

ROLE OF  $\beta$ -ADRENERGIC RECEPTORS IN THE ANTI-OBESITY  
AND ANTI-DIABETIC EFFECTS OF ZINC- $\alpha$ 2-GLYCOPROTEIN (ZAG)

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Short page-heading: Zinc- $\alpha$ 2-glycoprotein binds and activates  $\beta$ 3- and  $\beta$ 2-adrenergic receptors, but not  $\beta$ 1, and its effect on body weight and insulin sensitivity arise through this interaction.

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## Abstract

**Objectives:** The goal of the current study is to determine whether the  $\beta$ -adrenoreceptor ( $\beta$ -AR) plays a role in the anti-obesity and anti-diabetic effects of zinc- $\alpha$ 2-glycoprotein (ZAG).

**Material and Methods:** This has been investigated in CHO-K1 cells transfected with the human  $\beta$ 1-,  $\beta$ 2-,  $\beta$ 3-AR and in ob/ob mice. Cyclic AMP assays were carried out along with binding studies. Ob/ob mice were treated with ZAG and glucose transportation and insulin were examined in the presence or absence of propranolol.

**Results:** ZAG bound to the  $\beta$ 3-AR with higher affinity ( $K_d$   $46 \pm 1$  nM) than the  $\beta$ 2-AR ( $K_d$   $71 \pm 3$  nM) while there was no binding to the  $\beta$ 1-AR, and this correlated with the increases in cyclic AMP in CHO-K1 cells transfected with the various  $\beta$ -AR and treated with ZAG. Treatment of ob/ob mice with ZAG increased protein expression of  $\beta$ 3-AR in gastrocnemius muscle, and in white and brown adipose tissue, but had no effect on expression of  $\beta$ 1- and  $\beta$ 2-AR. A reduction of body weight was seen and urinary glucose excretion, increase in body temperature, reduction in maximal plasma glucose and insulin levels in the oral glucose tolerance test, and stimulation of glucose transport into skeletal muscle and adipose tissue, was completely attenuated by the non-specific  $\beta$ -AR antagonist propranolol.

**Conclusion:** The results suggest that the effects of ZAG on body weight and insulin sensitivity in ob/ob mice are manifested through a  $\beta$ -3AR, or possibly a  $\beta$ 2-AR.

Keywords: zinc- $\alpha$ 2-glycoprotein;  $\beta$ -adrenoreceptor; obesity; diabetes

**Abbreviations:**

AUC: area under the curve, AMPK: AMPkinase,  $\beta$ -AR: beta adrenergic receptor, BAT: brown adipose tissue, CHOK1: Chinese hamster ovary cells, DMEM: dulbecco's modified eagles medium, ECL: enhanced chemiluminescence, FCS: foetal calf serum, LMF: lipid mobilizing factor, PBS: phosphate buffered saline, NEFA: non-esterified fatty acids, TG: triglycerides, UCP: uncoupling protein, WAT: white adipose tissue, ZAG: zinc-alpha2-glycoprotein

## INTRODUCTION

Zinc- $\alpha_2$ -glycoprotein (ZAG) was first recognised to play a role in lipid metabolism when tryptic fragments of a lipid mobilizing factor (LMF), thought to be responsible for loss of adipose tissue in cancer cachexia, were shown to be identical in amino acid sequence to ZAG (1). Both ZAG and LMF were shown to be immunologically identical (1), and both stimulated lipolysis in murine adipocytes by the same amount, at the same concentration, by activation of adenylyl cyclase in a GTP-dependent process (2). Initial studies suggested that ZAG originated from the tumour, since tumours initiating cachexia showed high levels of expression, while other tumours which did not induce cachexia showed no expression (1). Later studies showed that ZAG was also produced in normal tissues including liver, brown adipose tissue (BAT) and white adipose tissue (WAT) (3), so that ZAG can be classified as an adipokine. Moreover, in both cachectic mice (3) and humans (4) expression of ZAG mRNA in WAT was found to be increased 10-fold (3) and 2.7-fold (4) respectively. In cachectic cancer patients ZAG mRNA showed a negative correlation with body mass index (BMI), but a positive correlation with weight loss and serum glycerol levels (4). In contrast ZAG mRNA levels in WAT have been shown to be down regulated in obesity and correlated negatively with fat mass, BMI, plasma insulin and leptin (5). Treatment of ob/ob mice with ZAG decreased body weight and fat mass and improved the parameters of insulin resistance including decreasing plasma levels of glucose, insulin and non-esterified fatty acids (NEFA), improving insulin sensitivity, and increasing muscle mass (6). Serum ZAG levels have been found to be significantly lower in mice fed a high fat diet than those fed a normal diet (7), as well as in obese humans and mice (8). While ZAG overexpression in mice reduced both the body weight and weight of epididymal fat (8), ZAG knock-out animals showed an increased body weight, especially when fed a high fat diet (9). These results suggest that ZAG, like leptin, is closely associated with fat mass. However, while leptin is positively correlated with fat mass, ZAG is negatively correlated.

