MECHANISM OF ACTION OF A TUMOUR DERIVED LIPID MOBILISING FACTOR

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'MECHANISM OF ACTION OF A TUMOUR DERIVED LIPID MOBILISING FACTOR': SUMMARY

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Cancer cachexia comprises unintentional and debilitating weight loss associated with certain tumour types.

Fat loss in cachexia is mediated by a 43kDa Lipid Mobilising Factor (LMF) sharing homology with endogenous Zinc-α2-Glycoprotein (ZAG). LMF and ZAG induced significant lipolysis in isolated epididymal adipose tissue. This is attenuated by co-incubation with 10μM of antagonist SR59230A and partially attenuated by 25μM PD098059 (indicating β_3-AR and MAPK involvement respectively). LMF/ZAG induced *in vitro* lipid depletion in differentiated 3T3-L1 adipocytes that seen to comprise a significant increase in lipolysis ($p<0.01$), with only a modest decrease in lipid synthesis ($p=0.09$).

ZAG significantly increased *in vitro* protein synthesis ($p<0.01$) in C_2C_{12} myotubes (without an effect on protein degradation). This increase was activated at transcription and attenuated by co-incubation with 10μM SR59230A. Proteolytic digestion of ZAG and LMF followed by sephadex G50 chromatography yielded active fragments of 6-15kDa, indicating the entire molecule was not required for bioactivity.

Cachexigenic MAC16 cells demonstrated significant *in vitro* ZAG expression over non-cachexigenic MAC13 cells ($p<0.001$). WAT and BAT excised from MAC16 mice of varying weight loss demonstrated increased ZAG expression compared to controls. Dosing of NMRI mice with s/c ZAG failed to reproduce this up-regulation, thus another cachectic factor is responsible.

0.58nM LMF conferred significant protection against hydrogen peroxide, paraquat and bleomycin-induced oxidative stress in the non-cachexigenic MAC13 cell line. This protection was attenuated by 10μM SR59230A indicating a β_3-AR mediated effect. In addition, 0.58nM LMF significantly up regulated UCP2 expression ($p<0.001$), (a mitochondrial protein implicated in the detoxification of ROS) implying this to be the mechanism by which survival was achieved. *In vitro*, LMF caused significant up-regulation of UCP1 in BAT and UCP2 and 3 in C_2C_{12} myotubes. This increase in uncoupling protein expression further potentiates the negative energy balance and wasting observed in cachexia.

KEY WORDS: CACHEXIA, ZINC-α2-GLYCOPROTEIN, BETA-3-ADRENORECEPTOR, REACTIVE OXYGEN SPECIES, UNCOUPLING PROTEIN.

To Mum

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AA	Arachadonic Acid
ADP	Adenosin-5'-diphosphate
AIDS	Acquired Immunodeficiency Syndrome
AIS	Anaemia-Inducing Substance
APPR	Acute Phase Protein Response
APS	Ammonium Persulphate
AR	Adrenergic Receptor
ATP	Adenosine-5'-Triphosphate
BAT	Brown Adipose Tissue
BMR	Basal Metabolic Rate
Bq	Becquerels
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CCK8	Cholecystokinin 8
CNTF	Ciliary Neurotrophic Factor
COX	Cyclooxygenase
CRE	cAMP Response Element
CRH	Corticotrophin Releasing Hormone
DAUDA	11-(dansylamino)undecanoic acid
DEAE	Diethylaminoethyl cellulose
2DG	2-Deoxyglucose
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol

ECL	Enhanced chemoluminescence
EPA	Eicosapentaenoic Acid
EZAG	Recombinant Zn-α2-glycoprotein
FCS	Foetal Calf Serum
FFA	Free Fatty Acid
5FU	5-Flourouracil
g	gram
GDP	Guanosine diphosphate
GC	Glucocorticoid
GTP	Guanoine triphosphat
GU	Glucose Uptake
h	Hour
HCAP	Human Cachexia Associated Protein
HETE	hydroxyeicosatetraenoic acid
HPLC	High Performance Liquid Chromatography
HSL	Hormone Sensitive Lipase
IEF	Isoelectric Focussing
IFN-γ	Interferon-gamma
IL	Interleukin
l	Litre
LIF	Leukaemia Inhibitory Factor
LPL	Lipoprotein Lipase
kDa	Kilo Dalton
Kg	Kilogram
μ	Micro

M	Mole
m	Milli
MAC	Murine Adenocarcinoma
min	Minute
MLPLI	Melanoma-derived Lipoprotein Lipase Inhibitor
MoAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic Acid
n	Nano
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NEFA	Non-Esterified Fatty Acid
NFκB	Nuclear Factor-κB
NPY	Neuropeptide Y
OSM	Oncostatin M
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PIF	Proteolysis Inducing Factor
PPAR	Peroxisome Proliferator-Activated Receptor
POMC	Proopiomelanocortin
PUFA	Polyunsaturated Fatty Acid
REE	Resting Energy Expenditure

ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
TEMED	N,N,N,N'-Tetra-methyl-ethylenediamine
TNF-α	Tumour Necrosis Factor-alpha
TPN	Total Parenteral Nutrition
Tris	Tris(hydroxymethy)methylamine
Tween 20	polyoxyethylene-sorbitan
TZD	Thiazolidinedione
U	Unit
UCP	Uncoupling Protein
UCP3l	Uncoupling protein 3 (long form)
UCP3s	Uncoupling protein 3 (truncated form)
WAT	White Adipose Tissue
ZAG	Zinc-α2-Glycoprotein

1. Introduction.

1.1 Cancer & Weight Loss.

Cancer patients frequently exhibit unintentional weight loss. In many cases, this weight loss is the presenting symptom of an otherwise asymptomatic malignancy.

Weight loss and poor nutritional status are associated with a lower quality of life, decreased response to chemotherapy and inferior survival. Even small amounts (<5% body weight) significantly worsen patient prognosis (De Wys & Begg (1980)). Patient weight loss greater than 30-40% results in death (Kotler *et al* (1989)).

Weight loss accompanying malignancy may result from cancer anorexia, cancer cachexia, or a combination of both.

1.2 Cancer Anorexia.

Anorexia is a noxious symptom experienced by over 50% of advanced cancer patients (Jatoi *et* al (2001)). Cancer patients become anorectic for a variety of reasons:

The psychological implications of cancer frequently reduce appetite and eating pleasure (Tisdale (2001)). Food aversions are common during cancer treatment; previously acceptable or preferred foodstuffs become associated with the unpleasant side effects of chemotherapy and avoided (Bernstein *et al* (1982)).

Carson & Cormican (1977) reviewed taste acuity and food attitudes of cancer patients. Such patients often perceive taste differently to healthy individuals. Cancer is often accompanied by decreased appetite, increased sweet recognition thresholds and decreased bitter recognition thresholds. A distaste for meat is frequently reported, and further taste abnormalities following 5FU treatment are common. Consideration of these factors is important and will allow preparation of appealing menus for the cancer patient with the goal of improving nutritional status.

Jatoi *et al* (2001) reviewed neuropeptide Y (NPY) and cholecystokinin 8 (CCK8) regulation in cancer patients. NPY is an orexigenic hormone, the release of which is seen to be impaired in the hypothalamus of some tumour models. Severe cancer-associated anorexia significantly correlates with lower circulatory NPY levels than in healthy controls.

The elevated levels of pro-inflammatory cytokines observed during malignancy also contribute to the anorexia. The satiety-inducing hormone CCK8 has been associated with the increase in the anorexia-inducing cytokines IL-1β, IL-6 and TNFα (Jatoi *et al* (2001)). Additionally, elevated IL-1β, IL-6 and TNFα stimulate the hypothalamic proopiomelanocortin (POMC) systems. The POMC gene product is the anorexigenic peptide α-melanocyte stimulating hormone (α-MSH), which activates the melanocortin receptors MC3R and MC4R (Samson & Taylor (2001)). Administration of the melanocortin antagonist SHU9119 to anorectic Lobund-Wistar tumour-bearers reversed weight loss in this model (Wisse *et al* (2001)).

Abnormalities in the hypothalamic-membrane adenylate cyclase system have been observed in tumour-bearing models and implicated in cancer anorexia (Tisdale (2001)).

Elevation of the serotonin precursor tryptophan may contribute to cancer weight loss (reviewed by Tisdale (2001)). Brain serotonin is reported to act via the hypothalamic-pituitary-adrenal axis, sympathetic and parasympathetic nervous systems. Serotonin stimulates catecholamines and corticol and also serves to inhibit insulin, leptin, ghrelin, neuropeptide Y, various anabolic hormones and paracrine-acting cytokines (reviewed by Nandi *et al* (2002)).

Mechanical interference of a tumour with organs or food absorption constitutes a more obvious and direct contribution to anorexia and weight loss (as reviewed by Tisdale (2001)).

1.3 Cancer Cachexia:

A major obstacle in cancer therapy is the dysmetabolism of host macromolecules. Catabolism of lipids, proteins and complex carbohydrates influence the ability of the host to tolerate and respond to therapeutic intervention. These complex metabolic abnormalities and auto-catabolism associated with cancer patients collectively form the condition known as cachexia.

Cachexia is a paraneoplastic syndrome characterised by marked weight loss, asthenia and anaemia. Cachexia will manifest (to a greater or lesser degree) in the majority of advanced cancer patients before death (Argiles *et al* (2003)). Warren (1932) speculated cachexia to be responsible for 22% of cancer mortalities. Cachexia accompanies several other distinct pathological disorders such as AIDS, Chaga's disease and following surgical trauma.

1.4 Cachexia & Starvation:

In contrast to simple starvation (in which 75% of body weight loss is from adipose stores and only 25% from muscle), cachectic emaciation comprises equal fat and muscle loss (Cohn *et al* (1981)). The starvation-induced switch to a fat fuel economy observed during normal weight loss (as reviewed by Stryer (1998)) does not occur during cancer cachexia.

Normal animals respond to dietary restriction and weight loss with an adaptive fall in the rate of tissue protein breakdown, however, the most devastating feature of cachexia is the emaciation of host skeletal muscle. Skeletal muscle comprises 45% total body weight (Alvarez *et al* (2002)), with significant depletion leading to diaphragm failure and death.

In vivo studies demonstrate cachexia to involve increased catabolism and decreased protein synthesis in the host. Quite why these abnormalities should occur during a disorder superficially similar to starvation prompted speculation. Several reasons were initially hypothesised: (i) The abnormalities in protein homeostasis that occur during cachexia may be due to demands of the tumour for the conversion of gluconeogenic precursors to glucose. (ii) The presence of a tumour directly increases the rate of fuel oxidation in brown adipocyte tissue (BAT). Other host tissues are thus degraded to produce metabolic substrates for the BAT. (iii) The depression of protein synthesis and enhanced protein catabolism seen during cachexia is due to hormonal-like factors secreted by the tumour (as reviewed by Emery *et al* (1984))

Cachexia – associated proteolysis will be reviewed in detail in **section 1.9**.

1.5 Cachexia and Resting Energy Expenditure (REE).

Contrary to simple starvation, patients with cancer cachexia frequently exhibit an increase in resting energy expenditure (Fredrix *et al* (1991), Falconer *et al* (1994)). Roe *et al* (1997) studied cachexigenic leukaemic Piebald Variegated (PVG) rats. PVG rodents have severe cachexia accompanied by an inappropriately high REE. Body temperature of this model is grossly elevated and resting oxygen expenditures (Vo_2) are 25% to 35% of pair fed controls. This increase in basal metabolic rate and REE promotes negative energy balance and enhances the wasting associated with cancer.

Although more efficacious retro-viral treatments now prevent catastrophic weight loss in HIV-associated cachexia, Roubenoff *et al* (2002) examined HIV-associated cachectic males and demonstrated a REE rise of >200kcal/day in 17.7% of subjects examined.

Under normal conditions, aerobic glucose metabolism consists of glycolysis, the TCA cycle and oxidative phosphorylation. A net gain of 36 ATP molecules is produced from the complete oxidation of one glucose molecule (Stryer (1998)).

Much evidence exists that solid tumours possess significant numbers of hypoxic cells (Palmer *et al* (1990)). Conventional aerobic metabolism cannot occur, thus anaerobic respiration takes place, resulting in the production of lactate (Stryer (1998)).

The MAC16 cachexia model (**section 1.7**) displays extensive hypoglycaemia resulting excessive glucose demands by the tumour (Bibby *et* al (1987)). These glucose requirements are fulfilled by activation of the futile Cori cycle – in which the liver converts lactate to first pyruvate and then glucose in a process of gluconeogenisis (Eden *et al* (1984)). This cycle has

a net yield of –4 ATP molecules per glucose molecule formed and thus contributes greatly to the overall negative energy balance seen in cachexia (**figure 1.5.1**)

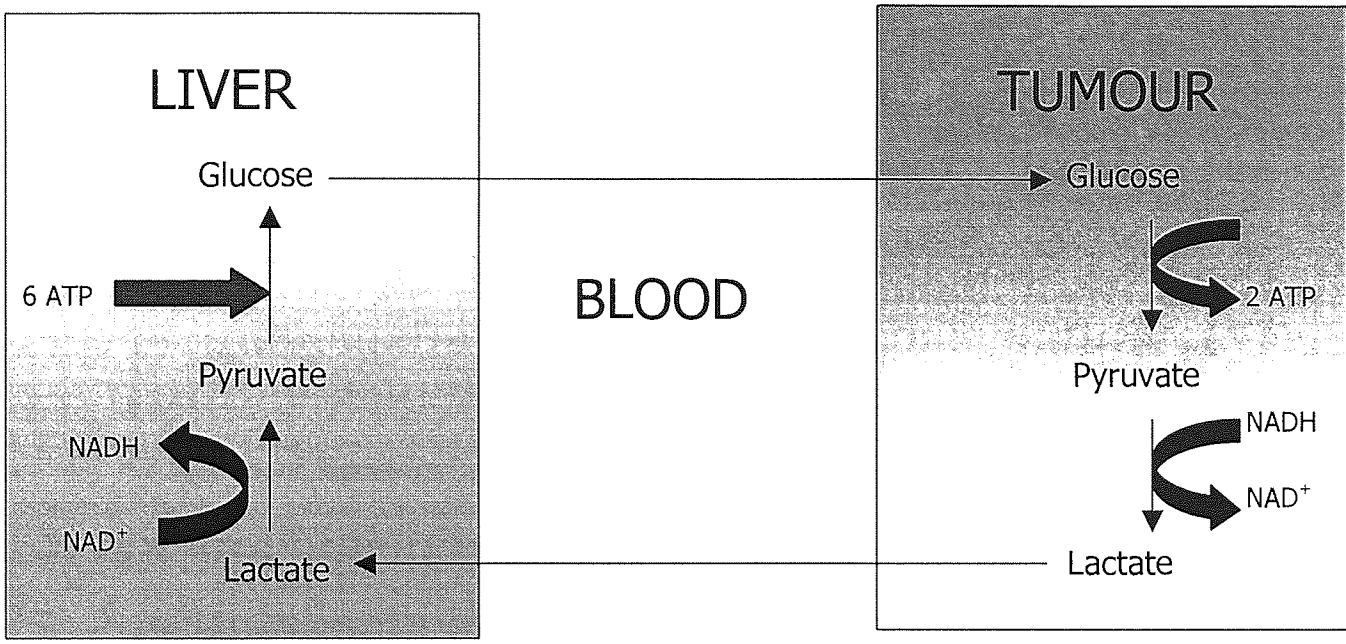


Figure 1.5.1. The Cori Cycle

1.6 The Acute Phase Protein Response (APPR).

The acute phase protein response is a host response to trauma, inflammation or infection. The APPR assists with tissue repair, blood clotting and facilitates the destruction of pathogens.

Acute phase protein responses occur in a significant portion of patients with lung, pancreas, oesophagus and kidney cancers. Why these patients should experience an increased APPR is unknown, and is potentially mediated by the increased pro-inflammatory cytokine response discussed in **section 1.10.12**. This cachexia-associated APP response contributes to the negative energy balance associated with cachexia and thus facilitates the emaciation (as reviewed by Fearon & Moses (2002)).

Mahmoud & Rivera (2002) positively correlated acute phase protein response with weight loss, anorexia-cachexia syndrome, extent of disease and recurrence in advanced cancer. The acute phase protein response as a predictor of survival has been demonstrated in multiple myeloma, lymphoma, ovarian, renal, pancreatic and gastrointestinal tumours. Measurement of patient acute phase protein response would provide valuable information prognosis and palliative care.

1.7 MAC16 – A Model for Cachexia.

The cachexigenic MAC16 model is a transplantable colon adenocarcinoma, passaged in NMRI mice (as characterised by Bibby *et al* (1987)).

Induction of weight loss in MAC16 mice occurs with comparatively low tumour volume (0.3% body weight). Losses of up to 30% body weight occur at only 3% tumour burden. This ratio of weight loss to tumour burden correlates with clinical cachexia in humans (Beck & Tisdale, (1987)). Moreover, the MAC16 model exhibits weight loss without reduction in fluid or calorific intake compared to non-tumour bearing controls. These criteria make it an excellent model for studying cachexia *in vivo*.

The related MAC13 adenocarcinoma is also passaged in NMRI mice. Mice transplanted with MAC13 cells display comparative tumour dynamics to that of MAC16-bearers, but do not experience cachexia. The MAC13 represents a non-weight losing cancer model for comparison with MAC16 mice during *in vivo* experiments.

1.8 Circulatory Factors & Cachexia.

Cancer-associated weight-loss has been the subject of investigation by many groups. The humoral nature of cancer cachexia was confirmed by Norton *et al* (1985). Norton demonstrated that a control rodent parabiotically joined to a tumour-bearer, exhibited homologous emaciation without evidence of metastasis.

These (and other) findings prompted the successful isolation and characterisation of numerous factors from various models.

1.8.1 Lipolytic Factors & Cancer Cachexia.

Gross depletion of lipid stores is a common feature of the cancer patient. Lipid loss often represents the greatest percentage of weight loss seen in the cancer-bearing state. Recently, a great many lipolytic factors have been isolated from various cachexia models and patients. The characterisation of such factors represents not only opportunity to develop novel intervention, but these molecules themselves may well offer therapeutic potential.

1.8.1.1 Lipolysis-Promoting Factor (Taylor *et al*).

Taylor *et al* (1992) identified a novel Lipolysis-Promoting Factor (LPF) produced by the human A375 melanoma line. This LPF was characterised as a 6kDa heat-stable molecule distinct from TNF-α. LPF produced loss of carcass lipid *in vivo* and dose/time-dependent lipolysis *in vitro* via a 2.5 – fold increase in the cellular lipase activity of isolated adipocytes.

1.8.1.2 Toxohormone-L (Masuno *et al*).

Masuno *et al* (1981) isolated a tumour-derived lipid mobilising factor (termed 'toxohormone-L') from the cachexigenic ascites sarcoma 180 rat model.

Masuno *et al* (1981) characterised Toxohormone-L as a 75kDa heat-labile protein with an isoelectric point of 4.7 which could be purified from patients bearing hepatomas or with Grawitz's tumour. Injection of isolated toxohormone-L into experimental animals resulted in lipid mobilisation, immunosupression and involution of the thymus gland (Masuno *et al* (1984)).

Interestingly, tryptic digestion of toxohormone-L yielded a heat-stable active moiety of <10kDa indicating that the entire protein was not required for lipolytic activity (Masuno *et al* (1981)).

1.8.1.3 Other Factors.

Costa & Holland (1962) induced loss of body fat in male Swiss mice following injection of non-viable Krebs-2-carcinoma (a tumour capable of producing extensive fat loss in the host).

Kitada *et al* demonstrated enhanced lipid mobilisation using serum isolated from mice bearing thymic lymphomas (Kitada *et al* (1980)) and from patients with advanced cancer (Kitada *et al* 1981). Initial characterisation of this factor indicated a molecular weight of approximately 5KDa. Later studies demonstrated this low molecular weight form to be inactive until it aggregated at lower temperatures (Kitada *et al* (1982)).

1.8.1.4 The 43KDa Tumour-Derived Lipid Mobilising Factor (LMF).

Groundwater *et al* (1990) speculated cancer weight loss to be mediated by tumour-produced factors. This group analysed serum and urine of weight-losing cancer patients, and, by means of control, compared it to that of weight-losing alzheimer's patients, as well as fluids from healthy individuals. Urinary and serum lipolytic activity was assessed according to the method of Weiland (1974). Control and alzheimer serum and urinary lipolytic activities were significantly lower than that of weight-losing cancer patients, implicating a circulatory lipolytic factor.

Similarly, weight loss in the MAC16 model correlated with a rise in serum lipolytic activity that peaked at 16% weight loss. Groundwater *et al* (1990) hypothesised a (then) unidentified humoral lipolytic factor to be associated with cancer weight-loss, but not necessarily weight loss accompanying other disorders.

A lipolytic factor was isolated from the MAC16 model. This was non-dialyzable and destroyed by acid and heat. Insulin and 3-hydroxybutyrate suppressed the lipolysis induced by this isolate. Administration of indomethacin to MAC16 mice failed to attenuate lipolysis *in vivo* and thus demonstrated this factor distinct from prostaglandins (Beck & Tisdale (1987))

A tumour-derived lipid mobilising factor was purified from excised MAC16 tumours (McDevitt *et al* (1995)) and later from the urine of cachectic pancreatic cancer patients (Hirai *et al* (1998)). This factor was visualised following 12% SDS-PAGE followed by Coomassie brilliant blue staining and found to have approximate molecular weight of 43KDa (McDevitt *et al* (1995), Hirai *et al* (1998)).

Khan & Tisdale (1999) demonstrated LMF-induced lipolysis to accompany stimulation of adenylate cyclase in adipocyte membrane preparations. Induction of lipolysis was attenuated by the adenylate cyclase inhibitor MDL_{12330A} and potentiated by the cAMP PDE inhibitor Ro 20-1724 suggesting a cyclic AMP-dependent mechanism (Russell, (2001)).

G proteins consisting of two families modulate the adenylate cyclase pathway. The Gs family stimulates adenylate cyclase activity, whilst the Gi family inhibit (as reviewed by Levitzki (1987)). The effect of LMF on adenylate cyclase is GTP sensitive, stimulation being observed at low concentrations and inhibition at high concentrations (Khan & Tisdale (1999)). The involvement of both stimulatory (Gs) and inhibitory (Gi) G-protein elements in the LMF pathway therefore seems probable (Islam Ali (2001)).

Islam-Ali *et al* (2001) analysed adipocyte membranes isolated from cachectic MAC16 models. Those animals who experienced up to 10% weight loss exhibited increased $G\alpha s$ expression and a reciprocal decrease in $G\alpha_i$.

Russell (2001) & Russell *et al* (2002) demonstrated the effects of LMF to be attenuated in the presence of the selective β_3-adrenoreceptor (β_3-AR) antagonist SR-59230A., suggesting that LMF was a β_3-AR agonist. β-Adrenorecpetors are implicated in body weight control and energy balance, with increased β-AR activity reported in both weight-losing cancer patients and in tumour-bearing animals (Hyltander *et al* (1999)).

The cytoplasmic fraction of adipocytes contains the enzyme Hormone Sensitive Lipase (HSL). This enzyme catalyses hydrolysis of triacylglycerols into free fatty acids (Stryer (1998))

HSL-mediated lipolysis is initiated by a complex combination of events. An increase in intercellular cAMP levels activates cAMP-depandant serine phosphorylations (as mediated by protein kinase A). HSL then translocates from the cytoplasm to the lipid droplet surface with a reciprocal shift of perilipin A (the lipid droplet protein that is also phosphorylated by PKA) to the cytoplasm – (Londos *et al*, (1999)). Interestingly, Khan & Tisdale (1999) demonstrated that H8, a cAMP-dependent protein kinase A inhibitor, almost completely attenuated the action of LMF implying PKA involvement.

Strong evidence exists that LMF-induced lipid mobilisation is analogous to that observed with the natural lipolytic hormones distinct from that facilitated by cytokines. Moreover, detectable LMF in the urine of weight-losing cancer patients provides convincing evidence for the involvement of this factor in clinical cachexia (McDevitt *et al* (1995)).

1.8.1.5 Zinc-α_2-Glycoprotein (ZAG).

Zinc-α_2-Glycoprotein is so termed because it precipitates with zinc ions and has electrophoretic mobility in the region of the α-globulins. ZAG is present in most body fluids including milk, CSF, sweat, saliva, serum, seminal plasma and amniotic fluid (Sanchez *et al* (1999))

The 43kDa tumour-derived LMF possesses identical amino acid sequence (Todorov *et al* (1998)), immunoreactivity (Sanders & Tisdale (2003)) and electrophoretic mobility on native and denaturing gels. Both molecules stain heavily for carbohydrate (Russell *et al* (2003), Hiari *et al* (1998)).

ZAG, like LMF, stimulates dose-dependent lipolysis *in vitro* and induces body fat loss *in vivo*. Similarly, these effects of ZAG are cyclic-AMP dependent (the cAMP PDE inhibitor Ro 20-1724 potentiates lipolysis) and attenuated by the selective β-$_3$AR antagonist SR-59230A (Russell *et al* (2002) & (2003)).

There is a plethora of further evidence to suggest that ZAG and LMF are the same molecule – ZAG antisera attenuates LMF-induced lipolysis *in vivo* (Hiari *et al* (1998)), ZAG and LMF are attributed similar molecular weights when analysed by MALDI-TOF (Zimmerman T, personal communication), and both molecules possess similar absorption spectra between 275-280nm (Russell (2001)). Further comparisons between the two molecules are drawn in **chapter 3.0**.

Despite the presence of LMF in cachectic urine (Hirai *et al.* (1998)), ZAG is also detectable in urine of healthy individuals (proteins of <67KDa being filtered by the glomerulus, thus may be excreted in the urine (Lafitte *et al* (2002)). It is likely only an elevation of urinary ZAG accompanies pathological cachexia. Dr. T. Zimmerman (Bayer Corporation) performed immunoblot analysis of cachectic and control urines – although ZAG was detectable in both, however, differences in expression could clearly be observed (Zimmerman T, personal communication).

ZAG shares 30-40% homology with the Class I Major Histocompatibility Complex (MHCI) antigen. MHC molecules present antigen to cytotoxic T lymphocytes as part of host immunosurveillance processes. ZAG lacks the transmembrane and cytoplasmic domains of MHCI molecules, leading some groups to hypothesise a soluble-HLA-like function (Araki *et al* (1998)). However, unlike MHCI molecules, spatial restrictions prevent ZAG associating with soluble β-$_2$ microglobulin (β-$_2$M) (Kennedy *et al* (2001)).

MHCI molecules possess a binding site between the α1 and α2 domains. Similarly, ZAG possesses a groove containing a small and as-yet unidentified proteinase-resistant compound whose injection induces glomerulonephritis in experimental animals (Sanchez *et al* (1999)).

Electron density maps of the ZAG groove (obtained by Sanchez *et al* (1999)) eliminate the possibility of a peptide ligand –, instead, it appears to be a small, non-covalently bound, hydrophobic molecule(s). Kennedy *et al* (2001) demonstrated ZAG binding of the flourophore-tagged fatty acid 11-(dansylamino)undecanoic acid (DAUDA), and, by competition, other FA's such as arachadonic, linoleic and eicosapentaenoic acid. This group speculated that the natural ligand for ZAG may be a fatty acid and postulated a role for ZAG in the natural lipid homeostasis of the body.

By way of comparison, LMF was subjected to similar analysis. Although less marked than was observed with ZAG, LMF bound DAUDA, causing a shift in peak fluorescence emission identical to that seen with ZAG. Arachadonic acid also competed to a decent degree (Kennedy M, Personal Communication). LMF preparations are often pale yellow in colour (unpublished observation), which may be indicative of an associated lipid with higher affinity for LMF than DAUDA (potentially explaining impaired DAUDA binding).

This unknown lipid had absorbance at 407nm and was detectable by GC-MS, MS and HPLC with UV detection (Kennedy M, Personal Communication). Covalent binding of this molecule to LMF is certainly a possibility, but further work is required to elucidate its true nature.

Roles for ZAG in the pathophysiology of cancer are now emerging. Hale *et al* (2001) reported sufficiently elevated ZAG in a prostate cancer subset to induce the cachexia associated with this tumour. Hyper-accumulation of ZAG has been observed in breast cysts (30 to 50 fold that

of normal plasma) and ZAG is over-expressed by 40-50% in breast carcinomas (Bundred *et al* (1987)). ZAG is also reported (Brysk *et al* (1999)) to be a marker of oral tumour differentiation – this having obvious implications for choice of therapy and marker of prognosis.

1.8.1.5.1 ZAG/LMF in the Treatment of Obesity.

Hirai *et al* demonstrated that the 43kDa tumour-derived LMF was capable of producing fat loss in isolated human WAT (although with lower maximal response than rodent WAT). Recently our laboratory has also demonstrated ZAG to possess activity in isolated human WAT *in vitro* (Russell ST, unpublished result).

The controlled use of these homologous factors as anti-obesity agents appears quite promising. Several other Beta-$_3$-Agonists have recently been evaluated as anti-obesity agents, with varying degrees of success (Arch (2002), Harper (2001), Hu & Jennings (2003), Kiso *et al* (1999)).

1.8.1.5.1.1 ObeX:™ .

A recent drug, Obex, (Ericsson, patent application # 385989), demonstrates the efficacy of ZAG at promoting weight loss *in vivo*.

The ObeX treatment regime comprises of α-interferon (50-5,000 units per day) and γ-interferon (50 to 5,000 units per day). ObeX is dispensed in 30ml spray bottles and sprayed directly into the oral cavity of patients. This formulation apparently induces the production and

release of ZAG from lymphocytes. Released ZAG then mediates weight loss in the manner previously described in **1.8.1.4 – 1.8.1.5**.

ObeX treatment produces superior weight loss (compared to fasting alone) in the initial period treatment, however, this then plateaus and stabilises. The ObeX treatment regime thus incorporates an active treatment period of 3-30 days, which is discontinued for a similar time and re-instated if desirable.

A trial comprising 17 obese adults produced encouraging results – the rate of weight loss in the study was encouraging at 0.83 pounds per day (fasting yields a loss of only 0.5 pound per day).

These findings are most encouraging. However, with an established method for ZAG manufacture, one suspects that direct ZAG administration will be more efficacious and acceptable to the relevant clinical authorities.

Although metabolic abnormalities which predispose individuals to weight-gain do exist (as cited in Stryer (1998)), perhaps more serious causative agents exist in today's society. The present 'high-calorie, junk food, no exercise, television and video game culture' that exists has contributed greatly to the prevalence of obesity. Ironically, if society would simply restrict daily calorific intake to recommended limits (1500-2000 Kcal/day (women) or 2000-2500Kcal/day (men)) and undertake 1.5 hours of cardiovascular exercise per week then such enormous monies would not have to be channelled into research of this nature.

1.9 A Tumour Derived Proteolysis Inducing Factor (PIF).

Cachectic MAC16 models were seen to exhibit decreased protein synthesis and increased protein degradation (Beck *et al* (1991)), leading to speculation about a circulatory proteolytic factor. Smith & Tisdale (1993) then demonstrated evidence for this factor in the serum of this cachexia model.

Todorov *et al* (1996) affinity-purified this proteolytic factor from excised MAC16 tumours. This extract was subjected to 12% SDS-PAGE electrophoresis, producing two immunoreactive bands with apparent molecular weights of 69kDa and 24kDa. Further purification elucidated the proteolytic factor to have a molecular weight of 24kDa. The factor bound non-covalently (Choudhary *et al* (1999)) to albumin, resulting in the 69kDa band (Todorov *et al* (1996)) and was distinct in amino acid sequence and immunoreactivity to any cytokine implicated in cancer cachexia (Todorov *et al* (1997)). This factor was named Proteolysis Inducing Factor (PIF).

Three major proteolytic pathways exist in skeletal muscle: (i) The lysosomal system (concerned largely with proteolysis of endocytosed proteins and cellular receptors), (ii) The cytosolic calcium-activated system (concerned with tissue injury, necrosis and autolysis) and (iii) The ATP-ubiquitin-dependent pathway. It is the ATP-ubiquitin-dependent-pathway which is believed responsible for the muscle wasting seen in sepsis, acute diabetes, starvation and cancer cachexia (as reviewed by Tisdale (2001)).

Injection of isolated factor into non-tumour bearing rodents induced a state of cachexia and significant weight loss. Lorite *et al* (1997) demonstrated twenty-four hour pre-treatment with factor-specific monoclonal antibody to be effective at attenuating this weight loss. Weight loss in PIF-dosed rodents occurred without accompanying reduction in food and water intake. Body

composition analysis revealed a significant reduction in carcass dry weight without alterations in body water. Decreases in blood glucose, threonine, serine, proline, glycine, alanine, methionine, isoleucine,. leucine. lysine, tryptophan and histidine were also observed.

In vitro this factor catabolises isolated gastrocenemius muscle, and induces protein degradation (Todorov *et al* (1996)) and decreased protein synthesis (Smith *et al* (1999)) in C_2C_{12} myotubes. A pre-incubation with 50μM of EPA abolished the increased degradation, but not the decreased synthesis, suggesting these to occur via different mechanisms (Smith *et al* (1999)).

Smith *et al* (1999) demonstrated PIF to induce a dose dependent release of [^{3}H]-arachadonic acid from prelabeled myoblasts. Released arachadonic acid was rapidly metabolised to prostaglandins E_2, $F_{2\alpha}$ and to 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETES). 15-HETE activates NF-κB (Whitehouse (2001), Wyke SM, (personal communication)), a pathway implicated in septic muscle breakdown (Gail-Penner *et al* (2001)), cachectic muscle degradation and the inflammatory response seen in cancer cachexia (Watchorn *et al* (2001), Watchorn *et al* (2002)). This induction of prostaglandin synthesis also contributes to cachexia (discussed further in **section 1.10.1**).

Structural investigations revealed PIF to be a sulphated glycoprotein of Mr 24kDa with a short 4kDa peptide core possessing GlcNAc residues (Todorov (1996)) and extensive glycosylation at Asn and Ser residues. Functional and immunological studies of PIF provide evidence that these *N*- and *O*- linked oligosaccharide chains mediate biological activity.

Todorov *et al* (1997) biosynthetically labelled MAC16 cells with $^{35}SO_4$, [^{3}H] GlcN, [^{3}H] His and $^{32}P_i$ then isolated MAC16-produced PIF via affinity purification. Fragments resulting from enzymatic deglycosylation demonstrated PIF molecule to consist of: (i)a 4KDa central polypeptide chain and a short GlcN-containing oligosaccharide, (ii)a 6KDa *O*-linked GlcN containing oligosaccharide, (iii)a 10KDa *N*-linked sulphated GlcN-containing oligosaccharide.

A possible human analogue of PIF, Human Cachexia-Associated Protein (HCAP), was recently identified from a breast cancer library during a homology search. HCAP appears 90% homologous to PIF and is absent from non-tumour libraries (Wang *et al*, 2003). Conversely, Watchorn *et al* (2002) report a role for PIF as an endogenous mediator, speculating its pathological role to be a tumour-induced abnormality. PIF expression is reported to peak in mice on embryonic day 8.5 – a time when cell differentiation, development and morphological transitions are occurring.

1.10 Mechanisms, Hormones, Factors & Molecules Contributing To Cachexia:

Numerous factors have been proposed, speculated and isolated as mediators or symptoms of cancer cachexia.

1.10.1 Prostaglandins.

Prostaglandins are produced in virtually all tissues from arachadonic acid, a fatty acid constituent of the plasma membrane. Prostaglandins (and the closely related arachadonic-acid derivatives the leukotrienes, thromboxanes and prostacyclins) are among the most active messengers in the human body. Prostaglandins are twenty-carbon fatty acid derivatives belonging to either PGA, PGE or PGF groups depending on structural classification (as reviewed by Sherwood (1993)).

Systemic bacterial or viral infection promotes skeletal muscle proteolysis, possibly due to IL-1 activation of PGE_2 synthesis.

IL-1 produced in response to a tumour (**section 1.10.12.2**) will stimulate PGE_2 production (Baracos *et al* (1983)). Roe *et al* (1997) suggested these peripherally produced prostaglandins to mediate cachexia-associated hypermetabolism. Prostaglandins act on hypothalamic chemical messenger systems to raise body temperature (Sherwood (1993)) which contributes to the fever and REE abnormalities observed in cachexia. Additionally PG synthesis mediates skeletal muscle atrophy in fever (Ruff & Secrist (1984)), suggesting these may contribute directly to skeletal muscle atrophy in cachexia.

IL-1 aside, a more direct stimulation of PG synthesis may occur during cancer cachexia. PIF is reported to promote the release of arachadonic acid from skeletal muscle cells (Smith *et al* (1999)). This arachadonic acid is metabolised to prostaglandins E_2, $F_{2\alpha}$ and to 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETES). The enzyme cyclooxygenase (COX) facilitates this conversion of arachadonic acid into prostaglandin endoperoxide. The resulting increase in circulatory prostaglandins contribute to the metabolic abnormalities, fever and muscle degradation associated with cachexia. Increased expression of cyclooxygenase isoforms COX-1 and COX-2 have been reported in various tumour models leading to speculation of a role for COX in inhibition of apoptosis and increased tumour invasiveness (Hussey & Tisdale (2000)).

The specific COX-2 inhibitor meloxicam was administered to mice bearing MAC13 and MAC16 tumours (Hussey & Tisdale (2000)). A refraction of tumour volume doubling time in both models was observed, in addition to attenuation of cachexia in MAC16 mice. *In vitro*, meloxicam successfully inhibited PIF-induced degradation of differentiated C_2C_{12} myotubes, suggesting this agent directly antagonises muscle catabolism in cancer cachexia

Similarly, Roe *et al* (1997) demonstrated acute systemic injections of the cyclo-oxeganase inhibitors indomethacin and flurbiprofen to significantly attenuate (14% and 10% respectively) metabolic rate increases in a cachexigenic PVG leukaemic rat model.

1.10.2 Lipoprotein Lipase (LPL) Inhibitors.

Tsujimoto *et al* (2000) postulated that impaired LPL activity might contribute to the cachectic state. LPL activity was reduced *in vivo* when C57BL/6 mice were transplanted with the cachexigenic EL-4 lymphoma. Mice bearing EL-4 displayed elevated blood triglyceride and non-esterified fatty acid content. EL-4-bearers also had decreased heparin-releasable plasma LPL activity; further suggesting impaired LPL function in cachexia.

Mori *et al* (1989) analysed the conditioned media of the cachexigenic human melanoma cell line, SEKI. A 40kDa protein factor capable of inhibiting LPL in 3T3-L1 adipocytes was isolated and designated melanoma-derived LPL inhibitor (MLPLI). Interestingly, amino acid sequencing of the MLPLI amino terminus revealed a sequence identical to that of leukaemia inhibitory factor (LIF). Mori *et al* (1991) then further characterised MLPLI and LIF. Four melanoma cell lines were analysed for LIF mRNA expression and the ability to induce cachexia *in vivo*. The SEKI and G361 cell lines that were able to induce cachexia following transplantation into nude mice and were found to express LIF mRNA. The A375 and MEWO cell lines were found to be non-cachexigenic *in vivo* and did not express LIF mRNA. Metcalfe *et al* (1990) administered recombinant LIF to experimental animals and observed body weight loss. Mori postulated that MLPLI and LIF represent either the same, or separate candidates for a cachectic factor. Kawakami *et al* (1991) also describes the purification (from SEKI) of a heat labile protein >25kDa that also suppresses LPL activity in fully differentiated 3T3-L1 adipocytes. This factor was not immunoreactive with antibodies raised against any cytokines implicated in cachexia, and one suspects that the two groups have purified the same molecule.

Zechner *et al* (2000) utilised 3T3-L1 adipocytes and demonstrate transcriptional down regulation of LPL by TNF-α. Greenberg (1992) used a similar model to demonstrate down

regulation of LPL by IL-6, again implicating the involvement of the cytokine network in cancer cachexia.

Conversely, Nara-Ashizawa *et al* (2001) analysed conditioned media from the cachexigenic human cell lines LS180, MKN-1, MMG-1, C32 and LX-1. Media from all five lines suppressed LPL activity in 3T3-L1 adipocytes. In addition, conditioned media from LX-1 and C32 lines promoted lipolysis in a dose-dependent manner. RT-PCR and ELISA were used to elucidate the cachexigenic factors produced by these lines. These factors were demonstrated not to be IL-1β, IL-6, IL-11, TNF-α, TGF-β1 or LIF. The mediators isolated were a 65kDa LPL-inhibiting factor and a <1kDa heat-stable Lipolytic Factor. Interestingly, in the same study Nara-Ashizawa could not generate significant *in vitro* lipolysis with ZAG at doses of up to 10mg/ml.

Conversely, Thompson *et al* (1993) demonstrated a two-fold increase in HSL mRNA in adipose tissue of weight-losing cancer patients. This was accompanied by a corresponding elevation in serum fatty acids and triacylglycerols. Additionally, Thompson demonstrated that LPL mRNA levels did not alter significantly in these patients. This suggests increased lipid mobilisation as opposed to deposition, and supports the role of the 43KDa LMF as the main mediator of fat loss in human cancer cachexia.

1.10.3 Leptin.

Leptin is the 16kDa product of the adipocyte *ob* gene. Leptin is closely associated with body weight homeostasis and provides a negative feedback signal critical to the normal control of food intake and body weight.

During starvation, leptin production falls away markedly with decreased body adiposity. Leptin acts hypothalamically to decrease NPY production, and thus decreased serum leptin results in increased NPY and hunger.

In rodent models, circulatory leptin is regulated by a variety of factors including insulin, glutocorticoids, catecholamines and cytokines. In humans however, insulin and glucose are believed to play a lesser role in *ob* gene regulation (Hardie *et al* (1996)).

Sarmiento *et al* (1997) administrated recombinant human leptin to C57BL/6 mice and observed marked depletion of epididymal WAT. Hyperleptinemia induced by injection of a leptin cDNA-containing adenovirus also resulted in triglyceride depletion *in vivo* (Chen *et al* (1996), Zhou *et al* (1999)).

Pro-cachectic cytokines stimulate the production of leptin. It was proposed therefore that cytokine-elevated leptin levels could contribute to the cancer anorexia-cachexia syndrome. To this end, Mantovani *et al* (2002) examined the role of leptin in stage IV cancer patients. Patients with advanced cancer possessed significantly lower leptin levels than healthy controls despite an inverse correlation of leptin with IL-6. Concurringly, Brown *et al* (2001) examined leptin levels in sixty-four patients undergoing surgery for pancreatic cancer (thirty of whom were cachectic). Again, these cancer patients possessed significantly lower leptin levels than controls. Similar findings were obtained by Aleman *et al* (2002) who demonstrated leptin levels in NSCLC patients to be dependent only on amount of body fat.

It would appear that although anorexia and reduced appetite contribute to cancer weight loss, elevated plasma leptin levels are not a contributing factor to cancer cachexia (Brown *et al* (2001)).

1.10.6 Ciliary Neurotrophic Factor (CNTF).

CNTF is a member of the IL-6 superfamily and is a 26kDa cytoplasmic protein. CNTF was initially characterised as a trophic factor for motor neurons in the ciliary ganglion and spinal cord, leading to its trial and evaluation in motor neuron disease sufferers. Unexpectedly, CNTF produced substantial weight loss, raising concerns that it may produce cachexia-like wasting (Lambert *et al* (2001)).

Espat *et al* (1996) administered 25μg/kg CNTF to healthy C57B1/6 mice over a seven-day period. CNTF induced profound muscle wasting (21% of carcass protein) and severe anorexia in addition to high fever and an acute phase protein response. Inui (1999) also implicates a cachectic role for CNTF. Similarly, Martin *et al* (1996) demonstrated the muscle wasting effect of recombinant human CNTF (rhCNTF) when administered to rats. Antibodies to pro-cachectic cytokines (TNF-α and IL-1) failed to attenuate the wasting, indicating an rhCNTF-specific effect.

Conversely, however, Lambert *et al* (2001) reported CNTF-induced weight loss to be distinct from that experienced by cachectic patients. In the experiments of this group, CNTF exhibited many similarities with leptin, acting through leptin-like receptors of similar hypothalamic distribution. CNTF induced weight loss not only in *ob/ob* mice but also in leptin-resistant diet-

induced obesity models. Lambert *et al* believe CNTF to act in a manner completely distinct from cachexia-inducing cytokines.

In light of conflicting evidence, it is difficult to propose a role for CNTF in cancer cachexia. Further studies are most certainly required to elucidate the exact mechanism of CNTF and to formulate interventional therapies that might monopolise this pathway.

1.10.7 Azaftig.

Interestingly Julio *et al* (2002) isolated a 24kDa chondroitin-SO_4-containing proteoglycan termed Azaftig (US patent # 6,274,550 B1 (2001)). Julio purified azaftig from the urine of cachectic-pancreatic cancer patients and demonstrated dose-dependent lipolytic activity *in vitro* using isolated human and rat adipocytes. Julio proposed azaftig-mediated lipolysis to be cyclic AMP and Hormone Sensitive Lipase-dependent and implicates the potential of this molecule as an anti-obesity agent.

It would appear that this group have simply co-purified LMF and PIF from cachectic urine (Tisdale MJ, Personal Communication). If such a case is true, administration of a PIF-containing preparation into humans could result in very serious consequences indeed.

1.10.8 Leukaemia Inhibitory Factor (LIF).

Mori *et al* (1989) isolated a 40kDa melanoma derived lipoprotein lipase inhibitor (MLPLI) sharing homology with LIF from the conditioned media of the SEKI melanoma cell line. Mori *et al* (1991) demonstrated that the cachexigenic SEKI and G361 cell lines expressed LIF mRNA.

LIF was not expressed in the non-cachexigenic A375 and MEWO cell lines. These findings suggest a potential role for LIF or MLPLI in certain cachexia models, and are covered in more detail in **1.10.2**.

Conversely, Barton and Murphy (2001) passaged the lung carcinoma line LL/2 in C57BL/6J mice. This tumour model induced the active expression of cytokines; however, at no time point or site was LIF induced during the cachexia in this model.

Once again, conflicting evidence make it difficult to postulate a role for this factor in cancer cachexia.

1.10.9 Glucocorticoids.

Glucocorticoids activate protein catabolism during fasting, causing impairment of myofibrillar protein synthesis and an increased protein catabolism. Excess glucocorticoid levels are evident in conditions such as Cushing's syndrome, and are implicated in muscle catabolism associated with trauma, sepsis and cancer cachexia (Llovera *et al,* (1996)).

Accelerated muscle emaciation in the Yoshida-AH-130 ascites hepatoma is mainly accounted for by an increase in protein degradation. (Llovera *et al* (1996)). Of particular interest in this model is the corresponding rise in host corticosterone plasma levels. (Tessitore *et al* (1993)) Llovera *et al,* (1996) attempted to reverse the muscle hypercatabolism associated with the cachexigenic Yoshida-AH-130 model using the glucocorticoid antagonist RU38486 (mifepristone) (an agent effective at reducing muscle degradation during sepsis). RU38486 failed to attenuate the wasting observed in this model.

Although glucocorticoids may produce emaciation conditions such as sepsis, further studies are required to elucidate an exact role (if any) in cancer-associated cachexia.

