STUDIES ON THE ANTIOBESITY ACTIVITY OF ZINC-α_2-GLYCOPROTEIN IN THE RAT

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Running Title: Zinc-α_2-glycoprotein and lipid loss

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Abstract

Zinc-α2-glycoprotein (ZAG) is a new adipokine with the potential to treat obesity. In this study, mature male Wistar rats (540±83g) were administered human recombinant ZAG, (50μg/100g body weight given i.v. daily) for 10 days, while control animals received an equal volume of PBS. Animals treated with ZAG showed a progressive decrease in body weight, without a decrease in food and water intake, but with a 0.4°C rise in body temperature. Body composition analysis showed loss of adipose tissue, but an increase in lean body mass. The loss of fat was due to an increase in lipolysis as shown by a 50% elevation of plasma glycerol, together with increased utilisation, as evidenced by the 55% decrease in plasma levels of non-esterified fatty acids. Plasma levels of glucose and triglycerides were also reduced by 36-37% and there was increased expression of glucose transporter 4 in both skeletal muscle and adipose tissue. Expression of the lipolytic enzymes adipose triglyceride lipase and hormone sensitive lipase in white adipose tissue (WAT) were significantly increased after ZAG administration. There was an increased expression of uncoupling proteins 1 and 3 in brown (BAT) and white adipose tissue (WAT), which would contribute to increased substrate utilisation. Administration of ZAG increased its expression in gastrocnemius muscle, BAT and WAT, which was probably necessary for its biological effect. These results show that ZAG produces increased lipid mobilization and utilization in the rat.

Keywords: Zinc-α_2-glycoprotein; lipolysis; uncoupling proteins; glucose transporter 4; adipose triglyceride lipase; hormone sensitive lipase.

Introduction

Obesity is a major problem of the twenty-first century with up to 25% of adults considered to be obese (21). Our previous studies (11, 25) have identified zinc-α_2-glycoprotein (ZAG), a soluble protein with sequence homology to histocompatibility antigens (1), as a potential treatment for obesity. ZAG was originally isolated as a lipid mobilising factor (LMF) responsible for fat loss in cancer cachexia, and was found to be expressed by tumours that produced a decrease in carcass lipids (29). Subsequent studies showed ZAG to be produced by a range of normal tissues including liver, heart and lung, as well as white (WAT) and brown (BAT) adipose tissue (5), suggesting that it was a new adipokine. Moreover, ZAG expression in liver, WAT and BAT was upregulated during the phase of weight loss in mice bearing a cachexia-inducing tumor, with the largest increase (10-fold) being in WAT (5). Both glucocorticoids and β3 agonists increased expression of ZAG mRNA in 3T3-L1 adipocytes (5), and glucocorticoids are probably responsible for the increase in ZAG expression seen in adipose tissue of cachexic mice (24).

In contrast with cachexia, adipose tissue of obese subjects shows only one-third the expression of ZAG seen in nonobese individuals (6). Recent studies (16) show that the ZAG mRNA level in visceral and subcutaneous fat of human subjects to be negatively correlated with BMI and fat mass, as well as parameters of insulin resistance. Studies using a mouse model of obese type 2 diabetes have also identified ZAG as a possible candidate gene for obesity (8). The effect of ZAG depletion has been studied in mice with inactivation of both ZAG alleles by gene targeting (20). ZAG depletion led to an increase in body weight compared with wild-type mice, especially when the animals were fed a high fat diet, while there was a decreased lipolytic activity in epididymal adipocytes in response to isoprenaline, a β3-agonist,

and agents which increase cyclic AMP (20). These results suggest that loss of ZAG may be responsible for obesity in some individuals.

To date all studies on the lipid mobilising effect of ZAG have been carried out in mice (11, 25), using human ZAG, although the identities in amino acid sequence of ZAG between mouse and human is only 58.6% (30). Since the sequence homology between rat and mouse is 88.5% (30) this suggests that if human ZAG binds to the mouse receptor it should also be effective in the rat. The current study investigates the anti-obesity effect of human ZAG in mature male Wistar rats.

