

Some pages of this thesis may have been removed for copyright restrictions.

If you have discovered material in AURA which is unlawful e.g. breaches copyright, (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please read our [Takedown Policy](#) and [contact the service](#) immediately

*The Mode of Action of a Lipid Mobilising
Factor in Cancer Cachexia*

STEVEN THOMAS RUSSELL
Doctor of Philosophy

Aston University
April 2002

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without proper acknowledgement.

Aston University

The mode of action of a lipid mobilising factor in cancer cachexia

Steven Thomas Russell

A thesis submitted for the degree of Doctor of Philosophy

2002

SUMMARY

Cachexia is characterised by a progressive weight loss due to depletion of both skeletal muscle and adipose tissue. The loss of adipose tissue is due to the production of a tumour-derived lipid mobilising factor (LMF), which has been shown to directly induce lipolysis in isolated epididymal murine white adipocytes. The administration of LMF to non-tumour bearing mice produced a rapid weight loss, with a specific reduction in carcass lipid with also some redistribution of lipid with the accumulation of lipid in the liver. There was also up-regulation of uncoupling protein-1 and -2 mRNA and protein expression in brown adipose tissue, suggesting that an adaptive process occurs due to increased energy mobilisation. There was also up-regulation of UCP-2 in the livers of LMF treated mice, suggesting a protective mechanism to the build up of lipid in the livers, which would produce free radical by-products. LMF was also shown to stimulate cyclic AMP production in CHO-K1 cells transfected with human β -3 adrenergic receptors and inhibited by the β -3 antagonist SR59230A. LMF binding was also inhibited by SR59230A in isolated receptors. This suggests that LMF mediates its effects through a β 3 adrenergic receptor. There were also changes in glucose and fatty acid uptake in LMF treated mice, which suggests metabolic changes are occurring. The study suggests that a tumour derived lipolytic factor acts through the β 3 adrenoceptor producing effects on lipid mobilisation, energy expenditure and glucose metabolism.

KEYWORDS: LIPID-MOBILIZING FACTOR, LIPOLYSIS, UNCOUPLING PROTEINS, β 3-ADRENERGIC RECEPTORS, ZINC- α 2-GLYCOPROTEIN, GLUCOSE UPTAKE.

To
My Parents

Preface

Dr Chen Bing at Liverpool University carried out the analysis of the uncoupling protein messenger RNA and plasma leptin levels and the histology of the brown adipose tissue and livers of the treated and untreated groups.

Dr Tom Zimmerman of the Bayer Corporation carried out the Isoelectrofocusing gel analysis of LMF and ZAG and his colleagues also at Bayer carried out the MALDI-TOF analysis on the isolated LMF and ZAG.

Acknowledgements

I would like to take this opportunity to thank Professor Tisdale for his guidance and support over the last three years. I would also like to thank my collaborators at Liverpool University in particular Dr Chen Bing and Professor Williams. Thank you also to Professor K.C. Fearon and Dr Moses from the Edinburgh Royal Infirmary, for urine samples from cachectic patients.

Also I wish to thank Dr Manara of the Sanofi Research Centre, Milan, Italy for kindly supplying me with SR59230A, Dr Zimmerman at the Bayer Corporation for supplying me with purified ZAG, Dr Bjorkman at Caltech Pasadena for supplying me with recombinant ZAG; also Dr Kennedy for providing a sample of 11-(dansylamino) undecanoic acid and Dr Waddell of Astra Zeneca for supplying me with the transfected CHO-K1 cells. Thank you also to Mike, Wayne and John in the biomedical facility for all their help.

Thanks also to Helen, Alison, Paul, Stacey, Jwan, Kam and Bill for all the good times in the lab and all those outside the lab especially Stewart, Ed, Conner, Rob, Mike and Sarah.

This PhD could not have been completed if it were not for the support of my mum and dad and of Peter and Wendy over all these years, so thanks. Lastly thanks to Lianne for coming into my life and keeping me sane during my write up.

Table of Contents

	Page Numbers
Title Page	1
Summary	2
Dedication	3
Preface	4
Acknowledgments	5
Table of Contents	6
List of Figures	13
List of Tables	15
Abbreviations	16
Chapter 1 – Introduction	20
1.1 General Introduction	20
1.1.1 Cachexia	20
1.1.2 Anorexia and Cachexia	20
1.2 Energy expenditure changes due to tumour burden	21
1.2.1 Carbohydrate metabolism	22
1.2.2 Lipid Metabolism	24
1.2.3 Protein Metabolism	26
1.3 Mediators of Cachexia	27
1.3.1 Role of cytokines in cancer cachexia	27

1.3.1.1 Tumour necrosis factor- α	28
1.3.1.2 Interleukin-1	30
1.3.1.3 Interleukin-6	31
1.3.1.4 Interferon Gamma	33
1.3.1.5 Leukaemia-Inhibiting factor	33
1.3.1.6 Interleukin-15	34
1.3.2 Catabolic factors in cancer cachexia	35
1.3.2.1 Proteolysis inducing factors	35
1.3.2.2 Lipid mobilising factors	38
1.3.2.3 Zinc- α 2-glycoprotein	43
1.3.3 Other putative cacachectic factors	45
1.4 Models of Cancer cachexia	46
1.5 Aims of this study	47
Chapter 2 – Materials	48
2.1 Animals	48
2.2 Chemicals	48
2.3 Buffers	52
2.4 Statistical Analysis	60
Chapter 3 – Purification of a tumour-derived lipid mobilising factor	61
3.1 Introduction	61
3.2 Method	62

3.2.1 Protein Purification	62
3.2.1.1 Patients	62
3.2.1.2 Purification of LMF	62
3.2.1.3 Protein determination	63
3.2.1.4 Lipolytic assay	64
3.2.2 Protein Identification	66
3.2.2.1 Gel electrophoresis	66
3.2.2.2 Coomassie brilliant blue staining	66
3.2.2.3 Bio-Rad silver staining	67
3.3 Results	67
3.3.1 Purification of a lipid mobilising factor	67
3.3.2 Analysis of the purified protein	70
3.4 Discussion	73
3.5 Conclusion	74
Chapter 4 – The effects of LMF <i>in vivo</i>	75
4.1 Introduction	75
4.1.1 Action of a tumour derived lipid-mobilising factor	75
4.1.2 Uncoupling proteins	76
4.2 Methods	81
4.2.1 <i>In vivo</i> investigation of LMF	81
4.2.2 Histology	81
4.2.3 Serum analysis	81
4.2.4 Expression of UCP-1, -2 and -3 mRNA	82

4.2.5 Western blotting analysis	83
4.2.5.1 Sample preparation	83
4.2.5.2 Blotting samples	83
4.2.6 Body temperature analysis	84
4.2.7 <i>In vivo</i> effects of LMF on NMRI mice transplanted with the MAC13 adenocarcinoma	84
4.2.8 Measurement of glycogen in LMF treated and control mice	85
4.3 Results	85
4.3.1 Body weight and tissue weights	85
4.3.2 Metabolite plasma concentrations in treated and untreated mice	87
4.3.3 UCP1 and UCP2 mRNA and protein expression	89
4.3.4 Histological examination of brown adipose tissue and liver samples	93
4.3.5 <i>In vivo</i> effects of LMF on mice with a MAC13 tumour burden	96
4.3.6 Glycogen levels in mice treated with LMF or vehicle control	98
4.4 Discussion	99
4.5 Conclusion	102
 Chapter 5- The role of β-3 adrenergic receptors in the action of LMF	 103
5.1 Introduction	103
5.1.1 β -adrenergic receptors	103
5.2 Methods	105
5.2.1 The attenuation of LMF action on lipolysis	105

5.2.2 CHO-K1 cell line	105
5.2.3 Cyclic AMP determination	105
5.2.4 Iodination of LMF with [Na ¹²⁵ I]	106
5.2.5 Binding studies	107
5.3 Results	108
5.4 Discussion	114
5.5 Conclusion	116
Chapter 6 – The effects of LMF on glucose and fatty acid uptake	117
6.1 Introduction	117
6.1.1 Glucose utilisation	117
6.1.2 Lipid utilisation	119
6.1.3 Distribution of LMF	120
6.2 Method	120
6.2.1 ¹⁴ CO ₂ production from ¹⁴ C-labelled glucose	120
6.2.2 Glucose utilisation <i>in vivo</i>	120
6.2.3 Treatment of animals	121
6.2.4 Accumulation of radioactive 2-deoxyglucose-6-phosphate by tissues (Cm*)	122
6.2.5 Determination of body weight	123
6.2.6 Lipid oxidation and accumulation	124
6.2.7 Extraction of Lipids	124
6.2.8 Total body radiography	125
6.3 Results	125

6.3.1	Glucose uptake	125
6.3.2	Fatty acid uptake	131
6.3.2.1	Absorption, oxidation and distribution of labelled lipid	131
6.3.3	I ¹²⁵ LMF distribution	133
6.4	Discussion	136
6.5	Conclusion	138
Chapter 7 – Characterisation of Zinc-α2-glycoprotein		139
7.1	Introduction	139
7.2	Method	140
7.2.1	Zinc- α 2-glycoprotein lipid mobilising activity	140
7.2.2	EZAG binding studies	140
7.2.3	Binding of EZAG with radio labelled noradrenaline	141
7.2.3.1	Binding of EZAG with radio labeled arachidonic acid	141
7.2.4	Spectral analysis of LMF and ZAG	141
7.2.5	Effects of Trypsin digestion on LMF	142
7.3	Results	143
7.3.1	Zinc- α 2-glycoprotein lipolytic activity	143
7.3.2	Zinc- α 2-glycoprotein and lipid mobilising comparisons	144
7.3.3.1	Recombinant zinc- α 2-glycoprotein lipolytic activity	147
7.3.3.2	Binding EZAG with H ³ -arachidonic acid	147
7.3.3.3	Tritated and non-tritated noradrenaline with EZAG	148
7.3.3.4	Incubation EZAG with the fluorogenic fatty acid DAUDA	151

7.3.3.5 Trypsin digestion of LMF	151
7.4 Discussion	151
7.5 Conclusion	152
Chapter 8 – Conclusion	153
Chapter 9 – References	157
<i>Appendix 1- Publications</i>	187

LIST OF FIGURES

	Page Number
1.2.1 The Cori Cycle	24
3.2.1.4 Conversion of glycerol to lactate corresponds to NAD ⁺ production	65
3.3.2 12% SDS-PAGE gel of LMF	70
3.3.3 HPLC trace of LMF purification	72
4.3.1 Body weight changes in LMF-treated and vehicle treated control mice	86
4.3.3.1 Western blot of UCP1 on brown adipose tissue	90
4.3.3.2 Western blot of UCP2 on liver tissue	91
4.3.3.3 Western blot of UCP1 on white adipose tissue	92
4.3.4.1 LMF or PBS treated murine liver tissue stained with orange red O - PBS (100µl)	94
4.3.4.2 LMF or PBS treated murine brown adipose tissue stained with HE stain	95
4.3.6 Glycogen levels in livers of LMF and PBS treated mice	98
5.3.1 The effect of the β3-AR antagonist SR59230A on lipolysis in murine white adipocytes, induced by LMF	109
5.3.2 Effect of LMF and Isoprenaline on cyclic AMP levels in CHO-K1β3 cells in absence and presence of propranolol or SR59230A	111
6.1.1 Metabolic interactions between tumour and host	118

6.3.1.1	Comparisons of various tissues Rg values in LMF and PBS treated mice	127
6.3.1.2	Oxidation of U- [¹⁴ C]-glucose to ¹⁴ CO ₂ in mice dosed with LMF 48h prior to injection of radiolabel	128
6.3.1.3	Ratio between ³ H glucose and ¹⁴ C glucose in various tissues after treatment with LMF or PBS over 48h	129
6.3.2.1.1	Absorption of an oral dose of [1- ¹⁴ C]-triolein in mice dosed with LMF or PBS over 48h prior to injection	132
6.3.2.1.2	Oxidation of [1- ¹⁴ C]-triolein to ¹⁴ CO ₂ in mice dosed with LMF or PBS over 48h prior to injection	132
6.3.2.1.3	Accumulation of [1- ¹⁴ C] –triolein in mice dosed with LMF or PBS over 48h prior to injection	133
6.3.3.1	The distribution of ¹²⁵ I LMF in various tissues at 6h and 24h after pre-treatment with LMF	135
7.3.2.1	Spectral analysis of LMF and ZAG	144
7.3.2.2	IEF PAGE (pH 4.0- 6.5) of ZAG and LMF western blot	146
7.3.3.2	EZAG incubated with ³ H-aracidonic acid	148
7.3.3.3	Noradrenaline/EZAG protein fractions from a G25 sephedex column	149
7.3.3.4	Fractions of EZAG and ³ H-Noradrenaline from a G25 sephedex column	150

LIST OF TABLES

	Page Numbers
3.2.1.1 Tumour type and corresponding weight loss	62
3.3.1.1 Protein concentration through the purification process and final glycerol release activity	69
3.3.2 LMF activity under different storage conditions	73
4.3.2 Effects of human LMF after 48h on body weight, fat mass, muscle mass and serum metabolites and leptin levels and temperature in NMRI mice	88
4.3.5 Effects of human LMF after 48h on MAC13 tumour-bearing mice on body weight, fat mass, muscle mass and serum metabolites in NMRI mice	97
5.3.1 Kd and Bmax values for LMF and other agonists of β 3-AR113	113
7.3.1 Glycerol release activity comparison for LMF, ZAG and EZAG with isoprenaline as a positive control	143

Abbreviations

AA	Arachidonic Acid
ADP	Adenosine-5'-diphosphate
AIDS	Autoimmune deficiency disease
APR	Acute phase response
APS	Ammonium persulfate
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
CHO	Chinese hamster ovary
C _m	Tissue glucose concentration
CP	Parameter of Blood glucose uptake
CRH	Corticotrophin-releasing hormone
DAUDA	11-(dansylamino)undecanoic acid
DEAE	Diethylaminoethyl cellulose
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
ECL	Emission chemoiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethyleneglycol-bis(β aminoethylether) N,N,N,N' tetraacetic acid
EPA	Eicosapentaenoic acid
EZAG	Recombinant Zinc- α 2-Glycoprotein
FCS	Foetal calf serum
FFA	Free fatty acid
g	gram
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
h	hour
H ₂ O	Water
HCAP	Human cachexia-associated protein
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HSL	Hormone-sensitive lipase
IEF	Isoelectrofocusing
IFN γ	Interferone- γ
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
i.p.	Intraperitoneal
i.v.	Intravenous
l	Litre
LC	Lumped constant

LIF	Leukaemia-inhibiting factor
LMF	Lipid Mobilising Factor
LPL	Lipoprotein lipase
kg	Kilogram
μ	Micro
M	Moles per litre
m	Milli
MAC	Murine adenocarcinoma of the colon
MHC	Major histocompatibility complex
min	Minutes
mRNA	messenger ribonucleic acid
NaCl	Sodium Chloride
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NEFA	Nonesterified fatty acid
P	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PIF	Proteolysis-inducing factor
PPAR γ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acid
QS	Q sepherose
REE	Resting energy expenditure

R _G	Glucose metabolic rate
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SNS	Sympathetic nervous system
SEM	Standard error of the mean
T3	Tri-iodothyronine
TEMED	N,N,N,N'-Tetra-methyl-ethylenediamine
TNF α	Tumour necrosis factor α
TNP	Total parenteral nutrition
TNF α	Tumour necrosis factor α
TNM	Tumour, node, metastasis
Tris	Tris (hydroxymethy) methylamine
Tween20	Polyoxyethylene-sorbitan
U	Unit
UCP	Uncoupling protein
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
ZAG	Zinc- α 2-Glycoprotein

Chapter 1. Introduction

1.1 General Introduction

1.1.1 Cachexia

Cachexia is a wasting disorder and has been observed in a number of disease states, including AIDS, septicaemia and cancer. In cancer patients this cachexia presents itself not just as severe weight loss, but also as nausea, anaemia, muscle weakness and anorexia. This condition is thought to occur in up to 50% of all cancer patients (De Wys, 1972), but is particularly prevalent in solid tumours such as pancreatic cancer. In non-Hodgkin's lymphoma 30% of patients will present with cachexia, whereas in pancreatic cancer 90% of patients present with this condition. Cancer cachexia has been shown to be responsible for up to 20% of all cancer deaths, normally due to the loss of lean muscle mass, particularly that of the respiratory system. It can also affect the patients' ability to cope with chemotherapy (DeWys and Begg, 1980).

Often it is the weight loss that is the presenting symptom before diagnosis of the tumour (Albrecht and Canada, 1996, Wigmore et al, 1997). This early onset of weight loss is probably due to a change in energy balance. It also differs from starvation, where most of the weight lost will be fat with little muscle loss, whereas with cachexia it is almost 50:50 (Cohn et al, 1981).

1.1.2 Anorexia and Cachexia

The weight loss observed in cachexia is different from that of anorexia nervosa in that there is loss of both skeletal muscle and adipose tissue, but not visceral protein. There is often a decrease in energy intake due to decreased food

consumption in cachectic patients, but other factors are involved in the presented weight loss. Both loss of muscle and adipose tissue precedes the fall in food consumption (Costa, 1977), and in malnourished cancer patients' food intake does not correlate with the degree of malnutrition (Costa et al, 1980). Studies aimed at increasing energy intake through dietary counselling failed to reverse cachexia (Grosvenor et al, 1989). Also administration of total parenteral nutrition (TPN) has failed to give long-term stabilisation of body weight (Evans et al, 1985). The transient weight gain seen in TPN is due to increased fat and water rather than skeletal muscle. Studies with the progestational agent megestrol acetate (Megace), an appetite stimulant, showed a weight gain of more than 5% in some 15% of treated patients (Loprinzi et al, 1994). This weight gain when examined by body composition analysis was not in lean muscle, but arose from an increased fat and water retention (Loprinzi et al, 1993). Cancer cachexia is not simply a case of calorie deficiency, but a more involved systemic metabolic change involving tumour burden, altered metabolism, anorexia, production of cytokines and specific tumour products.

1.2 Energy expenditure changes due to tumour burden

In starvation, the metabolic rate is reduced in an attempt to preserve tissues in a low-protein, low-calorie environment (Knox et al, 1983). In cancer patients there are variations in this energy change. Tumour type appears to determine whether there is an overall increase or decrease in energy expenditure. Lung, gastric, pancreatic, sarcoma and hepatocellular cancer all present an increase in energy expenditure in a proportion of patients (Fredrix et al, 1991, Harvie and Campbell 2000), whereas metabolic rates in weight losing patients with colon cancer,

oesophagus and in metastatic breast cancer tend to remain stable. Pancreatic cancer patients have increased resting energy expenditure (REE) relative to controls; patients that also had an acute phase response tended to have even greater increases in REE (Falconer et al, 1994). This might then explain differences between patients with the same type of cancer.

In some patients, hypermetabolism can be explained by loss of peripheral tissue, in an aim to maintain visceral tissue, but not in all cases (Lundmark et al, 1984). Thus, particular changes related to the disease may be responsible for abnormalities in energy expenditure in cancer patients.

1.2.1 Carbohydrate metabolism

In starvation, the body uses glycogen stores in the liver and muscle to provide energy requirements for the brain and other tissues (Mulligan and Bloch 1998). Once those stores have been depleted, muscle protein initially provides the fuel source, but as a protection mechanism fat will be converted to ketone bodies. These ketone bodies can provide 95% of the fuel needed by the brain, and in so doing protects skeletal muscle. Cancer patients may be able to use this adaptive mechanism.

There are marked alterations in tumour-free carbohydrate metabolism in cancer patients, particularly the liver. This is probably due to demand from the tumour for glucose as its primary fuel source (Holm et al, 1995), since solid tumours are anaerobic with poor internal vascularisation. Large amounts of lactic acid are then produced by the tumour leading to an increase in conversion of lactate to glucose in the liver, and an up regulation of the Cori cycle (**Fig 1.2.1**). The Cori cycle is

energy consuming since conversion of 2mol of lactate to glucose requires 6mol of ATP, while only 2mol of ATP is recovered in the conversion of glucose back to lactate (Tisdale, 1997), which causes energy wastage.

Cori cycle activity increased from 20% in normal control subjects to 50% of glucose turnover in cachectic patients and accounts for disposal of 60% of the lactate produced by the tumour (Holroyde et al, 1975). In addition to lactate other factors contribute to enhanced gluconeogenesis. Glycerol is released from active lipolysis, and amino acids released by activated proteolysis are both important in the augmented hepatic glucose production in cancer-bearing states (Waterhouse et al, 1979).

Weight loss in cancer is associated with glucose intolerance and an abnormal insulin response (Rofe et al, 1994). Glucose intolerance due to insulin resistance is common, and may even predate weight loss and tumour diagnosis. Cancer patients have an increased glucose flux, which could consume up to 40% of the ingested carbohydrate and may contribute to the weight loss observed by increasing energy expenditure (Burt and Brennan, 1984). Thus alterations in carbohydrate metabolism may contribute to cancer cachexia.

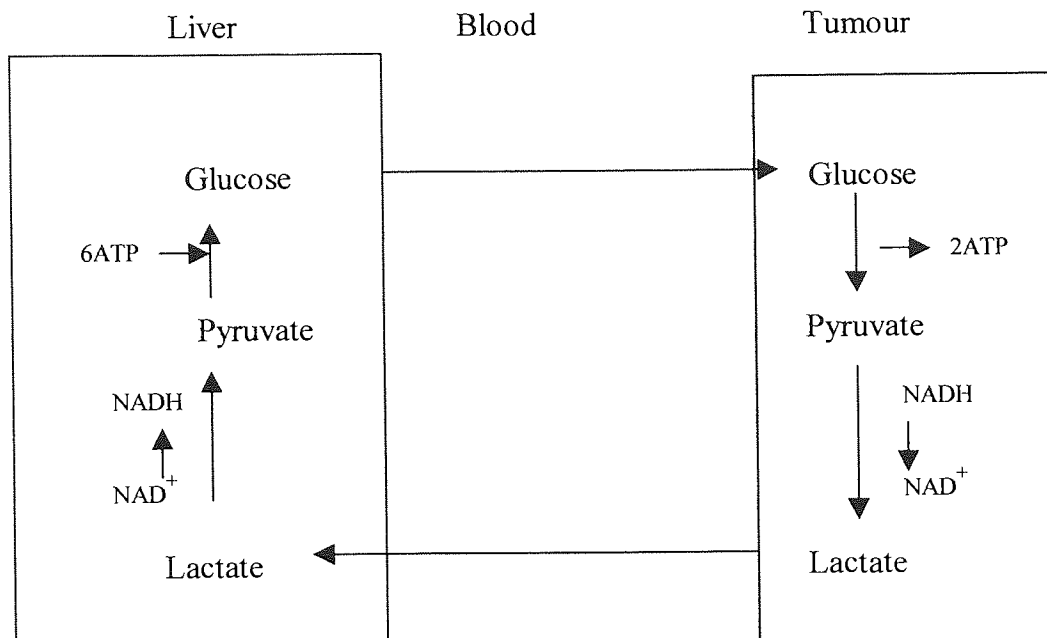


Fig 1.2.1 The Cori cycle (Stryer 1988)

1.2.2 Lipid Metabolism

Fat contributes around 90% of adult fuel reserves, and loss of this plays a major role in weight loss in cachectic cancer patients. With increased demands on fuel being placed on the host by the tumour the fat stores will be targeted. In addition, the metabolism of the polyunsaturated fatty acid (PUFA), linoleic acid through the 12-lipoxygenase pathway may be essential in protecting apoptotic cell death in tumour cells (Tang et al, 1996). The loss of fat is due to several processes; first there is increased lipolytic activity (Thompson et al, 1981), which releases glycerol and fatty acids. Glycerol is delivered to the liver where it is used as a

gluconeogenic substrate. Other tissues then use fatty acids as an alternative source of energy to glucose. There is also a decrease in the activity of lipoprotein lipase (LPL). This enzyme is responsible for the cleavage of triglycerides into glycerol and fatty acids and occurs in white adipose tissue (Lanza-Jacoby et al, 1984, Thompson et al, 1981). Inhibition of LPL causes lipid uptake to be severely disrupted. Also reduced *de novo* lipogenesis production is seen in tumour-bearing states (Thompson et al, 1981). This results in decreased esterification and therefore reduced lipid disposition. Hyperlipaemia and hypercholesterolaemia also appear to be features of cancer burden (Argiles et al, 1997).

Clinical studies have observed that increased mobilisation of fatty acids occurs before weight loss occurs (Costa et al, 1981). This suggests the production by either the tumour or the host tissues of lipid mobilising factors. They appear to act like polypeptide hormones and are present in the circulation, causing catabolism of adipose tissue by stimulation of cAMP formation (Tisdale and Beck, 1991). Increased fatty acid oxidation in the absence of an increase in dietary fat intake would lead to a depletion of fat stores, while an increased triglyceride fatty acid cycling and gluconeogenesis from glycerol could result in an increased metabolic rate. These would all contribute to a net loss of body weight.

1.2.3 Protein metabolism

It is the loss of protein and in particular skeletal muscle that determines the survival of cachectic cancer patients. The reduced survival is mainly associated with loss of respiratory muscle, whereas visceral protein is conserved (Fearon, 1992).

During fasting muscle proteins are degraded to provide amino acids which are used for gluconeogenesis; however during longer starvation periods protein breakdown decreases in order to conserve nitrogen and to maintain lean body mass (Argiles et al, 1997). This does not appear to be the case for cancer-bearing states where the loss of skeletal muscle results in an increased urinary excretion of nitrogen. There is also a reduction in the rate of protein synthesis and increased protein degradation in skeletal muscle biopsies from cachectic patients (Lundholm et al, 1976). This combination would result in a decrease of muscle mass.

There have been recent reports of a new proteolytic mechanism involved in skeletal muscle during cancer cachexia (Baracos et al, 1995, Llovera et al, 1995,1996). A non-lysosomal, ATP, and ubiquitin-dependent proteolytic system is activated in skeletal muscle of tumour-bearing rats. This system would appear to be up regulated in cachexia and would then lead to loss of skeletal muscle. This mechanism may be responsible for the actions of the proteolysis inducing factor (PIF). PIF has been isolated from the cachexia-inducing murine, MAC16 adenocarcinoma and has also been identified in the urine of weight losing cancer patients. It is a 24kDa sulphated glycoprotein and was recently, shown to have the ability to increase protein degradation both *in vivo* and *in vitro* (Todorov et al, 1996).

1.3 Mediators of cachexia

Much work has been carried out in trying to identify specific factors produced by the host and the tumour that produce a cachectic response. There has been good progress made into identification of the factors involved, but much has still to be done in understanding the mechanisms involved.

The factors involved can be separated into two main categories: those of tumour origin (produced and released by the tumour) and humoral (for example estrogen) factors. Cells of the immune system produce a large number of cytokines that act on a multitude of targets such as bone marrow, myocytes, hepatocytes, adipocytes, etc, producing a cascade of biological responses, and in so doing lead to wasting associated with cachexia. Among those cytokines involved in this type of cachectic response are tumour necrosis factor (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon- γ (IFN- γ) and leukaemia-inhibiting factor (LIF). These cytokines share the same metabolic effects and activity is closely inter-related, showing often-synergistic effects.

1.3.1 Role of cytokines in cancer cachexia

Tumour necrosis factor (TNF- α) was first designated the title cachectin when it was identified as a mediator of anorexia-cachexia in trypanosome-infected rabbits (Beutler et al, 1985). Since this was identified a number of other cytokines have been suggested to play a role in cachexia development. These include interleukins -1 and -6, interferon- γ and leukaemia-inhibiting factor (LIF). All cytokines will produce a profound anorexia and weight loss *in vivo*. The effect on adipose tissue could be mediated through inhibition of lipoprotein lipase (LPL). This would

prevent adipocytes from extracting fatty acids from plasma for storage. However it is unlikely that this alone would cause such a massive depletion in adipose tissue that is observed. In type 1 hyperlipidaemia there are elevated serum chylomicron levels caused by an inherent deficiency in LPL yet patients are not cachectic and appear to have normal fat stores (Tisdale, 1997).

1.3.1.1 Tumour Necrosis Factor- α

TNF- α has been shown *in vivo* to produce muscle degradation in healthy rats (Llovera et al, 1993), but this effect must be by an indirect mechanism, since Goldberg et al (1988) were unable to detect a catabolic effect when isolated skeletal muscle was incubated with TNF- α *in vitro*. Both IL-6 (Goodman et al, 1994) and IL-1 (Goldberg et al, 1988) have been shown incapable of inducing muscle degradation *in vitro*.

Elevated levels of TNF- α have been detected in serum from cachectic patients, but this is controversial (Maltoni et al, 1997). When increased levels of TNF- α have been identified in patients it appears to reflect the severity of the disease rather than the presence of cachexia. Ming et al (1997) showed that higher serum levels of TNF- α was linked to tumour size, TNM (tumour, node, metastasis), staging, and more advanced lymph status in patients with invasive breast cancer.

Attempts to reverse the effects of TNF- α by using anti-TNF- α antibodies have produced ambiguous results, probably due to difficulties measuring the local effects of cytokines. Also it has been hard trying to correlate serum levels of TNF- α with the extent of cachexia in cancer patients. In patients with solid tumours and weight loss between 8-40% no TNF- α was detected in serum samples (Socher et

al, 1988). However Balkwill et al (1987) showed serum levels correlated with the clinical status of the patient and a study of 91 patients with chronic B-cell lymphocytic leukaemia showed serum levels of TNF- α were elevated in all stages of the disease, with a progressive increase in relation to the stage (Adami et al, 1994). Although TNF- α has been shown to be a potent inhibitor of lipoprotein lipase (Berg et al, 1994), there is no difference seen between cancer patients and controls in either total lipoprotein lipase activity or the relative levels of the messenger RNA for lipoprotein lipase and fatty acid synthetase (Thompson et al, 1993). TNF- α has been shown to regulate adipose tissue function by increasing lipolysis by an inhibition of Gi protein. Treatment of isolated adipocytes with TNF- α stimulated lipolysis by blunting the endogenous inhibition of lipolysis produced by adenosine release and decreasing Gi activity (Gasic et al, 1999).

Adipocytes produce a number of cytokines including TNF- α , which causes increased insulin resistance and inhibits adipocyte differentiation. This may be due to TNF- α activation of chemokines, such as IL-8. These are low molecular weight peptides that play a role in inflammatory processes (Gerhardt et al, 2001). Chemokines are elevated in cancer, which may result in decreased fat content, this effect may be through a TNF- α /chemokine decrease in PPAR γ messenger RNA expression (Gerhardt et al, 2001). This TNF- α /chemokine causes increased leptin, which in turn can have an inhibitory effect on adipocyte differentiation (Zhou et al, 1999), and may indirectly control the anti-adipogenic actions of chemokines.

In rats injected with *Escherichia coli* weight loss occurs, but when they were given pentoxifylline, a potent TNF- α inhibitor, there was a decreased anorexia along with decreased rates of body weight loss and muscle protein degradation (Bruille et al, 1993). However, clinically pentoxifylline did not reduce the anorexia or cachexia in 35 cachectic cancer patients (Goldberg et al, 1995). TNF- α administration does induce cachexia in mice, but tolerance develops and food intake and body weights return too normal (Mullen et al, 1990). Thus the evidence for TNF- α involvement in cancer cachexia is as yet inconclusive, and it may be that TNF- α has a role, but does not act alone, but in conjunction with some other factor.

1.3.1.2 Interleukin-1

Several other cytokines have been thought to be involved in cachexia, for example IL-1, which stimulates TNF- α release from macrophages. IL-1 is thought to reduce appetite by inducing corticotrophin-releasing hormone (CRH) production in the hypothalamus, while suppressing neuropeptide Y, an appetite stimulant (Dunlop and Campbell, 2000). Strassman et al (1993) found that IL-1 may play a role in mediation of cachexia. They showed that cachexia associated with colon-26 tumours in mice could be improved by intratumoral injections of IL-1 receptor antagonist (IL-1ra); systemic administration of IL-1ra was not effective. An inhibitor of IL-1 synthesis and TNF- α , FR167653 when administered to rats bearing the ascites hepatoma Yoshida AH-130 did not prevent the anorexia or cachectic effects of the tumour (Busquets et al, 2000). Circulating triacylglycerols or other metabolites such as glucose or lactate were also not affected, and tumour

growth was not influenced. This suggests that factors other than IL-1 and TNF- α are involved in cachexia.

1.3.1.3 Interleukin-6

There has also been some evidence of IL-6 involvement for producing the cachectic signs seen in colon-26 adenocarcinoma bearing mice since both Strassman et al (1992) and Yasumoto et al (1995) showed that administration of anti-IL-6 antibodies did partially reverse weight loss. They concluded that IL-6 was involved in producing cachexia, but that other factors must also be present. IL-6 certainly has the potential to act as a cachectic factor, since atrophy of muscles has been observed in IL-6 transgenic mice and was blocked by the anti-mouse IL-6 antibody (Tsujinaka et al, 1996). These C57BL/6JLd-IL-6 transgenic mice developed IgG1, plasmocytosis and muscle loss despite overall weight not differing from controls (Suematsu et al, 1992). There were changes in proteolytic pathways with increased activity of cathepsins C and B+L increasing 20 fold and 6.5 fold respectively. There was also increased expression of cathepsin mRNA and these changes were abolished by treatment with the IL-6 receptor antibody (MR16-1) (Tsujinaka et al, 1995).

