ROLE OF β-ADRENERGIC RECEPTORS IN THE ANTI-OBESITY AND ANTI-DIABETIC EFFECTS OF ZINC-α2-GLYCOPROTIEN (ZAG)

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Short page-heading: Zinc-α2-glycotprotein binds activates β3- and β2-adrenergic receptors, but not b1, 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 β-adrenoreceptor (β-AR) plays a role in the anti-obesity and anti-diabetic effects of zinc-α2-glycoprotein (ZAG).

Material and Methods: This has been investigated in CHO-K1 cells transfected with the human β1-, β2-, β3-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 propranonol.

Results: ZAG bound to the β3-AR with higher affinity (Kd 46±1nM) than the β2-AR (Kd 71±3nM) while there was no binding to the β1-AR, and this correlated with the increases in cyclic AMP in CHO-K1 cells transfected with the various β-AR and treated with ZAG. Treatment of ob/ob mice with ZAG increased protein expression of β3-AR in gastrocnemius muscle, and in white and brown adipose tissue, but had no effect on expression of β1- and β2-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 β-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 β-3AR, or possibly a β2-AR.

Keywords: zinc-α2-glycoprotein; β-adrenoreceptor; obesity; diabetes

Abbreviations:

AUC: area under the curve, AMPK: AMPkinase, β-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-α_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 β3-adrenoreceptor (β3-AR) antagonist, SR59230A (10), and shown to bind to the β3-AR through a high affinity binding site (Kd 78±4.5nM) (11). These results suggest that lipolysis mediated by ZAG is mediated through a β3-AR. However, this conclusion has recently been questioned (9) based on the inability of β3-AR agonists to stimulate lipolysis in ZAG knock-out mice. The present study re-examines the role of the β3-AR in the action of ZAG, using human recombinant material, as well as determining the binding to human β_1- and β_2-AR, and the role of the β-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-^{14}C] deoxy-D-glucose sp.act. 1.85GBq mmol^{-1}) was purchased from American Radiolabeled Chemicals (Cardiff, UK). Na [^{125}I] (sp. Act. >17Ci mg^{-1})and [^{125}I] iodo($\pm$) Cyanopindodol (sp.act. 81.4 TBq mmol^{-1}) (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 (50μg, i.v. in 100μl PBS) or PBS daily with or without propranolol (40mgkg^{-1}, 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^{-1} in a volume of 100μl) 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 (50μgml^{-1}), under an atmosphere of 5% CO_2 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 Pyrogent single test kit (Lonza). The purified ZAG was stored at 4^{o}C in PBS.

[^{125}I] labelling of ZAG

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

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

Binding studies and cyclic AMP determination

CHO-K1 cells transfected with the human β1- and β2-AR were kind gifts from Dr Jillian Baker, University of Nottingham, UK, while CHOK1 cells transfected with the β3-AR were a gift from Dr Ian Waddell, Astra Zeneca, Macclesfield, Cheshire, UK. Gene expression was under the control of hygromycin, together with a β-gal reporter construct, selected for resistance to G418. They were maintained in DMEM supplemented with 2mM glutamine, hygromycin B (50mgml^{-1}), G418 (200mgml^{-1}), and 10% FCS, under an atmosphere of 10% CO_2 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 $MgCl_2$ 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 (500μg protein) in 0.4ml 50mM Tris HCl, pH 7.5, containing 0.5mM $MgCl_2$ for 60min with various concentrations of [^{125}I] ZAG (3000 to 15000 cpm) in the absence or presence of 100μM non-labelled ZAG. Binding of [^{125}I] iodo(±) 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 [^{125}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 β1-, β2- and β3-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±34ng total RNA was reverse transcribed, together with 2000pmol random hexamers as primers using Superscript II reverse transcriptase at 43°C for 50min. For reverse transcription and the following amplification by PCR, the primers used (sequences 5'-3') were, β1-AR-sense: CAGGTAGAACTCGAAGCCCAC antisense: CTCCCATCCCTTCCCAAACT β2-AR-sense CCGAAAGTTCCCGTACGTACGTCA antisense: CAGCCCGTGCTCTGAAGAA β3-AR-sense CTCCCCTGGTTCCATTCCTT antisense: TGGTCTTTTCTACCCTGCTGC GAPDH sense CCTGTTCGACAGTCAGCCG antisense CGACCAAATCCGTTGACTCC. The probe sequences were selected to obtain T_mS approximately 10°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°C for 10min, an annealing step at 42-65°C, and an extension step at 68°C for 2min, and with a final extension at 68°C for 10min. There were 40 cycles of amplification. Expression of β-AR mRNA was determined by the Δ-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 Phosphsafe™ Extraction reagent for 5 min at room temperature, followed by sonication at 4°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-^{14}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 β1, β2 and β3-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 β3-AR (Fig. 1A). However, at 580nM there was also a significant increase in cyclic AMP level in CHO cells transfected with the β2-AR, although the magnitude of the change was less than in cells transfected with the β3-AR. There was no increase in cyclic AMP in CHO cells transfected with the β1-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 β2- and β3-AR, but significantly less in cells transfected with β1-AR.