1.10.10 Anaemia-Inducing Substance (AIS).

Ishiko *et al* (2000) incubated plasma from advanced cachectic ovarian cancer patients with non-coated charcoal for one hour and observed a 40% drop in lipolytic activity. As IL-1α, IL-1β, IFN-γ, LIF, TNF-α and IL-6 do not adsorb significantly to non-coated charcoal, the loss of plasma lipolytic activity was hypothesised to be due to the 98.1% adsorbed anaemia-inducing substance.

As interesting as this study may be, there is little other evidence available to substantiate a role for this factor in cancer weight loss.

1.10.11 The Uncoupling Protein Homologues (UCPn).

Regulation of body weight and energy expenditure is a complex process involving multiple neural circuits and peripheral signals. WAT and BAT play a central role in energy expenditure. WAT is the storage site from which lipids are mobilised. BAT is able to dissipate energy as heat following uncoupling of respiration by the mitochondrial anion carrier Uncoupling Protein 1 (aka 'Thermogenin') (Research Diagnostics Review (2000)).

In cellular respiration, oxidation is coupled to the electron transfer chain by the pumping of protons from the mitochondrial matrix. This creates a so-called proton-motive force across the mitochondrial membrane. These protons pass down their gradient via the enzyme ATP

synthase, promoting phosphorylation of ADP to ATP. The uncoupling proteins render this coupling imperfect (Ricquier *et al* (2000)).

Initially, UCP1 was discovered in BAT, to which it is largely confined. UCP1 was initially termed 'thermogenin' because it facilitates non-shivering thermogenisis (Ricquier *et al* (2000)). Other mammalian homologues were identified and termed UCP2 and UCP3. UCP2 is ubiquitous but UCP3 is confined predominantly to skeletal muscle in humans (Ricquier *et al* (2000)).

Roles for UCP2 and UCP3 as mediators of thermogenisis were proposed but then discarded following the discovery of UCP2 mRNA in ectothermic vertebrates. This implies some other functional role(s) also exist (Stuart *et al* (1999)) A role for UCP2 is hypothesised in the control of body weight and energy expenditure – UCP2 expression is greatly elevated in the WAT of *ob/ob* and *db/db* mice for instance (Research Diagnostics 2000, (Review)). Additionally, UCP2 is implicated in the detoxification of reactive oxygen species (Jezek (2002), Sanders & Tisdale (2003), Pecqueur *et al* (2001)). A murine model which over-expressesUCP3 is hyperphagic and lean (Clapham *et al* (2000)), also implicating UCP3 in energy expenditure and body weight control. Other postulated roles for UCP2/3 include adaptive thermogenisis and the balancing of apoptotic processes (Jezek (2002)).

Various groups have demonstrated alterations in uncoupling protein expression in cachexigenic models (Bing *et al* (2000), Tonello *et al* (1998)) and by direct injection of a tumour derived LMF (Russell *et al* (2003), Bing *et al* (2002), Russell (2001)). In many cases, it has been unclear whether fluctuations in UCP expression were mediated by cachexia-associated starvation / fatty acid mobilisation or were the direct action of a tumour factor (or both).

Cachexia-induced increases in UCP expression will reduce host energy efficiency and contribute to the negative energy balance (and thus weight loss) associated with this syndrome. The role of the uncoupling proteins in cancer cachexia will be investigated later in these studies.

1.10.12 Cytokine Involvement in Cancer Cachexia.

Cytokines are low molecular weight (15-25kDa) secreted proteins that mediate cell growth, inflammation, immunity and host repair mechanisms. They are highly potent, with activity seen at 10^{-15}M at high affinity cell surface receptors (as reviewed by Roitt (1997))

In a healthy individual, cytokine responses are transient and tightly regulated - the presence of foreign material triggers a host response that dissipates when the stimulus is removed. Certain cytokines are indeed implicated with anti-tumour roles (e.g. tumour necrosis factors- α and β). However, the constant presence of a tumour, in addition to other metabolic aberrations that accompany cancer may lead to constant and inappropriate over-stimulation of the immune response and cytokine network.

Dysfunctions of the cytokine network will invariably be deleterious to the host. In addition, such hyper-stimulation potentiates the negative energy balance and elevated REE associated with cancer cachexia. This evidence has led some groups to propose cachexia in it's entirety to be a consequence of an ill-managed host-mediated inflammatory response against the tumour (Barton and Murphy (2001)).

The search for the humoral mediator(s) responsible for cachexia has therefore led many groups to strongly consider that a cytokine(s) may be responsible.

1.10.12.1 Tumour Necrosis Factor-α (TNF-α).

TNF-α is an endogenous 17kDa protein that mediates host immune, inflammatory and metabolic responses (Porter *et al* (2002)). TNF-α is produced by T-lymphocytes and macrophages. TNF-α has roles in tumour cytotoxicity, cachexia, acute phase protein induction, anti-viral and anti-parasitic actions, activation of phagocytic cells, endotoxic shock and induction of IFN-γ, IL-1, GM-CSF and IL-6 (as reviewed by Roitt (1997)).

TNF-α has long been implicated in cancer cachexia, and was once thought solely responsible for the condition (thus was termed 'cachectin)'. Karayiannakis *et al* (2001) demonstrated a significant relationship between serum TNF-α (sTNF-α) levels and nutritional status of 63 pancreatic cancer patients. Higher sTNF-α levels were associated with increased weight loss, lower serum total protein concentration and lower haemoglobin levels. This increase was particularly detectable in advanced disease.

TNF-α is implicated in cachexia associated with other pathological conditions also. Truyens *et al* (1995) infected BALB/c mice with *Trypanosoma cruzi*, the causative agent of Chaga's disease. These rodents experienced severe cachexia (approximately 20% weight loss) associated with dramatic fat depletion and water gain without significant alterations in food intake. Injection of anti-TNF-α monoclonal antibodies (MoAb) during the first two weeks of infection significantly altered the weight loss profile. Similar administration of anti-IL-6 or anti

IFN-γ MoAbs failed to attenuate the wasting. Once again, it was concluded that TNF-α is a key factor in this cachexia model.

Moreover, the effects of TNF-α induced cachexia appear reproducible *in vitro*. Porter *et al* (2002) evaluated glycerol release from isolated rat epidydimal fat. TNF-α at a concentration of 4nmol/L significantly increased fat pad glycerol release during 60 minute or 24 hour incubations. Furthermore, addition of 0.5mg/mL noradrenaline failed to augment this release, suggesting the cells were maximally stimulated.

However, whilst NMRI mice administered TNF-α do indeed lose adipose stores, the skeletal muscle emaciation seen in cachexia fails to occur (Beck & Tisdale (1990)). In addition, although TNF-α administration superficially mimics cachexia, tolerance soon develops, with food intake and body weight returning to normal (Argiles *et al* (2003))

Moreover, although TNF-α is strongly implicated in HIV-associated cachexia as the main causative agent of the weight loss (Roubenoff *et al* (2002)), Maltoni *et al* (1997)) failed to significantly correlate serum TNF-α (or other cytokine) levels with any parameter in 61 advanced weight-losing cancer patients.

Although TNF-α will stimulate lipolysis *in vivo*, it is via a pathway different to that initiated by the isolated 43kDa LMF associated with clinical cachexia. Green *et al* (1994) have demonstrated that TNF-α does not activate hormone-sensitive lipase, an enzyme activated by LMF (Khan & Tisdale (1999).

The contradictory evidence that exists makes it extremely unlikely that TNF-α is the factor solely responsible for cachexia as it was once thought to be (despite a prominent role in HIV- and Chaga's disease-associated cachexia). However, TNF-α is capable of producing some of the symptoms of cachexia *in vitro* and *in vivo*, and also of inducing pro-cachectic cytokines, so should not be ruled out as a contributory factor.

1.10.12.2 Interleukin-1 (IL-1).

IL-1 was originally termed 'leukcoytic protein' and is produced by macrophages and fibroblast cells. IL-1 induces macrophage production of cytokines and PGE2, proliferation of activated B- and T- cells and promotes the induction of neutrophil / T-cell adhesion molecules on endothelial cells. IL-1 also induces fever, acute phase protein response and bone reabsorption by osteoclasts.

In muscles incubated *ex vivo* at 37°C, IL-1 increased net protein degradation by 62-118 per cent without altering protein synthesis. IL-1 dramatically stimulates muscle production of PGE_2 that promotes skeletal muscle breakdown. IL-1 also acts at the hypothalamus to increase PGE_2 production causing fever (Baracos *et al* (1983)). A tumour-induced IL-1 response potentially accounts for emaciation of lean body mass, fever, malaise and REE increase observed in cancer cachexia.

Barber *et al* (2002) analysed genomic DNA from 64-pancreatic cancer patients compared to 101 healthy controls. A polymorphism in the IL-1β gene significantly affected the inflammatory response and patient prognosis. Homozygotes for allele 2 of the IL1-β gene exhibited significantly greater IL-1β and CRP production, hence shorter survival. Heterozygosity for

allele 2 was associated with significantly higher IL1-β and CRP production and inferior survival (although less significant than the homozygotes). These findings implicate a role for IL1-β in patient prognosis, cancer cachexia and acute phase protein response and may provide valuable information when determining patient therapy in future.

Nakatani *et al* (1998) examined IL-1β levels in C3H/He MH-134-bearing mice. Levels of IL-1β mRNA were significantly increased in the spleens of tumour bearers compared to pair fed controls. No other cytokine mRNA showed a significant increase. These results suggest IL-1β over-expression occurs in response to tumour burden.

1.10.12.3 Interferon-γ (IFNγ).

Interferon-γ is produced by T-lymphocytes. IFN-γ has anti-viral activity in addition to inducing MHCI and MHCII expression on macrophages and inducing differentiation of cytotoxic T lymphocytes. IFN-γ promotes synthesis of IgG2a by activated B cells and antagonises several actions of IL-4 (as reviewed by Roitt (1997)).

Matthys *et al* (1991) inoculated nude mice with either CHO tumour cells containing an expression plasmid with the MuIFN-γ cDNA, (CHO/IFN-γ cells) or with CHO cells containing the unmodified plasmid (CHO/control cells). A dose-dependent (and potentially lethal) cachexia was seen to develop in the CHO/IFN-γ mice, but not the CHO/control mice. This syndrome was attenuated by twenty-four hour pre-treatment with anti-IFN-γ-antibodies. Evidence from pair-fed controls demonstrated reduced food intake alone to be insufficient at explaining the emaciation. Matthys *et al* observed early and rapid disappearance of interscapular fat and suggested a prominent role for IFN-γ in this model of cachexia.

Kaplan *et al* (1990) documented a phase II trial of recombinant human IFN-γ in the treatment of cutaneous T-cell lymphoma. Kaplan *et al* noted that anorexia and weight loss were the most common side effects during this study, again suggesting that IFN-γ mediates weight loss of some description.

Perhaps of more interest to our group is the implication that IFN-γ stimulates lymphocytic release of ZAG, a homologue of the 43KDa LMF present in MAC16 tumours and the urine of weight-losing cancer patients. This is discussed in detail in **section 1.8.1.5.1.1**.

1.10.12.4 Interleukin 6 (IL-6).

Interleukin-6 is produced by CD4-bearing T lymphocytes, macrophages, mast cells and fibroblasts. IL-6 induces growth and differentiation of B- and T- cell effectors and haematopoietic precursors and induces the acute phase protein response (as reviewed by Roitt (1997)).

Scott *et al* (1996) observed that cachectic non-small cell lung cancer (NSCLC) patients with at least 5% weight loss consistently possessed higher circulating IL-6 concentrations than in non-weight losing patients. The authors demonstrated significant correlation ($p<0.001$) between circulatory IL-6 concentrations and C-reactive protein (CRP), implicating IL-6 as an important mediator of the acute phase response seen in cancer cachexia.

Barton & Murphy (2000) transplanted human myeloma cells into severe combined immunodeficient mice (SCID) mice. The splenocytes of these mice began expressing IL-6, and the IL-6 related-cytokines IL-11 and oncostatin M (OSM). The authors postulated that

tumour burden may induce IL-6 production at sites adjacent to and distant from the tumour, and that the release of these cytokines promotes cancer cachexia.

Barton and Murphy (2001) grew the lung carcinoma line LL/2 in C57BL/6J mice. This tumour model induced the expression of IL-6-like cytokines within a week. Cytokine expression in this model preceded cachexia (IL-6, IL-11 and OSM were induced on days 3, 1 and 2 respectively, and cachexia did not commence until day 7). The authors propose that cachexia is a consequence of host-mediated inflammatory response to the tumour.

Korner *et al* (2000) transplanted MCG 101 tumours onto wild type and IL-6 knockout C57Bl mice. Wild-type tumour bearers developed profound cachexia in conjunction with rapid tumour growth. Similarly, Ogata *et al* (1999) propose IL-6 to act as a growth factor for malignant cells.

Conversely, Soda *et al* (1994) transplanted the cachexigenic subclone 20 murine colon adenocarcinoma and another similar non-cachexigenic subclone (clone 5) into CDF_1 mice. Following induction of cachexia, clone 20 mice did indeed exhibit a rise in blood IL-6 levels; however, a similar rise was seen in the tumour-bearing clone 5 mice that did not experience cachexia. These findings suggest that although many groups implicate IL-6 in cancer cachexia, other factors are needed to produce the true symptoms of the disorder.

Soda *et al* (1994) also reported that IL-6 did not possess lipolytic activity *in vitro* when incubated with 3T3-L1 adipocytes. Similarly, Ishibashi *et al* (1993) reported that IL-6 administered to mice resulted in increased platelets and maturation of megakaryocytes but few adverse side effects.

Although it would appear that an IL-6 response is initiated as a result of malignancy, the degree to which this propagates the induction of cachexia remains unclear. There is convincing evidence that IL-6 initiates cachexia in some animal models, but the extent to which it is involved in clinical cachexia remains undetermined.

1.10.12.5 Oncostatin M (OSM).

Tanaka and Miyajima (2003) reviewed the role of OSM. OSM is a multifunctional cytokine belonging to IL-6 superfamily. OSM shares highest degree of homology with LIF and utilises the LIF receptor in addition to its own specific receptor.

Originally, OSM was recognised for it's ability to inhibit tumour cell proliferation, but new evidence points to roles for OSM in inflammation, haematopoiesis and development (Tanaka and Miyajima (2003))

Barton and Murphy (2000 & 2001) proposed a role for OSM as an IL-6 like cytokine in cancer cachexia (**section 1.10.12.4**). Interestingly, Repovic *et al* (2003) demonstrated that OSM acts synergistically with IL-1β to amplify COX-2 mRNA and increase PGE2 synthesis.

OSM could potentially be implicated in some models of cancer cachexia. The degree of influence of OSM in human cachexia has yet to be determined.

The MAC16 model implicates LMF and PIF as the main mediators of cachexia with minimal (though not necessarily none) cytokine involvement. Administration of monoclonal antibodies raised against TNF-α were ineffective in attenuating MAC16 cachexia. Moreover, no elevation of IL-6 was observed during the weight loss associated with this model (Mulligan *et al* (1992)).

The MAC16 model possesses apparent homology with clinical cachexia, where cytokine data cytokines is conflicting and incomplete (McDevitt *et al* (1995)). Indeed, PIF and LMF can be purified from the urine of cachectic patients and disappear when therapy is succeeding.

In reality, cancer cachexia is the consequence of many interrelated metabolic disorders and is unlikely to involve only a single cytokine or factor (Soda *et al* (1994)). The importance of considering all the factors *in vivo* in order to achieve better understanding and therapeutics is thus underscored.

1.10.11 Therapeutic Intervention.

The most effective intervention to cure cachexia is to remove the cancer. In many advanced cases this will obviously not be possible, with other means being required to ameliorate the cachectic state.

1.10.11.1 Anticytokine Therapy.

Haslett (1998) has reported a trial of the TNF-α inhibitors pentoxyfylline and thalidomide in cachexia. Although evidence of TNF-α inhibition was observed, pentoxyfylline was ineffective at reversing weight loss. Similarly, Fearon & Moses (2002) report pentoxyfylline- inhibited of

TNF-α induced protein degradation in a rat model but failed to demonstrate a beneficial effect in patients.

Thalidomide administration to patients with tuberculosis or HIV-associated cachexia has resulted in some weight gain and may prove beneficial to cachectic cancer patients (either alone or in combination). However, the complex interactions of thalidomide with the immune system remain poorly understood and this may well preclude it from such a study (Haslett (1998)).

1.10.11.2 Total Parenteral Nutrition (TPN).

If host weight loss resulted solely from anorexia or host-tumour competition for nutrients, administration of TPN should reverse the weight loss. TPN provides all the host nutrients and calorific requirements in a manner that by-passes the GI tract (thus ensures absorption).

Jordan *et al* (1981) reported inferior survival for NSCLC patients when combining TPN with conventional chemotherapy. Similarly, Nixon *et al* (1981) trialled 45 patients with colon cancer and observed a significant decrease in survival following TPN administration. Quite why TPN should result in a poorer prognosis is unknown: Some investigators suggest that by nutritional repletion of the protein-depleted host stimulates further tumour growth (Popp *et al* (1981)); however, explanations of this phenomenon remain equivocal.

This decreased survival raised concerns about administration of calorific support for weight-losing patients, especially in the absence of effective chemotherapy.

There is no question that TPN is highly beneficial to specific groups of cancer patients (for example short-term nutritional support helps to prevent post-surgical complications for patients with rescectable cancers). However, the suitability of a patient for Total Parenteral Nutrition must be very carefully considered if serious consequences are to be avoided.

1.10.11.3 Megestrol Acetate.

Megestrol acetate is a progestational agent that promoted weight gain when administered to metastatic breast cancer patients. Studies have demonstrated megestrol acetate to increase appetite, body mass and patient quality of life (Rowland *et al* (1996)).

Several randomised trials have suggested megestrol acetate to improve appetite and induce weight gain in various groups of cancer patients (Fearon & Moses (2002, Review)).

Megestrol acetate may provide some benefit as it is reported (Mantovani *et al* (1998)) to down-regulate the synthesis and release of cytokines, hence could attenuate the acute phase protein and REE response associated with cancer cachexia.

Rowland *et al* (1996) enrolled 243 weight-losing, extensive-stage small-cell lung cancer patients and orally administered 800mg/d megestrol acetate or placebo. This group reported non-fluid weight gain, with a reduction in nausea and vomiting. However, patients receiving megestrol acetate displayed significant oedema, an inferior response to conventional chemotherapy and inferior survival.

Problematically, megestrol acetate (and related agent medroxyprogesterone acetate) has numerous side effects, including venous thrombosis and peripheral oedema. The frequency of oedema, and the fact that patient weight gain is predominantly fat and water (with no arrest of lean tissue depletion) means that the use of megestrol acetate in cancer cachexia is questionable (Fearon & Moses (2002, Review)).

1.10.11.4 Ketogenic Diets.

Cancer cachexia involves extensive mobilisation of body fat stores but ketosis does not occur. Ketone bodies play an important role in the preservation of lean body mass in starvation. It was suggested a ketogenic diet (high fat / low carbohydrate) might preserve lean body mass during cancer cachexia (the tumour being dependent predominantly on glucose for it's energy requirements) (Tisdale & Brennan (1988)).

It was observed that MCT administration to MAC16 rodents induced a greater anti-cachectic effect than LCT administration, with a reduction in weight loss and tumour growth (Tisdale & Brennan (1988)). MCT-administered mice exhibited an increase in plasma acetoacetate and 3-hydroxybutyrate – a factor demonstrated previously to inhibit lipolytic and proteolytic factors associated with cachexia (Beck & Tisdale (1987)).

Fearon *et al* (1988) similarly reported weight gain following administration of a 3-hydroxybutyrate-supplemented 70% MCT ketogenic diet to cachectic cancer patients.

1.10.11.5 Non-Steroidal Anti Inflammatory Drugs (NSAIDs).

Administration of 400mg ibuprofen three times daily has proved effective at reducing REE, levels of acute phase proteins, IL-6 and cortisol. A randomised trial of the PG-synthesis inhibitor indomethacin (50mg twice daily) demonstrated this drug to significantly prolong patient survival (as reviewed by Fearon & Moses (2002)).

1.10.11.6 Amino Acids.

It has been speculated that during muscle wasting, amino acids that are not normally essential in a healthy individual become essential in a tumour bearing state. Thus it has been proposed that the amino acids derived biosynthetically from α-ketoglutarate (glutamine, glutamic acid, arginine, ornithine, citrulline and proline) be supplemented following stress, surgery or cancer (as reviewed by Baracos (2001)). However, relevant studies are sparse and inconclusive and the obvious danger is that administration of these molecules might stimulate further tumour growth.

1.10.11.6 Eicosapentaenoic Acid.

The fish-based diet of the Eskimo favours lower incidence of cancers than the western diet. Speculation that the omega-3 (ω-3) fatty acids in fish possessed anti-tumour properties led to the development of eicosapentaenoic acid as an anti-cachectic drug (Tisdale & Dhesi (1990)).

ω-3 fatty acids arrested tumour development *in vivo* for R323OAC mammary tumours, 7,12 dimethylbenz(a)anthracene induced mammary tumours, DU-145 prostatic tumours and the 1376MAT:B metastatic mammary carcinoma.

EPA reduces PGE2 synthesis, influences the direction of estrone hydroxylation, exerts an anti-metastatic effect and normalises levels of adenosine diphosphate ribosyl transferase (Kritchvsky (1996)). ω-3 FA's such as EPA possess immunomodulatory properties and suppress pro-inflammatory cytokine responses. Administration of EPA to pancreatic cancer patients reduced IL-1 and IL-6 production by peripheral-blood mononuclear cells (PBMC) and suppressed the ability of patients PBMC supernatants to stimulate CRP production *in vitro* (Wigmore (1997)).

EPA ameliorates the effects of the proteolysis inducing and lipid-mobilising factors isolated by this group. Whitehouse & Tisdale (2001) demonstrated excised gastrocenemius muscle from EPA treated MAC16 mice lack the increased ubiquitination associated with this model. Furthermore, EPA directly suppresses the production of proteasome subunits induced by PIF, possibly by suppression of 15-hydroxyeicosatetraenoic acid (Whitehouse & Tisdale (2001)). A two-hour preincubation with 50μM EPA significantly reduces lipolysis *in vitro*. The effect of EPA is mediated through plasma membrane Gi – either directly (through complex formation) or indirectly (through a modification of membrane fluidity) (Price SA (1998)).

Wigmore *et al* (1996) investigated dietary supplementation with MaxEPA, a complex fish oil containing EPA. Eighteen patients with unrescectable pancreatic cancer received 1X 1g fish oil capsule (18% EPA, 12% docosahexaenoic acid (DHA)) daily. Significant arrest of weight loss was seen, in addition to a decreased acute phase protein response and a reduced REE. Several small-scale trials (Barber *et al* (1999) & Wigmore *et al* (2000)) involving EPA have demonstrated further it's efficacy at reversing the weight loss and metabolic abnormalities associated with cachexia.

More recently, the BH80 clinical trial demonstrated the efficacy of EPA as an anti-cachectic agent: 200 patients with non-rescectable pancreatic adenocarcinoma (median age 68 (61-76 IQR)) with >5% body weight loss were enrolled. This nutritional supplement consisted 310Kcal and 16g protein, with or without 1.1g EPA> Patients were administered either 2X n-3 fatty acid enriched supplement per day or 2X placebo per day. Patient compliance was extremely poor, however, once accounted for, the EPA supplement significantly decreased weight loss when compared to placebo-administered controls ($p=0.0009$).

It is quite apparent that cachexia is a multifactoral condition that is difficult to treat. The complex nature of the syndrome means that TPN is relatively ineffective at reversing weight loss. ω-3 fatty acid administration shows great promise at attenuating cachexia, and future therapeutics are likely to at least include this in their treatment regimes.

1.11 Aims of Study.

Previous studies (Russell (2001), Russell *et al* (2002) (amongst others)) have characterised the tumour derived Lipid Mobilising Factor described in **section 1.8.1.4**. However, at present, the complete role for this glycoprotein in cancer-associated cachexia remains unclear.

The aim of this study therefore, is to further characterise this important factor. As our understanding has grown, a multifactoral role for this molecule in cachexia became evident. This work will therefore examine this molecule from a number of different angles, in order that we might better understand its role in cancer cachexia, which, in turn may generate more successful therapeutics.

LMF and ZAG are to be purified, co-characterised and compared, with specific examination of the carbohydrate moieties associated with these molecules (**chapter 3**). Bioassay of these molecules will be performed using isolated murine WAT (**chapter 3**), in addition to validation of an *in vitro* 3T3-L1 adipocyte bioassay (**chapter 6**). The involvement of an LMF-induced lipogenic reduction (if any) in the emaciation of fat stores will also be examined (**chapter 6**), as will the involvement of the mitogen-activated pathway (**chapter 6**).

The LMF homologue ZAG is endogenous (Russell (2001)). It is now known that host BAT and WAT tissue produce the ZAG adipokine (Bing *et al* (2003)). It is also known that MAC16 tumours produce ZAG directly (Todorov *et al* (1998)), but unknown whether host ZAG production rises during cachexia. The effect (if any) of the cachectic state and direct ZAG administration *in vivo* on host ZAG regulation will therefore be examined (**chapter 7**).

Previous studies (Wyke SM (unpublished data), Richardson S (unpublished data)) examined the proteolytic digest of the LMF and demonstrated the existence of a low molecular weight active fragment. Other instances of similar molecules are reported in the literature (**section 1.8.1**). These findings may imply that (at least some of) these groups have purified out LMF derivatives (presumably the molecule undergoes cleavage *in vivo*). An improved immobilised trypsin HPLC method (**chapter 5**) will be employed in order to further characterise this fragment, and produce sufficient yield (which previous protocols did not) to allow sequencing. ZAG will be co-assayed in order to provide further evidence of homology. As a useful aside, this study may yield important data regarding oral administration of this molecule – important in its development as an anti-obesity drug.

The uncoupling protein homologues are described in (**section 1.10.11**). Any increase in UCPn would potentiate energy loss and enhance the negative energy balance associated with cachexia. These molecules thus warrant investigation and may provide therapeutic targets. The up-regulation of these mitochondrial carriers is described in cachexia models (Bing *et al* (2000)), and more specifically following administration of the LMF (Bing *et al* (2002)). It is necessary to determine whether the LMF directly or in directly up-regulates these molecules (**Chapter 9**). UCP2 is implicated in ROS detoxification (discussed in **Chapter 10**). If LMF regulates this molecule, this may facilitate the detoxification of certain chemotherapeutic drugs, thus promoting tumour survival (examined in **chapter 10**).

Glucose aberrations in cancer cachexia have been reported (Russell & Tisdale (2002)). Similarly, some groups report insulin resistance to occur in certain cachexia models. The effect of effect of LMF +/- insulin on MAC16 cachexia will therefore be examined (**chapter 8**).

It is widely reported that β-AR agonists increase protein synthesis in host muscle (Mersmann (1998)). LMF/ZAG are reported β_3-AR agonists (Russell (2001) & Russell *et al* (2002)), thus may theoretically induce this effect, with important implications for the cachectic host (not to mention in the development of this molecule as an anti-obesity agent). Thus the effect of ZAG on protein synthesis *in vitro* warrants examination (**chapter 3**). Previous studies (Islam-Ali & Tisdale (2001)) have similarly examined the LMF, so this study should provide useful comparison.

These studies seek to promote insight into the underlying mechanisms of LMF and cachexia, provide further evidence for LMF/ZAG homology (wherever possible) and yield data to facilitate the development of ZAG as an anti obesity agent.

CHAPTER 2. MATERIALS.

2.1 Animals.

Pure strain NMRI mice were obtained from inbred colonies. Rodents were housed at an ambient temperature of 22°C+/-2°C under a 12h light/dark cycle. Animals were fed a standard chow diet from Special Diet Services, Lillico, Wonham Mill, Bletchworth, Surrey) with fresh tap water *ad libitium*. Animals were humanely sacrificed prior to 25% loss of body weight.

2.2 Chemicals.

All chemicals were obtained at highest available purity from Sigma-Aldrich, Dorset, UK unless otherwise stated below:

Amersham Biosciences (Buckinghamshire, UK)

ECL Western blotting detection reagents

HyBond nitrocellulose membrane

Hyperfilm ECL

Hypercassette

Peristaltic pump

L-[2,6-^{3}H]Phenylalanine (sp act 2.07 TBq/mmol)

2-deoxy-D-[2,6-^{3}H]glucose (sp act 1.63 TBq/mmol)

Rainbow coloured protein molecular weight markers

Resource Iso reverse-phase HPLC column

Autogen Bioclear (Wiltshire, UK)

Mouse Anti-Human ZAG monoclonal antibody.

•BDH (Poole, UK)

pH Indicator paper

Bio-Rad Laboratories (Richmond, CA, USA)

Ammonium persulphate (APS)

Electrophoresis and transferring apparatus

Inner & outer electrophoresis plates

Protein assay reagents

16.5% tris-tricene ready gels

Silver protein stain kit

British Oxygen Company ltd (London, UK)

5% CO_2 in air.

Calbiochem (San Diego, CA, USA)

Anti-Uncoupling Protein1 Antibody, Mouse (Rabbit)

Anti-Uncoupling Protein2 Antibody, Mouse (Rabbit)

Dako Cytomation (Glostrop, Denmark)

Rabbit α-Mouse HRP conjugated 2° Antibody

Goat α-Rabbit HRP conjugated 2° Antibody

Fischer Scientific Apparatus (England, UK).

Acetic Acid (Glacial)

Sodium Dihydrogen Orthophosphate

Water (HPLC-Grade)

Fison Scientific Equipment (Loughborough, UK)

Sodium dihydrogen orthophosphate

Magnesium sulphate

Gelman Sciences (Northampton, UK)

YM membranes, 10KDa MWCO

Microcon Concentrators, 10KDa MWCO

GIBCO BRL Life Technologies (Paisley, Scotland, UK)

DMEM (1X)

Foetal Calf Serum (FCS)

Glutamine (100X)

Horse Serum

Penicillin Streptomycin (100X)

RPMI 1640 (1X)

Trypsin (100X)

Oxoid (Basingstoke, UK)

Phosphate Buffered Saline (PBS) Tablets.

Perseptive Biosystems (Framingham, MA USA)

Poroszyme immobilised HPLC trypsin cartridge

Research Diagnostics Inc (Flanders, NJ, USA)

Rabbit anti-Rat UCP3 Antibody (100% Cross-Reactivity With Mouse (18/14aa))

Standard Laboratory Reagents

Acetone

Chloroform

Deionised Water

Ethanol

Hydrochloric Acid

Methanol

Methylated Spirits

Perchloric Acid

Whatman International Ltd (Maidstone, England, UK)

DEAE Cellulose

2.3 Buffers.

2.3.1 Purification of a Tumour-Derived LMF.

2.3.1.1 Phosphate Buffered Saline.

PBS Tablet	1 tablet/500ml dH_2O

2.3.1.2 Resource-Iso LMF Purification Buffer A.

50mM Phosphate Buffer in 1.5M Ammonium Sulphate

Anhydrous disodium orthophosphate dihydrate	7.1g/L 1.5M AMS
Sodium dihydrogen orthophosphate	7.8g/L 1.5M AMS

pH adjusted to 7.0 (dihydrogen added to disodium).

2.3.1.3 Resource-Iso LMF Purification Buffer B.

50mM Phosphate buffer in Deionised Water

Anhydrous disodium orthophosphate dihydrate	7.1g/L dH2O
Sodium Dihydrogen orthophosphate	7.8g/L dH2O

pH adjusted to 7.0 (Dihydrogen added to disodium)

2.3.2 Bioactivity Assessment.

2.3.2.1 Krebs Ringer Bicarbonate Buffer.

Sodium chloride	118mM
Potassium chloride	5mM
Calcium chloride	2mM
Potassium Dihydrogen orthophosphate	1mM
Sodium bicarbonate	25mM
Magnesium sulphate	1mM

2.3.2.2 Krebs Ringer/BSA Buffer.

3% Bovine serum albumin in Krebs-Ringer bicarbonate buffer.

2.3.2.3 Collagenase Solution.

4mg Collagenase in 1ml Krebs-Ringer/BSA buffer.

2.3.2.4 10% Perchloric Acid.

83.33ml Perchloric acid in 500ml deionised water.

2.3.2.5 40% Potassium Hydroxide.

40g potassium hydroxide in 100ml deionised water

2.3.2.6 Glycerol Estimation Spectrophotometer Buffer.

Triethanolamine	100mM
Magnesium sulphate	2mM
Phosphoenolpyruvate (PEP)	0.4mM
α-Nicotinamide adenine dinucleotide (NADH)	0.25mM
Adenosine 5' triphosphate (ATP)	1.2mM
Pyruvate kinase	1.0 Units/ml
Lactate dehydrogenase	7.0 Units/ml

Buffer prepared on ice at 4°C.

Volume adjusted to 100ml with deionised water.

pH is adjusted to 7.4 with 30% Perchloric acid.

2.3.3 12% SDS Electrophoresis.

2.3.3.1 Sample Denaturising Buffer.

20% SDS	4ml
Glycerol	2.4ml
2-mercaptoethanol	0.4ml
Brilliant Blue G (0.1%)	2ml
1M TRIS-HCl (pH 6.8)	1ml

Adjust volume to 20ml with deionised water.

2.3.3.2 12% Resolving Gel.

Deionised water	3.3ml
30% acrylamide/bis acrylamide	4.0ml
1.5M TIRS (pH 8.8)	2.5ml
10% SDS	0.1ml
10% ammonium Persulphate (APS)	0.1ml
TEMED	0.004ml

2.3.3.3 5% Stacking Gel.

Deionised water	2.5ml
30% acrylamide/bis acrylamide	0.67ml
1.0M TRIS (pH 6.8)	0.5ml
10% SDS	0.04ml
10% ammonium Persulphate (APS)	0.04ml
TEMED	0.004ml

2.3.3.4 10x Electrophoresis Buffer.

Trizma base	30.29g
Glycine	144.3g
10% SDS	100ml

Volume adjusted to 1000ml with deionised water.

Diluted 1:10 with deionised water for use.

2.3.3.5 Coomassie Brilliant Blue Stain.

Coomassie Brilliant Blue R250 Stain	0.1%
Glacial Acetic Acid	10%
Methanol	25%

Volume adjusted to 100ml with deionised water

2.3.3.6 Coomassie Blue Gel Destain Solution.

Glacial Acetic Acid	10%
Methanol	25%

Volume adjusted to 100ml with deionised water.

2.3.3.7 Silver Stain Fixing Solution.

Methanol	40%
Glacial Acetic Acid	10%
Deionised water	50%

2.3.3.8 Silver Stain Oxidising Solution.

Diluted 1:10 with deionised water for use.

2.3.3.9 Silver Stain Silver Nitrate Solution.

Diluted 1:10 with deionised water for use.

2.3.3.10 Silver Stain Developing Solution.

Developer	6.4g
Deionised water	200ml

2.3.3.11 Silver Stain Stopping Solution.

Glacial Acetic Acid	5%
Deionised Water	95%

2.3.4 LMF Carbohydrate Studies.

2.3.4.1 10x Blotting Transfer Buffer.

Trizma base	30.3g
Glycine	144.0g
SDS	15g

Diluted for use, 100ml buffer, 200ml methanol, 700ml dH_2O.

2.3.4.2a 0.1% PBS-Tween.

PBS	5 tablets
Tween 20	0.5g
Deionised water	500ml

2.3.4.2b 0.5% PBS Tween.

PBS	5 tablets
Tween 20	2.5g
Deionised water	500ml

2.3.4.3 Membrane Blocking Solution.

Marvel non-fat milk	5g
Deionised water	95ml

2.3.4.4 WGA Affinity Column Buffer.

Tris-HCl (pH 7.4)	10mM
NaN3	0.02%

2.3.4.5 WGA Affinity Column Elution Buffer.

Tris-HCl (pH 7.4)	10mM
NaN3	0.02%
N-acetylglucosamine	0.1M

2.3.4.6 Gelcode Glycoprotein Fixing Solution.

Methanol	50%
Deionised Water	50%

2.3.4.7 Gelcode Glycoprotein Wash Buffer

Glacial acetic acid	3%
Deionised water	97%

2.3.4.8 Gelcode Glycoprotein Oxidation Reagent.

Ready for use.

2.3.4.9 Gelcode Glycoprotein Stain Reagent.

Ready for use.

2.4 Effect Of ZAG on Protein Synthesis/Degradation & RNA Accretion.

2.4.1 C_2C_{12} Growth Media.

Dulbecco's modified eagles media (DMEM)	500ml
Foetal calf serum	10%
Penicillin streptomycin	1%
Glutamine	1%

2.4.2 C_2C_{12} Differentiation Media.

Dulbecco's modified eagles media (DMEM)	500ml
Horse serum	2%
Penicillin streptomycin	1%
Glutamine	1%

2.4.3 C_2C_{12} Chasing Media.

DMEM without phenol red	500ml
Horse serum	2%
Penicillin streptomycin	1%
Glutamine	1%

2.4.4 1X Trypsin Solution.

100X Trypsin Solution	10ml
Sterile PBS	90ml

2.4.5 [^{3} H] Phenylalanine Working Stock-Solution.

L-[2,6-^{3}H] phenylalanine	500µl
Phenylalanine	60mg
Sterile PBS	4.5ml

2.5 *In vitro* 3T3-L1 Lipolytic Factor Studies.

2.5.1 3T3-L1 Growth Media

Dulbecco's modified eagle media	500ml
Foetal calf serum	10%
Penicillin Streptomycin	1%
Glutamine	1%

2.5.2 3T3-L1 Differentiation Media 1.

Dulbecco's modified eagle media	500ml
Foetal calf serum	10%
Penicillin streptomycin	1%
Glutamine	1%
Methylisobutylxanthine	0.5mM
Dexamethasone	0.25µM
Insulin	1µg/ml

2.5.2 3T3-L1 Differentiation Media 2

Dulbecco's modified eagle media	500ml
Foetal calf serum	10%
Penicillin streptomycin	1%
Glutamine	1%
Insulin	1μg/ml

2.6 LMF Proteolytic Digestion Work.

2.6.1 Porozyme Cartridge Digestion Buffer.

1M TRIS	50ml
Deionised Water	550ml
$CaCl_2 2H_2O$	3.14g

pH adjusted to 8.0 with sodium hydroxide.

Volume adjusted to 1000ml with deionised water.

2.6.2 HPLC Digest Purification HPLC Mobile Phase 1

Triflouroacetic acid (TFA)	0.06%
HPLC-grade water	99.94%

2.6.3 HPLC Digest Purification HPLC Mobile Phase 2

Triflouroacetic acid	0.04%
HPLC-grade acetonitrile	99.96%

2.6.4 Low Mr Electrophoresis Cathode Buffer.

Trizma base	12.1g
Tricene	17.92
SDS	1g

Volume adjusted to 1litre with deionised water.

pH adjusted to 8.2.

2.6.5 Anion Buffer (X5)

Trizma base	1M

pH adjusted to 8.9.

2.6.6 Low Mr Electrophoresis Fixing Solution.

Methanol	40%
Glacial acetic acid	10%
Deionised water	50%

2.6.7 Low Mr Electrophoresis Staining Solution.

Coomassie Brilliant Blue G	0.025%
Glacial acetic acid	10%
Deionised water	89.975%

2.6.8 Low Mr Electrophoresis Destain Solution.

Glacial acetic acid	10%
Deionised water	90%

2.7 Effect of LMF on Soleus Glucose Transport.

2.7.1 Krebs-Ringer Henseleit Buffer.

$NaHCO_3$	25mM
NaCl	118mM
KCl	4.7mM
$MgSO_4$	1.2mM
NaH_2PO_4	1.2mM
$CaCl_3$	1.2mM

2.7.8 Krebs-Ringer Henseleit/BSA Buffer.

Krebs-Ringer Henseleit Buffer	99%
Bovine serum albumin	1%

2.8 Western Blotting Solutions

2.8.1 Uncoupling Protein Preparation / Sonication Buffer.

HEPES	100mM
Sucrose	10%
Tergitol (NP-40)	0.1%
Protease Inhibitor Cocktail	1 tablet/100ml

2.8.2 Tissue Homogenising Buffer.

Sucrose	250mM
EDTA	0.2mM
HEPES	238.3mg

Volume adjusted to 1000ml. pH adjusted to 7.0.

For further western blotting solutions, see also 2.3.3 and 2.3.4.1 - 2.3.4.3.

2.9 Reactive Oxygen Species Studies.

2.9.1 MAC13 Growth Media.

RPMI-1640	500ml
Foetal calf serum	10%
Penicillin streptomycin	1%
Glutamine	1%

For further solutions used in ROS study, see 2.3.3 and 2.3.4.1 - 2.3.4.3.

3.0 Primary Culture Media.

3.0.1 Primary Culture of WAT (Media A).

DMEM	500ml
HEPES	25mM
Collagenase	1mg/ml
Defatted BSA	4%
Penicillin Streptomycin	1%
Glutamine	1%

3.0.1 Primary Culture of WAT (Media B).

DMEM	500ml
FCS	2%
Defatted BSA	1%
Penicillin Streptomycin	1%
Glutamine	1%

3.1 Primary Culture of BAT.

3.1.1 Primary Culture of BAT (Media A)

DMEM	500ml
Newborn Calf Serum	10%
Sodium Ascorbate	25μg/ml
HEPES	10mM
Insulin	40Units/ml
Penicillin Streptomycin	1%
Glutamine	1%

3.1.2 Primary Culture of BAT Digestion Buffer.

Media A (**3.1.1**)	100ml
Collagenase	0.2g
HEPES	0.24g

3.1 Introduction:

Beck & Tisdale (1987) provided evidence for the operation of a lipid mobilising factor in the murine MAC16 cachexia model. This factor was later isolated (Hirai *et al*, 1998) from the urine of cachectic pancreatic cancer patients, and found homologous to the endogenous peptide Zinc-$_{\alpha 2}$-Glycoprotein with regards to amino acid sequence (Todorov *et al* (1998) & Russell *et al* (2003)), lipid mobilising activity (Hiari *et al* (1998)), electrophoretic mobility and immunoreactivity (**figure 3.1.1**). Moreover, antisera to ZAG neutralises LMF-induced lipolysis *in vivo* Hiari *et al* (1998)).

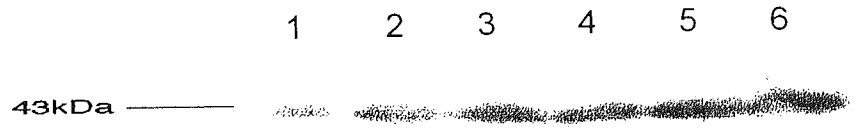


Figure 3.1.1. Western analysis of purified ZAG (lanes 1-3) and LMF (lanes 4-6). Detection was with polyclonal antibody to hZAG (Sanders & Tisdale (2003)).

ZAG stimulates lipolysis *in vitro* in a dose-dependent manner, enhanced by the cyclic AMP phosphodiesterase inhibitor Ro 20-1724, and attenuated by selective β_3 adrenorecpeptor antagonism with SR-59230A (Russell *et al* (2003)). Similarly LMF activity is cyclic-AMP dependent (Khan & Tisdale (1999)) and mediated through the β_3-adrenorecpetor (Russell (2001)). ZAG and LMF gave similar molecular weights by MALDI-TOF analysis (Zimmerman T, personal communication) and no difference was seen in spectral analysis of LMF and ZAG between 275-280nm (Russell (2001)).

An enormous amount of evidence therefore suggests these molecules to be the same. Some studies described herein employ LMF, some employ ZAG, and wherever possible both are employed, characterised and compared simultaneously. Unfortunately, problems concerning acquisition of purified ZAG and cachectic urine have meant that direct comparison has not always been possible. Individual chapters employing one factor or the other are valid in their own right, but note that the underpinning assumption of this thesis (for which more evidence will be presented in due course) is that ZAG and LMF are homologues.

This preliminary chapter aims to describe the purification of human LMF and ZAG from the cancer urine (**section 3.2.2**) and human plasma (**section 3.2.6**) respectively. The quantification, bioassay and further characterisation of each factor will also be described and performed (**section 3.2.3 – 3.3.5**), with particular attention being paid to analysis of the carbohydrate moieties associated with these molecules (**section 3.2.9 – 3.2.11**). This study seeks to lend further insight into the mechanisms of LMF/ZAG and to allow direct comparison of the two.

3.2 Methodology:

3.2.1 Patient Recruitment

LMF was purified from BH80-enrolled pancreatic cancer patients receiving either EPA or placebo. Urine was collected over 24 hours at timepoints 0 weeks, 4 weeks and 8 weeks. Patients had median age of 68 and had lost >5% body weight at baseline (trial details c/o Ross Nutrition). Urine was collected by medical staff (at Edinburgh Royal Infirmary), and stored at -20°C until required. Freeze-thaw cycles were avoided.

3.2.2 Purification Of LMF:

1 litre of cachectic urine was centrifuged (10,000 rpm, 15 minutes) to remove particulates. Urine was co-incubated (120 minutes, 4 °C with stirring) with 9 litres 10mM Tris.HCl (pH 8.0) and 20g pre-activated DEAE cellulose. DEAE cellulose was pelleted using low speed centrifugation (4,500 rpm, 3 minutes) and washed twice with 10mM Tris.HCl (pH 8.0). LMF was eluted from the DEAE cellulose using 0.5M NaCl in 10mM Tris.HCl (pH8.0).

The resulting solution was dialysed and concentrated against Phosphate Buffered Saline (PBS) using a medium (100ml capacity) Amicon stirred ultrafiltration cell fitted with a 10kDa molecular weight cut off (MWCO) membrane. Further concentration was achieved as required using 10kDa MWCO microcon units.

Individual 50µl extract injections were subjected to reverse-phase High Performance Liquid Chromatography (HPLC). Separation was achieved using a Resource-Iso™ column against a decreasing $(NH_4)_2SO_4$ gradient. LMF had a retention time of 13-15 minutes with elution at 0.6M $(NH_4)_2SO_4$ (Russell (2001)). LMF fractions were transferred to a small stirred Amicon ultrafiltration cell fitted with a 10kDa MWCO membrane. Eluate was washed then desalted against PBS five times and concentrated to a final volume of 1ml.

3.2.3 Total Protein Quantification:

Total protein was assessed using Bio-Rad protein reagent. This kit utilises the method of Bradford (1976). Briefly, 10µl of test solution was mixed with 790µl deionised water and 200µl of protein reagent. Following a 5-minute reaction, the solution was transferred to a cuvette and absorbance estimated at 595nm. Unknowns were calibrated against a deionised water control.

Protein concentration was calculated using the following formula:

$$(Abs_{595}/0.053^{a}) \times 0.1^{b} = [Protein]\ (\mu g/\mu l)$$

a= gradient obtained from BSA calibration curve

b= dilution factor

3.2.4 Homogeneity Assessment (12% SDS-PAGE):

Purity was assessed by means of 12% SDS-PAGE followed by Coomassie brilliant blue staining. Briefly, a LMF preparation was diluted with sample buffer (**section 2.3.3.1**)to 5µg/10µl and denatured for 5 minutes at 90°C. 12% resolving and 5% stacking gels (**section 2.3.3.2** and **2.3.3.3**) were prepared and Mini Protean II electrophoresis apparatus (or equivalent) was constructed. 10µl (5µg) of diluted sample was loaded and electrophoresed at 180V in 1X electrophoresis buffer (**section 2.3.3.4**) against protein molecular weight markers.