Also administration of IL-6 to rats showed acute activation of total and myofibrillar protein degradation in skeletal muscle (Goodman et al, 1994). *In vitro*, using C₂C₁₂ myotubes, IL-6 shortened the half life of long lived proteins and increased the activation of the 26S proteasome proteolytic pathway (Ebisui et al, 1995), suggesting direct up-regulation of this protein degradation pathway. IL-

6 is the main cytokine involved in the acute phase response (APR), and levels of IL-6 were elevated in the serum of colon cancer patients with an ongoing APR (Fearon et al, 1991). In this study though, the patients had lost some weight and further work will be necessary to determine whether IL-6 is elevated in cancer cachectic patients.

The cancer cachexia syndrome seen in intracerebral tumours was examined by injecting human A431 epidermal carcinoma and OVCAR3 ovarian carcinoma intracranially, which produced a cachectic response within 7-10 days. The serum levels of various cytokines were measured, but they were observed not to predict development of the tumour. RT-PCR analysis revealed A431 tumour-bearing mice expressed IL-1, IL-6 and LIF. Serum murine IL-6 was also increased in the A431 mice and mice injected with IL-6 receptor antibodies showed increased survival time. IL-6 may be important centrally even when tumour cell number is low (Negri et al, 2001). IL-6 has been strongly implicated in cachexia, but it is probable that it does not act alone, either inducing or acting in synergy with other factors. Also Wing et al (1993) suggested that IL-6 activity was more due to activation of lysosomal proteolysis in muscle, which may mean that its contribution to overall muscle proteolysis is negligible, since cathepsin activity does not affect overall protein degradation or myofibrillar proteolysis in fasting.

1.3.1.4 Interferon Gamma

A role for interferon Gamma (IFN- γ) in cachexia has been suggested, since observations confirmed it has properties similar to that of TNF- α in respect to metabolism *in vitro* (Patton et al, 1986). Also in mice bearing the Lewis lung tumour, which produces large amounts of IFN- γ and produces weight loss, the loss of fat was reduced using anti- IFN- γ antibody without affecting protein stores (Mattys et al, 1991). Langstein et al (1991) showed that in rats transplanted with MCG101 sarcoma, anti- IFN- γ antibody reduced weight loss and anorexia but this was partial and short term, suggesting other mediators are involved.

1.3.1.5 Leukaemia-inhibiting factor

In cachexia there is a decrease in lipoprotein lipase activity, which has been suggested is due to leukaemia-inhibiting factor (LIF). Athymic nude mice implanted with the human cell line SEKI cells that express LIF in large amounts present severe cachexia (Mori et al, 1991). LIF mRNA has also been observed in cachexia-inducing melanoma xenografts and not present in non-cachectic inducing xenografts. However, it is unlikely that the decrease in lipoprotein lipase activity alone could cause the depletion of fat observed in cachexia, and there are no reports of LIF affecting skeletal muscle protein reserves.

1.3.1.6 Interleukin-15

Interleukin-15 (IL-15) may play a role in muscle fibre growth and anabolism. When administered to rats over 7 days IL-15 did not increase muscle mass or muscle protein content, but it did produce changes in the rates of synthesis and degradation. There was also a 33% decrease in white adipose tissue mass and a 20% drop in circulating triacylglycerols. This was associated with a 47% decrease in hepatic lipogenic rate and a decrease of 36% in plasma VLDL triacylglycerol content. There was no change in the rate of lipolysis observed. This suggests that IL-15 may participate in the regulation of muscle and adipose tissue mass and is therefore a target for playing a role in the development of cachexia (Carbó et al, 2001).

Other groups have shown no evidence for increased cytokine serum levels and associated cachexia. Maltoni et al (1997) studied 61 patients with weight loss; serum levels of IL-1, IL-2 and TNF- α were undetectable in most samples. There was no correlation between these serum levels and the cachexia suggesting that other factors are involved. There is some evidence that cytokines play a role in the development of cachexia, at least in experimental models. In nude mice bearing human tumour xenografts cytokines were associated with four out of eight models tested (Kajimura et al, 1996). It would appear that cytokines alone are unlikely to produce cachexia and may work in harmony with each other and other non-cytokine factors, such as the recently isolated catabolic factors.

1.3.2 Catabolic factors in cancer cachexia

1.3.2.1 Proteolysis inducing factors

The major factor in cachectic patient survival is the rate of skeletal muscle loss. This loss of muscle mass in cancer patients (Belizario et al, 1991), and in an experimental model of cachexia in the mouse (Smith and Tisdale, 1993b), has been shown to correlate with the presence in serum of bioactivity capable inducing protein degradation in isolated gastrocnemius muscle, as measured by tyrosine release. Using the MAC16 tumour model for cachexia Smith and Tisdale (1993) were able to show that serum from the cachectic animals was able to increase protein degradation in isolated gastrocnemius muscle. This effect appeared to be due to the presence of cachexia since serum from mice bearing MAC13 adenocarcinoma, which is non-cachectic, did not increase tyrosine release. Evidence for a circulating catabolic agent in patients with weight loss greater than 10%, was shown by Belizario et al (1991). There appears to be a catabolic factor present in the circulation that mediates muscle degradation.

This tumour product has since been identified as a sulphated glycoprotein of M_r 24,000 (Todorov et al, 1996). This has been named proteolysis inducing factor (PIF). It consists of a central polypeptide chain of M_r 4kDa with phosphate residues attached, one O-linked sulphated oligosaccharide chain of M_r 6kDa containing glucosamine and one N-linked sulphated oligosaccharide chain of M_r 10kDa also containing glucosamine. Thus most of the structure of PIF resides in the carbohydrate component. The polypeptide core was shown phosphorylated (Todorov et al, 1997), and showed no structural homology with other materials in the protein database. Wang et al (2001) have shown that there may be a possible

homologue from a human breast cancer library, using the available 20 amino acid sequence of PIF. The homologue was given the title human cachexia-associated protein (HCAP). It is unknown at this time if they are the same molecule. Wang et al (2001) examined HCAP expression in human prostate cancer, and it was identified by RT-PCR in cell lines CU145, LNCap and its metastatic sublines C4-2, C4-2b and PC-3, as well as in prostate cancer tissue and bone marrow from prostate cancer patients with cachexia.

In PIF it is the carbohydrate chains that are responsible for bioactivity and antigenic activity (Todorov et al, 1997), since both the ability of protein degradation in isolated muscle, and antibody activity, were destroyed by incubation with enzymes capable of removing the N- and O-linked oligosaccharide chains. Western blotting using the MAC16 monoclonal antibody showed that PIF was present in the urine of pancreatic cancer patients with weight loss. PIF was not present in the urine of patients with weight loss due to major burns, multiple injuries or surgery-associated catabolism and sepsis, or even cancer patients with little or no weight loss. This material was then isolated from the urine and analysed. It was found to be chemically, immunologically and functionally the same as that isolated from MAC16 mice.

PIF either from the MAC16 tumour or from patient urine when administered to non-tumour bearing mice, produced cachexia with rapid loss of 10% of body weight in 24h. This was achieved without decreased water or food consumption, suggesting cachexia occurred without the presence of anorexia (Todorov et al, 1996, Cariuk et al, 1997). Weight loss was attenuated by pre-treatment with the MAC16 monoclonal antibody to PIF, showing the specificity of the effect. Body

composition analysis showed the weight loss was due to loss of skeletal muscle mass and there was no change in water content. *In vitro* PIF was shown to induce direct release of tyrosine from isolated gastrocnemius muscle. This effect was inhibited by the MAC16 monoclonal antibody.

The protein degradation produced by PIF was reduced in mice by the pre-treatment with the polyunsaturated fatty acid eicosapentaenoic acid (EPA). This also inhibited the rise of PGE₂ (Tisdale, 1996). EPA has been shown to counter the weight loss in pancreatic cancer patients (Wigmore et al, 1996). In these patients there was stabilisation of protein and fat reserves accompanied by a reduction in acute phase protein production and stabilisation of resting energy expenditure. It is possible that PGE₂ is an intracellular mediator of the protein degradation by PIF, or that another metabolite of arachidonic acid formed at the same time as PGE₂, is responsible (Tisdale, 1998).

Body composition changes seen with PIF were similar to those seen in cachectic cancer patients. Thus there was no change in the weight of the heart or kidney, but there was an increase in the liver (Lorite et al, 1998). The progressive decrease in skeletal muscle mass involved a decrease in muscle protein synthesis and an increase in protein degradation (Cariuk et al, 1997). The enhanced protein degradation produced by PIF has been attributed to activation of the ATP-ubiquitin-dependent proteolytic pathway (the predominant pathway of muscle protein degradation) in animals bearing the MAC16 tumour (Lorite et al, 1998). Increased levels of mRNA were found for the ubiquitin carrier protein (E₂) and the C9 proteasome catalytic subunit in gastrocnemius muscle, and a monoclonal antibody to PIF attenuated the enhanced protein degradation in soleus muscle

from MAC16 bearing mice confirming that PIF was responsible for the loss of skeletal muscle. Lorite et al 2001 also showed that there was up-regulation of the ATP ubiquitin-dependent proteolytic pathway by PIF in NMRI mice and in murine myoblasts. The degradation was also inhibited by anti-PIF antibody and by the proteasome inhibitors MG115 and lactacystin. This suggests that PIF activity is via the activation of the ATP-ubiquitin-dependent proteolytic pathway.

1.3.2.2 Lipid mobilising factors

Lipid-mobilising activity was identified in the urine of cancer patients in 1958 by Chalmers et al. Chalmers also identified an identical factor in the urine of fasted but not fed normal subjects. Sub-cutaneous administration to non-tumour bearing mice of an extract purified from the urine of starved subjects, resulted in increased mobilisation of adipose tissue, due to an increase in total metabolic turnover of fat, hence producing a decrease in weight without anorexia.

A number of factors have since been identified from the serum and urine of cancer patients. Neoplastic cells were shown to be capable of elaborating a lipid mobilising substance by implantation of non-viable preparations of Krebs-2 carcinoma in Swiss mice where a decrease in carcass fat was observed (Costa and Holland, 1966).

There was also a decrease in carcass neutral lipids in Walker 256 carcinoma implanted in rats (Kralovic et al, 1977), suggesting a tumour derived lipolytic factor. Theologides (1976) detected the presence of anorexigenic and lipolytic peptides in the urine of patients with widespread neoplastic disease. Thompson et al (1981) observed a drop in adipose tissue LPL activity with a rise in basal

lipolysis in mice bearing the preputial gland tumour ESRE 586 at low tumour burdens and they suggested this was possibly due to humoral factors from the growing tumour.

A lipid-mobilising factor was isolated by Kitada (1980) from the serum of non-anorectic, cachectic tumour-bearing mice which induced a profound mobilisation of adipose tissue stores when administered to non-tumour bearing mice. An identical heat stable 5kDa protein fraction was isolated from the serum of a patient with advanced cancer and could be isolated from the culture media of a human lymphoma cell line (Kitada et al, 1981). This suggests that the factor was directly produced by the tumour cells, rather than as a result of the host response to the tumour burden.

Studies using a thymic lymphoma transplanted into AKR mice identified a tumour-derived factor, which was heat stable and had a molecular weight of 10kDa (Kitada, 1982), Kitada suggested that since the factor was not found in healthy subjects and that it must be a product of the lymphoma cells, and that it induced lipolysis in isolated adipocytes via a receptor mediated mechanism in a similar fashion to catecholamines.

Masuno et al (1981) described the isolation of a lipolytic substance from ascites fluid of sarcoma 180-bearing mice, which was observed to be a single 75kDa band on an SDS PAGE gel, following ammonium sulphate fractionation. This was termed toxohormone-L. It was also identified in patients bearing hepatomas or Grawitz's tumour, and was believed to be responsible for the reduced host adipose tissue and increased plasma FFA observed in these patients. Masuno et al (1984) reported the purification of toxohormone-L from the ascites fluid of hepatoma,

lymphoma and ovarian cancer patients. The administration of toxohormone-L into the lateral ventricle of the rat brain produced a decreased intake of food and water, together with a stimulation of adipose tissue lipolysis. Appetite suppression by toxohormone-L was also observed by Okabe et al (1992), who suggested that the direct anorexigenic effects of toxohormone-L might result in the induction of anorexia in cancer patients. Thus lipid-depleting mechanisms would appear to occur more as a consequence of its ability to induce a state of semi-starvation, rather than direct lipid mobilising activity.

A heat stable 6kDa glutamic acid rich peptide was isolated from the extracellular environment of cultured human tumour cells. This peptide induced lipolysis in isolated adipocytes, in a time and dose dependent manner, which correlated with the *in vivo* loss of body lipids in tumour-bearing animals (Taylor et al, 1992). This factor was designated the name lipolysis promoting factor (LPF) and was also shown to be present in the serum and ascites fluid of ovarian cancer patients and was associated with increased HSL activity in normal adipose tissue (Gercel-Taylor 1996).

The MAC16 adenocarcinoma was reported to produce a factor that was able to induce lipolysis in isolated adipocytes via the activation of a signal transduction pathway classically associated with adrenergic receptor agonists. Incubation of a single cell suspension of WAT with crude MAC16 tumour supernatants gave an enhanced, linear and time-dependent release of FFA (Beck and Tisdale, 1987). Conversely supernatant from the closely related non-cachectic colon adenocarcinomas MAC13 and 15a had one-third the lipolytic activity. A protein

was then isolated and designated the term lipid-mobilising factor (LMF). This protein was also identified in the serum and urine of cachectic cancer patients, but not in normal patient serum or even in starvation (Beck and Tisdale, 1990, Groundwater et al, 1990). The *i.p.* administration of LMF to both MAC13 tumour-bearing (Beck 1990) and nontumour-bearing mice (Cariuk et al, 1997) induced a dose dependent decrease in host body weight due to fat loss, without alterations in either total body water or lean body mass. Chromatographic purification of LMF produced peaks of lipolytic activity at 20, 3, 1.5 and 0.7kDa (Beck and Tisdale, 1991). The low molecular weight fractions could be due to proteolytic digestion. Further purification and characterisation studies using protease inhibitors revealed LMF to be a protein with an apparent molecular weight 40kDa, which demonstrated sequence homology with a known protein zinc- α 2-glycoprotein (ZAG) (McDevitt, 1996, Todorov et al, 1998b). Harai et al (1998) showed that both LMF and ZAG caused stimulation of cyclic AMP in isolated white adipocytes in a dose dependent manner.

The activity of LMF appears to be through stimulation and up-regulation of adenylate cyclase. Islam-Ali et al (2001) showed that there was up-regulation of $G_{\alpha s}$ with a decrease in $G_{\alpha i}$ in adipose tissue and that the activity of LMF may be due to increased receptor number. These G protein changes were observed in NMRI mice treated with LMF and *in vitro* using 3T3-L1 adipocytes. The G protein changes were attenuated by eicosapentaenoic acid (EPA), suggesting the tumour-derived lipolytic factor acts to sensitise adipose tissue to lipolytic stimuli. The effect of EPA in cachexia is well documented and has been shown to preserve the adipose tissue in cancer patients. EPA was recently compared in a clinical

study to another FFA γ -linolenic acid in cachectic patients with non-resectable pancreatic cancer (Wigmore et al, 1996). The majority of γ -linolenic acid patients still lost weight, whereas EPA stabilised weight and showed a temporary reduction in APP and stabilisation of resting energy expenditure. The *in vitro* analysis of LMF showed fat mobilisation activity could be attenuated by EPA (Tisdale and Beck, 1991) and *in vivo* studies of mice with MAC16 tumour showed EPA to completely prevent the weight loss (Tisdale and Beck, 1991).

LMF has also been shown to affect protein synthesis (Islam-Ali and Tisdale, 2001). They showed that LMF caused a concentration-dependent stimulation of protein synthesis and a decrease in muscle catabolism. There was also a concentration-dependent stimulation of 2-deoxyglucose uptake in C₂C₁₂ myoblasts, myotubes and tumour cells in keeping with the increased protein synthesis. LMF was also shown to increase intracellular cyclic AMP levels. The activity of LMF was inhibited by the use of the adenylate cyclase inhibitor MDL_{12330A}, which would suggest activity via adenylate cyclase. The stimulation of protein synthesis was also attenuated by the use of propranolol and the β -adrennergic antagonist SR59230A. This would suggest that LMF activity is affected via a classical catecholamine receptor mechanism. It also suggests that LMF is acting in opposition to PIF and may be attempting to conserve skeletal muscle.

1.3.2.3 Zinc- α 2-glycoprotein

Zinc- α 2-glycoprotein (ZAG) has a molecular weight of approximately 40kDa. It is a human protein originally isolated from plasma by Bürgi and Schmid (1961), with the biological function still remaining unknown. The protein can be precipitated by the addition of zinc ions, shows electrophoretic mobility in the α 2 region, and contains 18% carbohydrate, hence the name. The amino acid sequence analysis revealed a single polypeptide chain of 276 amino acid residues having a high degree of similarity with antigens of the major histocompatibility complex (36-39%) (Araki et al, 1988) including the class I proteins and CD1 (which presents lipidic antigens to T cells). ZAG does lack the transmembrane and cytoplasmic domains characteristic of MHC antigens (Bjorkman and Parham, 1990) raising the possibility that it is a soluble HLA-like antigen. Although the biological function of ZAG has yet to be identified ZAG is found in high concentrations in cyst fluid from women with gross cystic breast disease and may play a role in the development of the disease (Haagensen et al, 1986). It is also inducible in a breast cancer cell line by glucocorticoids and androgens. ZAG has also been identified in most body fluids including serum, sweat, saliva, cerebrospinal fluid, seminal plasma and urine (Tada et al, 1991). It has also been shown to be a potential serum marker for prostate cancer (Hale et al, 2001) ZAG production in malignant tissue was measured immunohistochemically using anti-ZAG antibodies. High-grade tumours expressed significantly less ZAG than moderate-grade tumours and men with ZAG producing tumours had elevated ZAG serum levels.

The crystal structure of ZAG shows an overall fold that is similar to that of the MHC class 1 molecules (Sanchez et al, 1999). This groove appears to be open in ZAG and contains an additional electron density, which may belong to an unidentified ligand (Sanchez et al, 1999). This ligand does not appear to be a peptide or carbohydrate (Kennedy et al, 2001) and like CD1, ZAG may bind a hydrophobic ligand, although the groove in ZAG is not as deep as that of CD1, and could not accommodate lipids of the size and complexity of those bound by this protein. Kennedy et al 2001, using fluorescent methods and a fluorescent fatty acid analogue, showed that ZAG did appear to bind natural fatty acids, such as arachidonic acid and eicosapentaenoic acid. If like class 1 MHC molecules and CD1, ZAG binds a variety of ligands *in vivo*, even of a single generic class (peptides for class 1, large complex lipids for CD1) then it is possible various fatty acids bound to ZAG determine its biological effect. Eicosapentaenoic acid and other PUFAs have notable pharmacological effects and Kennedy (2001) found that DHA bound best to ZAG, which is known to activate the retinoid X receptor in mouse brain (de Urquiza et al, 2000).

ZAG was shown to have an identical sequence to LMF (Todorov et al, 1998), and a ZAG antibody was shown to identify both of the proteins. They also migrated together on denaturing and non-denaturing gels. ZAG was also shown to stimulate glycerol release from isolated adipocytes and showed cAMP stimulation in a dose dependent manner, similar to that of LMF (Harai et al, 1998).

The seminal form of ZAG was found not to be glycosylated and does not contain the N terminal blocking found in plasma purified ZAG and did not appear to have

lipolytic activity (Harai et al, 1998). Glycosylation would appear to be very important to activity and may affect the 3D structure of the molecule in presenting the unknown ligand. The structural and sequential similarities of LMF and ZAG warrant further investigation. Perhaps different ZAG: ligand combinations act as do CD1: lipid and MHC class 1: peptide combinations at a variety of receptors, performing different biological actions.

1.3.2.4 Other putative cachectic factors

A number of other mediators of cachexia have been suggested. With the role of hormones in the intermediate metabolism of carbohydrates they have been postulated to be involved. Insulin, corticotrophin, adrenaline, human growth hormone, and insulin-like growth factor have all been suggested to play a role in cachexia (Puccio and Nathanson, 1997). Infusions of hydrocortisone or cortisol, glucagon and adrenaline result in cyclic adenosine monophosphate activation of a protein kinase that phosphorylates and activates hormone-sensitive lipase (HSL). A failure of this normal mechanism may account for the fact that patients with cancer that are weight losing have increased rates of glycerol and FFA turnover compared with starved healthy patients.

1.4 Models of cancer cachexia

A number of animal models have been used to study the mechanisms of cancer cachexia. Only a few tumours have been shown to produce cachectic symptoms in experimental animals. The model in this study was a transplantable mouse adenocarcinoma (MAC16), which was derived from prolonged administration of 1,2-dimethylhydrazine to NMRI mice, (Double et al, 1975, Bibby et al, 1987). Implanted subcutaneously into the flanks of NMRI mice the tumour produces weight loss, which starts to appear after a period of 10-12 days after transplantation (Beck and Tisdale, 1987). Weight loss occurs with a small tumour burden less than 1% to 3% of host weight and produces a 30% decrease in body weight without a decrease in food and water intake this compares well with human patients where the tumour represents 1% of the body weight. Other cachectic models only produce weight loss when the tumour burden reaches 10-20% of the host body weight, and here anorexia appears to be the major contributor to the weight loss. In the MAC16 model both carcass and muscle mass decrease in direct proportion to weight of the tumour. This suggests tumour-derived products are responsible for the cachexia observed. When using the MAC16 tumour model there is also a very convenient MAC13 tumour that has a similar pattern of differentiation, but without the weight loss (Bibby et al, 1987). Other models used in examining cachexia include the Walker 256 transplantable carcinosarcoma (Morrison et al, 1984), murine colon-26 adenocarcinoma (Stassman et al, 1993), MCG 101 a transplantable sarcoma (Svaniger et al, 1983), DMH colorectal tumours (Emery et al, 1989) and Yoshida A130 ascites hepatoma (Sanchis et al, 1998).

1.5 Aims of this study

The aim of this study was to examine the mechanism of action of the tumour-derived cachectic factor, lipid mobilising factor (LMF). In cancer patients there is often an increase in energy expenditure and a major component of that may be the up-regulation of uncoupling proteins. This was examined *in vivo* (**Chapter 4**). It had also been previously suggested that LMF action might be due to activation of a receptor mechanism similar to that of catecholamines. LMF was examined *in vitro* for binding to isolated membranes and activation of cAMP via the β_3 -adrenoceptor on CHO-K1 cells (**Chapter 5**). LMF has also been shown to produce a decrease in plasma glucose levels and causes massive lipid mobilisation. These changes were examined using radioactive labelling of metabolic substrates (**Chapter 6**). Finally LMF was previously shown to have sequence homology with a known protein zinc- α_2 -glycoprotein and was also identified by an anti-ZAG antibody. LMF was compared to a recombinant ZAG and a plasma purified ZAG (**Chapter 7**).

Chapter 2. Materials

2.1 Animals

Pure strain NMRI mice were obtained from our own inbred colonies, they were housed at an ambient temperature of $22\pm 2^{\circ}\text{C}$ under a 12:12h light/dark cycle. All animals were fed a standard chow diet from Special Diet Services, Lillico, Wonham Mill, Bletchworth, Surrey, UK) with tap water *ad libitum*. All animal work was terminated before weight loss exceeded 25% of total body weight.

2.2 Chemicals

All chemicals used have been supplied by Sigma-Aldrich, Dorset, UK, at the highest purity available unless stated bellow:

Amersham Life Sciences, UK

Q Sepharose Fast Flow

Rainbow Molecular weight markers

Amersham Pharmacia Biotech UK Ltd

Iodine-125; NaI in NaOH solution 3.6 GBq/ml. Specific activity $> 0.6\text{TBq/mg}$

Iodide

1-[7,8- ^3H]Noradrenaline 37MBq/ml. Specific activity 444GBq/mmol

2-Deoxy-D- [1- ^{14}C]-Glucose. Specific activity 2.072 GBq/mmol

2-Deoxy-D- [2,6- ^3H]-Glucose. Specific activity 1.63 TBq/mmol

D- [U ^{14}C] Glucose Specific activity 11.0 GBq/mmol

Triolein [Carboxyl – ^{14}C] Specific activity 3.8GBq/mmol

[8-³H] Adenosine 3',5' cyclic phosphate NH₄ Salt Specific activity

888GBq/mmol

Arachidonic acid [5,6,8,11,12,14,15 -³H] Specific activity 7.6TBq/mmol

Autogenbioclear, Wiltshire, UK

Mouse uncoupling protein 1 (UCP1), Mouse (Rabbit)

Bio-Rad Laboratories, Richmond, CA, USA

Bio-Rad Protein assay dye reagent concentrate

Ammonium persulfate

Silver stain kit

BDH limited, Poole, UK

Potassium dihydrogen orthophosphate

Sodium hydrogen carbonate

BOC Ltd, London, UK

Air: CO₂, 95%: 5%

Nitrogen

Calbiochem-Novabiochem Corporation, San Diego, CA, USA

Anti-Uncoupling Protein 2 (UCP2), Mouse (Rabbit)

DAKO A/S, Glostrup, Denmark

Peroxidase-Conjugated Goat Anti-Rabbit Immunoglobulins

Fisher Scientific, Loughbrough, UK

Glacial acetic acid

Hydrochloric Acid

Perchloric acid

Ethanol

Methanol

Sodium dihydrogen orthophosphate

Magnesium Sulphate

Optiphase Hisafe 3

Sucrose

Gelman Sciences, Northampton, UK

Astrodisc 0.2 μ m syringe filters

YM membranes, 10KDa cut-off

Microcon concentrators, 10KDa cut-off

Gibco BRL Life Technologies, Paisley, Scotland, UK

Bovine foetal calf serum (FCS)

Dulbecco's Modified Eagles Medium (DMEM) with glutamax-I

Penicillin-Streptomycin

Trypsin/EDTA

Oxoid, Basingstoke, Hampshire, UK

Phosphate Buffered Saline tablets

Pierce, Rockford, IL, USA

iodo-BEADS, iodination reagent

Pharmacia Biotech, UK

Resource™ISO column 1ml

Research Diagnostics, Inc, Flanders, NJ, USA

Rabbit anti-Rat Uncoupling Protein 3 (UCP-3)

Sanofi Winthrop S.p.A., Milan, Italy

[3-(2-ethylphenoxy)-1-[(1 S)-1,2,3,4-tetrahydronaphth-1-ylaminol]]-(2S) 2-propanol oxalate, SR59230A

WAKO Chemicals GmbH, Neuss, Germany

Non-Esterified Fatty Acid detection in serum (NEFA C) Kit

Whatman International Ltd, Maidstone, Kent, UK

DEAE cellulose

2.3 Buffers

2.3.1 Protein Purification

2.3.1.1 QS Buffer 1

Tris HCl pH 8.0	10mM
DTT	1mM
PMSF	0.5mM
EGTA	0.5mM

2.3.1.2 QS Buffer 2

Tris HCl pH 8.0	10mM
DTT	1mM
PMSF	0.5mM
EGTA	0.5mM
NaCl	0.2M

2.3.1.3 Wash Buffer

Phosphate Buffered Saline (PBS)	1 tablet/100ml deionised H ₂ O
---------------------------------	-------------------------------------------

2.3.1.4 Resource-Iso HPLC Buffer A

50mM Phosphate Buffer in 1.5M Ammonium Sulfate

Anhydrous disodium orthophosphate dihydrate	7.1g/L 1.5M AMS
Sodium dihydrogen orthophosphate	7.8g/L 1.5M AMS
pH7 by adding dihydrogen to the disodium	

2.3.1.5 Resource-Iso HPLC Buffer B

50mM Phosphate Buffer in deionised water

Anhydrous disodium orthophosphate dihydrate 7.1g/L

Sodium dihydrogen orthophosphate 7.8g/L

pH7 by adding dihydrogen to the disodium

2.3.2 Lipolytic Bioassay

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 Buffer

Krebs Ringer Bicarbonate Buffer / 3% Bovine Serum Albumin

2.3.2.3 Collagenase Solution

4mg Collagenase in 1ml Krebs Buffer

2.3.2.4 10% Perchloric Acid

83.33ml in 500ml deionised H₂O

2.3.2.5 40% Potassium Hydroxide

40g in 100ml deionised H₂O

2.3.2.6 Glycerol Assay Buffer

Triethanolamine	100mM
Magnesium Sulphate	2mM
Phosphoenolpyruvate	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

Mixed with deionised water and pH 7.4 with perchloric acid

2.3.3 SDS-Page gels

2.3.3.1 Sample Buffer 1

Tris HCl pH6.8	50mM
2% SDS	
Dithiothreitol	100mM
10% Glycerol	
0.1% Bromophenol Blue	

2.3.3.2 12% Resolving Gel

30% Acrylamide/ Bis acrylamide	4ml
Deionised H ₂ O	3.3ml
1.5M Tris HCl pH8.8	2.5ml
10% SDS	0.1ml
10%APS	0.1ml
TEMED	0.004ml

2.3.3.3 5% Stacking Gel

30% Acrylamide/ Bis acrylamide	1.7ml
Deionised water	6.8ml
1M Tris HCl pH6.8	1.25ml
10% SDS	0.1ml
10%APS	0.1ml
TEMED	0.01ml

2.3.3.4 Running Buffer (10x) pH 8.3-8.5

Trizma base	32.29g
Glycine	144.3g
10% SDS	100ml
Distilled Water	to 1 litre

2.3.3.5 Coomassie Blue Gel Stain

Coomassie Brilliant Blue R250 Stain	0.1%
Glacial Acetic Acid	10%
Methanol	25%
Make up with deionised H ₂ O	

2.3.3.6 Coomassie Blue Destain solution

Glacial Acetic acid	10%
Methanol	25%
Deionised H ₂ O	65%

2.3.3.7 Fixing Solution 1

Methanol	40%
Glacial acetic acid	10%
Deionised H ₂ O	50%

2.3.3.8 Oxidising Solution 1:10 dilution

2.3.3.9 Silver Nitrate Solution 1:10 dilution

2.3.3.10 Developer Solution

Developer	6.4g
Deionised H ₂ O	200ml

2.3.3.11 Stopping Solution

Glacial acetic acid	5%
Deionised H ₂ O	95%

2.3.4 Trypsin Digestion

2.3.4.1 Cathode Buffer

Trizama Base HCl pH 8.2	12.1g
Tricine	17.92g
SDS	1g
Make to 1 litre with deionised H ₂ O	

2.3.4.2 Anion Buffer (x5)

Trizama Base HCl pH8.9	1M
------------------------	----

2.3.4.3 Fixing Solution 2

Methanol	50ml
Glacial Acetic Acid	10ml
Make to 100ml with deionised H ₂ O	

2.3.4.4 Coomassie Destain 2

Glacial Acetic Acid	10%
Make to 1 litre with deionised H ₂ O	

2.3.4.5 Brilliant Blue G Stain

Brilliant Blue G	0.025%
5mg/ 20mls 10% Glacial Acetic Acid	

2.3.4.6 Sample Buffer 2

20% SDS	4ml
Glycerol	2.4ml
2-mercaptoethanol	0.4ml
Brilliant Blue G (0.1%)	2ml
1M Tris- HCl (pH6.8)	1ml
Make up to 20 ml with deionised H ₂ O	

2.3.5 Western Blotting

2.3.5.1 Western Blotting Wash Buffer 1

PBS	1 table/100ml
Tween-20	0.5ml

2.3.5.2 Western Blotting Wash Buffer 2

PBS	5 tables/500ml
Tween-20	0.5ml

2.3.5.3 Blocking Buffer

Marvel	5g
Wash buffer 2	100ml

2.3.6 Chinese Hamster Ovary Cell Culture Media

Dulbecco's modified Eagles medium	500ml
Foetal calf serum	10%
Pen/ Strep	1%
Glutamate	2mM
HT Supplement	10ml
Non-Essential Amino Acids	5ml
G418 (200mg/ml)	0.5ml
Hygromycin B (50mg/ml)	5ml

2.3.7 Cyclic AMP analysis

2.3.7.1 cAMP Assay Buffer

HEPES pH7.5	20mM
EDTA	5mM
Isobutylmethyloxanthine	0.1mM

2.3.7.2 Binding Protein

cAMP dependent kinase 0.02% w/v

Dissolved in 1mM sodium citrate pH6.5 and 2mM DTT

2.3.7.3 Charcoal suspension

Charcoal	5%w/v
BSA	0.1% w/v in assay buffer

2.3.8 Homogenising buffer

Sucrose	250mM
EDTA	0.2mM
pH to 7 with Concentrated HCl	
HEPES	238.3mg

2.5 Statistical Analysis

Unless otherwise stated, values are reported as means and standard error of means. Data were analysed by analysis of variance (ANOVA) followed by Student's t-test where appropriate. Two tailed tests were used on all occasions and p values <0.05 were considered significant. The analysis was performed using GraphPad InStat version3.00 for windows 95, GraphPad Software, San Diego, California USA, www.graphpad.com

Chapter 3. Purification of a tumour-derived lipid mobilising factor

3.1 Introduction

Previously a tumour-derived lipid mobilising factor (LMF) was identified as a product of the cachectic tumour, MAC16 adenocarcinoma. It was also identified in the urine of weight losing pancreatic cancer patients (Todorov et al, 1998). On administration to non-tumour bearing mice a severe weight loss was observed, the majority of which was due to the loss of fat. In this study LMF was purified from both weight losing pancreatic cancer patients and lung cancer patients, whose weight loss was not as severe. The method previously used was modified to enable a greater yield without effect on purity.

3.2 Method

3.2.1 Protein purification

3.2.1.1 Patients

Urine was collected over a 24h period from patients with unresectable pancreatic cancer and with a weight loss. No patient had received radiotherapy or chemotherapy. Urine samples were stored at -20°C in the absence of preservatives prior to use. Samples used were from times 0, 4 and 8 weeks of a trial on the use of eicosapentaenoic acid (EPA) in the treatment of cancer cachexia. Urine was also collected from lung cancer patients over a 24h period these patients had a less severe weight loss (table 3.2.1.1)

Tumour type	Weight loss kg month^{-1}
Pancreatic cancer	1.8 ± 0.6
Lung cancer	0.25 ± 0.09

Table 3.2.1.1 Tumour type and corresponding weight loss, Data presented mean \pm SEM for 3 patients per group.