To determine whether expression of the β-AR was the same in the three cell lines mRNA levels of β1-AR, β2-AR and β3-AR was determines by RT-PCR as described (13). The data in Fig. 1C show that the level of expression of each β-AR is the same in relation to the housekeeping gene GAPDH, although Western blotting (Fig. 1D) suggested that there was twice the level of β3-AR as β1- and β2-AR. However, the problem with Western blotting was that it was clearly not quantitative, and the affinity of the antibodies to each of the β-AR may not be the same. The level of adenylate cyclase, as be the 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 β-AR in the three cell lines may not be valid if the Western blots represent the true amounts of the three β-AR, although it is clear from Fig. 1A that the major effects of ZAG are on β2-and β3-AR, and RT.PCR suggests equal amounts of mRNA for the three β-AR.

The affinity of binding of ZAG to the three β-AR was determined using ^{125}I labelled ZAG and crude membranes from CHO-K1, β1, β2 and β3 cells (Fig. 1F). The data was evaluated using non-linear regression analysis and the Kd and Bmax 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 β3-AR (high Bmax and lowest Kd), less so to β2-AR (Bmax 20% of β3-AR and Kd twice β3-AR), and not at all to β1-AR (no Bmax and infinity high Kd). Non-specific binding was determined by the binding of [^{125}I] ZAG in the presence of 100μM 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 β-AR two experiments were performed: (i) Freeze-thaw [^{125}I] ZAG was used in the binding studies, which completely attenuated binding to the β2- and β3-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 β2-AR or β3-AR. These results suggest that freeze/thawing ZAG destroys biological activity by preventing binding to β-AR.

To compare ZAG binding with other ligands we employed iodocyanopindidol, which has been shown to bind to β1-, β2-and β3-AR (14). The data shown in Tables 1 and 2 indicates that ZAG binds to the β3-AR with similar affinity to iodocyanopindidol, but with a lower Bmax, while the affinity for the β2-AR is less than iodocyanopindidol. Moreover, ZAG displaces binding of iodocyanopindidol from β2-AR and β3-AR, raising the Kd, and lowering the Bmax, while having no effect on binding to β1-AR. These results confirm that ZAG does not bind to the β1-AR.

To determine whether the effects of ZAG on body weight and insulin sensitivity were due to interaction with a β-AR, ob/ob mice were treated with ZAG (50μg, iv, daily), as previously reported (6), in the absence and presence of the non-specific β-AR antagonist propranolol (40mg kg^{-1}, po, daily). This dose level is higher than that commonly employed with β2-AR agonists, since higher levels are required to counteract the effect of β3-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 β-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 β3-agonists are known to increase expression of the β3-AR (16, 17, 18). To determine whether ZAG had the same effect, tissue β3-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 β3-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 β1-AR

or β2-AR in either gastrocnemius muscle (Fig. 5A) or WAT (Fig. 5B) and no change in expression of β1-AR in heart (Fig. 5C), but a small increase in β2-AR which just reached significance (Fig. 5C).