The lipolytic effect of ZAG was shown to be attenuated by the  $\beta$ 3-adrenoreceptor ( $\beta$ 3-AR) antagonist, SR59230A (10), and shown to bind to the  $\beta$ 3-AR through a high affinity binding site ( $K_d$   $78 \pm 4.5$  nM) (11). These results suggest that lipolysis mediated by ZAG is mediated through a  $\beta$ 3-AR. However, this conclusion has recently been questioned (9) based on the inability of  $\beta$ 3-AR agonists to stimulate lipolysis in ZAG knock-out mice. The present study re-examines the role of the  $\beta$ 3-AR in the action of ZAG, using human recombinant material, as well as determining the binding to human  $\beta$ <sub>1</sub>- and  $\beta$ <sub>2</sub>-AR, and the role of the  $\beta$ -AR in the anti-obesity and anti-diabetic effects.

## EXPERIMENTAL

### Materials

FCS (foetal calf serum) was from Biosera (Sussex, UK), while DMEM (Dulbecco's modified Eagle's medium) was from PAA (Somerset, UK) and Freestyle media and Superscript II reverse transcriptase were purchased from Invitrogen (Paisley, UK). 2-[1-<sup>14</sup>C] deoxy-D-glucose sp.act. 1.85GBq mmol<sup>-1</sup>) was purchased from American Radiolabeled Chemicals (Cardiff, UK). Na [<sup>125</sup>I] (sp. Act. >17Ci mg<sup>-1</sup>) and [<sup>125</sup>I] iodo(±) Cyanopindodol (sp.act. 81.4 TBq mmol<sup>-1</sup>) (Cambridge, UK) was purchased from Perkin Elmer Limited. Chicken polyclonal antibody to β3-AR and rabbit polyclonal antibodies to β1-AR and β2-AR were purchased from Abcam (Cambridge, UK) and peroxidase-conjugated goat anti-chicken antibody was from Santa Cruz (USA). Polyclonal rabbit antibodies to UCP1 and UCP3 were from Calbiochem (via Merck Chemicals, Nottingham, UK). Peroxidase-conjugated goat anti-rabbit antibody was from Dako (Cambridge, UK). Polyclonal rabbit antibody to mouse β-actin, Tri-Reagent and propranolol were from Sigma Aldrich (Dorset, UK). Hybond A nitrocellulose membranes were from GE Healthcare (Bucks, UK). The Parameter cyclic AMP assay kit was purchased from New England Biolabs (Hitchin, UK). The iodo beads and enhanced chemiluminescence (ECL) development kits were purchased from Thermo Scientific (Northumberland, UK). A mouse insulin ELISA kit was purchased from DRG (Marburg, Germany) and glucose measurements were made using a Boots (Nottingham, UK) plasma glucose kit. Primers for reverse transcription and Easy-A one tube RT.PCR system were from Agilent Technologies (Cheshire, UK).

### Animals

Obese (ob/ob) hyperglycaemic mice having an average weight of 71g were bred in our own colony. The background of these animals has been previously described (12), and they exhibit a more severe form of diabetes than C57BL/6J ob/ob mice. Male mice (about 20 weeks of age) were grouped into three per cage and 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. Mice were administered ZAG (50 $\mu$ g, i.v. in 100 $\mu$ l PBS) or PBS daily with or without propranolol (40mgkg<sup>-1</sup>, po, daily) and body weight and food and water intake were determined, as well as urinary glucose excretion and body temperature, determined by the use of a rectal thermometer (RS Components, Northants, UK). A glucose tolerance test was performed on day 3. Glucose (1gkg<sup>-1</sup> in a volume of 100 $\mu$ l) was administered orally to animals which had been fasted for 12h. Blood samples were removed from the tail vein at 15, 30, 60 and 120 min after glucose administration and used for the measurements of glucose and insulin. At the end of the experiment the animals were terminated by cervical dislocation, tissues removed and rapidly frozen in liquid nitrogen, and maintained at -80°C. All animal experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

#### Production of recombinant human ZAG

Human HEK293F cells, which had been transfected with pcDNA3.1 containing human ZAG were maintained in Freestyle medium, containing neomycin (50 $\mu$ gml<sup>-1</sup>), under an atmosphere of 5% CO<sub>2</sub> in air. After 2 weeks of growth cells were removed by centrifugation (700g for 15min) and the medium was concentrated into a volume of 1ml sterile PBS using an Amicon Ultra-15 centrifugal filter with a M.W. cut-off of 10kDa. The ZAG was purified as described (6), by binding to DEAE cellulose, since ZAG has a high electronegativity, and was eluted with 0.3MNaCl. The ZAG produced by this method was greater than 95% pure as determined by SDS PAGE, and was free of endotoxin, as determined by the LAL Pyrogen single test kit (Lonza). The purified ZAG was stored at 4°C in PBS.