3.2.1.2 Purification of LMF

LMF was purified from human urine using a combination of batch extraction on DEAE-cellulose and hydrophobic interaction chromatography (Todorov et al, 1998). Urine was centrifuged at 3000g for 10 min to remove particulate material

and was then diluted with 4 vol 10mM Tris.HCl, pH 8.0. DEAE-cellulose, previously activated by washing in 100mM Tris.HCl, pH 8.0 for 5 min, was added to the diluted urine (10g l^{-1} of original urine) and the mixture was stirred for 2h at 4°C . The DEAE-cellulose was recovered by sedimentation by low speed centrifugation, and the LMF was eluted with 0.5M NaCl in 10mM Tris.HCl, pH8.0. The eluate was equilibrated and concentrated to 1ml by ultra-filtration, in an Amicon filtration cell containing a membrane filter with a molecular weight cut-off of 10kDa (Millipore, UK), against phosphate Buffered Saline (PBS). Further purification was achieved using a Resource-Iso HPLC column (Pharmacia Biotech, St Albans, Herts, UK), employing a decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration from 1.5M. Active fractions containing LMF eluted at 0.6M $(\text{NH}_4)_2\text{SO}_4$, and were desalted before use by washing 5-times against PBS using an Amicon filtration cell (Bing et al, 2002, Russell et al, 2002).

3.2.1.3 Protein determination

Protein concentration was determined by the method of Bradford (1976) using Bio-Rad reagent, a dye-binding assay based on the differential colour change of coomassie Brilliant Blue G-250 in response to various concentrations of protein. Dilutions of protein samples (1:200 and 1:1000) were made. 0.8ml of the diluted protein sample was mixed with 0.2ml of dye reagent and allowed to stand for 5min. The absorbance was read on a Beckman DU7 spectrophotometer at 595nm. All samples were calibrated against a control containing distilled water in place of

the protein sample. The protein concentration was calculated using the following formula derived from a standard curve of a solution of BSA:

$$\frac{\text{Sample Absorbance} - \text{Control Absorbance}}{0.053} * 0.1 \text{ (dilution factor)} = \mu\text{g of protein}/\mu\text{l}$$

3.2.1.4 Lipolytic assay

Lipolysis was measured as described by McDevitt et al (1995). A single cell suspension of white adipocytes was prepared from the epididymal adipose tissue of ex-breeder male NMRI mice using collagenase digestion (Beck and Tisdale 1987). Lipolytic activity was determined by measuring glycerol release after incubation of LMF with 10^5 - 2×10^5 adipocytes for 2h at 37°C in 1ml Krebs-Ringer bicarbonate buffer (**section 2.3.2.1**), pH 7.2. Control samples containing adipocytes alone were analysed to determine the spontaneous glycerol release. Glycerol was measured by the method of Weiland (1974). Briefly 830µl of glycerol assay buffer (**section 2.3.2.6**) was added to 200µl of sample and the reaction was started using 1.0 Unit of glycerokinase (10µl, 2mg/ml) and allowed to proceed for 15min. The reaction was measured using a Beckman DU7 spectrophotometer at 340nm. The reaction followed the scheme below (**Fig 3.2.1.4**):

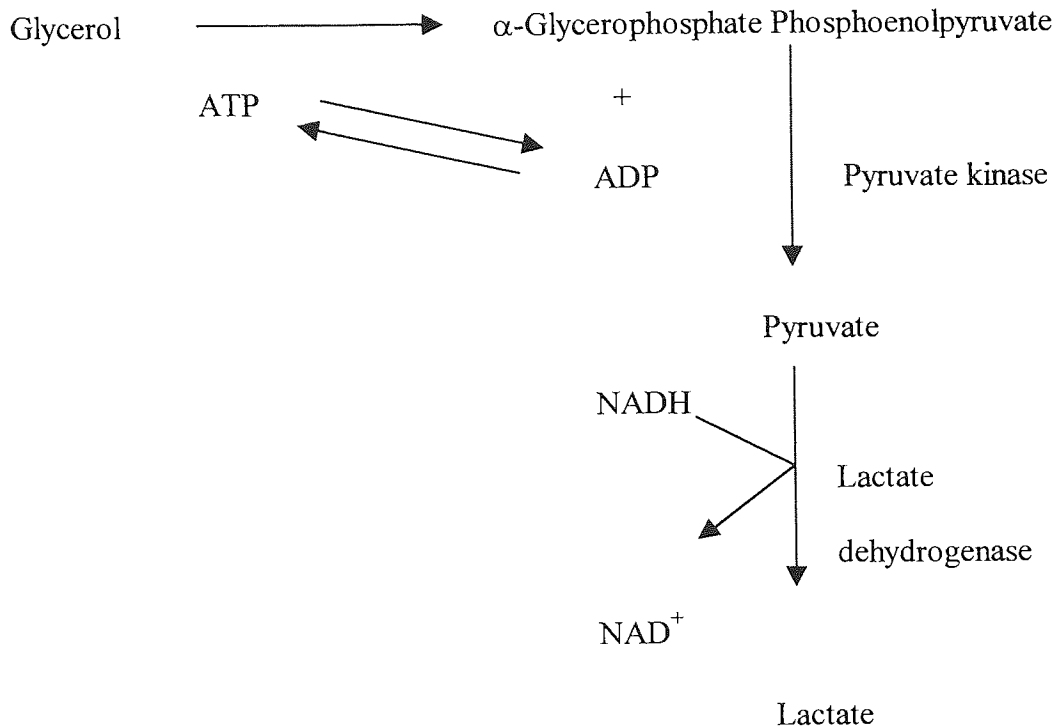


Fig 3.2.1.4 Conversion of glycerol to lactate corresponds to NAD⁺ production

Glycerol release was calculated using the following formula, glycerol being equal to the conversion of NADH to NAD⁺.

(Change in sample absorbance) – (Change in control absorbance) * dilution factor (10)

Extinction

coefficient

for NADH (6.22)

Lipid mobilizing activity was expressed as μmole glycerol released/ 10^5 adipocytes/2h).

3.2.2 Protein identification

3.2.2.1 Gel Electrophoresis

Samples for SDS-PAGE were prepared as 10µg protein/10µl PBS with 5µl sample buffer 1 heated at 95°C using a Scotlab Dri-Block DB-1. Gels were prepared according to the method of Lemmeli (1970) and consisted of a 5% stacking gel and a 12% resolving gel, in 1.5mm slabs and run at 180V for 1h in running buffer using a Mini-Protean II apparatus (Bio-Rad; Richmond, CA). Rainbow molecular marker weight standards consisting of rabbit muscle myosin (M_r 205,000), phosphorylase b (M_r 97,000), BSA (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 29,000), trypsin inhibitor (M_r 20,000), and α -lactalbumin (M_r 14,200) and were purchased from Amersham Life Sciences. Protein was then identified either using coomassie brilliant blue staining (section 3.2.3.2) or a more sensitive Bio-Rad silver stain kit (section 3.2.3.3). The gels were then stored in a 10% glycerol solution for one week before being preserved by the use of a Promega drying kit.

3.2.3.2 Coomassie brilliant blue staining

Protein samples on SDS-PAGE gels were washed in destain solution 1 (section 2.3.3.6) for 30min before being stained with coomassie brilliant blue R-250 (section 2.3.3.5), a sulphated trimethylamine dye, for 1h. Gels were then destained using destain solution 1 over 2h, changing solution every 20min.

3.2.3.3 Bio-Rad silver staining kit

This is a more sensitive alternate to coomassie staining. After electrophoresis the gels were fixed for 1h using fixing solution 1 (section 2.3.3.7). The gels were washed in a 10% oxidising solution (section 2.3.3.8) and then rinsed using deionised water until the water became clear. The gels were then washed in a 10% Bio-Rad silver nitrate solution (section 2.3.3.9) for 20min, before being washed in deionised water for 30sec. The gels were then developed using Bio-Rad developing solution (section 2.3.3.10). When the developer was added at around 30sec a precipitate appeared. The solution was then changed until the desired intensity of bands was obtained. The reaction was stopped using a solution of 5% acetic acid (section 2.3.3.11).

3.3 Results

3.3.1 Purification of a lipid mobilising factor

A lipid-mobilising factor was purified from the urine of pancreatic cancer patients undergoing a clinical trial into the use of eicosapentaenoic acid (EPA) in the treatment of cachexia. Previous studies showed that EPA had no effect on LMF production (Price et al, 1997). Table 3.2.1.1 shows that there was a difference between the pancreatic cancer patients and the lung cancer patients in terms of weight loss.

The yield seemed to differ slightly for each sample (table 3.3.1.1) suggesting there is some difference in the levels due to the progression of the disease or due to isoforms of the protein; protein concentration was measured at each stage of purification using the method of Bradford (1976) (section 3.2.3). Whether the urine was from weeks 0, 4 or 8 of the trial LMF was still produced at more or less the same level. LMF was also obtained from lung cancer patient urine, these patients had some weight loss, but not to the extent of the pancreatic cancer patients. Suggesting that overall weight loss was independent of the presence of LMF. There was also a significant difference in glycerol release activity between the initial T0 group and T8 week group suggesting the possibility LMF has less involvement as the disease progresses and as the adipose stores decrease in size (table 3.3.1.1).

	Initial Urine µg/µl	Conc. Urine µg/µl	DEAE Extract µg/µl	Post HPLC µg/µl	Final Protein µg/µl	Yield µg	Glycerol Release µmol/ 10 ⁵ cells/2h
T0 a	0.09± 0.02	5.5±0.8	-	0.32± 0.2	2.5±0.5	530±39	0.012± 0.003
T4 a	0.06± 0.03	2.3±0.9	-	0.83± 0.35	1.7±0.35	420±26	0.023± 0.004
T8 a	0.06± 0.02	1.1±0.3	-	0.39± 0.15	0.72±0.2	380±32	0.05±** 0.02
T0 b	0.035± 0.03	-	1.03± 0.2	0.9± 0.23	1.76±0.5	320±26	0.011± 0.005
T4 b	0.05± 0.02	-	1.06± 0.3	0.8± 0.3	1.3±0.25	700±82	0.026± 0.005
T8 b	0.07± 0.02	-	1.0±0.4	0.35± 0.04	1.2±0.23	620±35	0.023± 0.008
Lung b	0.08± 0.02	-	1.2± 0.03	0.79± 0.3	0.83±0.26	300±45	0.015± 0.004

Table 3.3.1.1 Protein concentrations throughout the purification process and final glycerol release activity (**a**) sephadex method (**b**) batch extraction method with final yields and glycerol release activity. T samples are from 24h urine collections from pancreatic cancer patients with weight loss, samples were collected at 0, 4 and 8 weeks. Lung **b** samples are from 24h urine collections from lung cancer patients with slight weight loss. Data presented as mean ± SEM for 3 patients per group. Statistically significant difference between T8**b** vs. T0**b** **, p<0.01, analysed by Student's t-test.

3.3.2 Analysis of the purified protein

Purification was carried out as in section 3.2.1.2 and gave a band at 43KDa on a 12% SDS-Page gel and this is in keeping with that found by Todorov et al (1998) and the band was detectable with coomassie blue staining (Fig 3.3.2), there were also several very faint lower weight bands identified by staining but these have yet to be sequenced, the overall purity was around 90%.

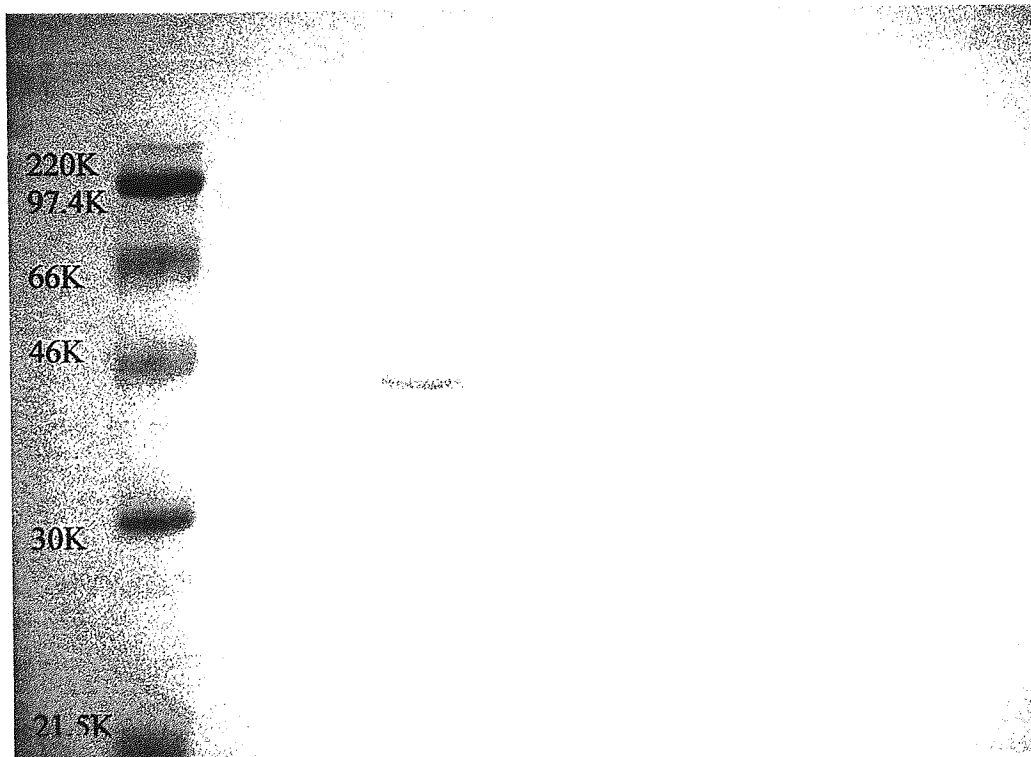


Fig 3.3.2 12% SDS-PAGE gel of LMF, stained using coomassie blue stain: Lane 1 molecular weight markers. Lane 2: LMF purified from cachectic patient urine.

The method of extraction of LMF was adapted from Todorov et al (1998). Several changes were made, in particular a batch extraction for the first step was found to be simpler and quicker than concentration of the urine and then using a sephadex column. Originally the 2l of urine was concentrated in an ultra-filtration unit with a 10kDa cut-off and equilibrated in QS1 buffer (section 2.3.1.1). Before being placed on a sephadex column, fractions were then taken by increasing salt gradient by the addition of QS2 buffer (section 2.3.1.2). Fractions were then analysed for lipolytic activity (section 3.2.1.4). Active samples were then added to the Resource-Iso hydrophobic column. This system produced a high purity of sample (table 3.3.1.1), but it was also very time consuming. By starting with an initial batch extraction on DEAE cellulose meant a faster purification with no significant difference in yield (table 3.3.1.1), with only a slight drop in purity and showed no change in lipolytic activity.

There do appear to be several isoforms of LMF, since there are often two distinct peaks very close together on the HPLC traces that appear to differ in intensity depending on the urine sample and both peaks are biologically active (Fig 3.3.3).

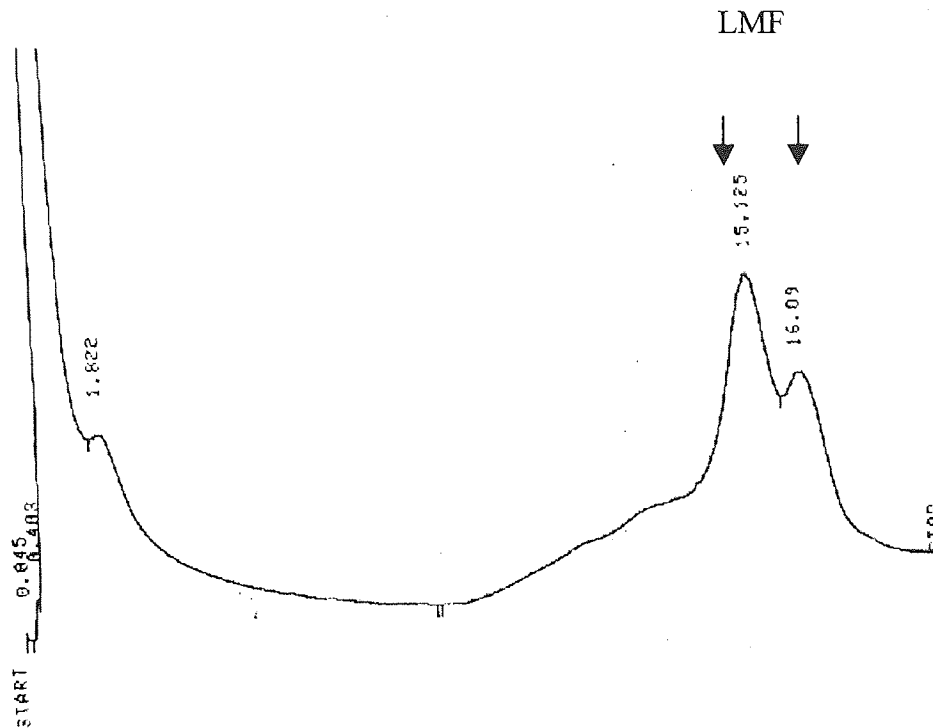


Fig 3.3.3 HPLC trace of LMF purification

The purification product was then tested for bioactivity as in section 3.2.1.4. At a concentration of $1\mu\text{g}/\mu\text{l}$ of protein, the product was able to stimulate lipolysis in isolated murine adipocytes as determined by glycerol release. The activity of LMF appeared significantly to lose its bioactivity after 2-3 weeks at 4°C probably due to the presence of proteases in the sample (table 3.3.2). Freezing was also seen to inactivate the protein (table 3.3.2) since there was a very significant decrease in activity, probably due to structural changes in the carbohydrate component, since the unglycosolated seminal ZAG is also inactive (section 7.3.1).

Sample	Glycerol Release $\mu\text{moles}/10^5 \text{ adipocytes}/2\text{h}$
New LMF 4°C	0.026 \pm 0.005
3 weeks post purification 4°C	0.005 \pm 0.002***
Frozen sample -20°C	0.002 \pm 0.001***

Table 3.3.2 LMF activity under differing storage conditions. Data presented as mean \pm SEM n=6, ***P<0.001 statistically significant from new batch of LMF, analysed by Student's t test.

3.4 Discussion

Purification of a lipid-mobilising factor was achieved by an adaptation of the method of Todorov et al (1998). The need for a higher yield and a decrease of through put time, dictated changes. There was no difference seen in yield between the two extraction methods. There was a significant decrease in glycerol release activity between weeks 0 and 8 using the sephadex method of extraction and this may be an indication that as fat deposits decrease LMF activity decreases, this will have to be further investigated.

On comparison between two groups of cancer patients there was no difference in yield or activity (table 3.3.1.1). The pancreatic cancer patients had a severe weight loss whereas the lung cancer patients only had slight weight loss suggesting LMF occurs early in the development of the disease.

Activity of the LMF purification was significantly reduced when stored at 4°C for three weeks probably due to the presence of proteases. The activity was also reduced by freeze thawing the sample, which may produce a conformational change in the tertiary structure of the protein (table 3.3.2).

3.5 Conclusion

The production of LMF appears to be not affected by the severity of weight loss and may therefore indicate that LMF production occurs early in the tumour development. The use of a batch extraction method in the purification process decreases the through put time and makes purification of LMF simpler without seriously affecting the yield.

Chapter 4. The effects of LMF *in vivo*

4.1 Introduction

4.1.1 Action of a tumour derived lipid-mobilizing factor

Cancer patients with weight loss show fat depletion, increased free fatty acid (FFA) levels and glycerol turnover, whereas those with stable weight do not (Heymsfield and McManus, 1985, Puccio and Nathanson, 1997). A tumour derived lipid-mobilising factor (LMF) was previously identified in MAC16 adenocarcinoma bearing mice and human cancer patients (Todorov et al, 1998). When LMF was given to mice over three days rapid weight loss was observed without an effect on food and water consumption. LMF appears to act selectively on body fat, since carcass lipid was dramatically reduced (40%) without affecting muscle mass. LMF enhances lipolysis in adipocytes and increases in serum FFA, by the activation of hormone sensitive lipase (HSL) through increased intracellular cyclic AMP levels (Hirai et al, 1998).

Fat breakdown will cause weight loss only if the products of lipolysis cannot be reutilised to resynthesize triglycerides. In cancer cachexia there would appear to be mechanisms for utilization of lipolytic products. LMF was further examined *in vivo* to understand its role in cachexia. LMF was examined for effects on body weight, plasma serum levels of a number of metabolites, and core body temperature. LMF action was also examined on tumour-burdened mice to determine if the increased availability of nutrients had any effect on tumour growth.

4.1.2 Uncoupling proteins

In cancer patients there is often an increase in resting energy expenditure observed (Fredrix et al, 1990, Falconer et al, 1994). One possible mechanism for this is the up-regulation of uncoupling proteins. These proteins appear to have the function of uncoupling the oxidative phosphorylation process. In mitochondrial ATP synthesis from ADP this process is driven by an electron flow coupled to the electron transport chain, which pumps protons from the mitochondrial matrix, and in doing so generates a proton motive force across the inner membrane of the mitochondria. This force drives the protons back into the matrix through ATP synthase, which couples proton transport across the membrane to phosphorylation of ADP (Nicholls and Rial, 1984). When ADP phosphorylation was inhibited there was still oxygen consumption present, which suggested that the coupling of respiration from ATP synthesis was not perfect. This impairment was thought to be due to the existence of leaks through the mitochondrial membrane (Rolfe and Brand, 1997). It now seems clear that these leaks are due to uncoupling proteins, the first of which was originally called thermogenin (Cannon and Nedergaard, 1985) and has now been designated uncoupling protein-1 (UCP1). This was found as a result of thermogenic studies on brown adipose tissue (BAT), in which it was observed that there was increased substrate oxidation and heat production in BAT when animals were exposed to cold. UCP1 is a 32kDa protein and is found exclusively in BAT (Ricquier and Kader, 1976).

There is a biological use for the uncoupling of respiration in that there is activation of substrate oxidation and the dissipation of energy in the form of heat.

This dissipation of energy and heat production represents a mechanism for energy balance and body weight control (Brown, 1992).

Since UCP1 was identified several other related proteins have been found. Uncoupling protein-2 (UCP2) has a 59% similarity to UCP1 (Fleury et al, 1997) and Uncoupling protein-3 (UCP3) has a homology of 72% with UCP2 (Boss et al, 1997). Other UCPs have been identified in plants, fish and other mammals, and there are at least 110 mouse, rat and human sequences from various cDNA sources (Fleury and Sanchis 1999). The proteins all have a conserved motif that is present in other mitochondrial carriers, and secondary structure analysis has provided evidence of 6 transmembrane domains linked by polar loops (Aquila et al, 1985, Laloi et al, 1997).

The mRNA of UCP2 has been identified in a number of tissues whereas UCP3 and UCP1 are much more tissue specific (Ricquier and Bouillaud, 2000). In fact since UCP1 is only expressed in BAT its role in human energy expenditure must be limited due to lack of substantial amounts of BAT in adult humans. Therefore UCP2 and 3 may play a more prominent role in energy expenditure. Certainly positive correlations have been observed in adipose tissue UCP2 mRNA levels and resting metabolic rate in obese women (Barbe et al, 1998), and a study of 79 obese subjects and 17 lean controls showed that there was a decrease in UCP mRNA in the obese subjects consistent with the decrease in energy expenditure (Oberkofler et al, 1997). Transgenic mice over-expressing UCP3 mRNA in skeletal muscle have been found to be hyperphagic, but weighed less than their wild type littermates (Clapham et al, 2000), suggesting an up-regulation of energy

expenditure. UCP3 knockout mice showed a decreased proton leak in muscle but not liver, providing some evidence that UCP3 accounts for much of the proton leak in muscle (Gong et al, 2000). The overexpression of UCP2 and UCP3 reduces the mitochondrial membrane potential in yeast and is consistent with uncoupling activity (Fleury et al, 1997, Gimeno et al, 1997), and are therefore candidate thermogenic mediators in tissues that lack UCP1.

Paradoxically, however, in muscle the expression of UCP2 and 3 are both increased during fasting when energy expenditure falls (Weigle et al, 1998) raising the possibility that they may serve other physiological roles? Since UCP2 mRNA was identified in carp and zebrafish, which are ectothermic, which means they do not raise their body temperature above ambient temperature this presents the possibility that UCP2 must perform some other function (Stuart et al, 1999).

Alternative functions may include the regulation of lipid metabolism (Boss et al, 2000). With high-fat diets and other situations that raise circulating levels of FFA, such as obesity and hyperinsulinemia (Fleury et al, 1997), there are increases in both UCP2 and UCP3 expression in white fat, skeletal muscle and liver (Matsuda et al, 1997). The up-regulation of UCP2 expression, particularly, may play a role in lipid accumulation where it appears that UCP2 expression increases in rat hepatocytes due to lipid accumulation (Cortez-Pinto et al, 1999). This was also observed in hepatic steatosis, where there is increased triglyceride accumulation and free fatty acids in hepatocytes (Mezey, 1991, Maverlis et al, 1983). The accumulation of fatty acids means there is potential for the production of metabolites that are hepatotoxic and so UCP2 and 3 may provide a mechanism to

dispose of the excessive fatty acids and protect against reactive oxygen species (ROS) that would be produced (Cortez-Pinto et al, 1999).

A possible mechanism for UCP up-regulation is the stimulation of the sympathetic nervous system via a β -adrenergic receptor, since uncoupling proteins are induced by cold exposure (Champigny and Ricquier, 1990), and by catecholamines, such as CL316, 243, that increased UCP2 in BAT (Yoshitomi et al, 1998). When Klaus et al 1994 used a HIB-1B cell line which expresses uncoupling proteins they suggested that 30-40% of the catecholamine stimulation via β 3 receptors and the rest by the classical β 1/ β 2 pathway.

Sanchis et al (1998) has shown that cachectic rats bearing the Yoshida AH-130 ascites hepatoma had increased UCP2 and 3 mRNA in skeletal muscle and that was a result of the anorexia associated with tumour burden. Also Basquets et al (2001) showed that tumour growth increased expression of UCP2 mRNA in brain tissue, suggesting that the brain may contribute to the increased energy expenditure associated with hypermetabolic states.

It is possible that the tumour-derived factor LMF initiates lipolysis and concomitantly activates UCPs and the mitochondrial uncoupling pathway, increasing energy expenditure and lipid disposal and in doing so accelerates the fat catabolic cascade. Recently it has been reported that there is increased gene expression of UCP1 in BAT, and UCP2 and UCP3 in skeletal muscle in mice bearing the MAC16 tumour, which produces LMF (Bing et al, 2000), but does not show anorexia. It was therefore necessary to examine the effects of LMF

stimulation in normal mice, to examine the role of uncoupling proteins in the metabolic changes seen in cancer cachexia.

4.2 Methods

4.2.1 *In Vivo* investigation of LMF

NMRI ex-breeder male mice (30-40g) were injected twice daily with either 8µg LMF in 100µl PBS or 100µl PBS via the tail vein, LMF was prepared as described in section 3.2.1.2, both LMF and PBS had been filter sterilised before injection, using a 0.2µM filter. At each injection the mice were weighed, and food and water were also measured. At the end of 48h the mice were anaesthetised with Halothane; blood was then removed by cardiac puncture, before being analysed. The mice were then killed immediately by cervical dislocation.

Tissues were removed for analysis of uncoupling protein mRNA and protein levels. Samples were snap frozen in liquid nitrogen before storage at -70°C. The hypothalamus was also removed for the measurement of neuropeptide Y, which was carried out by Dr Bing of Liverpool University, along with the analysis of the uncoupling protein mRNA.

4.2.2 Histology

Liver and brown adipose tissues were sectioned using a cryostat. Frozen sections were then stained with hematoxylin and eosin. Comparisons for the liver sections were made using Oil Red O staining, Dr Bing carried out this work.

4.2.3 Serum analysis

Serum concentrations of glucose and triglyceride and β-hydroxybutyrate, glycerol were measured using diagnostic kits (Sigma, Poole, UK). Non-Esterified fatty acids were measured also using a kit (Wako chemicals GmbH, Germany).

4.2.4 Expression of UCP-1, -2 and -3 mRNA

The total RNA was extracted from BAT and gastrocnemius muscle using Tri-reagent (Sigma, Poole, UK) and RNA concentration determined from the absorbance at 260nm. Twenty μg of total RNA per sample was applied to a 1% agarose-formaldehyde gel and separated by electrophoresis. RNA was transferred overnight to a charged nitrocellulose membrane by capillary blotting and then cross-linked under UV light. Northern Blotting detected UCP-1, -2 and -3 mRNAs in conjunction with the chemiluminescence method. The membranes were pre-hybridised in Easyhyb[®] solution (Boehringer Mannheim) at 42°C for 1h and hybridised in the same solution with a digoxigenin-labelled 32-mer antisense oligonucleotide probe for mouse UCP-1, or digoxigenin-labelled 30-mer oligo probes for mouse UCP-2 (5'-ACTGTTTGACAGAGTCGTAGAGGCCAATGC-3'; Genebank accession number: U69135) and UCP-3 (5'-CGTAGGTCACCATCTCAGCACAGTTGACAA-3'; Genebank accession number: AB008216) respectively. Each blot was stripped and re-probed for 18S rRNA with a 31-mer digoxigenin -labelled oligonucleotide. The mRNA signals were quantified by autoradiography and gel image analysis. The amount of mRNA was expressed as the ratio of UCP mRNA/18S rRNA signals

4.2.5 Western Blot Analysis

4.2.5.1 Sample preparation

Animals were injected with either LMF or PBS as with section 4.2.1. Tissues were extracted before being snap frozen in liquid nitrogen and then stored at -70°C. The samples were homogenised in homogenising buffer (section 2.3.8) using a Teflon glass homogeniser. The samples were then centrifuged at 4500 rpm for 15min. The supernatants were then centrifuged at 13,000 rpm for a further 15min; the pellets were then sonicated. The samples were then measured for protein using the method of Bradford (section 3.2.1.3).

4.2.5.2 Blotting samples

30µg of protein was loaded per lane and separated on a 12% SDS-polyacrylamide gel electrophoresis, as in section 3.2.2.1, under reducing conditions, and transferred onto nitrocellulose membranes (Hybond C) (Amersham Inc. Buckinghamshire, UK) by electroblotting for 2h at 80V. The transfer of protein and equality of loading in all lanes was verified using reversible staining with Ponceau S. Membranes were blocked using 5% Marvel in 0.1% Tween-20 in PBS (section 2.3.5.3) for 1h at room temperature. The membranes were washed in 0.5% Tween-20 in PBS (section 2.3.5.2), once for 15min and twice for 5min. Uncoupling protein 1,2 and 3 were identified using monoclonal antibodies at a dilution of 1:1000-fold, overnight at 4°C. UCP-1 was identified with a monoclonal antibody (Autogenbioclear, Wilts, UK), UCP-2 (Calbiochem, San Diego, CA, USA) and UCP-3 were identified using a monoclonal antibody from

Research Diagnostics, Inc, USA (Boss et al 1997). These were found to be the optimal antibodies with the least non-specific binding available. The membranes were washed as before but in 0.1% Tween-20 in PBS (section 2.3.5.1). Blots were then incubated with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:2000-fold dilution) from DAKO A/S, Glostrup, Denmark. Bands were then detected on Kodak photographic film using an emission chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK).

4.2.6 Body temperature analysis

NMRI Ex-breeder mice (30-40g) were injected with LMF or PBS as in section 4.2.1. The mouse rectal temperatures were first measured just prior to termination. Then previous to the first injection and at each injection time and until termination, rectal temperature was measured using a RS digital thermometer.

4.2.7 *In vivo* effects of LMF on NMRI mice transplanted with the MAC 13 adenocarcinoma.

NMRI ex-breeder male (30-40g) were transplanted with a fragment of mouse adenocarcinoma (MAC13) in the flank, by means of a trocar (Bibby et al 1987). Eight days after transplantation mice were injected every 12h for 72h with 8µg of LMF in 100µl PBS or 100µl PBS via the tail vein. The mice were then treated as in section 4.2.1.

4.2.8 Measurement of Glycogen in LMF treated and control mice

Mice were injected twice daily with LMF or PBS as described in section 4.2.1. The mice were killed by cervical dislocation and the livers were excised and snap frozen in liquid nitrogen. Glycogen was measured by the method of Keppler and Decker (1974) determined with Amyloglucosidase. Briefly the livers were homogenised with 70% Perchloric acid at 4°C. The homogenate was then incubated with a potassium hydrogen carbonate solution and amyloglucosidase solution for 2 hours at 40°C, which hydrolyses the glycogen to glucose. The glucose concentration was measured using hexokinase with the subsequent conversion of NADP⁺ to NADPH measured using a Beckman spectrophotometer at 340nm for the presence of NADPH.

4.3 Results

4.3.1 Body weight and tissue weights

Body weight, composition and blood metabolite levels are shown in Table 4.3. LMF injected b.d. over 48h caused a rapid weight loss, reaching 10% (P=0.03) below controls. The weight loss over the 48h is also shown in Fig. 4.1. LMF-treated mice also showed significant reductions in both gonadal fat mass (20%, P<0.01) and interscapular BAT (23%, P<0.01), whereas skeletal muscle mass was not altered. Food and water consumption did not show significant differences between the two groups (Table 4.3.1). Core temperature at the time of termination was slightly higher (+0.6±0.2°C) in mice treated with LMF, but was seen not to be statistically significant.