Proteins were visualised by 30 minutes destaining (**section 2.3.3.6**), followed by 1 hour Coomassie blue staining (**section 2.3.3.5**). Gels were destained further until background was reduced as desired. The presence of a single band at 43kDa was indicative of a homogenous preparation; other bands were the result of protein contamination. Transfer into a 10% glycerol solution (7 days) and subsequent preparation using Promega gel-drying apparatus achieved gel preservation.

A poor protein yield necessitated use of the more sensitive silver staining kit. Briefly, fixer (40% MeOH, 10% acetic acid, 50% dH_20) was added to the gel on a shaker for 30 minutes. Fixer was removed and (1:10) oxidase reagent added (10 minutes), after which the gel was

washed (3 x 5 minutes with dH_2O). Water was removed and 1:10 silver nitrate reagent added (20 minutes). Following a 30-second wash, 6.4g/ml developer reagent was added and replaced every 30 seconds until gel development. The reaction was terminated with 5% acetic acid (15 minutes). Gels were preserved in the usual manner.

3.2.5 Bioactivity Assessment:

An *in vitro* bioassay was prepared according to the method of McDevitt *et al* (1995). Briefly, epidydimal fat pads of sacrificed male ex-breeder mice were excised under sterile conditions. Fat pads were macerated in 3%Krebs BSA /collagenase solution, equilibriated with 2% CO_2 and incubated at 37°C for 30 minutes. Addition of Krebs buffer floated mature adipocytes to the solution surface, allowing isolation. Following washing, the adipocyte suspension was added to 3%Krebs BSA to produce a cell concentration of 10^5 cells/ml. Cell aliquots were transferred to reaction vessels as required by the assay (**Table 3.2.5.1**):

	Positive Control	Negative Con	LMF Test Sampl
Cell Suspensi	900µl	900µl	900µl
10µM Isopren	5µl	/	/
Krebs	95µl	100µl	90µl
LMF (8µg)	/	/	10µl

Table 3.2.5.1 Preparation of lipolysis assay reaction vessels for bioassay according to the method of McDevitt et al (1985). See ***section 3.2.5*** *for further methodology.*

Following equilibration with 2% CO_2, samples were agitated at 37°C for 120 minutes. Proteins were removed by perchloric acid precipitation and centrifugation (13,000 rpm), and subsequent isolates were neutralised with 40% KOH.

Adipocyte glycerol release was assumed a function of bioactivity and monitored according to the method of Weiland (1974). Briefly, 830 μl of spectrophotometer buffer (**section 2.3.2.6**) was added to 200μl sample at 4°C. The assay reaction (**Fig 3.2.5.1**) was initiated by addition of 1.0 Units glycerol kinase

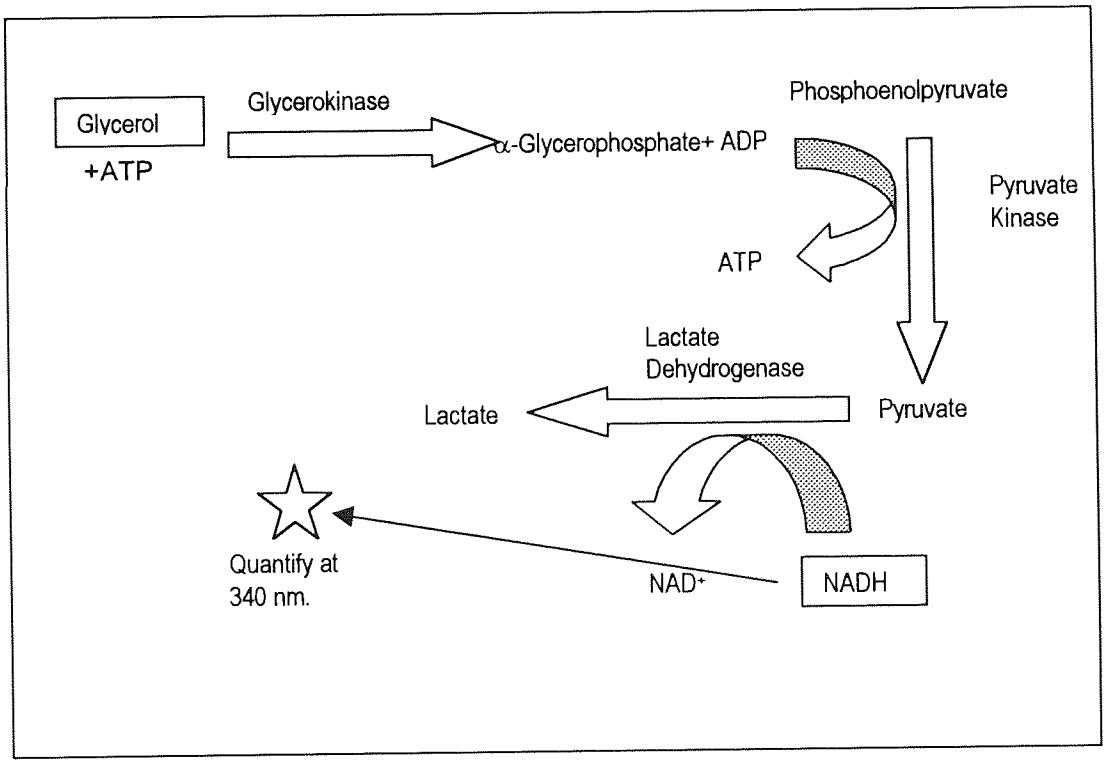


Fig 3.2.5.1; Reaction profile of glycerol assay (as described by Weiland (1974)). Initial glycerol concentration correlates with NAD+ production, which is measured spectrophotometrically at 340nm (adapted from Russell (2001)).

The following equation adjusts for dilution and NADH extinction coefficients and calculates LMF bioactivity as $\mu g/10^5$ cells / 120 minutes.

$$\text{(Control adjusted change in sample } Abs_{340}) * \frac{\text{Dilution Factor}^a}{6.22^b}$$

a – Dilution factor = 10
b – Extinction coefficient for NADH

3.2.6 Purification of Zinc-α_2-Glycoprotein.

ZAG was purified by Bayer Corporation from human plasma as described by Russell *et al* (2003]). Briefly, fraction V effluent was diluted to 16mM with 1.0M zinc acetate. This mixture was stirred for 30 minutes at 0°C and then centrifuged at 16,900g at 4°C for a further 30 minutes. Addition of 1.0M barium sulphate precipitated the supernatant and achieved a final concentration of either 4.0mM or 20mM (depending on the behaviour of each batch of fraction V effluent). Following stirring (30', 0°C) and centrifugation (60,900g, 4°C, 30'), the barium precipitate was dissolved to approximately 450ml in 10mM Tris-HCl (pH8). pH was adjusted to pH7.7 – pH 7.9 using 1M Tris-HCl (pH8) and the solution passed through a 0.22μm filter.

Redissolved barium precipitate was loaded onto a 5.0 x 17cm Q Sepharose FF column (pre-equilibrated with 10mM Tris-HCl (pH 8.0). Fraction elution utilised either a 1500ml linear gradient of 0-0.23 M NaCl or a 500ml isocratic gradient of 0.17M NaCl (both in 10mM Tris-HCl (pH 8.0). Fractions were pooled for further analysis depending on impurity (assessed by 8-25% native PAGE). Selected fractions were concentrated to 3-5ml on Amicon YM3 membranes, and the concentrate loaded onto a 2.6 x 60 cm Superdex 75 column (pre-

equilibrated with sterile PBS). Collection of 5ml fractions ensued. Residual endotoxin was removed by passage through a 1.6 x 18cm acticlean ETOX column against sterile PBS.

3.2.7 Bioassay of Purified Zinc-α_2-Glycoprotein.

It was necessary to determine whether purified ZAG (**section 3.2.6**) would possess/retain homologous lipolytic activity to LMF. Concerns were raised about potential glycerol and endotoxin contamination. Bioassay (**section 3.2.5**) was therefore performed on ZAG batch # 11-12 with low (0.001 μg/μl) and high (0.01 μg/μl) endotoxin controls (these represent levels that could potentially be co-purified). It has been reported (Russell (2001)) that ZAG possesses 2-fold less activity than LMF (**section 3.3.3**). Therefore, 25μg ZAG/assay is used for analysis.

3.2.8 Bioassay of ZAG Q Sepharose Pooled Fractions:

Five pools were obtained following purification (**section 3.2.6**) and Q Sepharose fractionation of ZAG 27-29. These pools analysed by Iso-Electric Focussing (IEF), pH 3-6 (**figure 3.2.8.1**). Each pooled fraction was subjected to bioassay (**section 3.2.5**) to examine any differences in isoformic activity.

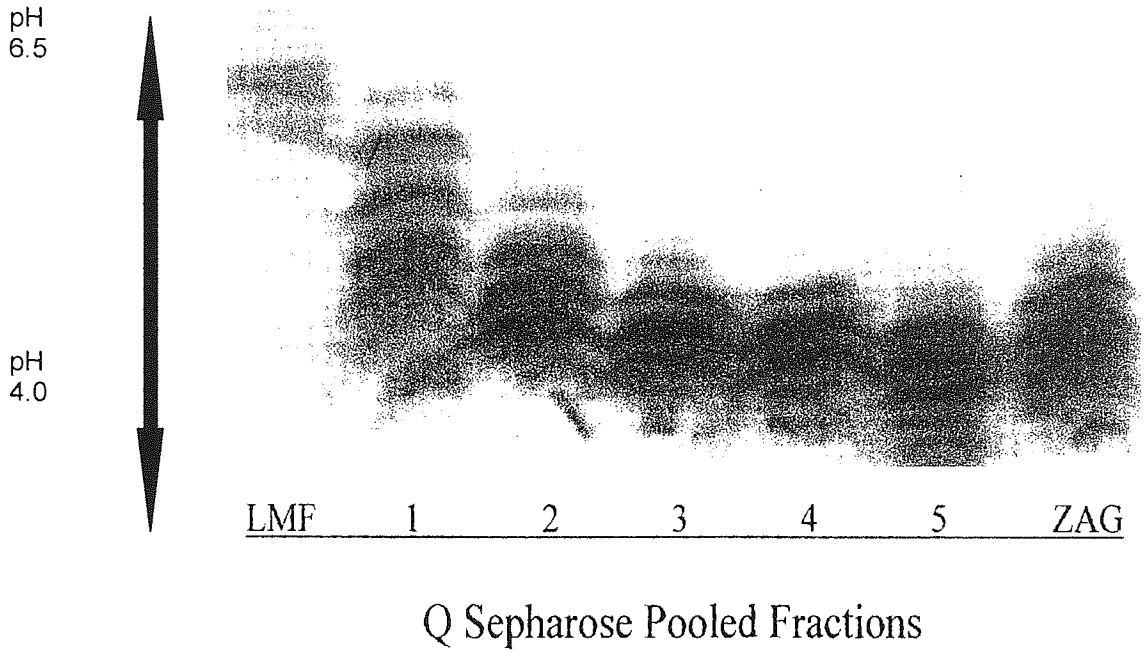


*Figure 3.2.8.1; Five pools of ZAG were obtained following purification and Q Sepharose fractionation pf ZAG 27-29 (see **section 3.2.6**). Samples were subjected to iso-electric focussing (IEF) PAGE (pH 4.0-6.5) and visualised following probing with α-ZAG (IEF PAGE kindly performed by Bayer Corporation).*

3.2.9 Characterisation of LMF Carbohydrate Moieties.

Carbohydrate analysis of LMF was performed by lectin blotting (as described by Todorov *et al* (1996) & in 'Glycobiology, a practical approach' (1993 (Fukada & Kobata (Eds))). Briefly, LMF was prepared and resolved on a 12% gel (as described in **section 3.2.4**) and transferred in 1X transfer buffer (**section 2.3.4.1**) onto 0.45μm Hybond A nitrocellulose membrane (80V, 120

minutes). Membranes were incubated in 5% block (**see 2.3.4.3**) at 4°C overnight to prevent non-specific binding, and then washed in 0.5% PBS-Tween 20 (**section 2.3.4.2b**) (PBS-T) for 3 x 15 minutes.

Membranes were probed (90 minutes, room temperature) with peroxidise labelled lectins - either wheat germ agglutinin (WGA) or concavalin A (ConA) at 1:500 dilutions. Membranes were washed for 4 x 15 minute intervals with 0.1%PBS-T (**section 2.3.4.2a**). Bands were detected on Kodak photographic film following treatment with ElectroChemiLuminesence (ECL) reagents.

3.2.10 Wheat Germ Agglutinin Affinity Purification of LMF.

It was investigated whether WGA interactions with the *N*-acetyl-glucosamine moieties of LMF could be used in an affinity purification procedure.

A column was packed with sepharose-immobilised WGA and pre-equilibrated with 10mM Tris-HCl (pH 7.4) / 0.02% NaN_3. 50μg of protein from a heavily contaminated LMF preparation was loaded and the column washed with 20 bed volumes of 10mM Tris-HCl (pH 7.4) / 0.02% NaN_3. Bound material was eluted by addition of 10ml 0.1M *N*-acetyl-glucosamine in 10mM Tris-HCl (pH 7.4) / 0.02% NaN_3.

3.2.11 Glycoprotein Staining of WGA Column Eluate:

Samples obtained from **section 3.2.10** were assayed for protein content (**section 3.2.3**), denatured, electrophoresed (**section 3.2.4.**) and subjected to Glycoprotein analysis. Carbohydrate-containing proteins were visualised using the Gelcode™ Glycoprotein staining kit. Gels were fixed in 50% MeOH (30 minutes) then washed (2 x 10 minutes) in 3% acetic acid. Gels were transferred into oxidation reagent (15 minutes) then washed again with 3% acetic acid (3 x 5 minutes). Gelcode™ Glycoprotein stain was added (15 minutes), followed by reduction reagent (5 minutes). Finally, gels were washed with 3% acetic acid followed by deionised water. Visualisation of magenta bands indicated the presence of carbohydrate-containing proteins.

3.3 Results & Discussion.

3.3.1 Purification of LMF.

The reverse-phase component of LMF purification gave distinct retention times and good separation from other proteins. Two isoforms of LMF are apparent (having retention times of approximately 13 and 15 minutes). Which isoform predominates will vary with the patient and no isoform is preferentially purified as both are validated to have biological activity (Russell 2001). An example trace is shown in **figure 3.3.1.1**

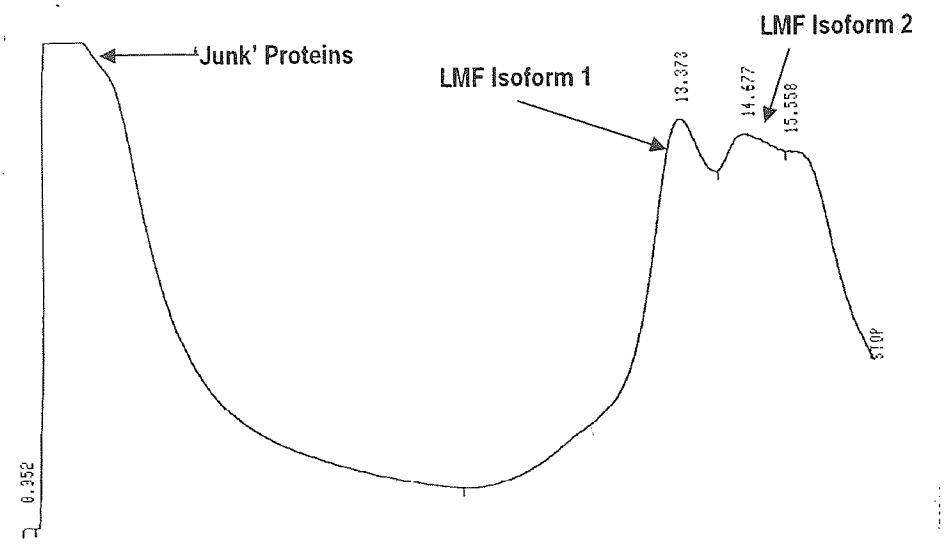


***Figure 3.3.1.1**; Example HPLC LMF elution profile. Separation was achieved using reverse-phase HPLC against a decreasing $(NH_4)_2SO_4$ gradient. LMF was eluted at 0.6M $(NH_4)_2SO_4$ with a retention time of 13-15 minutes. Trace clearly shows presence of two distinct LMF isoforms.*

Following purification procedure, electrophoresis and staining (**section 3.2.4**), homogenous preparations yield a single band at 43kDa (**Figure 3.3.1.2**)

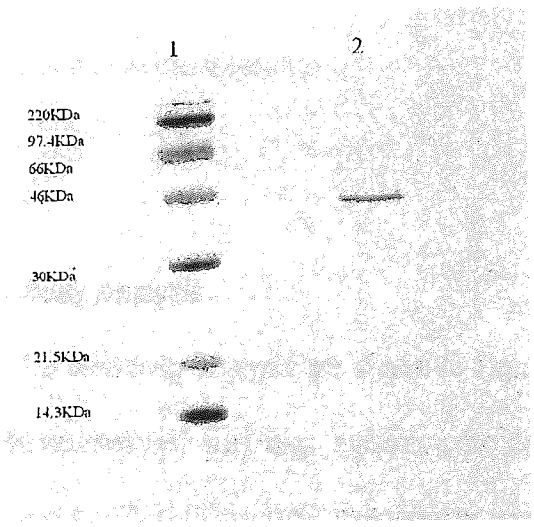


*Figure 3.3.1.2; 12% SDS-PAGE analysis of LMF purified from human urine (**section 3.2.2**). Lane 1 MW markers; Lane 2 LMF. Detection was by Coomassie brilliant blue staining followed by background destaining (see **section 3.2.4**)*

If both isoforms observed in **figure 3.3.1.1** are obtained, a double band at 43kDa appears on the gel above. ZAG homology is seen once again by the presence of a doublet of similar

molecular weight following 12% SDS-PAGE and Coomassie staining (Zimmerman T, personal communication).

Final preparation quality was seen to vary with the patient. Occasionally, other bands appear on **figure 3.3.1.2**, the most commonly observed impurities being albumin (66kDa) and an unknown contaminant of approximately 30kDa. The advanced catabolism associated with terminal cancer produces high concentrations of protein and breakdown products in the urine, some of which are difficult to remove from the final preparation. Any circulatory proteins of <67kDa are filtrated from the blood by the glomerulus and thus appear in urine (Lafitte *et al* (2002)) and hence may be co-purified with LMF. The similar HPLC retention times of albumin (12 minutes) and LMF (13-15 minutes) make this difficult to separate and hence is the most frequent contaminant.

The sequencing and characterisation of commonly observed LMF preparation impurities may well yield interesting and valuable results.

3.3.2 ZAG Bioactivity Analysis.

Results of the ZAG bioactivity analysis are shown in **Figure 3.3.2.1**. Endotoxin (at potential co-purification concentrations) was also subjected to bioassay. Neither concentration of endotoxin displayed significant bioactivity compared to negative controls ($p > 0.05$). Although higher endotoxin concentrations would certainly produce activity in this assay, these concentrations represent the 'worst case scenario' co-obtained with ZAG despite the great measures that were taken to eliminate endotoxin entirely (**section 3.2.3**).

Both 10μM isoprenaline and 25μg ZAG demonstrated statistically greater bioactivity than negative controls ($p < 0.01$), with no difference between the active groups ($p > 0.05$). These findings strongly support a lipolytic mechanism for ZAG and are very comparable with those previously obtained for LMF (Russell (2001) & Hiari *et al* (1998)).

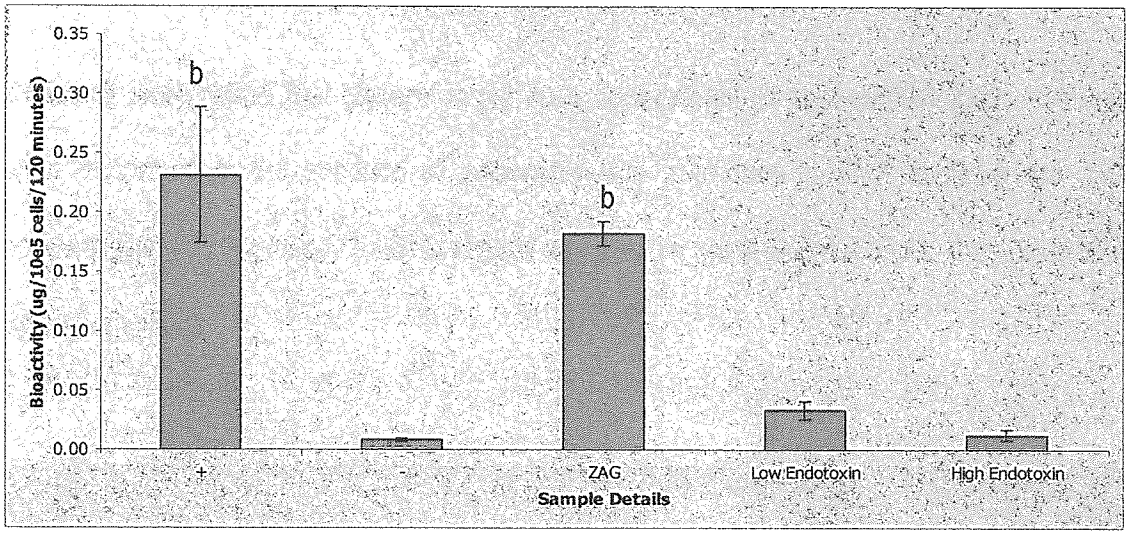


*Figure 3.3.2.1; Bioassay of ZAG versus potential endotoxin contaminants using the method of McDevitt et al (1985) (**section 3.2.5**). Data are means (n=3) +/- SEM. One way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from negative control are shown as b, $p<0.01$.*

Inter-assay variation for glycerol release does occur due to variations in adipocyte viability. The most creditable way to perform inter-assay comparison is as percent positive control. Here ZAG is seen to be 78.9% of the isoprenaline response.

3.3.3 Bioactivity of ZAG Q-Sepharose Separated Samples.

Homology between LMF and ZAG is demonstrated in **Figure 3.2.8.1** – both factors were resolved by IEF PAGE followed by ZAG antibody probing. Despite isoformic variation, both factors resolve similarly and display identical immunoreactivity.

Q-Sepharose pooled samples (**Figure 3.2.8.1**) were analysed for bioactivity (**section 3.2.5**). Unfortunately, fraction yield following this procedure was extremely poor – pool 1 contained only enough protein for one assay. The other fractions provided sufficient for *n*=3 (all at 25μg/assay).

Concerns were raised that glycerol might be a contaminant of post-purified ZAG. Controls were performed in the absence of adipocytes and a mixture of ZAG fractions with final concentration of 25μg/assay (*n*=3) to ensure any positive result was not due to the presence of external glycerol.

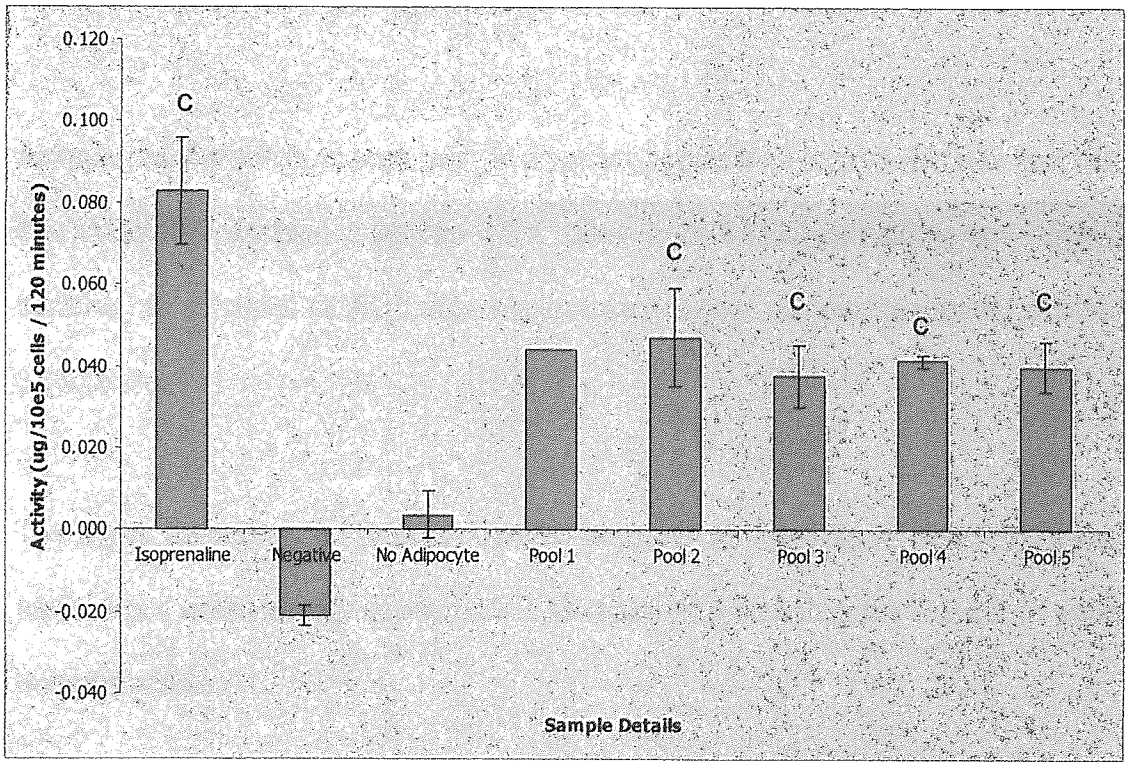


*Figure 3.3.3.1; Bioactivity analysis (method of McDevitt et al (1985)) of ZAG Q Sepharose pooled samples (obtained from **figure 3.2.8.1**) data are n=3 +/- SEM except pool 1 due to poor yield (not included in statistical analysis). Pools 2-5 analysed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Difference from negative control are shown as c, p<0.001*

Poor yield of pool 1 (hence n=1) precluded it from statistical analysis. However, it appears of similar activity to pools 4-5 which are all extremely significant against negative controls ($p<0.001$). Encouragingly, bioactivity does not appear to result simply from glycerol contamination (Pools 2, 3, 4 and 5 all being significantly different from no adipocyte controls ($P<0.01$, <0.05, <0.05 and <0.05 respectively)).

Speculation was that pool 1 would display greatest bioactivity due to the greatest degree of isoformic homology with LMF. It may be that only some 5% of a total ZAG preparation possesses activity (i.e. those fractions sharing isoformic homology with LMF). This correlates to a certain extent with observations made in this laboratory.

As expected, the activity of each pool (as a percent isoprenaline control) was less than that of the 'whole' ZAG assayed in **section 3.3.2**. Thus, a ZAG batch constituting any number of fractions has activity of 78.9% control, whereas each fraction has reduced activity of 51% +/- 5.6% control.

The slight differences in isoformic activity accounts for inter-batch bioactivity variation (unpublished observations), as each ZAG/LMF batch may constitute any number of different isoform fractions.

Figure 3.2.8.1 demonstrates different isoformic distributions between LMF and ZAG. As only one gene for ZAG is reported (Araki *et al* (1998)), it is likely these isoforms result from differences in post-translational modification (Russell (2001)). Kamboh & Ferrell (1986) demonstrated ZAG isoforms to differ in sialic acid content, the more acidic LMF apparently

possessing greater sialic acid content. Glycosylation and post-translational modification are essential for bioactivity (Alberts *et al* (Eds (1998)). The 2-fold increase of LMF activity over ZAG probably results from further tumour-induced glycosylation of ZAG peptides.

The exact mechanism by which the tumour modifies ZAG (or ZAG expression) in clinical cachexia is poorly understood. This is discussed in more detail in **Chapter 7**.

3.3.4 Glycoprotein Analysis of LMF.

LMF lectin probing was performed to investigate surface moieties. Surface carbohydrates are essential for bioactivity – unglycosylated seminal ZAG is lacks bioactivity (Hirai *et al* (1998)), whilst ZAG (EZAG) produced by *E.coli* lacking in post-translational apparatus is also inactive (Russell (2001)).

A heavily contaminated (to determine the nature of other protein contaminants also) LMF preparation was electrophoresed and transferred (**section 3.2.9**) then probed with peroxidase-labelled wheat germ agglutinin. The results are shown in **figure 3.3.4.1**.

Figure 3.3.4.1; Glycoblotting of heavily contaminated LMF preparation. Following electrochemiluminsence and developing, two bands are clearly seen at approximately 43kDa on a relatively clear gel.

Two bands at approximately 43-46kDa are clearly visible on this membrane. It would appear that the WGA associated with both isoforms, but failed to interact with the other proteins in the contaminated prep. *Triticum vulgaris* wheat germ agglutinin binds *N*-acetylglucosamine residues (Todorov *et al* (1996)). Both isoformic variants of LMF therefore appear to have associated *N*-acetylglucosamine. This moiety is also attached to the 24kDa proteolysis-inducing factor, where it is thought to confer bioactivity (Todorov *et al* (1997)). Because WGA does not appear to bind to other proteins present in the contaminated preparation, this can perhaps be monopolised for affinity purification.

Peroxidase-labelled concavalin A also demonstrated an affinity for LMF. ConA binds to mannose residues, indicating these too were present on the LMF molecule. Unfortunately this probe gave high background, hence these blots are not suitable for inclusion here (this problem has also been encountered by previous researchers (Tisdale MJ, personal communication)). Con A did not exclusively interact with LMF – the presence of many other bands indicating a great number of mannose-containing proteins in a preparation. Con A is not a candidate therefore for an affinity purification procedure.

3.3.5 Affinity Purification of LMF Using Immobilised Wheat Germ Agglutinin.

It was determined whether high-affinity interactions between LMF and WGA could be utilised to affinity purify LMF as described in **section 3.2.10**. The LMF prep used was of extremely poor quality (homogeneity analysis shown in **Figure 3.3.5.1**). This investigation was performed to enhance purification at a time when patient urine consistently yielded inferior LMF preparations.

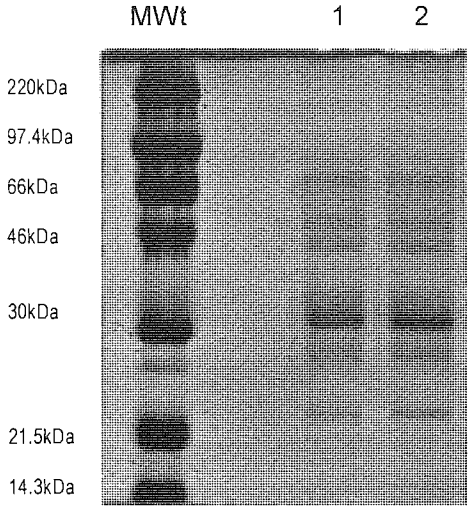


Figure 3.3.5.1. Coomassie blue staining of a heavily contaminated LMF preparation following 12% SDS-PAGE.

The eluate was analysed. A poor yield of protein necessitated silver staining to excess - this resulted in extremely high background value, however, a band was clearly observed at 30KDa (with faint bands at higher molecular weights also). Additionally, eluate was further stained for glycoprotein as described in **section 3.2.11**. Results in **Figure 3.3.5.2**

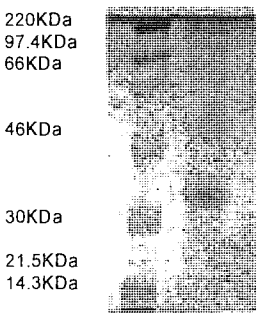


Figure 3.3.5.2. Glycoprotein staining of eluate from sepharose-immobilised WGA column. Note presence of strong contaminant band at approx 30kDa.

LMF clearly has associated carbohydrate moieties – certainly *N*-acetylglucosamine and probably mannose. The potential presence of other similarly glycosylated molecules in cachectic urine precludes an affinity chromatography procedure as part of LMF purification.

3.4 Conclusions.

ZAG and LMF have been successfully purified (from plasma and urine respectively) with minimal loss of bioactivity. LMF and ZAG are homologous although slight differences in carbohydrate moieties are apparent. Issues regarding endotoxin and glycerol contamination of ZAG appear unfounded, with this factor demonstrating 'real' bioactivity. Resolution of ZAG / LMF on IEF PAGE followed by hZAG antibody probing reveals a number of isoforms - a large proportion of which display significant bioactivity. Lectin probing revealed valuable information about the activity-conferring carbohydrate groups but the presence of other glycosylated contaminants precluded instigation of an affinity purification step (although this has yet to be repeated with ZAG).

The similarities between ZAG and LMF demonstrated in this chapter add further credibility to the theory that the two are homologues. Furthermore, this chapter produced important information regarding the structure and functioning of these two important molecules.

CHAPTER 4. EFFECT OF ZINC α-2-GLYCOPROTEIN ON PROTEIN SYNTHESIS / DEGRADATION AND RNA ACCRETION.

4.1 Introduction.

Cancer cachexia comprises unintentional weight loss of both host muscle and proteins. Plasma from MAC16 tumour-bearers possesses twice the lipolytic activity (1.29+/- 0.04 µmol FFA/h/ml plasma) of control animals (0.63 +/- 0.05 µmol FFA/h/ml) ($p<0.001$), and from animals bearing the MAC13 tumour (0.596µmol FFA/h/ml of plasma). This plasma lipolytic activity was observed to peak at 16% body weight loss (Groundwater *et al* (1990)), then decline.

Cell-free extracts of the MAC16 tumour also caused enhanced release of amino acids from mouse diaphragm, whereas the extracts from the non-cachexigenic MAC13 and MAC15A tumours did not. This proteolytic factor is present in the urine of patients whose weight loss exceeds 1.5kg/month (as is the lipolytic factor), and this decreases when the patient is responding to chemotherapy (Smith HJ, personal communication).

It is the 24kDa proteolysis-inducing factor that mediates skeletal muscle protein wasting in clinical cachexia, without an apparent effect on adipose tissue (Todorov *et al* (1996)). The associated 43kDa Lipid Mobilising Factor depletes host fat stores, but, antagonistically to PIF, actually causes an increase in lean carcass mass (Hirai *et al* (1998)). Interestingly, it is only after the decline in serum lipolytic activity (i.e. at 16% body weight loss) in MAC16-bearers, that the effects of PIF become apparent, further indicating antagonism by these two factors (Groundwater *et al* (1990)).

It is well known (Mersmann (1997) among others) that administration of β-AR agonists promotes an increase in lean body mass. It is hardly surprising therefore, that LMF, a reported (Russell (2001)) β_3-AR agonist, also induces this effect.

Previous studies (Islam-Ali & Tisdale (2001)) report purified LMF to induce a dose and cyclic AMP-dependant increase in protein synthesis *in vitro* using C_2C_{12} myoblasts. C_2C_{12} myoblasts lack many of the functional receptors of mature myocytes (Whitehouse AS, personal communication). The aim of this study is to therefore employ a 'more appropriate' model (i.e. fully differentiated C_2C_{12} myotubes). As this study employs ZAG, comparison with the work performed with LMF by Islam-Ali & Tisdale (2001) should also provide data regarding homology of these two factors.

This study therefore examine the effect of the LMF homologue ZAG on fully differentiated C_2C_{12} myotubes with regards to protein synthesis and RNA accretion.

4.2 Methodology.

4.2.1 Maintenance of C_2C_{12} Myoblasts.

C_2C_{12} myoblasts were maintained in 75cm^3 flasks using DMEM supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. At semi-confluence, cells were treated with 1x trypsin and sub-cultured. Passage number was not permitted to exceed seventeen.

4.2.2 Measurement of Protein Degradation in C_2C_{12} Myotubes.

ZAG was purified from fraction V effluent as described in **section 3.2.6**. ZAG antibody was isolated as follows; two rabbits were immunised with 1mg >99% pure ZAG. The rabbits were bled and confirmation of a high sera titre against ZAG was followed by purification of antibody by Protein A chromatography (Zimmerman T, personal communication).

Six-well multiwell plates were seeded with $2x10^5$ C_2C_{12} myoblasts, which were grown as described in **section 4.2.1**. Following achievement of 90% confluence, differentiation was induced by addition of DMEM supplemented with 2% horse serum, 1% penicillin streptomycin and 1% glutamine under similar atmospheric conditions. Myotubes were used experimentally no later than four days post-differentiation.

500µl of L-[2,6-^{3}H] phenylalanine was added to 60mg phenylalanine (unlabelled) and dissolved in 4.5ml sterile PBS to produce a working stock solution. 10µl of working stock was added to each experimental well and incubated for 24 hours (37°C, 5% CO_2) to allow cellular incorporation.

Cells were washed twice with sterile PBS and chased for 2 hours with DMEM (without phenol red) supplemented with 2% horse serum, 1% penicillin streptomycin and 1% glutamine. Media was discarded and substituted for fresh. Inhibitors (SR-59230A and rabbit α-human (polyclonal) ZAG antibody) were added 2 hours prior to experimentation. Experimental wells were set up to contain either 50μg ZAG (n=3), 50μg ZAG with 10μM SR-59230A (n=3), 50μg ZAG with 1:500 α-ZAG (n=3) or 50μl PBS (n=3).

Following a 24-hour incubation, 1ml of media was transferred into a 20ml scintillation vial and mixed thouroughly with 8ml Ultima Gold XR scintillation fluid. Sample radioactivity was assessed as disintegrations per minute (dpm) over two minutes using a 2000CA Tri-Carb liquid scintillation analyser.

4.2.3 Protein Synthesis Assessment.

Six-well multiwell plates were seeded with $2x10^5$ C_2C_{12} myoblasts, which were grown as described in **section 4.2.1**. Following achievement of 90% confluence, differentiation was induced by addition of DMEM supplemented with 2% horse serum, 1% penicillin streptomycin and 1% glutamine under similar atmospheric conditions. Myotubes were used experimentally no later than four days post-differentiation.

Protein synthesis was assessed according to the method of Southourn & Palmer (1990). Eighteen hours prior to experimentation, media was substituted with unsupplemented DMEM. Inhibitors (SR-59230A and rabbit α-human (polyclonal) ZAG antibody) were added as required 2 hours prior to experimentation. Experimental wells were prepared to contain either 50μg

ZAG (n=3), 50μg ZAG with 10μM SR-59230A (n=3), 50μg ZAG with 1:500 α-ZAG (n=3) or 50μl PBS (n=3). Plates were incubated for 30 minutes (5% CO_2 in air, 37°C), after which 10μl/well of phenylalanine working stock (**section 4.2.2.**) was added and the samples incubated for a further 60 minutes (5% CO_2 in air, 37°C).

The reaction was arrested by washing three times with 1ml of ice-cold sterile PBS (pH 7.4). Following removal of PBS, 1ml/well of ice-cold 0.2M perchloric acid (PCA) was added and samples incubated for 20 minutes at 4°C. The PCA was then substituted for 1ml of 0.3M NaOH/well for 30 minutes at 4°C. This reaction was then removed to an incubator and maintained at 37°C for a further 20 minutes.

The NaOH (which now contained dissolved cellular proteins) was pipetted into a 15ml centrifuge tube. Each well was further washed with 1ml 0.3M NaOH, which was also transferred to the same tube. 0.5ml of 0.2M PCA was added to the mixture, which was placed on ice for 20 minutes to precipitate the cellular proteins.

Sample tubes were centrifuged (700g, 5 minutes at 4°C), and the RNA-containing supernatant decanted and estimated as described in **section 4.2.4**. The protein-containing pellet was dissolved in 1ml 0.3M NaOH. 0.5ml of solution was transferred into a 20ml scintillation vial and mixed thouroughly with 8ml Ultima Gold XR scintillation fluid. Sample radioactivity was assessed as disintegrations per minute (dpm) over two minutes using a 2000CA Tri-Carb liquid scintillation analyser.

4.2.4 Measurement of RNA Accretion.

RNA samples were prepared and isolated as described in **section 4.2.3**. RNA was estimated as reported by Morrison (1995), the absorbance of the sample being assessed spectrophotometrically at 232nm and 260nm. The following equation corrects for the presence of aromatic amino acids and calculates the RNA content/ml of supernatant (and thus the RNA content per well).

$$\text{RNA cellular content } (\mu g/ml) = [Abs_{260}*(32.9)] * [Abs_{232}*(6.11)]$$

4.3 Results and Discussion.

4.3.1 Protein Degradation Estimation.

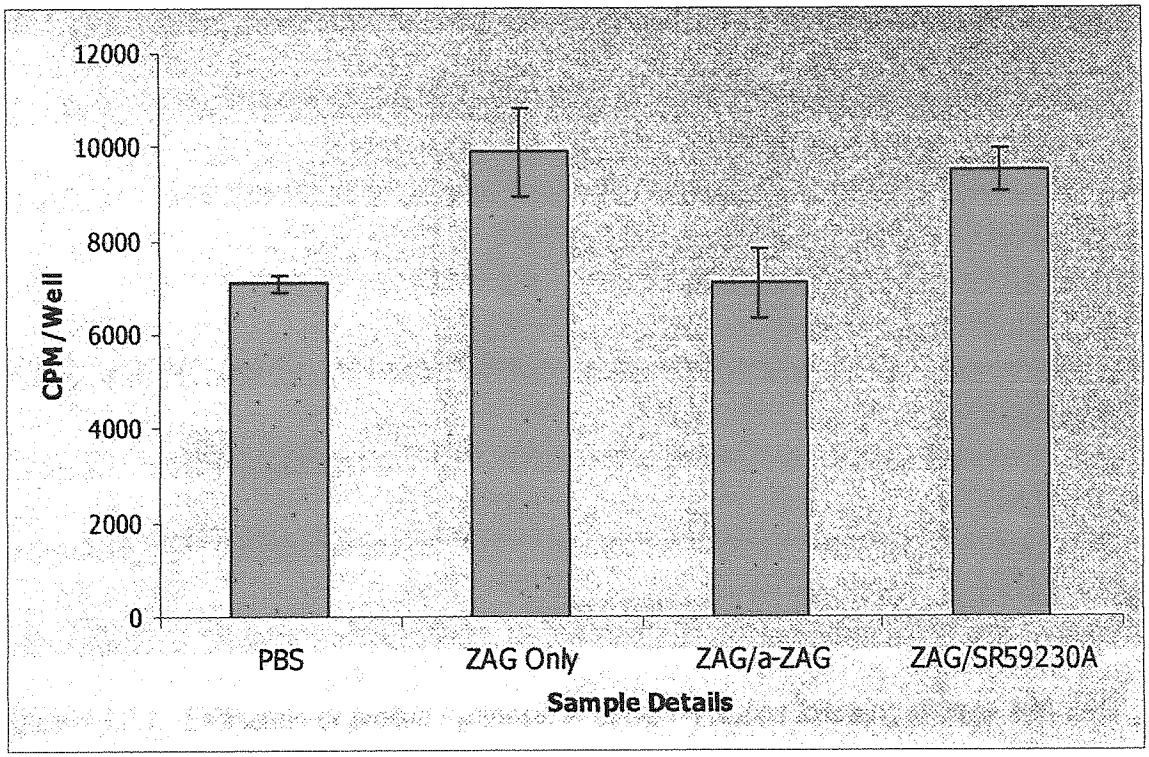


Figure 4.3.1.1. Estimation of protein degradation in C_2C_{12} myotubes following dosage with ZAG (25μg/ml) in the presence of various inhibitors. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test.

One-way ANOVA followed by Tukey-Kramer multiple comparison revealed no significant difference between experimental groups. LMF/ZAG has never been implicated in the induction of protein degradation. However, Islam-Ali & Tisdale (2001) report a slightly significant ($p<0.05$) decrease in 'chymotrypsin-like' proteolytic activity of C_2C_{12} myoblasts and a similar decrease ($p<0.05$) in tyrosine release in soleus muscle excised from exbreeder mice receiving LMF. However, these assays are distinct from the phenylalanine release assay – perhaps the lower sensitivity of which (Wyke SM, personal communication) explains these discrepancies.

4.3.2 Protein Synthesis Estimation.

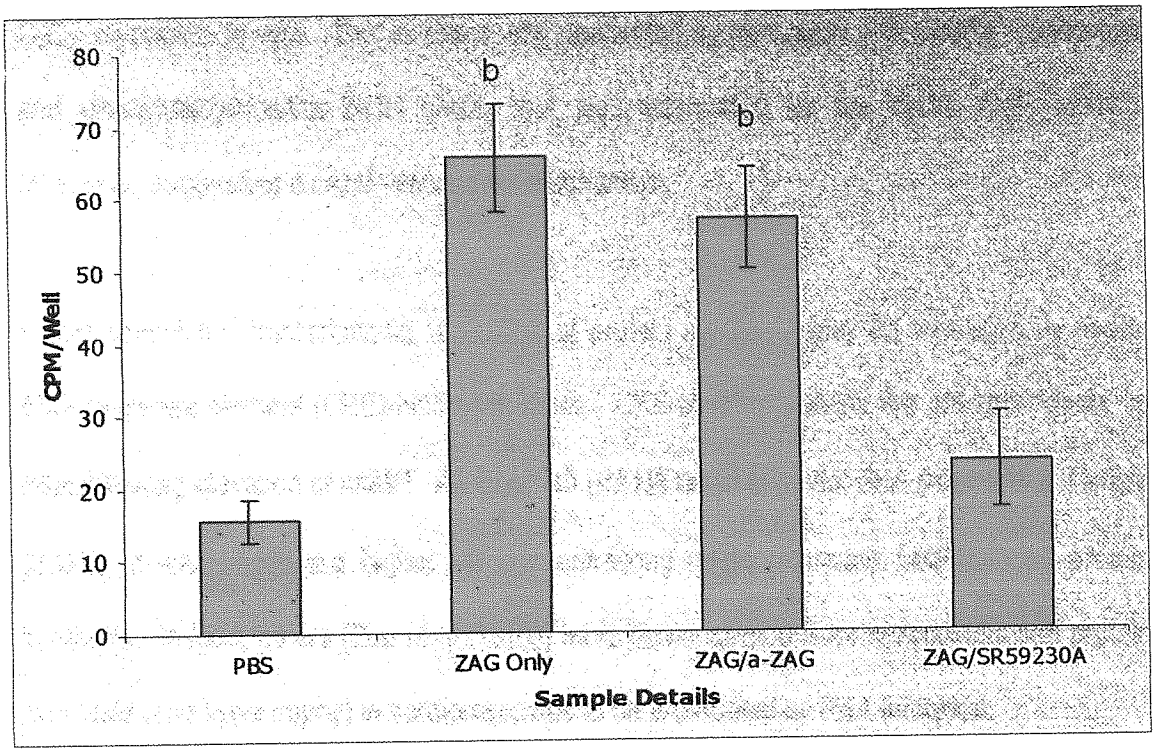


Figure 4.3.2.1. Estimation of protein synthesis in C_2C_{12} myotubes following dosage with ZAG (25μg/ml) in the presence of various inhibitors. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from PBS control are shown as b, $p<0.01$.