#### [<sup>125</sup>I] labelling of ZAG

One iodo bead that had been washed and dried was incubated with Na [<sup>125</sup>I] (1mCi per 100 $\mu$ g protein) for 5min in PBS, then ZAG (100 $\mu$ g protein) was added and left for a further 15min. The reaction was terminated by removal of the iodo bead, while free Na [<sup>125</sup>I] was removed using a

Sephadex G25 column eluted with 0.1MNaI. The [<sup>125</sup>I] ZAG was concentrated against PBS using a Microcon microconcentrator with a filter cut-off of Mr 10,000. The specific activity of the [<sup>125</sup>I] ZAG was 2.17  $\mu\text{Ci}/\text{mmol}$  assuming one iodine per protein.

#### Binding studies and cyclic AMP determination

CHO-K1 cells transfected with the human  $\beta$ 1- and  $\beta$ 2-AR were kind gifts from Dr Jillian Baker, University of Nottingham, UK, while CHOK1 cells transfected with the  $\beta$ 3-AR were a gift from Dr Ian Waddell, Astra Zeneca, Macclesfield, Cheshire, UK. Gene expression was under the control of hygromycin, together with a  $\beta$ -gal reporter construct, selected for resistance to G418. They were maintained in DMEM supplemented with 2mM glutamine, hygromycin B ( $50\text{mgml}^{-1}$ ), G418 ( $200\text{mgml}^{-1}$ ), and 10% FCS, under an atmosphere of 10% CO<sub>2</sub> in air. To determine the effect of agonists on cyclic AMP production, cells were grown in 24-well plates containing 1ml of nutrient medium. ZAG at the concentrations shown in Fig. 1 was added to the cells and incubation was continued for 30min. The medium was removed and replaced with 0.5ml of 20mM HEPES, pH 7.5, 5mM EDTA and 0.1mM isobutylmethylxanthine, and the plates were heated on a boiling water bath for 5min and cooled on ice for 10min. The concentration of cyclic AMP was determined with an ELISA assay.

For binding studies cells were sonicated in 2mM Tris HCl, pH7.5, containing 0.5M MgCl<sub>2</sub> and crude total membranes were pelleted by centrifugation (13,000g; 15min) at 4°C. Binding studies were carried out at 37°C by incubating membranes (500 $\mu\text{g}$  protein) in 0.4ml 50mM Tris HCl, pH 7.5, containing 0.5mM MgCl<sub>2</sub> for 60min with various concentrations of [<sup>125</sup>I] ZAG (3000 to 15000 cpm) in the absence or presence of 100 $\mu\text{M}$  non-labelled ZAG. Binding of [<sup>125</sup>I] iodo( $\pm$ ) Cyanopindodol (ICI25) (200pM) was also determined in the absence or presence of 580nM non-labelled ZAG. The membranes were then precipitated by centrifugation at 13000g for 20min, the supernatant was removed and the [<sup>125</sup>I] bound to the pellet was quantitated using a Packard Corbra Model 5005



Auto-gamma counter. Binding was analysed using non-linear regression analysis (GraphPad Prism, Version 5.04). Specific binding was regarded as the amount of labelled ZAG displaced by non-radioactive ZAG.

#### RNA isolation and RT-PCR

Quantitation of the mRNA transcripts for  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR in the three CHO-K1 cells was based on the methodology already described (13). Total RNA was extracted with Tri Reagent and quantitated by spectrophotometry,  $800 \pm 34$  ng total RNA was reverse transcribed, together with 2000 pmol random hexamers as primers using Superscript II reverse transcriptase at  $43^\circ\text{C}$  for 50 min. For reverse transcription and the following amplification by PCR, the primers used (sequences 5'-3') were,  $\beta$ 1-AR-sense: CAGGTAGAACTCGAAGCCCAC antisense: CTCCCATCCCTTCCCAAAC  $\beta$ 2-AR-sense CCGAAAGTCCCGTACGTACGTCA antisense: CAGCCCGTGCTCTGAAGAA  $\beta$ 3-AR-sense CTCCCCTGGTTCCATTCCTT antisense: TGGTCTTTTCTACCCTGCTGC GAPDH sense CCTGTTTCGACAGTCAGCCG antisense CGACCAAATCCGTTGACTCC. The probe sequences were selected to obtain  $T_m$ s approximately  $10^\circ\text{C}$  lower than the matching primer pair. PCR was carried out using Easy-A one tube RT.PCR system according to the manufacturer's instructions. The PCR conditions included a denaturing at  $95^\circ\text{C}$  for 10 min, an annealing step at  $42$ - $65^\circ\text{C}$ , and an extension step at  $68^\circ\text{C}$  for 2 min, and with a final extension at  $68^\circ\text{C}$  for 10 min. There were 40 cycles of amplification. Expression of  $\beta$ -AR mRNA was determined by the  $\Delta$ -CT method using Stratagenes MxPro, QPCR software v3.00.

#### Western blot analysis

WAT, BAT, heart and gastrocnemius muscle were thawed, washed in PBS, and lysed in Phosphosafe™ Extraction reagent for 5 min at room temperature, followed by sonication at  $4^\circ\text{C}$ . The supernatant

formed by centrifugation at 18,000g for 5min at 4°C was used for Western blotting. Cytosolic protein (5µg for UCP's and 20µg for β-AR) was resolved on 12% sodium dodecylsulphate polyacrylamide gels by electrophoresis at 180V for about 1h and transferred on 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. Prior to adding the primary antibodies membranes were washed for 15min in 0.1% Tween 20-buffered saline. Both primary and secondary antibodies were used at a dilution of 1:1000. Incubation was for 1h at room temperature and development was by ECL. Blots were scanned by a densitometer to quantify differences.