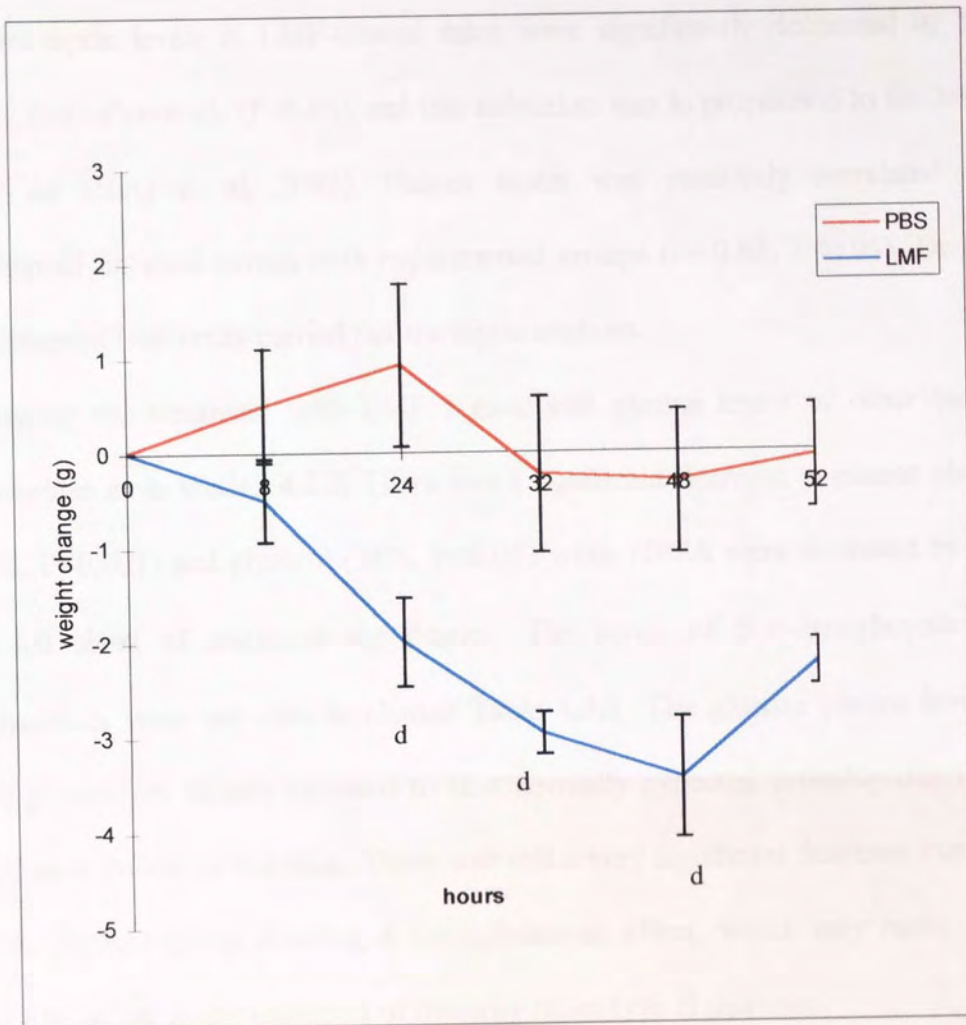


Fig 4.3.1: Body weight changes in LMF-treated and vehicle-treated control mice. Data are mean \pm SEM for six mice per group. Statistically significant differences between LMF vs. controls d, $p < 0.001$, analysed by Student's t-test.

4.3.2 Metabolite plasma concentrations in treated and untreated mice

Plasma leptin levels in LMF-treated mice were significantly decreased by 59% below that of controls ($P < 0.01$) and this reduction was in proportion to the loss of body fat (Bing et al, 2002). Plasma leptin was positively correlated with epididymal fat mass across both experimental groups ($r = 0.83$, $P < 0.01$). Dr Bing of Liverpool University carried out the leptin analysis.

Following the treatment with LMF I measured plasma levels of other various metabolites as in section 4.2.3. There was a significant decrease in plasma glucose (58%, $P < 0.001$) and glycerol (54%, $P < 0.05$) while NEFA were increased by 10% but fell short of statistical significance. The levels of β -hydroxybutyrate and triglycerides were not seen to change Table 4.3.2. The glucose plasma levels of both groups are slightly elevated to that normally expected probably due to the short term effects of halothan. There was still a very significant decrease from that of the control group showing a hypoglycaemic effect, which may mean LMF, might be of use in the treatment of maturity onset type II diabetes.

Also of interest is the ratio between that of glycerol levels and NEFA, with the control group there is a 1:3 ratio but it is slightly higher for the treated group which is suggestive of some other mechanism utilising the glycerol has occurred.

	Control	Treated	P
Food intake (g)	7.4± 0.9	7.0± 0.4	NS
Water intake (ml)	9.4± 3.6	10.8± 1.6	NS
Final body weight (g)	37.6± 1.1	33.7±1.2	0.03
Body weight change (g)	-0.06± 0.5	-2.24± 0.2	0.01
Epididymal fat mass (g)	0.76± 0.02	0.61± 0.04	0.01
Interscapular Brown fat mass (mg)	91.2± 7.7	70.0± 5.6	0.05
Gastrocnemius muscle (g)	0.37± 0.06	0.35± 0.04	NS
Glycerol (mM)	0.15± 0.03	0.09± 0.03	0.05
NEFA (mEq/l)	0.46± 0.02	0.49± 0.02	NS
Glucose (mmol/l)	11.64± 0.37	6.67±0.95	0.001
Triglycerides (mmol/l)	1.5± 0.31	1.46±0.4	NS
β-hydroxybutyrate (mmol/l)	0.72± 0.23	0.65± 0.21	NS
Leptin (ng/ml)	7.0± 0.7	2.9± 0.6	0.01
Core temperature (°C)	37.6± 0.8	38.2± 0.5	NS

Table 4.3.2: Effects of human LMF after 48h on body weight, fat mass, muscle mass and serum metabolite and leptin levels and temperature in NMRI mice. Data are mean± S.E.M for six mice in each group. Differences from control values were analysed by Student's t test.

NS, not statistically significant

4.3.3 UCP1 and UCP2 mRNA and protein expression

Uncoupling protein-1 and -2 were measured as in section 4.2.4 the mRNA expression analysis was carried out by Dr Bing of Liverpool University. LMF treatment over 48h significantly increased UCP1 mRNA levels in BAT (+96%, $P < 0.01$), and also up-regulated UCP2 mRNA in BAT (+57%, $P = 0.02$) and skeletal muscle (+146%, $P < 0.05$). UCP3 mRNA in BAT was increased by 37% ($p > 0.05$) but this was not significant. In liver UCP2 mRNA and its competitor mRNA were detected and quantified by RT-PCR, because the signal was too low to be detected by Northern blotting. There was a significant increase in liver UCP2 mRNA expression in LMF-treated mice compared with PBS controls (+142%, $P = 0.03$). The protein levels for UCP-1 and -2 were measured as in section 4.2.5. There was an apparent up-regulation of UCP-1 protein in BAT (+67%, $P = 0.02$) (Fig 4.3.3.1), while UCP-2 protein levels also in liver appeared to be up regulated (+49%, $P = 0.02$) (Fig 4.3.3.2). Polyclonal antibodies for UCP2 have in the past bound to other 32KDa proteins, but Stuart et al 2001 showed that the UCP2 antibody used in this study detected UCP2 levels in mammalian mitochondria and it was also verified in wildtype and UCP2 knockout mice (Pecqueur et al, 2001).

An attempt at measuring UCP-3 protein in mouse muscle was unsuccessful, the polyclonal antibody used produced so much non-specific binding it was impossible to determine bands at 32KDa. UCP1 protein was measured in white adipose tissue, to examine if there was appearance of brown adipocytes in the tissue but no bands were detected Fig (4.3.3.3).

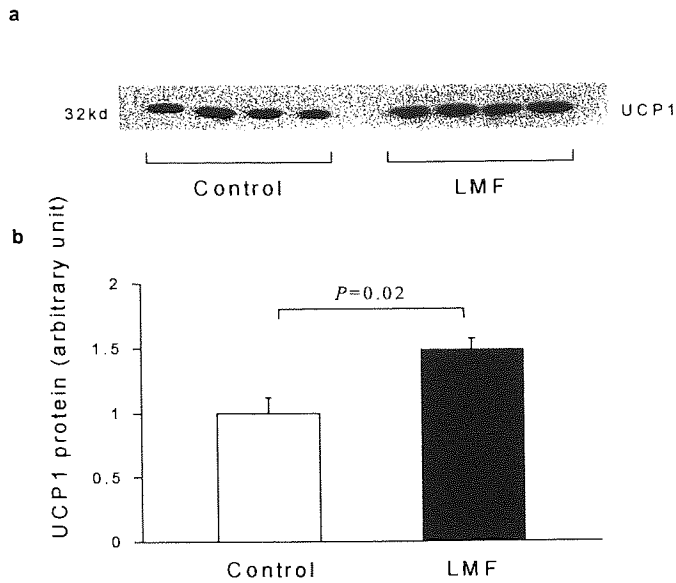


Fig 4.3.3.1 Western blot of UCP1 on Brown adipose tissue.

(a) Western blot showing UCP1 concentrations in mitochondrial preparations of brown adipose tissue from vehicle-treated and LMF-treated mice (b) UCP1 protein content in control and LMF-treated mice (mean \pm SEM of the signals shown in a).

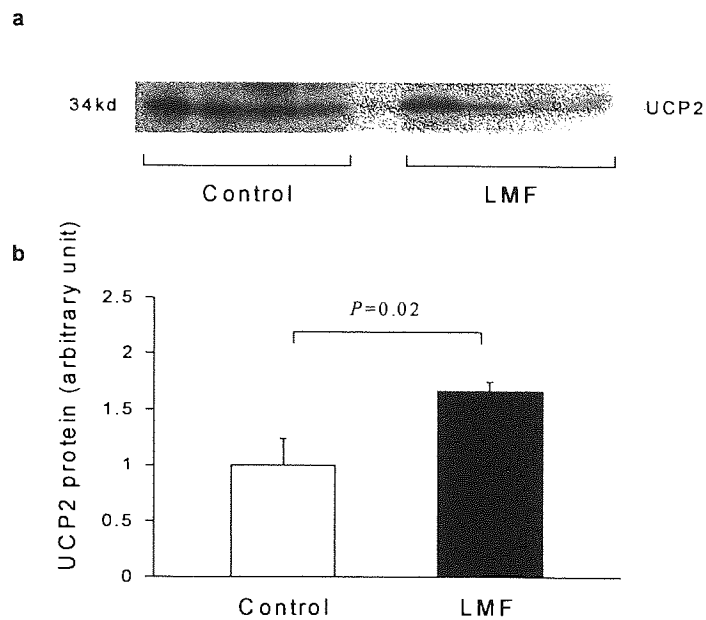


Fig 4.3.3.2. Western blot of UCP2 on Liver tissue

(a) Western blot showing UCP2 content in mitochondrial preparations of liver from vehicle-treated and LMF-treated mice. (b) UCP1 protein content in control and LMF-treated mice (mean \pm SEM of the signals shown in a).

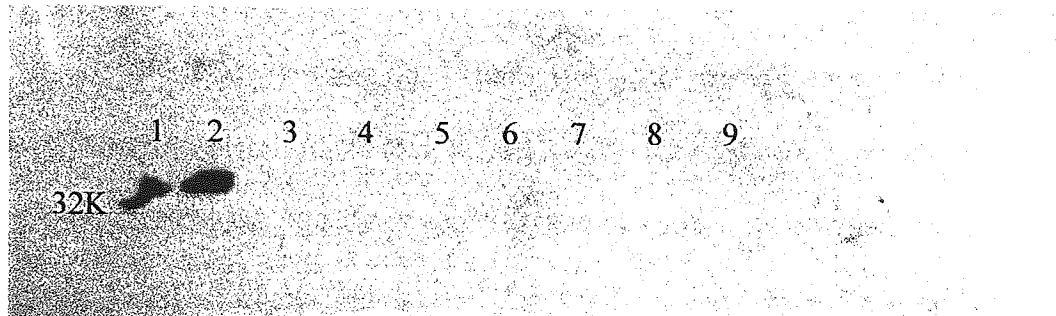


Fig 4.3.3.3 Western blot of UCP1 on white adipose tissue

Lane 1-2: Brown adipose tissue control

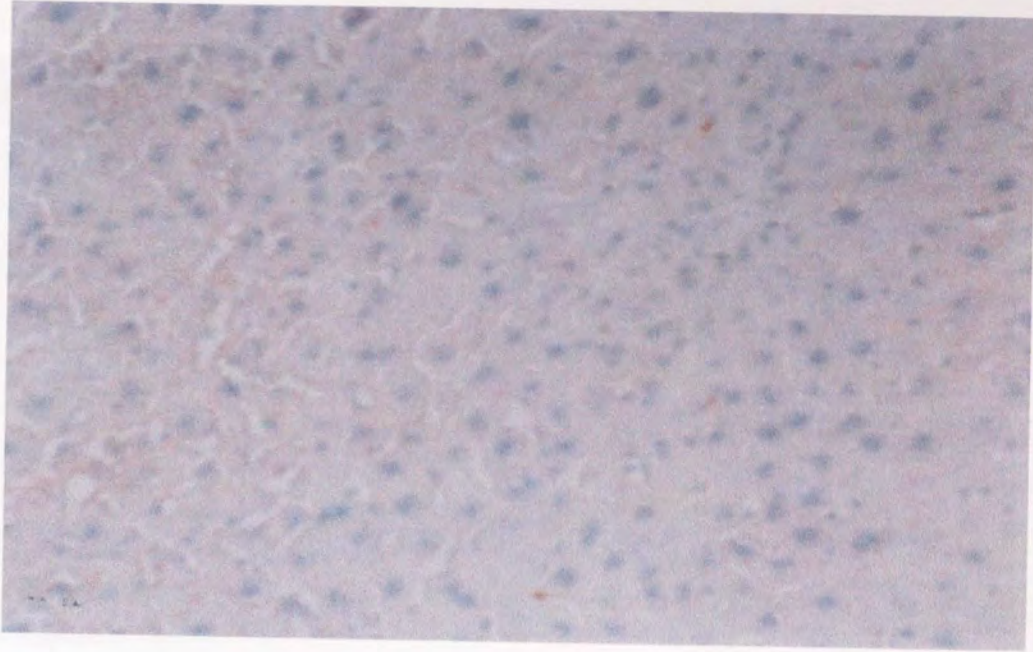
Lanes 3-5: White adipose tissue in PBS control mice

Lanes 6-9: White adipose tissue in LMF mice

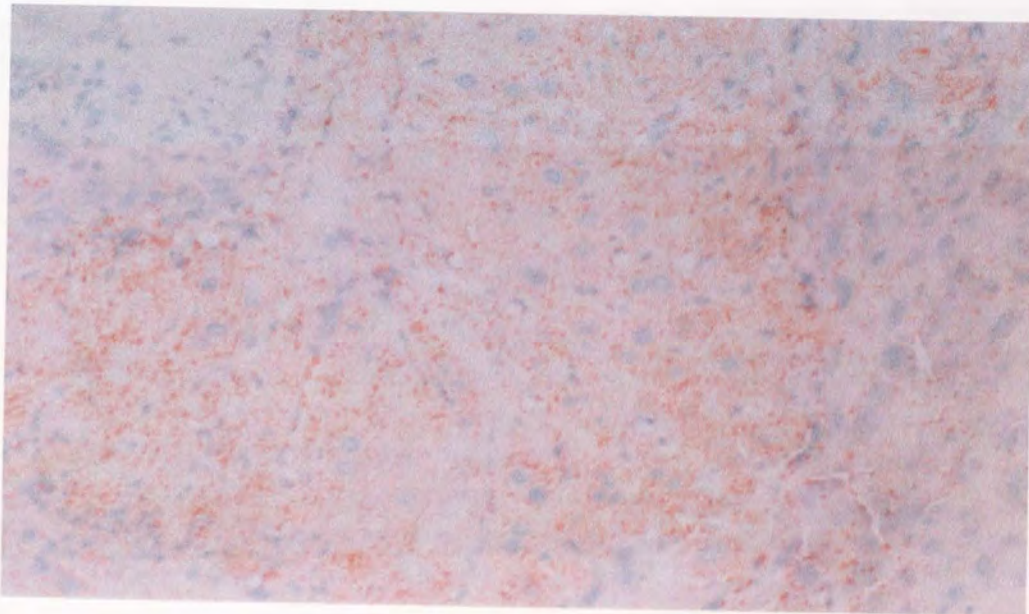
4.3.4 Histological examination of brown adipose tissue and liver samples

Haematoxylin and eosin staining of liver sections from LMF-treated mice carried out by Dr Bing of Liverpool University showed no obvious signs of inflammation or other abnormality, while Oil Red O staining showed large amounts of cytoplasmic micro-droplets of lipid deposited in hepatocytes in all zones of the hepatic lobule. By contrast, the hepatocytes of the control mice containing only a few, small droplets of lipid (Fig 4.3.4.1). This may indicate a condition similar to fatty liver disease, which is seen in non-alcoholic obesity and alcoholic lean humans and in ob/ob and ethanol fed mice. In fatty liver there is also an up regulation of UCP2, which may be a protective mechanism (Rachid et al, 1999) against the reactive oxygen species produced as metabolites of free fatty acids.

The brown adipose tissue samples do show some changes occurring in the LMF-treated mice. There appears to be more vascularisation in the LMF-treated mice as compared to the PBS controls (Fig 4.3.4.2). This may be due to increased need to dissipate heat due to increased energy expenditure.

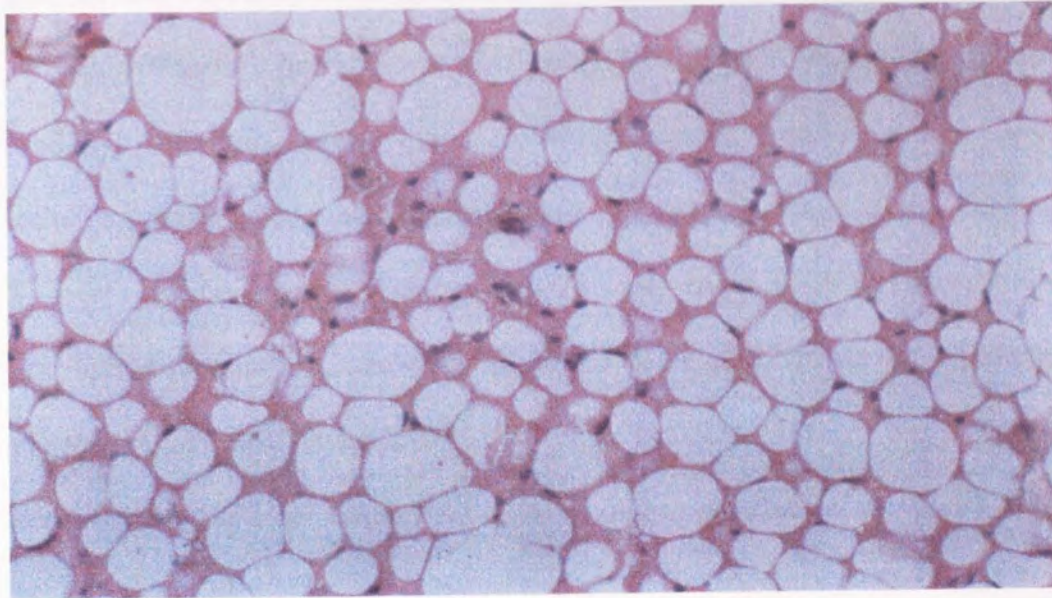


A

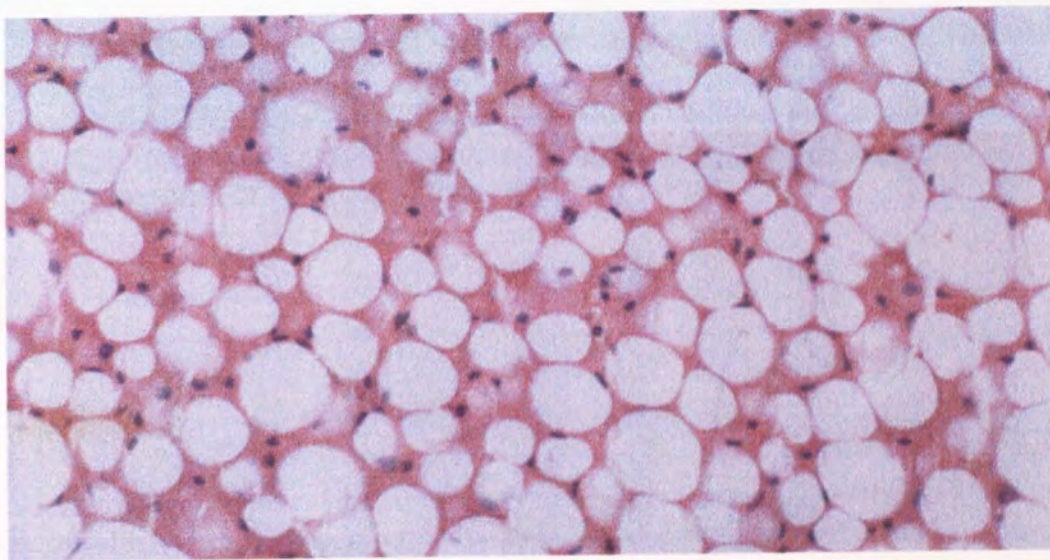


B

Fig 4.3.4.1 Histology of mouse liver. Fresh frozen tissue from five mice per group were sectioned on a cryostat and stained with Oil Red O. There was marked intracytoplasmic lipid accumulation in the liver from LMF-treated mice (B) but little in the vehicle-treated mice (A). Hepatocytes from all zones of the hepatic lobule were affected equally.



A



B

Fig 4.3.4.2 Histology of brown adipose tissue, fresh frozen tissue from five mice per group were sectioned on a cryostat and stained with Haematoxylin and eosin. There was increased vascularisation in the BAT from LMF-treated mice (**B**) but little in the vehicle-treated mice (**A**).

4.3.5 *In vivo* effects of LMF on mice with a MAC13 tumour burden

As with non-tumour bearing mice LMF produced a weight loss, without effects on food and water consumption over 72h, with significant decreases in body weight including epididymal fat and brown adipose tissue mass. Glucose also decreased as before, as did glycerol (Table 4.3.5). The messenger RNA was measured for uncoupling protein-1, -2 and -3 as in section 4.2.4. LMF treatment significantly increased UCP1 mRNA levels in BAT (+56%, $P < 0.02$) and there was a slight up-regulated of UCP2 mRNA in BAT (+20%) and in skeletal muscle UCP3 mRNA was up regulated (+116%, $P < 0.05$).

Tumour burden was measured throughout, but there did not appear to be any differences between the treated and non-treated groups (Table 4.3.5). This would suggest that LMF does not affect tumour burden and may play some other role in the development of the tumour.

	MAC13- Control	MAC13-Treated	P
Food intake (g)	11± 1.1	10.2± 0.5	NS
Water intake (ml)	16± 0.3	16.3± 0.5	NS
Final body weight (g)	36.2± 1.1	32.9±0.9	0.02
Body weight change (g)	-1.4± 0.2	-2.5± 0.4	0.01
Epididymal fat mass (g)	0.63± 0.04	0.52±0.06	0.01
Interscapular Brown fat (mg)	90.3± 6.5	72.0±3.6	0.05
Gastrocnemius muscle (g)	0.4± 0.04	0.32±0.05	NS
Tumour mass (g)	1.09±0.96	1.1±0.5	NS
Glycerol (mmol/l)	0.29± 0.05	0.15±0.03	0.05
NEFA (mEq/l)	0.52± 0.06	0.45±0.04	NS
Glucose (mmol/l)	9.5± 0.25	7.5±0.09	0.001
Triglycerides (mmol/l)	1.0± 0.2	0.8±0.03	NS
β-hydroxybutyrate (mmol/l)	0.62± 0.24	0.63±0.29	NS

Table 4.3.5 Effect of human LMF after 48h on MAC13 tumour-bearing mice, on body weight, fat mass, muscle mass and serum metabolites in NMRI mice. Data are mean± S.E.M for six mice each group. Differences from control values were analysed by Student's t test.

NS, not statistically significant

4.3.6 Glycogen levels in the livers of treated and untreated mice

With the decrease in the plasma levels of glucose an examination of liver glycogen levels was carried out. For this the mice were injected with LMF or PBS as before (section 4.2.1), except that the mice were not anaesthetised with halothane, since it can affect glucose levels. Glycogen was measured as in section 4.2.8. The LMF-treated group showed no statistical difference from that of the controls (Fig 4.3.6.). With the decrease in blood glucose levels glycogen would be expected to decrease so that the body could compensate which did not occur and may mean that other mechanisms are involved.

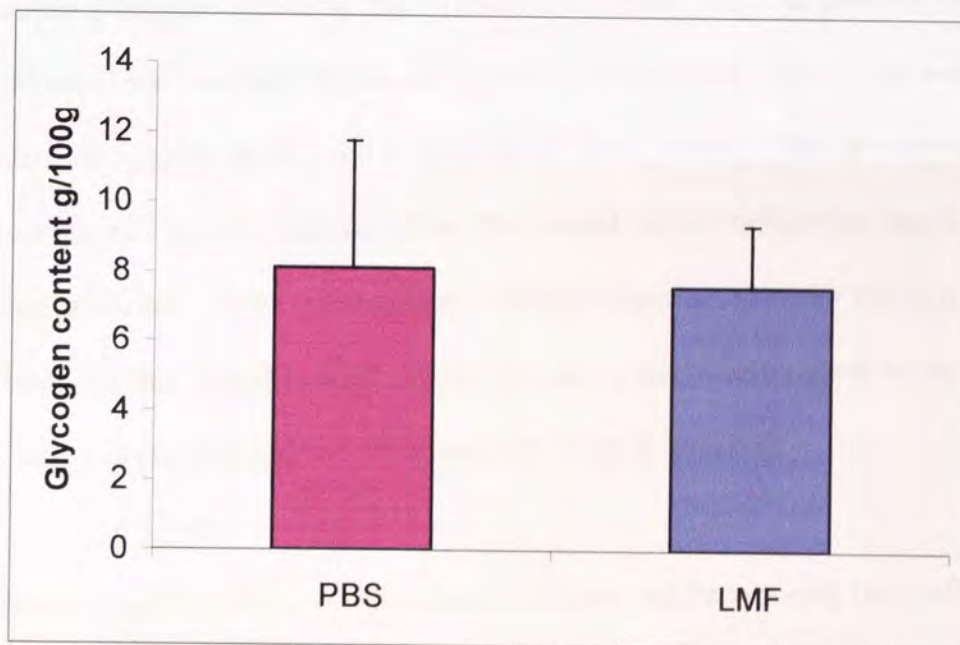


Fig 4.3.6. Glycogen levels in Livers of LMF and PBS treated mice. Data presented as mean \pm SEM for six mice in each group.

4.4 Discussion

The administration of LMF to normal mice caused a rapid reduction in weight, primarily due to loss of body fat, since muscle mass was unaffected. There did appear to be almost a redistribution of fat in the LMF-treated mice as there was accumulation of fat in the liver (section 4.3.4). LMF therefore induces massive adipose catabolism, and must also act to prevent the resynthesis of triglycerides in the tissue.

There is evidence that lipid utilization is increased following treatment, such as elevated plasma β -hydroxybutyrate (indicating enhanced ketogenesis) and increased oxygen uptake by BAT (Hirai et al, 1998), but it is possible that other pathways are involved, which are as yet undetermined. There was a dramatic effect on glucose levels with a decrease of 58%, $P < 0.01$. This is substantial and may suggest an anti-diabetic effect that would be of therapeutic use. LMF has been previously shown to stimulate 2-deoxyglucose uptake into C₂C₁₂ myoblasts (Islam-Ali and Tisdale, 2000). There was also a decrease in leptin levels of 59%, which was proportional to loss of body fat (Bing et al, 2002).

There appeared to be no difference in tumour weight between the LMF treated and untreated group bearing the MAC13 tumour. There appears to be no increase in tumour burden, but there were similar decreases in glucose or glycerol plasma levels and body weight to that seen in non-tumour burdened mice. It would seem that since the effects of LMF do not include increasing tumour mass and thus LMF might provide some other function.

With an increase in mRNA and protein expression in BAT, and previous data showing LMF causes enhanced oxygen consumption in BAT (Harai et al, 1998), this suggests that there is increased BAT thermogenesis. The increase in UCP1 gene expression has also been observed in mice bearing the MAC16-adenocarcinoma, which produces LMF (Bing et al, 2000). There is also up-regulation of uncoupling proteins observed in cachectic rats (Sanchis et al, 1998). The elevation in BAT thermogenesis has also been observed in several rat models of malignancy, such as T-cell leukaemia and the Yoshida sarcoma although this may be due to sympathetic stimulation rather than a lipolytic factor (Roe et al, 1996, Oudart et al, 1995).

The uncoupling protein mechanism appears to be controlled by sympathetic afferents via β_3 -adrenoceptors located on the brown adipocytes (Ricquier and Bouillaud, 2000). It is therefore possible that LMF-induced lipolysis, mediated by β_3 -receptors, up regulates expression of BAT UCP1 (Russell et al, 2000), which in turn increases heat production while utilizing additional oxidative substrates.

There was an increase of UCP2 gene expression in BAT, muscle and liver as well as up regulation of UCP2 protein in the liver. UCP2 and UCP3 have been implicated in the regulation of lipids as a fuel substrate in skeletal muscle and adipose tissue in rodents and humans. Fasting triggers the mobilisation of stored fat to provide a major source of fuel to meet ongoing energy requirements, particular that of skeletal muscle (Boss et al, 1997). Enhanced lipolysis leads to significant increases in circulating FFA levels within 24h of fasting in rats, and this is accompanied by marked increases in UCP3 in muscle (Wiegler et al, 1998). There also appears to be a role for UCP2 in enhanced lipid utilisation induced by

LMF. Certainly LMF caused massive lipid deposition in the liver that was associated with increases in UCP2 mRNA and protein levels. Hepatic UCP2 has recently been implicated in the regulation of lipid utilization, in that exposure to intralipid emulsions leads to increases in UCP2 mRNA and UCP2 protein levels in cultured hepatocytes (Cortez-Pinto et al, 1999). It could be possible that UCP2 provides a mechanism that allows tissues to adapt to increased lipid supply. UCP induction may serve a function in tissues forced to oxidise excess FFA. Lipid oxidation is known to increase intracellular reactive oxygen species (ROS) that can cause cell death, while the uncoupling of respiration can be a powerful mechanism limiting ROS formation. It has been observed that by inhibiting BAT UCP1 there is a stimulation of H₂O₂ production, while both the ROS-inducing agent tetra-butyl hydroperoxide (TBHP) and tumour necrosis factor alpha (TNF- α), which increases mitochondrial oxidant production, can induce hepatocyte UCP2 mRNA; this suggests that UCP1 and -2 may provide a protective antioxidant mechanism (Negre-Salvayre et al, 1997, Arsenijevic et al, 2000, Cortez-Pinto et al, 1999).

The fatty livers from ob/ob mice or ethanol-fed lean mice show the up-regulation of UCP2, while hepatocyte death is not increased (Rashid et al, 1999). Induction of UCP1 in BAT and UCP2 in BAT, muscle and liver, when there is an excessive lipid accumulation caused by LMF could also limit ROS production in these tissues, thereby preventing cell death. Rashid et al (1999) showed that in mice with fatty liver the up-regulation of UCP2 expression was accompanied by up regulation of anti-apoptotic proteins such as Bcl-2, this might indicate a protective

mechanism. Certainly there appears not to be any evidence of apoptosis in the fatty liver from LMF-treated mice (Bing et al, 2002). The correlation between hepatic steatosis and UCP2 expression raises the possibility that lipid or lipid metabolism may regulate UCP2 expression (Rashid, 1999).

It would appear that uncoupling proteins are involved in the enhanced lipolysis in cancer cachexia. Induction of UCP1, -2 and -3 expression by LMF probably provides a mechanism for excessive fat disposal, which in turn facilitates the fat catabolic cascade in malignancy. UCP induction may protect tissues such as the liver against the oxidative damage that would result from enhanced FFA oxidation.

4.5 Conclusion

Treatment of mice with LMF caused a substantial weight loss and redistribution of body fat. There was also up-regulation of UCP-1 and -2 mRNA and protein in BAT and UCP-2 in liver suggesting that the mice are adapting to an increase in energy mobilization. The increase in UCP-2 in liver may also produce a protective mechanism against the build up of lipid. Structural changes were also observed in sections of liver and BAT.

Chapter 5. The role of β -3 adrenergic receptors in the action of LMF

5.1 Introduction

5.1.1 β -adrenergic receptors

With increased resting energy expenditure often reported in cancer patients (Fredix et al, 1990, Falconer et al, 1994) and increased fat oxidation also observed (Hyltander et al, 1993), there appears to be evidence that a classical catecholamine pathway is involved. This was supported by the administration of the non-specific β -blocker propranolol to cancer patients, which produced a decrease in the basal metabolic rate (BMR) (Gambaroldella et al, 1999).

There are several subtypes of beta-adrenergic receptors (AR). Classically it is the β 1 and β 2-AR subtypes that mediate the response of noradrenaline release from the sympathetic nervous system. There is now evidence of a third β -AR subtype (Howe, 1993), which shares only 40-50% amino acid sequence identity with β 1 and β 2, and is referred to as a β 3-AR. These receptors mediate lipolysis in white adipose tissue in mice and rats (Arch and Wilson 1996), and thermogenesis in BAT (Arch et al, 1989). The β 3-adrenoceptor belongs to the R7G superfamily of G protein coupled receptors, which in turn stimulates cyclic AMP (cAMP) via adenylate cyclase (Kauman et al, 1997, Tota et al, 1999). Kauman et al 1997 also suggests evidence that a β -4 adrenoceptor also exists based on pharmacological evidence but this has yet to be sequenced.