The increased expression of the β3-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 β3-AR, with intermediate binding to the β2-AR, and no binding to the β1-AR. The human β3-AR is 51% homologous in amino acid sequence to the β_1-AR and 46% homologous to the β2-AR (21). The Bmax for ZAG binding to the β3-AR is about three times that for the β2-AR, while the Kd is about half. The Kd for ZAG for binding to the β3-AR is about 100-fold lower than that of CGP12177, a partial agonist, while the Bmax is only slightly lower (22), while the Kd for ZAG binding is similar to iodocyanopindidol, but the Bmax 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 Kd. While most of the studies with ZAG have been carried out in rodents there is a difference between human and rodent β3-AR. Thus BRL37344 is less effective at stimulating adenylyl cyclase via human than rodent β3-AR (23), while CGP12177 is an effective agonist at human β3-AR, but a poor partial agonist at the rat β3-AR (23, 24). How ZAG binds to the β3-AR and β2-AR, while not binding to the β1-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 Kd 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 β2- and β3-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 β3-AR. Thus β3-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 β3-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 β3-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 β3-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 β3-AR responsive tissues in humans, or down-regulation of β3-AR with chronic dosing. Studies in human subcutaneous abdominal adipose tissue show that β3-AR play a weaker role in the control of lipolysis than found in rodents, and that mobilisation of lipids is mainly through β1 and β2-AR subtypes (31). Thus ZAG may exert its effect in humans via a β2-AR rather than β3-AR.

This study has shown that propranolol, a non-specific β-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 β2- and β3-AR. These results confirm that the anti-obesity and anti-diabetic effects of ZAG are mediated through a β-AR. Further studies will utilise specific “knock-out” animals to determine which β-AR is involved.

This study has also shown that administration of ZAG to ob/ob mice increases the expression of β3-AR protein in BAT, WAT and skeletal muscle. This effect is also seen with someβ3-AR agonists. Thus chronic treatment of ob/ob mice with the β3-AR agonist BRL35135 resulted in a two-fold increase in β3-AR mRNA in BAT (15). Similar effects were reported with another β3-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 β3-AR would enhance its effect on obesity and diabetes. The reduced β-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 β3-AR agonist, BRL 37344 (19). The ability of ZAG to induce expression of β3-AR could explain the reduced response of adipose tissue from ZAG 'knock-out mice' to the lipolytic effect of the β3-AR agonist CL316243 (9), although the authors reported no change in β3-AR mRNA. It is possible that ZAG induces changes at the protein level of β3-AR rather than mRNA.

Using knock-out mice the antiobesity effect of β3-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. β3-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 β3-AR in the induction of UCP1 by ZAG. β3-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) γ coactivator 1 (PCG-1α), 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 AMPKα 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 β3-AR. Very few proteins display such activity, although the hypotensive peptide adrenomedullin may also activate β3-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 β3-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 β3-AR. Molecular modelling studies may provide further information on the interactions involved. β3-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 β3-AR (43). Thus the β3-AR is important in both the production and biological effects of ZAG, and ZAG is a natural agonist of β2- and β3-AR.

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References

1. Todorov PT, McDevitt TM, Meyer DJ, Ueyama H, Ohkubo I, Tisdale MJ, Purification and characterization of a tumor-lipid mobilizing factor. Cancer Res 58 1998; 58:2353-2358

2. Hirai K, Hussey HJ, Barber MD, Price SA, Tisdale MJ, Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. Cancer Res 1998;58:2359-2365

3. Bing C, Bao Y, Jenkins J, Sanders P, Manieri M, Cinti S, Tisdale MJ, Trayhurn P, 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 2004; 101:2500-2505