#### Glucose uptake into adipose tissue and skeletal muscle

Uptake of 2-[1-<sup>14</sup>C] deoxy-D-glucose (2-DG) into freshly isolated epididymal adipocytes and gastrocnemius muscle was determined as previously described (6).

#### Statistical analyses

Results are shown as mean ±SEM for at least three replicate experiments. Differences in means between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. p values <0.05 were considered significant.

## RESULTS

The effect of human ZAG on cyclic AMP production in CHO cells transfected with human  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3-AR in comparison with non-transfected cells is shown in Fig. 1. At low concentrations (up to 460nM) there was specific stimulation of cyclic AMP production only in cells transfected with the  $\beta$ 3-AR (Fig. 1A). However, at 580nM there was also a significant increase in cyclic AMP level in CHO cells transfected with the  $\beta$ 2-AR, although the magnitude of the change was less than in cells transfected with the  $\beta$ 3-AR. There was no increase in cyclic AMP in CHO cells transfected with the  $\beta$ 1-AR at any concentration of ZAG (Fig. 1A). The effect of isoprenaline on cyclic AMP production in the transfected cell lines is shown in Fig. 1B. There was no difference in cyclic AMP levels in CHO cells transfected with  $\beta$ 2- and  $\beta$ 3-AR, but significantly less in cells transfected with  $\beta$ 1-AR.

To determine whether expression of the  $\beta$ -AR was the same in the three cell lines mRNA levels of  $\beta$ 1-AR,  $\beta$ 2-AR and  $\beta$ 3-AR was determined by RT-PCR as described (13). The data in Fig. 1C show that the level of expression of each  $\beta$ -AR is the same in relation to the housekeeping gene GAPDH, although Western blotting (Fig. 1D) suggested that there was twice the level of  $\beta$ 3-AR as  $\beta$ 1- and  $\beta$ 2-AR. However, the problem with Western blotting was that it was clearly not quantitative, and the affinity of the antibodies to each of the  $\beta$ -AR may not be the same. The level of adenylate cyclase, as determined by cyclic AMP production in the presence of forskolin, was similar in the three cell lines (Fig. 1E). These results suggest that a comparison between the extent of stimulation of the  $\beta$ -AR in the three cell lines may not be valid if the Western blots represent the true amounts of the three  $\beta$ -AR, although it is clear from Fig. 1A that the major effects of ZAG are on  $\beta$ 2- and  $\beta$ 3-AR, and RT-PCR suggests equal amounts of mRNA for the three  $\beta$ -AR.

The affinity of binding of ZAG to the three  $\beta$ -AR was determined using  $^{125}$ I labelled ZAG and crude membranes from CHO-K1,  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3 cells (Fig. 1F). The data was evaluated using non-linear regression analysis and the  $K_d$  and  $B_{max}$  values are shown (Table 1). The binding data reflect

the stimulation of cyclic AMP production by ZAG as shown in Fig. 1A. Thus ZAG bound predominantly to  $\beta$ 3-AR (high Bmax and lowest Kd), less so to  $\beta$ 2-AR (Bmax 20% of  $\beta$ 3-AR and Kd twice  $\beta$ 3-AR), and not at all to  $\beta$ 1-AR (no Bmax and infinity high Kd). Non-specific binding was determined by the binding of [ $^{125}$ I] ZAG in the presence of 100 $\mu$ M non-labelled ZAG, and these values were subtracted from the total binding to give the specific binding values (Fig. 1F and table 1). The lipolytic activity of ZAG was shown to be destroyed by a single freezing and thawing cycle (Fig. 1G), probably due to a change in conformation of the protein. To determine whether this disrupted binding to  $\beta$ -AR two experiments were performed: (i) Freeze-thaw [ $^{125}$ I] ZAG was used in the binding studies, which completely attenuated binding to the  $\beta$ 2- and  $\beta$ 3-AR (results not shown). (ii) Freeze/thawed non-labelled ZAG was used in a competition assay with [ $^{125}$ I]ZAG, as in the determination of non-specific binding above. In contrast with fresh non-labelled ZAG this had no effect on either the Kd or Bmax for binding to  $\beta$ 2-AR or  $\beta$ 3-AR. These results suggest that freeze/thawing ZAG destroys biological activity by preventing binding to  $\beta$ -AR.

To compare ZAG binding with other ligands we employed iodocyanopindolol, which has been shown to bind to  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR (14). The data shown in Tables 1 and 2 indicates that ZAG binds to the  $\beta$ 3-AR with similar affinity to iodocyanopindolol, but with a lower Bmax, while the affinity for the  $\beta$ 2-AR is less than iodocyanopindolol. Moreover, ZAG displaces binding of iodocyanopindolol from  $\beta$ 2-AR and  $\beta$ 3-AR, raising the Kd, and lowering the Bmax, while having no effect on binding to  $\beta$ 1-AR. These results confirm that ZAG does not bind to the  $\beta$ 1-AR.

