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MECHANISM OF THE ANTI-OBESITY EFFECT OF ZINC-α_2-GLYCOPROTEIN IN THE OB/OB MOUSE

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Abstract

The mechanism of the antiobesity effect of zinc-α_2-glycoprotein (ZAG) has been investigated in the ob/ob mouse. The lipolytic effect of both isoproterenol and ZAG was lower in epididymal adipocytes from ob/ob than lean mice, but at higher concentrations of ZAG (>0.23μM) there was no differences between the groups. There was a lower lipolytic response by adipocytes from subcutaneous and visceral deposits, but there was no difference between isoproterenol and ZAG, and no difference between lean and ob/ob mice. ZAG increased expression of hormone sensitive lipase (HSL) in isolated epididymal adipocytes after 3h incubation, and this was completely attenuated by PD98059, an inhibitor of the extracellular signal-regulated kinase (ERK) pathway. Treatment of ob/ob mice with ZAG for 5 days increased ZAG expression in epididymal, subcutaneous and visceral adipose tissue about two-fold, and this remained elevated in tissue culture in the absence of ZAG for a further 3 days. Expression of HSL and adipose triglyceride lipase (ATGL) was also elevated after ZAG administration, but only in epididymal adipose tissue, as was ERK. The increased expression of HSL correlated with the increased lipolytic response to ZAG, and the β3-adrenergic receptor (β3-AR) agonist BRL37344, suggesting that ZAG may act synergistically with β3-AR agonists to mobilise lipids. This was strengthened by the observation that ZAG increased the expression of the β3-AR in brown (BAT) and white adipose tissue (WAT), as well as skeletal muscle. There was also an increased expression of uncoupling protein 1 (UCP-1) in both BAT and WAT, which would provide an energy sink for the fatty acids released during lipolysis. These results suggest that ZAG may overcome some of the metabolic alterations associated with obesity.

Keywords: zinc-α_2-glycoprotein; lipolysis; β3-adrenoreceptor; hormone sensitive lipase

Introduction

Obesity and its associated health effects is a major problem for the Western World, and is thought to arise through both genetic and environmental influences (32). Obesity has been linked with insulin resistance and type 2 diabetes through release from adipocytes of non-esterified fatty acids (NEFA), glycerol and pro-inflammatory cytokines (25). The current approach to management involves lifestyle alteration combined with pharmacological intervention, although the options for treatment are limited.

We have investigated the potential use of zinc-α_2-glycoprotein (ZAG) for the treatment of obesity using ex-breeder male NMRI mice (36). ZAG was initially identified as the lipid-mobilizing factor (LMF) associated with loss of adipose tissue in cancer cachexia (19), and was shown to be tumor-derived (41). However, later studies showed that ZAG was produced by a range of normal tissues including both white (WAT) and brown (BAT) adipose tissue (7), with major increases in expression in mice bearing a cachexia-inducing tumor, which induced loss of fat mass. In contrast with cachexia expression of ZAG in WAT is low in obese human subjects (11) and correlated negatively with body weight, BMI, fat mass, waist and hip circumference, as well as plasma insulin levels (15, 30). The expression level of ZAG may be responsible for some of the effects of obesity, since ZAG 'knock-out' animals gain more weight, especially on a high fat diet, while adipocytes from these animals showed a decreased lipolytic response to various agents including catecholamines, β3-adrenoreceptor (β3-AR) agonists and agents which increase cyclic AMP (31). In contrast overexpression of ZAG in mice was associated with a reduced body weight and percentage of epididymal fat when they were fed a high fat diet (15). A reduced catecholamine-induced lipolysis and fat oxidation is seen in obese individuals (23),

and in first-degree relatives of obese subjects (18), and may play a role in the development and maintenance of the increased fat stores. A reduced hormone-sensitive lipase (HSL) expression is the best characterized defect contributing to this resistance to catecholamines (22). ZAG has been suggested as a possible candidate gene for obesity using the KK/Ta mouse as an animal model of spontaneous type 2 diabetes (14).