Figure 4.3.2.1 shows significant up-regulation of cellular protein synthesis in the presence of 25μg/ml ZAG. This is attenuated ($p<0.01$) by co-incubation with 10μM of the selective β_3AR-antagonist SR-59230A, suggesting β_3 agonistic properties of ZAG. Although co-incubation with 1:500 α-ZAG also attenuates the increase in protein synthesis, this does not reach significance ($p>0.05$). This suggests that blocking the action of ZAG directly at the β_3-AR is more effective than relying on ZAG - α-ZAG interactions to occur in solution away from the receptor. Homology between ZAG and LMF is once again emphasised, with very comparable results (Islam-Ali & Tisdale (2001)) being obtained in a similar study utilising LMF.

Islam-Ali & Tisdale (2001) demonstrated LMF to significantly increase protein synthesis in C_2C_{12} myoblasts *in vitro*. This increase was unaffected by rapamycin, PD-98059, wortmannin and phosphatidylinositide-3-OH kinase but was attenuated by the cyclic AMP inhibitor MDL_{12330A}, suggesting a cAMP-dependant mechanism.

It was speculated transcriptional regulation of protein synthesis may be mediated by cyclic-AMP response element (CRE)-binding proteins. CRE-binding proteins are phosphorylated by PKA following elevation of cAMP. Although 10 μM H8 failed to inhibit PKA (Islam-Ali & Tisdale (2001)), it was suggested higher H8 concentrations might attenuate LMF-induced protein synthesis. Indeed, others (Zou *et al* (1999)) have demonstrated isoproterenol-induced protein synthesis (and hypertrophy) in cardiomyocytes to be attenuated by PKA inhibition.

Similar findings are reported *in* vivo. Administration of β_3-AR agonists results in loss of lipid only, with lean body mass actually increasing (as reviewed by Arch (2002)). Oral administration of β-AR agonists to cattle, pigs, poultry and sheep resulted in increased muscle and decreased fat accretion (as reviewed by Mersmann (1997)). Corresponding data has been published regarding growth modulation in animals administered clenbuterol, cimaterol, ractopamine, L 664 969 and salbutamol (Ricks *et al* (1984) & Lafontan *et al* (1988)). Such an agent, if approved, would raise the market value of livestock enormously.

Seemingly antagonistic effects of ZAG/LMF and PIF are apparent. Previous studies (Islam-Ali & Tisdale (2001)) demonstrated LMF not only to up-regulate protein synthesis but also to attenuate proteosome catalytic activity. Interestingly, lipolytic activity in the MAC16 model peaks at 15% weight loss (Groundwater *et al* (1990)). Only after 16% weight loss (when LMF production falls away) do the actions of PIF become apparent (Smith & Tisdale (1993)). This

suggests that LMF/ZAG itself may modulate the degree of skeletal muscle loss during cancer cachexia.

4.3.3 Estimation of RNA Accretion

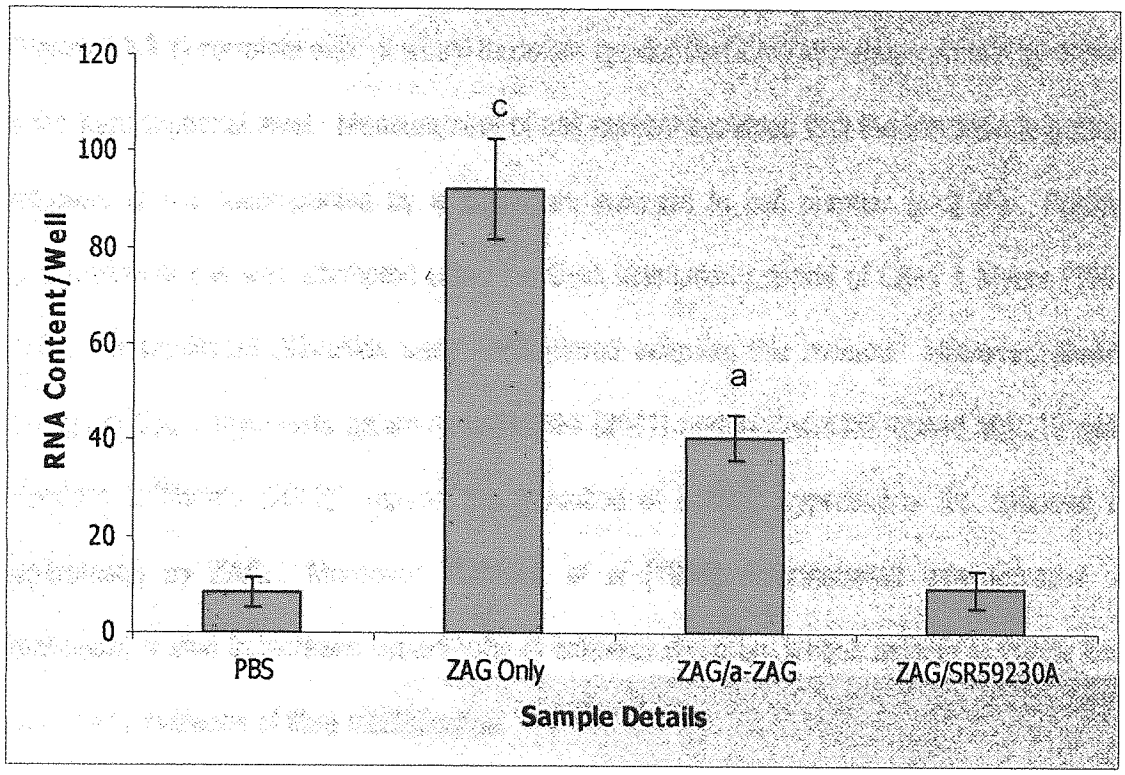


Figure 4.3.3.1. Estimation of RNA accretion in C_2C_{12} myotubes following dosage with ZAG (25μg/ml) in the presence of various inhibitors. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from PBS control are shown as a, $p<0.05$, c, $p<0.001$.

Figure 4.3.3.1 shows significant up-regulation of cellular RNA content in the presence of 25μg/ml ZAG. This is attenuated ($p<0.001$) by co-incubation with the selective β_3-AR antagonist SR-59230A, suggesting β_3 agonistic properties of ZAG. Co-incubation with 1:500 α-ZAG also attenuates the increase in RNA cellular content, albeit to a lesser degree ($p<0.05$). As in **section 4.3.2.1**, this suggests that blocking the action of ZAG directly at the β_3-AR is

more effective than relying on ZAG-α-ZAG interactions to occur in solution away from the receptor.

The effect of 25μg/ml ZAG on protein synthesis (**Figure 4.3.2.1**) and RNA cellular content (**Figure 4.3.3.1**) correlate well. It would therefore appear that ZAG stimulates protein synthesis at the transcriptional level. Measurement of cell number revealed that the increase in protein synthesis is not accompanied by a significant increase in cell number ($p>0.05$). Further confirmation of this was attempted using the DNA estimation method of Giles & Myers (1965 [170]), but significant difficulties were encountered adapting this method. However, similar findings in C_2C_{12} myoblasts (Islam-Ali & Tisdale (2001) and in ZAG/LMF-dosed MAC13 cells (Sanders & Tisdale (2003)) support the induction of cellular hypertrophy (as opposed to hyperplasty) by ZAG. Moreover, Rehfeldt *et al* (1994) demonstrated administration of clenbuterol *in vivo* to increase hypertrophy of extensor digitorum longus muscle in Wistar rats without any evidence of fibre multiplication.

4.3.4 Conclusion.

25μg/ml Zinc-α2-Glycoprotein induces increased protein synthesis in C_2C_{12} myotubes at the transcriptional level. In contrast to previous work (Islam-Ali & Tisdale (2001), no accompanying decrease in protein degradation was observed in this study. This increase in protein synthesis results in cellular hypertrophy as opposed to cellular hyperplasty.

Despite small discrepancies, this study further demonstrates homology between ZAG and LMF when compared to previous studies (Islam-Ali & Tisdale (2001)). This study also demonstrates

ZAG (and hence LMF) to possess a property the specific to β-AR agonists, i.e. to increases lean body mass. This adds further credibility to its role as a β_3-AR agonist.

The rationale as to why LMF and PIF should possess apparently antagonistic properties is unknown. Unsupported speculation (Smith HJ & Russell ST, personal communication) has been voiced that an increase in ZAG/LMF expression that accompanies cachexia might be a host response to the actions of 24kDa proteolytic factor.

Alternatively, as LMF is seen to induce protein synthesis in tumour cells also (Islam-Ali & Tisdale (2001)), LMF / ZAG may function to increase overall tumour bulk without affecting cellular proliferation.

5.1 Introduction.

Loss of adiposity in cancer cachexia is mediated by LMF/ZAG, protein molecules of approximately 40kDa (Hirai *et al* (1998)).

The interaction of these molecules with β_3-AR is poorly understood. LMF/ZAG induce G-Protein interactions (described in detail in **section 1.8.1.4**) leading to the stimulation of adenylate cyclase, an elevation in cyclic AMP, and the activation PKA (cyclic AMP dependant kinase) hence of HSL and the lipid droplet protein perilipin (**section 1.8.1.4**).

It is extremely likely that only a small proportion of the molecule is required for receptor activation (hence lipolysis). In order to understand this and facilitate the development of ZAG as an anti-obesity agent, it was determined whether proteolytic digestion would yield a fragment that retained bioactivity.

Administration of oral ZAG to NMRI mice induced significant weight loss (unpublished observations). Despite this, ZAG could not be detected in their sera (Zimmerman T, personal communication). A parallel study administering subcutaneous ZAG resulted in both weight loss and detectable serum ZAG. It is probable that digestion of ZAG occurred in the stomach of mice mediated by either trypsin or chymotrypsin. The resultant low molecular weight peptide proceeded to mediate the weight loss *in vivo* (Tisdale MJ, personal communication).

A review of the literature reveals lipolytic molecules that possess similar properties to LMF and/or a low molecular weight peptide derivative. For instance, Toxohormone-L (Masuno *et al* (1981)) is a lipolytic molecule which is cleaved by trypsin to form an active fragment of <10kDa. Similarly Li & Adrian (1999) purified a low molecular weight peptide from pancreatic and colon cell lines. This peptide was found to stimulate glucose utilisation in a manner similar to LMF (Li & Adrian (1999)). It is very possible that these molecules represent LMF or LMF cleavage product.

Correspondingly, previous studies (Wyke SM (unpublished results), Richardson D (unpublished results), & Russell (2001)) incubated LMF with trypsin protease to simulate this digestion *in vitro*. An active fragment was indeed produced and molecular weight estimated at <10kDa.

However, problems encountered with this method were numerous. The main difficulties arose due to trypsin autodigestion (hence detection of trypsinised trypsin), contamination with trypsin inhibitor (used to terminate the reaction), pH denaturation of fragment (also used to terminate the reaction) and extremely poor final yield. These studies did not produce the yield or homogeneity required for detailed fragment characterisation.

The aim of this work was to successfully fragment LMF and ZAG (to allow comparison) in a manner such that sufficient yield was obtained to permit further characterisation. As stated, previous methods have met with limited success, to this end, an immobilised HPLC method was instituted to overcome these problems. This HPLC method would overcome trypsin contamination, and would not necessitate the reaction to be terminated by acid or trypsin inhibitor. It also was hoped the sufficient start quantities now available due to ZAG production *en mass* would allow improved throughput and thus sequencing. This study also aims to further

demonstrate homology between isolated ZAG and LMF; furthermore, characterisation of this fragment would give important receptor mechanistic data.

As a secondary aim, this study may yield important data in the development of ZAG as an anti-obesity agent as it is desirable for any new agent to be administered orally (this has optimal patient compliance). Insight therefore, into what occurs *in vivo* following oral administration is essential.

Knowledge of the cleavage products that mediate weight loss *in vivo* is vital at this stage.

5.2 Methodology.

5.2.1 Factor Purification.

LMF was purified from the urine of cachectic cancer patients as described previously in **section 3.2.2**. ZAG was purified from fraction V effluent as described in **section 3.2.6**. ZAG was concentrated prior to HPLC injection using microcon units fitted with 10kDa MWCO membranes.

5.2.2 HPLC Immobilised Digestion of Factor

A Porozyme™ immobilised trypsin cartridge was installed on a Shimadzu HPLC chromatograph and equilibrated with 95% Digestion buffer (**section 2.6.1**):5% acetonitrile at a flow rate of 3ml/minute.

Flow rate was adjusted to 0.1ml/minute. Following injection of 150µl protein, flow was halted to promote digestion of factor on the column. Flow was restarted when desired, and the eluate collected over 20 minutes. Repetition several times was required to acquire adequate yield for later stages. The samples were washed and concentrated against PBS using a small Amicon stirred cell fitted with a 3kDa MWCO membrane.

Eluate was prepared, electrophoresed and visualised (**section 3.2.4**) to ensure adequate digestion of LMF/ZAG.

5.2.3 Size-Exclusion Chromatography of HPLC Eluate.

A sephadex G50 column was prepared (at 4°C and kept at such) and a peristaltic pump attached. The flow rate was adjusted to 1ml/min and the column equilibrated with phosphate buffered saline (PBS).

Void volume was estimated using blue dextran (Mr 2,000,000), and the column calibrated using the known protein standards albumin (Mr 66,000), trypsin inhibitor (Mr 20,000), cytochrome C (Mr 12,400) and aproptonin (Mr 6,500). The emergence of these peptides from the column was monitored (against fraction number) using total protein assay (**section 3.2.3**). This calibration allowed for molecular weight determination of subsequent unknowns.

Crude trypsinised eluate was loaded onto the column and eluted with PBS at a flow rate of 1ml/minute. 1ml fractions were collected.

5.2.4 HPLC Purification of Digest.

A reverse-phase HPLC method was investigated to assess and purify eluate obtained in **section 5.2.2**. A C8 column was fitted to a Shimadzu HPLC machine and the flow rate was adjusted to 0.2ml/minute. 50μl of digest eluate was then injected. The mobile phase used was 0.06% triflouroacetic acid (TFA) (in HPLC-grade water) and 0.04% TFA in HPLC-grade acetonitrile.

5% acetonitrile was run for 5 minutes, then peaks eluted using two increasing gradients of acetonitrile (5-65% (over 30 minutes) and 65-100% (over 15 minutes)). (This method was adapted from McDevitt (1986)).

Following fractionation, samples were dialysed out of the solvents against PBS in an Amicon stirred cell fitted with a 3kDa MWCO membrane.

5.2.5 Bioassay of Purified Fractions.

Each fraction was subjected to bioassay as described in **section 3.2.5**.

5.2.6 Active Fragment Electrophoresis.

Fractions demonstrating bioactivity were assayed for total protein (**section 3.2.3**), mixed 1:20 with 1X sample buffer denatured at 65°C for 2 minutes.

Samples were loaded (5µl/well) onto a 16.5% Tris-Tricine gel and resolved against ultra-low molecular weight markers (triosephosphatase isomerase (26,600Da), myoglobin (17,000Da), α-lactalbumin (14,200Da), aproptonin (6,500Da) insulin chain B (3,496Da) and bradykinin (1,060Da)). A cathode buffer (**section 2.6.4**) and an anode buffer (**section 2.6.5**) were used to resolve the gel at constant ampage of 20mA (1 hour) and then 30mA (until completion).

The gel was then fixed in 40% methanol/10% acetic acid (30 minutes) and stained with 0.025% brilliant blue G stain in 10% acetic acid for an hour. The gel was destained over a further hour with several changes of 10% acetic acid destain solution. If low fragment concentrations were too low to visualise by this method, the gel was further destained (10% acetic acid) overnight, and then subjected to silver staining (**section 3.2.4**).

5.2.7 Further Active Fragment Characterisation.

Active fractions were pooled, washed and concentrated against PBS in a medium Amicon stirred cell fitted with a 3kDa MWCO membrane. Samples were couriered to Dr. T Zimmerman (Bayer Corporation) who kindly performed mass spectrometry and MALDI-TOF analysis.

5.3 Results & Discussion.

5.3.1 Tryptic Digestion of LMF and ZAG.

LMF was trypsinised according to **section 5.2.2.** Trypsin cleaves polypeptide chains at the carboxyl side of basic L-amino acids such as arginine or lysine (Marana *et al* (2002)). Effluent from the HPLC immobilised trypsin cartridge was monitored at 214nm and peptide peaks collected over 20 minutes. An example trace is shown below in **figure 5.3.1.1**

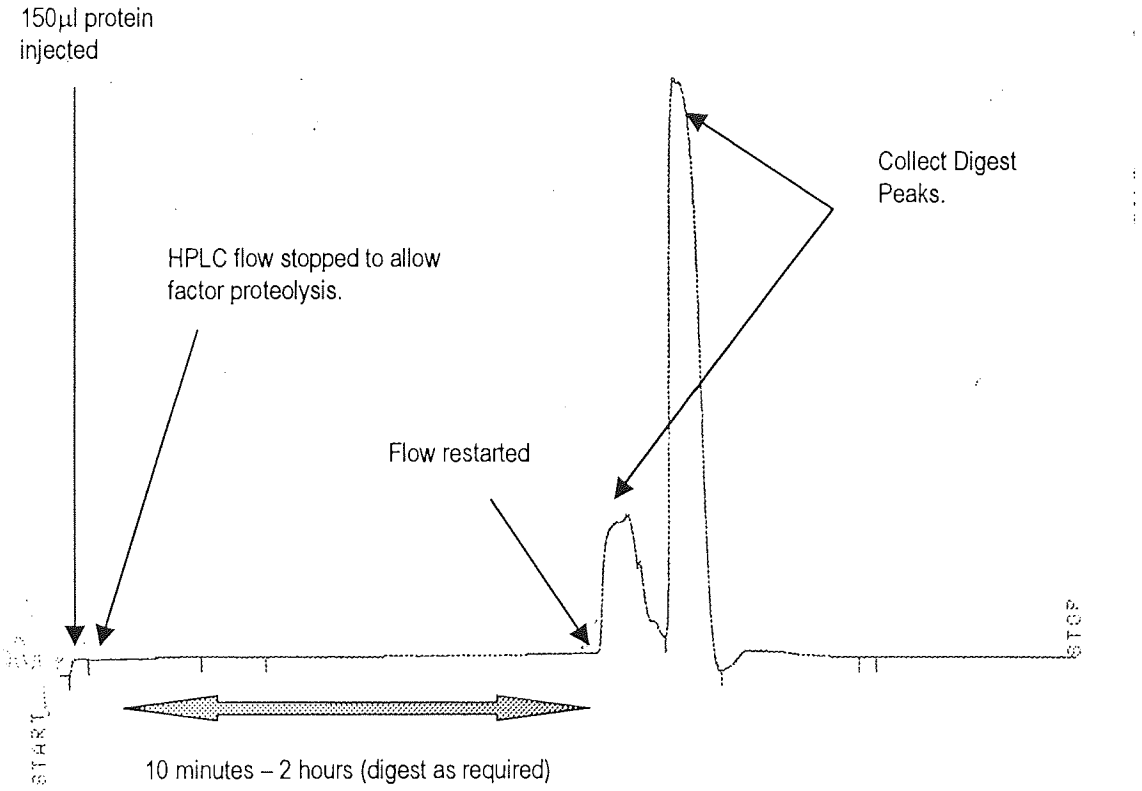


Figure 3.1.1. HPLC profile of tryptic digest. 150µl of sample was loaded onto the immobilised trypsin column, and flow halted to allow digestion. Following flow restart, all peaks were collected over 20 minutes. The presence of two effluent peaks may indicate two fragment groups of slightly different properties or a small amount of undigested factor. Peaks elute immediately following restart of flow for approximately 7 minutes.

To confirm adequate digestion of LMF, pre- and post-fragmentation samples were prepared and resolved on a 12% SDS-PAGE gel (**section 3.2.4**). Results are shown in **figure 5.3.1.2**.

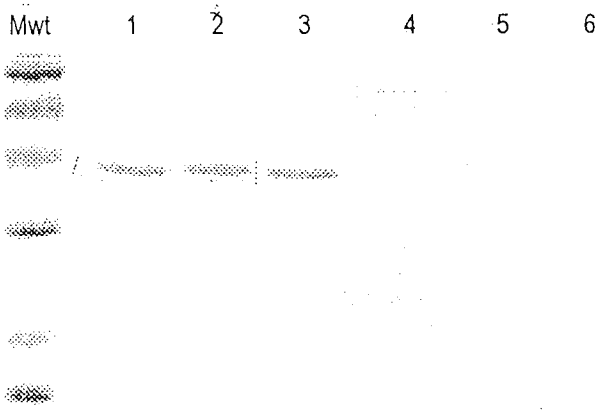


Figure 5.3.1.2. 12% SDS-PAGE gel demonstrating factor digestion. Prior to LMF digestion (lanes 1-3), a homogenous 43kDa band is observed. The majority of LMF appears trypsinised - no such band appears in the post LMF digest effluent (lanes 4-6).

It was unlikely that active fragments would be visible in lanes 4-6 of **figure 5.3.1**. The low molecular weight and small quantity of any fragment preclude visualisation on this gel.

Reverse-phase chromatography could provide excellent separation of fragments (in addition to time-effective homogeneity assessment). Unfortunately, this technique failed to purify an active peptide. The unknown characteristics of the digestion products made choice of a suitable column difficult. Initially a mid-range C8 column was instigated as opposed to utilising more hydrophobic (e.g. C18) or hydrophilic (e.g. C2) columns – this was not necessarily the most appropriate choice, and further optimisation is required. In addition, the mobile phase utilised may promote denaturation of any proteins initially present. Even if separation were achieved, this technique provides little insight into molecular weights, and the 'harsh' solvents used means bioactivity would never be

retained (thus active and inactive peptides would be indistinguishable).. To this end, eluate from **section 2.2** was subjected to sephadex G50 size exclusion chromatography.

Sephadex G50 chromatography allows determination of molecular weight determination (and allows the retention of any bioactivity), but lacks the separation resolution of reverse-phase HPLC. Results from fraction bioassay are presented in **figure 5.3.2**

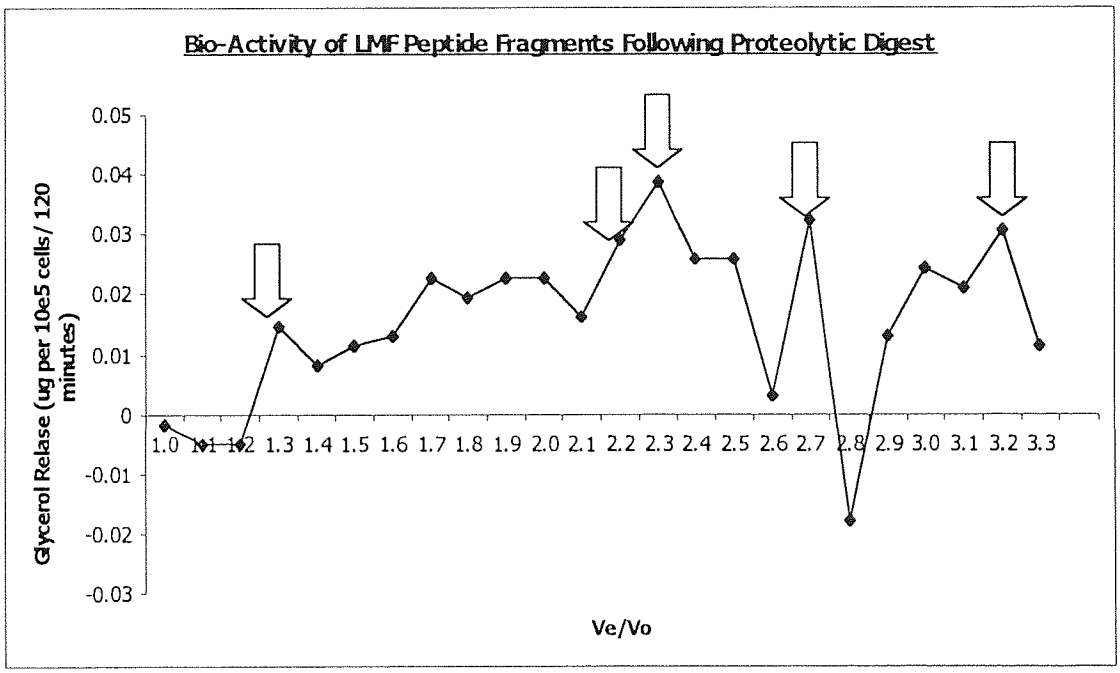


Figure 5.3.2. Bioactivity analysis of sephadex G50-eluted fractions. Trypsinised eluate was loaded onto a calibrated sephadex G50 column and size-separated. 1ml fractions were collected and assayed according to the method of McDevitt et al (1995). Apparent active fragments denoted with arrows. (Ve = elution volume, Vo = void volume). Calibration ***(section 5.2.3)*** *with known standards enables molecular weight to be estimated from Ve:Vo ratio.*

Bioactivity is not simply due to undigested factor – a small peak indicative of intact LMF is present at Ve/Vo = 1.3 (this approximates to 45kDa). Activity distinct from that of LMF is observed at Ve/Vo ratios of 2.1, 2.2 and 2.6 (equating to fragments of molecular weight 12-14KDa, 8-10kDa and 6kDa respectively).

Unfortunately, Low start quantities of LMF and an inefficient procedure resulted in too poor a yield to permit further analysis and sequencing. The LMF homologue ZAG could be purified (c/o Bayer Corporation) in much greater quantity, so a higher ZAG start concentration was used in the hope of gaining better fragment yield. Bioassay of sephadex-separated ZAG fractions is shown in ***figure 5.3.3.***

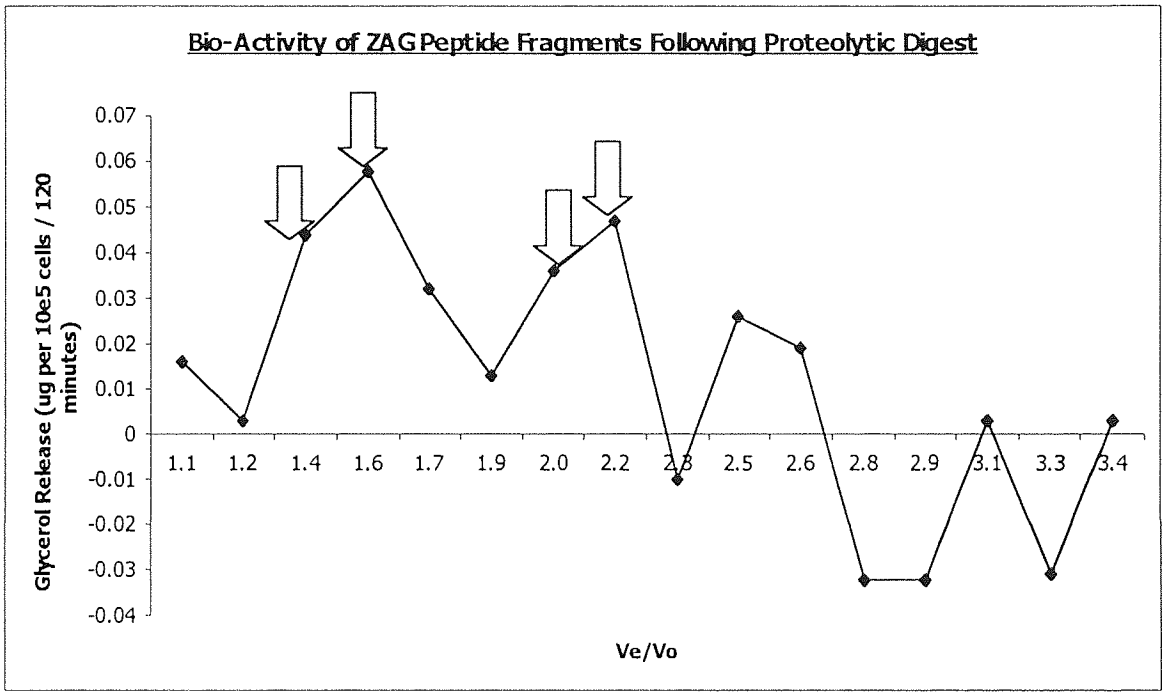


Figure 5.3.3. Bioactivity analysis of sephadex G50-eluted fractions. Trypsinised eluate was loaded onto a calibrated sephadex G50 column and size-separated. 1ml fractions were collected and assayed according to the method of McDevitt et al (1995). Apparent active fragments denoted with arrows. (Ve = elution volume, Vo = void volume). Calibration ***(section 5.2.3)*** *with known standards enables molecular weight to be estimated from Ve:Vo ratio.*

A peak at Ve/Vo 1.4-1.6 (approximately40-45kDa) indicates the presence of intact ZAG. The injection of large start quantities of ZAG onto the immobilised trypsin has resulted in greater amounts of undigested factor. The loading of larger amounts of ZAG will obviously saturate the trypsin so future experiments should employ longer digestion interval if using such increased start quantities.

Activity is seen (distinct from that of undigested factor) at Ve/Vo 2.0, 2.1 and 2.2 indicating fragments of 14-15kDa, 12kDa and 10kDa respectively. The amino acid sequences of ZAG and LMF are reported to be identical (Todorov *et al* (1998) & Russell *et al* (2003) & see also **figure 5.3.4**), thus, similar peptides would be expected following trypsinisation (which is what was observed here).

ZAG fragmentation products are of marginally higher weight distribution than those obtained with LMF – probably due to a less efficient digestion, (greater initial concentrations likely saturated trypsin sites). Nonetheless, extremely similar fragments are observed between LMF and ZAG adding yet more credibility to the pair's homology. Encouragingly, similar findings were obtained following low molecular weight electrophoresis of fragments. Minute protein concentrations necessitated silver staining to excess, but bands of apparent Mr 6-12kDa can be observed.

The complete amino acid sequence of ZAG/LMF is reported in **figure 5.3.4**. Peptides resulting from trypsin digestion are also indicated. With the present data however, we cannot ascertain which of these fragments possess bioactivity and which do not.

Greater starting material afforded by ZAG resulted in a superior final yield (approximately 40μg) compared to LMF (approximately 15μg). Unfortunately, delays (and hence degradation) *en route* to analysis meant that neither sample was adequate to provide further characterisation than is detailed here. Further work is currently in progress.

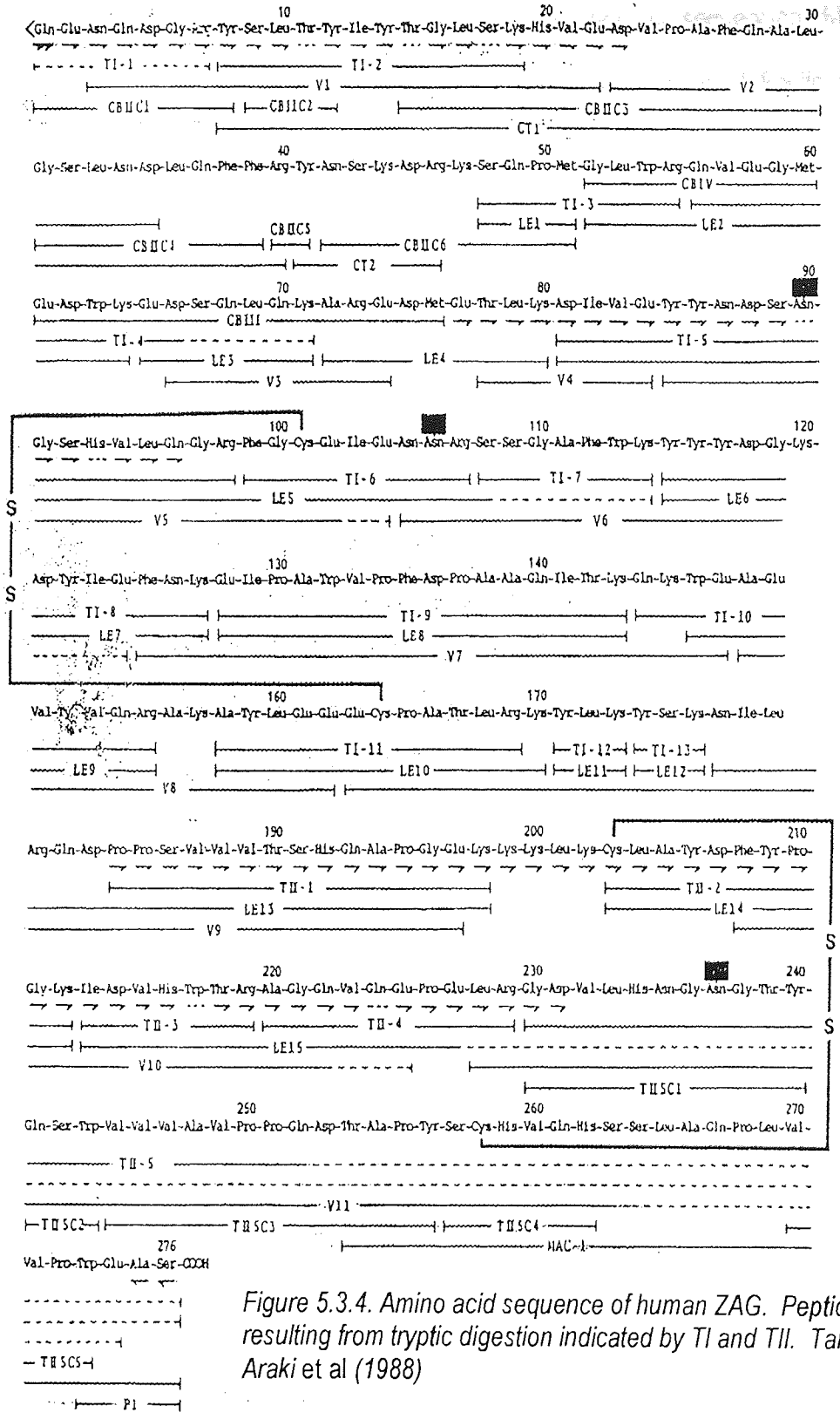


Figure 5.3.4. Amino acid sequence of human ZAG. Peptides resulting from tryptic digestion indicated by TI and TII. Taken from Araki et al *(1988)*

The limitations of these procedures are very evident. At each stage, dilution and inferior throughput has made final yield extremely poor. *n* =1 throughout because a great a

possible of final sample was required for further characterisation and sequencing. Moreover, the purpose of these bioassays was merely to provide an indication as to which fractions possessed activity was, as opposed to any kind of statistical quantification.

Li & Adrian (1999) purified a factor from pancreatic and colon cancer line lines that stimulates glucose utilisation in a manner analogous to LMF (Hiari *et al* (1998)). Islam-Ali & Tisdale (2001) noted this factor to be of similar molecular weight to fragments produced during LMF tryptic digestion. However, the factor isolated by Li & Adrian is unglycosylated (Li & Adrian (1999)) therefore unlikely to be an LMF-derivative. Interestingly, many of the other lipolytic factors isolated from cachexia models (as described in **section 1.8.1)** are either low molecular weight species or will retain activity following tryptic digestion (Kitada *et al* (1980), Kitada *et al* (1981), Masuno *et al* (1981) & Taylor *et al* (1992)). Is it possible that at least some of this 'coincidental evidence' points to involvement of an LMF-cleavage product in cancer cachexia?

Interestingly, there are no other examples of a biologically active glycoprotein (Fearon K, personal communication). Despite this fact, experimental evidence in this (and other) chapter (in addition to previous studies) point to LMF/ZAG being an exception to this rule. Caution is required however, as it may be that the non-peptide ligand (described in **section 1.8.1.5**) is released following proteolysis, and that it is this moiety, and not a peptide fragment, that mediates the observed bioactivity. The bioactivity *in vivo* may also be due to a secondary effect on the gut. Care with inferences is therefore required here, and further work is currently underway to elucidate exact mechanisms.

5.4 Conclusions.

Following tryptic digestion, ZAG and LMF cleave to form apparent peptide products that retain biological activity. Although insufficient yields were produced to allow sequencing, homologous fragments were formed following proteolysis of both ZAG and LMF. This finding adds to the plethora of mounting evidence that ZAG and LMF represent the same factor.

Previous studies (Richardson D & Wyke SM (unpublished data) report an LMF active fragment of approximately 7kDa. The results obtained in this study were comparable (6-15kDa). The slightly larger fragments probably result from less time in contact with immobilised trypsin (previous studies have incubated trypsin with LMF for up to 8 hours). It may be the case that fragments such as these (as opposed to the 43kDa molecule) mediate cachexia *in vivo*, although further evidence is required to substantiate this.

Further studies are definitely required. Dr. S Russell is currently working on the C8 HPLC purification of ZAG digests, and hopes to obtain peptide sequence data in the very near future.

CHAPTER 6. MOLECULAR CHARACTERISATION OF A LIPID MOBILISING FACTOR AND VALIDATION OF AN *IN VITRO* 3T3-L1 ADIPOCYTE MODEL.

6.1 Introduction.

ZAG and LMF mediate dose-dependent *in vitro* lipolysis in a cyclic-AMP manner through apparent Beta-$_3$-Adrenoreceptor interactions (Russell (2001) & Russell *et al* (2002)).

Inadera *et al* (2002) analysed plasma derived from cachexigenic colon-26 clone 20-bearing mice, and, by way of comparison, plasma from rodents bearing related non-cachexigenic clone 5 tumours. Clone 20 plasma promoted *in* vitro lipolysis and glycerol release in fully differentiated 3T3-L1 adipocytes. This glycerol was depleted from the cellular triglyceride fraction. Inadera *et al* (2002) reported the lipid-depleting activity of this factor to be due not only to lipolytic induction but also the inhibition of lipogenesis

Many lipolytic stimuli mediate lipolysis through stimulation of adenylate cyclase. This results in increased intracellular cAMP and hence activation of cyclic AMP-dependent protein kinase (PKA), which phosphorylates perilipins and HSL (discussed in detail in section **1.8.1.4**). There is evidence however, that in addition to activating PKA, G-protein coupled receptors and cyclic AMP can also activate mitogen activated protein kinase (MAPK) pathways (Greenberg *et al* (2001)). The involvement of this pathway in LMF-induced lipolysis remains uninvestigated.

The aims of this study are as follows: Firstly, our colleagues at Bayer Corporation were experiencing difficulty acquiring WAT to bioassay purified ZAG. The evaluation of a 3T3-L1 *in vitro* bioassay was investigated as an alternative to the standard *in vitro* bioassay described in

section 3.2.5. The standard bioassay of McDevitt *et al* (1995) utilises isolated WAT in a primary culture system. This has the disadvantage that the cells expire after 6 hours - precluding studies which exceed this duration. As many LMF studies require a 24-hour incubation, the 3T3-L1 model was validated as an alternative.

Secondly, thus far it had not been determined (Tisdale MJ, personal communication) whether LMF/ZAG inhibits *in vitro* lipogenesis in addition to stimulating lipolysis. A modified version of the method described by Inadera *et al* (2002) was instituted to determine the effect of LMF/ZAG on cellular lipogenisis and to allow further characterisation and comparison of LMF and ZAG. LMF/ZAG was also compared to the factor isolated by Inadera *et al* (2002) to determine any similarity or homology.

Thirdly, the involvement of the mitogen activated (MAPK) pathway in β_3-AR agonism has been reported (Greenberg *et* al (2001)). Involvement of the MAPK pathway in LMF-mediated lipolysis remains uninvestigated thus was determined using the standard *in vitro* bioassay +/- the MAPK inhibitor PD098059.

6.2 Methodology.

6.2.1 Purification of Lipolytic Factors.

LMF was purified from the urine of patients with advanced pancreatic cancer (**section 3.2.2**). ZAG was kindly purified by Bayer Corporation from fraction V effluent (**section 3.2.6**). These studies employed ZAG batch # 11-12.

6.2.2 Maintenance and Subculture of 3T3-l1 Preadipocytes.

3T3-L1 preadipocytes were maintained in $75cm^3$ flasks using DMEM supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine. Cells were cultured in a humidified atmosphere of 10% CO_2 in air at 37°C. At semi-confluence, cells were treated with 1X trypsin and sub-cultured. In contrast to other cell lines, a higher passage number was desirable due to improved characteristics and response of cells (Richardson D, personal communication).

6.2.3 Differentiation of 3T3-L1 Preadipocytes.

Six-well multiwell plates were seeded with $2x10^5$ 3T3-L1 preadipocytes. These were grown as described in **section 6.2.3**. Differentiation was induced according to the method of Frost *et al* (1985).

Two days post-confluence (day 0), differentiation was induced with DMEM supplemented with 10% FCS, 1% penicillin streptomycin, 1% glutamine, 0.5mM methylisobutylxanthine, 0.25μM dexamethasone and 1μg/ml insulin. On day 2, methylisobutylxanthine and dexamethasone were removed but insulin maintained for a further two days. On day 4 (and thereafter), insulin

was removed and the cells maintained in DMEM supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine. This was replaced every two days.

Cells ($2x10^6$ per well) were used experimentally between days 8-12. At this time, 95% of the cells express the adipocyte phenotype (Frost *et al* (1985)). Confirmation that differentiation had occurred was confirmed by staining with Oil Red O (Islam-Ali 2001). Two hours prior to each procedure, adipocyte monolayers were incubated in serum-free DMEM.

6.2.4 Modified *in vitro* Bioassay.

3T3-L1 adipocytes were differentiated as described in **section 6.2.4**. A modified version of the bioassay detailed in **section 3.2.5** was employed. Briefly, isolated epididymal WAT was substituted with 3T3-L1 cells in culture. Inhibitors were applied 2 hours prior to experimentation and allowed to equilibrate. Cells were incubated 25μg/ml ZAG +/- 10μM SR59230A and +/- 1:500 α-ZAG (co-assayed with positive and negative controls) in Krebs Ringer solution (**section 2.3.2.2**) for 120 minutes at 37°C in an atmosphere consisting 10% CO_2 in air (with occasional agitation). 0.5ml of supernatant is removed and assayed for glycerol using the method of Weiland (1974) previously described (**section 3.2.5**).

6.2.5 Estimation of Lipid Synthesis.

3T3-L1 preadipocytes were grown to confluence in six-well plates and differentiated as described in **section 6.2.4**. Cells were dosed with either 25μg/ml ZAG, 25μg/ml LMF or phosphate buffered saline as a vehicle control. Cells were co-incubated with 0.5μCi of [^{14}C] glucose for 24 hours at 37°C in a humidified atmosphere of 10% CO_2 in air.

Total lipids were extracted according to the method described by Mulligan (1991). Cells were harvested using a rubber policeman and transferred to glass scintillation vials containing 3ml of 30%w/v potassium hydroxide. Samples were heated (with agitation) to 70°C for 15 minutes. 3ml of 95%w/v ethanol was added to each vial and the samples returned to 70°C for a further two hours.

Scintillation vials were removed to room temperature and adequate cooling allowed. Acidification of saponified material was achieved by addition of 3ml 9M sulphuric acid. Acid soluble lipids were extracted by shaking with light petroleum ether (b.p 40-60 °C) and removal of supernatants following sedimentation. This extraction was repeated three times, the petroleum fractions combined and evaporated under nitrogen.

Lipid extracts were reconstituted with 10ml of Optiphase Hi-Safe II scintillation fluid, and the radioactivity of each sample assessed using a Packard TRI-CARB 2000CA liquid scintillation counter.

6.2.6 Assessment of 3T3-L1 Triglyceride Fraction.

3T3-L1 preadipocytes were grown to confluence in six-well plates and differentiated as described in **section 6.2.4**. Cells were dosed with either 25μg/ml ZAG, 10μM isoprenaline or phosphate buffered saline as a vehicle control. Cells were incubated for 2 hours at 37°C in a humidified atmosphere of 10% CO_2 in air.

Media was removed, and the cells harvested using a rubber policeman. Cells were added to a solution of chloroform:methanol (2:1), the organic layer removed and dried under nitrogen. The resulting pellet was dissolved in 1% Triton X-100 in PBS. Cellular triglyceride content was assessed using the Sigma Triglyceride Kit. Briefly, triglyceride reagent was reconstituted and 1ml added to 10μl of test sample. Reactions were allowed to proceed over ten minutes (at 37°C) after which absorbance was read at 500nm against deionised water.

Triglyceride content was then estimated using the following equation:

$$\frac{A_{test} - A_{blank}}{A_{cal} - A_{blank}} \quad X \text{ [calibrator] (250mg/dL)}$$

6.2.7 Involvement of Mitogen Activated Pathway Kinase in ZAG-Induced Lipolysis.

To estimate MAPK involvement in LMF induced-lipolysis, the standard *in vitro* bioassay (**section 3.2.5**) was performed in the presence of 25μM of the MAP kinase inhibitor PD98059.

6.3 Results and Discussion.

6.3.1 *In Vitro* 3T3-L1 Lipolysis Assay.

Results obtained by *in vitro* bioactivity assessment are shown in **figure 6.3.1.1**.

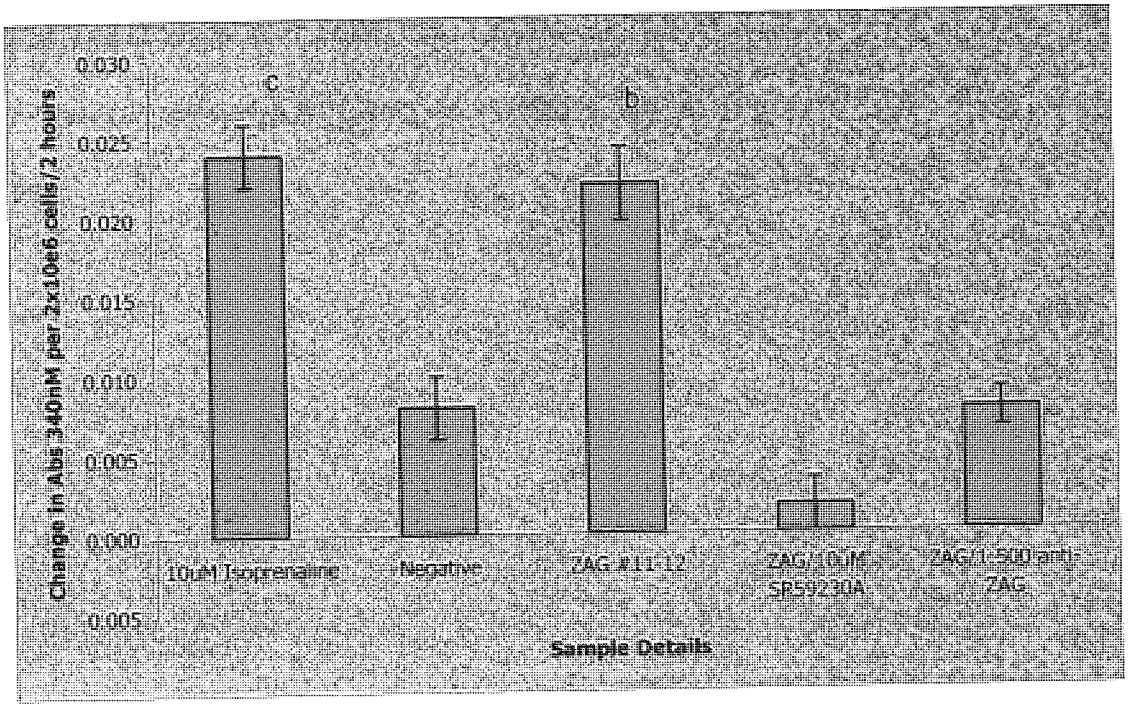


Figure 6.3.3.1; Bioassay of ZAG (according to the method of McDevitt et al (1995)) against positive and negative controls, and in the presence of inhibitors. Data are means (n=3) +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from negative control are shown as b, p <0.01, c, p<0.001. All n=3.