To determine whether the effects of ZAG on body weight and insulin sensitivity were due to interaction with a  $\beta$ -AR, ob/ob mice were treated with ZAG (50 $\mu$ g, iv, daily), as previously reported (6), in the absence and presence of the non-specific  $\beta$ -AR antagonist propranolol (40mg kg $^{-1}$ , po, daily). This dose level is higher than that commonly employed with  $\beta$ 2-AR agonists, since higher levels are required to counteract the effect of  $\beta$ 3-AR agonists (15). Propranolol completely

attenuated the decrease in body weight produced by ZAG (Fig. 2A), although animals treated with propranolol alone did not show such a large weight gain as did PBS controls. There was no effect of propranolol on food intake. As previously reported (6) mice treated with ZAG showed an increased body temperature (Fig. 2B), and this was completely attenuated by propranolol, as was the reduction in the urinary excretion of glucose (Fig. 2C). ZAG alone had no effect on liver lipids, although there was some increase in glycogen (Fig. 2D). Propranolol also blocked the reduction in peak plasma glucose levels, and the area under the glucose curve (AUC) induced by ZAG in the oral glucose tolerance test (Fig. 3A), as well as the corresponding reduction in peak plasma insulin levels (Fig. 3B). Animals treated with ZAG showed an increased glucose uptake into gastrocnemius muscle in the presence of insulin (10nM) (Fig. 3C), and this was completely attenuated in gastrocnemius muscle from mice receiving propranolol. Epididymal adipocytes from mice treated with ZAG also showed an enhanced glucose uptake in the absence and presence of insulin (Fig. 3D), and this was also completely attenuated in animals treated with propranolol. The decrease in serum levels of triglycerides (TG) and non-esterified fatty acids (NEFA) produced by ZAG were also attenuated by propranolol (Fig. 3E and F). These results suggest that the biological effects of ZAG are mediated through a  $\beta$ -AR. To determine whether ZAG can increase insulin signalling the effect on Glut4 expression was determined. Both insulin and ZAG increased expression of Glut4 in gastrocnemius muscle (Fig. 3G) and WAT (Fig. 3H), but the combination did not produce an increase over that of insulin alone. These results suggest that ZAG influences the same signalling pathways as insulin, but does not increase insulin signalling.

A number of  $\beta$ 3-agonists are known to increase expression of the  $\beta$ 3-AR (16, 17, 18). To determine whether ZAG had the same effect, tissue  $\beta$ 3-AR expression was quantified by Western blotting after 5 days of treatment of ob/ob mice with ZAG. The results in Fig. 4 show a two-fold increase in  $\beta$ 3-AR expression in gastrocnemius muscle (Fig. 4A) an 89% increase in BAT (Fig. 4B) and an almost 3-fold increase in WAT (Fig. 4C). In contrast there was no change in expression of  $\beta$ 1-AR

or  $\beta$ 2-AR in either gastrocnemius muscle (Fig. 5A) or WAT (Fig. 5B) and no change in expression of  $\beta$ 1-AR in heart (Fig. 5C), but a small increase in  $\beta$ 2-AR which just reached significance (Fig. 5C).

The increased expression of the  $\beta$ 3-AR in BAT and WAT (Fig. 4) would be expected to lead to an increased expression of UCP1, which is observed in both BAT (Fig. 6A) and WAT (Fig. 6B) after ZAG administration. In vitro experiments have shown that induction of expression of UCP3 by ZAG was attenuated by the mitogen activated protein kinase kinase (MAPKK) inhibitor PD98059, suggesting the involvement of MAPK in this process (19). Previous studies (20) have shown an increase in expression of ERK in WAT of ZAG-treated mice. This would be expected to lead to an increase in expression of UCP3 in WAT, as was observed (Fig. 6C). We have also previously reported an increase in UCP3 in skeletal muscle of ob/ob mice after administration of ZAG (6). The increased expression of UCP's would provide a sink for the NEFA released from adipose tissue, generating heat as previously reported (6).

In addition treatment with ZAG produced an increase in expression of AMPK in skeletal muscle (Fig. 6D), which would lead to an increased oxidation of long-chain fatty acids, decreasing the availability for the synthesis of triglycerides, as well as stimulating glucose uptake through increased expression of GLUT4 (6).