ZAG has been shown to induce loss of adipose tissue through a lipolytic effect on WAT, combined with an increased expression of uncoupling protein-1 (UCP-1) in BAT, which would result in an increase in energy expenditure (36). The lipolytic effect arises from activation of adenylyl cyclase to produce cyclic AMP in a GTP-dependent process (19), which is attenuated by the specific β3-AR antagonist SR59230A (33), suggesting that it is mediated through the β3-AR. This study evaluates the mechanism by which ZAG could reduce fat mass in obese subjects using the ob/ob mouse as an experimental model.

RESEARCH DESIGN AND METHODS

Materials Freestyle media and RPMI 1640 were purchased from Invitrogen (Paisley, UK) and fetal calf serum was from Biosera (Sussex, UK). Rabbit polyclonal antibodies to phospho (Thr-202) and total ERK1, phospho (Ser-552) HSL and phospho (Ser-563) adipose triglyceride lipase (ATGL), and chicken polyclonal antibody to β3-adrenergic receptors (β3-AR) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody to human ZAG and peroxidase-conjugated goat anti-chicken antibody were from Santa Cruz (California, USA). Polyclonal rabbit antibodies to UCP1 and UCP3 and Phosphosafe™ Extraction Reagent 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, PD98059, BRL 37344, endotoxin standard, endotoxin free water and the triglyceride assay kit were purchased from Sigma Aldrich (Dorset, UK). Hybond A nitrocellulose membranes and enhanced chemiluminescence (ECL) development kits were from GE Healthcare (Bucks, UK). A WAKO colorimetric assay kit for NEFA was purchased from Alpha Laboratories (Hampshire, UK), and a mouse insulin ELISA kit was purchased from DRG (Marburg, Germany). Endotoxin was measured with a LAL Pyrogent single test kit from Lonza (Bucks, UK). Glucose measurements were made using a Boots (Nottingham, UK) plasma glucose kit.

Animals Obese hyperglycaemic (ob/ob) mice (average weight 90g) were bred in our own colony and the origin and characteristics of these animals have been described in detail previously (2). Expression of the ob gene on this background produces a more severe form of diabetes than C57BL/6J ob/ob mice. Mice were housed in a air-

conditioned room at 22 ± 2°C and fed ad libitum a rat and mouse breeding diet (Special Diet Services, Witham, UK) and tap water. Male mice (20-21 weeks old) were grouped into three per cage and administered ZAG (35μg daily) by i.v. administration. Both body weight and food and water intake were monitored daily, as was body temperature, with use of a rectal thermometer (RS Components, Northants, UK).

Production and purification of recombinant human ZAG Human HEK 293F cells were transfected with the mammalian cell expression vector pcDNA 3.1, containing human ZAG, and selected for growth in neomycin (50μg/ml) in Freestyle medium under an atmosphere of 5% CO_2 in air at 37°C. Protein levels in the culture medium increased progressively with time reaching plateau levels within about 2 weeks of seeding. Cells were removed by centrifugation at 700g for 15min, and the medium (200ml) was concentrated into a volume of 1ml of sterile PBS using an Amicon Ultra-15 centrifugal filter with a cut-off of 10kDa. The concentrate (containing about 2mg protein) was then added to DEAE cellulose (2g) suspended in 20ml 10mM Tris, pH8.8, and stirred at 4°C for 2h. ZAG bound to the DEAE cellulose, which was sedimented by centrifugation (1500g for 15min), and was eluted by stirring for 30min at 4°C with 20ml 10mM Tris, pH8.8, containing 0.3M NaCl. After sedimentation the supernatant fluid, containing ZAG, was concentrated to a volume of 1ml in sterile PBS using the Amicon centrifugal filter. The ZAG was free of endotoxin as determined by a LAL Pyrogent single test kit (Lonza, Bucks, UK). The purity of the recombinant ZAG has previously been reported (34).

Preparation of human and murine adipocytes Adipose tissue was minced into small fragments and digested in Krebs-Ringer bicarbonate containing 1g/L collagenase and 4% bovine serum albumin under an atmosphere of 95% oxygen : 5% CO_2 at 37°C, as described (5). After 30min the adipocytes were filtered through nylon mesh (pore size 250µm), centrifuged at 500g for 2min, and washed three times with PBS before suspension in RMPI 1640 medium containing 10% FCS and maintained under an atmosphere of 5% CO_2 in air at 37°C. The culture medium was replaced daily.