ZAG displays significant bioactivity in this *in vitro* assay system ($p<0.01$ against negative control). The effects of ZAG at the β_3-AR were potently inhibited ($p<0.001$) by the selective β_3-AR antagonist SR59230-A. ZAG is inhibited to a slightly lesser degree ($p<0.01$) by co-incubation with 1:500 α-ZAG. As before, inhibition by direct β_3-AR antagonism proves slightly more effective than inhibition with antibody.

These results correlate extremely well with those obtained using isolated epididymal WAT in **section 3.3.2**. The 3T3-L1 adipocyte line is reported (Shimizu *et* al (1996)) to be extremely

suitable for such lipolytic assessment. Upon differentiation, 3T3-L1 cells acquire the morphological and metabolic characteristics of isolated adipocytes. Comparative experiments have demonstrated fully differentiated 3T3-L1 adipocytes to respond in a manner homologous to that of isolated human and murine WAT. Furthermore, the 3T3-L1 system lends ideal comparison to human fat cells, with differentiated 3T3-L1's possessing three functional β-Adrenoreceptor subtypes (Shimizu *et* al (1996)), agonism at the β_3 subtype being observed here.

6.3.2 Effect of LMF/ZAG on Lipid Synthesis.

The effect of 25μg/ml ZAG on 3T3-L1-lipid synthesis is reported in **figure 6.3.2.1**. A small amount of LMF was available at this time and thus co-assayed (25μg/ml) for comparative purposes.

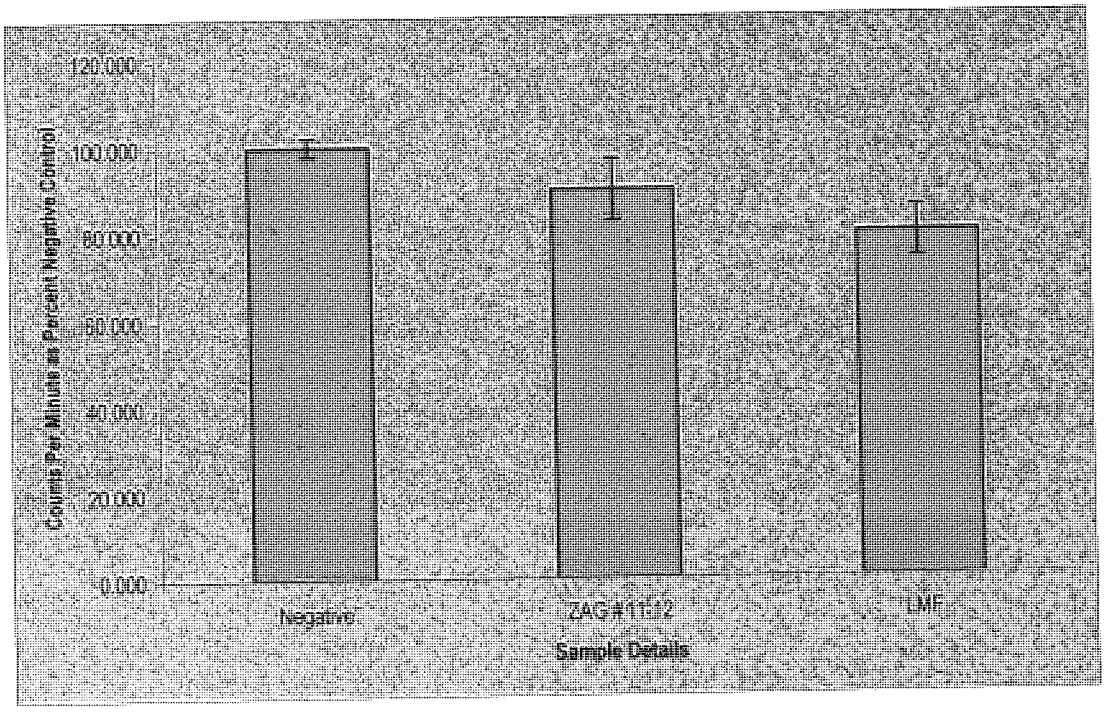


Figure 6.3.2.1. Lipid synthesis (estimated by [^{14}C] glucose incorporation into cellular lipid fraction over 24 hours) of 3T3-L1 cells in vitro. Data are means (n=3) +/- SEM. Statistical analysis was via one-way ANOVA.

The data described in **figure 6.3.2.1** did not quite reach significance (p=0.09). ZAG dosing decreased lipogenesis by 10.3% and LMF by 20.7%. As reported previously (Russell (2001)), ZAG is demonstrated here to be two-fold less active than isolated LMF.

Cachexigenic colon-26 clone 20 plasma isolated by Inadera *et al* (2002) produced similar results, achieving a slightly significant ($p<0.05$) 25-30% inhibition of [^{14}C] glucose incorporation

into lipid fractions. it would appear that lipid depletion by LMF/ZAG is mediated primarily by lipolysis with little / no reduction in lipid synthesis.

6.3.3 Estimation of ZAG-Induced Depletion of Cellular Triglyceride Fraction.

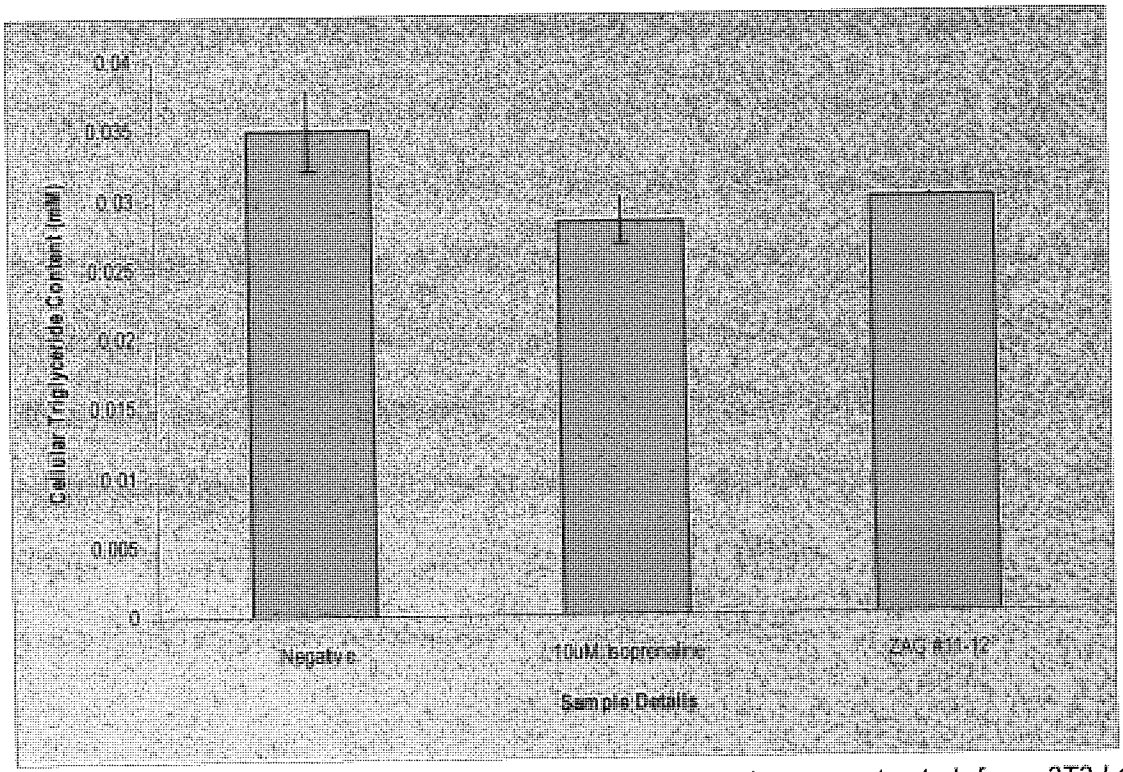


Figure 6.3.3.1. Measurement of cellular triglyceride fraction as extracted from 3T3-L1 adipocytes following 24 hour incubation with 25 μg/ml ZAG, 10μM isoprenaline or vehicle control. Data are means (n=3) +/- SEM. Statistical analysis was via one-way ANOVA.

A slight depletion of the triglyceride fraction was observed. This is not seen to reach significance (p=0.1106). It is highly probable that LMF/ZAG do mediate lipolysis through depletion of the triglyceride fraction. Cachexigenic plasma from the colon-26 clone 20 model is reported to deplete triglyceride fractions of differentiated 3T3-L1 adipocytes (Inadera *et al* (2002)). It is likely that cells harvested from low-volume multiwell plates, coupled with the 'harsh' TG extraction procedure resulted in insufficient triglyceride concentration to meet assay sensitivity.

Repetition of these experiments utilising 75cm3 flasks (as opposed to multiwell plates of much lower volume) should overcome this poor TG yield. Alternatively, the same *in vitro* system could be incubated with [^{14}C] glucose and lipolytic factor. Cellular lipids could be extracted and subjected to thin layer chromatography. Areas corresponding to the triglyceride fraction could be scraped from the TLC plates and counted in a much more sensitive procedure.

Adipogenisis is controlled at the transcriptional level by several transcription factors. PPAR-γ, C/EBP-α and SREBP-1 all interplay to directly influence fat cell development, induce and sustain differentiation (Rosen *et al* (2000) & Auwerx (1999, review). An unidentified circulatory factor described by Inadera *et al* (2002) induced lipid depletion through post-translational interactions with SREBP-1 (a member of the bHLH transcription factor family, concerned with adipocyte development and cholesterol homeostasis). It is yet unclear whether the factor isolated from colon-26 clone 20 bearers is LMF/ZAG, or whether LMF/ZAG will regulate SREBP-1 in adipocyte models. Antibodies are currently available against SREBP-1, and results of such investigations should prove very interesting indeed.

6.3.4 Involvement of Mitogen Activated Pathway Kinase in ZAG-Induced Lipolysis.

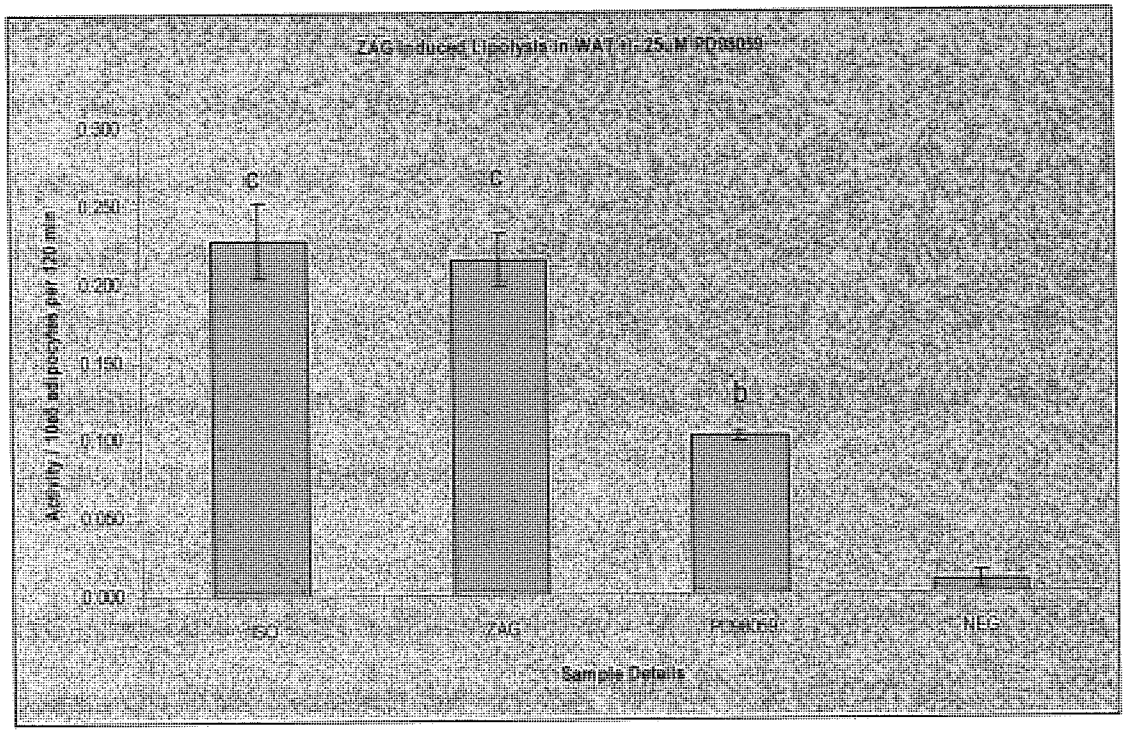


Figure 6.3.4.1. Lipolytic bioassay (according to the method of McDevitt et al (1995)) of 10μM isoprenaline, 25μg ZAG/assay +/- 25μM PD098059 and negative. Data are means (n=3) +/- SEM. Statistical analysis was one-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from negative control are shown as b, p<0.01 and c, p<0.001.

Partial inhibition of ZAG is seen when incubated with 25μM PD98059 ($p<0.01$). Greenberg *et al* (2001) utilised PD098059 and U0126 (two specific MEK (MAP kinase kinase) inhibitors) in an *in vitro* 3T3-L1 system, Greenberg *et al* (2001) demonstrated this MAP kinase inhibition to reduce β_3-AR mediated lipolysis by approximately 41%. This study achieved 52.56% inhibition of lipolysis by PD098059. Given that two distinct assay systems have been utilised, these results are very comparable. Greenberg *et al* (2001) speculated that ERK activation may regulate lipolysis to a degree by phosphorylation of the Ser^{600} residues of hormone sensitive lipase. Despite speculation, these hypothesis await further evidence.

6.4 Conclusion.

Administration of ZAG *in vitro* to fully differentiated 3T3-L1 adipocytes induces glycerol release. The 3T3-L1-adipocyte system would appear a viable alternative to the isolated epididymal WAT currently employed in bioassay. Ideally, both the primary culture (which represent highest homology between *in vitro* and *in vivo* systems) and 3T3-L1 systems should be employed to ensure accuracy – longer studies preclude this however, and this work validates the 3T3-L1 model as an alternative. Moreover, the 3T3-L1 line has been documented as possessing marked similarity with human fat (Shimizu *et* al (1996)), and thus represents a valuable model in clinical cachexia research.

LMF-induced lipid depletion in differentiated 3T3-L1 cells appears to be primarily mediated by lipolysis with little/no decrease in lipid synthesis. This is true for both LMF and ZAG, between which further evidence for homology has been presented. This lipid depletion appears to primarily originate from the triglyceride fraction, although further work will be necessary to substantiate this.

Interestingly, the results obtained in this study are comparable to those obtained by Inadera *et al* (2002) using a lipolytic factor isolated from the colon 26 clone 20 model. From the data presently available, one is not able to ascertain whether the two factors are homologous, derivatives or simply unrelated.

Evidence is also accumulating implicating the MAP kinase pathway in classical lipolysis. From work in this study, and by others, it would certainly appear involvement of this pathway might account for at least a portion of the lipolysis observed during β_3-AR agonist administration.

Although the exact mechanisms here remain undiscovered, further work should elucidate interesting results in this field.

7.1 Introduction

Cancer cachexia comprises unintentional emaciation mediated by a 24kDa Proteolysis Inducing Factor and a 43kDa Lipid Mobilising Factor sharing homology with Zinc-α2-Glycoprotein (ZAG). *In vivo* and *in vitro* administration of ZAG induces lipolysis in a manner analogous to LMF (Russell *et al* (2003)).

Adipocytes secrete a wide range of local, autocrine, paracrine or endocrine proteins now termed 'adipokines'. These regulatory molecules include leptin, adiponectin, resistin, adipsin, TNF-α and IL-6 (Bing *et al* (2003)). It has been speculated (Bing *et al* (2003)) that endogenous ZAG may be produced locally in adipose tissue, and may itself act as an autocrine/paracrine signal to influence lipid metabolism within the tissue.

The MAC16 model exhibits extensive lipid mobilisation (Beck & Tisdale (1987)). Increased ZAG mRNA (Todorov *et al* (1998)) and protein expression (**Chapter 10**) is observed in MAC16 cells over the non-cachexigenic MAC13 line. These findings further support ZAG as the putative lipid mobilising factor in cancer cachexia. It may be that cachexia-associated lipolysis is the result not only of tumour-produced ZAG/LMF, but also of increased ZAG expression in host adipocytes. This study therefore examines endogenous ZAG production, and determines whether this is raised to pathological levels during MAC16 cancer cachexia.

In addition, adipocyte ZAG regulation in cachexia models will be compared to that of mice administered ZAG to determine whether it is ZAG, or another cachexia-associated factor that mediates ZAG expression *in vivo*.

7.2 Methodology.

7.2.1 Housing, Preparation & Maintenance of MAC16 Cachexia Models.

Female NMRI mice (20-22g) from Aston University's inbred colony were maintained at ambient temperature (22°C (+/-2°C)) under a 12:12 hour light:dark cycle. Mice were fed a standard diet (SDS economy breeder, Lillico, UK) with tap water *ad libitium*. The MAC16 adenocarcinoma was passaged in mice within the colony. Non-transplanted NMRI mice were housed under identical conditions.

Under general anaesthesia, tumour fragments were transplanted subcutaneously in the flank using a trocar. Weight loss was monitored, and the mice sacrificed 16 days post-transplantation by cervical dislocation.

7.2.2 Preparation of Murine Adipose Tissue.

MAC16 mice and healthy NMRI mice were sacrificed as described in **section .2.1**. Inter-scapular BAT and epidydimal WAT was removed from the mice under sterile conditions.

Tissue was macerated (under sterile conditions) and homogenised with a hand-held homogeniser in ice-cold tissue homogenising buffer (**section 2.8.2**). Samples were sonicated on ice at 4°C for 3 intervals of 10 seconds (with 60 second breaks between pulses). Total protein content was determined according to the method of Bradford (**section3.2.3**) and concentration adjusted to 5µg/10µl with sample denaturing buffer (**section2.3.3.1**). Following this, samples were denatured at 90°C for 5 minutes in a water bath.

Samples were resolved on 12% gels by SDS-electrophoresis (**section 3.2.4**) then transferred (1X transfer buffer (**section 2.3.4.1**), 80v for 2h) onto o.45µm Hybond A nitrocellulose membrane. Post-transfer, non-specific binding was prevented by 1 hour incubation with 5% non-fat milk (**section 2.3.4.3**). Membranes were washed for 45 minutes in 0.5% PBS-Tween (**section 2.3.4.2.b**) with three changes of buffer. Transferred proteins were probed with a mouse α-human monoclonal primary antibody (2 hours, 1:1000 dilution at room temperature) then with rabbit α-mouse peroxidase-conjugated secondary antibody (1 hour, 1:2000 dilution at room temperature). Membranes were washed with 0.1% PBS Tween (**section 2.3.4.2.a**) for 1 hour (with four changes of wash buffer) before and after incubation with secondary. Development was by enhanced chemiluminescence (ECL). Blots were subjected to densitometric scanning and analysed using 'Phoretix ID Advanced' software. Equal loading was confirmed following Ponceau S staining.

7.2.3 Analysis of Adipose Tissue from ZAG/PBS-Dosed Rodents.

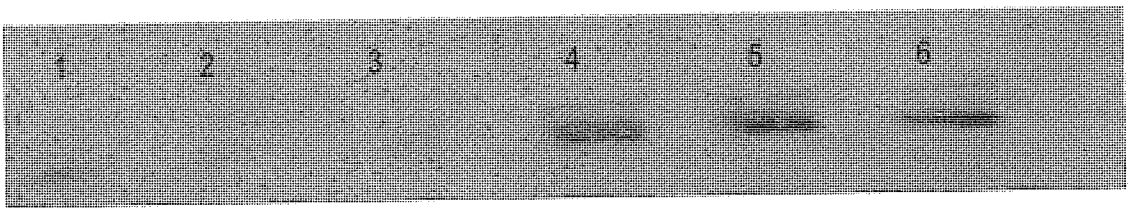
Non-tumour bearing NMRI mice were administered either 25µg ZAG (in 100µl sterile PBS) or 100µl PBS by way of control. Dosing was subcutaneous and twice daily (dosed c/o Dr. Steven Russell). Brown adipocyte tissue was removed, prepared, blotted and analysed as described in **section 7.2.3**.

7.3 Results & Discussion.

Differential ZAG expression by MAC13 and MAC16 cell lines are reported in **figure 7.3.1**. Western blot and densitometric analysis of BAT and WAT ZAG expression are reported in **figures 7.3.2.1 – 7.3.2**

Tissues excised were from MAC16 mice exhibiting a range of weight losses to determine whether adipocyte ZAG expression correlated with this parameter. Controls samples (i.e. 0% weight loss) were isolated from age/weight equivalent NMRI mice.

7.3.1 MAC13 and MAC16 ZAG Expression *in vitro.*



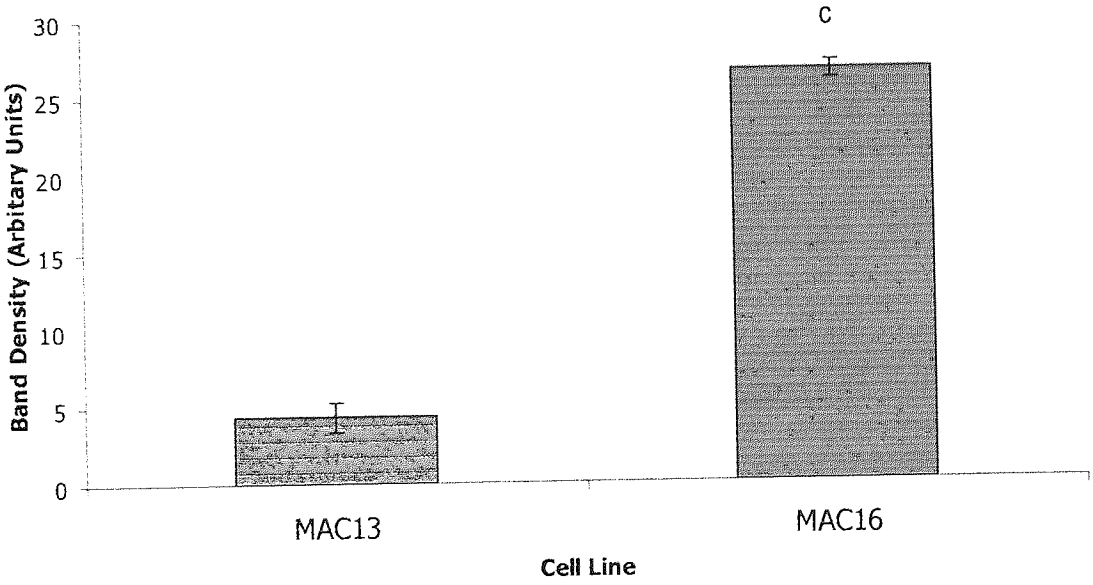
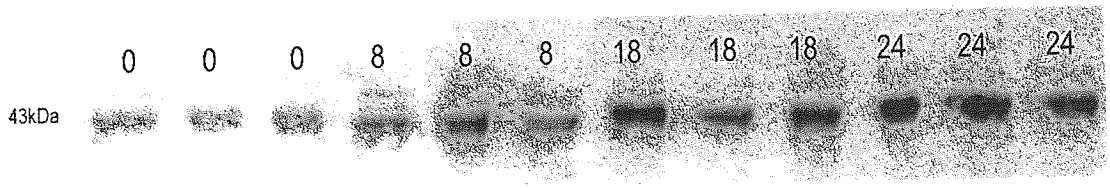


Figure 7.3.1. ZAG expression by MAC13 (lanes 1-3) and MAC16 (lanes4-6) cell lines as determined by western blotting utilising α-ZAG antibody. Data are shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparisons test. Results are shown as c, p<0.001.

7.3.2 BAT and WAT Immunoblot Analysis From MAC 16 Cachexia Model.



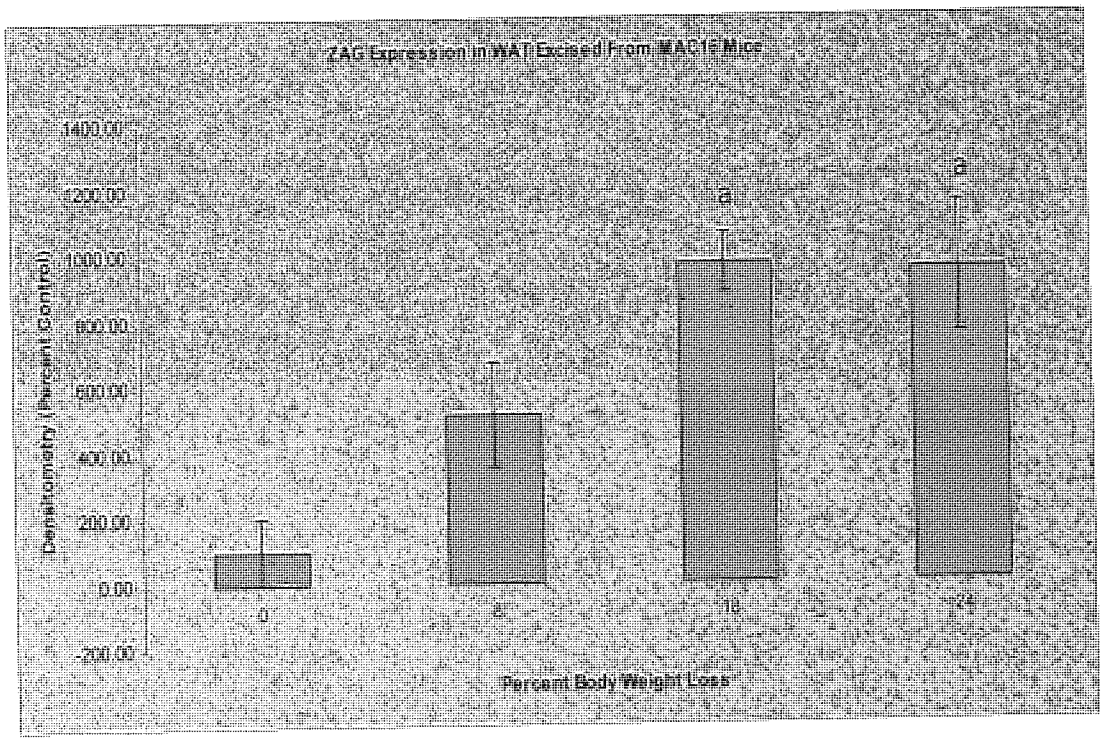
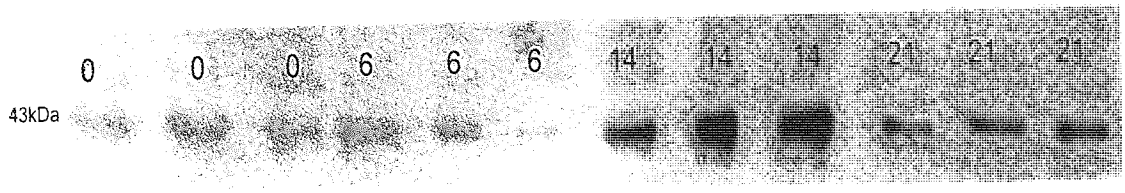


Figure 7.3.2.1. ZAG expression in WAT excised from mice exhibiting 0, 8, 18 and 24% body weight loss (as determined by western blotting utilising α-ZAG antibody). Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as a, $p<0.05$ compared to 0% weight loss



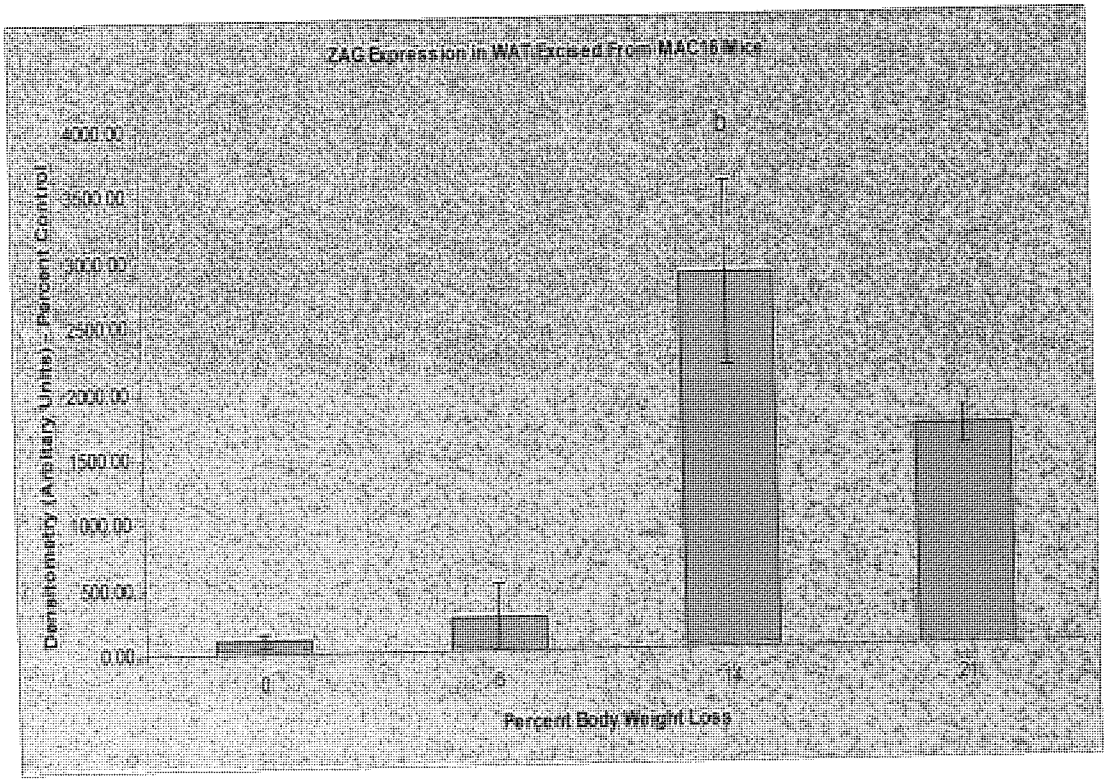
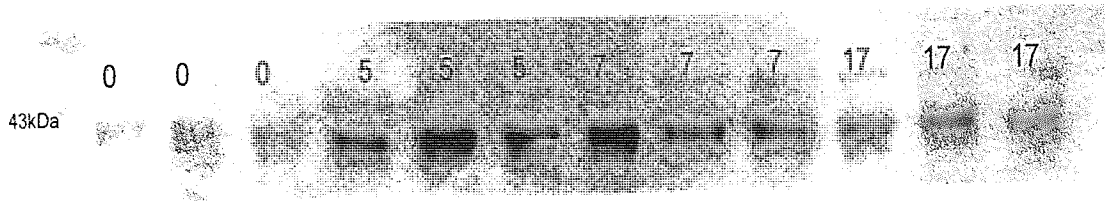


Figure 7.3.2.2. ZAG expression in WAT excised from mice exhibiting 0, 6, 14 and 21% body weight loss (as determined by western blotting utilising α-ZAG antibody). Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as b, $p<0.01$ compared to 0% weight loss



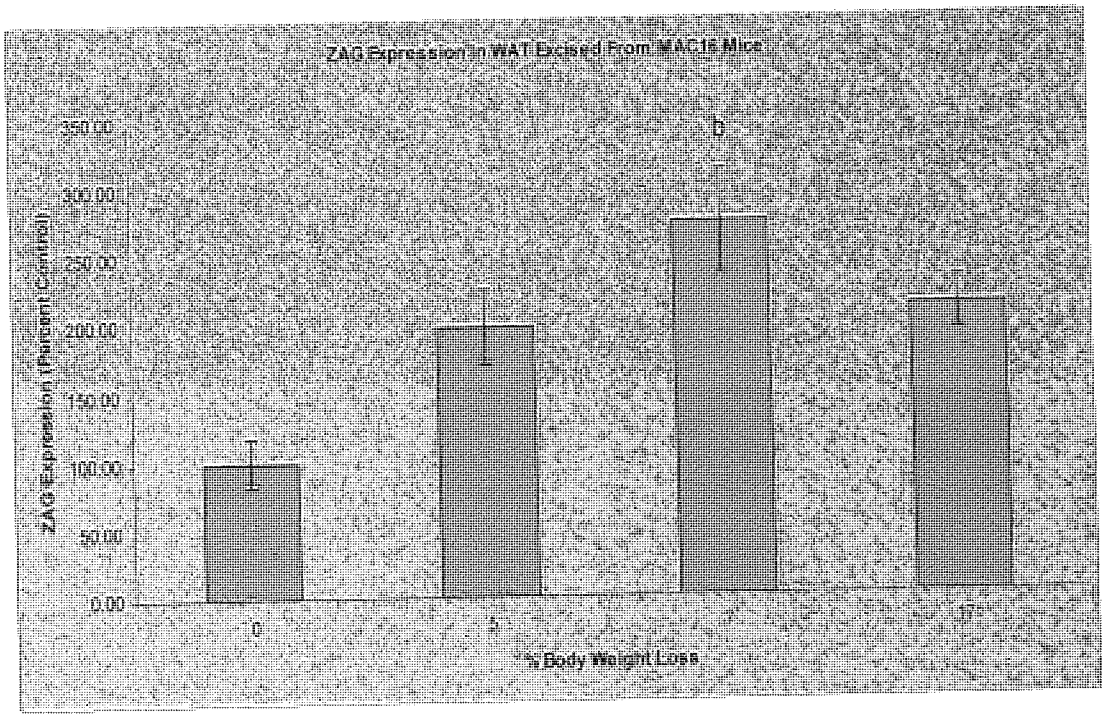


Figure 7.3.2.3. ZAG expression in WAT excised from mice exhibiting 0, 5, 7 and 17% body weight loss (as determined by western blotting using α-ZAG antibody). Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as b, $p<0.01$ compared to 0% weight loss

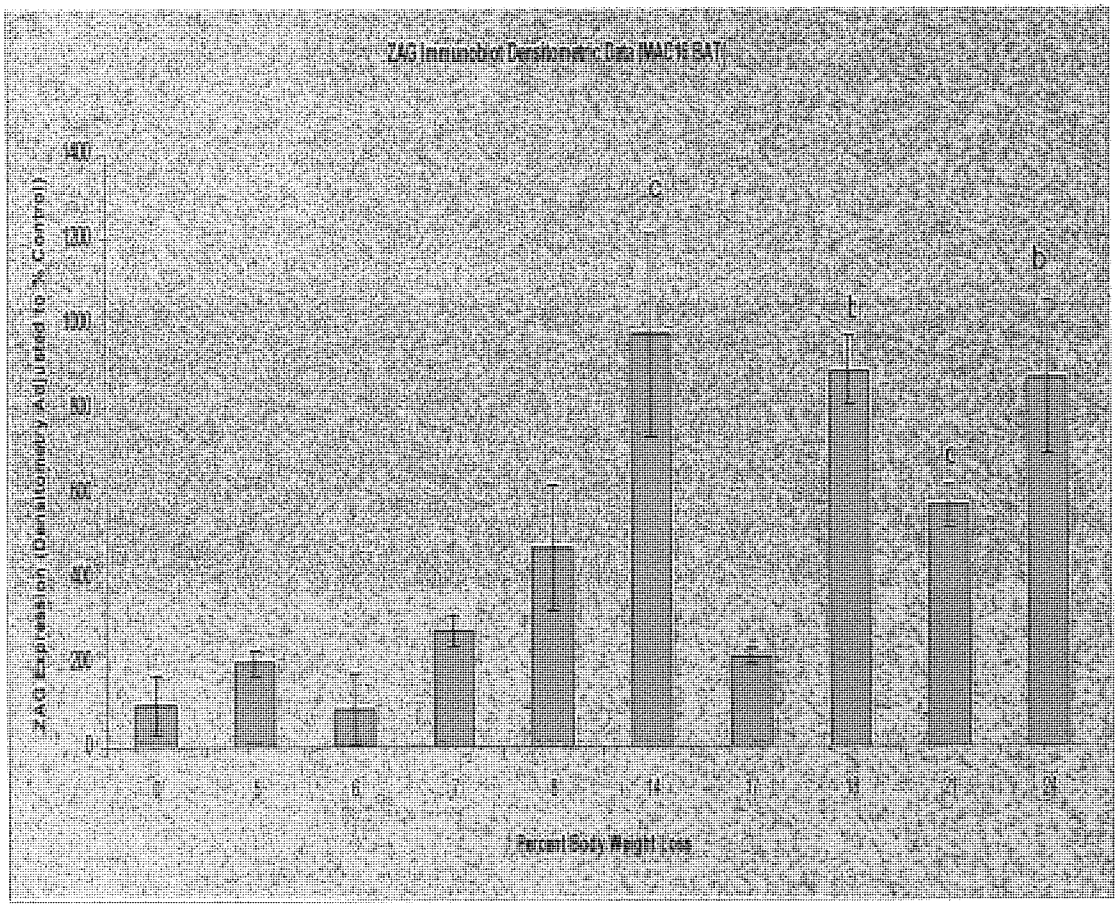
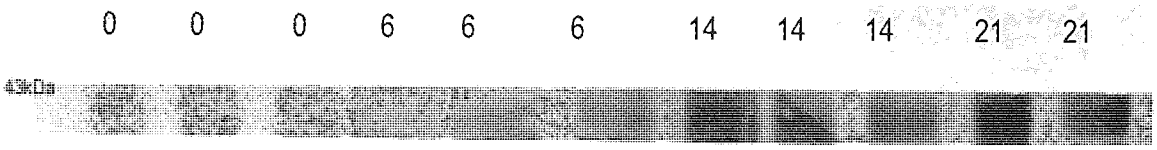


Figure 7.3.2.4. Cumulative graph showing ZAG expression in WAT excised from mice exhibiting all analysed body weight losses. Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as; b, $p<0.01$, c, $p<0.001$ compared to 0% weight loss



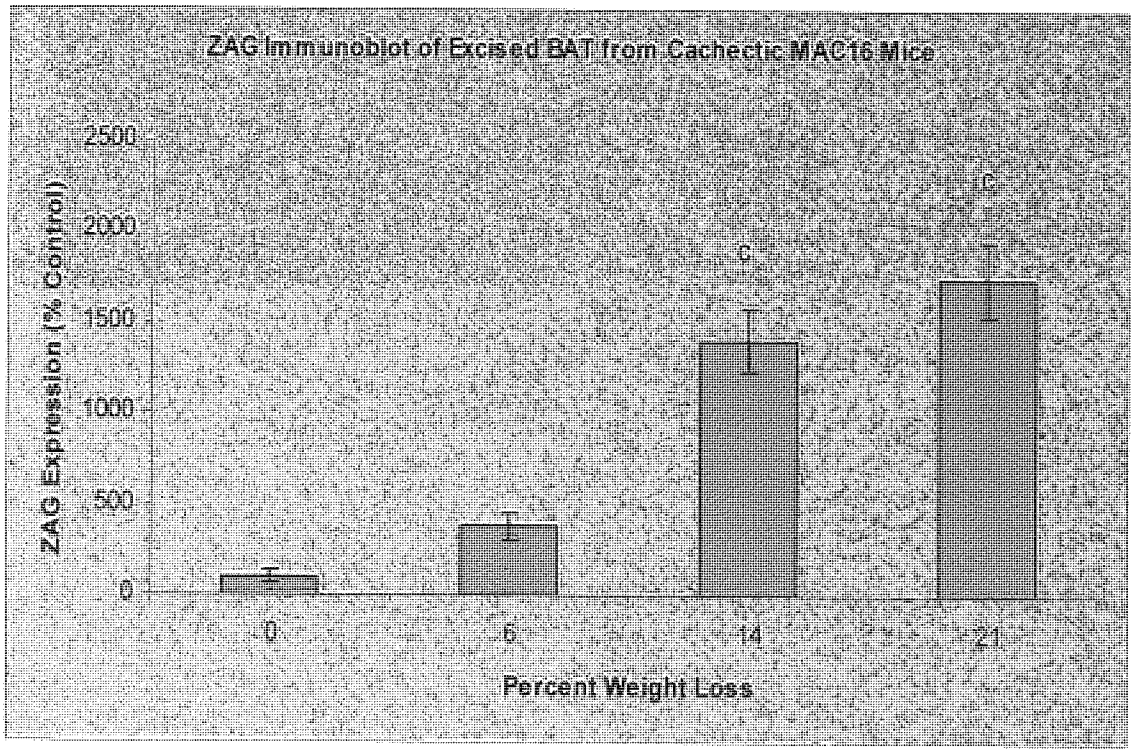
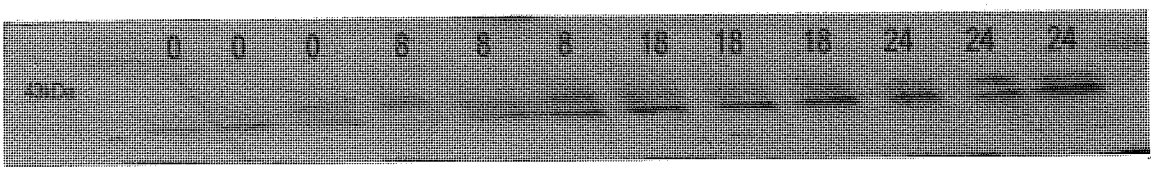


Figure 7.3.2.5. ZAG expression in BAT excised from mice exhibiting 0, 6, 14 and 21% body weight loss (as determined by western blotting utilising α-ZAG antibody). Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as c, $p<0.001$ compared to 0% weight loss



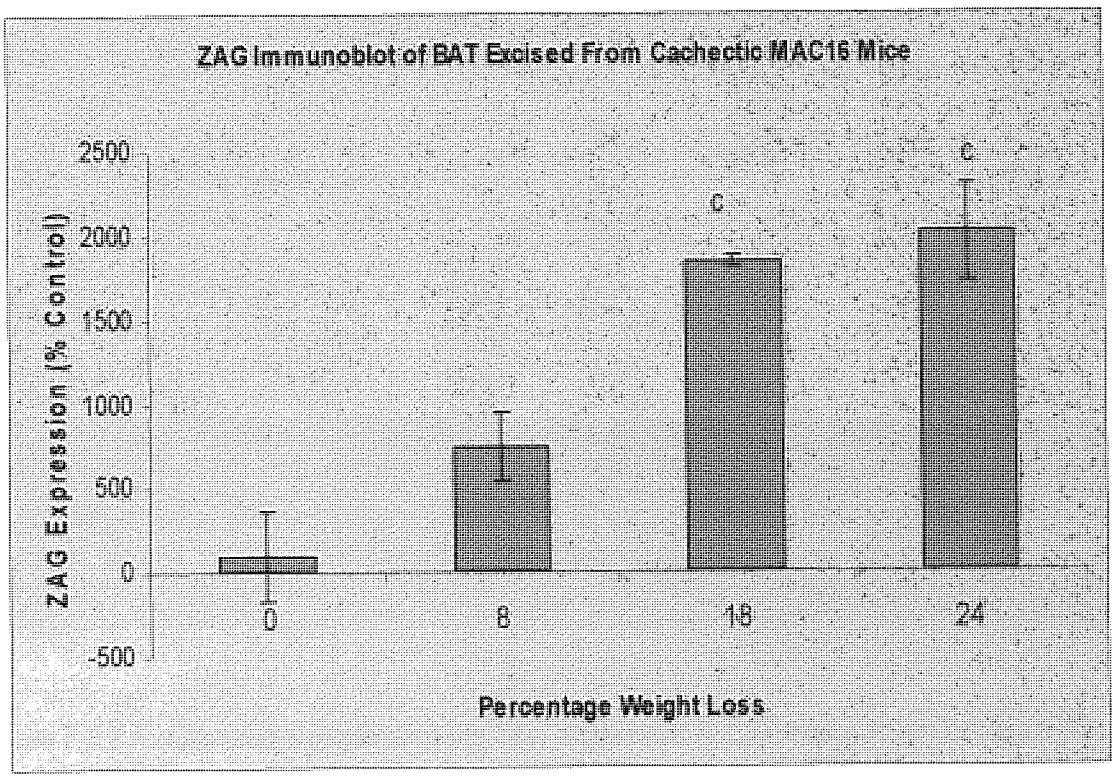


Figure 7.3.2.6. ZAG expression in BAT excised from mice exhibiting 0, 8, 18 and 24% body weight loss. Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as c, p<0.001 compared to 0% weight loss

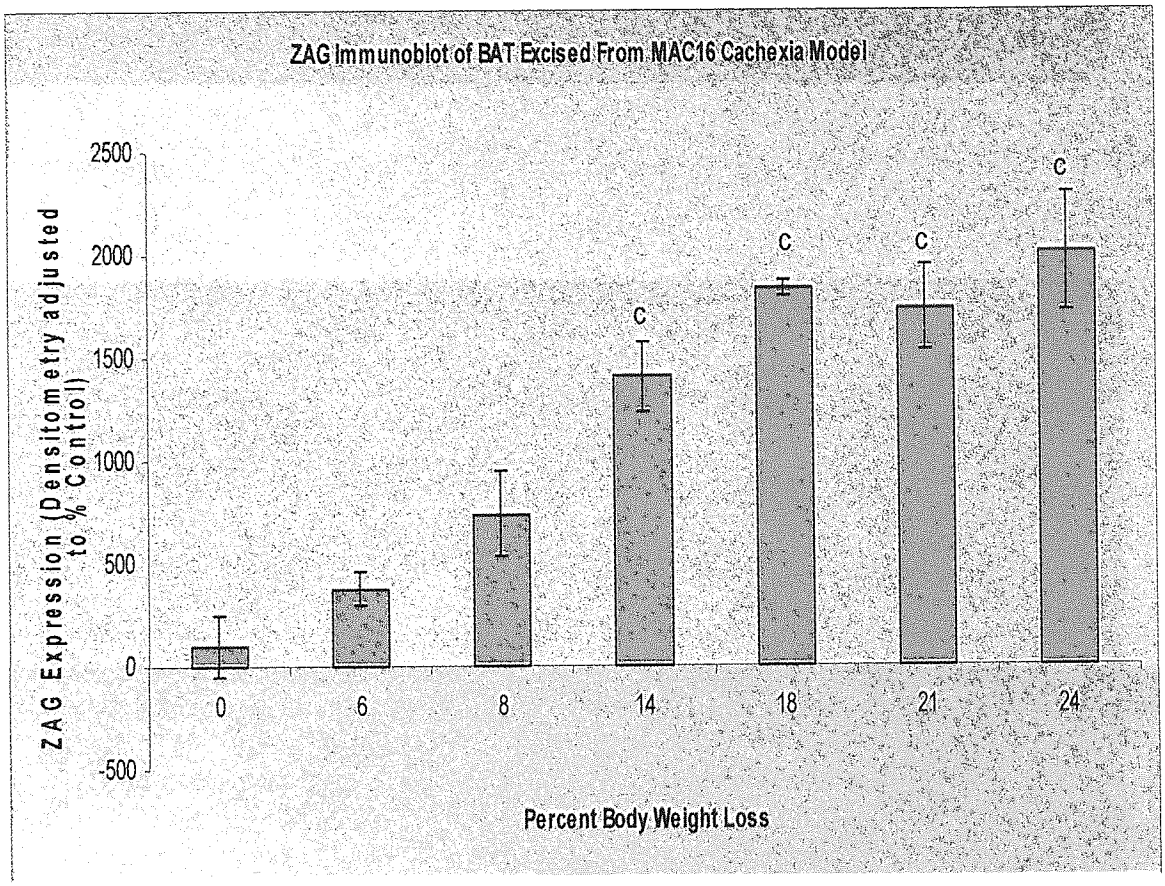
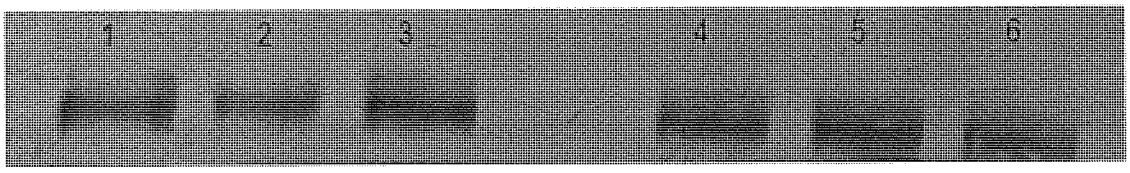


Figure 7.3.2.7. Cumulative graph showing ZAG expression in BAT excised from mice exhibiting all analysed body weight losses. Results shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as c, p<0.001 compared to 0% weight loss

7.3.3 ZAG Expression in BAT of PBS- and ZAG-dosed NMRI Mice



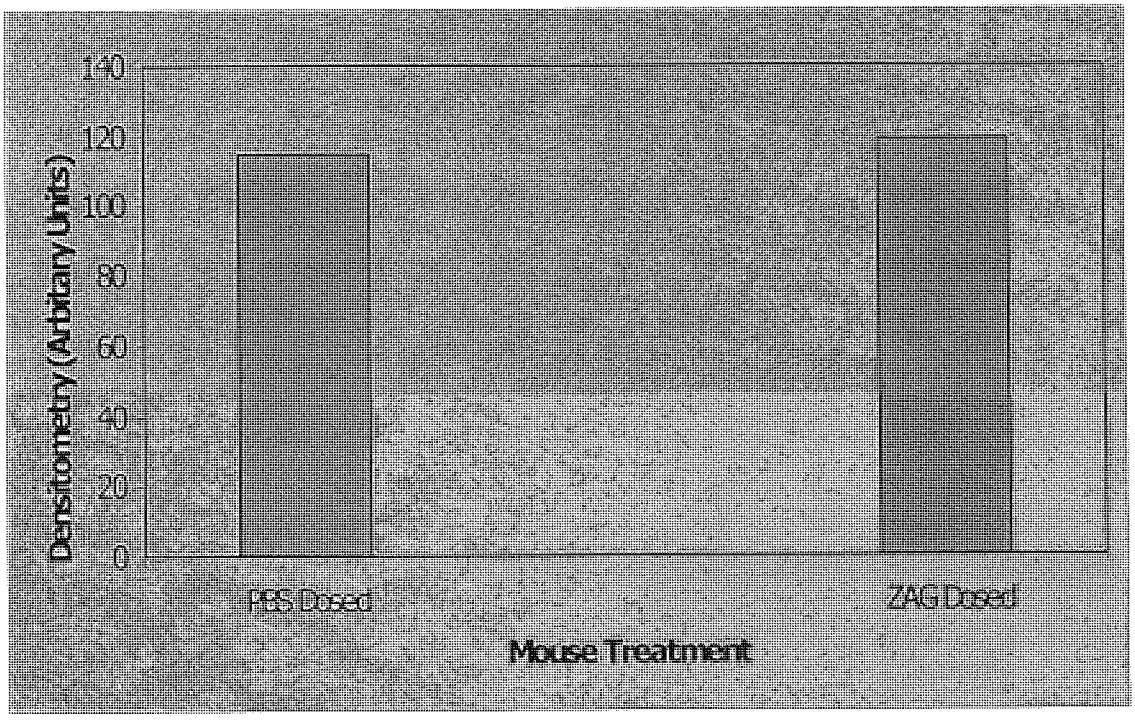


Figure 7.3.2.8. Immunoblot of BAT extracted from mice receiving twice daily 100μl PBS (lanes 1-3) or 25μg ZAG/100μl PBS (lanes 4-6). Data are means +/- SEM. Students t test revealed no significant difference between the two groups.

