane represents serum from an individual mouse. Differences from PBS treated animals are shown as *, p<0.05 or ***, P<0.001, while differences from ZAG treated animals are shown as ##, 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 (■) or PBS (□). Differences
from PBS treated animals are shown as ***, P<0.001.