Lipolytic assay For lipolytic assays 10^5-$2x10^5$ adipocytes were incubated with the lipolytic agent for 2h in 1ml Krebs-Ringer bicarbonate buffer, pH 7.2, and the extent of lipolysis was determined by measuring glycerol released (42). Control samples containing adipocytes alone were analysed to determine the spontaneous glycerol release.

Western blot analysis Freshly excised WAT, BAT and gastrocnemius muscle were washed in PBS and lysed in Phosphosafe™ Extraction Reagent for 5min at room temperature, followed by sonication at 4°C. Samples of cytosolic protein formed by centrifugation at 18,000g for 5min at 4°C were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 180V for approximately 1h, and transferred to 0.45µm nitrocellulose membranes, which were blocked with 5% Marvel in Tris-buffered saline, pH 7.5, at 4°C overnight. 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.

Statistical analysis 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

Human recombinant ZAG induced lipolysis in murine epididymal adipocytes in a dose-related manner (Fig. 1A). For isoproterenol and low concentrations of ZAG (up to 0.23μM) the lipolytic effect was reduced in adipocytes from obese (ob/ob) mice, although at higher concentrations of ZAG there was no significant difference in the extent of lipolysis between lean and obese animals. The lipolytic response to both isoproterenol and ZAG was significantly less in adipocytes from both subcutaneous and visceral deposits (Fig. 1B), although there was no significant difference in response of adipocytes from obese and non-obese animals. Freshly isolated human subcutaneous adipocytes also showed lipolysis in the presence of ZAG (Fig. 1C) and isoproterenol, and the lipolytic response was comparable with murine adipocytes (Fig. 1B). The lipolytic response of human adipocytes to ZAG was comparable with that for isoproterenol (Fig. 1C). To determine the mechanism for the increased lipolysis by ZAG, adipocytes from non-obese animals were incubated with ZAG for 3h and the expression of HSL was determined by Western blotting (Fig. 1D). ZAG, but not isoproterenol increased the expression of phospho HSL, and this was completely attenuated by the selective and cell permeable inhibitor of mitogen activated protein kinase kinase (MAPKK) PD98059 (10μM) (26). It is known that the extracellular signal-regulated kinase (ERK) pathway increases lipolysis and phosphorylates HSL at Ser^{600} (16). Indeed, lipolysis induced in isolated adipocytes by ZAG and to a lesser extent isoproterenol was attenuated by PD98059, although this did not return to basal values (Fig. 1E).

Treatment of ob/ob mice with ZAG produced a decrease in body weight over a 5 day period (Fig 2A) due to loss of adipose tissue and this was associated with a 0.4°C increase in body temperature, (Fig. 2B), as previously reported (34) (Fig. 2A).

After 5 days treatment with ZAG, ZAG expression in epididymal, subcutaneous and visceral deposits was increased about two-fold compared with PBS controls (Fig. 2C). Moreover ZAG expression remained elevated in adipocytes in tissue culture in the absence of ZAG for a further 3 days (Fig. 2D). As in vitro experiments had shown (Fig. 1D) ZAG administration to ob/ob mice also caused an increased expression of phospho HSL in epididymal adipocytes, and this also remained elevated in tissue culture in the absence of ZAG for 3 days (Fig. 2E). In addition adipocytes from ZAG treated mice showed an increased response to the lipolytic action of isoproterenol, and this was retained for 3 days when the adipocytes were maintained in tissue culture in the absence of ZAG (Fig. 2F).

There was a differential response to ZAG in the different adipose depots. Thus expression of both phospho HSL (Fig. 3A) and adipose triglyceride lipase (ATGL) (Fig. 3B) was significantly upregulated by ZAG in epididymal (ep), but not in subcutaneous (sc) or visceral (vis) adipose tissue. This correlated with expression of the active (phospho) form of ERK, which showed selective upregulation only in epididymal adipose tissue (Fig. 3C). This, together with the data in Fig. 1D, suggests that ERK may be responsible for the increase in phospho HSL. The increased expression of HSL and ATGL in epididymal adipocytes would correlate with the increased lipolytic response to ZAG (Fig. 1A) compared with subcutaneous and visceral adipocytes (Fig. 1B). This effect is also seen with the β3-adrenergic receptor (β3-AR) agonist, BRL 37344 (Fig. 4A), which caused an increased stimulation of lipolysis in epididymal adipocytes from ZAG-treated animals, while in subcutaneous and visceral adipocytes pretreatment with ZAG had no effect on the lipolytic response. These results suggest that ZAG may act synergistically with β3-AR agonists to mobilise lipids. The sensitisation of adipocytes to BRL 37344 was seen

even in short-term culture after 2h incubation with ZAG, but not with isoproterenol (Fig. 4B). The sensitizing effect may arise from the ability of ZAG to induce expression of the β3-AR in BAT, WAT and gastrocnemius muscle (Fig. 4C).