Basal level ZAG expression is apparent in both WAT and BAT of healthy NMRI mice. This has been confirmed by immunoblotting (this study) and RT-PCR of both *in vivo* and *in vitro* 3T3-L1 adipocyte samples (Bing *et al* (2003)). However, this expression is seen to increase during MAC16 model-associated cachexia.

There is some correlation of WAT ZAG expression with weight loss experienced by the MAC16 mice. Due to inter-mouse variation, this correlation is not rigid, however induction of significant ZAG expression commences at approximately 14% weight loss (although the expression at 17% weight loss appears spurious).

Similarly, the cachectic syndrome is seen to steadily increase BAT ZAG expression (induced to a significant degree at approximately 14%). Unlike WAT, ZAG expression is does not fall away slightly at higher weight losses, but plateaus at maximal expression after approximately 18% weight loss.

Both BAT and WAT begin to demonstrate significant up-regulation of ZAG at approximately 14% weight loss. This correlates well with the findings of Groundwater *et al* (1990), who demonstrated the lipolytic activity of MAC16 plasma to peak at 16% body weight loss.

It is unclear why WAT ZAG expression falls away slightly at higher weight loss but BAT expression does not. This could be the result of preferential WAT desensitisation (i.e. to the inducing factor). It is possible that during cachexia, WAT stores are preferentially mobilised over BAT (of which there is comparatively little (Russell ST, personal communication)). In actuality, it is very likely that following such enormous loss of body weight, this sample merely comprises

endothelial cells and macrophages as opposed to any functional adipocytes (Fearon K, personal communication).

Initially, it was suspected that tumour-synthesised ZAG was inducing alterations in host adipocyte ZAG expression. This theory is credible; the sympathetic nervous system regulates adipocyte tissue, and is the dominant stimuli in regulating rodent lipolysis. Additionally, the selective β_3-AR agonist BRL37344 increases 3T3-L1 ZAG mRNA levels *in vitro* (Bing *et al* (2003)). Similarly, ZAG itself is reported (Russell *et* al (2002)) to be agonistic at the β_3-AR so it is certainly conceivable for tumour-produced ZAG to induce adipocyte ZAG expression *in vivo* Such an increase would obviously potentiate weight loss associated with cancer cachexia.

However, **figure 7.3.2.8** demonstrates subcutaneous dosing with active concentrations of ZAG not to increase ZAG expression over those dosed with PBS ($p>0.05$). This implies that the increased adipocyte ZAG expression associated with MAC16 cachexia is not simply due to the action of tumour-produced ZAG. This result was expected to some degree as healthy NMRI mice dosed with ZAG fail to lose as much weight as predicted from their MAC16 counterparts. Furthermore, host desensitisation to administered ZAG is quick to set in, and the weight-loss plateaus (unpublished observation). Thus, although tumour-produced ZAG may mediate increased lipolysis, it is another factor apparently promoting ZAG expression in the adipocyte tissue of the host.

Other factors implicated in cancer cachexia (**section 1.8 – section 1.10.10**) were considered in an effort to assign responsibility for this adipocyte regulation. The 24kDa Proteolysis Inducing Factor is unlikely to interact such, due to the absence of PIF receptors on adipocyte tissue

(unpublished observations). It is not outside the realms of possibility that a PIF or muscle breakdown product may cause this increase in ZAG expression, although it is unlikely.

It may be that a cytokine is responsible; presently TNF-α would appear most likely candidate, however, as established in **section 1.10.12**, the MAC16 model possesses little/no cytokine contribution to the cachexia. Interestingly however, the newly developed anti-obesity agent Obex™ utilises administered interferon to induce ZAG expression in lymphocytes (reviewed in section **1.8.1.5.1.1.**), although whether this induction also occurs in adipose tissue remains undetermined.

It is conceivable that the glucose alterations associated with cachexia could alter adipocyte metabolite expression. However, the reduction of glucose levels associated with cancer make this unlikely to modulate ZAG expression in such a manner (Tisdale MJ, personal communication).

Leptin is a regulatory adipokine involved in body fat homeostasis (as reviewed in **section 1.10.3**). However, WAT leptin expression and circulatory levels of leptin markedly decrease during clinical cachexia (Brown *et al* (2001), the MAC16 tumour and the cachexigenic Yoshida hepatoma model (Bing *et al* (2003)). This argues against a role for leptin in mediating cachexia-associated lipolysis.

Another adipokine, resistin, inhibits adipocyte differentiation, therefore may potentially also influence ZAG synthesis (Kim *et al* (2001)). However, this too is reported unchanged in the tumours of MAC16 mice (Gomez-Ambrosi *et* al (2002)), making it an unlikely candidate as a mediator of lipolysis.

Glucocorticoids (GCs) are lipophilic molecules of low Mr. GCs are able to diffuse through cellular membranes where they bind their intracellular receptor (GR). GRs are hormone-activated, dual zinc finger transcription factors. Depending on the target gene, activated GR's may then stimulate (e.g. GC transactivation of the NFkB inhibitor IkB) or inhibit (e.g. GC transrepression of IL-2 and other cytokines) gene expression (as reviewed by Almawi & Melemedjian (2002)).

The roles of glucocorticoids in cancer cachexia (reviewed in **section 1.10.9**) are speculative to say the least. However, these may represent best candidates for regulating adipocytes in this manner. Bing *et al* (2003) demonstrated glucocorticoids to produced increased ZAG mRNA levels in 3T3-L1 adipocytes *in vitro*. Stimulatory effects of glucocorticoids on ZAG expression have also been reported in human breast cancer cells (Lopez-Boado *et al* (1994)). Adipocyte-derived ZAG expression is up regulated by dexamethasone, further implicating glucocorticoids to be regulators of adipose ZAG synthesis (Bing *et al* (2003)). Further work is required to determine the exact role of glucocorticoids on adipocyte ZAG expression.

Whatever the mechanism, the up-regulation of ZAG in host adipocyte tissue will induce marked lipolysis (BAT and WAT) and stimulate a local (BAT) increase in UCP1 (Bing *et al* (2002)). A rise in UCP1 expression further enhances the negative energy balance and weight loss associated with cachexia.

Bio-informatics analysis of the ZAG promoter locus indicated the presence of an octomer, one TATA box and multiple SP1 binding sites (unpublished results). It may be that glucocorticoids are discovered to interact with one of these elements. Interestingly, ω-3 fatty acids are

implicated in the regulation of SP1 binding domains – this could partially explain the anti-cachectic effect of EPA (a ω-3 oil reviewed in **section 1.10.11.6**) via action at this domain.

Unpublished experiments demonstrate ZAG to possess a degree of RNase activity. It was initially proposed that this activity conferred a percentage of ZAGs activity. However, the RNase angiogenin was apparently devoid of lipolytic activity (Russell ST, unpublished observation). This suggested that some aspect of RNase activity (such as the ability to enter cells) may be important for the functioning of ZAG.

7.4 Conclusions.

Lipolysis in cachexia is mediated by LMF/ZAG. This study demonstrates that this lipolytic factor to arise not only from the tumour itself but also following induction in the adipose tissue of the cachectic host.

Significant expression of ZAG by host adipocyte tissue appears at approximately 14% weight loss. This correlates very well with the findings of Groundwater *et al* (1990). It is impossible however to speculate at this stage whether it is the tumour or adipocyte-derived ZAG (or a combination of both), which is responsible for the fat loss in cachexia.

Quite which cachectic factor induces the synthesis of adipocyte-derived ZAG is unclear. It was speculated initially that tumour-manufactured ZAG might itself induce further ZAG expression in WAT and BAT. This was demonstrated not to be the case, and, despite speculation and hypothesis, a candidate molecule remains experimentally unproven. Further work is currently in progress to elucidate this factor, and results will prove interesting.

8.1 Introduction.

Skeletal muscle glucose uptake is essential for glucose homeostasis and the contraction/relaxation process. This is outlined in **figure 8.1.1**.

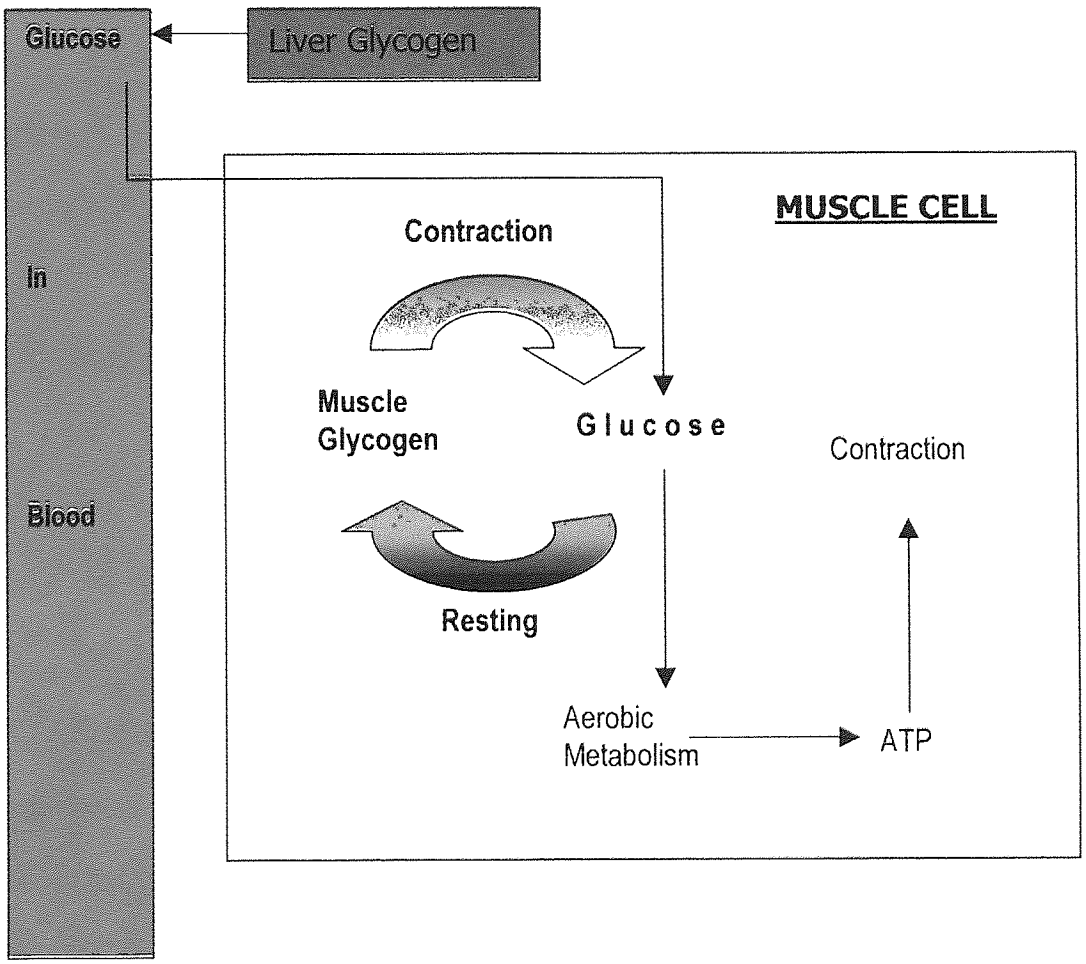


Figure 8.1.1. Metabolic pathways in muscle resulting from glucose uptake in contraction and relaxation (adapted from Sherwood (1993)).

The process of glucose metabolism (**figure 8.1.1**) is fundamental to the normal homeostasis of skeletal muscle. In pathological conditions such as obesity and cachexia, abnormalities in **figure 8.1.1** may become apparent. Islam-Ali & Tisdale (2001) demonstrated dose-dependent LMF-induced 2-Deoxyglucose uptake into C_2C_{12} myotubes and MAC16 tumour cells. Similarly, Li & Adrian (1999) demonstrated a bioactive cachexia-associated factor to facilitate glucose utilisation.

Similarly, administration of LMF *in vivo* is seen to significantly alter host glucose metabolism (Russell & Tisdale (2002)). Additional studies have examined the effects of β_3-AR agonists +/- insulin (Liu *et al* (1996), Tanishita *et al* (1997) & Roy *et al* (1998)) on 2-deoxyglucose (2DG) uptake of *in vivo* and *in vitro*. A study that examines LMF under similar experimental conditions, would provide interesting comparison, and potentially add credibility to its β_3-AR agonist 'status'.

This study aims to provide further insight into muscle glucose transport in MAC16 cancer cachexia +/- administration of additional LMF. This will be performed in the presence and absence of a maximal concentration of insulin, and should add credibility to the theory that insulin resistance does not occur in this model.

Therefore, the effect of LMF on basal 2DG uptake in isolated soleus muscle (+/- insulin) will be examined in MAC16 mice. This should provide further mechanistic data for this catabolic factor. The data produced by this study may also be of specific use in the development of LMF/ZAG as an anti-obesity agent.

8.2 Methodology.

8.2.1 Purification of Lipid Mobilising Factor.

LMF was purified from the cachectic urine of advanced pancreatic cancer patients as described in section **3.2.2**.

8.2.3 The Effect of LMF on Glucose Transport In The Soleus Muscle of MAC16 Mice.

The method of Borst and Hennessy (2001) was adapted. Briefly, MAC16 mice were administered intravenously either vehicle control (PBS) or LMF (8μg) every 12 hours for 72 hours. Food and water was available *ad libitium* for the initial 48 hours, and then the rodents fasted for the final day. Mass, food and water intake was recorded throughout.

Mice were sacrificed in accordance with Schedule 1 of the Animals (Scientific Procedures) Act. Strips of soleus muscle (approximately 25mg each) were removed under aseptic conditions and incubated in sterile 1%BSA/Krebs-Ringer Henseleit buffer (**section 2.7.1**) for 60 minutes.

Muscle strips were removed and transferred into sterile Krebs-Ringer Henseleit buffer in the presence of 5mM 2-deoxy-D-[2,6-^{3}H]glucose (2DG) and 20mM sorbitol +/- a maximal concentration of insulin (16U/ml).

Muscles were removed from Krebs Henseleit buffer, macerated entirely and transferred into 20ml scintillation vials. Samples were mixed thouroughly with 8mls of Ultima Gold XR scintillation fluid. Sample radioactivity was assessed as disintegrations per minute (dpm) over four minutes using a 2000CA Tri-Carb liquid scintillation analyser.

8.3 Results And Discussion.

8.3.1 [^{3} H] Deoxyglucose Uptake in Soleus Muscle.

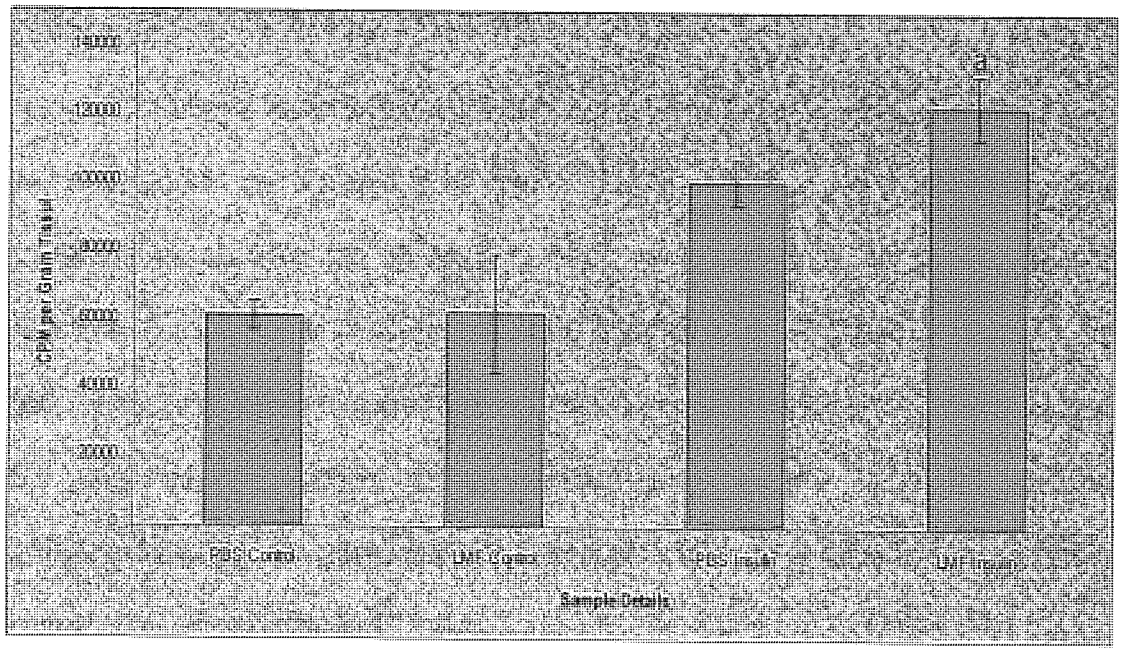


Figure 8.3.1.1. Tritiated 2 Deoxyglucose (2DG) uptake by isolated soleus muscle. Data are shown as +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison test. Results are shown as a, $p<0.05$ compared to LMF and PBS controls.

The presence of 16U/ml insulin raises soleus muscle 2DG uptake of [^{3} H] deoxyglucose from basal activity in both PBS (164.2% of control) and LMF (197.5% of control)-dosed groups. Roy *et al* (1998) studied glucose utilisation in various tissues also reports augmentation of skeletal muscle glucose uptake by insulin in a nitric oxide (NO) dependent manner. However, statistical significance here over basal uptake is only achieved following LMF administration, implying this factor also to mediate an increase in soleus glucose uptake.

Liu *et al* (1996) studied the effects of the selective β_3-Adrenorecptor agonist BRL37344 on glucose uptake (GU) and phosphorylation in isolated soleus muscle. BRL37344 significantly increased GU in a manner not attenuated by selective β_1-AR or β_2-AR antagonists (implying a

'true' β_3-AR-specific effect). Liu *et al* (1996) demonstrated similar effects with CL316,243 (another selective β_3-AR agonist).

Tanishita *et al* (1997) studied the effects of BRL37344 and insulin on glucose transport in L6 myotubes. Insulin was demonstrated to increase glucose transport in a dose-dependent manner (maximal stimulation was achieved at 10^{-7} M). BRL37344 was also observed to potentiate *in vitro* glucose transport (in the absence of insulin) at concentrations of 10^{-7} to 10^{-5} M. Furthermore, Tanishita *et al* (1997) demonstrated the effects of BRL37344 and insulin to be additive, suggesting different mechanisms for stimulating glucose transport. Tanishita *et al* (1997) suggested insulin-induced glucose transport to be mediated by GLUT4 induction. BRL37344-induced glucose uptake was independent of GLUT4, but possibly due to an increase in intrinsic plasma membrane GLUT activity.

Similar *in vitro* soleus muscle glucose transport stimulation is reported by Board *et al* (2000) using the selective β_3-AR agonist SB226552. Interestingly, one selective β_3-AR agonist CGP12177A did not have any effect on glucose transport in such a system (Board *et al* (2000)).

These findings correlate well with the results of this study. There is abundant evidence (Russell (2001), Russell *et* al (2002) & Russell *et al* (2003)) that LMF and ZAG are also agonists at the β_3-AR, further credibility to which has therefore been added here.

It may well be that LMF would enhance glucose uptake to even greater significance if the experiment was repeated with non-cachectic mice (this would overcome the potential desensitisation (discussed in **section 8.3.1**). This experiment does demonstrate however, that the MAC16 model is not insulin-resistant (Tisdale MJ, personal communication).

Quite why LMF should stimulate 2DG uptake in soleus (and skeletal) muscle remains unclear. Hiari *et al* (1998) demonstrated that LMF administration to NMRI mice to result in decreased blood glucose levels (suggesting that LMF stimulates glucose utilisation). Islam-Ali & Tisdale (2001) obtained *in vitro* dose-dependent LMF-induced 2DG uptake into the surrogate muscle C_2C_{12} myotube line, and also into MAC16 tumour cells. Similarly, Li & Adrian (1999) demonstrated a pancreatic cancer-associated bioactive factor to facilitate glucose uptake. Tumours associated with cachexia often have high glucose demands (discussed in **sections 1.5-1.6**). The LMF-associated alterations in glucose metabolism may therefore be a mechanism by which the substrate demands of the tumour are met (Islam-Ali & Tisdale (2001) & Islam-Ali (2001)).

Such properties of LMF/ZAG at the β_3-AR can be monopolised further in the development of a potential anti-obesity agent. Insulin resistance is a common problem associated with obese individuals. Borst and Hennessy (2001) examined insulin resistance in obese Sprague-Dawley rats. CL316,243 administration reversed the insulin resistance associated with this model. The mechanism of this reversal was unclear, however, the authors hypothesised that a reduction in visceral fat may be accompanied by a decrease of some fat-derived mediator of insulin resistance.

Similarly Pan *et al* (2001) report clenbutarol administration to obese Zucker rats. Chronic clenbutarol treatment restored insulin sensitivity, adipose and muscle glucose uptake. Again, this was achieved by a re-partitioning of body weight between tissues as opposed to any direct effect. If Beta Agonists are indeed capable of restoring insulin resistance in obesity, then ZAG should prove an invaluable tool in the treatment of this disorder.

8.4 Conclusions.

Insulin raises 2DG uptake of isolated soleus muscle obtained from both LMF and PBS dosed MAC16 mice. The effects of LMF and insulin appear additive, resulting in a maximal response in the presence of both. Previous *in vivo* and *in vitro* studies utilising MAC16 and C_2C_{12} cells dosed with LMF provide evidence to substantiate these findings.

These alterations in glucose metabolism may be induced in order for the tumour to meet its glucose demands. The mechanisms remain unclear but it may be that B_3-AR agonists alter intrinsic plasma membrane GLUT activity.

Further studies should repeat this protocol utilising NMRI mice, MAC13 mice, and *ob/ob* mice. It would certainly be interesting to determine whether LMF/ZAG would also reverse insulin resistance associated with an obesity model. This would certainly be of value when marketing as an anti-obesity drug.

CHAPTER 9. EFFECTS OF A LIPID MOBILISING FACTOR ON UCPn EXPRESSION.

9.1 Introduction.

A review of the structure and function of the uncoupling protein homologues is provided in **section 1.10.11**. This study aims examines specifically the effect of LMF on UCPn in a variety of *in vitro* models.

Uncoupling protein 1 uncouples mitochondrial respiration from ATP synthesis in brown adipocyte tissue (BAT). Energy is dissipated as heat, resulting in non-shivering thermogenesis. The function of uncoupling protein 1 is summarised in **figure 9.1.1.**

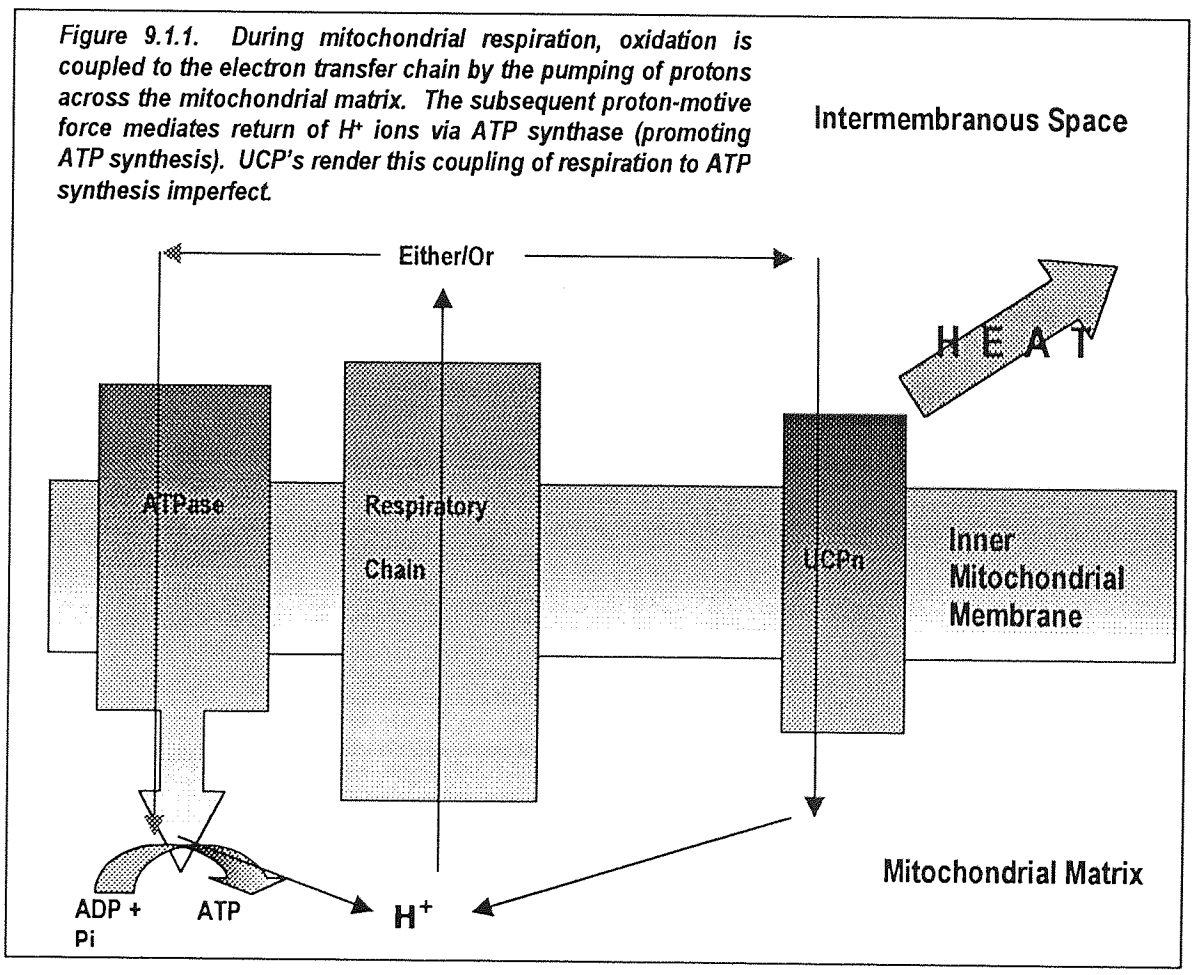


Figure 9.1.1. *During mitochondrial respiration, oxidation is coupled to the electron transfer chain by the pumping of protons across the mitochondrial matrix. The subsequent proton-motive force mediates return of H^+ ions via ATP synthase (promoting ATP synthesis). UCP's render this coupling of respiration to ATP synthesis imperfect.*

Adapted from Ricquier & Bouillaud (2000).

Isolated UCP1 consists of 306 amino acids with a calculated molecular weight of 33.2kDa. UCP1 is divided into 3 approximately equal domains of 100 amino acids each, and possesses six transmembrane helices (as reviewed by Klingenberg & Huang (1999)).

The uncoupling proteins are implicated in energy balance of body weight regulation. Maintenance of body weight depends on the difference between energy intake and energy expenditure. If the energy balance is positive, it can lead to obesity. Conversely, a negative energy balance contributes to cachexia (Argiles *et al* (2002)).

Unlike UCP2 or UCP3, UCP1 is confined exclusively to the brown adipocyte tissue of rodents and human neonates (Bing *et al* (2000) & Ricquier & Bouillaud (2000)). UCP1 is modulated sympathetically through the Beta-$_3$-Adrenoreceptor (β_3-AR) (Bing *et al* (2000) & Yoshitomi *et al* (1998)). UCP1 is also activated by fatty acids and purine nucleotides (Argiles *et al* (2002)). Argiles *et al* (2002) postulate that surface agonist-β-AR interactions promote increased cAMP and thus activation of PKA. This results in enhanced lipolytic activity and increased intracellular fatty acid content. The internal FA increase enhances both UCP1 gene transcription and UCP1 expression (Argiles *et al* (2000)).

Recently, other proteins sharing homology with UCP1 (UCP2, UCP3 and UCP4) were discovered (Ricquier & Bouillaud (2000)). UCP2 is expressed ubiquitously throughout the body. The tissue distribution of UCP2 is different from that of UCP1 or UCP3, and UCP2 is expressed in sites not traditionally associated with adaptive thermogenesis, indicating some other function (Yoshitomi H *et al* (1999)).

UCP2 shares 59% homology with UCP1.Although some groups attribute similar thermogenic roles to that of UCP1 for UCP2, the presence of this carrier implies other functions also. UCP2 is activated by PPARγ agonists (such as TZDs) in a manner independent to the differentiation process (Fleury & Sanchis (1999)) and fatty acids (Fleury *et al* (1997)). UCP2 expression is greatly elevated in the WAT of *ob/ob* and *db/db* compared to lean animals – thus UCP2 is implicated in energy regulation and body weight balance (as reviewed by Research Diagnostics inc. (2000)). UCP2 becomes up-regulated in starvation and the role of UCP2 in detoxification of reactive oxygen species appears important and has been reported (Pecqueur *et al* (2001) amongst others) and investigated (see also **Chapter 10**).

Uncoupling protein 3 shares 71% and 57% homology with UCP2 and UCP1 respectively. UCP3 expression is highly specific for skeletal muscle. The long and mature form of UCP3 is designated UCP3L ;UCP3s (the truncated isoform) lacks the sixth potential transmembrane domain. Both forms are expressed in muscle (as reviewed by Research Diagnostics inc. (2000)). Like UCP2, UCP3 is also implicated in the detoxification of reactive oxygen species (Garvey (2003), Pecqueur *et al* (2001)), and is heavily implicated in regulation of resting energy expenditure (Garvey (2003)). Genetically modified mice over-expressing UCP3 are of hyperphagic and lean phenotype (Clapham *et al* (2000)). Additionally, an exon 6 splice donor/acceptor polymorphism in the UCP3 gene (especially prevalent in Gullah-speaking African Americans) is associated with a marked reduction in basal metabolic rate (Garvey (2003)). It is thought this may be an evolutionary adaptation to the frequent starvation encountered during the history of these people (Russell ST, personal communication). UCP3 therefore appears of importance in the regulation of energy expenditure and basal metabolic rate.

Groups have reported increased UCP2 and UCP3 mRNA levels in skeletal muscle of rodent cancer cachexia models (Bing *et al* (2000) & Sanchis *et al* (1998)). Skeletal muscle contributes up to 40% of whole-body adrenaline-induced thermogenesis in humans (Research Diagnostics inc., Review (2000)). Because skeletal muscle contributes to thermogenic response to cold, the reported up-regulation by the tumour burden could point to enhanced energy production that could exacerbate tissue losses (Bing *et al* (2000)). However, this notion is challenged by the fact that pair feeding induced similar increases in muscle UCP2 and UCP3 mRNA expression.

Cachexia and starvation (+/- the associated mobilisation of free fatty acids) may therefore promote this skeletal muscle UCP up-regulation. Studies point to a relationship between lipid metabolism and UCP expression in various tissues, however, a great deal of data divergence exists presently (Fleury & Sanchis (1999)), making exact mechanisms more difficult to elucidate.

Bing *et al* (2000) investigated whether increased UCP1 contributed to the overall negative energy balance (hence weight loss) in MAC16-associated cachexia. BAT UCP1 mRNA increased in tumour-bearing mice compared to controls. However as hypothermia accompanies cachexia in this model, was not possible to determine whether increased UCP1 mRNA was a compensatory measure to the cold or the action of a tumour product.

Bing *et al* (2002) then directly injected tumour-derived LMF into NMRI mice and observed increased UCP mRNA expression in a variety of tissues. This particular experimental model exists in the absence of starvation, thus this cannot be the cause of increased UCP2 mRNA in skeletal muscle here. It was postulated (Bing *et* al (2002)) that LMF-induced fatty acid mobilisation may up-regulate UCP2 mRNA in skeletal muscle.

The molecular mechanisms which regulate the UCP homologues are generally accepted. However, some controversy and a great deal of conflicting data exist. Fatty acids and purine nucleotides activate and inhibit UCP1 respectively. It was therefore suggested that GDP could also inhibit UCP2, and that all the uncoupling proteins functioned with similar mechanisms (Negre-Salvayre *et al* (1997)). However, this proposal was based only on analogy with UCP1, as opposed to direct experimental evidence. The sequence similarities between UCP2 and UCP1 are not sufficient to presume synergistic mechanisms (Fleury & Sanchis (1999)). Fleury *et al* (1997) demonstrated regulators of UCP1 to have absolutely no effect on UCP2, and Rial & Gonzalez-Barraso (2000) report further mechanistic differences between UCP homologues. UCP1 and UCP2 are regulated by retinoids (Rial & Gonzalez-Barraso (2000)) whilst UCP2 and UCP3 were either unaffected or only mildly affected by fatty acids (Rial *et al* (1999) & Klinenberg (2001) respectively). These data highlight regulatory differences between the UCP homologues.

Interestingly, some cachexia-associated factors have been implicated in uncoupling protein regulation. Leptin, in addition to cytokines such as TNF-α and IL-1 result in increased UCP2 mRNA (Fleury & Sanchis (1999)). Thus, increased skeletal muscle UCP2/UCP3 mRNA in the cachexigenic Yoshida AH-130 ascites hepatoma model (Sanchis *et al* (1998)) may not simply be the result of cachexia-associated starvation and/or FFA mobilisation. Busquets *et al* (1998) performed a single intravenous injection of the cachexia-associated cytokine TNF-α. This injection was sufficient to induce a fourfold and twofold increase in muscular UCP2 and UCP3 expression respectively, confirming a direct relationship between this pyrogen cytokine and UCP expression (Fleury & Sanchis (1999)). This implies that circulatory cachectic factors may modulate UCP expression directly.

Interestingly Cortright *et al* (1999) also report an increase in UCP2 mRNA in dennervated gastrocenemius. This finding is of interest because denervation induces atrophy of muscle cells in a manner similar to that observed during cancer cachexia.

This review is by no means exhaustive. The distribution, regulation and functioning of the uncoupling proteins are highly complex. Herein, this study will specifically examine the roles of uncoupling proteins in cancer cachexia. For a broad and comprehensive review, readers are advised to study; 'The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP' (Ricquier & Bouillaud, Biochemical Journal 345:161-179 (2000)) and 'Homologues of the uncoupling protein from brown adipocyte tissue (UCP1):UCP2, UCP3, BMUCP1 and UCP4' (Bouillaud *et al*, Biochimica et Biophysica Acta 1504:107-109 (2001)).

Until now, studies examining UCP1/2/3 regulation during cancer cachexia have focussed predominantly on mRNA data obtained from *in vivo* models (Bing *et al* (2000), Bing *et al* (2002) & Sanchis *et al* (1998)). The disadvantage with these models is that *in* vivo, it is difficult to distinguish direct from indirect effects. In addition, quantification of protein expression is arguably of greater value than mRNA data (especially as regulation of UCP2 is reported to be translational (Pecqueur *et al* (2001))).

This study employs various *in vitro* models to elucidate the exact interactions between a tumour-derived lipid mobilising factor and the uncoupling protein homologues. C_2C_{12} myotubes were used as a surrogate skeletal muscle model, differentiated 3T3-L1 adipocytes as a surrogate adipocyte model, and primary cultures of WAT and BAT will also be employed.

10.2 Methodology.

10.2.1 Preparation of LMF.

LMF was purified from the cachectic urine of pancreatic cancer patients as described in **section 3.2.2**).

10.2.2 Preparation of C_2C_{12} Myotubes.

C_2C_{12} myoblasts were maintained in 75cm^3 flaks using DMEM supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were subcultured at semi-confluence, and passage number was not permitted to exceed seventeen.

For experimental purposes, 50cm3 flasks were seeded with 2x10^5 myoblasts. Following achievement of 90% confluence, differentiation was induced by the addition of DMEM supplemented with 2% horse serum, 1% penicillin streptomycin and 1% glutamine. Myotubes were used experimentally no later than four days post-differentiation. The effect of LMF on UCPn expression in this cell system was examined as described in **10.2.6**.

10.2.3 Differentiation of 3T3-L1 Preadipocytes.

50cm3 flasks were seeded with 2x10^5 3T3-L1 preadipocytes. These were grown to confluence in DMEM supplemented with 10% CO_2 in air at 37°C. Cells were differentiated according to the method of Frost *et al* (1985).

Two days post-confluence (day 0), differentiation was induced with DMEM supplemented with 10% FCS, 1% penicillin streptomycin, 1% glutamine, 0.5mM methylisobutylxanthine, 0.25μM dexamethasone and 1μg/ml insulin. On day 2, methylisobutylxanthine and dexamethasone were removed but insulin maintained for a further two days. On day 4 (and thereafter), insulin was removed and the cells maintained in DMEM supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine. This was replaced every two days.

Cells were used experimentally between days 8-12. At this time, 95% of the cells express the adipocyte phenotype (Frost *et al* (1985)). Confirmation that differentiation had occurred was confirmed by staining with Oil Red O (Islam-Ali (2001)). Two hours prior to each procedure, adipocyte monolayers were incubated in serum-free DMEM. The effect of LMF on UCPn expression in this cell system was examined as described in **9.2.6**.

9.2.4 Primary Culture of Brown Adipocytes.

Primary culture of brown adipocyte tissue was performed using a method adapted from that of Nechad *et al* (1983). Briefly, following sacrifice, inter-scapular BAT was dissected from NMRI mice under sterile conditions. 3ml of sterile 3%Krebs BSA collagenase solution (**section 2.3.2.3**) was added and the tissue macerated. Samples were gassed with 10% CO_2 in air and incubated at 37°C for 30 minutes with agitation. Tissue remnants were removed by filtering with a 250μM nylon screen and the preparation incubated at 4°C on ice for a further 30 minutes to allow separation of mature adipocytes.

The infranatant containing adipocyte precursors was removed using a syringe and filtered further through a 25μM nylon screen. Cells were pelleted by centrifugation at 700g for ten minutes and

re-suspended DMEM supplemented with 10% newborn calf serum, 1% penicillin streptomycin, 25μg sodium ascorbate/ml, 10mM HEPES and 40U/ml insulin. BAT cells were allowed to adhere and form a monolayer (about 7 days). Two hours prior to experimentation, insulin was removed from the culture media and replaced with 5mM IBMX. The effect of LMF on UCPn expression in this cell system was examined as described in **9.2.6**.

9.2.5 Primary Culture of White Adipocytes.

Epidydimal fat pads were removed from sacrificed NMRI mice under sterile conditions. 2-4g of tissue was macerated in DMEM supplemented with 25mM HEPES, 1mg/ml collagenase, 4% defatted BSA, 1% penicillin streptomycin and 1% glutamine. This preparation was gassed with sterile 5% CO2 in air, and incubated for 30 minutes at 37°C with agitation. Fat cells separated to the surface of the media, were collected and washed twice with DMEM supplemented with 2% FCS, 1% defatted BSA, 1% penicillin streptomycin and 1% glutamine. Mature adipocytes were allowed to float to the surface and isolated with a pasteur pipette. These cells were transferred into 50cm3 flasks in DMEM supplemented with 2% FCS, 1% defatted BSA, 1% penicillin streptomycin and 1% glutamine. Experimental procedures were completed within 16 hours, after which adipocyte characteristics were lost. The effect of LMF on UCPn expression in this cell system was examined as described in **9.2.6**.

9.2.6 Effect of LMF on UCPn Expression *in vitro*.

Two hours prior to LMF addition, SR59230A (10μM), α-ZAG (1:500), IBMX (0.5mM) or PD98059 (25μM) were added to culture systems as desired. Experimental concentrations of LMF (0, 0.23, 0.35, 0.46 and 0.58μM) were added and co-incubated for a further 24 hours.

Cells were washed twice with ice-cold PBS and scraped from the substratum into uncoupling protein sonication buffer (**section 2.8.1**) using a rubber policeman. Isolate was sonicated on ice at 4°C three times for ten seconds (with intervals between each pulse).

Protein concentration was estimated according to the method of Bradford (**section 3.2.3**), and the samples appropriately diluted and denatured (**section 3.2.4**). Western blotting was performed employing rabbit α-mouse 1° antibody (UCP1 (1:1000, 3h at RT) and UCP2 (1:1000, 2h at RT)) and rabbit α-rat (UCP3 (1-10μg/ml, 3h at RT). Following four 15 minute washes with 0.1% PBS-T (**section 2.3.4.2a**), secondary probing was performed with peroxidase-conjugated goat α-rabbit 2° antibody (1:2000, 1h at RT). Development was by enhanced chemiluminescence (ECL). Blots were subjected to densitometric scanning and analysis using 'Phoretix ID Advanced' software. Equal loading was confirmed following Ponceau S staining.

9.2.6 Skeletal Muscle UCP2 Expression following *in vivo* ZAG Administration.

Healthy NMRI mice were dosed subcutaneously with 30μg ZAG (in 100μl sterile PBS) or 100μl sterile PBS twice daily over a 5 day period. Another group of NMRI mice were administered ZAG (90μg/300ml water) or PBS orally over an identical duration. Injections were performed c/o Dr. Russell.

Following sacrifice, the gastrocenemius was excised under sterile conditions and macerated in ice-cold EDTA/sucrose tissue homogenising buffer (**section 2.8.2**). Samples were centrifuged at 4500rpm for 10 minutes to removes cellular debris. 3-5ml of supernatant is removed, balanced and ultrafuged at 40,000 rpm for 10 minutes to isolate microsomes. The pellet is resuspended in

1ml homogenising buffer, and prepared for western blot analysis as previously described in 7.2.2.

9.3 Results & Discussion.

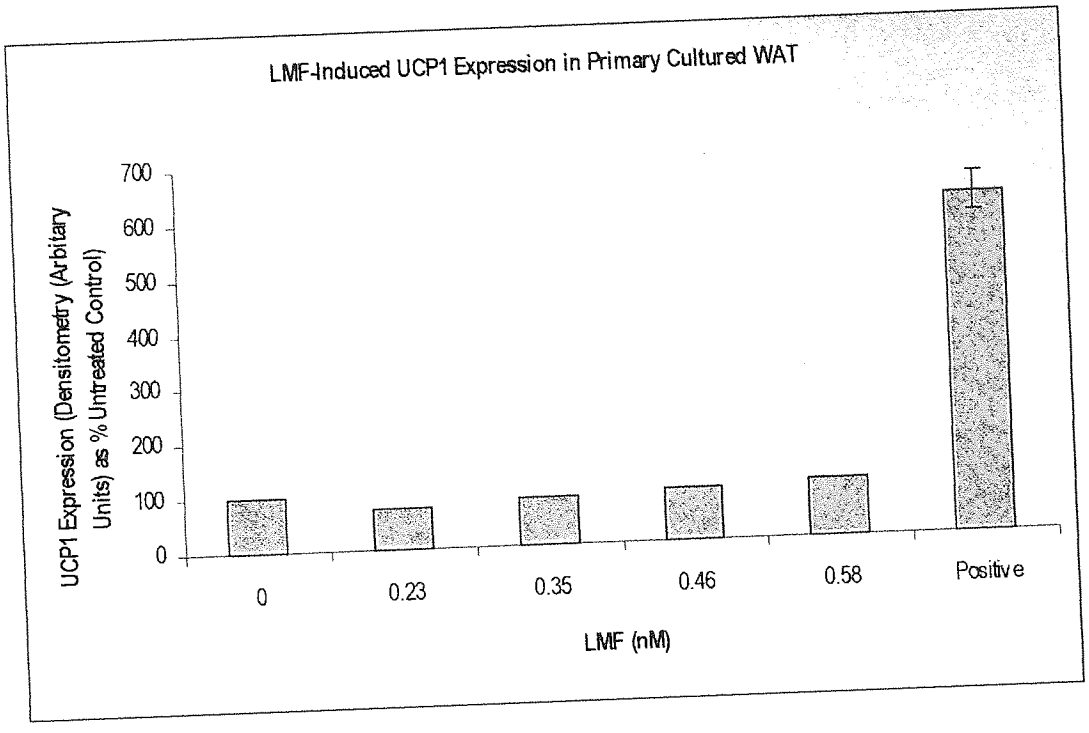
9.3.1 Immunological Detection of Uncoupling Proteins.