β 3-adrenoceptors were previously shown stimulated by β -agonists causing a decrease in body weight in diet-induced obesity. Since the receptors are primarily found in brown and white adipose tissue, it was therefore suggested as a target for obesity (Galitzky, 1993, Arch et al, 1984). The β 3-AR's also appear to activate

uncoupling proteins and therefore act directly on energy expenditure (Toriello et al, 1998). Toriello showed that the β 3-AR specific antagonist SR59230A blocked the effects of the agonist SR8611A in brown adipocytes and also inhibited UCP1 up-regulation. The identification and function of the β -3 AR was restricted until the identification of a specific β -3 AR antagonist SR59230A by Manara et al (1996) and which was further characterised by Nisoli et al (1996) who showed that SR59230A specifically inhibited the action of β -3 AR agonists in isolated rat brown adipocytes.

Treatment of ob/ob mice with LMF, not only produced a specific depletion of the adipose mass together with an elevation of serum glycerol levels, but also an increased oxygen uptake by interscapular brown adipose tissue (BAT) (Hirai et al, 1998). Pharmacological studies indicate that the β -receptor responsible for the stimulation of oxygen consumption in BAT is mostly of the β 3-subtype (Howe, 1993). Induction of lipolysis in epididymal adipocytes by LMF was attenuated by the β -adrenergic receptor blocker propranolol (Khan and Tisdale, 1999). The biphasic effect of GTP on cyclic AMP production by LMF in adipocyte plasma membranes suggested a receptor associated with both Gs and Gi. Since GTP stimulated at specific concentrations and had no effect or inhibited at other concentrations. This corresponds to the findings of Muramayma and Ui (1983) who showed that low concentrations of GTP activated adenylate cyclase through Gs and at higher concentrations inhibited through activation of Gi. Only β 3 and not β 1-AR interact with Gi in adipocyte membranes (Granneman, 1995), suggesting that it is the β 3-AR subtype that is involved.

In the present study the ability of LMF to interact with the β 3-AR has been studied both in white adipocytes and in CHO-K1 cells transfected with the human β 3-AR.

5.2 Methods

5.2.1 The Attenuation of LMF action on Lipolysis

LMF was incubated with isolated murine white adipocytes in a volume of 1 ml as described in section 3.2.1.4. Prior to the addition of 20 μ g of LMF, varying concentrations of the specific β -3 adrenergic antagonist SR59230A were incubated with the isolated adipocytes for 30min. SR59230A was kindly supplied by Dr Manara, of the Research Centre Sanofi Midy, Milan, Italy.

5.2.2 CHO-K1 Cell line

CHO-K1 cells transfected with the human β 3-AR, under the control of Hygromycin, together with the b-gal reporter construct selected for by resistance to G418, were a gift from Dr Ian Waddell, Astra Zeneca, Macclesfield, Cheshire, UK. They were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 2mM glutamine, 50mgml⁻¹ Hygromycin B and 200mgml⁻¹ G418, under an atmosphere of 10% CO₂ in air.

5.2.3 Cyclic AMP determination

CHO-K1 Cells were seeded in 24 well multi-dishes at a concentration of 5x10⁴ cells/ml. Cells were then left for 48h before varying concentrations of LMF or

isoprenaline were added with or without the non-specific β -adrenergic receptor antagonist propranolol, or the specific β 3-adrenergic antagonist SR59230A was added to the wells and incubated for 30min, after which the medium was removed and 0.5ml assay buffer was added to each well. The plates were placed in a boiling water bath for 5min to stop the reaction and lyse the cells. The dishes were then cooled on ice for 10min. To 50 μ l of the cell extract was added 925Bq of [8- 3 H] cyclic AMP (Amersham Pharmacia Biotech, UK) and 20 μ g of binding protein and incubated for 2h at 4°C. 100 μ l charcoal suspension was added to the samples before being vortexed and left to stand for a few minutes. Tubes were then centrifuged at 10,000rpm for 5min at 4°C. 185 μ l of supernatant was then added to 10ml Optiphase Hi-safe II before analysis using a Packard TRI-CARB 2000CA scintillation analyser. The concentration of cyclic AMP in the samples was determined by comparison with known concentrations of cyclic AMP, using a standard curve.

5.2.4 Iodination of LMF with [125 I]

One Iodo-bead (Pierce and Warriner, Chester, UK), washed and dried, was incubated with Na [125 I](1mCi per 100 μ g protein sp. Act. > 15Ci mg $^{-1}$ iodide; Amersham Pharmacia Biotech, UK) for 5min in 100 μ l PBS. LMF (100 μ g protein) was then added for 15min. The iodo-bead was physically removed and unbound Na [125 I] was removed using a Sephadex G25 column eluted with 0.1M NaI. The [125 I] LMF was then concentrated using a Microcon microconcentrator with a 10kDa cut-off against PBS.

5.2.5 Binding Studies

CHO-K1 cells transfected with the human β 3-AR (section 5.2.2) were lysed by sonication in 0.5M MgCl₂, 2mM Tris.HCl, pH 7.5 and crude membranes were pelleted by centrifugation (45,000g, 15min, 4°C). Binding studies were conducted in 400 μ l 0.5mM MgCl₂ 50mM Tris.HCl, pH 7.5 by incubation of membranes (50 μ g protein) with various concentrations of [¹²⁵I] LMF for 60min at 37°C. [¹²⁵I] LMF was also tested against 10 μ M SR59230A, 10 μ M propranolol and 100 μ M of cold LMF. All inhibitors were added 30min before the addition of [¹²⁵I] LMF.

The samples were then centrifuged at 13000 rpm for 20min, the supernatant was removed and the radioactivity of the pellet was determined using a Packard Cobra Model 5005 Auto-gamma counter. Binding was analysed using non-linear regression analysis (GraphPad Prism, Version 3.00 for windows, GraphPad Software, San Diego, CA, USA).

5.3 Results

LMF induced a direct lipolytic response in murine white adipocytes, (section 3.2.1.4) and this effect was attenuated by low concentrations (10^{-5} - 10^{-7} M) of SR59230A (Fig. 5.3.1), which has been reported to have 10-fold selectivity for the β 3-AR over the β 1-AR (Nisoli et al, 1996). Induction of lipolysis by LMF was associated with a stimulation of adenylate cyclase in isolated adipocyte membranes in the presence of 0.1 μ M GTP, and this action was almost completely inhibited by SR59230A at concentrations as low as 10^{-9} M (Russell et al, 2002). The difference in sensitivity of intact adipocytes and plasma membranes may be related to access of SR59230A to the β 3-AR. These results suggest that LMF stimulates lipolysis through interaction with a β 3-AR.

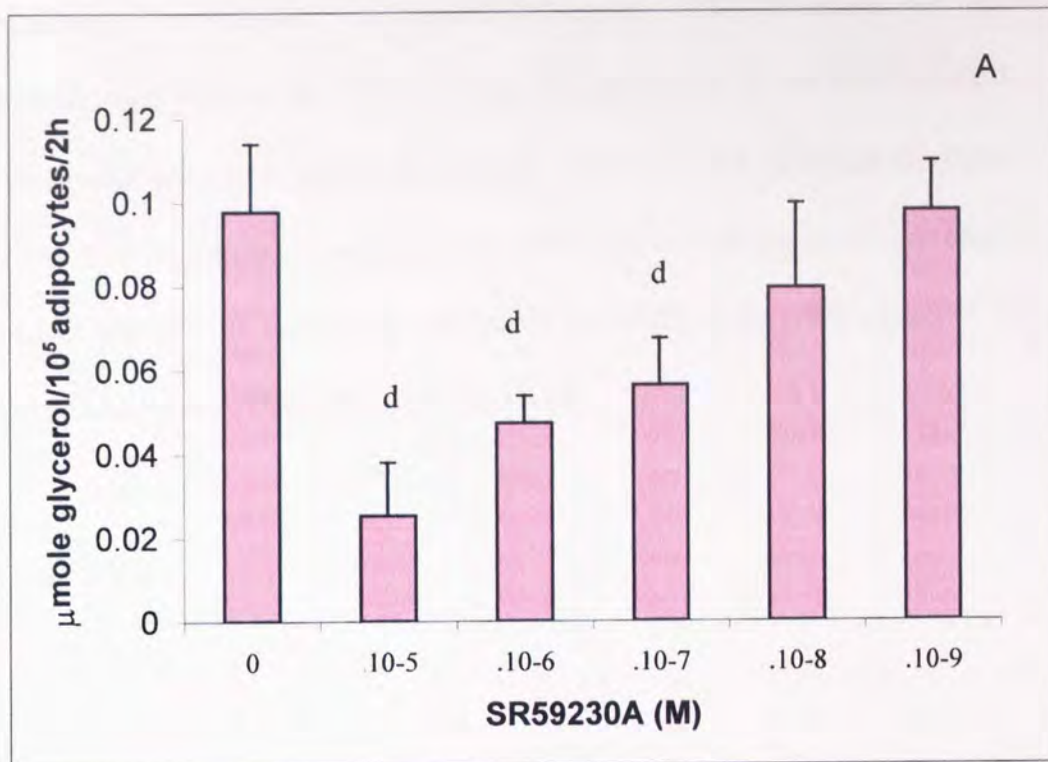


Figure 5.3.1. The effect of the β 3-AR antagonist SR59230A on lipolysis in murine white adipocytes, induced by LMF. Adipocytes were preincubated with the indicated concentration of SR59230A for 30min prior to the addition of LMF (465nM). Data are mean \pm SEM for n=6. Statistically significance d, $p < 0.001$ was determined by Student's t-test.

To investigate this possibility, the effect of LMF on cyclic AMP production was determined in CHO-K1 cells, which had been transfected with the human β 3-AR. The data presented in **Fig. 5.3.2** shows that both isoprenaline and LMF stimulated cyclic AMP production, which reached a comparable maximum level of 25 pmoles per 10^6 cells with both agents. However maximal cyclic AMP production was achieved with much lower concentrations of isoprenaline (1nM) than LMF (250nM), suggesting that LMF had a lower affinity for the β 3-AR than

isoprenaline. The increase in intracellular cyclic AMP produced by both isoprenaline and LMF in the CHO-K1 cells was attenuated by the non-specific β -AR antagonist propranolol (10 μ M), while the effect on LMF, although significant, was less than complete. However, cyclic AMP production by both isoprenaline and LMF was almost completely attenuated by SR59230A, confirming that the action of LMF was mediated through a β 3-AR

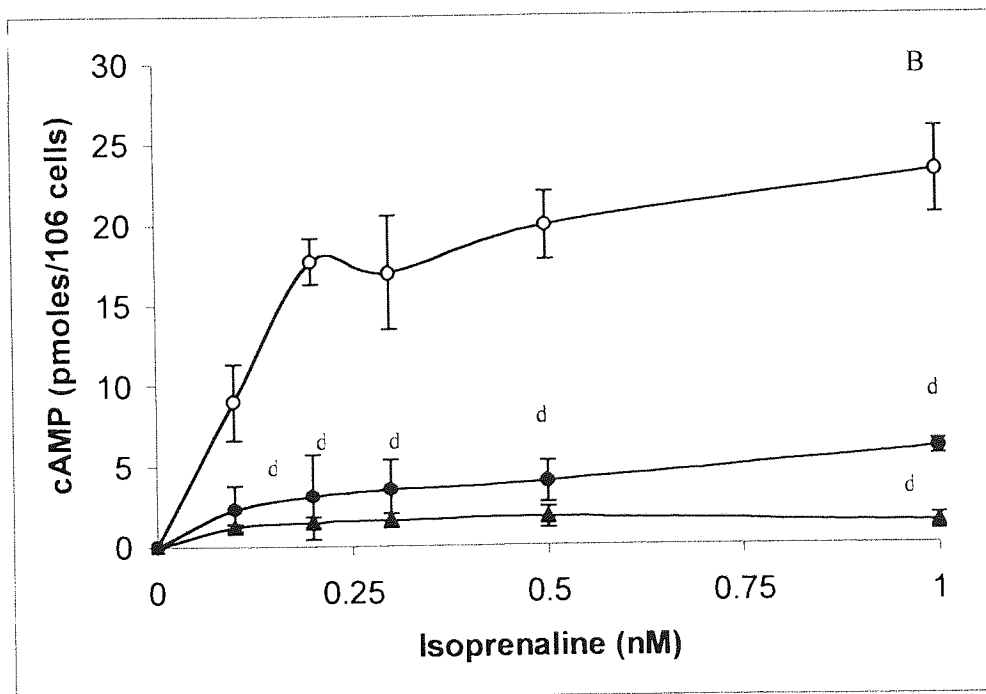
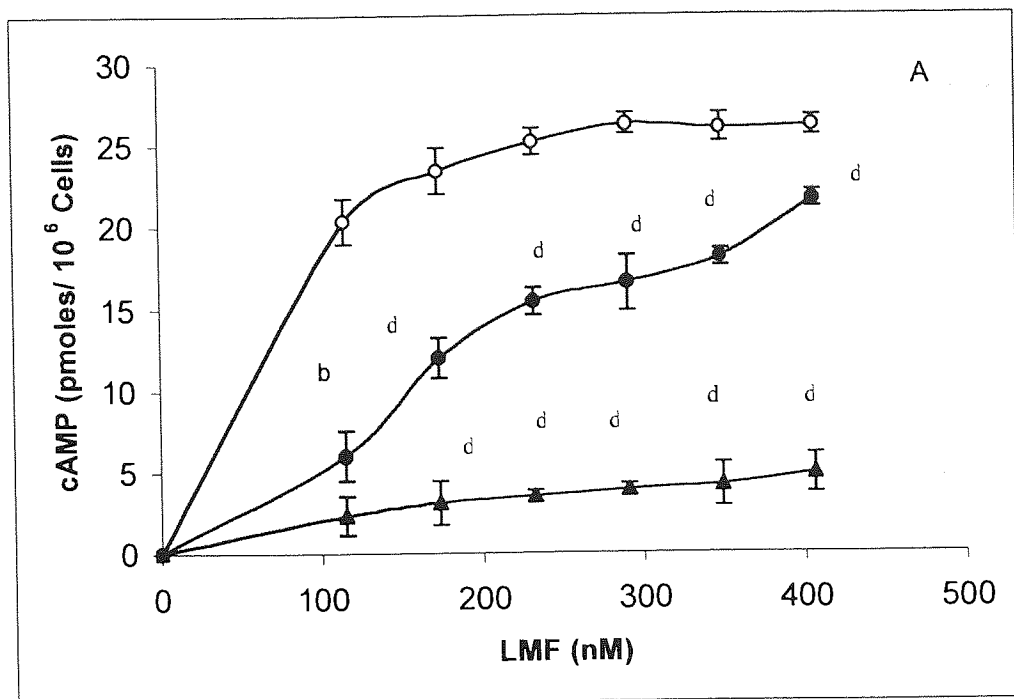


Figure 5.3.2 Effect of LMF (A) and isoprenaline (B) on cyclic AMP levels in CHO-K1β3 cells in the absence (○) or presence of 10μM propranolol (●) or 10μM SR59230A (▲) Differences from controls are indicated as b, $p < 0.01$ and d, $p < 0.001$ as determined by ANOVA.

To determine the affinity of binding of LMF to the β 3-AR, LMF was radioiodinated with ^{125}I and binding to crude plasma membranes from CHOK1 β 3 cells was determined using non-linear regression analysis of binding showed a high affinity binding site for LMF with a K_d value about one hundred fold lower than that of CGP 12177, a partial agonist of β 3-AR (Kubo et al, 1997) and [^{125}I] iodocyanopindolol (Hutchison et al, 2000), commonly used in binding studies with β 3-AR. However, the B_{max} value for LMF was similar to that for other β 3-AR agonists. Binding of [^{125}I] LMF was significantly reduced in the presence of non-labelled LMF, the non-specific β -AR antagonist propranolol and the selective β 3-AR antagonist SR59230A (Table 5.3.1). These results confirm that LMF binds to a β 3-AR.

Agent	Kd (nM)± S.E.M	Bmax (fmolmgprotein ⁻¹) + S.E.M	Reference
CGP 12177	0.42±0.06	312±22	Kubo et al, 1997
[¹²⁵ I]-CYP	0.78±0.28	63±10	Hutchinson et al, 2000
[¹²⁵ I]-LMF	78±4.5	282±1	This Study
[¹²⁵ I]-LMF+ propranolol (10µM)	123±6.1	3.9±0.2	This Study
[¹²⁵ I]-LMF+ SR59230A (10µM)	182±3.98	2.7±0.75	This Study
[¹²⁵ I]-LMF+ Cold LMF (100µM)	109±13	1.7±0.6	This Study

Table 5.3.1 Kd and Bmax values for LMF and other agonists to β 3-AR. Binding was analysed using non-linear regression analysis (GraphPad Prism , Version 3.00 for Windows) data presented as mean \pm SEM for n=12.

5.4 DISCUSSION

LMF has been previously shown to stimulate lipolysis like a classical lipolytic hormone through increases in intracellular cyclic AMP, as a result of the stimulation of adenylate cyclase (Hirai et al, 1998). Islam-Ali and Tisdale (2001) showed that in cachexia there is modulated G-protein expression with a reduction in $G_{\alpha i}$ and an increase in $G_{\alpha s}$, changes that favour mobilization of lipid stores. Similar changes were involved with LMF on 3T3L1 adipocytes *in vitro*. LMF was also shown to stimulate hormone sensitive lipase (HSL). Islam-Ali et al (2001) also showed that there was increased protein synthesis in gastrocnemius muscle. This increase in muscle was also seen in rats treated for 1-2 weeks with the β -agonist clenbuterol where a 10-20% increase in muscle mass was observed (Emery et al, 1984).

In this study LMF appears to exert its effect through a β 3-AR, although the affinity for this receptor appears to be less than seen with classical β 3-AR agonists. However, evidence that β 3-AR can mediate lipolysis in human adipocytes is controversial, since β 3-AR mRNA is expressed at a much lower level than in rat or mouse (Langin et al, 1991), although lipolysis has been induced in human omental fat cells by the selective β 3-AR agonist CGP 12177 (Hoffstedt et al, 1995), and LMF (Hirai et al, 1998).

In white adipocytes both the induction of lipolysis and the stimulation of adenylate cyclase were attenuated by the β 3-AR antagonist SR59230A (Nisoli et al, 1996), while in CHO-K1 cells transfected with the human β 3-AR LMF stimulated cyclic AMP production in a similar manner to isoprenaline, although the concentration required to produce maximal stimulation was 250-fold greater.

In addition SR59230A attenuated the increase in cyclic AMP confirming the effect was mediated through a β 3-AR. The effect of propranolol was less complete than with isoprenaline suggesting that the mechanism of stimulation by LMF may be different. Intact cells have been used here, since the coupling efficiency of β 3-AR to adenylate cyclase is highly dependent upon the integrity of the cells (Granneman, 1995). However, it is known that the coupling efficiency of β 3-AR is greater than that for β 1-AR, thus offsetting the low binding affinity. Also unlike β 1 and β 2-AR the β 3-AR has fewer potential phosphorylation sites and is resistant to agonist-induced desensitization (Grannemann, 1995). The β 3-AR mediated coupling of LMF to lipolysis would explain the lowered maximal response of human omental adipocytes to lipolysis when compared with murine white adipocytes (Hirai et al, 1998).

However, the increased coupling efficiency together with the induction of UCP1 in brown adipose tissue (BAT) (Russell et al, 2000, Chen et al, *in press*) would ensure maximum fat mobilization and utilization together with a net increase in energy expenditure. There also appears to be a role for β 3 receptors in obesity, since populations with the polymorphism W64R in β 3-adrenoceptor have accelerated development of obesity and it may contribute to decreased BMR (Evans et al, 2000). These results suggest that selective β 3-AR antagonists may be useful in controlling energy expenditure and fat mobilisation in cancer cachexia and may also be of some use in the treatment of obesity.

5.5 Conclusion

This study provides further evidence that LMF action is mediated via the β 3-AR, and hence this receptor may provide a therapeutic target for the treatment of cachexia and obesity.

Chapter 6. The effects on LMF on Glucose and Fatty acid uptake

6.1 Introduction

6.1.1 Glucose utilisation

There are alterations to host carbohydrate metabolism that occur in animals and patients with cancer. This is probably due to the demand from the tumour for glucose as its primary energy source (Holm et al, 1995), since solid tumours are anaerobic with poor vascularisation. Warburg in 1930 observed that slices of tumour appeared to utilise glucose with production of lactate. This production of lactate would cause an up regulation of the Cori cycle (**Fig 1.2.1**). The Cori cycle is a heavy consumer of ATP and increases the energy utilisation and so it may contribute to the weight loss experienced (Tisdale, 1997) (**Fig 6.1.1**). Decreased blood glucose levels have also been shown in tumour bearing animals (Shapot, 1972), suggesting increased glucose demand on the host by the tumour.

Previously it has been shown that serum glucose levels and utilisation have been affected *in vivo* by PIF (Todorov et al, 1996, Hussey and Tisdale, 1999). LMF has also been shown to cause depressed serum glucose levels (section 4.3.2) and has been shown to enhance glucose uptake in C₂C₁₂ myoblasts (Islam-Ali and Tisdale, 2001). Using the 2-deoxyglucose-tracer technique the effect of LMF was examined on utilisation of glucose *in vivo*.

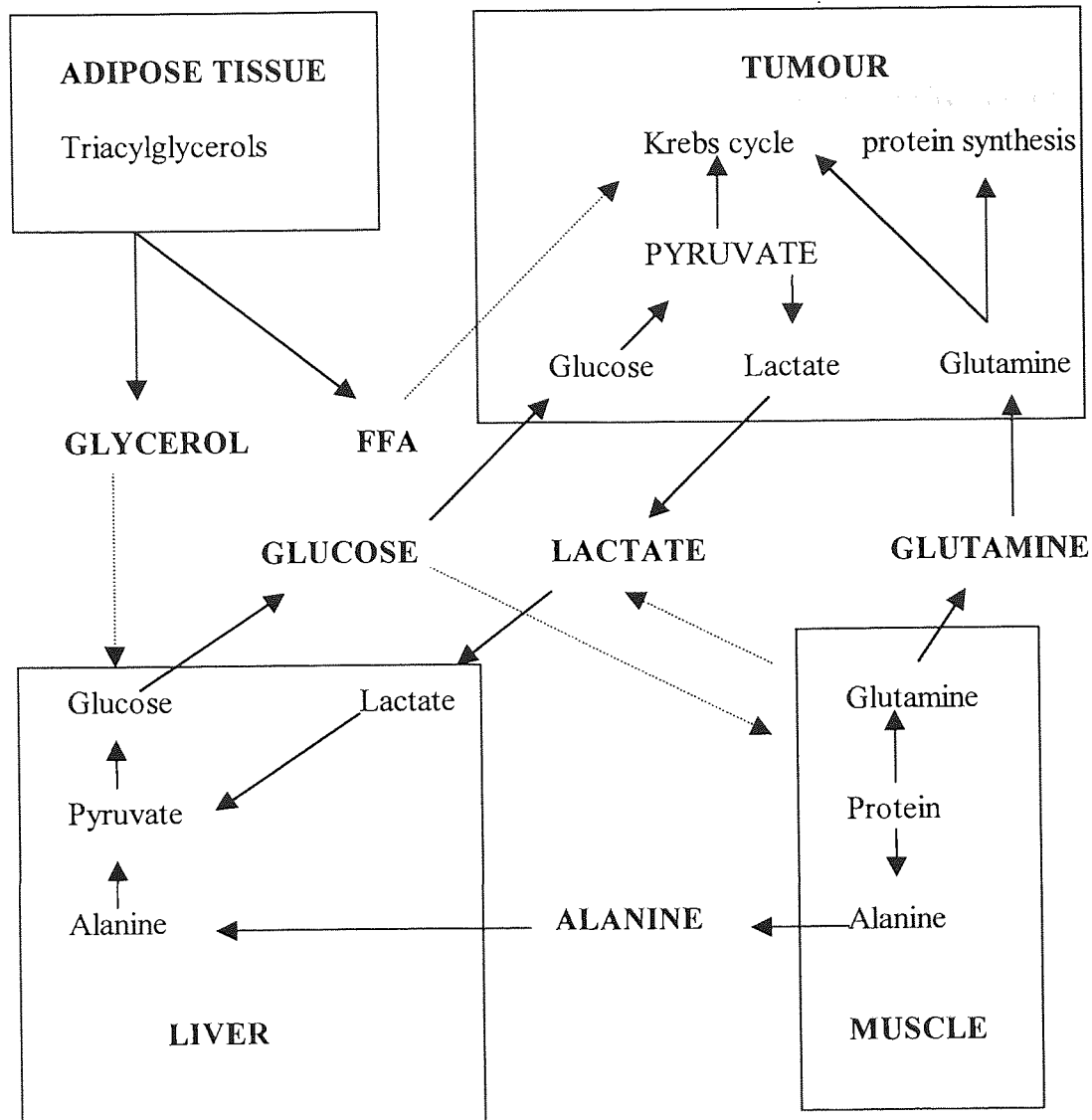


Figure 6.1.1 Metabolic interactions between tumour and host (taken from Argiles et al 1997). The three main metabolic trends associated with cancer cachexia are (1) lactate recycling, which is associated with increased liver gluconeogenesis and thus Cori cycle activity; (2) lipid mobilisation, due to an inhibition of LPL and increased lipolysis and leading to hypertriglyceridemia, and (3) muscle waste as a result of enhanced protein degradation which leads to release of amino acids taken up by the tumour to sustain growth and by the liver for the synthesis of acute-phase proteins. (4) There is also increased glucose uptake in the muscle with corresponding increases in glycolysis and subsequent release of lactate.

6.1.2 Lipid utilisation

When given to NMRI mice over a 48h period LMF produces a mobilisation of carcass lipid, including loss of white adipose tissue and build up of fat in the liver (section 4.3.4). The depletion of host adipose stores is a common factor in cancer cachexia. Anorexia does not account for this fat depletion as shown by the results from pair-feeding experiments (Lundholm et al, 1981). Hyperlipidaemia has also been reported in some models of cachexia (Devereux et al, 1982), but plasma levels of free fatty acids (FFA) and triacylglycerides are reduced in the MAC16 adenocarcinoma model (Mahony et al, 1988), possibly due to an elevated level of lipoprotein lipase in skeletal muscle (Bridson et al, 1991). This suggested that the fatty acids liberated during excessive lipid breakdown in this model are rapidly oxidised. The FFA liberated during the cachexia may also be required to maintain tumour growth. The mobilisation of fat stores as a result of acute fasting (Sauer and Dauchy, 1987) stimulates tumour growth, and inhibition of host fat mobilisation in cancer cachexia is associated with inhibition of tumour growth (Tisdale and Beck, 1991). This suggests that tumour growth *in vivo* may be limited by substances present in fat stores, and provides further evidence for the theory that fatty acids are important fuels in neoplastic processes. LMF has been shown to produce a dramatic fat redistribution and to understand this ^{14}C triolein was used to examine fatty acid uptake and the conversion to $^{14}\text{CO}_2$.

6.1.3 Distribution of LMF

To examine if the distribution of LMF compares with the tissue specific effects of glucose and fatty acid uptake, Iodinated LMF was given *i.v.* to mice previously treated with either LMF or PBS over a period of 48h (section 4.2.1). By the iodination of LMF it should be possible to examine the bioavailability. The iodination process (section 5.2.4) should produce enough labelled LMF to use *in vivo* since there are a large number of tyrosine groups.

6.2 Method

6.2.1 ^{14}C production from ^{14}C -labelled glucose

Mice were injected *i.p.* with $50\mu\text{Ci/kg}$ of D-[U- ^{14}C]-glucose (Specific Activity 11.0GBq/mmol) in $200\mu\text{l}$ of 0.9% NaCl. Animals were then placed in airtight metabolic cages into which air was pumped through solid calcium carbonate to absorb any CO_2 . Metabolically produced CO_2 was trapped in glass test tubes containing 20ml of a mixture of ethanamine: ethoxyethanol (1:4). At specified time intervals (0.5, 1, 2, 4, 8h), $500\mu\text{l}$ aliquots were taken and transferred to scintillation vials containing 10ml Optiphase Hi-safe II. The vials were shaken and the radioactivity was measured in a Packard TRI-CARB 2000CA scintillation analyser. The mice had previously been injected *i.v.* either with PBS or LMF over 48h as in section 4.2.1.

6.2.2 Glucose utilisation *in vivo*

The extent of glucose utilisation by different tissues was investigated using the method of Sokoloff et al (1977), which involves the *in vivo* administration of [^3H]

2-deoxy-D-glucose (2DG). The transport, cellular uptake and phosphorylation by hexokinase of this analogue correlate with those of glucose, but, because 2-deoxyglucose-6-phosphate (2DGP) cannot readily be metabolised further, it can be detected in tissues containing little or no glucose-6-phosphate activity, such as brain and muscle (Lackner et al, 1984 and Jenkins et al, 1986). Glucose utilisation was calculated according to the following equation:

$$R_g = \frac{C_m^* (T)}{LC \int_0^T \frac{C_p^* dt}{C_p}}$$

Where R_g is the tissue glucose metabolic rate (nmol/g/min); $C_m^* (T)$ is the concentration of phosphorylated metabolites of 2-deoxyglucose in the tissue (dpm/g) at $t=60$ min; C_p is the blood glucose concentration (nmol/ml); C_p^* is the concentration of radioactive 2-deoxyglucose in the blood (dpm/ml) and LC is the lumped constant which was determined using the method of Ferre et al (1985). Previous workers (Mahony and Tisdale 1988, Mulligan and Tisdale 1991) have reported an LC value of 0.46 for NMRI mice. This figure was used in the following determinations of rates of glucose utilisation.

6.2.3 Treatment of animals

NMRI mice were treated over 48h with either PBS or LMF. The mice were given food and water *ad libitum* throughout the experiment. After 48h the mice were injected *i.v.* with $50\mu\text{Ci/kg}$ 2-deoxy-D-[2,6- ^3H]-glucose (Specific Activity 1.63

TBq/mmol) in 200µl of 0.9% NaCl. So retention of 2-deoxyglucose-6-phosphate could be determined in the different tissues, an *i.v.* injection of 5µCi/kg 2-[1-¹⁴C]-]deoxy-D-glucose (Specific Activity 2.072 GBq/mmol) in 200µl of 0.9% NaCl was administered 35min after the injection of the tritiated deoxyglucose.

6.2.4 Accumulation of radioactive 2-deoxyglucose-6-phosphate by tissues (Cm*)

The accumulation of phosphorylated metabolites of 2-deoxyglucose was measured in selected tissues 60min after the injection of 2-deoxy-D- [2,6-³H]-glucose. Mice were killed by cervical dislocation and blood was removed directly from the heart. Serum was analysed for various metabolites as in section 4.3.2, 10µl of serum was then collected and added to 6ml Optiphase Hisafe scintillation fluid before analysis in a Packard TRI-CARB 2000CA liquid scintillation analyser. The following tissues were then carefully dissected out and weighed; liver, brain, spleen, kidney, lung and colon. Each tissue was homogenised in 0.4ml/100mg tissue weight of ice-cold 0.5N perchloric acid using a CAMLAB 563C homogeniser (speed 8) fitted with a Teflon pestle. The homogenate was then transferred to a centrifuge tube and centrifuged at 3000rpm in a Beckmann bench centrifuge for 15min. The supernatant was transferred to a clean centrifuge tube, neutralised to pH 7 with 10%w/v potassium hydroxide solution, re-centrifuged at 3000rpm for 5min and the final volume of supernatant was measured. 100-500µl of the neutral extract was then transferred to scintillation

vials containing 10ml Optiphase scintillation fluid and the radioactivity was measured in duplicate using a dual $^{14}\text{C}/^3\text{H}$ channel analyser by means of a Packard TRI-CARB 2000CA liquid scintillation analyser. This gave the total radioactivity of 2-deoxyglucose and its metabolites present in the tissue.

Removal of 2-deoxyglucose-6-phosphate from the neutral extract was accomplished by use of the Somogyi (1945) reagent; 200 μl of neutral extract was transferred to a clean microfuge tube and 100 μl of 0.175M zinc sulphate solution, followed by 0.15M barium hydroxide, were added, yielding a neutral mixture. The resulting precipitate, which absorbed any 2-deoxyglucose-6-phosphate, was then sedimented in a Beckmann microfuge for 30 sec. 100 μl of the supernatant was transferred to a scintillation vial containing 10ml Optiphase scintillation fluid and the radioactivity was measured in duplicate. This gave a measure of the free 2-deoxyglucose content of the tissue.

The difference between the total radioactivity of the neutral extract and the radioactivity present after the zinc sulphate/ barium hydroxide treatment; represented the 2-deoxyglucose-6-phosphate content of the tissue.

6.2.5 Determination of body weights

Various organs (brain, lung, liver, kidneys, spleen, colon, white fat and brown fat) were removed and carefully rinsed in 0.9% w/v NaCl and gently blotted before weighing.