## Discussion

This study has found that ZAG binds predominantly to the  $\beta$ 3-AR, with intermediate binding to the  $\beta$ 2-AR, and no binding to the  $\beta$ 1-AR. The human  $\beta$ 3-AR is 51% homologous in amino acid sequence to the  $\beta$ 1-AR and 46% homologous to the  $\beta$ 2-AR (21). The  $B_{max}$  for ZAG binding to the  $\beta$ 3-AR is about three times that for the  $\beta$ 2-AR, while the  $K_d$  is about half. The  $K_d$  for ZAG for binding to the  $\beta$ 3-AR is about 100-fold lower than that of CGP12177, a partial agonist, while the  $B_{max}$  is only slightly lower (22), while the  $K_d$  for ZAG binding is similar to iodocyanopindolol, but the  $B_{max}$  is lower. These results were obtained using [ $^{125}$ I] ZAG which may have non-equivalent binding activity to native ZAG, which could lead to over or under estimation of the  $K_d$ . While most of the studies with ZAG have been carried out in rodents there is a difference between human and rodent  $\beta$ 3-AR. Thus BRL37344 is less effective at stimulating adenylyl cyclase via human than rodent  $\beta$ 3-AR (23), while CGP12177 is an effective agonist at human  $\beta$ 3-AR, but a poor partial agonist at the rat  $\beta$ 3-AR (23, 24). How ZAG binds to the  $\beta$ 3-AR and  $\beta$ 2-AR, while not binding to the  $\beta$ 1-AR, is not known, but the conformation of the protein is very important, since binding is destroyed by a single freeze/thaw cycle, as is also the ability of ZAG to stimulate lipolysis in murine adipocytes. The  $K_d$  values are in the range expected, both from studies on the stimulation of lipolysis (10), and cyclic AMP production by ZAG. However, they are more than 10-times lower than the human plasma concentration reported using an ELISA (600nM), (25), but are comparable with that reported using mass spectrometry (85nM) (26). If the former value was true ZAG would be maximally stimulating the  $\beta$ 2- and  $\beta$ 3-AR at normal plasma concentrations, which is clearly not correct. Care must be taken in interpreting plasma concentrations of ZAG using an ELISA, since there may be other components which bind to the anti-ZAG antibody, giving apparently higher concentrations. Thus ZAG has been shown to non-specifically bind to a monoclonal antihuman erythropoietin antibody giving apparently higher values in samples containing increased amounts of urinary ZAG (27).

The effect of ZAG on obesity and diabetes in the ob/ob mouse model (6) may be due to its ability to bind to  $\beta$ 3-AR. Thus  $\beta$ 3-AR agonists show anti-obesity effects in rodent models similar to ZAG, which induced an increased mobilisation of triglycerides from WAT depots (2), increased fat oxidation (6), and increased BAT-mediated thermogenesis (6), resulting in a selective reduction in body fat and preservation of fat-free mass (2). As with ZAG the anti-diabetic effects of  $\beta$ 3-AR agonists are independent of the anti-obesity effects, and occur at dose levels which do not induce weight loss. Treatment of ob/ob mice with the  $\beta$ 3-AR agonist BRL 35135 normalised plasma glucose levels and significantly decreased plasma insulin and non esterified fatty acid (NEFA) levels (15). As with ZAG BRL 35135 stimulated glucose uptake into three types of skeletal muscle, BAT, WAT, heart and diaphragm, which was independent of the action of insulin (28). Another  $\beta$ 3-agonist L-796568 increased lipolysis and energy expenditure in obese men when administered as a single dose (29). However, treatment for 28 days had no major lipolytic or thermogenic effect, although it lowered triacylglycerol concentration (29, 30). This may be due to insufficient recruitment of  $\beta$ 3-AR responsive tissues in humans, or down-regulation of  $\beta$ 3-AR with chronic dosing. Studies in human subcutaneous abdominal adipose tissue show that  $\beta$ 3-AR play a weaker role in the control of lipolysis than found in rodents, and that mobilisation of lipids is mainly through  $\beta$ 1 and  $\beta$ 2-AR subtypes (31). Thus ZAG may exert its effect in humans via a  $\beta$ 2-AR rather than  $\beta$ 3-AR.

This study has shown that propranolol, a non-specific  $\beta$ -AR antagonist attenuates the effect of ZAG in reducing body weight and urinary glucose excretion, increasing body temperature, improving the response to glucose in the oral glucose tolerance test and increasing glucose uptake into skeletal muscle and WAT of ob/ob mice, when administered at high dose levels. In addition freeze-thawing, which destroyed the ability of ZAG to induce lipolysis in WAT, and reduce body fat in aged obese mice (10) also completely attenuates its ability to bind to human  $\beta$ 2- and  $\beta$ 3-AR. These results confirm that the anti-obesity and anti-diabetic effects of ZAG are mediated through a  $\beta$ -AR. Further studies will utilise specific “knock-out” animals to determine which  $\beta$ -AR is involved.



This study has also shown that administration of ZAG to ob/ob mice increases the expression of  $\beta$ 3-AR protein in BAT, WAT and skeletal muscle. This effect is also seen with some  $\beta$ 3-AR agonists. Thus chronic treatment of ob/ob mice with the  $\beta$ 3-AR agonist BRL35135 resulted in a two-fold increase in  $\beta$ 3-AR mRNA in BAT (15). Similar effects were reported with another  $\beta$ 3-AR agonist CL 316,243 in Zucker fa/fa rats (17), and in adipocytes of adult humans (18). Thus the ability of ZAG to induce expression of the  $\beta$ 3-AR would enhance its effect on obesity and diabetes. The reduced  $\beta$ -AR mediated lipolysis and fat oxidation seen in obese subjects (32) may be due to low levels of ZAG (5), and that administration of ZAG could improve sensitivity. Certainly ZAG administration to ob/ob mice increased sensitivity of epididymal adipocytes to the lipolytic effect of the  $\beta$ 3-AR agonist, BRL 37344 (19). The ability of ZAG to induce expression of  $\beta$ 3-AR could explain the reduced response of adipose tissue from ZAG 'knock-out mice' to the lipolytic effect of the  $\beta$ 3-AR agonist CL316243 (9), although the authors reported no change in  $\beta$ 3-AR mRNA. It is possible that ZAG induces changes at the protein level of  $\beta$ 3-AR rather than mRNA.