Optimisation and utilisation of these antibodies was to prove extremely difficult, fickle and unpredictable. The inferior quality of available antibodies to detect UCP homologues is documented extensively in the literature (Fleury & Sanchis (1999), Gavery (2003), Pecqueur *et al* (2001) & Ricquier & Bouillaud (2000) among others), with little convincing immunological data existing in at the moment (Ricquier & Bouillaud (2000)).

Although of poor quality, the UCP2 and UCP3 antibodies employed in this study have been utilised previously by our laboratory (Russell, 2001) and published (Bing *et al* (2002)). Initially, immumodetection of the UCP homologues proved impossible. Optimisation of cellular preparation procedures was performed in order to attain maximum yield of mitochondrial protein fraction (optimised procedure reported in **9.2.6**). Similarly, many antibodies were trialled to ensure optimum and accurate detection. The immunoreactive band detected by this antibody increases in murine skeletal muscle is up-regulated with LMF-induced *in vivo* fatty acid mobilisation and corresponds with UCP2 mRNA data obtained in the same study (Bing *et al* (2002)). Similarly, the UCP3 antibody utilised is reported to be of high quality (Russell ST, personal communication after attending International Obesity Conference (2003)). All antibodies detected immunoreactive bands at approximately 30kDa (as would be expected). In addition to this, all experiments were repeated to ensure reproducibility and accuracy throughout.

Given adequate time, RT-PCR and northern blotting could have been performed. However, the reported translational regulation of UCP2 (Pecqueur *et al* (2001)) may have rendered such an exercise fruitless.

9.3.1 Effect of LMF on UCP1 Expression in Primary Cultured WAT.



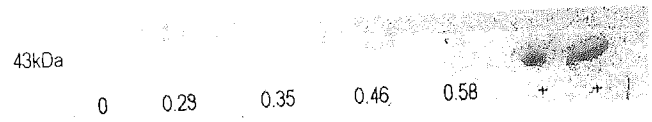


Figure 9.3.1.1. LMF-induced expression of UCP1 in primary cultured white adipocyte tissue (as detected following 16 hour incubation by western blotting). Densitometric data are means (n=1) and n=2 +/- SEM for positive control. Example blot also shown. No significance found between experimental groups.

9.3.2 Regulation of UCP1 in Primary Culture (BAT).

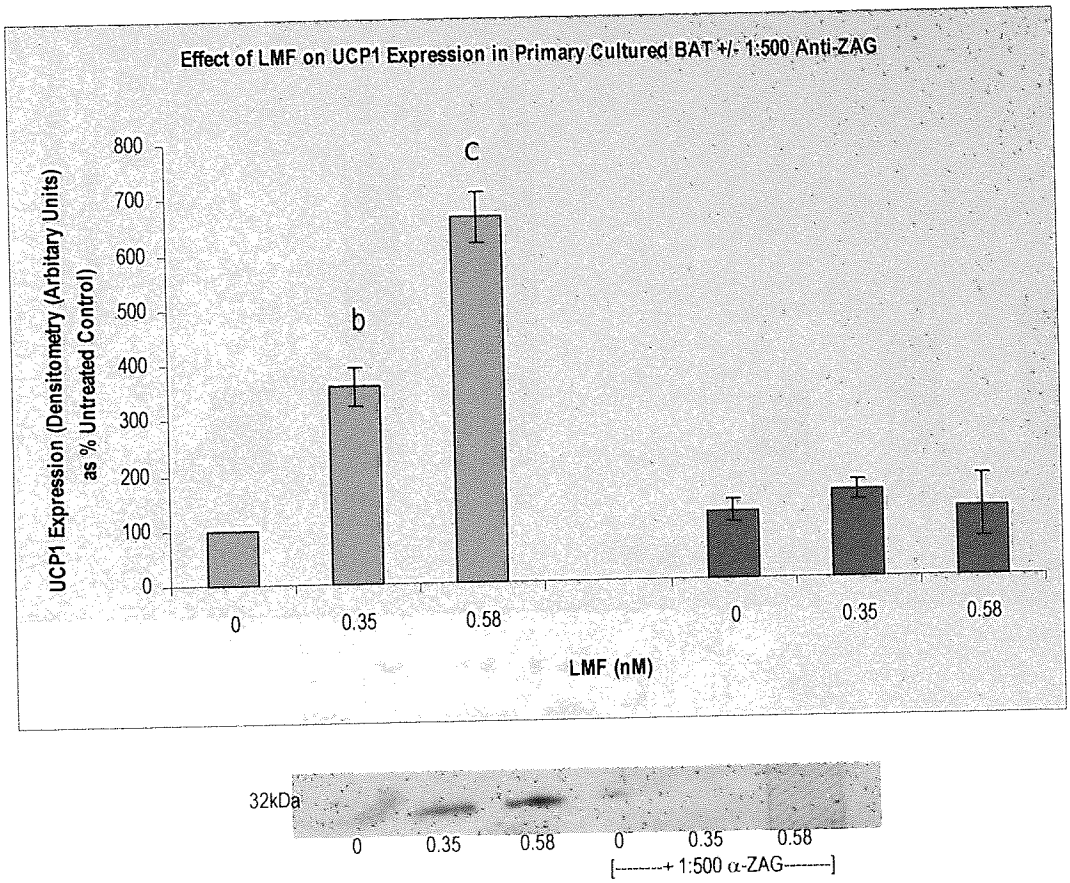


Figure 9.3.2.1. LMF-induced expression of UCP1 in primary cultured brown adipocyte tissue in the presence (red bars) and absence (blue bars) of 1:500 α-ZAG (24 hour LMF incubation, bands detected utilising α UCP-1). Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by b, $p<0.01$ and c, $p<0.001$.

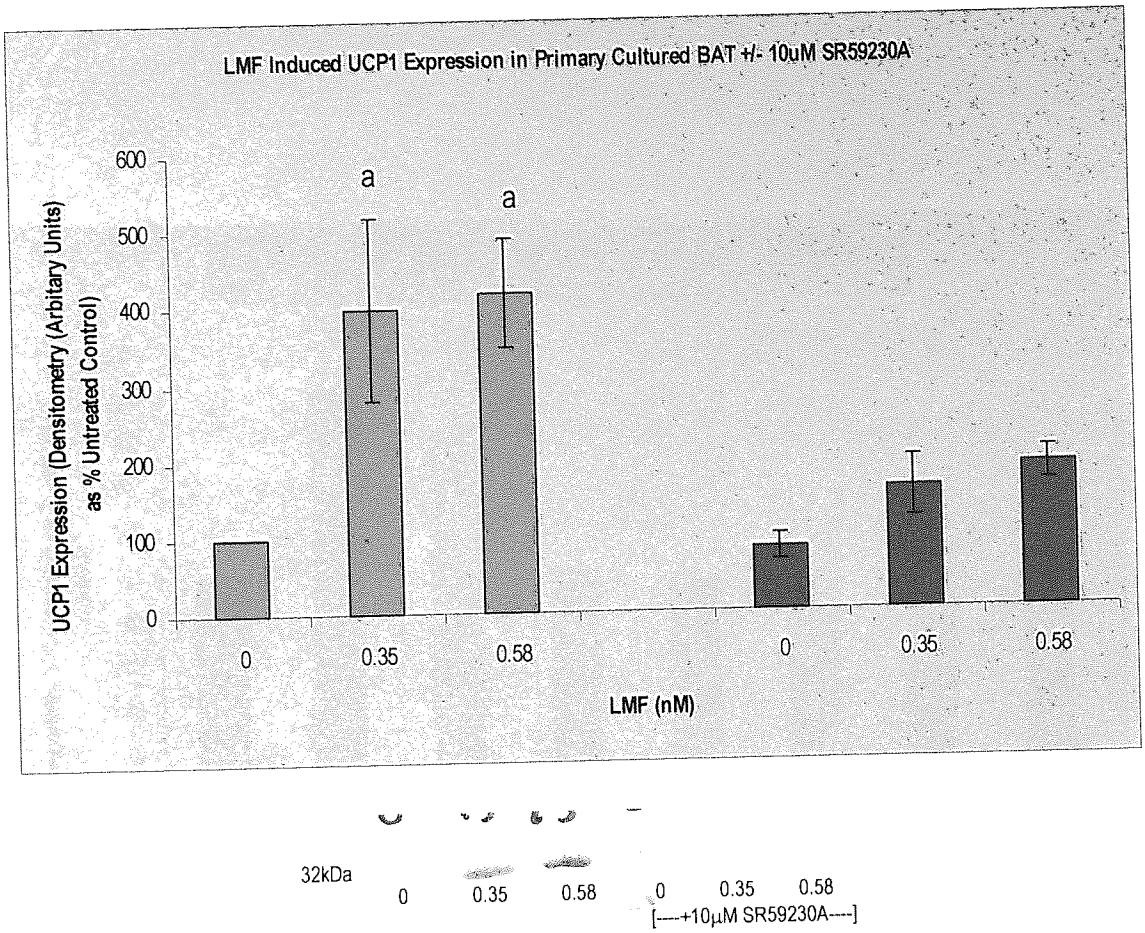
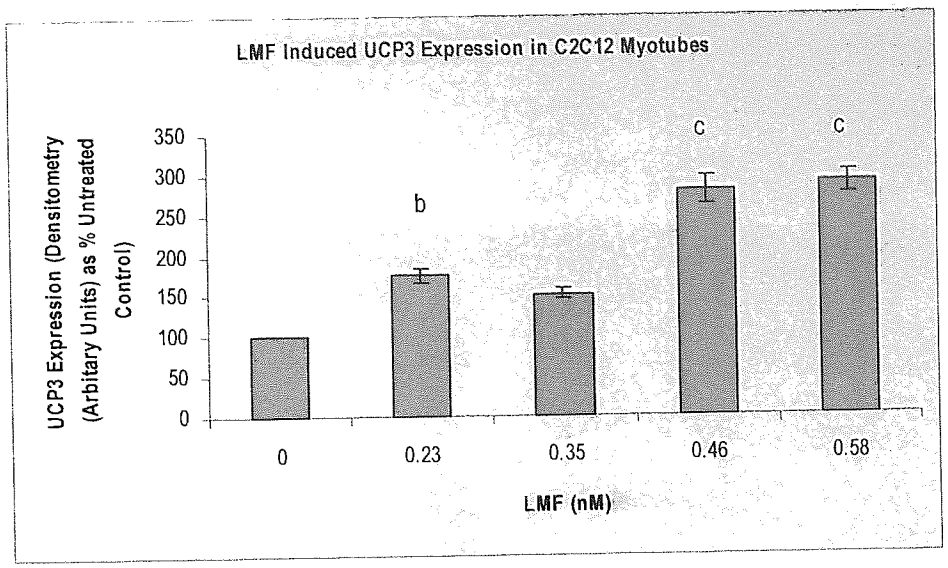
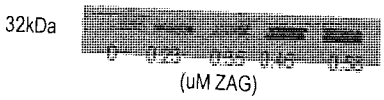


Figure 9.3.2.2. LMF-induced expression of UCP1 in primary cultured brown adipocyte tissue in the presence (red bars) and absence (blue bars) of 10μM SR59230A. Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by a, $p<0.05$.

9.3.3 The Effect of LMF on UCP3 Expression in C_2C_{12} Myotubes.

A





C

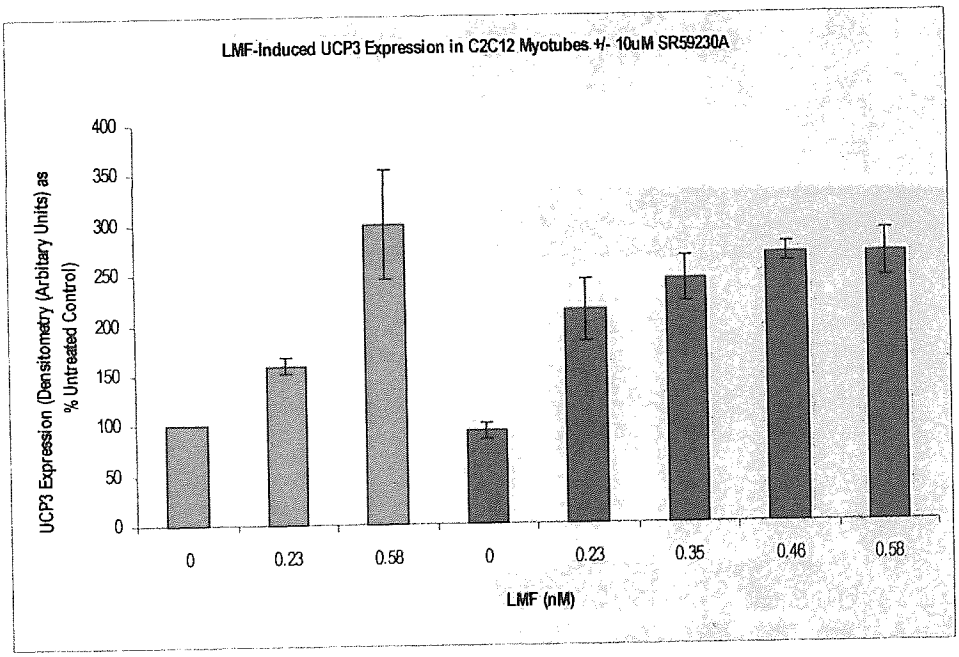
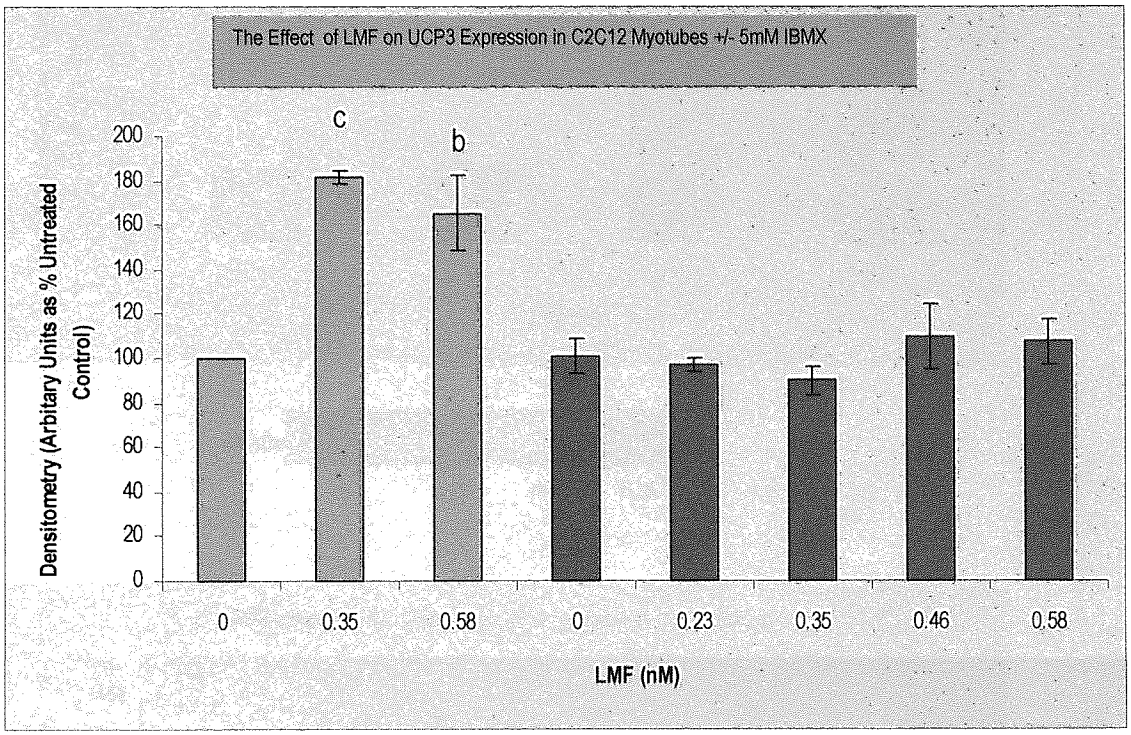


*Figure 9.3.3.1 Effect of LMF on UCP3 expression in skeletal muscle in the absence (blue bars on **A** and **C** and blot **B**) and presence (red bars on **C**) of 10μM SR59230A (following 24 hr LMF incubation, bands detected using α-UCP3). **A** is a densitometric representation of **B** and **C** is densitometric representation of an identical experiment performed +/- 10μM SR59230A. Data are means (n=3 (**A**) and n=2 (**B**))+/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison. Differences from untreated control are shown as a, $p<0.05$, b, $p<0.01$ & c, $p<0.001$.*

9.3.4 Effect of LMF on C_2C_{12} UCP3 Expression in the Presence/Absence of 5mM IBMX.



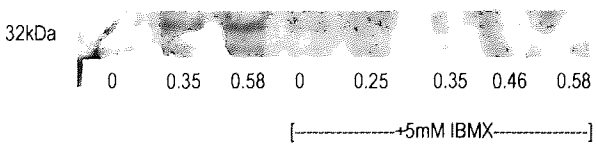
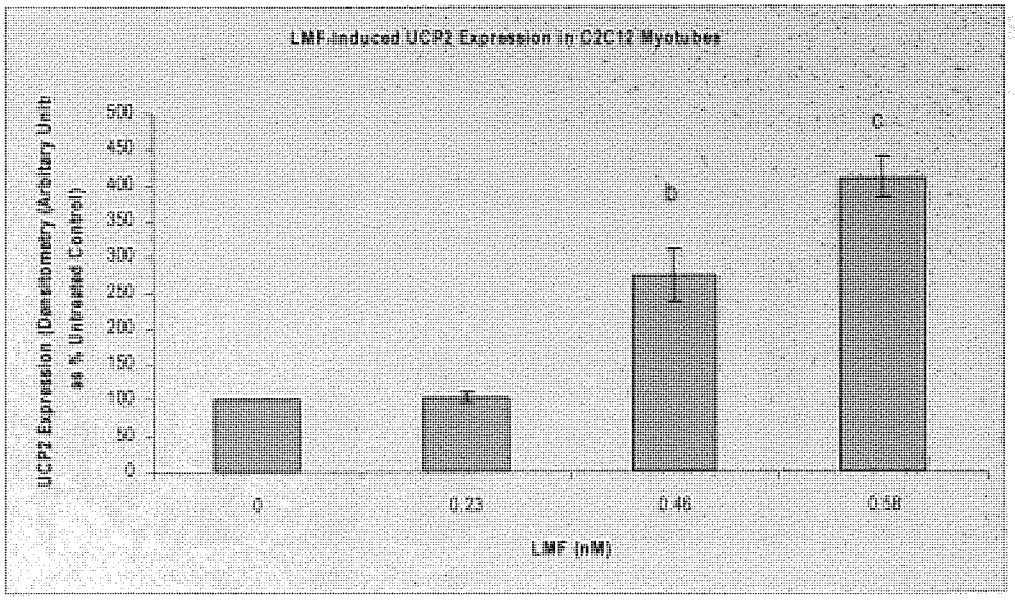
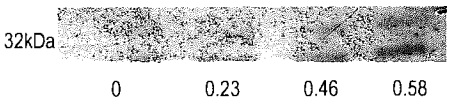


Figure 9.3.4.1 LMF-induced expression of UCP3 in C_2C_{12} myotubes in the presence (red bars) and absence (blue bars) of 5mM IBMX (LMF incubation over 24 hours, bands detected by α UCP3). Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by b, $p<0.01$, c, $p<0.001$.

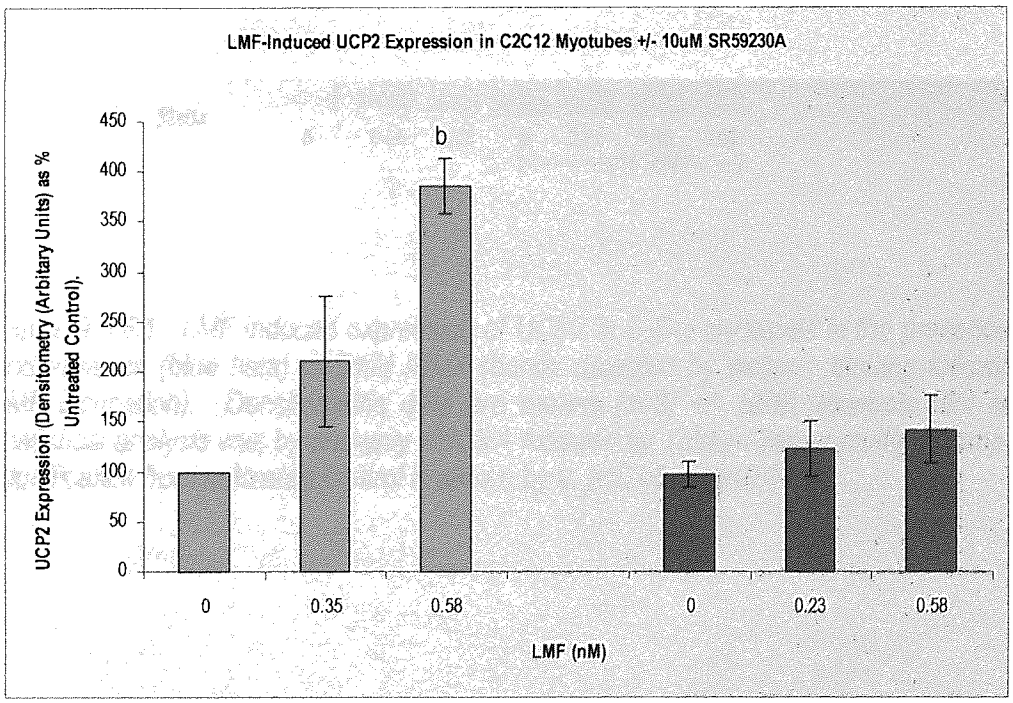
9.3.5 Effect of LMF on UCP2 Expression in C_2C_{12} Myotubes.



A



B



C

*Figure 9.3.5.1. Effect of LMF on UCP2 expression in skeletal muscle in the absence (blue bars on **A** and **C** and blot **B**) and presence (red bars on **C**) of 10μM SR59230A. **A** is a densitometric representation of **B** and **C** is densitometric representation of an identical experiment performed +/- 10μM SR59230A. Data are means (n=3)+/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparison. Differences from untreated control are shown as b, p<0.01, c, p<0.001.*

9.3.6 Effect of LMF (+/- 5mM IBMX) on UCP2 Expression in C_2C_{12} Myotubes.

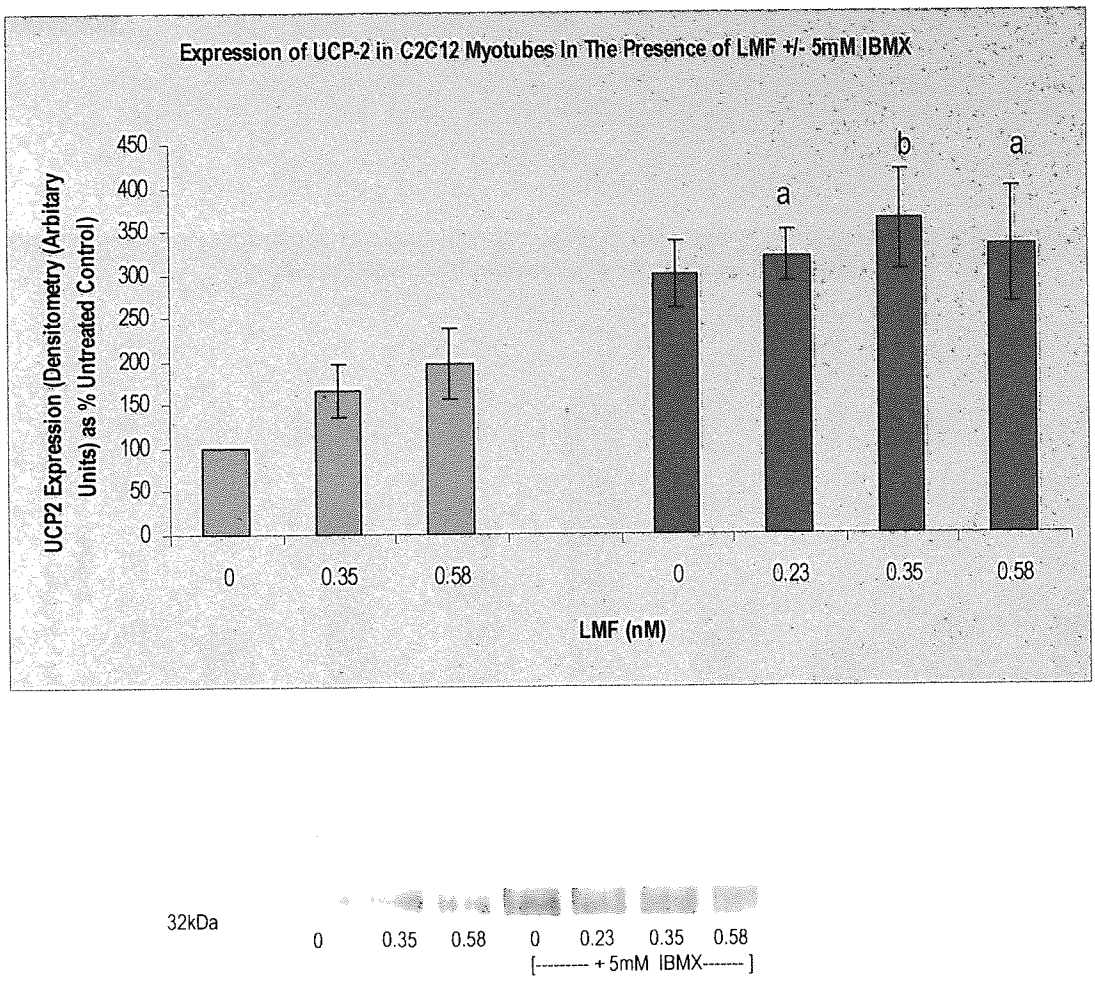
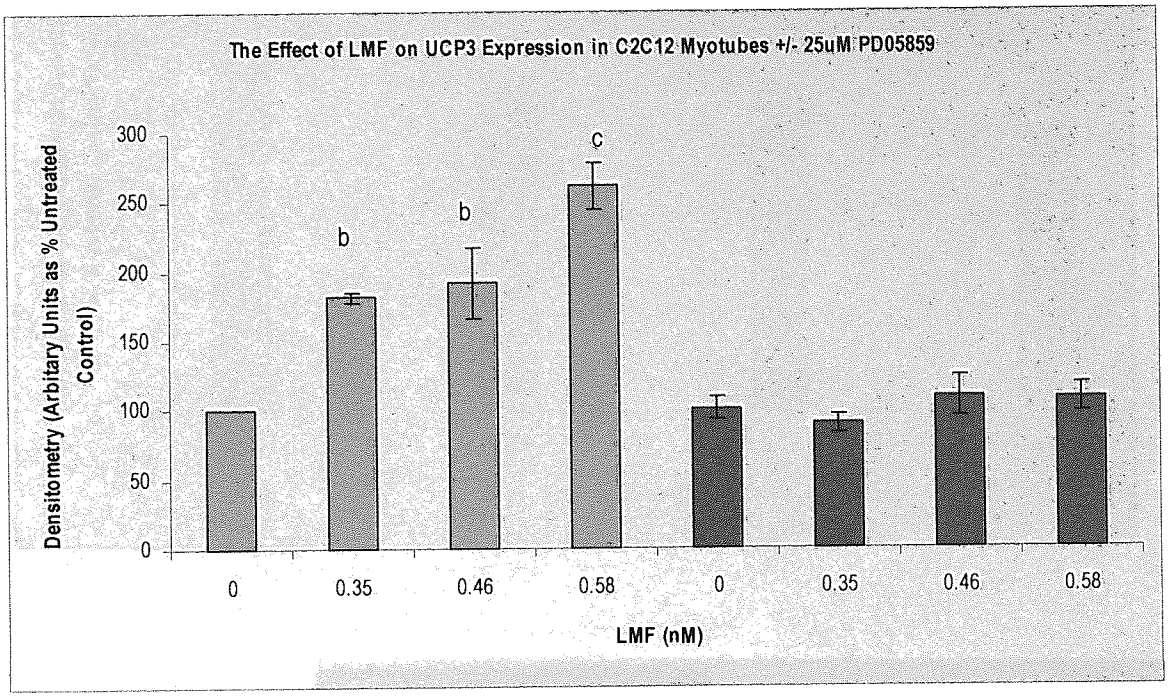


Figure 9.3.6.1. LMF-induced expression of UCP2 in C_2C_{12} myotubes in the presence (red bars) and absence (blue bars) of 5mM IBMX (bands detected by western blotting following 24 hour LMF incubation). Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by a, $p<0.05$, b, $p<0.01$.

9.3.7 Effect of LMF on UCP3 in C_2C_{12} Myotubes in the Presence/Absence of 25μM PD098059.



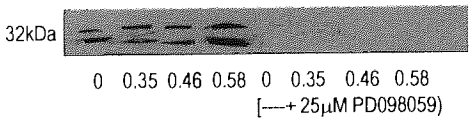
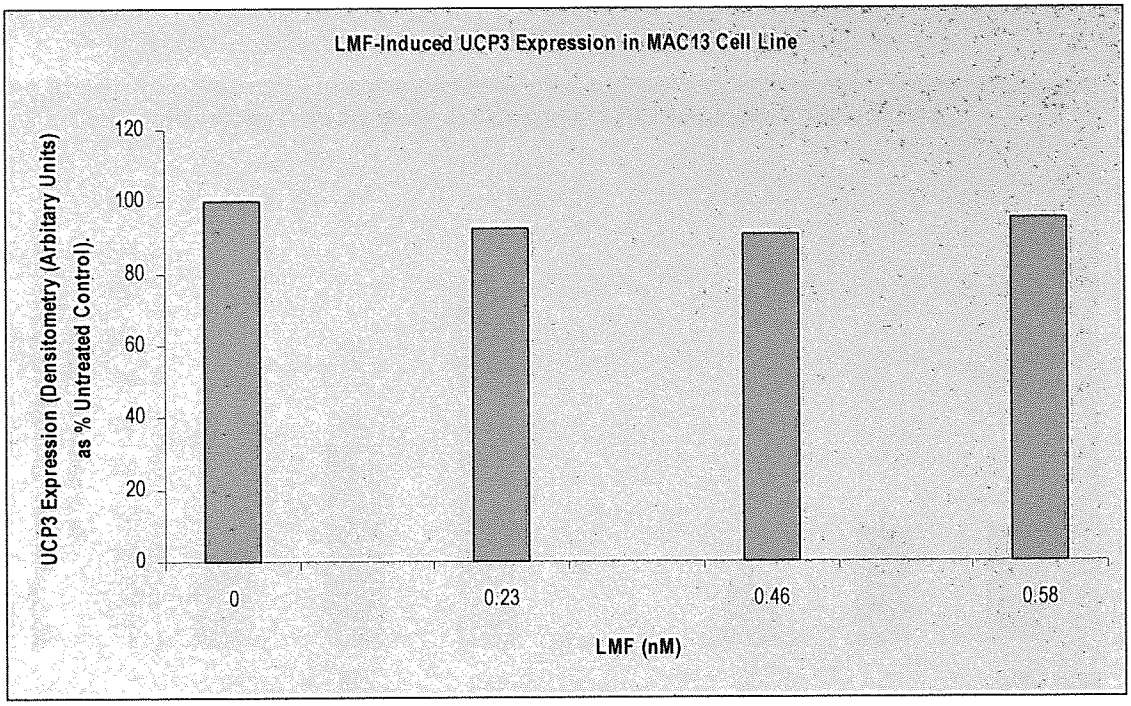


Figure 9.3.7.1. LMF-induced expression of UCP3 in C_2C_{12} myotubes in the presence (red bars) and absence (blue bars) of 25μM PD098059 (bands detected by western blotting following 24 hour LMF incubation). Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by b, $p<0.01$, c, $p<0.001$.

9.3.8 Effect of LMF on UCP3 Expression in MAC13 Cell Line.



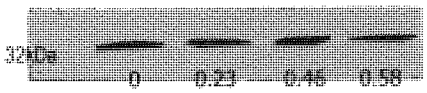


Figure 9.3.8.1. LMF-induced expression of UCP3 MAC13 cell line (as detected by western blotting following 24 hour LMF incubation). Blot and densitometric representations are presented.

9.3.9 Effect of LMF on UCP3 Expression in Differentiated 3T3-L1 Cells.

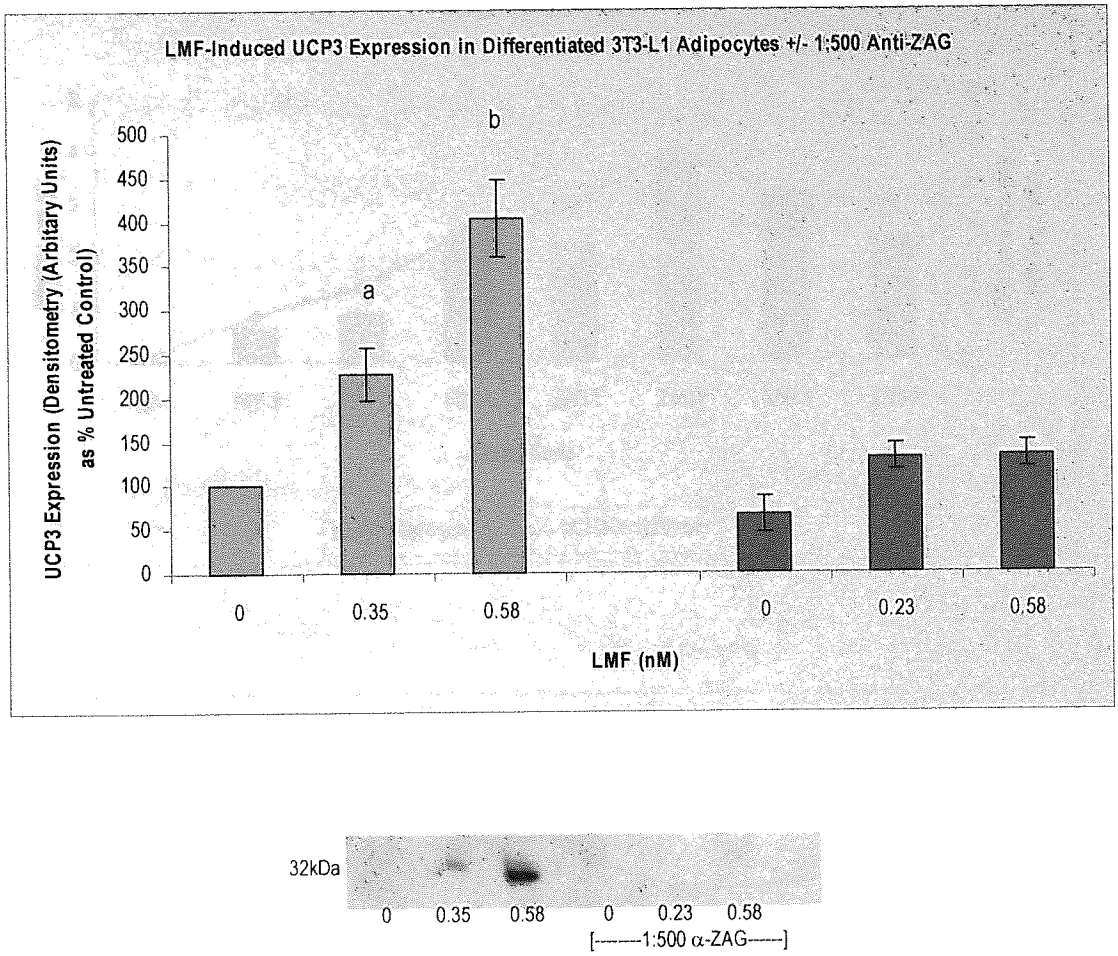
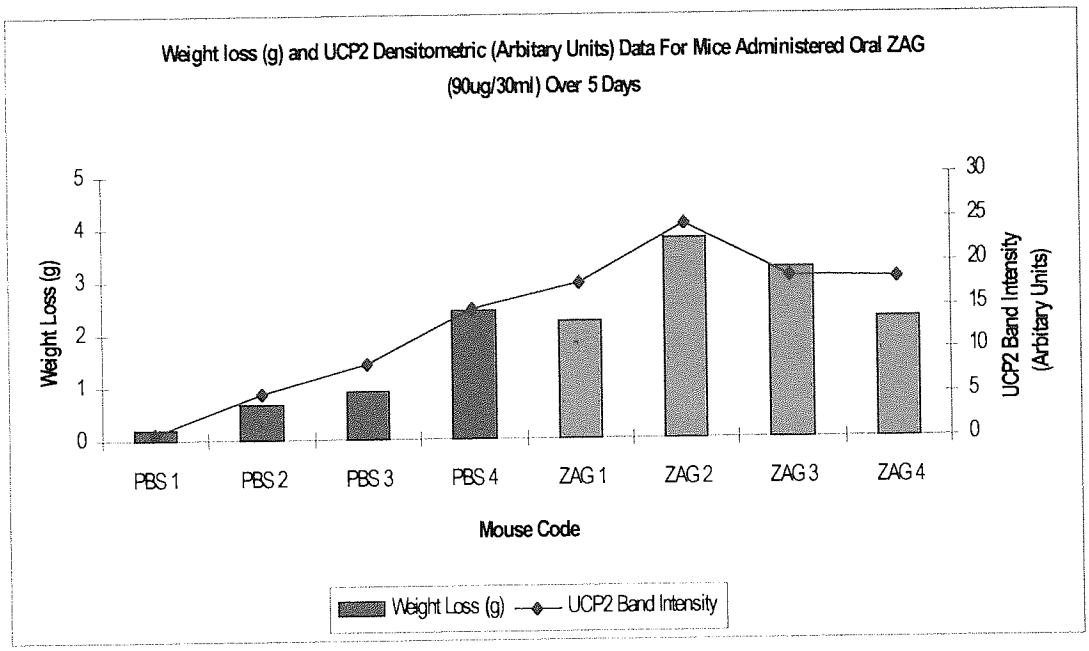


Figure 9.3.9.1. LMF-induced expression of UCP3 in 3T3-L1 adipocytes. Data in presence (red bars) and absence (blue bars) of 1:500 α-ZAG (bands detected by western blotting following 24 hour LMF incubation). Densitometric data are means (n=3) +/- SEM, example blot also shown. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Significance from untreated control is shown by a, $p<0.05$, c, $p<0.001$.

9.3.10 Gastrocenemius UCP2 Expression Following Administration (Oral & s/c) of ZAG to NMRI Mice.



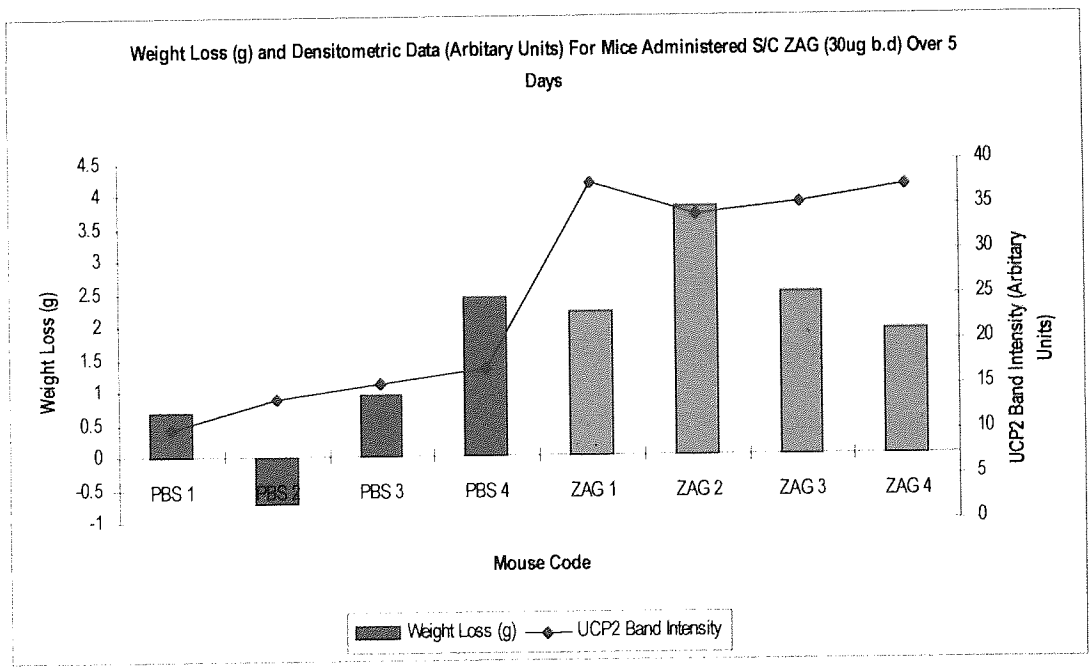


*Figure 10.3.10.1. Final weight loss plot of mice administered PBS (purple bars) or oral (**A**) or sub-cutaneous (**B**) ZAG (pink bars) over 5 days. UCP2 band intensity is indicated by line plot (UCP2 quantified by western analysis using α-UCP2).*

The regulation of the uncoupling protein homologues has been discussed in detail **section 9.1**. What follows therefore is a brief examination of the results obtained and an attempt to explain the results in the context of **section 9.1** and **chapter 10**.

UCP1 was up regulated by LMF in primary cultured brown adipocyte tissue. This up-regulation is attenuated by co-incubation with 1:500 α-ZAG (**figure 9.3.2.1**) (indicating successful inhibition away from the receptor) and with 10μM SR59230A (**figure 9.3.2.2**). This indicates that the UCP1 up-regulation observed here is achieved through interactions of LMF with the β_3-adrenoreceptor. This result was expected, as adipocyte tissue is under sympathetic (and thus adrenergic) regulation (Bing *et al* (2003)).

Correspondingly, Tonello *et al* (1998) report noradrenaline and the selective β_3-AR agonist SR58611A to up-regulate UCP1 mRNA expression in an *in vitro* BAT 1° culture system. Forskolin was demonstrated to mimic this stimulation, indicating such an increase in UCP1 to be cAMP-mediated.

UCP1 could not be detected in primary cultured WAT, where, if present at all, it is likely to be well below the sensitivity of this antibody (Tisdale MJ, personal communication). There is some evidence to suggest that treatment of WAT with β_3-AR agonist may cause it to adopt the characteristics of BAT (Russell ST, personal communication) thus RT-PCR and northern blotting could be employed as an extension to this work.

Regulation of UCP2 has been discussed in **chapter 10**. Here its regulation in the surrogate skeletal muscle C_2C_{12} *in vitro* model has been examined. A dose-dependent increase in UCP2 expression was observed following incubation of C_2C_{12} myotubes with 0-0.58nM LMF. This

increase was attenuated by co-incubation with 10μM SR-59230A **(figure 9.3.5.1)** and potentiated by 5mM IBMX co-incubation **(figure 9.3.6.1)**. This implies a β_3-AR mediated cyclic AMP-dependant mechanism.

Many factors (e.g. fatty acids, TZD's, purine nucleotides, starvation *in vivo*, reactive oxygen species etc.) reportedly contribute to the regulation of UCP2. Interestingly, and in agreement with this study, some authors also report β-adrenoreceptor regulation. Yoshitomi *et al* (1999) report a cyclic AMP response element flanking the UCP2 gene – this is in concordance with β-AR regulation of UCP2. Moreover, Yoshitomi *et al* (1999) demonstrated cyclic AMP analogues to up-regulate UCP2 mRNA in a 3T3-L1 *in vitro* model. In the same study, TZD's (which induce UCP2 via the PPAR pathway) also up regulated UCP2 mRNA; indicating more than one pathway may exist.

Nagase *et al* (2001) reported *in vitro* beta-adrenoreceptor regulation of UCP2 in L6 myotubes (a model bearing similarity to C_2C_{12} myotubes). More specifically, the β_3-AR agonist BRL37344 up-regulated UCP2 mRNA in this model (although the authors hypothesised this was due to cross-reactivity with the β_2-AR in this instance).

The regulation of UCP2 has been discussed further in **section 9.1**. The exact regulation of this mitochondrial carrier is presently unclear, and the scientific literature contradictory and incomplete. Further work is most definitely required in this field.

For mice administered oral or sub-cutaneous ZAG, the gastrocenemius UCP2 expression correlates extremely well with weight loss **figures 9.3.10.1a/b**. These results correspond to

mRNA data previously reported by Bing *et al* (2000) & Bing *et al* (2002). Interestingly, where control (PBS) mice have also experienced weight loss, their UCP2 expression also reflects this.

As was seen with UCP2, UCP3 expression is seen to increase in a dose dependant manner in C2C12 myotubes incubated with 0-0.58nM LMF (**figure 9.3.3.1**). This increase is very slightly attenuated by co-incubation with 10μM SR59230A (**figure 9.3.3.1**), but fails to be potentiated by 5mM IBMX (**figure 9.3.6.1**).. This implies only minimal involvement of the cyclic AMP-mediated pathways in the regulation of this mitochondrial protein.

Garvey (2003) cites β-AR regulation (in addition to other processes involved with energy expenditure) to be important in the regulation of UCP3 in humans, and interestingly, co-incubation (**figure 9.3.7.1**) of 25μM PD098059 (a MAP kinase inhibitor) attenuates the LMF-induced up regulation in C_2C_{12} myotubes. The involvement of MAPK has been investigated and reported in **Chapter 6**. Greenberg *et al* (2001) also demonstrated the MAPK pathway to contribute to the effects of both β-AR agonists and β_3-AR agonists. The results obtained in this study suggest that the MAPK pathway may be primary in regulating UCP3 as observed.

The up-regulation of UCP3 observed in 3T3-L1 adipocytes (**figure 9.3.9.1**) could be a direct (i.e. β_3-AR mediated) or indirect (i.e. activated by mobilised fatty acids) effect. Further work is required to determine the exact mechanisms.

LMF fails to induce UCP3 expression in MAC13 cells. The tissue distribution of UCP3 (Ricquier & Bouillaud (2000)) is such that UCP3 should not be present at functional concentrations in colon cells, potentially explaining UCP2 induction (**chapter 10**) but not UCP3 induction in this cell line.

The α-UCP3 antibody fails to distinguish between the long and truncated isomers of UCP3. Both are present in skeletal muscle (hence visualisation of two separate bands), whereas in 3T3-L1 and MAC13 cells, one isomer seems to predominates. UCP2 functions as a dimer to facilitate proton transport, thus explains the two bands observed following western analysis (NCBI sequence viewer database (accession # p70406)).

As reported by Yoshitomi *et al* (1999), these proteins may potentially be subject to regulation by more than one pathway. If, as Kennedy *et al* (2001) speculate, the non-peptide ligand associated with LMF is indeed a fatty acid, then this could also contribute to the modulation of these carriers by LMF (Fleury *et al* (1997)).