6.2.6 Lipid oxidation and accumulation

The absorption, accumulation and oxidation of an oral dose of triolein was determined using the method of Oller do Nascimento and Williamson (1986). ^{14}C -Trolein (0.33 μCi in 100 μl normal saline) (Specific Activity 3.8GBq/mmol) was administered by intragastric intubation to LMF and PBS dosed NMRI mice. Immediately after administration of the oral dose, the mice were placed in airtight metabolic cages and metabolically produced CO_2 was collected at the following time points (0.5, 1,2,3,4,5 and 24h), using the method described in section 6.2.1. After 5h and 24h the mice were anaesthetised and blood was collected by cardiac puncture. The complete gastrointestinal tract was removed and homogenised in 5ml of 3% perchloric acid. The liver, adipose tissue, etc were removed. Lipids were extracted from the extracted organs, blood and the gastrointestinal tract as described in section 6.2.7. Triolein absorption was calculated by subtracting the total gastrointestinal tract radioactivity from that administered.

6.2.7 Extraction of lipids

Lipids were extracted from the organs and the blood by the method of Stansbie et al (1976). Briefly, the organs and blood samples were transferred to glass test-tubes containing 3ml 30% w/v potassium hydroxide and heated to 70°C for 15min. 3ml of 95%w/v ethanol was then added to each test-tube and heating at 70°C was continued for a further 2h. The samples were cooled and the saponified material was acidified with 3ml of 9M sulphuric acid. Acid soluble lipids were

extracted three times by shaking with light petroleum ether (b.p. 40°C –60°C), centrifuging at 3000rpm in a Beckman bench centrifuge for 15min and removing the supernatants. The three petroleum fractions were combined and evaporated to dryness under a stream of nitrogen. The lipid extracts were then redissolved in 10ml of Optiphase Hi-safe II scintillation fluid and the radioactivity was measured using a Packard TRI-CARB 2000CA liquid scintillation analyser.

6.2.8 Total body radiography

NMRI ex-breeder mice (30-40g) were dosed with either LMF or PBS as in section 4.2.1. After 48h the mice were injected with 8µg [¹²⁵I] LMF (section 5.2.4) or Na¹²⁵I and maintained in metabowls. The mice had free access to both food and water *ad libitum*, had a supply of air, and were left for either 6h or 24h. The mice were killed by cervical dislocation and the tissues were removed. The tissues were then analysed for radioactivity using a Packard Cobra Model 5005 Auto-gamma counter.

6.3 Results

6.3.1 Glucose uptake

Changes in glucose utilisation have been analysed in mice that had undergone treatment with LMF for 48h against those that had a placebo of PBS as in section 4.2.1.

Glucose utilisation was investigated using the 2-deoxyglucose (2-DG)-tracer method (section 6.2.2). The transport, cellular uptake and phosphorylation by hexokinase of 2DG correlate with those of glucose, resulting in the production of 2-deoxyglucose-6-phosphate (2-DGP). Glucose-6-phosphate normally undergoes isomerisation to fructose-6-phosphate, but the absence of an oxygen atom on the 2 position of the pyranose ring of 2DGP hinders this. Metabolism of 2-DGP is therefore blocked and so it accumulates within the tissues that lack sufficient glucose-6-phosphatase, such as brain and muscle (Sokoloff et al, 1997 and Meszaros et al, 1987a). Glucose-6-phosphatase cleaves glucose-6-phosphate to form glucose. 2-DGP would not be expected to accumulate in those tissues with abundant glucose-6-phosphate such as the liver or kidneys. By using a sequential double-labelling technique it has been shown to be possible to measure this metabolite (Meszaros et al, 1987b, Hussey et al 1999).

Glucose utilisation in the LMF treated mice differed from that of PBS controls (Fig 6.3.1.1), with a significant increase in R_g values for the brain, brown adipose, gastrocnemius and heart. There was also a significant decrease in R_g value for the diaphragm. This difference between the gastrocnemius muscle and diaphragm may be due to differences in the muscle fibre types since LMF was shown to increase protein synthesis in C2C12 Myoblasts and in isolated gastrocnemius muscle but not in isolated soleus muscles (Islam-Ali 2001), with increased protein there will be an increased demand for glucose. The massive uptake in the brain was unexpected and will have to be further investigated perhaps using ^{13}C -Glucose as a marker.

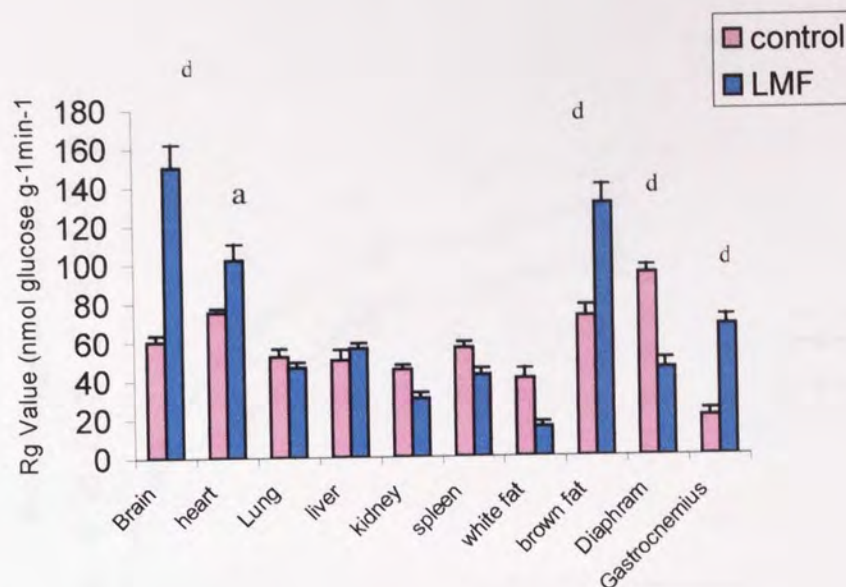


Fig 6.3.1.1 Comparison of various tissues Rg values in LMF-treated and vehicle-treated mice, Data presented as mean \pm SEM for 12 mice per group. Statistically significant a $p < 0.05$, d $p < 0.001$ as determined by ANOVA.

The conversion of D- [U-¹⁴C]-glucose to ¹⁴CO₂ was significantly higher in the LMF treated group than the control was over a period of 8h (**Fig 6.3.1.2**). This suggests that glucose oxidation is increased due to treatment with LMF, but it is important to remember that a bolus injection of D- [U-¹⁴C]-glucose with the measurement of ¹⁴CO₂ production does not strictly measure glucose oxidation. Recycling of the label, and its transfer to other substrates such as lipids must also be considered, since they may contribute to the amount of ¹⁴CO₂ produced.

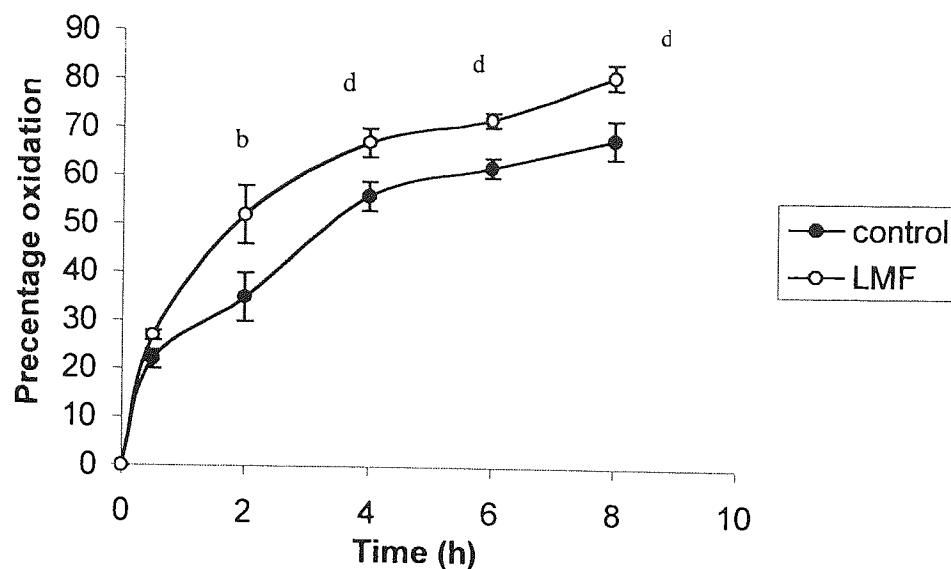


Fig 6.3.1.2 Oxidation of U- [^{14}C]-glucose to $^{14}\text{CO}_2$ in mice dosed with LMF or vehicle control 48h prior to injection of radiolabel. Data presented as mean \pm SEM for 12 mice per group. Statistically significant b $p < 0.01$, d $p < 0.001$ as determined by ANOVA for 12 animals per group.

The effect of LMF treatment on the retention of 2DGP in various organs was examined using a sequential double-labelling technique followed by an analysis of the two labels in 2DGP (section 6.2.4). The bulk of the [^3H] 2DGP was synthesised in the tissues during the initial 35min of the labelling period, and the $^3\text{H}/^{14}\text{C}$ ratio of 2DGP was measured at the end of the experiment. Loss of 2DGP from the tissue would therefore affect the ^3H component of the ratio more than the ^{14}C component. The $^3\text{H}/^{14}\text{C}$ ratio of 2DGP in the tissues was a measure of the

retention of 2DGP, i.e. a low ratio indicates a high rate of loss. Since 10-times as much ^3H radioactivity was administered as ^{14}C , the $^3\text{H}/^{14}\text{C}$ ratio would be expected to be near 10.

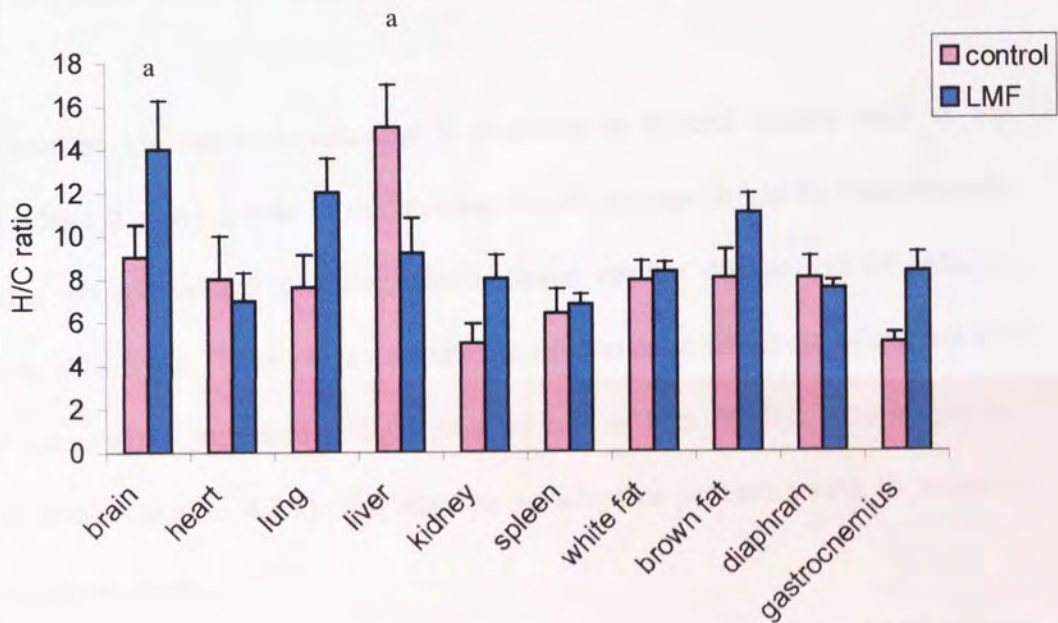


Fig 6.3.1.3 Ratio between ^3H glucose and ^{14}C glucose in various tissues after previous treatment with LMF or vehicle over 48h, a $p < 0.05$. Data presented as mean \pm SEM for 12 mice per group. Statistically significant a $p < 0.05$, d $p < 0.001$ as determined by ANOVA.

In the LMF treated mice, the $^3\text{H}/^{14}\text{C}$ ratio was significantly higher in the brain (Fig 6.3.1.3). The ratio was also higher in gastrocnemius muscle, brown adipose tissue and lung, but did not reach significance, suggesting that the brain is able to adapt to use metabolic substrates other than glucose which may possibly be due to an adaption to the sudden influx of glucose and a switching away from ketone bodies as a fuel source but this requires further investigation. Adaption may also occur to a lesser extent in the gastrocnemius muscle, brown adipose and lung.

The enzyme glucose-6-phosphatase is abundant in several tissues such as the liver, where it plays a role in maintaining blood glucose levels by hydrolytically cleaving phosphorylated glucose, which cannot readily diffuse out of cells, to glucose, which can. There was a significant difference in the livers of control and LMF mice, which indicated a slightly higher rate of loss. With a drop in glucose serum levels (section 4.3.2), this may be an adaptive process trying to maintain blood plasma levels.

The conversion of D- $[\text{U}-^{14}\text{C}]$ -glucose to $^{14}\text{CO}_2$ was higher in the LMF treated group than in the control group (section 6.2.1) at all time points, with a higher total conversion over a 8 hour period (Fig 6.3.1.2). Although this suggest that glucose oxidation has increased due to treatment with LMF, a bolus injection of $[\text{U}-^{14}\text{C}]$ -glucose with measurement of $^{14}\text{CO}_2$ production does not strictly measure glucose oxidation. Re-cycling of the label, and its transfer to other substrates such as lipids must also be considered, since they may contribute to the amount of $^{14}\text{CO}_2$ produced.

6.3.2 Fatty acid uptake

6.3.2.1 Absorption and oxidation and distribution of a labelled lipid

The mice were dosed with LMF for 48h previous to the injection of [$1\text{-}^{14}\text{C}$]-triolein (section 4.2.1). There was no difference between the two groups for the amount of triolein absorbed (Fig 6.3.2.1.1).

The rate of oxidation of [$1\text{-}^{14}\text{C}$] triolein to CO_2 significant increased ($p < 0.001$), with the LMF-treated group when compared with PBS treated mice over 24h (Fig 6.3.2.1.2). The pattern of labelled lipid between the treated and untreated groups showed some significant differences. In particular there was increased accumulation of triolein in the liver, plasma, brown and white adipose tissue. There was also a slight, but not significant increase in brain lipid (Fig 6.3.2.1.3). With a 2-fold increase in the accumulation of lipid in the livers of the LMF-treated mice, this corresponds to the fat redistribution caused by LMF treatment (section 4.3.4).

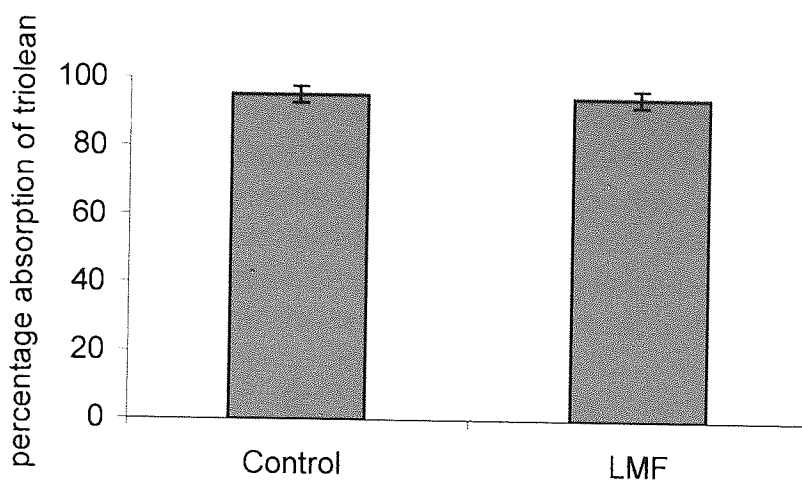


Fig 6.3.2.1.1 Absorption of an oral dose of $[1-^{14}\text{C}]$ - triolein in mice previously treated with LMF or vehicle control over 48h prior to the triolein injection. Data presented as mean \pm SEM for 12 animals per group.

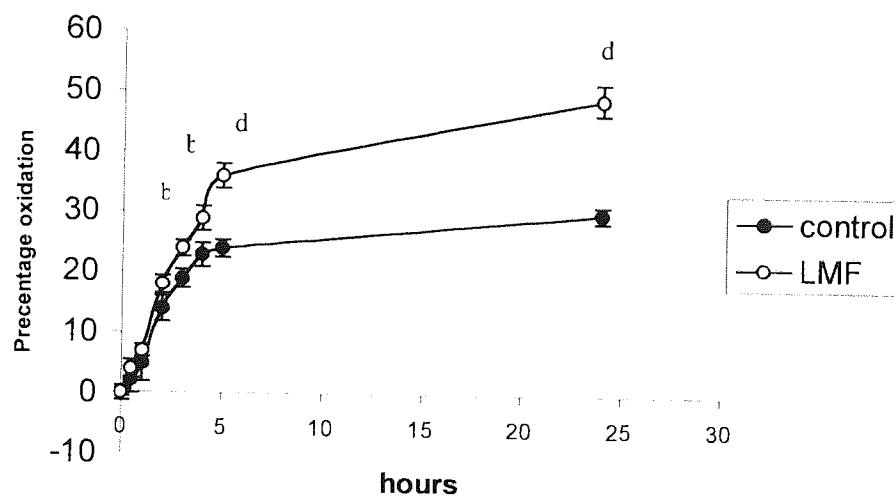


Fig 6.3.2.1.2 Oxidation of $[1-^{14}\text{C}]$ - triolein to $^{14}\text{CO}_2$ in mice pretreated with LMF or vehicle over 48h prior to injection Data presented as mean \pm SEM for 12 mice per group. Statistically significant a $p < 0.05$, d $p < 0.001$ as determined by ANOVA.

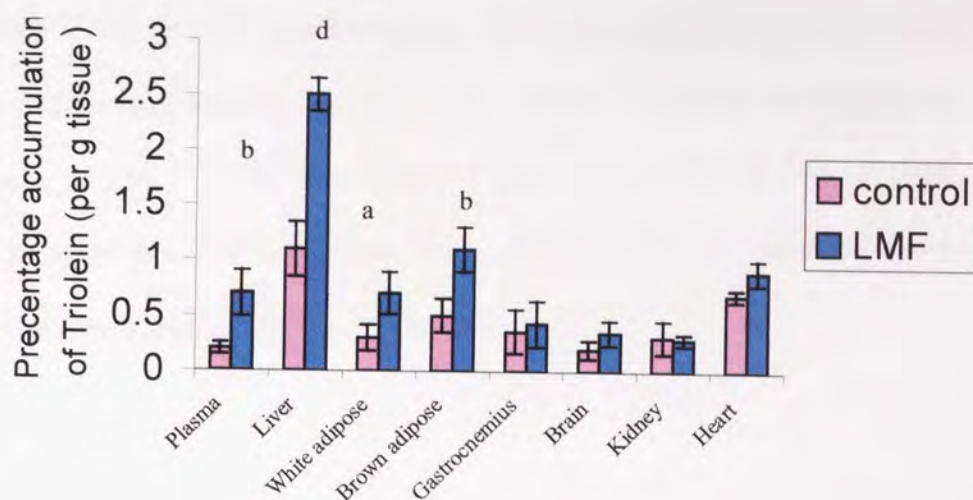


Fig 6.3.2.1.3 Accumulation of $[1-^{14}\text{C}]$ -triolein in mice previously treated with LMF or vehicle control over 48h prior to injection. Data presented as mean \pm SEM for 12 mice per group. Statistically significant a $p < 0.05$, b $p < 0.01$, d $p < 0.001$ as determined by ANOVA.

6.3.3 I^{125} LMF distribution

When iodinated LMF was administered to LMF treated and untreated mice (section 4.2.1), there was evidence of distribution in specific organs. In particular there appeared to be a significant increase in the livers of the treated mice at 6h, with that decreasing over 24h (Fig 6.3.3.1). This increase in the liver may correspond to the effects of increased lipid and up-regulation in UCP2 in the liver tissue of these mice. An increase in ^{125}I LMF was also seen for the gastrocnemius

muscle, which would correspond to the effects observed by Islam-Ali and Tisdale (2001), with effects on protein synthesis. There was a significant increase in accumulation in the thyroid, even though the mice had been dosed on potassium iodide from three days prior to the experiment. There was also an increased level in the urine, as the $^{125}\text{ILMF}$ was excreted (Fig 6.3.3.1). There was a small apparent increase in $^{125}\text{ILMF}$ in brain tissue. This was further examined using slices of brain tissue, but no binding was evident.

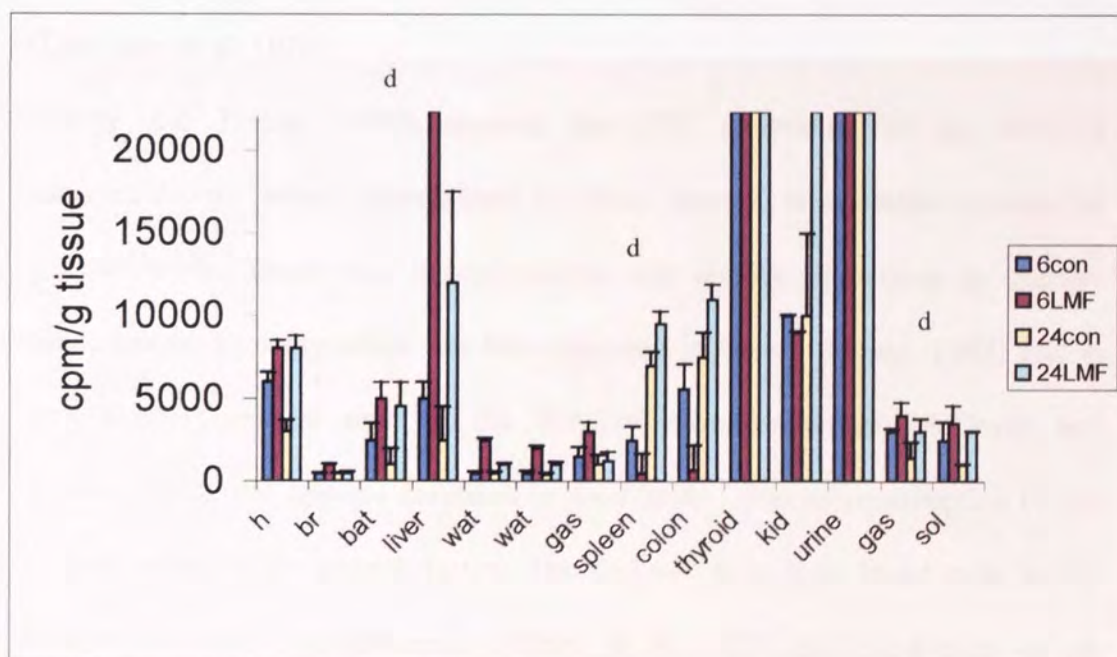
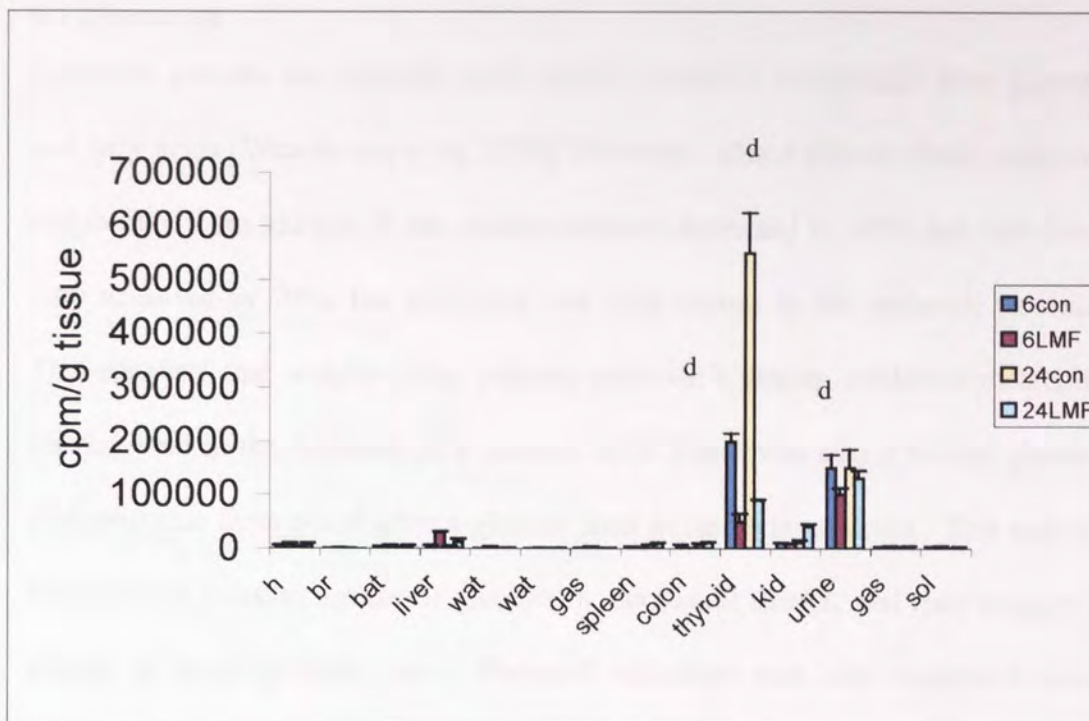


Fig 6.3.3.1 The distribution of ^{125}I ILMF in various tissues 6h and 24h after pretreatment with LMF or vehicle control for 48h. Data presented as mean \pm SEM for 12 mice per group. Statistically significant a $p < 0.05$, d $p < 0.001$ as determined by ANOVA.

6.4 Discussion

Cachectic patients and controls show similar oxidative metabolism from glucose and fatty acids (Waterhouse et al, 1979). However, after a glucose load, oxidative metabolism from glucose in the control subjects increased by 62% and that from fatty acids fell by 70%, but still there was little change in the cachectic patients. This suggests that weight-losing patients maintain a fasting oxidative metabolic profile, even in the presence of a glucose load. There was also a slower glucose disappearance from blood after a glucose load in cachectic patients. This may be indicative of reduced uptake of glucose by peripheral tissues, and may suggest a degree of glucose intolerance. Reduced utilisation was also suggested from decreased activity of glycolytic and glucose oxidative enzymes in the biopsies from rectus abdominus muscle of patients with heterogenous malignancies (Lundholm et al, 1976).

Hussey and Tisdale (1999) showed that PIF, a product of the MAC16 adenocarcinoma, when administered to mice, showed homeostatic changes in glucose levels. There was hypoglycaemia and specific alterations in glucose consumption. Hypoglycaemia has been reported in human (Nolop, 1987) and in experimental tumours such as the MAC16 adenocarcinoma (McDevitt and Tisdale, 1992), and appears unrelated to food intake, glucose consumption by the tumour or insulin-like growth factors. Insulin levels have been found to be low in tumour-associated hypoglycaemia (Bibby et al, 1987) and production of an insulin-like factor by the tumour was suggested as the cause of the enhanced glucose uptake.

LMF appears to have similar effects to that of PIF, in that glucose consumption was increased in the brain, brown fat and heart. They did differ slightly, since PIF

produced a decrease in both gastrocnemius and diaphragm glucose consumption, whereas LMF gives increased levels in the gastrocnemius. This may be due to the fact that PIF causes decreased weight in the soleus and gastrocnemius muscles, with no effect on the heart weight, PIF was also shown to have a direct inhibitory effect on the glucose consumption of skeletal muscle, since uptake of 2-deoxyglucose by C₂C₁₂ myoblasts *in vitro* was inhibited (Hussey and Tisdale, 1999). On the other hand LMF causes increased protein synthesis in gastrocnemius muscle and increased glucose uptake in C₂C₁₂ myoblasts (Islam-Ali and Tisdale, 2001).

There was a greater oxidation of fatty acid for the LMF treated mice, which may relate to the induction of uncoupling proteins. There was also an accumulation of ¹⁴C triolein in white adipose, brown adipose and also the liver, which corresponds to the accumulation of lipid in the livers of treated mice. The induction of UCP2 in the livers of these mice may be a response to this accumulation of lipid, which would lead to the production of free radicals.

The distribution of ¹²⁵I LMF showed that there was accumulation in the liver and gastrocnemius muscle and also a slight increase in brown adipose tissue, corresponding to the effects seen with glucose and fatty acid uptake.

6.5 Conclusion

The increased glucose consumption by brown adipose tissue, heart and gastrocnemius muscle seen with both PIF and LMF is indicative of an increased energy expenditure, which would contribute to the weight loss. This would provide thermogenic activity, which fits with the increased expression of UCP1 mRNA and protein and the increased vascularisation, seen in BAT (section 4.3.4). The accumulation of lipid particularly in the liver is also indicative of a role for uncoupling proteins.

Chapter 7. Characterisation of Zinc- α 2-glycoprotein

7.1 Introduction

LMF has previously been shown to have a high level of homology with a known protein zinc-alpha2-glycoprotein (ZAG). ZAG has homology and structure similar to the MHC class 1 family. A comparison of the LMF and ZAG gave similar migration on denaturing and non-denaturing gels. Also a polyclonal antibody for ZAG was able to identify LMF (Todorov et al, 1998).

ZAG was examined for lipolytic activity in isolated adipocytes (Hirai et al, 1998) and found to be active; this was a plasma-purified form. Our group was supplied with both recombinant ZAG (EZAG) and ZAG purified from human plasma. These were then analysed for lipolytic activity. It has been suggested that ZAG may be also be a carrier protein for some lipolytic component, and so EZAG was incubated with various compounds such as T3 and noradrenaline to examine such an interaction. Dr Zimmerman of the Bayer Corporation using MALDI-TOF also examined LMF for similarities to ZAG and by the use of electro-focusing gels.

Kennedy et al (2001) suggested that ZAG was a carrier protein possibly for arachidonic acid. To try and identify the active part of LMF the protein was digested with trypsin and bioactive fraction were then identified.

7.2 Methods

7.2.1 Zinc- α 2-glycoprotein lipid mobilising activity

Dr Bjorkman of the University of California, Pasadena, supplied recombinant Zinc- α 2-glycoprotein (EZAG). EZAG was incubated with isolated white adipocytes using the method described in section 3.2.1.4. ZAG isolated from human plasma by Dr Zimmerman was also analysed for lipolytic activity.

7.2.2 EZAG Binding studies

EZAG was incubated overnight at 4°C with 100mM each of T3, adrenaline, arachidonic acid, noradrenaline and 11-(dansylamino) undecanoic acid (DAUDA) supplied by Dr Malcolm Kennedy of Glasgow University. To separate the bound from the unbound EZAG a sephadex G25 column was used. Sephadex G25 was incubated in PBS overnight before being placed in a Pasteur pipette with glass wool at the bottom. The V_0 peak was measured using Blue Dextrose, the V_0 being the time for the Blue Dextrose to pass through. The samples were then passed through the column and 100 μ l fractions were collected for analysis (Section 3.2.1.4). Alternatively, the samples were placed in a Microcon ultra-filtration device (Millipore, UK) with a 10kDa cut-off and centrifuged at 13,000 rpm for 15min. The unbound ligand passed through the membrane, and the remainder was analysed for activity (Section 3.2.1.4).

7.2.3 Binding of EZAG with radio labelled Noradrenaline

To assess if EZAG could bind radio labelled noradrenaline they were incubated together overnight at 4°C before being run on a non-denaturing gel. 50µM EZAG was incubated with 100mM 1-[7,8-³H]-Noradrenaline (Sp. Activity 444GBq/mmol). Once the gel was run half the gel was stained for protein. The other half of the gel, a section of gel corresponding to the EZAG, was removed, along with a section of gel not corresponding to any protein bands. The removed sections were chopped up and paced in scintillation vials containing 6ml Optiphase Hi-safe II scintillation fluid and the radioactivity was measured using a Packard TRI-CARB 2000CA liquid scintillation analyser.

7.2.3.1 Binding of EZAG with radio labelled arachidonic acid

EZAG was incubated overnight at 4°C with 100mM H³-arachidonic acid (Sp.Act 7.6TBq/mmol). The sample was then placed on a G25 sephedex column (section 7.2.2) and fractions were collected for analysis using a Packard TRI-CARB 2000CA liquid scintillation analyser.

7.2.4 Spectral analysis of LMF and ZAG

Samples of both LMF and ZAG were analysed in a Beckman DU7 spectrophotometer. Analysis of the entire spectra of LMF and ZAG was performed. 100µM of each protein in PBS was analysed from 530nm to 340nm for absorbance. The samples were then compared to one another and a PBS blank.

7.2.5 Effects of Trypsin digestion on LMF

Several samples were prepared with 100µg LMF in 10mM Tris.HCl (pH8) and trypsin (0.002g/ml), as well as controls –Trypsin + Tris.HCl and LMF + Tris.HCl. The samples were incubated for 4h and 8h at 37°C. The reactions were stopped by the addition of trypsin inhibitor (0.002g/ml). Each sample was filtered using an ultrafiltration unit (Millipore, USA) with a 10kDa cut off. The flow through was then placed on a sephadex-50 gel exclusion column, after being mixed with a small amount of sucrose. The sephadex-50 column was calibrated with Albumin (M_r 66,000), Trypsin Inhibitor (M_r 20,000), Cytochrome C (M_r 12,400) and Aprotinin (M_r 6,500). The V_o was measured by the addition of dextrane blue to the column and timing how long this took to pass through the column. The column was first equilibrated using 10mM Tris.HCl (pH8). Samples were collected for 30min using a fraction collector FRAC100 (Pharmacia) and analysed for protein (section 3.2.1.3) and lipolytic activity (section 3.2.1.4). Alternatively the samples were passed down a smaller G25 column or the digested material was separated from the undigested, by passing the sample through a microcon (Millipore, USA) with a 10kDa cut-off. The active samples were run on a 16.5% ready-made Tris-Tricine gel (Bio-Rad, CA) against ultra-low molecular weight markers (Sigma). A cathode buffer (section 2.3.4.1) and an anode buffer (section 2.3.4.2) were used to run the gel at constant amps of 20mA and then 30mA once the sample buffer front was on the resolving gel. The gel was fixed using a fixing solution (section 2.3.4.3) for 30min and then washed with coomassie destaining solution 2 (section 2.3.4.4) for 15min. The gel was then stained for 1h using Brilliant Blue G staining solution (section 2.3.4.5). The gel was then washed in coomassie destaining solution 2 for 2h.