Using knock-out mice the antiobesity effect of  $\beta$ 3-AR stimulation has been shown to be through the UCP-1 dependent degradation of fatty acids released from WAT (33,34). Until recently BAT was considered to be restricted to rodents and neonatal humans. However three independent *studie* (35, 36, 37) conclusively identified BAT in adult humans primarily behind the muscles of the lower neck and collar bone, as well as along the spine of the chest and the abdomen.  $\beta$ 3-AR agonists have been shown to stimulate remodelling of WAT into BAT, determined histologically (16), or by the appearance of UCP1 (17). The appearance of UCP1 in WAT in response to ZAG would suggest that it initiates a similar process. Previous studies (18) have suggested a role for the  $\beta$ 3-AR in the induction of UCP1 by ZAG.  $\beta$ 3-AR agonists have been shown to induce upregulation of UCP1 in BAT through stimulation of p38 mitogen activated protein kinase (p38 MAPK) downstream of cyclic AMP/protein kinase A, leading to activation (phosphorylation) of peroxisome proliferator-activated receptor (PPAR)  $\gamma$  coactivator 1 (PCG-1 $\alpha$ ), as well as ATF-2, allowing the CRE and PPAR elements of

the UCP1 enhancer to be occupied (38). The increased expression of AMPK in skeletal muscle would also be expected to increase oxidation of fatty acids through phosphorylation and inactivation of acetyl CoA carboxylase (ACC) and phosphorylation and activation of malonyl CoA decarboxylase, decreasing malonyl CoA levels. Using human skeletal muscle cells ZAG has been recently shown (39) to activate both AMPK $\alpha$  and ACC, Activation of AMPK also increases expression of the Glut 4 gene (40).

ZAG is a naturally occurring ligand with selective agonist activity towards the  $\beta$ 3-AR. Very few proteins display such activity, although the hypotensive peptide adrenomedullin may also activate  $\beta$ 3-AR leading to relaxation of ileal muscle [41]. Since ZAG is much larger than the normal catecholamine agonists it is possible that activation occurs through allosteric modulation. However, previous studies using LMF (11) have shown binding to be completely attenuated by propranolol, suggesting direct interaction with a  $\beta$ 3-AR. It is likely that only part of the ZAG molecule is required for binding, since evidence from our own laboratory, and that of others (42) have suggested that tryptic fragments of a lipolytic factor (Mr about 5kDa) were still biologically active. It is possible that certain groups of amino acids, such as serine hydroxyl, can mimic the hydrogen bonding interactions seen between catecholamines and the  $\beta$ 3-AR. Molecular modelling studies may provide further information on the interactions involved.  $\beta$ 3-AR agonists such as BRL37344 have been shown to increase ZAG expression in adipocytes (3), and induction of ZAG expression by ZAG has also been suggested to occur through a  $\beta$ 3-AR (43). Thus the  $\beta$ 3-AR is important in both the production and biological effects of ZAG, and ZAG is a natural agonist of  $\beta$ 2- and  $\beta$ 3-AR.

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## FIGURE LEGENDS

Figure 1 Effect of ZAG and isoprenaline on cyclic AMP production in CHO-K1 cells transfected with the human  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR. (A) Effect of ZAG concentration and (B) isoprenaline (10 $\mu$ M) on cyclic AMP production in CHO cells transfected with  $\beta$ 1-AR (■),  $\beta$ 2-AR (□) and  $\beta$ 3-AR (▣). Differences from basal levels of cyclic AMP in the absence of ZAG are indicated as either \*,  $p < 0.05$  or \*\*\*,  $p < 0.001$ , while difference from CHO cells transfected with  $\beta$ 2- and  $\beta$ 3-AR is indicated as a,  $P < 0.05$ . (C) Expression of  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR in CHO-K1 cells transfected with the respective human genes as absolute numbers of  $\beta$ -AR mRNA molecules/ $\mu$ g of total RNA, measured by RT- real time PCR (closed boxes) in comparison with expression of GAPDH in the same sample (open boxes). (D) Western blots showing expression of  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR in relation to actin in  $\beta$ -AR transfected CHO-K1 cells. The numbers underneath the blots are densitometric values given in arbitrary units. (E) Cyclic AMP production in CHO-K1 cells transfected with human  $\beta$ 1-,  $\beta$ 2- and  $\beta$ 3-AR in response to forskolin (20 $\mu$ M). (closed boxes) in relation to basal levels (open boxes). (F) Specific binding curve of ZAG in CHO-K1 cells transfected: with human  $\beta$ 1- (X),  $\beta$ 2- ( ) and  $\beta$ 3-AR (■). (G) Lipolytic activity of ZAG (0.58 $\mu$ M), either fresh (□), or frozen and thawed once (▣), in comparison with isoprenaline (Iso; 10 $\mu$ M) in murine epididymal adipocytes. Differences from control are shown as c,  $p < 0.001$ , while differences between fresh and frozen ZAG and in the presence of SR59230A are shown as †††,  $p < 0.001$ .