ZAG has been shown to produce upregulation of the expression of UCP1 in a process mediated through a β3-AR (37). 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. 5A) and WAT (Fig. 5B) after ZAG administration. In vitro experiments have shown that induction of expression of UCP3 by ZAG was attenuated by PD98059, suggesting the involvement of MAPK (37). The increase in expression of ERK in WAT in ZAG-treated mice would therefore be expected to lead to an increase in expression of UCP3 as observed (Fig. 5D). ZAG also produced an increase in expression of UCP3 in BAT (Fig. 5C). The increased expression of UCP's would provide a sink for the NEFA released from adipose tissue and generate heat, as previously observed (34), since NEFA are the principal substrates for thermogenesis in BAT (39).

Discussion

This study shows ZAG to be as efficient as isoproterenol in inducing lipolysis in WAT, although its effectiveness against adipocytes from subcutaneous and visceral depots was less than that in epididymal adipocytes, as was also observed with isoproterenol. The decreased lipolytic effect of catecholamines and β-AR agonists towards subcutaneous and visceral adipocytes has been previously reported (28), and may be due to differences in number of β3-AR (40). However, epdidymal adipocytes show a more marked reduction in lipolysis after isoproterenol pretreatment than those from subcutaneous fat (28). In addition subcutaneous adipocytes possess a lower steady-state level of mRNA for HSL consistent with the reduced lipolysis rate (40). Differences in expression of HSL could explain the lower lipolytic response of epididymal adipocytes from ob/ob mice to both isoproterenol and ZAG. Treatment of epididymal adipocytes with ZAG, but not isoproterenol, increased expression of HSL, although both would be expected to increase intracellular cyclic AMP. Previous studies have shown that catecholamines do not increase expression of HSL, but instead translocate it to its substrate on the surfaces of lipid droplets in fat cells (29). A recent study (15) has reported an increased expression of HSL mRNA in epididymal adipose tissue in mice overexpressing ZAG. Activation of ERK is required for the induction of phospho HSL by ZAG, since it was attenuated by the specific inhibitor PD98059. In addition expression of HSL was increased in epididymal adipose tissue of ob/ob mice administered ZAG, but not in visceral or subcutaneous adipose tissue, and this correlated with expression of phosphor ERK. Mice lacking MAPK phosphatase-1 have increased activities of ERK and p38MAPK in WAT, and are resistance to diet-induced obesity, due to enhanced energy expenditure (43).

HSL was initially considered to be the rate-limiting enzyme for lipolysis, but recent data (17) suggests that ATGL may be rate-limiting. As with HSL (22) levels of ATGL in subcutaneous adipose tissue of obese subjects has been shown to be reduced despite an increase in mRNA expression (38), although other studies (6, 24) report a decrease in both HSL and ATGL mRNA and protein. There is a significant correlation between mRNA expression of ATGL and HSL in both visceral and subcutaneous adipose tissue suggesting a common regulatory mechanism for their expression (6, 24). This may be related to activation of the ERK pathway, since like HSL, expression of ATGL was only increased in epididymal adipose tissue of ZAG treated mice in which ERK was activated. The lack of induction of HSL and ATGL in visceral and subcutaneous adipose deposits could be due to lower levels of the β3-AR (40). Both insulin resistance and hyperinsulinemnia in obese subjects have been shown to be negatively correlated with ATGL and HSL protein expression independent of fat mass (24). Thus the ability of ZAG to increase expression of both HSL and ATGL would correlate with its ability to attenuate insulin resistance (34).