Research methods and procedures

Materials FreeStyle media was purchased from Invitrogen (Paisely, UK). 2-[1-14C]Deoxy-D-glucose (sp.act. 1.85GBq $mmol^{-1}$) was from American Radiolabelled Chemicals (Cardiff, UK). Rabbit polyclonal antibody to total p38MAPK and phospho PLA_2 (Ser-505) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody to full length human ZAG was from Santa Cruz (California, USA) and mouse monoclonal antibody to myosin heavy chain type II was from Novocastra (via Leica Biosytems, Newcastle, UK). Mouse monoclonal antibody to 20S proteasome α-subunits was from Affinity Research Prodcts (Exeter, UK). Mouse monoclonal antibody to phospho (Thr-180/Tyr-182) p38MAPK and rabbit polyclonal antisera to total and phospho (Thr-451) PKR, phospho (Ser-162) eIF2α and to total eIF2α were from New England Biosciences (Herts, UK). Polyclonal rabbit antibodies to UCP1 and UCP3 were from Calbiochem (via Merck Chemicals, Nottingham, UK). Peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse antibodies were purchased from Dako (Cambridge, UK). Polyclonal rabbit antibody to mouse β-actin and the triglyceride assay kit were purchased from Sigma Aldrich (Dorset, UK).

Hybond A nitrocellulose membranes and enhanced chemiluminescene (ECL) development kits were from GE Healthcare (Bucks, UK). A WAKO colorimetric assay kit for measurement of NEFA was purchased from Alpha Laboratories (Hampshire, UK), and a mouse insulin ELISA kit was purchased from DRG (Marburg, Germany). Glucose measurements were made using a plasma glucose assay kit (Boots, Nottingham, UK).

Production of recombinant ZAG HEK293 cells were transfected with full length human ZAG cDNA in the expression vector pcDNA 3.1, and maintained in FreeStyle medium under an atmosphere of 5% CO_2 in air at 37°C. ZAG was secreted into the medium, which was collected, and maximal protein levels (16μgml^{-1}) were obtained after 14 days of culture. To purify ZAG, media (200ml) was centrifuged at 700g for 15min to remove cells, and concentrated into a volume of 1ml sterile PBS using an Amicon Ultra-15 centrifugal filter with a 10kDa cut-off. The concentrate (about 2mg protein) was added to 2g DEAE cellulose suspended in 20ml 10mM Tris, pH 8.8 and stirred for 2h at 4°C. The DEAE cellulose bound ZAG and it was sedimented by centrifugation (1500g for 15min) and the ZAG was eluted by stirring with 20ml 10mM Tris, pH8.8 containing 0.3M NaCl for 30min at 4°C. The eluate was washed and concentrated into a volume of 1ml in sterile PBS using an Amicon centrifugal filter. The purified ZAG was free of endotoxin, as determined with a LAL Pyrogent single test kit (Lonza, Bucks, UK)

Lipolytic activity in rat adipocytes White adipocytes were prepared from finely minced epididymal adipose tissue of male Wistar rats (400g) using collagenase digestion, as described (4). Lipolytic activity was determined by incubating 10^5-

$2x10^5$ adipocytes for 2h in 1ml Krebs-Ringer bicarbonate buffer, pH7.2, and the extent of lipolysis was determined by measuring the glycerol released (33). Spontaneous glycerol release was measured by incubating adipocytes alone. Lipolytic activity was expressed as μmol glycerol released/10^5 adipocytes/2h.

Glucose uptake into adipocytes Isolated adipocytes ($5x10^4$) were washed twice in 1ml Krebs-Ringer bicarbonate buffer, pH7.2 (KRBS) and further incubated for 10min at room temperature in 0.5ml KRBS containing 18.5MBq 2-[1-^{14}C] deoxy-D-glucose and non-radioactive 2-deoxy-D-glucose to a final concentration of 0.1mM. Uptake was terminated by the addition of 1ml ice-cold glucose-free KRBS, and the cells were washed three times with 1ml KRBS, lysed by addition of 0.5ml 1M NaOH, and left for at least 1h at room temperature before the radioactivity was determined by liquid scintillation counting.

Glucose uptake into gastrocnemius muscle Gastrocnemius muscles were incubated in Krebs-Henseleit bicarbonate buffer for 45min at 37°C and then incubated for a further 10min in 5ml Krebs-Henseleit buffer containing 185MBq 2-[1-^{14}C] deoxy-D-glucose and non-radioactive 2-deoxy-D-glucose to a final concentration of 0.1mM. the muscles were then removed and washed in 0.9% NaCl for 5min followed by dissolution in 0.5ml 1MNaOH and the radioactivity was determined by liquid scintillation counting.