4. Mracek T, Stephens NA, Gao D, Bao Y, Ross JA, Rydén M, Arner P, Trayhurn P, Fearon KC, Bing C, Enhanced ZAG production by subcutaneous adipose tissue is linked to weight loss in gastrointestinal cancer patients.Br J Cancer 2011; 104(3):441-7

5. Mracek T, Ding Q, Tzanavari T, Kos K, Pinkney J, Wilding J, Trayhurn P, Bing C, The adipokine zinc-α2-glycoprotein is downregulated with fat mass expansion in obesity. Clin Endocrinol 2010; 72(3) 334-41

6. Russell ST and Tisdale MJ, Antidiabetic properties of zinc-α2-glycoprotein in ob/ob mice. Endocrinol 2010, 151: 948-957

7. Gong F-Y, Deng J-Y, Zhu H-J, Pan H, Wang L-J, Yang H-B , Fatty acid synthase and hormone sensitive lipase expression in liver are involved in zinc-α2-glycoprotein-induced body fat loss in obese mice. Chin Med Sci J 2010; 25: 169-175

8. Gong F-Y, Zhang S-J, DingJ-Y, Zhu H-J, Pan H, Li N-S, Shi Y-F, Zinc-α2-glycoprotein is involved in regulation of body weight through inhibition of lipogenic enzymes in adipose tissue. Int J Obese 2009; 33:1023-1030

9. Rolli V, Radosavljevic M, Astier V, Macquin C, Castan-Laurell I, Visentin V, Guigne C, Carpene C, Valet P, Gilfillan S, Bahram S, Lipolysis is altered in MHC class I zinc-α2-glycoprotein deficient mice. FEBS Lett 2007;581: 394-400

10. Russell ST, Zimmerman TP, Domin BA, Tisdale MJ, Induction of lipolysis in vitro and loss of body fat in vivo by zinc-α2-glycoprotein. Biochim Biophys Acta 2004; 1636: 59-68

11. Russell ST, Hirai K, Tisdale MJ, Role of β3-adrenergic receptors in the action of a tumour lipid mobilizing factor. Br J Cancer 2002;86: 424-428

12. Bailey CL, Flatt PR, Atkins TA Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. Int J Obes 1982; 6: 11-21

13. Moniotte S, Vaerman J-L, Kockx MM, Larrovy D, Langin D, Noirhomme P, Balligand J-L**.** Real-time RT-PCR for the detection of beta-adrenoceptor messanger RNAs in small human endomyocardial biopsies. J Mol Cell Cardiol 2001;33:2121-2133

14. Tate KM, Briend-Sutren M-M, Emorine LJ, Delavier-Klutchko C, Matullo S, Strosberg AD, Expression of three human beta-adrenergic-receptor subtypes in transfected Chinese hamster ovary cells. Eur. J. Biochem 1991; 196:357-361

15. Liu YL and Stock MJ, Acute effects of the beta 3-adrenoreceptor agonist, BRL 35135, on tissue glucose utilisation. Br J Pharmacol 1995; 114:888-894

16. Arbeeny CM, Meyers DS, Hillyer DE, Bergquist KE, Metabolic alterations associated with the antidiabetic effect of β3-adrenergic agonists in obese mice. Am J Physiol Endocrinol Metab 1995;268: E678-E684

17. Ghorbani M and Himms-Hagen J, Appearance of brown adipocytes in white adipose tissue during CL316, 243-induced reversal of obesity and diabetes in Zucker fa/fa rats. Int J Obes 997; 211: 465-475

18. Champigny O. and Ricquier D. Evidence from in vitro differentiating cells that adrenoreceptor agonists can increase uncoupling protein mRNA level in adipocytes of adult humans: an RT-PCR study. J Lipid Res 1996; 37: 1907-1914

19. Sanders PM and Tisdale MJ, Effect of zinc-α_2-glycoprotein (ZAG) on expression of uncoupling proteins in skeletal muscle and adipose tissue. Cancer Lett 2004; 21: 71-81

20. Russell ST and Tisdale MJ, Studies on the anti-obesity effect of zinc-α2-glycoprotein in the ob/ob mouse. Int J Obes 2011; 35:345-354