Treatment of ob/ob mice with ZAG also increased expression of ZAG in epididymal, subcutaneous and visceral adipose tissue. Although expression of ZAG is low in obesity (11, 15, 30) its expression in WAT has been shown to be increased 10-fold in mice with cachexia (7). This has been shown to be due to an increase in serum cortisol (35), while TNF-α has been shown to result in a 4-fold decrease in ZAG expression in human SGBS adipocytes (3), providing a potential mechanism to explain the low levels of ZAG found in adipose tissue of obese subjects (11, 15, 30). Induction of ZAG in 3T3-L1 adipocytes by administration of exogenous ZAG has been shown to be attenuated by the selective β3-AR antagonist SR59230A, suggesting that it is mediated through a β3-AR (35).

If this is so, then repeated administration of ZAG might lead to an increase in expression, since ZAG has been shown to increase expression of the β3-AR in BAT, WAT and skeletal muscle. This effect is also seen with β3-AR agonists. Thus chronic treatment of ob/ob mice with the β3-AR agonist BRL 35135 resulted in a two- fold increase in β3-AR mRNA in BAT (1). Similar effects were reported with the b3-AR agonist CL316,243 in Zucker fa/fa rats (13) and in adipocytes of adult humans (8). Using knock-out mice the antiobesity effect of b3-AR stimulation has been through the UCP 1-dependent degradation of fatty acids released from WAT (20). The ability of ZAG to induce expression of the β3-AR would enhance its effect on obesity and diabetes. This may be important for ZAG to exert a therapeutic effect in humans where β3-AR play a weaker role in the control of lipolysis than in the mouse (4). The presence of brown adipocytes has recently been reported in adult humans (10), suggesting that the same process may be operative.

ZAG may be necessary for optimal β3-AR action, since ZAG knock-out mice showed a lower response to the specific β3-AR agonist CL316243 (31). Since ZAG levels are low in obesity (11, 15, 30), the expression of β3-AR may also be suboptimal. Thus β3-AR agonists may require ZAG for optimal activity, as evidenced by the increased lipolytic effect of both isoprenaline and BRL 37344 in adipocytes from ob/ob mice treated with ZAG. In addition many of the effects of ZAG in diabetes in this model (34) possibly related to its β3-AR agonist activity (27). β3-AR agonists induce lipolysis in WAT, both through the classical cyclic AMP and PKA pathway, and through the ERK pathway, which accounts for between 15 and 25% of total lipolysis (16).

Previous studies (37) have suggested a role for the β3-AR in the induction of UCP1 expression in BAT by ZAG. β3-AR agonists have been shown to induce upregulation of UCP1 in BAT through stimulation of p38MAPK downstream of cyclic AMP/protein kinase A leading to activation (phosphorylation) of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 (PGC-1α), as well as ATF-2, allowing the CRE and PPAR elements of the UCP1 enhancer to be occupied (9).

Adipocytes from obese mice also express two-fold lower levels of Gαs, a stimulatory subunit of the GTP-binding protein, which stimulates adenylyl cyclase (12). ZAG has been shown to increase the expression of Gαs and decrease the expression of the inhibitory G-protein, Gαi, in 3T3 adipocytes (21), suggesting a mechanism by which ZAG could increase lipolytic responsiveness, in addition to the induction of HSL and ATGL. These results suggest that ZAG may overcome some of the metabolic alterations associated with the obese state.

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References

1. **Arbeeny CM, Meyers DS, Hillyer DE, Bergquist KE**. Metabolic alterations associated with the antidiabetic effect of β3-adrenergic receptor agonists in obese mice. *Am J Physiol Endocrinol Metab* 268: E678-E684, 1995.
2. **Bailey CJ, Flatt PR, Atkins TW**. Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. *Int J Obes* 6:11-21, 1982.
3. **Bao Y, Bing C, Hunter L, Jenkins JR, Wabitsch M, Trayhurn P**. Zinc-α_2-glycoprotein, a lipid mobilizing factor, is expressed and secreted by human (SGBS) adipocytes. *FEBS Lett* 579: 41-47, 2005.
4. **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 subcutaneous adipose tissue. *Br J Pharmacol* 117: 907-913, 1996.
5. **Beck SA, Tisdale MJ**. Production of lipolytic and proteolytic factors by a murine tumor-producing cachexia in the host. *Cancer Res* 47: 5919-5923, 1987.
6. **Berndt J, Kralisch S, Kloting N, Ruschke K, Kern M, Fasshauer M, Schon MR, Stumvoll M, Beüher M.** Adipose triglyceride lipase gene expression in human visceral obesity. *Exp Clin Endocrinol Diabetes* 116: 203-210, 2008.
7. **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 upregulated in mice with cancer cachexia. *Proc Natl Acad Sci USA* 101: 2500-2505, 2004.