Animal studies Mature male Wistar rats (one year old from our own colony) weighing 540±82.5g were housed individually and treated once daily i.v., with either ZAG in PBS (100μl) (50μg per 100g body weight), or with PBS (100μl) as a control. Both

food and water intake and body weight were measured daily. Animals were given free access to food (Special Diet Services, Essex, UK) and water ad libitum. The animal experiment was carried out under the welfare conditions imposed by the British Home Office. After 10 days treatment the animals were terminated and the body composition determined. Animals were heated to 80-90°C for 7 days until constant weight was achieved. The water content was then determined from the difference between the wet and dry weight. Lipids were extracted from the dry carcass using a sequence of chloroform:methanol (1:1), ethanol/acetone (1:1) and diethyl ether (120ml of each) as described by Lundholm et al (14). The solvents were evaporated and the fat weighed. The non-fat carcass mass was calculated as the difference between the initial weight of the carcass and the weight of water and fat.

Western blotting Samples of epididymal WAT, BAT and gastrocnemius muscle excised from rats treated with ZAG or PBS for 5 days were homogenised in 0.25M sucrose, 1mM HEPES, pH 7.0 and 0.2MEDTA, and then centrifuged for 10min at 4,500 r.pm. Samples of cytosolic protein (10μg) were resolved on 12% sodium dodecylsulphate polyacrylamide gel electrophoresis and the proteins were then transferred onto 0.45μm nitrocellulose membranes, which had been blocked with 5% Marvel in Tris-buffered saline, pH7.5, at 4°C overnight, and following four 15 min washes with 0.1% Tween in PBS, incubation with the secondary antibody was performed for 1h at room temperature. Development was by ECL.

Statistical analysis Results were expressed as mean ± s.e.m. Differences were determined by one-way ANOVA, followed by Tukey-Kramer multiple comparison test. P-values less than 0.05 were considered significant.

Results

The lipolytic effect of human ZAG towards rat epididymal adipocytes in comparison with isoprenaline is shown in Figure 1. At concentrations between 233 and 700nM ZAG produced a dose-related increase in glycerol release, which was attenuated by anti-ZAG monoclonal antibody showing the specificity of the action. The extent of lipolysis in rat adipocytes was similar to that previously reported in the mouse (25). As in the mouse the lipolytic effect of ZAG was completely attenuated by the β3-adrenergic receptor (β3-AR) antagonist SR59230A (17), suggesting that the action of ZAG was mediated through β3-AR. These results suggest that ZAG may be effective in inducing fat loss in rats.

The effect of single daily i.v. injection of ZAG (50μg/100g b.w.) on the body weight of mature male Wistar rats (540±83g) is shown in Figure 2A. Compared with control rats administered the same volume of solvent (PBS), rats administered ZAG showed a progressive decrease in body weight, such that after 10 days, while rats treated with PBS showed a 13g increase in body weight, animals treated with ZAG showed a 5g decrease in body weight (Table 1). There was no difference in food (ZAG: 102±32g; PBS:98±25g) or water (ZAG: 135±35ml; PBS: 125±25ml) intake between the two groups during the course of the study, but ZAG-treated animals showed a consistent 0.4°C elevation in body temperature, which was significant within 24h of the first administration of ZAG (Figure 2B), indicating an elevated energy expenditure. Body composition analysis (Table 1) showed that the loss of body weight induced by ZAG was due to a loss of carcass fat, which was partially offset by a significant increase in lean body mass. There was a 50% increase in plasma glycerol concentration in rats treated with ZAG (Table 2), indicative of an increased lipolysis, but a 55% decrease in plasma levels of non-esterified fatty acids

(NEFA), suggesting an increased utilisation. Plasma levels of glucose and triglycerides were also reduced by 36-37% (Table 2), also suggesting an increased utilisation. Plasma levels of insulin fell in proportion to the decrease in blood glucose levels. There was a significant increase in the uptake of 2-deoxygluocse into epididymal adipocytes of rats treated with ZAG for 10days, which was increased in the presence of insulin (Figure 2C). However, there was no significant difference in glucose uptake into adipocytes from ZAG or PBS treated animals in the presence of insulin (Figure 2C). There was a small, non-significant increase in glucose uptake into gastrocnemius muscle and BAT of rats treated with ZAG in comparison with PBS controls, but a significant increase in uptake in the presence of insulin (Figure 2D). These results suggest that the decrease in blood glucose is due to increased utilisation by BAT, WAT and skeletal muscle, and this is supported by an increased expression of glucose transporter 4 (GLUT4) in all three tissues (Figure 3).