7.3 Results

7.3.1 Zinc- α 2-glycoprotein lipolytic activity

Plasma purified ZAG appeared to have lipolytic activity and this was in keeping with the findings of Hirai et al (1998). In contrast the recombinant ZAG (EZAG) did not appear to have any activity and this was probably due to the lack of glycosylation (table 7.3.1). Certainly previous work showed that although plasma ZAG was active, seminal ZAG, which is non-glycosylated, was inactive and therefore it seems that the glycosylation is vital to activity.

Sample	Glycerol Release $\mu\text{moles}/10^5$ adipocytes/2h
LMF (8 μg)	0.154 \pm 0.06
ZAG (25 μg)	0.183 \pm 0.05
EZAG (25 μg)	0.003 \pm 0.001***
Isoprenaline (10 μM)	0.278 \pm 0.08

Table 7.3.1 Glycerol release activity comparison for LMF, ZAG and EZAG, with Isoprenaline as a positive control Data presented as mean \pm SEM for n=6. ***P<0.001 statistically significant decrease in activity from isoprenaline control, LMF or ZAG.

7.3.2 Zinc- α 2-glycoprotein and Lipid mobilising comparisons

Dr Zimmerman, using coomassie blue staining on native gels, carried out comparison studies between ZAG and LMF. Two bands for ZAG were recognised by an anti-ZAG antibody, while a LMF doublet migrated slightly slower but was also recognised by the anti-ZAG antibody (personnel communication). It would appear LMF contains several isoforms as does ZAG. LMF two isoforms can also be seen during the purification process of LMF where there are often two peaks very close together on the HPLC trace (Fig 3.3.3). Spectral analysis of LMF and ZAG showed no difference between the two both giving peaks at between 275-280nm (Fig 7.3.2.1).

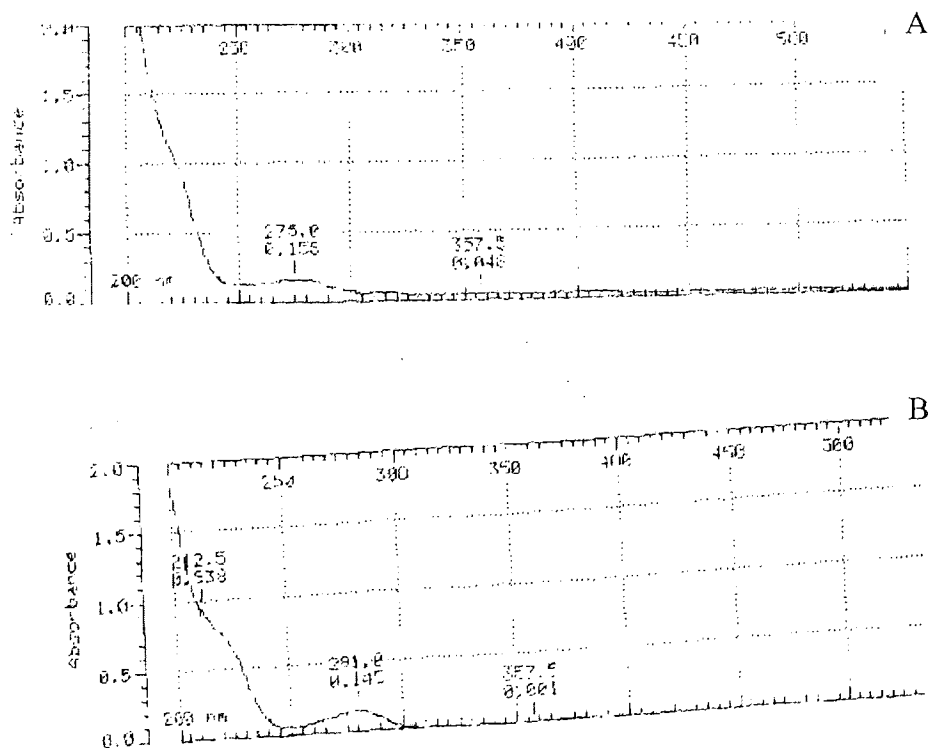


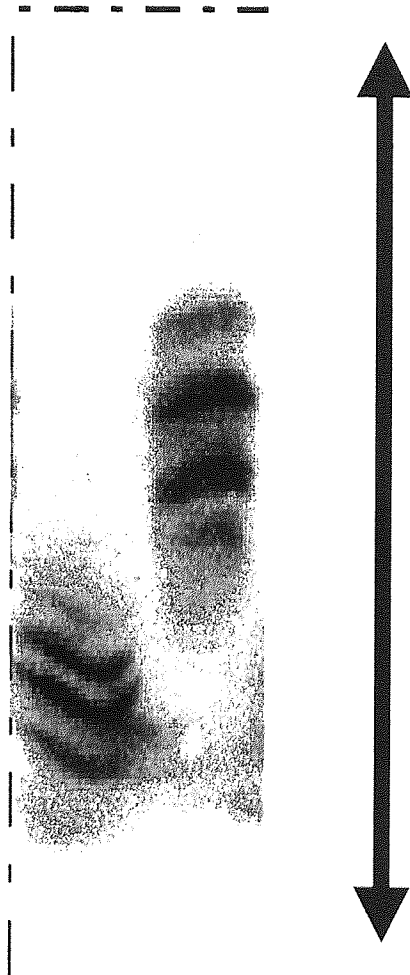
Fig7.3.2.1 Spectral analysis of LMF (A) and ZAG (B) was carried out using a Beckman Spectrophotometer. 100 μ g of sample in 1ml PBS analysed from 200nm-550nm.

Further comparisons were made between ZAG and LMF using MALDI-TOF; ZAG giving a mass of 38,850 while LMF gave masses of 34,764 and 40,429 (Dr Zimmerman, personnel communication). This provided further evidence that LMF was an isoform of ZAG.

Both LMF and ZAG were resolved on an isoelectro-focusing PAGE gel (IEF) carried out by Dr Zimmerman, followed by western blotting with an anti-ZAG antibody (fig 7.3.2.2). There was a different distribution of isoforms between ZAG and LMF. LMF appeared to be more acidic, and this may be due to differences in the glycosylation of the proteins. Since there is one gene for ZAG (Araki et al, 1988), any differences in isoforms must be due to post-translational modification, probably due to changes in the glycosylation of the protein. This could also explain the difference in molecular weight between LMF and ZAG.

ZAG LMF

High pH



Low pH

Fig 7.3.2.2 IEF PAGE (pH 4.0 – 6.5) of ZAG and LMF Western Blot

7.3.3.1 Recombinant Zinc- α 2-glycoprotein lipolytic activity

Since EZAG appeared to be lipolytically inactive, an attempt was made to bind a number of lipolysis inducing compounds. When T3, adrenaline, arachidonic acid and noradrenaline were incubated with EZAG overnight they were placed on a G25 column as in section 7.2.2. The fractions were then analysed for lipolytic activity but no activity was observed.

7.3.3.2 Binding EZAG with ^3H -arachidonic acid

^3H -Arachidonic acid was incubated overnight with EZAG at 4°C on passing through a G25 sephedex column (section 7.2.2) and fractions were then collected for analysis in a Packard 2000A scintillation counter (section 7.2.3.1). There was only one major peak observed suggesting no binding had taken place (Fig 7.3.3.2).

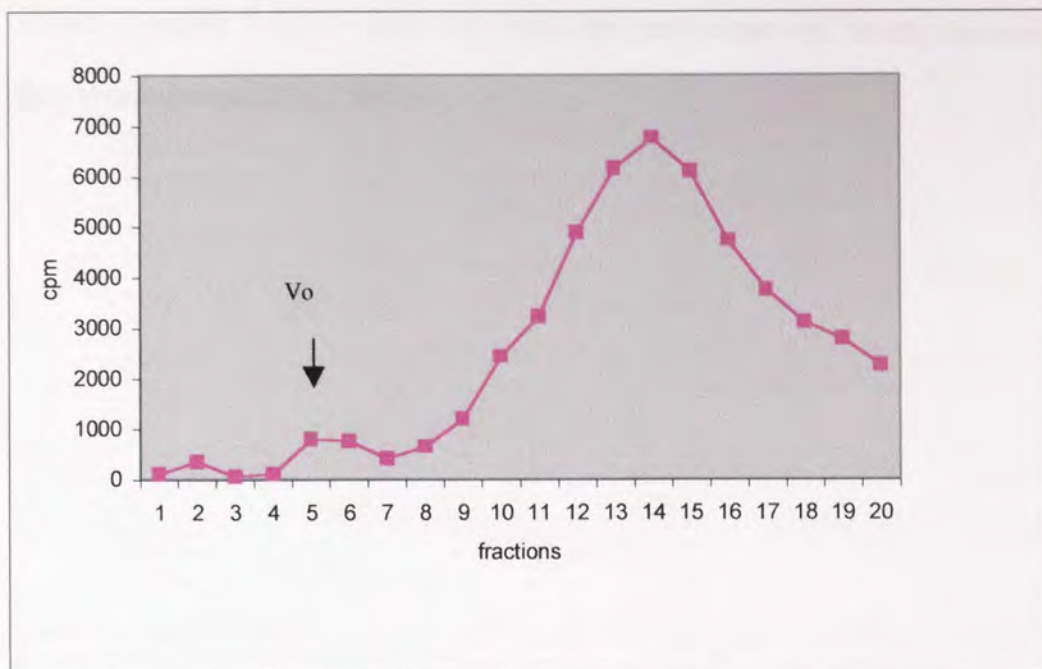


Fig 7.3.3.2 EZAG incubated overnight at 4°C with ^3H -arachidonic acid before bound from unbound was separated using a sephadex G25 column. Fractions were measured in a Packard TRI-CARB 2000CA liquid scintillation analyser.

7.3.3.3 Tritiated and non-tritiated noradrenaline incubated with EZAG

EZAG was incubated, as in section 7.2.2, with noradrenaline and passed through a G25 sephedex column. Fractions were then collected and measured for protein concentration at 214nm and noradrenaline at 260nm (**Fig 7.3.3.3**). No binding appeared to take place since the large peak at the V_0 for 260nm was free noradrenaline, and there was no corresponding protein peak. When ^3H -noradrenaline was incubated with EZAG and then run on a non-denaturing (section 7.2.3) gel there did not appear to be any binding to the EZAG, when fragments of gel were cut out and analysed in a Packard 2000A scintillation counter. Also when ^3H -noradrenaline and EZAG were run down a G25 Sephedex

column (section 7.2.3.1) there was only one peak observed, which represented free noradrenaline (Fig 7.3.3.4).

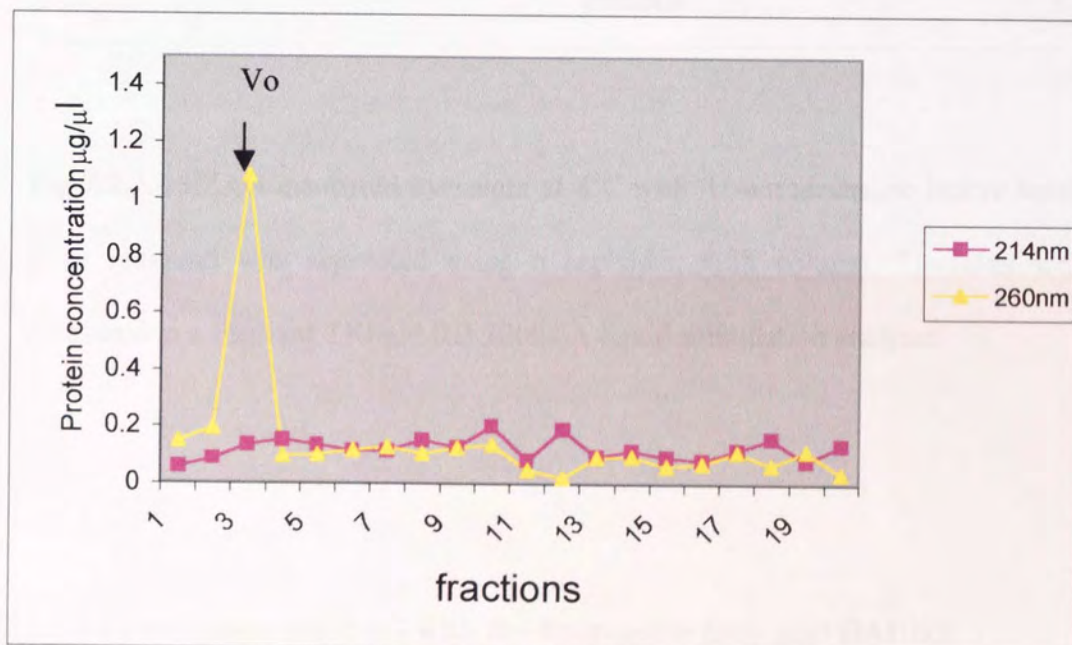


Fig 7.3.3.3 EZAG incubated overnight at 4°C with noradrenaline before bound from unbound was separated using a sephadex G25 column. Fractions were measured for protein content by the method of Bradford (section 3.2.1.3).

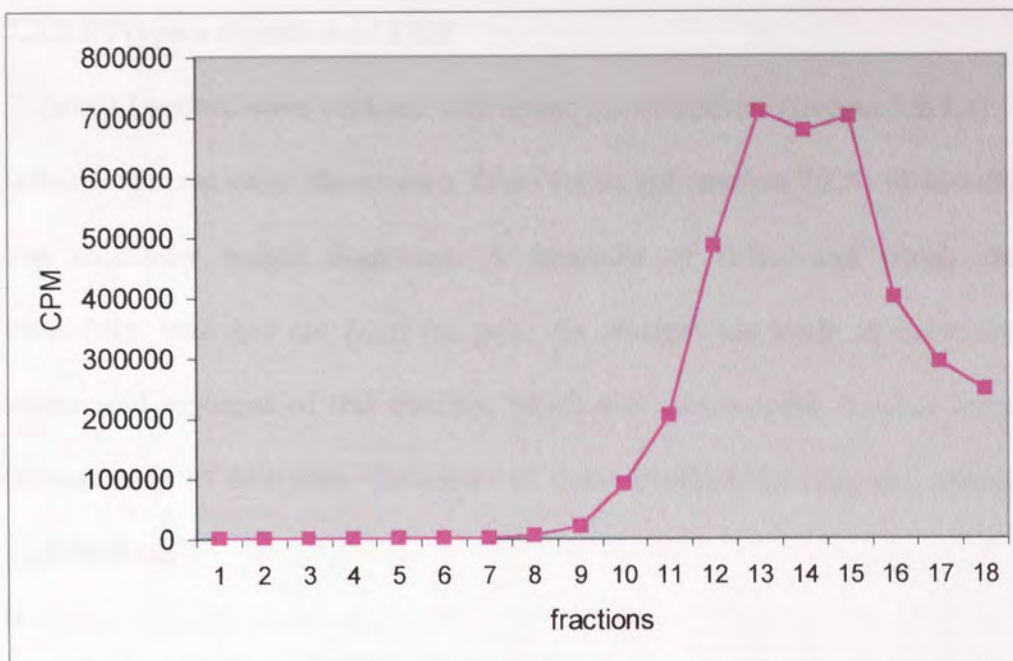


Fig 7.3.3.4 EZAG incubated overnight at 4°C with ^3H -noradrenaline before bound from unbound was separated using a sephadex G25 column. Fractions were measured in a Packard TRI-CARB 2000CA liquid scintillation analyser.

7.3.3.4 Incubation of EZAG with the fluorogenic fatty acid DAUDA

When EZAG was incubated with DAUDA (section 7.2.2) supplied by Dr Kennedy there was no lipolytic activity. Kennedy et al (2001) showed that DAUDA does bind to EZAG at micromolar concentrations and competed with natural fatty acids such as arachidonic acid. Possibly this causes some other biological function.

7.3.3.5 Trypsin digestion of LMF

Digested fractions were obtained and tested for bioactivity (section 3.2.1.4). Then active fragments were placed on a Tris-Trysine gel (section 7.2.5) to identify any low molecular weight fragments. A fragment of 7kDa, and which showed bioactivity, was then cut from the gels. An attempt was made to determine the amino acid sequence of this fraction, which was unsuccessful. Further work will be necessary to determine the origin of these fractions for example staining for carbohydrate.

7.4 Discussion

Zinc- α 2-glycoprotein appears to have lipolytic activity with around 2-fold less activity than LMF. The EZAG on the other hand was not active, which was probably due to the lack of glycosylation. Certainly plasma derived ZAG was previously shown to be lipolytically active, but seminal derived ZAG was not. Since seminal ZAG is not glycosylated it may perform another function. Kennedy et al (2001) have shown that DAUDA binds to recombinant ZAG in the micromolar range in competition with arachidonic acid and other natural fatty acids. This may mean that different fatty acids and other compounds bind to ZAG and may then provide variety in function. Attempting to bind other possible ligands did not appear to provide a lipolytically active fraction. Also attempting to bind radiolabelled noradrenaline and aracidonic acid did not appear to show binding. Although there does appear to be active fragments from LMF digested with trypsin this has yet to be identified and further work will be required.

7.5 Conclusion

When LMF is compared with ZAG there does appear to be evidence that both are isoforms of the same protein, since an antibody to ZAG identifies them both. They have similar mobility on denaturing and non-denaturing gels and HPLC often shows two peaks for LMF bioactivity, while IEF provides evidence for a different isoform distribution. With only one gene for ZAG the differences are possibly be due to posttranslational modification. It would appear that in particular glycosylation appears to be the major factor, and it may be that differences in glycosylation and possibly the involvement of different fatty acids changes the proteins biological function.

Chapter 8. Conclusion

Patients with cancer cachexia have a severe loss of muscle and fat as the condition progresses. These patients have been found to have an elevated level of a lipid mobilising factor (LMF) in both serum and urine (Groundwater et al, 1990), which was characterised by the ability to stimulate lipolysis directly in isolated murine adipocytes. The mechanism of action appeared to be stimulation of adenylate cyclase in a GTP-dependent manner (Hirai et al, 1998). The activation of adenylate cyclase stimulates of cAMP production and so increases HSL expression, which has been shown to correlate with increased mobilisation of stored triacylglycerols with subsequent increases in FFA serum levels (Thompson et al, 1993, Hirai et al, 1998). In cachexia there is modulation of G protein expression with a decrease in $G_{\alpha i}$ and an increase in $G_{\alpha s}$, which favours mobilisation of lipid stores. These changes have also been identified in 3T3L1 adipocytes when LMF was added *in vitro* (Islam-Ali et al, 2001). There is also evidence that there is an increase in protein synthesis in the gastrocnemius muscle when mice were dosed with LMF. This increase in muscle protein synthesis has also been observed when rats were dosed with the β agonist Clenbuterol over 1-2 weeks (Emery et al, 1984).

With the activation of adenylate cyclase, cAMP and the effects seen on muscle it has been suggested that LMF acts via a classical catecholamine receptor mechanism. This study provides evidence that this is specifically via the β_3 -adrenegic receptor mediated pathway. LMF has been shown to stimulate cAMP production in CHO-K1 cells transfected with human β_3 -AR, both propranolol and the specific β_3 -AR antagonist SR59230A inhibited this activation. LMF was also

shown to bind to isolated β 3-ARs and again this binding was inhibited by SR59230A.

In cancer patients there are often changes in energy expenditure, which may be an adaptation to the effects of tumour burden. A major component of energy expenditure up-regulation is the expression of uncoupling proteins. Previously UCP1 was shown to be up-regulated in MAC16 adenocarcinoma bearing mice (Bing et al, 2000) and in cachectic rats (Sanchis et al, 1998), LMF was also shown to cause increased oxygen consumption in brown adipocytes (Hirai et al, 1998) which suggests that an up-regulation of energy expenditure occurs in cachexia. This study has shown that there is up-regulation of UCP1 and β -2 mRNA in the brown adipose tissue, which would suggest that there is an adaptation to the increased energy mobilisation. With little brown adipose tissue in adult humans the up-regulation of UCP1 may not be as important as that of rodents, and it may be UCP2 and β -3 are more involved. The increase in both UCP2 mRNA and protein in the livers of LMF treated mice may provide a mechanism of protection for the liver to the accumulation of lipid. Structural changes were observed in the livers of LMF treated mice that indicated a massive redistribution of lipid in these mice. The accumulation of lipid provides a source of ROS, which would lead to cell death. Therefore uncoupling proteins may provide a mechanism to dispose of these ROS. Certainly the inhibition of BAT UCP1 has previously been shown to stimulate the production of peroxides (Salvayre et al, 1997). UCP activity appears to be controlled by the sympathetic nervous system via β 3-ARs located in brown adipose tissue, providing more evidence that LMF produces its effects through the β 3-AR.

Hussey and Tisdale (1999) showed that PIF, a product of the MAC16 adenocarcinoma, produced marked homeostatic changes in glucose levels in non-tumour bearing mice. There was a marked hypoglycaemic effect and specific alterations in glucose consumption. This study shows that this is also the case with the administration of LMF. There was increased glucose consumption in the brain, BAT and heart. There was a slight difference in that whereas PIF produced changes in the gastrocnemius and diaphragm muscles, LMF produced an increase in the gastrocnemius muscle. This may be due to fact that PIF produces a decreased weight in soleus and gastrocnemius, with no effect on heart muscle. PIF has also been shown to have a direct inhibitory effect on glucose consumption of skeletal muscle, since uptake of 2-deoxyglucose by C₂C₁₂ myoblasts *in vitro* was inhibited by PIF (Hussey and Tisdale, 1999), while LMF causes increased glucose uptake in myoblasts (Islam-Ali and Tisdale, 2001).

This study also showed there was increased lipid uptake in Liver, BAT and WAT and also an increased lipid oxidation in LMF treated mice, similar to that seen in MAC16 adenocarcinoma (Mulligan, 1991), thus providing evidence that there is mobilisation of lipid in the LMF treated mice.

LMF has previously been shown to be similar to zinc- α 2-glycoprotein (ZAG), both migrate together on both denaturing and non-denaturing gels, and an anti-ZAG antibody identified both (Hirai et al, 1998). This study shows that LMF is 2 fold more active than ZAG at producing lipolysis in isolated murine adipocytes. This study also shows that plasma purified ZAG and LMF are isoforms with LMF being more acidic. It would appear that it is the glycosylation that is important in the bioactivity of the protein since the recombinant form of ZAG was inactive.

The clinical implications from the mechanism of LMF's action could lead to important treatments not only for cachexia but also diabetes and obesity. Stimulation or inhibition of this β -3AR pathway may prove important as new therapies are developed. The fact that LMF not only stimulates lipid metabolism but also increases energy expenditure may provide an avenue for obesity research with the added bonus of increased muscle protein. Also the increased glucose uptake observed with LMF treatment may prove important in diabetes particularly if GLUT transporters are involved, further investigation is required. Finally in cachexia if LMF can be inhibited and patient weight maintained survival times for cancer patients might improve, they may also respond to chemotherapy better.

This study has shown that the tumour-derived product LMF causes lipid mobilisation via the β 3-AR. The effects of LMF also produce adaptive changes to increase energy expenditure, with structural changes in BAT and the livers of the treated mice, and up-regulation of uncoupling proteins. The up-regulation of the uncoupling proteins in particular UCP2 and -3 may also provide a mechanism for protection of the lipid accumulation in the liver. This study has also shown that LMF and ZAG are isoforms and that it is the glycosylation that is important in the bioactivity.

Chapter 9. References

Adami F., Guarini A., Pini M., Siviero F., Sancetta R., Massaia M. (1994) Serum levels of tumour necrosis factor- α in patients with B-cell chronic lymphocytic leukaemia. *Eur J cancer* 30A: 1259- 1263

Albrect J.T. and Canada T.W. (1996) Cachexia and anorexia in malignancy. *Paraneoplastic syndromes* 10(4): 791- 800

Arsenijevic D., Onuma H., Pecqueur C., Raimbault S., Manning B.S., Miroux B., Couplan E., et al (2000) Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nature Genetics* 26: 435-439

Aquila H., Link T.A., Klingenberg M. (1985) The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequence homologies and of folding of the protein in the membrane. *EMBO J* 4: 2369- 1376

Araki T., Gejyo F., Takagaki K., Haupt H., Gerhard Schwick H., Bürgi W., Marti T., Schaller J., Rickli E., Brossmer R., Atkinson P.H., Putnam K.W. and Schmid K. (1988) Complete amino acid sequence of human plasma Zn α 2 glycoprotein and its homology to histocompatibility antigens. *Proc Natl Acad Sci USA* 85: 679- 683

Arch J.R.S. (1989) The brown adipocyte beta-adrenoceptor. *Proc Nutr Soc* 48:215-223

Arch J.R.S., Ainsworth A.T., Cawthorne M.A., Piercy V., Sennitt M.V., Thody V.E., Wilson C. and Wilson S. (1984) Atypical beta-adrenoceptor on brown adipose as target for anti-obesity drugs. *Nature* 309: 163-165

Arch J.R.S., Cawthorne M.A., Concy K.A., Gusterson B.A., Piercy V., Sennitt M.V., Smith S.A., Wallace J. and Wilson S. (1991) β -adrenoceptor-mediated control of thermogenesis, body composition and glucose homeostasis. In *Obesity and Cachexia*, Rothwell NJ and Stock MJ (eds) pp 241-268, John Wiley and Sons Ltd

Arch J.R.S. and Wilson S. (1996) β 3-adrenoceptors and the regulation of metabolism in adipose tissue. *Biochem Soc Trans* 24: 412-418

Argiles J.M., Alvarez B. and Lopez-Soriano F. (1997) The metabolic basis of cancer cachexia. *Med Res Rev* 17(5): 477- 498

Balkwill F., Osborne R., Burke F., Naylor S., Talbot D. and Durbin H. (1987) Evidence for tumor necrosis factor cachectin production in cancer. *Lancet* 2i: 1229-1232

Baracos V.E., De Vivo C., Hoyle D.H.R. and Goldberg A.L. (1995) Activation of the ATP-Ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing hepatoma. *Am J Physiol* 268: E996- E1006

Barbe P., Millet L., Larrouy D., Galitzky J., Berlan M., Louvet J.P. and Langin D. (1998) Uncoupling protein-2 messenger ribonucleic acid expression during very-low-calorie diet in obese premenopausal women. *J Clin Endocrinol Metab* 83: 2450- 2453

Beck S.A. and Tisdale M.J. (1987) Production of lipolytic and proteolytic factors by a murine tumor-producing cachexia in the host. *Cancer Res* 47: 5919-5923

Beck S.A. and Tisdale M.J. (1989) Nitrogen excretion in cancer cachexia and its modification by high fat diet in mice. *Cancer Research* 47: 5919- 5923

Beck S.A. and Tisdale M.J. (1990) Effect of megestrol acetate on weight loss induced by tumour necrosis factor $-\alpha$ and a cachexia $-\text{inducing}$ tumour (MAC16) in NMRI mice. *Br J Cancer* 62: 420- 424

Beck S.A. and Tisdale M.J. (1991) Inhibition of tumour induced lipolysis in vitro and and cachexia and tumour growth in vivo by eicosapenaenoic acid. *Biochem Pharmacol* 41(1): 103- 107

Belizario J.E., Katz M. and Raw R.I. (1991) Bioactivity of skeletal muscle proteolysis-inducing factors in the plasma proteins from cancer patients with weight loss. *Br J Cancer* 63: 705- 710

Berg M., Fraker D.L. and Alexander H.R. (1994) Characterization of differentiation factor/ leukaemia inhibitory effect in lipoprotein lipase activity and mRNA in 3T3-L1 adipocytes. *Cytokines* 6: 425-432

Beutler B., Greenwald D., Hulmes J.D., Chang M., Pan Y.C.E., Mathison J., Ulevitch R. and Cerami A. (1985) Identify of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature* 316: 552-554

Bibby M.C., Double J.A., Ali S.A., Fearon K.C.H., Brennan R.A. and Tisdale M.J. (1987) Characterization of a transplantable adenocarcinoma of the mouse colon producing cachexia in recipient animals. *Journal of the national cancer institute* 78: p539-546

Bing C., Brown M., King P., Collins P., Tisdale M.J. and Williams G. (2000) Increased gene expression of brown fat UCP1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. *Cancer Res* 60: 2405-2410

Boss O., Samec S., Paolini-Giacobino A., Rossier C., Dullo A., Seydoux J., Muzzin P. and Giacobino J.P. (1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 408: 39-42

Boss O., Hagen T. and Lowell B.B. (2000) Uncoupling proteins 2 and 3. *Diabetes* 49: 143- 156

Bradford M.M.(1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principal of protein-dye binding. *Anal. Biochem.* 72, 248-254

Bridson S., Beck S.A. and Tisdale M.J. (1991) Changes in activity of lipoprotein lipase, plasma free fatty acids and triglycerides with weight loss in a cachexia model. *Cancer Letters* 57: 49-53

Brown G.C. (1992) Control of respiration and ATP synthesis in mammalian mitochondria and cells. *Biochem J* 284: 1-13

Bruille D., Forge M.C., Rose F., Arnal M., Attaix D. and Obled C. (1993) Pentoxifylline decreases body weight loss and muscle protein wasting characteristic of sepsis. *Am J Phys* 265: E660-666

Bürki W. and Schmid K. (1961) Preparation and properties of Zn- α 2-glycoprotein of normal human plasma. *J Biol Chem* 236(40): 1066- 1073

Burt M.E. and Brennan M.F. (1984) Nutritional support of the patient with esophageal cancer. *Semin Oncol* 11: 127

Busquets S., Alvarez B., Van Rogen M., Carbó N., Lopez-Soriano F.J. and Argiles J.M. (2000) Lack of effect of the cytokine suppressive agent FR167653 on tumour growth and cachexia in rats bearing the yoshida AH-130 ascites hepatoma. *Cancer Letters* 157: 99- 103

Busquets S., Alvarez B., Van Royen M., Figueras M.T., Lopez-Soriano F.J. and Argiles J.M. (2001) Increased uncoupling protein-2 gene expression in brain of lipopolysaccharide-injected mice: role of tumour necrosis factor- α ? *Biochimica et Biophysica Acta (BBA)- Molecular Cell Research* 1499(3): 249-256

Carbó N., Lopez-Soriano J., Costelli P., Alvarez B., Busquets S., Baccino F.M., Quinn L.S., Lopez-Soriano F.J. and Argiles J.M. (2001) Interleukin-15 mediates reciprocal regulation of adipose and muscle mass: a potential role in body weight control. *Biochimica et Biophysica Acta* 1526: 17-24

Cariuk P., Lorite M.J., Todorov P.T., Field W.N., Wigmore S.J. and Tisdale M.J. (1997) Induction of cachexia in mice by a product isolated from the urine of cachectic cancer patients. *Br J Cancer* 76(5): 606-613

Chalmers T.M., Kekwick A. and Dawan G.L.S (1958) On the fat-mobilising activity of human urine. *Lancet* i: 866-869

Champigny O. and Ricquier D. (1990) Effects of fasting and refeeding on the level of uncoupling protein mRNA in rat brown adipose tissue: evidence for diet-induced and cold-induced responses. *J Nutrition* 120: 1730-1736

Chance W.T., Balasubramaniam A. and Dayal R. (1994) Hypothalamic concentration and release of neuropeptide Y into microdialysates is reduced in anorectic tumor bearing rats. *Life Sci* 54: 1869-1874

Clapham J.C., Arch J.R.S., Chapman H., Haynes A., Lister C., Moore G.B.T., Piercy V., Carter S.A., Lehner I., Smith S.A., Beeley L.J., Godden R.J., Herrity N., et al. (2000) Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. *Nature* 406: 415- 418

Cohn S.H., Gartenhaus W. and Sawgivitsky A. (1981) Compartmental body composition of cancer patients with measurement of total body nitrogen, potassium and water. *Metabolism* 30: 222- 229

Cortez-Pinto H., Lin H.Z., Yang S.Q., Da Costa S.O. and Diehl A.M. (1999)
Lipids up-regulate uncoupling protein 2 expression in rat hepatocytes.
Gastroenterology 116: 1184- 1193

Costa G. (1977) Cachexia, the metabolic component of neoplastic disease. Cancer
Res 37: 2327- 2335

Costa G., Lane W.W., Vincent R.G., Siebold J.A., Aragon M. and Bewley P.T.
(1980) Weight loss and cachexia in lung cancer. Nutr Cancer 2:98- 103

Costa G., Bewley P. and Aragon M. (1981) Anorexia and weight loss in cancer
patients. Cancer Treat Rep 65(Suppl): 3- 7

Costa G. and Holland J.F. (1996) Effects of krebs-2 carcinoma on the lipid
metabolism of male swiss mice. Cancer Res 22: 1081- 1083

de Uriquiza A.M., Liu S., Sjöberg M., Zetterström R.H., Griffiths W., Sjövall J.
and Perlmann T. (2000) Science 290: 2140- 2144

Devereux D.F., Roby C., Deckers P.J., Pietrafitta J. and Mozden P. (1982) Effects
of tumour bearing and removal on lipid metabolism in the rat. Surg Forum 33:
406-408

De Wys W.D. (1972) Anorexia as a general effect of cancer. *Cancer* 45, 2013-19

De Wys W.D. and Begg C.(1980) Prognostic effect of weight loss prior to chemotherapy in cancer patients. *American journal of medicine* 69: 491-7

Double J.A., Ball C.R. and Cowen P.N. (1975) Transplantation of adenocarcinomas of the colon in mice. *J Natl Cancer Inst* 54(1): 271- 275

Double J.A., Ball C.R. and Cowen P.N. (1975) Transplantation of adenocarcinomas of the colon in mice. *J Natl Cancer Inst* 54(1): 271- 275