Figure 2 Effect of propranolol on body weight (A), body temperature (B) and urinary glucose excretion (C) in ob/ob mice treated with ZAG. Animals were divided into 4 groups (n=5 per group) to receive daily administration of ZAG (50mg, iv) (■), ZAG + propranolol (40mgkg<sup>-1</sup>, po) (▲), while controls received either PBS (◆) or PBS and propranolol (X).

Differences from PBS are shown as \*\*\*,  $p < 0.001$ , while differences from ZAG alone are shown as †††,  $p < 0.001$ . Liver histology after 60 days ZAG administration is shown in (D).

Figure 3 Effect of propranolol on glucose and insulin tolerance and glucose uptake into skeletal muscle and WAT of ob/ob mice. (A) Plasma glucose levels and total areas under the glucose curves (AUC) in arbitrary units during a glucose tolerance test 3 days after initiation of ZAG (■) or ZAG with propranolol (▲) in comparison with PBS (◆) or PBS and propranolol (X) as described in Figure 2, and in methods. (B) Plasma insulin during the glucose tolerance test described in (A). (C) Glucose uptake into isolated gastrocnemius muscle of ob/ob mice in the absence or presence of insulin (100nM). Ob/ob mice were treated with ZAG with or without propranolol for 7 days prior to excision of muscle. (D) Glucose uptake into epididymal adipocytes of ob/ob mice in the absence or presence of insulin (10nM). Animals received the treatments indicated for 7 days prior to excision of WAT. Serum levels of TG (E) and NEFA (F) in ob/ob mice treated with PBS or ZAG, with or without propranolol for 7 days. Western blots shows Glut 4 expression in Gastrocnemius (G) and WAT (H) from ob/ob mice in the presence of ZAG or Insulin or both. Differences from PBS controls are shown as \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  or \*\*\*,  $p < 0.001$ , while differences from ZAG alone are shown as ††,  $p < 0.05$  or †††,  $p < 0.001$ .

Figure 4 Expression of  $\beta 3$ -AR after treatment of ob/ob mice with ZAG ( $35\mu\text{g}$ ; i.v.  $\text{day}^{-1}$ ) for 5 days. Western blots showing expression of  $\beta 3$ -AR in gastrocnemius muscle (A), BAT (B) and WAT (C) of ob/ob mice treated with either PBS or ZAG. The densitometric analysis is the average of three separate Western blots. Differences from control as shown as \*\*\*,  $p < 0.001$ .

Figure 5 Expression of  $\beta$ 1- and  $\beta$ 2-AR in gastrocnemius muscle (A), WAT (B) and heart (C) after treatment of ob/ob mice with ZAG (35 $\mu$ g; i.v., daily) for 5 days. Differences from PBS treated animals is shown as \*,  $p < 0.05$ .

Figure 6 Effect of ZAG on expression of uncoupling proteins. Western blots showing expression of UCP1 showing expression of UCP1 in BAT (A) and WAT (B), and expression of UCP3 in WAT (C) and AMPK in gastrocnemius muscle (D) in ob/ob mice after treatment with either PBS or ZAG (35 $\mu$ g; i.v., daily) for 5 days. The densitometric analysis is the average of three separate blots. Differences from PBS treated animals are shown as \*\*\*,  $p < 0.001$ .

Human $\beta$ -AR	Bmax (fmolmgprotein <sup>-1</sup> )	Kd (nM)
$\beta$ 1-AR	0	$\infty$
$\beta$ 2-AR	51 $\pm$ 6	71 $\pm$ 3
$\beta$ 3-AR	262 $\pm$ 14	46 $\pm$ 1

Table 1: Bindings studies on CHO-K1 cells transfected with human  $\beta$ 1-AR,  $\beta$ 2-AR or  $\beta$ 3-AR

[ <sup>125</sup> I] iodocyanopindidol (200pM)	[ <sup>125</sup> I] iodocyanopindidol (200pM)  +ZAG 580nM
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CHO	Bmax(fmolmgprotein <sup>-1</sup> )	Kd (nM)	Bmax(fmolmgprotein <sup>-1</sup> )	Kd (nM)
B1	234 <sub>±</sub> 23	40 <sub>±</sub> 3.5	220 <sub>±</sub> 45	41.3 <sub>±</sub> 4.5
B2	356 <sub>±</sub> 37	35 <sub>±</sub> 2.9	290 <sub>±</sub> 3.5	69.3 <sub>±</sub> 3.5
B3	356 <sub>±</sub> 21	51 <sub>±</sub> 2.4	259 <sub>±</sub> 62	70.4 <sub>±</sub> 4.5

Table 2 Competitive binding studies of ZAG and iodocyanopindidol to β1-AR, β2-AR and β3-AR