ZAG administration increased expression of the uncoupling proteins (UCP)-1 and -3 in both BAT and WAT (Figure 3A and 3B), which would contribute to increased substrate utilisation. In rats treated with ZAG there was also an increased expression of the lipolytic enzymes adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in epididymal adipose tissue (Figure 5). ATGL is mainly responsible for the hydrolysis of the first ester bond in a triacylglycerol molecule forming diacylgylcerol, while its conversion to monacylglycerol is carried out by HSL. Expression of ZAG was also significantly increased in skeletal muscle, (Figure 6A), WAT (Figure 6B) and BAT (Figure 6C) of rats treated with ZAG for 10 days, showing that exogenous ZAG boosts its own production in peripheral tissues.

Discussion

ZAG has been attributed a number of biological roles (10), but its role as an adipokine regulating lipid mobilisation and utilisation is most important in regulating body composition. Our previous studies (11) identified ZAG as a lipid mobilising factor capable of inducing lipolysis in white adipocytes of the mouse in a GTP-dependent process, similar to that induced by lipolytic hormones. This study shows that ZAG has a similar lipolytic effect in rat adipocytes, and, moreover, produces a decrease in body weight and carcass fat in mature male rats, despite the fact that the sequence homology between rat and human ZAG is only 59.4% (30).

The results of this study show that ZAG administration to the rat also increases the expression of ATGL and HSL in the rat. ATGL may be important in excess fat storage in obesity, since ATGL knockout mice have large fat deposits and reduced free fatty acids release from WAT in response to isoproterenol although they did display normal insulin sensitivity (9). In contrast HSL null mice, when fed a normal diet, had body weights similar to wild-type animals (18). However, expression of both ATGL and HSL are reduced in human WAT in the obese insulin-resistant state compared with the insulin sensitive state, and weight reduction also decreased mRNA and protein levels (13).

Stimulation of lipolysis alone would not deplete body fat stores, since without an energy sink the liberated NEFA would be resynthesised back into triglycerides in adipocytes (9). To reduce body fat ZAG not only increases lipolysis, as shown by an increase in plasma glycerol, but also increases lipid utilisation, as shown by the decrease in plasma levels of triglycerides and NEFA. This energy is channelled into heat, as evidenced by the 0.4°C rise in body temperature in rats treated with ZAG. The increased energy utilisation most likely arises from the increased expression of

UCP1, which has been shown in both BAT and WAT after administration of ZAG. An increased expression of UCP1 would be expected to decrease plasma levels of NEFA, since they are the primary substrates for thermogenesis in BAT (28). BAT also has a high capacity for glucose utilisation (15), which could partially explain the decrease in blood glucose. In addition there was increased expression of GLUT4 in skeletal muscle and WAT, which helps mediate the increase in glucose uptake in the presence of insulin (27). In mice treated with ZAG there was an increased glucose utilisation by brain, heart, BAT and gastrocnemius muscle, and increased production of $^{14}CO_2$ from D-[U-^{14}C] glucose, as well as [^{14}C carboxy] triolein (23). There was also a three-fold increase in oxygen uptake by BAT of ob/ob mice after ZAG administration (11).

Induction of lipolysis in rat adipocytes by ZAG is suggested to be mediated through a β3-AR, and the effect of ZAG on adipose tissue and lean body mass may also be due to its ability to stimulate the β3-AR (22). Induction of UCP1 expression by ZAG has been shown to be mediated through interaction with a β3-AR (26). The increased expression of UCP1 in WAT may also be a β3-AR effect through remodelling of brown adipocyte precursors, as occurs with the β3-AR agonist CL316,243 (7). Using knock-out mice the antiobesity effect of β3-AR stimulation has been mainly attributed to UCP1 in BAT, and less to UCP2 and UCP3 through the UCP1-dependent degradation of NEFA released from WAT (12). Glucose uptake into peripheral tissues of animals is stimulated by cold-exposure, an effect also mediated through the β3-AR (31). However, targeting the β3-AR has been more difficult in humans than in rodents, since β3-AR play a less prominent role than β1 and β2-AR subtypes in the control of lipolysis and nutritive blood flow in human subcutaneous abdominal adipose tissue (2). However, despite this the β3-AR agonist

CL316,243 has been shown to increase fat oxidation in healthy young male volunteers (32). This may be due to the ability of β-adrenergic agonists to increase the number of β3-AR in plasma membranes from BAT (32).