9.4 Conclusions.

The UCP homologues are important proteins in the control and maintenance of energy balance and body weight.

In addition to the normal regulatory mechanisms, it is likely that some of the factors associated with cancer cachexia may also modulate the expression of UCPn. Induction of these carriers during cachexia increases the negative energy balance and potentiates the wasting process. Understanding the underlying mechanisms was therefore of importance.

This study demonstrates the ability of a tumour-derived lipid mobilising factor to directly induce expression of the uncoupling proteins in an *in vitro* system. It is likely that a combination of both direct and indirect factors associated with cachexia modulate the expression of UCPn *in vivo*.

Much further work is required to elucidate the exact mechanisms. Once these mechanisms are understood, perhaps better clinical attenuation of cachexia will become possible.

CHAPTER 10. THE ROLE OF LIPID MOBILISING FACTOR (LMF) IN PROTECTING TUMOUR CELLS FROM OXIDATIVE DAMAGE.

10.1 Introduction.

Cancers accompanied by cachexia are associated with an inferior response to conventional chemotherapy. It is currently not investigated whether any tumour survival advantage is conferred by the production of catabolic factors such as LMF.

A large percentage of chemotherapeutic drugs (e.g. adriamycin, bleomycin and mitomycin C) exert their action through the generation of free radicals (Sanders & Tisdale (2003)), moreover, cachexia itself is accompanied by a significant increase in free radical concentration (Gomes-Marcondes & Tisdale (2002) & Mantovani *et al* (2002)). A role has been suggested for the ubiquitous mitochondrial anion carrier Uncoupling Protein 2 (UCP-2) in detoxifying such species (Lee *et al* (1999), Pecqueur *et al* (2001) & Li *et al* (2001)).

This study investigates whether LMF confers a chemotherapeutic survival advantage to the non-cachexigenic MAC13 line. The mechanism of this survival (if any) will be investigated (with particular attention being paid to LMF-induced regulation of UCP2 (as determined in **chapter 9**)

10.2 Methodology.

10.2.1 Purification of Lipid Mobilising Factor.

LMF was purified from the urine of patients with advanced pancreatic cancer as described in **section 3.2.2**. Bioactivity and homogeneity were confirmed as described in **section 3.2.4** and **3.2.5**.

10.2.1 Maintenance of MAC13 Cell Line

MAC13 is a colon adenocarcinoma induced in mice by prolonged administration of 1,2-dimethylhdrazine. Cells derived from the solid tumour were maintained *in vitro* in RPMI-1640 media supplemented with 10% FCS, 1% penicillin streptomycin and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were sub-cultured at semi-confluence, and passage number was not permitted to exceed seventeen.

10.2.2 MAC13 Cellular Survival Assay in The Presence of LMF.

Confirmation was required that LMF itself did not affect MAC13 proliferation under the conditions of this assay. MAC13 cells were seeded in six-well multiwell dishes at a density of 1.6 x 10^4 cells/well. Cells were allowed to reach semi-confluence prior to experimentation. 0-0.58μM LMF was incubated for 2 hours (5% CO_2 in air at 37°C), removed, and fresh media added. Cell number was assessed after a further 96 hours (5% CO_2 in air at 37°C) with a Z1 Coulter Counter.

10.2.3 Determination of Suitable Drug Concentrations For Experimental Use.

MAC13 cells were seeded at 1.6 x 10^4 cells/well in six-well multiwell dishes. Cell density was permitted to reach semi-confluence prior to experimentation. Hydrogen peroxide (0-800μM), 1,1'-dimethyl-4, 4'-bipyridinium dichloride (paraquat) (0-200μM) and chlorambucil (0-200μM) were added to the cells for 120 minutes (5% CO_2 in air at 37°C). Experimental media was removed and replaced with fresh RPMI-1640 supplemented with 10% FCS in the absence of agent. Cell numbers were determined after a further 96 hours using a Z1 Coulter Counter. This assessment was not performed with bleomycin, as suitable concentration data was provided (Sigma Aldrich).

10.2.4 Determination of MAC13 vs. MAC16 ZAG Production.

ZAG/LMF production was evaluated in the MAC13 and MAC16 cell lines. MAC13 and MAC16 cells were both grown to confluence in 75cm^3 flasks. Cells were washed twice with ice-cold sterile PBS and scraped from the substratum into homogenising buffer (**section 2.8.2**) using a rubber policeman. Isolate was sonicated at 4°C on ice (3 times for 10 seconds (with intervals between each pulse)). Samples were assayed for total protein (**section 3.2.3**), concentration adjusted to 5μg/10μl and then denatured (**section 3.2.4**).

Prepared samples were immunoblotted. Briefly, 10μl of sample was resovled on 12% SDS PAGE (180v 45 minutes) then transferred (80V, 120 minutes) onto 0.45μm nitrocellulose membranes. Mouse monoclonal α-human ZAG antibody was used at 1:1000 dilution (2 hours, room temperature), whilst secondary antibody (peroxidase-conjugated rabbit anti-mouse) were utilised at 1:2000 (1 hour, room temperature). Development was by enhanced chemiluminescence (ECL). Blots were subjected to

densitometric scanning and analysed using 'Phoretix ID Advanced' software. Equal loading was confirmed following Ponceau S staining.

10.2.5 MAC13 Uncoupling Protein 2 Expression in The Presence of LMF.

MAC13 UCP2 expression was examined the presence and absence of LMF. MAC13 cells were subcultured and grown to confluence in 25cm^3 flasks. LMF (0-0.58μM) was added and left for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were washed twice with ice-cold PBS and scraped from the substratum into uncoupling protein sonication buffer (**section 2.8.1**) using a rubber policeman. Isolate was sonicated on ice at 4°C three times for ten seconds (with intervals between each pulse).

Protein concentration was estimated according to the method of Bradford (**section 3.2.3**), and the samples appropriately diluted and denatured (**section 3.2.4**). Western blotting was performed as described in **section 9.2.4** using rabbit α-mouse 1° antibody (1:1000, 2 hours, room temperature) and goat α-rabbit peroxidase conjugated 2° (1:2000, 1 hour, room temperature). Detection and analysis was performed as described previously (**section 9.2.4**).

10.2.6 MAC13 Cellular Survival Assay.

MAC13 cells were seeded at 1.6 x 10^4 cells/well in six-well multiwell dishes. Cellular density was allowed to reach semi-confluence prior to experimentation.

10μM SR59230A and was added as required 2 hours prior to experimentation to allow equilibration. Experimental concentrations of bleomycin (0-100μg/ml), hydrogen peroxide (0-20μM), 1,1'-dimethyl-4, 4'-bipyridinium dichloride (paraquat) (0-1.0μM) and chlorambucil (0-25μM) were incubated with cells for 120 minutes +/- 0.58μM LMF. Experimental media was removed and replaced with supplemented RPMI-1640/10% FCS without drug or factor. Quantification of cell number was achieved 96 hours subsequently using a Z1 Coulter Counter.

10.2.7 Malondialdehyde (MDA) Assay.

MAC13 cells were grown and treated as described in **section 10.2.6**. Following 24 hour drug/factor incubation, cells were washed twice with ice-cold PBS and scraped directly into 20mM Tris, pH 7.5, 2mM ATP, 5mM $MgCl_2$ and 1mM dithiothreitol. Cell samples were sonicated on ice (in the presence of 5mM butylatedhydroxytoluene) and centrifuged at 15,000 rpm for 10 minutes, and the supernatant used for the MDA assay.

Cell homogenate (0.2ml), or 1,1,3,3-tetramethoxypropan (0.2ml, standard) or water (0.2ml, blank) were added to *N*-methyl-2-phenylindole (0.2ml). The mixtures were vortexed prior to the addition of 46μl 12N HCl, followed by another mix. The tubes were sealed and the samples incubated at 45°C for one hour, followed by centrifugation at 15,000 g for 10

minutes. Samples were transferred to a microtiter plate and the absorbance measured at 570nm. Absorbance readings were adjusted for sample protein concentration.

10.3 Results & Discussion.

10.3.1 ZAG Expression in MAC13 and MAC16 Lines.

Homology between ZAG and LMF has been established previously (**Chapter 3**).Todorov *et al* (1998) utilised RT-PCR to demonstrate MAC16 cells (which induces cachexia *in vivo*), to express high levels of ZAG mRNA, whilst MAC13 cells (which do not induce cachexia *in vivo*) does not. Immunoblot analysis of MAC13 and MAC16 cellular extracts result in statistically significant ($p<0.001$) MAC16 ZAG/LMF production compared to MAC13. Results are presented in **figure 9.3.1.1**

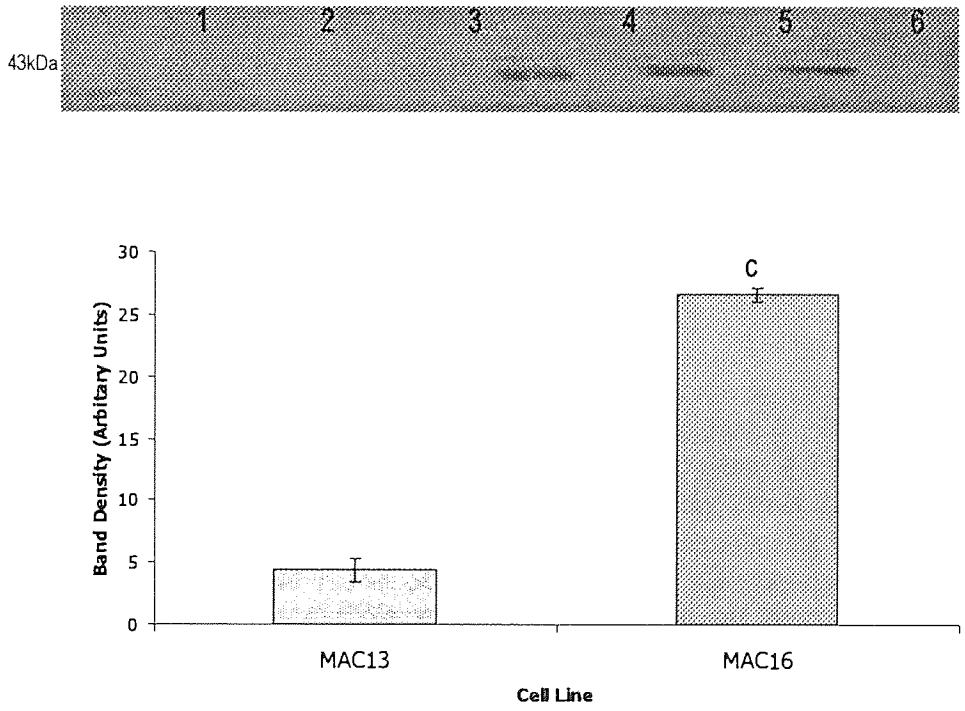
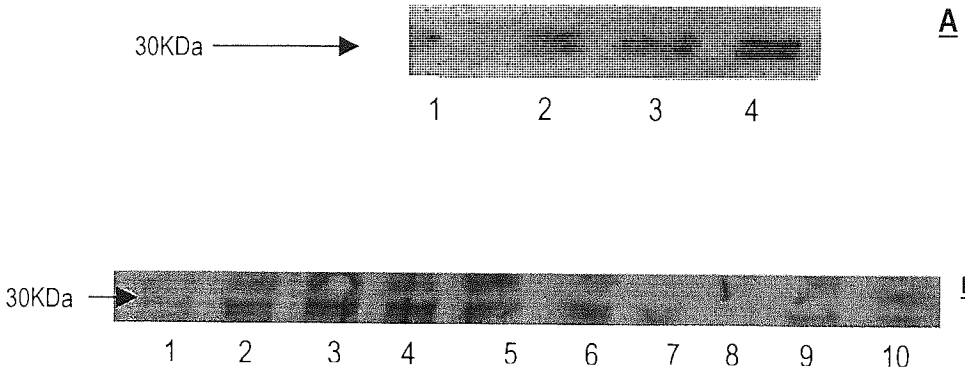


Figure 10.3.11. ZAG expression by MAC13 (lanes 1-3) and MAC16 (lanes4-6) cell lines (as measured by western blotting analysis). Data are shown as means +/- SEM. Statistical analysis was via one-way ANOVA followed by Tukey-Kramer multiple comparisons test. Results are shown as c, $p<0.001$.

9.3.2 Effect Of LMF on MAC13 UCP-2 Expression.



C

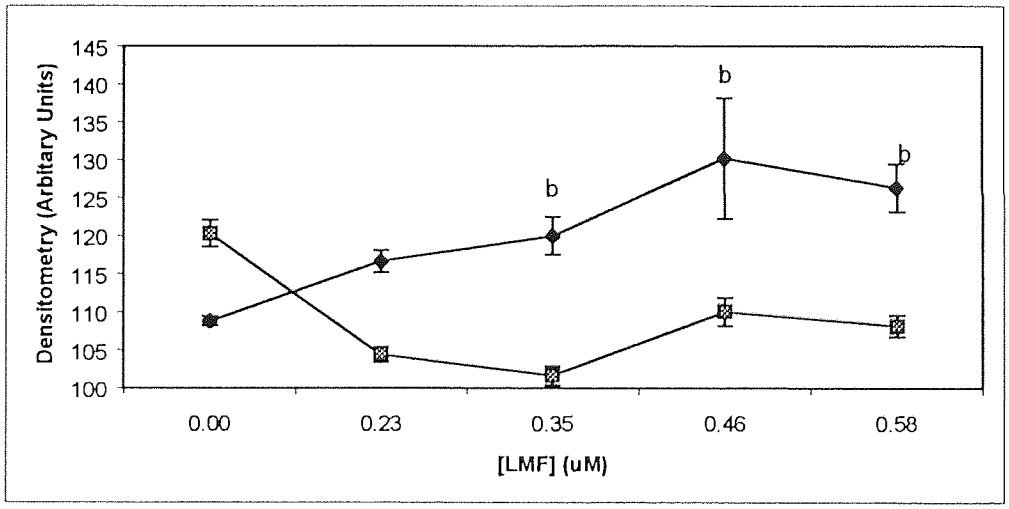


*Figure 10.3.2.1 Immunoblot of UCP-2 expression in MAC13 line (**A**) in the presence of 0 (lane 1), 0.23 (lane 2), 0.35 (lane 3) and 0.58 (lane 5) μM LMF after 24h incubation and (**B**) in the presence of 0 (lanes 1 & 6), 0.23 (lanes 2 & 7), 0.35 (lanes 3 & 8), 0.46 (lanes 4 & 9) and 0.58 (lanes 5 & 10)μm LMF for 24h in the absence (lanes 1-5) and presence (lanes 6-10) of 10μM SR59230A. Densitometric analysis of blot **B** is shown in **C**. Data are* n=3 +/- *SEM. Statistical analysis was by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences from control shown as b, p<0.01, c, p<0.001.*

LMF induces a concentration-dependant increase of UCP2 expression in the MAC13 cell line. This increase was significantly attenuated by co-incubation with the selective β_3-adrenoreceptor antagonist SR59230A (**figures 9.3.2a, 9.3.2b** and **9.3.2c**). This implies this action of LMF to be mediated through the β_3-AR. The concentrations of LMF seen to induce UCP2 here have previously been demonstrated as active (Hiari *et al* (1998)). Since maximal stimulation of UCP2 was achieved at 0.46 -0.58μM LMF, this concentration was employed for further studies

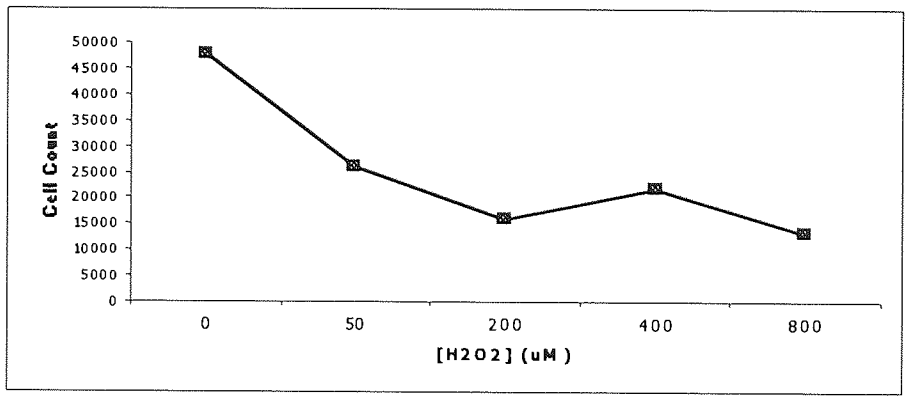
Control of UCP2 expression remains somewhat unclear. Up regulation of UCP2 in liver by polyunsaturated fatty acids appears to involve a prostaglandin/peroxisome-proliferator-activated receptorα (PPARα)-mediated pathway (Armstrong and Towle, 2001), while TZDs appear to work through a PPARα pathway in muscle and adipose tissue. The β_3-AR agonist Teratol increased UCP2 expression in WAT (Margarets *et al* (2002)), but this was probably due to the action of released free fatty acids (reported to regulate UCP expression (Fleury *et al* (1997)). Interestingly, Kennedy *et al* (2001) report that the non-peptide ligand in the groove of LMF may be a fatty acid, potentially contributing to the regulation of UCP2 by LMF observed here.

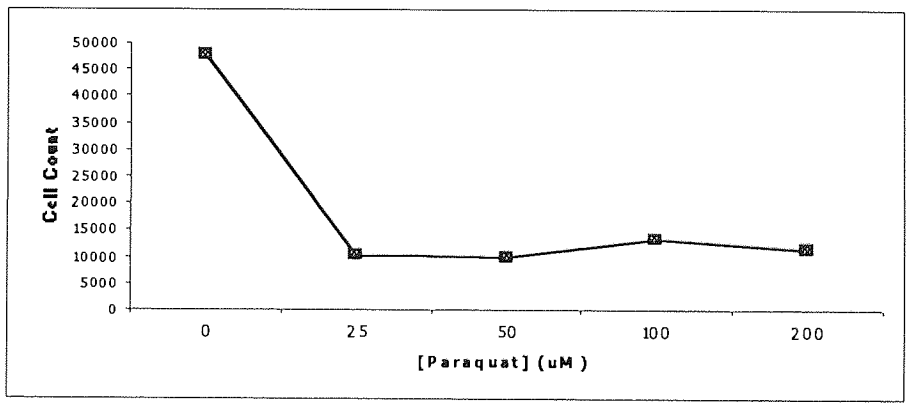
Evidence implicating β_3-AR agonists in the regulation of UCP2 also exists. Nagase *et al* (2001) demonstrated adrenaline and the selective β_3–AR agonist BRL37344 to upregulate UCP2 mRNA in L6 myotubes. Yoshitomi *et al* (1999) reported a cyclic AMP-response element (CRE) flanking the UCP2 gene and demonstrated cyclic AMP analogues to increase the UCP2 mRNA of 3T3-L1 adipocytes *in vitro*.

The regulation of UCP2 and the effect of a tumour-derived LMF are discussed in more detail in **chapter 9**. The effect of LMF here was in the absence of fatty acids, suggesting a direct effect through the β_3-AR. The mechanism of this effect is not known, may involves a cyclic AMP-mediated process.

10.3.3 MAC13 Cell Survival In The Presence Of Chemotherapeutic Agents.

10.3.3.1 Determination of Optimal Drug Concentrations for Experimental Use.





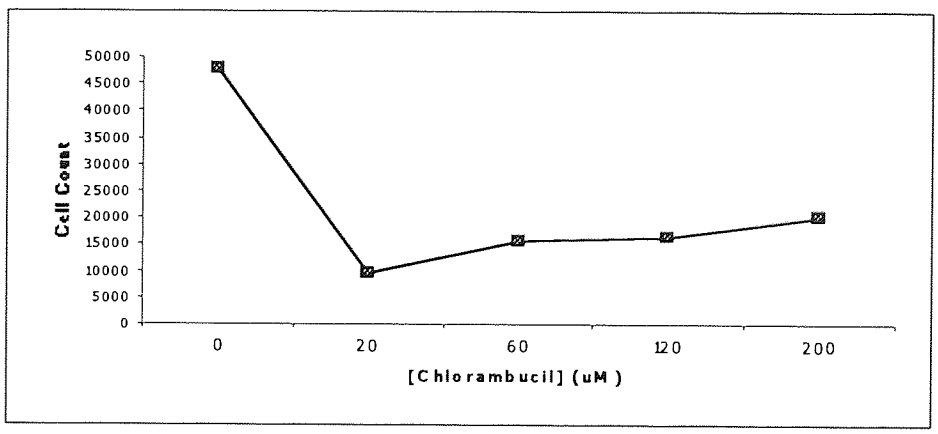


*Figure 10.3.3.1. MAC13 cellular survival curves 96 hours following 2h incubation with 0-800μM H_2O_2 (**A**), 0-200μM paraquat (**B**) and 0-200μM chlorambucil (**C**). Concentrations for experiment use thus become 0-20μM H_2O_2, 0-1μM paraquat and 0-25μM chlorambucil. Final cell numbers assessed using a Z1 coulter counter.*

10.3.4 Effect of LMF Only on Cell Proliferation.

Prior to these studies, it was necessary to confirm that LMF did not induce MAC13 proliferation.

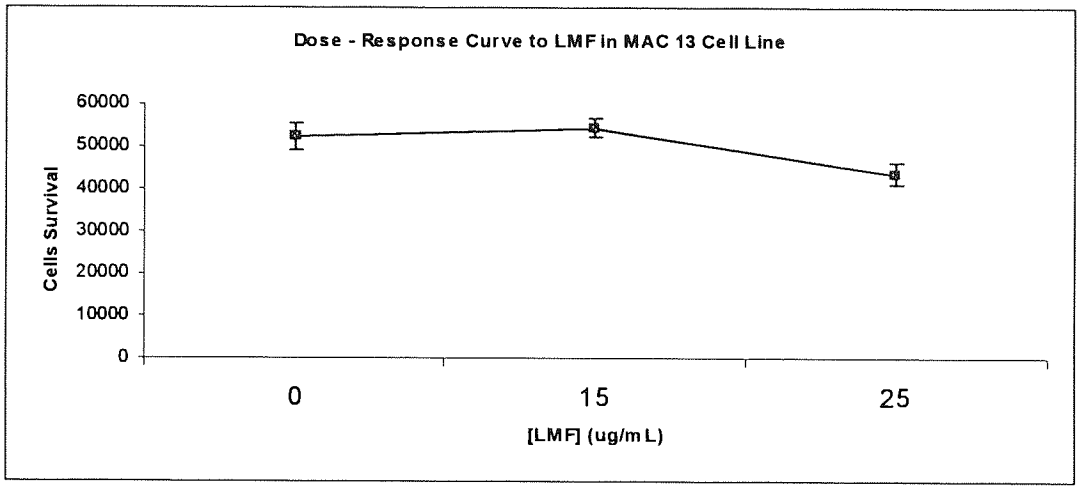


Figure 10.3.4.1. MAC13 cell numbers 96 hours following a 2h incubation with LMF (0-0.58μM) incubation. Data are means (n=4) +/- SEM. Statistical analysis (one-way ANOVA followed by Tukey-Kramer multiple comparison test) revealed no difference between experimental groups.

LMF alone did not affect the MAC 13 growth curve. This has correlates with previous findings (Wyke SM, unpublished results).

10.3.5 MAC13 Chemotherapy Survival Assay.

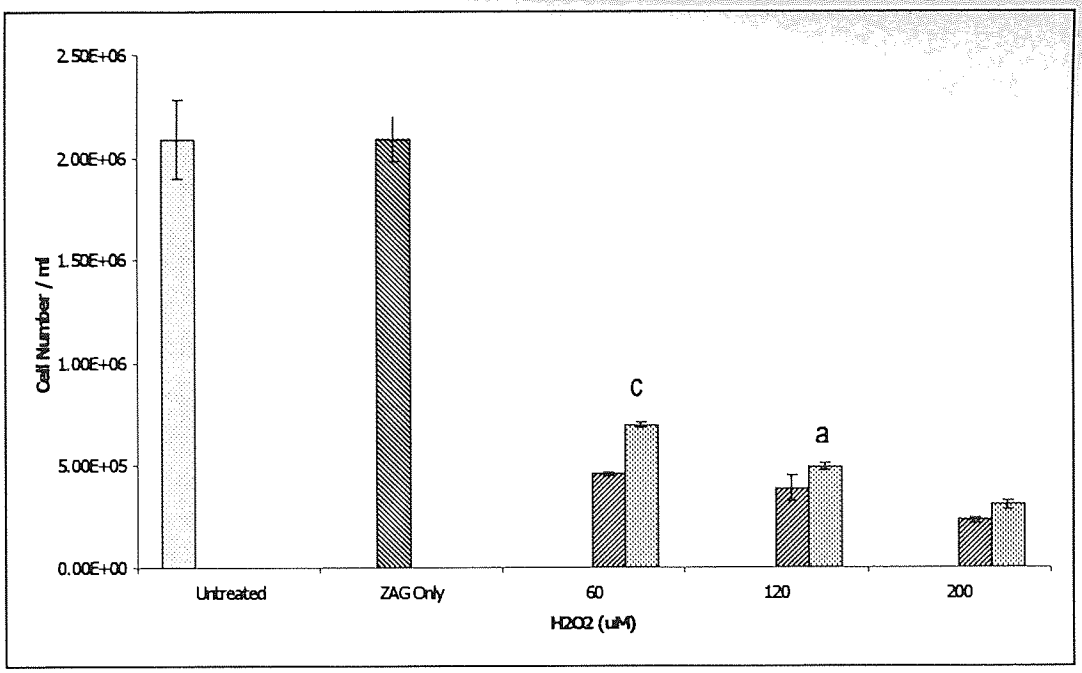


Figure 10.3.5.1. Effect of 2 hour hydrogen peroxide insult on the growth of MAC13 cells alone (striped boxes) and in the presence of 0.58μM LMF (hatched boxes). Cellular survival assessed 96h post incubation using a coulter counter. Data are means (n=3) +/- SEM. Results indicated as, p<0.05 and b, p<0.01

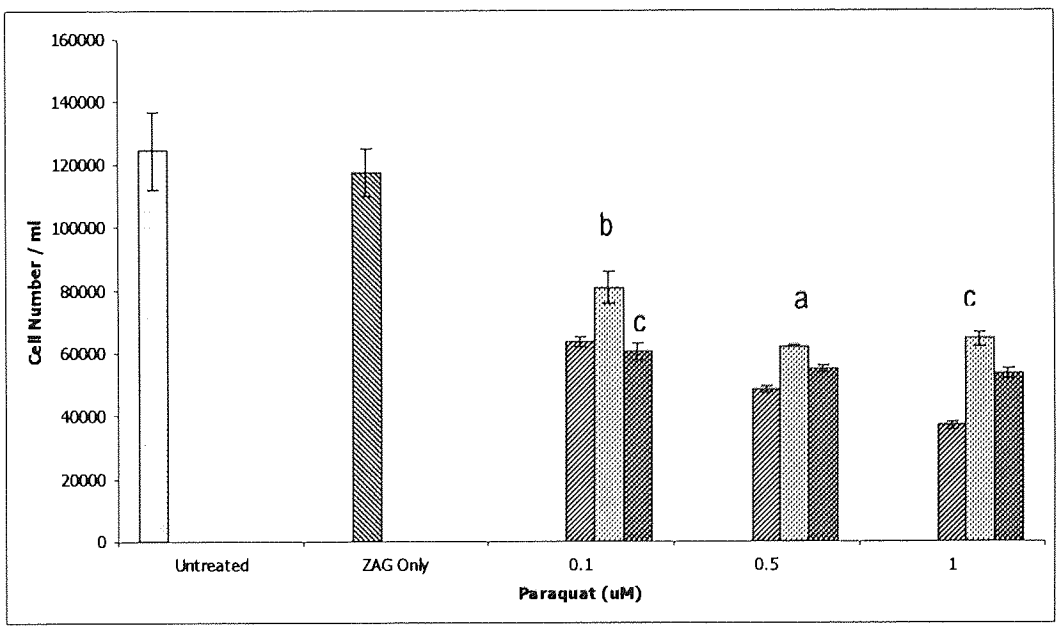


Figure 10.3.5.2. Effect of 2h paraquat incubation on growth of MAC13 cells alone (striped boxes), in the presence of 0.58μM LMF (dashed boxes) and in the presence of 0.58μM LMF and 10μM SR50230A (squared boxes). Cell survival assessed after 96h using a coulter counter. Data are means (n=3) +/- SEM. Differences in values in the presence of paraquat alone are indicated as a, p<0.05 and b, p<0.01 while differences from paraquat + LMF are indicated as c, p<0.001.

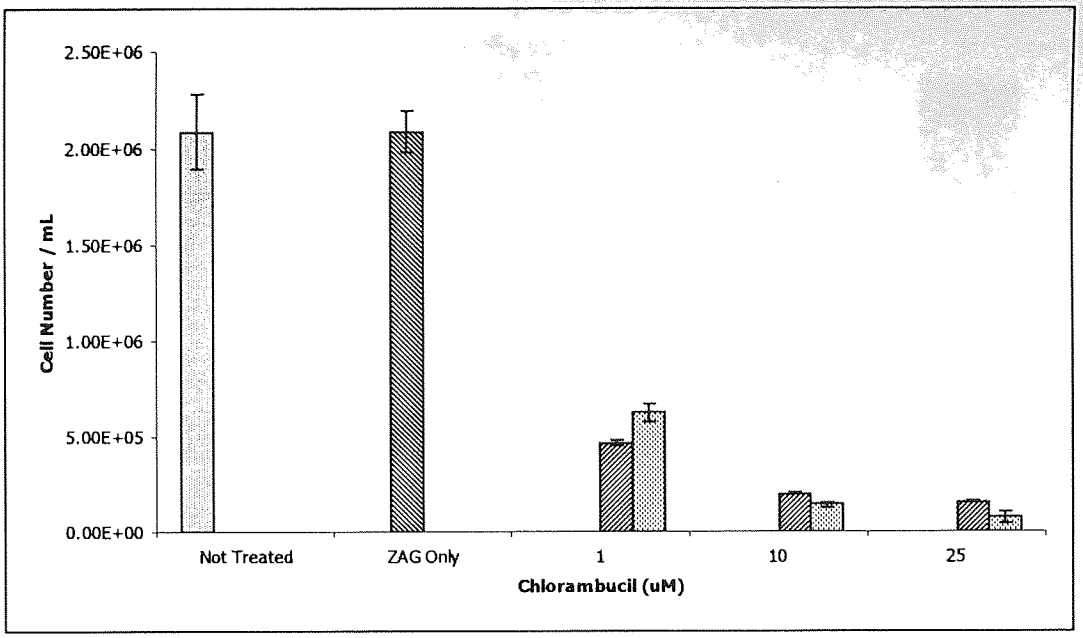


Figure 10.3.5.3. Effect of a 2h chlorambucil insult on the growth of MAC13 cells alone (striped boxes) and in the presence of 0.58μM LMF (dashed boxes). Cellular survival was assessed 96h post-insult using a coulter counter. Data are means (n=3) +/- SEM. There was no significant difference between the two groups

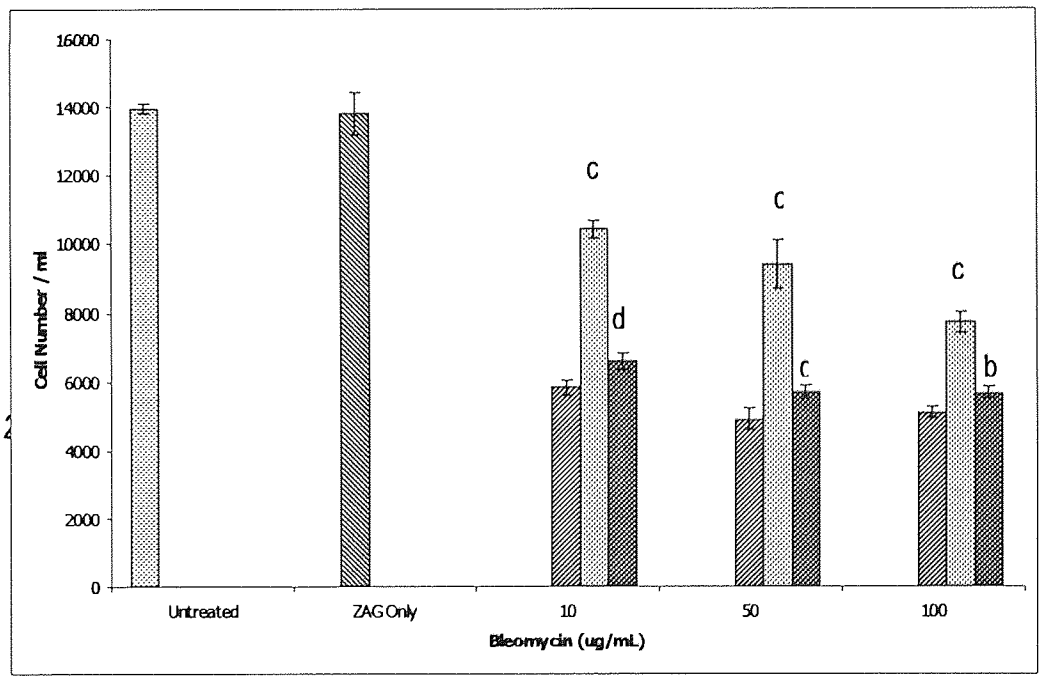


Figure 10.3.5.4. Effect of a 2h bleomycin insult on the growth of MAC13 cells alone (striped boxes), in the presence of 0.58μM LMF (dashed boxes) and in the presence of 0.58μM LMF and 10μM SR50230A (squared boxes). Cellular survival assessed 96h post insult using a coulter counter. Data are means (n=3) +/- SEM. Differences from values in the presence of bleomycin alone are indicated as c, p<0.001, while differences from bleomycin + 0.58nM LMF are indicated as b, p<0.01 and d, p<0.001

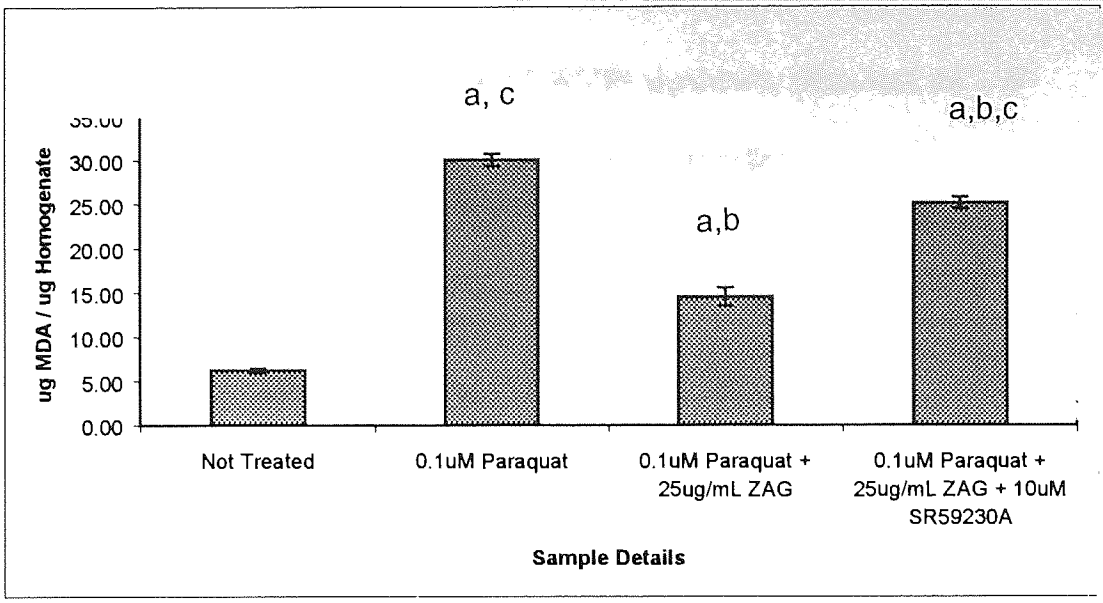


Figure 10.3.5.5; MDA assay of MAC13 cells as an indicator of oxidative stress. Cells were exposed to paraquat for 2 hours in the presence/absence ZAG +/- 10μM SR59230A. Media was changed and cellular survival assessed 96 hours post insult. Data represent means +/- SEM. All n=6. Statistical analysis by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Difference from negative control shown as a, p<0.001. Difference from 0.1um paraquat only shown by b, p<0.001. Difference from 0.1uM paraquat + 25ug/mL ZAG shown by c, p<0.001.

0.58μM LMF conferred a significant protective effect against hydrogen peroxide-induced growth inhibition (although this is seen to decrease the concentration of hydrogen peroxide concentration increases and was not apparent at the maximal). It is well established that hydrogen peroxide treatment promotes ROS formation that induces cellular damage (as reviewed by Roitt (1997)). This led to speculation that LMF may confer protection against such species.

Paraquat is another potent pro-oxidant, and subsequent ROS production rapidly oxidises cellular lipids, proteins and nucleic acids (Saibara *et al* (2003)). Paraquat similarly produced dose-dependant decrease in MAC13 proliferation, which was successfully attenuated by co-incubation with 0.58μM LMF (**figure 10.3.5.2**). To determine whether such effects were a result of the action of LMF at the β_3-AR, 10μM SR59230A was also

included in experimental protocol. 10μM SR59230A significantly attenuated the LMF-conferred survival, indicating a β_3-AR mechanism for this protection.

Figure 10.3.5.5 provides evidence that LMF is promoting MAC13 cellular protection via a reduction of the oxidative stress present in this system. MDA is a known marker of oxidative stress, and MDA-lysine adducts in skeletal muscle are seen to increase under such conditions (Gomes-Marcondes & Tisdale (2002)). Thus co-incubation of 0.58μM LMF with 0.1μM paraquat significantly attenuates the rise in MDA induced by this agent. Again, this protection is significantly attenuated by co-incubation with 10μM SR59230A, indicating a β_3-AR-dependant mechanism.

Chlorambucil, an alkylating agent which inhibits cellular proliferation by inducing DNA cross-links (Harrap & Gascoigne (1976)) also promoted a significant decrease in MAC13 survival. Co-incubated LMF failed to confer a protective effect in the presence of this agent.

Significant reversal of bleomycin-induced (all concentrations) growth inhibition was achieved by co-incubation with 0.58μM LMF. Bleomycin cleaves DNA in an oxygen-dependant manner.. However, in addition to this, redox cycling of bleomycin itself also produces superoxide (O^{2-}) and hydroxyl (OH^{-}) radicals. These reactive oxygen species produced by this cycling are reported to be lethal (Davermann *et al* (2000)). The increase in cellular survival promoted by LMF with bleomycin was completely attenuated by co-incubation with 10μM SR59230A. This suggests the protective effect of LMF to be mediated through the β_3-AR. Results obtained thus far suggest LMF to attenuate the

ROS as opposed to DNA damage associated with bleomycin (although why the latter remains unapparent at experimental concentrations remains unclear).

Although similar thermogenic roles to UCP1 have been suggested for UCP2, this is not the primary function of this anion carrier (Li *et al* (2001)). Evidence is accumulating for a role of UCP2 in detoxification of free radicals and reactive oxygen species (Jezek (2002)).

Mice given partial hepatectomy (PH) demonstrated a corresponding rise in TNF-α production (Lee *et al* (1999)). This rise in TNF-α contributed to the potentially lethal mitochondrial oxidant production following PH. Consistent with this finding was the corresponding rise in hydrogen peroxide production by liver mitochondria 5-180 minutes post-PH. This rise preceded transient increases in hepatic NFkB (peaked 30 minute post-PH) and UCP2 (commencing 30 minutes following PH and peaking 6-24 hours post-PH). The authors suggested NFkB to be anti-apoptotic and a role for UCP2 in detoxifying oxidant species as a cellular defence mechanism (Lee *et al* (1999)).

Li *et al* (2001) also suggest a role for UCP2 in cellular defence against oxidative stress. Li *et al* (2001) studied UCP2mRNA expression in pancreatic β cells. β cells of diabetic individuals are destroyed by autoimmune and cytokine assaults, thus their cellular defences (or lack of) were therefore investigated by the authors. UCP2 mRNA was seen to significantly decrease when clonal β-cells were co-cultured with the antioxidants α-tocopherol (vitamin E) and selenium. INS-1 cells exposed to 200μM hydrogen peroxide demonstrated increased UCP2 mRNA compared to controls. Similarly, INS-1 cells transfected for 30% UCP2 over-expression demonstrated significantly greater survival

($p<0.004$) following a 200μM H_2O_2 (5 minute) insult than non-transfected cells (Li *et al* (2001)).

Pecqueur *et al* (2001) studied UCP2 in regulation of ROS. This particular role for UCP2 is supported by greater amounts of reactive oxygen species in the muscles of UCP2-knockout mice. UCP2 protein expression is increased in the lung and stomach following LPS treatment and starvation (these organs are routinely exposed to pathogens and toxic substances). Moreover, the kinetics of UCP2 up-regulation following LPS injection are consistent with a primary immune response leading to an oxidative burst in the lung (Pecqueur *et al* (2001)).

0.58μM LMF induces *in vitro* up-regulation of UCP2 (**figures 10.3.2a**, **10.3.2b** and **10.3.2c.**) in MAC13 cells. This concentration of LMF also appears to confer protection of the MAC13 cell line against reactive oxygen species *in vitro*. It is therefore plausible in the light of the existing evidence that such protection is mediated through UCP2 regulation.

Similar increases in UCP2 expression are reported in the cachexia models *in vivo*. Bing *et al* (2002) demonstrated injection of LMF to NMRI mice to up-regulate UCP2 mRNA in skeletal muscle and liver (+146%, ($p<0.05$) and +142%, ($p=0.03$) respectively). Sanchis *et al* (1998) report a similar increase (194%) in skeletal muscle in the cachexigenic Yoshida AH-130 model.

10.4 Conclusion

LMF produced by MAC16 cells may be a protective mechanism for the tumour. The related MAC13 cells produce no LMF and it was investigated whether addition of LMF would protect this tumour from simulated chemotherapy *in vitro*. LMF conferred a beneficial effect for MAC13 cells against those agents generating reactive oxygen species, but not those with other mechanisms. This protection coincided with an LMF-induced increase in UCP2, which potentially explains this survival advantage.

This study suggests therefore that one reason for the poor response of cachexia inducing tumours to conventional chemotherapy may be due to increased UCP2 expression and detoxification of free-radical generating agents by tumour-produced LMF. It should be stressed however, that many other factors may also account for the inferior survival accompanying cachexia, and that this study merely presents a hypothesis.

Chapter 11. Conclusions.

Cancer cachexia is a wasting syndrome that accounts for a significant proportion of cancer-related deaths. Despite the reported involvement of cytokines (and other factors), cachexia is mediated predominantly by a 43kDa lipid mobilising factor and a 24kDa Proteolysis inducing factor.

The LMF associated with cancer cachexia shares homology with Zinc-α2-Glycoprotein, an endogenous protein found in most body fluids. Both LMF and ZAG mediate lipolysis in an identical fashion, bioactivity (monitored by glycerol release from isolated WAT) are similar. Glycosylation (hence carbohydrate moieties) appear important for the functioning of both factors, and isoformic variation exists. Interest is rife for the monopolisation of this factor as an anti-obesity agent.

Interestingly, the LMF homologue ZAG increased protein synthesis at translation in the surrogate skeletal muscle C_2C_{12} *in vitro* model. These effects are apparently antagonistic to PIF, and why they should occur remains unclear. LMF enhances glucose transport (+/- insulin) in the soleus muscle of the MAC16 model. This may be through alteration of intrinsic plasma membrane GLUT activity and may function to facilitate glucose uptake in the tumour.

Both LMF and ZAG are tryptically cleaved to yield an active fragment of approximately 10kDa (as determined by sephadex G50 chromatography). This indicates that the whole molecule is not required to mediate lipolysis *in vitro*. It is likely that such tryptic (or chymotryptic) digestion occurs in the stomach when ZAG is orally administered (thus intact ZAG is not detectable in the plasma of orally-dosed NMRI mice, although weight loss still occurs). The effects of LMF

appear, in part, to be mediated through MAP Kinase pathways, in addition to the classical cAMP pathway.

Lipolysis obtained using the standard *in vitro* epidydimal WAT bioactivity assay is reproducible with differentiated 3T3-L1 adipocytes. However inducing differentiation in 3T3-L1 adipocytes is difficult and time consuming, thus the former system is the more desirable. Lipid depletion by ZAG/LMF was predominantly via lipolysis, with only a modest reduction in lipid synthesis.

Cachexigenic MAC16 cell lysate test positive for ZAG/LMF production when subjected to western blotting. The non-cachexigenic MAC13 line does not. Incubation of LMF/ZAG with MAC13 cells appears to confer some protection against certain ROS-generating chemotherapeutic drugs (possibly via up regulation of UCP2). This may go some way to explaining the inferior chemotherapeutic response of cachexia-associated tumours. Given adequate time, further drugs (with a variety of mechanisms) could be examined in both MAC16 and MAC13 cell lines, and the response compared.

ZAG expression in WAT and BAT is increased in the cachexigenic MAC16 model. An increase in levels of this adipokine further potentiates the wasting associated with this model. This increase of adipocyte ZAG is absent in healthy NMRI rodents dosed with ZAG, indicating that another cachexia-associated factor is responsible. At present, glucocorticoids would appear the most likely candidates for mediating this increase.

During cachexia *in vivo*, anomalies in the regulation of the uncoupling protein homologues are seen to occur. Despite difficulties with regards to the antibodies currently available, LMF was observed to up-regulate these carriers directly *in vitro* (although in an *in vivo* situation, indirect

factors also come into play). Gastrocenemius isolated from ZAG dosed rodents show good correlation between weight loss and the expression of UCP2. This increase in UCPn potentiates energy wastage and thus the cachexia-associated emaciation.

Understanding the mechanisms of this 43kDa glycoprotein is essential for the knowledge and treatment of cancer cachexia. Furthermore, development of this agent as an anti obesity agent necessitates detailed knowledge of it's functioning. Further work is required (and in many cases underway) and is detailed at the end of each appropriate chapter.

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ABSTRACTS & PUBLICATIONS:

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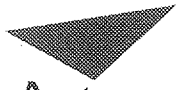

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EFFECT OF ZINC-α2-GLYCOPROTEIN (ZAG) ON EXPRESSION OF UNCOUPLING PROTEINS IN SKELETAL MUSCLE AND ADIPOSE TISSUE.

Paul M. Sanders & Michael J Tisdale (*Article in Press*).

PROTEOLYTIC DIGEST OF A TUMOUR-DERIVED LIPID MOBILISING FACTOR AND SUBSEQUENT IDENTIFICATION OF AN ACTIVE FRAGMENT.

Paul M Sanders & Michael J Tisdale. *British Journal of Cancer 88 (Supplement 1):S68*

ZINC-α2-GLYCOPROTEIN, A LIPID MOBILISING FACTOR, IS EXPRESSED IN ADIPOCYTES AND UPREGULATED IN MICE WITH CANCER CACHEXIA'.

Chen Bing, Yi Bao, John Jenkins, **Paul Sanders**, Savero Cinti, Michael J Tisdale and Paul Trayhurn. (*Article in Press*).