8. **Champigny O, 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* 37: 1907-1914, 1996.
9. **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* 18: 2123-2131, 2004.
10. **Cypress AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng Y-H, Doria A, Koloday GM, Kahn CR**. Identification and importance of brown adipose tissue in adult humans. *N Eng J Med* 360: 1509-1517, 2009.
11. **Dahlman I, Kaaman M, Olsson T, Tan GD, Bickerton AST, Wåhlén K, Andersson J, Nordström EA, Blomqvist L, Sjogren A, Forsgren M, Attersand A, Arner P**. A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *J Clin Endocrinol Metab* 90: 5834-5840, 2005.
12. **Gettys TW, Ramkumar V, Uhing RJ, Singer L, Taylor IL**. Alterations in mRNA levels, expression and function of GTP-binding regulatory proteins in adipocytes from obese mice (C57BL/6J-ob/ob). *J Biol Chem* 266: 15949-15955, 1991.
13. **Ghorbani M, 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* 21: 465-475, 1997.

14. **Gohda T, Makita Y, Shike T, Tanimoto M, Funabiki K, Horikoshi S, Tomino Y**. Identification of epistatic interaction involved in obesity using the KK/Ta mouse as a type 2 diabetes model. Is Zn-α_2-glycoprotein-1 a candidate gene for obesity? *Diabetes* 52: 2175-2181, 2003.

15. **Gong F-Y, Zhang S-J, Ding J-Y, Zhu H-J, Pan H, Li N-S, Shi Y-F**. Zinc-α_2-glycoprotein is involved in regulation of body weight through inhibition of lipogenic enzymes in adipose tissue. Int J Obes 33: 1023-1030, 2009.

16. **Greenberg AS, Shen WJ, Muliro K, Patel S, Sruza SC, Roth RA, Kraemer FB**. Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-related kinase pathway. *J Biol Chem* 276: 45456-45461, 2001.

17. **Haemmerle G, Lass A, Zimmerman R, Gorkiewicz G, Meyer C, Rozman J, Heldmaier G, Maier R, Theussl C, Eder S, Krathy D, Wagner EF, Klingenspor M, Hoefler G, Zechner R**. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312: 734-737, 2006.

18. **Hellstrom L, Langin D, Reynisdottir S, Dauzats M, Arner P**. Adipocyte lipolysis in normal weight subjects with obesity among first-degree relatives. *Diabetologica* 39: 921-928, 1996.

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

20. **Inokuma K, Okamatsu-Ogura Y, Omachi A, Matsushita Y, Kimura K, Yamashita H, Saito M**. Indispensable role of mitochondrial UCP1 for

the antiobesity effect of β3-adrenergic stimulation. *Am J Physiol Endocrinol Metab* 290: E1014-E1021, 2006.

21. **Islam-Ali B, Khan S, Price SA, Tisdale MJ**. Modulation of adipocyte G-protein expression in cancer cachexia by a lipid-mobilizing factor (LMF). *Br J Cancer* 85: 758-762, 2001.
22. **Jocken JWE, Blaak EE**. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. *Physiol Behav* 94: 219-230, 2008.
23. **Jocken JWE, Goosens GH, van Hees AMJ, Frayn KN, van Baak M, Stegen J, Pakbiers MTW, Saris WHM, Blaak EE**. Effect of beta-adrenergic stimulation on whole-body and abdominal subcutaneous adipose tissue lipolysis in lean and obese men. *Diabetologica* 51: 320-327, 2008.
24. **Jocken JWE, Langin D, Smit E, Saris WHM, Valle C, Hul GB, Holm C, Arner P, Blaak EE**. Adipose triglyceride lipase and hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. *J Clin Endocrinol Metab* 92: 2292-2299, 2007.
25. **Kahn SE, Hull RL, Utzschneider KM**. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444: 840-846, 2006.
26. **Kultz D, Madhany S, Burg MB**. Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmo-sensing via stress-associated protein kinase 2. *J Biol Chem* 273:13645-13651, 1998.
27. **Liu X, Perusse F, Bukowiecki LJ**. Mechanisms of the antidiabetic effects of the β3-adrenergic agonist CL-316243 in obese Zucker-ZDF rats. *Am J Physiol Renal Physiol* 274: R1212-R1219, 1998.