Recent results (16) suggest that ZAG expression in adipose tissue may be more important locally than circulating ZAG, by acting in a paracrine manner. Thus in humans, while mRNA levels of ZAG in visceral and subcutaneous fat correlated negatively with BMI, fat mass and insulin resistance (16), serum levels, determined by ELISA, correlated positively with parameters of adiposity (BMI and waist circumference) and insulin resistance (31). Thus the ability of ZAG to induce its own expression in gastrocnemius muscle, WAT and BAT may be critical for its ability to increase lipolysis and energy utilisation.

These results provide evidence for the ability of ZAG to mobilise and utilise lipid in rats, confirming a species independent effect, and suggest that it may be useful as an antiobesity agent in man.

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Figure Legends

Figure 1 Lipolytic activity of human ZAG in isolated rat epididymal adipocytes, compared with isoprenaline (10μM) in the absence or presence of SR59230A (10μM) or anti-ZAG antibody (1:1000) (IgG). Each value is an average of 5 separate studies. Differences from control are shown as b, $p<0.01$ or c, $p<0.001$, while differences from ZAG alone are indicated as e, $p<0.01$ or f, $p<0.001$.

Figure 2 (A) Effect of daily i.v. administration of either ZAG (50μg/100g b.w.) in 100μl PBS (■) or PBS alone (◆) on body weight of male Wistar rats over a 10 day period. The protocol for the experiment is given in the methods section. (B) Body temperature of male Wistar rats administered either ZAG (■) or PBS (◆) as described in (A). (C) Uptake of 2-deoxy-D-glucose into epididymal adipocytes of male Wistar rats after 10 days treatment with either ZAG or PBS for 10 days, as shown in (A), in the absence (closed box) or presence (open box) of insulin (60μU/ml). (D) Glucose uptake into gastrocnemius muscle and BAT of male Wistar rats after 10 days treatment with either ZAG (closed box) or PBS (open box), in the absence or presence of insulin (60μU/ml). Differences between ZAG and PBS treated animals are shown as b, $p<0.01$ or c, $p<0.001$, while differences in the presence of insulin are shown as d, $p<0.05$ or f, $p<0.001$.

Figure 3 Western blots showing expression of GLUT4 in BAT (A) and WAT (B) and gastrocnemius muscle (C) of male Wistar rats treated with either PBS or ZAG for 10 days as shown in Figure 2.

Figure 4 Western blots showing expression of UCP1 and UCP3 in BAT (A) and WAT (B) of male Wistar rats treated with either PBS or ZAG for 10 days as shown in Figure 2.

Figure 5 Expression of ATGL (A) and HSL (B), detected by Western blotting, in epididymal adipose tissue of male Wistar rats treated with either PBS or ZAG for 10 days as shown in Figure 2.

Figure 6 Expression of ZAG in gastrocnemius muscle (A), WAT (B) and BAT (C), detected by Western blotting of tissues excised from male Wistar rats treated with either PBS or ZAG for 10 days as shown in Figure 2.

Table 1 Body composition of male rats after treatment with either PBS or ZAG

Treatment	Starting weight (g)	Final weight (g)	Weight change (g)	Water		Fat		Non fat	
				(g)	(%)	(g)	(%)	(g)	(%)
PBS	510±30	523±2	+13±3	326±32	62±2	105±14	20±3	90±6	17±3
ZAG	530±45	525±1	-5±1	331±5	63±3	92±5[b]	18±1	96±2[a]	18±2

Differences from animals treated with PBS are shown as a, $p<0.05$ or b, $p<0.01$

Table 2 Plasma metabolite and insulin levels in rats treated with either PBS or ZAG for 10 days

	PBS	ZAG
Glucose mmol/l	25.5±2.3	16.2±2.1[c]
Trigylcerides mmol/l	1.75±0.01	1.1±0.09[a]
Glycerol umol/l	300±52	450±51[c]
NEFA mEq/l	0.58±0.008	0.26±0.06[b]

Differences from animals treated with PBs are shown as either a, $p<0.05$; b, $p<0.01$ or c, $p<0.001$