21. Emorine LJ, Marullo S, Briend-Suttren M-M, Patey G, Tate K, Delavier-Klutchko C, Strossberg AD, Molecular characteristics of the human β3-adrenergic receptor. Science 1989; 245: 1118-1121

22. Kubo S, Matsuda A, Ohnuki T, Hattori K, Suzuki J, Nagatomo T. Assessment of β2- and β3-adrenoreceptors in rat white adipose tissue by radioligand binding assay. Biol Pharm Bull 1997;20: 142-148

23. Liggett SB. Functional properties of the rat and human β3-adrenergic receptors, differential agonist activation of recombinant receptors in Chinese hamster ovary cells. Mol Pharmacol 1992;42: 634-637

24. Granneman JG, Lahners K, Chaudry A, Characterisation of the human β3-adrenergic receptor gene. Mol Pharmacol 1993; 44:264-270

25. Stejskal D, Karpisek M, Reutova H, Stejskal P, Kotolova H, Kollar P, Determination of serum zinc-alpha-2-glycoprotein in patients with metabolic syndrome by a new ELISA. Clin Biochem 2008;41:313-316

26. Bondar OP, Barnidge DR, Klee EW, Davis BJ, Klee GG. LC-MS/MS quantification of Zn-α2-glycoprotein: a potential serum biomarker for prostate cancer. Clin Chem 2007; 53: 673-678

27. Reichel C, Identification of zinc-alpha-2-glycoprotein binding to clone AE7A5 antihuman EPO antibody by means of nano-HPLC and high-resolution high-mass accuracy ESI-MS/MS. J Mass Specrom 2008; 43:916-923

28. Abe H, Minokoshi Y, Shimazu T, Effect of a β3-adrenergic agonist, BRL 35135A on glucose uptake in rat skeletal muscle in vivo and in vitro. J Endocrinol 1993;139:479-486

29. Van Baak M, Hul GBH, Toubros S, Astrup A, Gottesdiene KM, DeSmet H, Saris WHM. Acute effect of L-796568, a novel β3-adrenergic receptor agonist, on energy expenditure and body composition in obese men. Clin Pharmacol Ther 2002;71: 272-279

30. Larsen TM, Toubro S, van Baak M, Gottesdiener KM, Larson P,. Saris WHM, Astrup A **,** Effect of a 28-d treatment with L-79568, a novel β3-adrenergic receptor agonist, on energy expenditure and body composition in obese men. Am J Clin Nutr 2002; 76: 780-788

31. Barbe P, Millet L, Galitzky J, Lafontan M, Berlan M**.** In situ assessment of the role of the β1-, β2-and β3-adrenoreceptors in the control of lipolysis and nutritive blood flow in human subcutaneous adipose tissue. Br J Pharmacol 11996; 117: 907-913

32. Blaak EE, Van Baak MM, Kemerick GJ, Pakbiers MT, Heidendal GA, Saris WH, Beta-adrenergic stimulation of energy expenditure and forearm skeletal muscle metabolism in lean and obese men. Am J Physiol Endocrinol Metab 1994;267: E306-E315

33. Inokuma K, Okamatsu-Ogura Y, Omachi A, Matushita Y, Kimura K, Ymashita H, Saito M, Indispensable role of mitochondrial UCP1 for the antiobesity effect of β3-adrenergic stimulation. Am J Physiol Endocrinol Metab 2006;290: E1014-E1021

34. Grujic D, Susulic VS, Harper M-E, Himms-Hagen J, Cunningham BA, Corkey BE, Lowell BB. B3-adrenergic receptors on white and brown adipocytes mediate β3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. 1997;272:17686-17693

35. van Markin Lichtenbelt WD, Vanhomnerig JW, Smulders NM, Drossaerts JMAFL, Kemerink GJ, Bouvy ND, Schrauwen P, GJJ Teule**.** Cold-activated brown adipose tissue in healthy men, New Engl J Med 2009;360:1500-1508

36. Cypress AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng Y-H, Doria A, Kolodny GM, Kahn R, Identification and importance of brown adipose tissue in adult humans. New Engl J Med 3 2009; 360: 1509-1517.

37. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto N-J, Enerback S, Nuutila P, Functional brown adipose tissue in healthy adults. New Engl J Med 2009; 360:1518-1525

38. Collins S, Cao W, Robidoux J, Learning new tricks from old dogs: β-adrenergic receptors teach new lessons on firing up adipose tissue metabolism. Mol Endocrinol 2004;18: 2123-2131

39. Eckardt K, Schober A, Platzbecker B, Mracek T, Bing C, Trayhurn P, Eckel J, The adipokine zinc-α2-glycoprotein activates AMP kinase in human primary skeletal muscle cells. Arch Physiol Biochem 2011; 117: 88-93

40. Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL, Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001; 91: 1073-1083

41. Kravtsov GM, Hwang IS, Tang F, Inhibitory effect of adrenomedullin in the rat ileum: cross-talk with beta 3-adrenoreceptor in the serotonin-induced muscle contraction. J Pharmacol Exp Ther 2004; 308: 241-248

42. Masuno H, Yamaski N, Okuda H, Purification and characterisation of a lipolytic factor (toxochrome-L) from cell-free fluid of ascites sarcoma 180. Cancer Res 1981;41: 284-288

43. Russell ST and Tisdale MJ, The role of glucocorticoids in the induction of zinc-α_2-glycoprotein expression in adipose tissue in cancer cachexia. Br J Cancer 2005; 92: 876-8

FIGURE LEGENDS

Figure 1 Effect of ZAG and isoprenaline on cyclic AMP production in CHOK1 cells transfected with the human β1-, β2- and β3-AR. (A) Effect of ZAG concentration and (B) isoprenaline (10μM) on cyclic AMP production in CHO cells transfected with β1-AR (■), β2-AR (□) and β3-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 β2- and β3-AR is indicated as a, $P<0.05$. (C) Expression of β1-, β2- and β3-AR in CHO-K1 cells transfected with the respective human genes as absolute numbers of β-AR mRNA molecules/μg 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 β1-, β2- and β3-AR in relation to actin in β-AR transfected CHOK1 cells. The numbers underneath the blots are densitometric values given in arbitrary units. (E) Cyclic AMP production in CHO-K1 cells transfected with human β1-, β2- and β3-AR in response to forskolin (20μM). (closed boxes) in relation to basal levels (open boxes). (F) Specific binding curve of ZAG in CHO-K1 cells transfected: with human β1- (X), β2-(△) and β3-AR (■). (G) Lipolytic activity of ZAG (0.58μM), either fresh (□), or frozen and thawed once (▨), in comparison with isoprenaline (Iso; 10μM) 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 ($40mgkg^{-1}$, 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 or 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 Gastronemius (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 β3-AR after treatment of ob/ob mice with ZAG (35μg; i.v. day^{-1}) for 5 days. Western blots showing expression of β3-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 β1- and β2-AR in gastrocnemius muscle (A), WAT (B) and heart (C) after treatment of ob/ob mice with ZAG (35μg; 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 (35μg; 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 β-AR	Bmax (fmolmgprotein^{-1})	Kd (nM)
β1-AR	0	∞
β2-AR	51±6	71±3
β3-AR	262±14	46±1

Table 1: Bindings studies on CHO-K1 cells transfected with human β1-AR, β2-AR or β3-AR

[^{125}I] iodocyanopindidol (200pM)	[^{125}I] iodocyanopindidol (200pM) +ZAG 580nM

CHO	Bmax(fmolmgprotein^{-1})	Kd (nM)	Bmax(fmolmgprotein^{-1})	Kd (nM)
B1	234±23	40±3.5	220±45	41.3±4.5
B2	356±37	35±2.9	290±3.5	69.3±3.5
B3	356±21	51±2.4	259±62	70.4±4.5

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