28. **Mori S, Nojiri H, Yoshizuka N, Takema Y**. Rapid desensitization of lipolysis in visceral and subcutaneous adipocytes of rats. *Lipids* 42: 307-314, 2007.

29. **Morimoto C, Kameda K, Tsujita T, Okuda H**. Relationships between lipolysis induced by various lipolytic agents and hormone-sensitive lipase in rat fat cells. *J Lipid Res* 42: 120-127, 2001.

30. **Mracek T, Ding Q, Tzanavari T, Kos K, Pinkney J, Wilding J, Trayhurn P, Bing C**. The adipokine zinc-α_2-glcyoprotein is downregulated with fat mass expansion in obesity. *Clin Endocrinol* doi. 10.1111/j.1365-2265.2009.03658.x.

31. **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 1 zinc-α2-glycoprotein deficient mice. *FEBS Lett* 581: 394-400, 2007.

32. **Rössner S**. Obesity: The disease of the twenty-first century. *Int J Obes Relat Metab Disord* 26 (sup 4): S2-S4, 2002.

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

34. **Russell ST, Tisdale MJ**. Antidiabetic properties of zinc-α_2-glycoprotein (ZAG) in ob/ob mice. *Endocrinol*, in press 2010.

35. **Russell ST, Tisdale MJ**. The role of glucocorticoids in the induction of zinc-α_2-glycoprotein expression in adipose tissue in cancer cachexia. *Br J Cancer* 92: 876-881, 2005.

36. **Russell ST, Zimmerman TP, Domin BA, Tisdale MJ**. Induction of lipolysis in vitro and loss of body fat in vivo by zinc-α_2-glycoprotein. *Biochim Biophys Acta* 1636: 59-68, 2004.

37. **Sanders PM, Tisdale MJ**. Effect of zinc-α_2-glycoprotein (ZAG) on expression of uncoupling proteins in skeletal muscle and adipose tissue. *Cancer Lett* 212: 71-81, 2004.

38. **Steinburg GR, Kemp BE, Watt MJ.** Adipocyte triglyceride lipase expression in human obesity. *Am J Physiol Endocrinol Metab* 293: E958-E964, 2007.

39. **Susulic VS, Frederick RC, Lawitts J, Tozze E, Kahn BB, Harper ME, Himms-Hagen J, Flier JS, Lowell BB**. Targeted disruption of the β3-adrenergic receptor gene. *J Biol Chem* 270: 29483-29492, 1995.

40. **Tavernier G, Galitzky J, Valet P, Remaury A, Bouloumie A, Lafontan M, Langin D**. Molecular mechanisms underlying regional variations of catecholamine-induced lipolysis in rat adipocytes. *Am J Physiol Endocrinol Metab* 31: E1135-E1142, 1995.

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

42. **Wieland O**. Glycerol UV method in *Methods of Enzymatic Analysis* (ed. Bergemeyer HU) London, UK, Academic Press, pp1404-1409, 1974.

43. **Wu JJ, Roth RJ, Anderson EJ, Hong E-G, Lee M-K, Choi CS, Neufer PD, Shulman GI, Kim JK, Bennett AM**. Mice lacking MAP kinase phosphatase-1 have enhanced MAP kinase activity and resistance to diet-induced obesity. *Cell Metabolism* 4:61-73, 2006.

Figure Legends

Fig. 1 Lipolytic activity of ZAG in adipocytes from lean and ob/ob mice, human adipocytes and effect of ZAG on expression of HSL. (**A**) Induction of lipolysis in murine epididymal adipocytes from lean (■) and ob/ob (□) mice by isoproterenol or ZAG at the concentrations indicated over a 2h period. Differences from lean animals are shown as a, $p<0.05$; b, $p<0.01$ or c, $p<0.001$. (**B**) Lipolysis in adipocytes from subcutaneous (sc) and visceral (vis) deposits from lean and ob/ob mice in the presence of isoproterenol (10μM; □) or ZAG (0.58μM; ▨) compared with basal levels (■). Differences from epididymal adipocytes in (A) are shown as b, $p<0.01$. (**C**) Lipolysis in human sc adipocytes in response to isoproterenol or ZAG over a 2h time period. Differences from control are shown as b, $p<0.01$, or c, $p<0.001$. (**D**) Western blot showing expression of phospho HSL in mouse epididymal adipocytes after incubation for 3h with isoproterenol (10μM) or ZAG (0.58μM), alone, or in the presence of PD 98059 (10μM). Actin was used as a loading control. The densitometric analysis is an average of three separate Western blots and is expressed as a percentage of control in the absence of stimulation. (**E**) Lipolytic effect of isoproterenol (10μM) and ZAG (0.58μM) in the absence and presence of PD98059 (10μM). Differences from control are shown as c, $p<0.001$, while differences in the presence of PD98059 are shown as e, $p<0.01$ or f, $p<0.001$.

Fig. 2 Effect of ZAG on body weight of ob/ob mice and expression of ZAG and HSL in adipose tissue. (**A**) Effect of ZAG (35μg; iv; ■) or PBS (◆) on body weight of ob/ob mice over 5 days. (**B**) Rectal temperature of ob/ob mice administered PBS (■) or ZAG (□). (**C**) Western blot showing expression of

ZAG in epididymal (ep), subcutaneous (sc) and visceral (vis) adipose tissue of ob/ob mice after 5days continuous administration (35μg; iv/day). Actin served as a loading control. (**D**) Adipocytes (ep) were removed from mice at the end of 5days treatment (day 0) and maintained in RPMI 1640 medium containing 10% fetal calf serum in the absence of ZAG for a further 4 days. ZAG expression was determined by Western blotting. (**E**) Western blot showing expression of phospho HSL in ep adipocytes directly after removal from mice (day 0) and after a further 4 days in tissue culture in the absence of ZAG. (**F**) Lipolytic response of ep adipocytes from ob/ob mice treated with either PBS (■) or ZAG (▤) with no additions, or after the addition of isoprenaline (10μM) to PBS (□) or ZAG (▨) treated mice. Differences from the respective controls are shown as c, $p<0.001$, while the difference in response to isoprenaline between ZAG and PBS treated mice are shown as e, $p<0.01$ or f, $p<0.001$.

Fig. 3 Expression of HSL, ATGL and phospho ERK in WAT of ob/ob mice treated with ZAG. Western blots showing expression of phospho HSL (**A**), ATGL (**B**) and phospho ERK (**C**) in epididymal (ep), subcutaneous (sc) and visceral (vis) adipocytes of ob/ob mice treated with either PBS or ZAG for 5 days as described in the legend to Fig. 2A. The densitometric analysis represents an average of three separate blots. Differences from PBS treated animals are shown as c, $p<0.001$.

Fig. 4 Effect of ZAG treatment on response to β3-AR agonists. (**A**) Glycerol release from epididymal adipocytes treated with PBS (■) or ZAG (□), subcutaneous adipocytes treated with PBS (▨) or ZAG (▤) and visceral adipocytes from ob/ob mice treated with PBS (░) or ZAG (▩) in response to BRL 37344.

Differences from PBS treated mice are shown as c, $p<0.001$. (**B**) Lipid mobilising effect of BRL 37344 in epididymal adipocytes pretreated with either isoprenaline (1μM) or ZAG (0.58μM) for 2h prior to measurement of lipolytic activity in the absence (■), or presence (□) of BRL 37344 at the concentrations shown. Differences from control are shown as c, $p<0.001$, while differences in the presence of BRL 37344 are shown as f, $p<0.001$. (**C**) Western blots showing expression of the β3-AR in gastrocnemius muscle, BAT and WAT of ob/ob mice treated with either PBS or ZAG for 21 days. The densitometric analysis is the average of three separate Western blots. Differences from control are shown as c, $p<0.001$.

Fig. 5 Effect of ZAG on expression of uncoupling proteins. Western blots showing expression of UCP1 in BAT (A) and WAT (B) and expression of UCP1 in BAT (C) and WAT (D) in ob/ob mice after treatment with PBS or ZAG. The densitometric analysis is the average of three separate blots. Differences from PBS treated animals are shown as c, $p<0.001$.