Drott C., Waldenström A. and Lundholm K.(1987) Cardiac sensitivity and responsiveness to beta-adrenergic stimulation in experimental cancer and undernutrition. *J Mol Cell Cardiol* 19: 675-683

Ebisui C., Tsujinaka T., Marimoto T., Kan K., Iijima S. and Yano M. (1995) Interleukin-6 induces proteolysis by activating intracellular proteases (Cathepsins B and L, proteasome) in C₂C₁₂ myotubes. *Clin Sci (Colch)* 89: 431-439

Emery P.W., Ward M.W.N. and Lewin M.R. (1989) Effect of nutritional supplementation on protein synthesis in tumour and host tissues of rats with colonic cancer. *Br J Surgery* 76: 790

Evans W.K., Makuch R., Clamon G.H., Feld K., Weiner R.S., Moran E., Blum R., Shepherd F.E., Jeejeebhoy K.N. and De Wys W.D. (1985) Limited impact of total parenteral nutrition on nutritional status during treatment for small cell lung cancer. *Cancer Res* 45: 3347- 3353

Falconer J.S., Fearon K.C.H., Pleater C.E., Ross J.A. and Carter D.C. (1994) Cytokines, the acute-phase response and resting energy expenditure in cachectic patients with pancreatic cancer. *Ann Surg* 219: 325-331

Fearon K.C.H., McMillan D.C., Preston T., Winstanley F.P., Cruikshank A.M. and Shenkin A. (1991) Elevated circulating interleukin-6 is associated with an acute-phase response but reduced fixed hepatic protein synthesis in patients with cancer. *Annals of Surgery* 213: 26- 31

Fearon K.C.H. (1992) The mechanisms and treatment of weight loss in cancer. *Proc Nutr. Soc.* 51: 251-265

Fredrix E.W.H.M., Soeters P.B., Wouters E.F.M., Deerenberg I.M., von Meyenfeldt M.F. and Saris W.H.M. (1990) Energy balance in relation to cancer cachexia. *Clin Nutr* 9: 319-324

Fredrix E.W., Saris W.H., Soeters P.B., Wouters E.F., Kester A.D., von Meyenfeldt M.F. and Westerterp K.R. (1990) Estimation of body composition by bioelectrical impedance in cancer patients. *Eur J Clin Nutr* 44(10): 749- 752

Ferre P., Leturque A., Burnol A., Penicaud L. and Girard J. (1985) A method to quantify glucose utilisation *in vivo* in skeletal muscle and white adipose tissue of the anaesthetised rat. *Biochem. J.* 228, 103-110

Fleury C., Neverova M., Collins S., Rainbault S., Champigny O., Levi-Meyervies C., Bouillard F., Seldin M.F., Surwit R.S., Ricquier D. and Warden C.H. (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nature Genetics* 15: 269- 272

Gambardella A., Tortoriello R., Peace L., Tagliamonte M.R., Paolisso G. and Varricchio M. (1999) Intralipid infusion combined with propranolol administration has favourable metabolic effects in elderly malnourished cancer patients. *Metabolism* 48:291-297

Garcia-Martinez C., Costelli P., Lopez-Soriano F.J. and Argiles J.M. (1997) Is TNF really involved in cachexia? *Cancer Investigations* 15: 47-54

Gasic S., Tian B. and Green A. (1999) Tumor necrosis factor α stimulates lipolysis in adipocytes by decreasing Gi protein concentration. *Journal of Biological Chemistry* 274(10): 6770-6775

Gauthier C., Tavernier G., Charpentier F., Langin D. and Le Marec H. (1996) Functional β_3 -adrenoceptor in the human heart. *J Clin Invest* 98: 556-562

Gerhardt C.C., Romero I.A., Canello R., Camoin L. and Strosberg A.D. (2001) Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Molecular and cellular endocrinology* 175: 81-92

Gercel-Talyor C., Doering D.L., Kraemer F.B. and Taylor D.D. (1996) Aberrations in normal systemic lipid metabolism in ovarian cancer patients. *Gynecologic Oncology* 60:35-41

Gimeno R.E., Dembski M., Weng X., Deng N., Shyjan A.W., Gimenco C.J., Iris F., Ellis S.J., Woolf E.A. and Tartaglia L.A. (1997) Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* 46: 900- 906

Goldberg A.L., Kettlehut I.C., Foruno K., Fagan J.M. and Baracos V. (1988) Activation of protein breakdown and prostaglandin E₂ production in rat skeletal muscle in fever is signalled by a macrophage product distinct from interleukin-1 or other known monokines. *J Clin Invest* 81: 1378-83

Goldberg R.M., Loprinzi C.L., Malliard J.A., O'Fallon J.R., Krook J.E. and Ghost C. (1995) Pentoxifyllino for treatment of cancer anorexia and cachexia? A randomised, double-blind, placebo-controlled trial. *J Clin Oncol* 13: 2856-2859

Goodman M.N. (1994) Interleukin-6 induces skeletal muscle protein breakdown in rats. *Proc Soc Exp Biol Med* 205: 182-185

Gong D-W., Monemdjou S., Gavrilova O., Leon L.R., Marcus-Samuels B., Chou C.J., et al. (2000) *The Journal of Biological Chemistry* 275: 16251- 16257

Granneman J.G. (1995) Why do adipocytes make the β 3-adrenergic receptor. *Cell Sig* 7: 9-15

Grosvenor M., Balcavage L. and Chlebowski R.T. (1989) Symptoms potentially influencing weight loss in a cancer population. *Cancer* 63: 330- 335

Groundwater P., Beck S.A., Barton C., Adamson C., Ferrier I.N. and Tisdale M.J. (1990) Alteration of serum and urinary lipolytic activity with weight loss in cachectic cancer patients. *Br J cancer* 62: 816-821

Haagensen D.E. and Mazoujian G.R. (1985) Biochemistry, and immunohistochemistry of fluid proteins of the breast. In : Diseases of the breast, 3rd Edition (C.D. Haagensen Ed) p474- 500, Saunders, Philadelphia

Hadri K.E., Charon C., Pairault J., Hauguel-de Mouzon S., Quignard-Boulangé A. and Fève B. (1997) Down regulation of β 3-adrenergic receptor expression in rat adipose tissue during the fasted/fed transition: Evidence for a role of insulin. *Biochem J* 323: 359-364

Hale L.P., Price D.T., Sanchez L.M., Demark-Wahnefried W. and Madden J.F. (2001) Zinc-alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. *Clinical Cancer Research* 7(4): 846- 853

Harvie M.N. and Campbell I.T. (2000) Energy balance, cancer and the sympathetic nervous system. *Eur J Cancer* 36: 289- 292

Heymsfield S.B. and McManus C.B. (1985) Tissue components of weight loss in cancer patients: a new method of study and preliminary observations. *Cancer* 55: 238- 249

Hirai K., Ishiko O. and Tisdale M.J. (1997) Mechanism of depletion of liver glycogen in cancer cachexia. *Biochem Biophys Res Commun* 241: 49-52

Hirai K., Hussey H.J., Barber M.D., Price S.A. and Tisdale M.J. (1998) Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. *Cancer Res* 58: 2359-2365

Hoffstedt J., Shimizu M., Sjöstedt S. and Lönnquist F. (1995) Determination of β 3-adrenoceptor mediated lipolysis in human fat cells. *Obesity Res* 3: 447-457

Howe R (1993) β 3-Adrenergic agonists. *Drugs Future* 18: 529-549

Holmn E., Hagmuller E., Staedt U., Schlickeiser G., Gunther H.J. and Leweling H. (1995) Substrate balances across colonic carcinomas in humans. *Cancer Res* 55: 1373-8

Holroyde C.P., Gabazda T.G., Putnam R.C., Paul P. and Reichard G.A. (1975) Altered glucose metabolism in metastatic carcinoma. *Cancer Res* 35: 3710- 3714

Hussey H.J. and Tisdale M.J. (1999) Effect of a cachectic factor on carbohydrate metabolism and attenuation by eicosapentaenoic acid. *British journal of cancer* 80: 1231-1235

Hutchinson D.S., Evans B.A. and Summers R.J. (2000) β 3-Adrenoceptor regulation and relaxation responses in mouse ileum. *Br J Pharmacol* 129: 1251-1259

Hyltander A., Drott C., Körner U., Sandström R. and Lundholm K. (1991) Elevated energy expenditure in cancer patients with solid tumours. *Eur J Cancer* 27:9-15

Islam-Ali B. and Tisdale M.J. (2001) Effect of a tumour-producing lipid-mobilizing factor on protein synthesis and degradation. *British Journal of Cancer* 84(12): 1648- 1655

Islam-Ali B., Khan S., Price S.A. and Tisdale M.J. (2001) Modulation of adipocyte G-protein expression in cancer cachexia by a lipid-mobilizing factor (LMF) *British Journal of Cancer* 85(5): 758-763

Jenkins A.B., Furler S.M., and Kraegen E.W. (1986) 2-Deoxy-D-glucose metabolism in individual tissues of the rat *in vivo*. Int. J. Biochem., 18, 311-318

Kajimura N., Iseki H., Tanaka R., Ohue C., Otsubo K., Gyoutoku M., Sasaki K., Akiyama Y. and Yamaguchi K. (1996) Toxohormones responsible for cancer cachexia syndrome in nude mice bearing human cancer cells. Cancer Chemother Pharmacol 38(Suppl): 548- 552

Kauman A. (1997) Four b-adrenoceptor subtypes in the mammalian heart. Trends in Pharmaceutical Sciences 18, 70-76

Kennedy M.W., Heikema A.P., Cooper A., Bjorkman P.J. and Sanchez L.M. (2001) Hydrophobic ligand binding by ZAG, a soluble fat-depleting factor related to Major Histocompatibility complex proteins. J. Bio. Chem. 276 (37): 35008-35013 .

Keppler D. and Decker K. Glycogen.(1974) Determination with amyloglucosidase. Methods of enzymatic analysis, volume 3, pp.1127- 1131. Ed. Bergmeyer H.U.

Khan S. and Tisdale M.J. (1999) Catabolism of adipose tissue by a tumour-produced lipid mobilizing factor. Int J Cancer 80: 444-447

Kitada S., Hays E.F. and Mead J.F. (1980) A lipid-mobilizing factor in serum of tumour bearing mice. *Lipids* 15: 168- 174

Kitada S., Hays E.F. and Mead J.F. (1981) Characterisation of a lipid mobilising factor from tumours. *Prog Lipid Res* 28; 823- 826

Kitada S., Hays E.F., Mead J.F. and Zabin I. (1982) Lipolysis induction in adipocytes by a protein from tumors. *Progress in Lipid Research* 28: 823-826

Klaus S., Choy L., Champigny O., Cassard-Doulcier A-M., Ross S., Spiegelman B. and Ricquier D. (1994) Characterization of the novel brown adipocyte cell line HIB 1B: Adrenergic pathways involved in regulation of uncoupling protein gene expression. *Journal of Cell Science* 107: 313- 319

Knox L.S., Crosby L.O., Feurer I.D., Buzby G.P., Miller C.L. and Muller J.L. (1983) Energy expenditure in malnourished cancer patients. *Ann Surg* 197: 152-162

Kralovic R.C., Zepp E.A. and Cenedella R.J. (1977) Studies of the mechanism of carcass fat depletion in experimental cancer. *European J of Cancer* 13: 1071- 1079

Kubo S., Matsuda A., Ohnuki T., Hattori K., Suzuki J. and Nagatomo T. (1997) Assessment of β 2-and β 3-adrenoceptors in rat white adipose tissue by radioligand binding assay. *Biol Pharm Bull* 20:142-148

Lackner R.A., Challiss R.A.J., West D. and Newsholme A.A. (1984) A problem in the radiochemical assay of glucose-6-phosphate in muscle. *Biochem. J.*, 218:649-651

Laloli M., Klein M., Riesmeier B., Muller R., Fleury C., Bouillaud F. and Ricquier D. (1997) A plant cold-induced uncoupling protein. *Nature* 389(6647): 135- 136

Lamelli U.K. (1970) Cleavage of structural proteins during the assembly of the heat bacteriophage T4. *Nature (London)*, 227:680-685

Langin D., Portillo M., Saulnier-Blanche J.S. and Lafontan M. (1991) Coexistence of three β -adrenoceptor subtypes in white fat cells of various mammalian species. *Eur J Pharmacol* 199: 291-301

Langstein H.N., Doherty G.M., Fraker D.L., Burest C.M. and Norton J.A. (1991) The roles of γ -interferon and tumor necrosis factor α in an experimental rat model of cancer cachexia. *Cancer Res* 51: 2302-2306

Lanza-Jacoby S., Lansey S.C., Miller E.E. and Cleary M.P. (1984) Sequential changes in the activities of lipoprotein lipase and lipogenic enzymes during tumour growth in rats. *Cancer Res* 44: 5062- 5067

Leibowitz S.F. (1992) Neurochemical-neuroendocrine systems in the brain controlling macronutrient intake and metabolism. *Trends in Neuroscience* 12: 491-497

Llovera M., Lopez-Soriano F.J. and Argiles J.M. (1993) Effects of tumour necrosis factor- α on muscle protein turnover in female wistar rats. *J Natl Cancer Inst* 85: 1334-1339

Llovera M., Garcia-Martinez C., Agell N., Lopez-Soriano F.J. and Argiles J.M. (1995) Muscle wasting associated with cancer cachexia is linked to an important activation of the ATP-dependent-Ubiquitin-mediated proteolysis. *Int J Cancer* 61: 138- 141

Llovera M., Garcia-Martinez C., Costell P., Agell N., Carbo N., Lopez-Soriano F.J. and Argiles J.M. (1996) Muscle hypercatabolism during cancer cachexia is not reverse by the glucocorticoid receptor antagonist RU38486. *Cancer Lett* 99: 7-14

Loprinzi C.L., Michalak J.C., Schaid D.J., Mailliard J.A., Athmann L.M, Goldberg R.M., Tschetter L.K., Hatfield A.K. and Morton R.F. (1993) Phase III evaluation of four doses of megestrol acetate as therapy for patients with cancer anorexia and/or cachexia. *J Clin Oncol* 762-767

Loprinzi C.L., Schaid D.J., Dose A.M., Burnham N.L. and Jensen M.D. (1994) Body composition changes in patients who gain weight while receiving megestrol acetate. *J Clin Oncol* 11: 152- 154

Lorite M.J., Thompson M.G., Drake J.L., Carling G. and Tisdale M.J. (1998) Mechanism of muscle protein degradation induced by a cancer cachectic factor. *Br J Cancer* 78(7): 850- 856

Lorite M.J., Smith H.J., Arnold J.A., Morris A., Thompson M.G. and Tisdale M.J. (2001) Activation of ATP-ubiquitin-dependent proteolysis in skeletal muscle in vivo and murine myoblasts in vitro by a proteolysis-inducing factor (PIF). *Br J Cancer* 85(2): 297- 302

Lovell B.B. and Flier J.S. (1997) Brown adipose tissue, beta3-adrenergic receptors and obesity. *Ann Rev Med Chem* 48: 307-316

Lundholm K., Bylund A.C., Holm J. and Schersten T. (1976) Skeletal muscle metabolism in patients with malignant tumour. *Eur J Cancer*, 12, 465-473

Lundmark L., Bennyard K., Eden E., Ekman L., Schersten T., Svaninger G. and Lundholm K. (1984) Resting energy expenditure in malnourished patients with and without cancer. *Gastroent* 87: 402- 408

Mahony S.M. and Tisdale M.J. (1988) Induction of weight loss and metabolic alterations by human recombinant tumour necrosis factor. *Br. J. Cancer*, 58, 345-349

Maltoni M., Fabbri L., Nanni O., Scarpi E., Pezzi L., Flamini E., Riccobon A., Dernis S., Palliti G. and Amadori D. (1997) Serum levels of tumour necrosis factor alpha and other cytokines do not correlate with weight loss and anorexia in patients. *Support care cancer* 5: 130- 135

Manara L., Badone D., Baroni M., Boccardi G., Cacchi R., Croci T., Giudice A., Guzzi U., Lundi M. and Le Fur G. Functional identification of rat atypical b-adrenoceptors by the first b3-selective antagonist aryloxypropanolaminotetralins. *Br J Pharmacology* 117: 435-442

Marvelis P.G., Ammon H.V., Gleysteen J.J., Komorowski R.A. and Charaf U.K. (1983) Hepatic free fatty acids in alcoholic liver disease and morbid obesity. *Hepatology* 3: 226- 231

Masuno H., Yoshimura H., Ogawa N. and Okuda H. (1984) Isolation of lipolytic factor (toxohormone-L) from ascites fluid of patients with heptoma and its effect on feeding behaviour. *Eur J cancer Clin Oncol* 20: 1177-1185

Matthys P., Heremans H., Opendalker G. and Billiau A. (1991) Anti-interferon- γ antibody treatment, growth of lewis lung tumours in mice and tumour associated cachexia. *Eur J Cancer* 27: 182-7

Matsuda J., Husoda K., Itoh H., Son C., Doi K., Tanaka T., Fukunaga Y., Inoue G., Nishimura M., Yoshimasa Y., Yamari Y. and Nakoa K., (1997) Cloning of rat uncoupling protein-3 and uncoupling protein-2 cDNAs : their gene expression in rats fed high-fat diet. *FEBS Lett* 412: 111- 114

M^cDevitt T.M., Todorov P.T., Beck S.A., Khan S.H. and Tisdale M.J. (1995) Purification and characterisation of a lipid-mobilising factor associated with cachexia-inducing tumours in mice and humans. *Cancer Research*, 55, 1458-1463

M^cDevitt T. (1996) Purification and characterisation of 2 cancer cachexia factors. PhD Thesis Aston University

Meier M.K., Alig L., Burgi-Saville M.E. and Muller M. (1984) Phenylethanolamine derivatives with calorogenic and antidiabetic qualities. *Int J Obest* 8 (sup1): 215-225

Meszaros K., Bagby G.J., Lang C.H. and Spitzer J.J. (1987a) Increased uptake and phosphorylation of 2-deoxyglucose by skeletal muscles in endotoxin-treated rats. *Am J Physiol* 253: E33-39

Meszaros K., Lang C.H., Bagby G.J and Spitzer J.J. (1987b) Contribution of different organs to increased glucose consumption after endotoxin administration. *J Biol Chem* 262: 10965-10970

Mezey E. (1991) Interactions between alcohol and nutrition in the pathogenesis of alcoholic liver disease. *Semin Liver Dis* 11: 340- 348

Ming S., Chen S., Chen W-J., Eng H-L. and Chen F-F (1997) Serum concentration of tumour necrosis factor in patients with breast cancer. *Breast Cancer Res Treat* 43: 21- 215

Mori M., Yamaguchi W., Honda S., Nagasaki K., Ueda M. and Abe O. (1991) cancer cachexia syndrome developed in nude mice bearing melanoma cells producing leukaemia-inhibiting factor. *Cancer Res* 51: 6656-6659

Morrison S.D. (1984) Contributions of reduced hunger and premature satiety to cancerous hypophagia in rats. *Cancer Res* 44: 1041

Muller B.J., Harris R.B.S., Patton J.S. and Martin R.T. (1990) Recombinant tumour necrosis factor α chronically administered in rat: lack of cachectic effect. *Proc Soc Exp Biol Med* 193: 318-325

Mulligan H.D. (1991) Mechanisms of tumour-induced cachexia. PhD Thesis Aston University.

Mulligan H.D. and Tisdale M.J. (1991) Metabolic substrate utilization by tumour and host tissues in cancer cachexia. *Biochem Journal* 277: 321-326

Mulligan K. and Bloch A.S. (1998) Energy expenditure and protein metabolism in human immunodeficiency virus and cancer cachexia. *Semin Oncol* 25(2, Suppl 6): 82-91

Muramayma T and Ui M (1983) Loss of the inhibitory function of guanine regulatory component of adenylate cyclase due to its ADP-ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J Biol Chem* 258: 3319-3326

Negre-Salvayre A., Hirtz C., Carrera G., Cazenave R., Trolly M., Salvayre R., Penicaud L. and Casteilla L. (1997) A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J* 11: 809- 815

Negri D.R., Mezzananza D., Sacco S., Gadina M., Benigni F., Cajola L., Finocchiaro G., Ghezzi P. and Canevari S. (2001) Role of cytokines in cancer cachexia in a murine model of intracerebral injection of human tumours. *Cytokine* 15(1): 27-38

Nicholls D.G. and Rial E. (1984) Brown fat mitochondria. *TIBS* 2: 489-91

Nisoli E., Tonello C., Landi M. and Carruba M.O. (1996) Functional studies of the first selective β_3 -adrenergic receptor antagonist SR59230A in rat brown adipocytes. *Mol Pharmacol* 49: 7-14

Nixon D.W., Heymsfield S.B., Cohen A.E., Kutner M.J., Ansley J., Lawson D.H. and Rudman D. (1980) Protein-caloric undernutrition in hospitalised cancer patients. *Am J Med* 68: 683- 690

Nolop K.B., Rhodes C.G., Brudin L.H., Beaney R.P., Kravsz T., Jones T. and Hughes J.M. (1987) Glucose utilisation in vivo by human pulmonary neoplasm. *Cancer*, 43, 2682-2689

Oberkofler H., Liu Y-M., Hell E., Krempler F. and Patsch W. Uncoupling protein gene quantification of expression levels in adipose tissue of obese and non-obese humans. *J of Lipid Research* 38: 2125- 2133

Okube Y., Sakata T., Kurokawa M., Fujimoto K., Ueda K., Masuno H. and Okuda H. (1992) Anorexia induced by toxohormone-L isolated from ascites fluid of patients with hepatoma. *Physiology and Behaviour* 52: 333- 337

Oller do Nascimento C.M. and Williamson D.H. (1986) Evidence for conservation of dietary lipid in the rat during lactation and the immediate period after removal of the litter: Decreased oxidation of oral (1-¹⁴C)-triolein. *Biochem. J.*, 239, 233-236

Oudart H., Calgari C., Andriamampandry M., LeMaho Y. and Malan A. (1995) Stimulation of brown adipose tissue activity in tumour-bearing rats. *Can J Physio Pharmacol* 73: 1625-1631

Patton J.S., Shepard H.M., Wilking H., Lewis G., Aggareval B.B. and Eessalu T.E. (1986) Interferons and tumor necrosis factors have similar catabolic effects on 3T3-1 cells. *Proc Nat Acad Sci USA* 83: 8313-8317

Pecqueur C., Alves-Guerra M.C., Gelly C., Levi-Meyruesis C., Couplan E., Collins S., Ricquier D., Bouillaud F. and Miroux B. *Journal of Biological Chemistry* 276 (12): 8705-8712

Price SA (1997) The role of eicosapentaenoic acid in cancer cachexia. PhD Thesis Aston University

- Puccio M. and Nathanson L. (1997) The cancer cachexia syndrome. *Semin Oncol* 24: 277- 287
- Rashid A., Wu T.C., Huang C.C., Chen D.H., Lin H.Z., Yang S.Q., Lee F.Y., Diehl A.M. (1999) Mitochondrial proteins that regulate apoptosis and necrosis are induced in mouse fatty liver. *Hepatology* 29: 1131- 1138
- Ricquier D. and Kader J.C. (1976) Mitochondrial protein alteration in active brown fat: a SDS polyacrylamide gel electrophoretic study. *Biochem Biophys Res Commun* 73: 577- 583
- Ricquier D., and Bouillaud F. (2000) The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem J* 345: 161- 179
- Roe S., Cooper A.L., Morris I.D. and Rothwell N.J. (1996) Mechanisms of cachexia induced by T-cell leukaemia in the rat. *Metabolism* 45: 645- 651
- Rofe A.M., Bourgeois C.S., Coyle P., Taylor A. and Abdi E.A. (1994) Altered insulin response to glucose in weight-losing cancer patients. *Anticancer Res* 4: 647-650
- Rolfe D.F. and Brand M.D. (1997) The physiological significance of mitochondrial proton leak in animal cells and tissues. *Biosci Rep* 17: 9-16
- Russell S.T., Tisdale M.J. and Bing C. (2000) Regulation of uncoupling protein-1 and -2 gene expression by a lipid mobilizing factor. *Br J Cancer (Suppl July)* A212 (Abstract)
- Salomon Y., Londos C. and Rodbell M. (1973) A highly sensitive adenylate cyclase assay. *Anal Biochem* 58: 541-548

Sanchis D., Busquets S., Alvarez B., Ricquier D., Lopez-Soriano F.J. and Argiles J.M. (1998) Skeletal muscle UCP2 and UCP3 gene expression in a rat cancer cachexia model. FEBS Letters 436: 415- 418

Sanchez L.M., Chirino A.J. and Bjorkman P.J. (1999) Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. Science 283: 1914- 1919

Sauer L.A. and Dauchy R.T. (1987) Blood nutrient concentrations and tumour growth in vivo in rats: Relationships during the onset of a acute fast. Cancer Res 47: 1065-1068

Shapot V.S. (1972) Some biochemical aspects of the relationship between the tumour and the host. Adv Cancer Res 15: 253-285

Smith K.L. and Tisdale M.J. (1993b) Increased protein degradation and decreased protein synthesis in skeletal muscle during cancer cachexia. Br J Cancer 67: 682- 685

Smith K.L. and Tisdale M.J. (1993) Mechanism of muscle protein degradation in cancer cachexia. Br J Cancer 68: 314- 318

Socher S.H., Martinez D., Craig J.B., Kuhn J.G. and Oliff A. (1988) Tumour necrosis factor not detectable in patients with clinical cancer cachexia. J of the National Cancer Institute 80: 595-598

Sokoloff L., Reivich M., Kennedy C., Des Rosiers M.H., Patlak C.S., Pettigrew K.D., Sakurada O. and Shinohara M. (1977) The (¹⁴C)-Deoxyglucose method for the measurement of local cerebral glucose utilisation: Theory procedure and normal values in the conscious and anaesthetised albino rat. J. Neurochemistry, 28, 897-916

Somogyi M. (1945) Determination of blood sugar. J. Biol. Chem, 160, 69-73

Stansbie D., Brownsey R.W., Crettaz M. and Denton R.M. (1976) Acute effects *in vivo* of anti-insulin serum on rates of fatty acid synthesis and activities of acetyl-Coenzyme A carboxylase and pyruvate dehydrogenase in liver and epididymal adipose tissue of fed rats. *Biochem. J.* 160 413- 416

Strassman G., Jacob C.O., Evans R., Beall D. and Fong M. (1992) Mechanisms of experimental cancer cachexia. Interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance in IL-6 mediated cancer cachexia. *J Immunol* 148: 3674- 3679

Strassman G., Masui Y., Chizzonite R. and Fong M. (1993) Mechanisms of experimental cancer cachexia: local involvement of IL-1 in colon-26 tumour. *J Immunol* 150: 2341- 2345

Stryer L. (1988) *Biochemistry* 3rd Edition. W.H. Freeman and Company, New York, p445

Stuart J.A., Harper J.A., Brindle M.D. and Brand M.D. (1999) Uncoupling protein 2 from carp and zebrafish, ectothermic vertebrates. *Biochimica et Biophysica Acta* 1413: 50- 54

Stuart J.A., Harper J.A., Brindle K.M., Jekabsons M.B. and Brand M.D. (2001) Physiological levels of mammalian uncoupling protein 2 do not uncouple yeast mitochondria. *Journal of Biological Chemistry* 276 (21): 18633-18639

Suematsu S., Matsusaka T., Matsuda T., Ohno S., Miyazaki J., Yamamura K., Hirano T. and Kishimoto T. (1992) Generation of plasmocytomas with the chromosomal translocation t(12,15) in interleukin 6 transgenic mice. *Proc Natl Acad Sci USA* 89: 232-235

Svaniger G., Bennegard K., Ekman L., Ternell M. and Lundholm K. (1983) Lack of evidence for elevated breakdown rate of skeletal muscle in weight losing, tumour-bearing mice. *J Nat Cancer Inst* 71: 341

Tada T., Ohkubo I., Niwa M., Sasaki M., Tateyama H. and Eimoto T. (1991) Immunohistochemical localisation of Zn-alpha-2-glycoprotein in normal human tissue. *J Histochem Cytochem* 39: 1221-1226

Taga T. and Kishimoto T. (1993) Cytokine receptors and signal transduction. *FASEBJ* 7:3387-3396

Tang D.G., Chen Y.O. and Honn K.V. (1996) Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. *Proc Natl Acad Sci USA* 93: 5241

Taylor D.D., Gercel-Taylor C., Jenis L.G. and Devereux D.F. (1992) identification of a human tumour-derived lipolysis promoting factor. *Cancer Res* 82: 829-834

Theologides A. (1976) Anorexia-producing intermediary metabolites. *American J of Clinical Nutrition* 29: 552- 558

Thompson M.P., Koons J.E., Tan E.T.H. and Grigor M.R. (1981) Modified lipoprotein lipase activities, rates of lipogenesis and lipolysis as factors leading to lipid depletion in C57BL-mice bearing the preputial gland tumour ESR-586. *Cancer Res* 41: 3228- 3232

Thompson M.P., Cooper S.T., Parry B.R. and Tuckey J.A. (1993) Increased expression of the mRNA for the hormone-sensitive lipase in adipose tissue of cancer patients. *Biochim Biophys Acta* 1180: 236-242

Tisdale M.J. and Beck S.A. (1990) Cancer cachexia. *International Journal of Pancreatology*. The Humana Press 141-150

Tisdale M.J. and Beck S.A. (1991) Inhibition of tumour-induced lipolysis in vitro and cachexia and tumour growth in vivo by eicosapentaenoic acid. *Biochem Pharmacol* 41: 103- 107

Tisdale M.J. (1997) Isolation of a novel cancer cachectic factor. Proc Nutr Soc 56:
777-783

Tisdale M.J. (1998) New cachectic factors. Curr Opin Clin Nutr Metab Care 1:
235-256

Todorov P., Cariuk P., McDevitt T., Fearon K. and Tisdale M.J. (1996)
Characterisation of a cancer cachectic factor. Nature 379: 739- 742

Todorov P.T., Deacon M. and Tisdale M.J. (1997) Structural analysis of a tumour-
produced sulfated glycoprotein capable of initiating muscle protein degradation. J
Biol Chem 272: 12279- 12288

Todorov P.T., McDevitt T.M., Meyer D.J., Ueyama I., Ohkubo I. and Tisdale M.J.
(1998) Purification and characterization of a tumour lipid mobilizing factor.
Cancer Res 58:541-548

Todorov P.T., McDevitt T.M. Meyer D.J., Ueyama H., Ohkubo I. And Tisdale
(1998b) Purification and characterisation of a tumour lipid-mobilising factor.
Cancer Research 58: 2353- 2358

Tsujinaka T., Ebisui C., Fujita J., Kischibuchi M., Morimoto T., Ogana A.,
Katswe A., Ohsugi Y., Kominami E. and Monda M. (1995) Muscle undergoes

atrophy in association with increase of lysosomal cathepsin activity in interleukin-6 transgenic mouse. *Biochem Biophys Res Com* 207: 168-174

Tsujinaka T., Fujita J., Ebisui C., Yano M., Kominami E, and Suzuki K. (1996) Interleukin 6 receptor antibody inhibits muscle atropy and modulates proteolytic systems in interleukin 6 transgenic mice. *J Clin Invest* 97: 244-249

Wang Z., Hass G.M., Tisdale M.J. and Vessella R. (2001) Differential expression of the human cachexia-associated protein (HCAP) gene and protein in prostate cancer. *Proceedings of the AACR 42: Abstract 1418*

Waterhouse C., Jean petre N. and Keilson J. (1979) Gluconeogenesis from alanine in patients with progressive malignant disease. *Cancer Res* 39: 1968- 1972

Weigle D.S., Selfridge L.E., Schwartz M.W., Seeley R.J., Cummings D.E., Havel P.J., Kujiper J.L. and Beltrandelrio H. (1998) Elevated free fatty acids induce uncoupling protein-3 expression in muscle. *Diabetes* 47: 298-320

Wieland O. (1974) Glycerol. UV-method. *Methods of enzymatic analysis*, volume 3, pp.1404- 1409. Ed. Bergmeyer H.U.

Wigmore S.J., Ross J.A., Falconer J.S., Plester C.E., Tisdale M.J., Carter D.C. and Fearon K.C.H. (1996) The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer. *Nutr* 12(1): 527- 530

Wing S.S. and Goldberg A.L. (1993) Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *Am J Physiol* 264: E668-676

Yasumoto K., Mukaida N., Harada A., Kuno K., Akiyama M., Nakashima E., Fujioka N., Moi M., Kasahara t., Fujimototoouchi K., Mori K., Tanak Y. and Matsushima K. (1995) Molecular analysis of the cytokine network involved in cachexia in colon-26 adenocarcinoma-bearing mice. *Cancer Res* 55: 921- 927

Yoshitomi H., Yamazaki K., Abe S. and Tanaka I. (1998) Differential regulation of mouse uncoupling proteins among brown adipose tissue, WAT, and skeletal muscle in chronic beta 3 adrenergic receptor agonist treatment. *Biochim Biophys Res Commun* 253: 85- 91

Zhou Y.T., Wang Z.W., Higa M., Newgard C.B., Unger R.H. (1999) Reversing adipocyte differentiation: implications for treatment of obesity. *Proc. Natl. Acad. Sci. USA* 96, 2391-2395

Appendix

Publications

Role of β -3 adrenergic receptors in the action of a tumour lipid mobilizing factor.
S.T. Russell, K. Hirai and M.J. Tisdale, British journal of cancer 86, 424-428
(2002)

Expression of uncoupling protein-1, -2 and -3 mRNA is induced by a
adenocarcinoma-derived lipid-mobilizing factor. C.Bing, S.T. Russell, E.B.
Beckett, P. Collins, S. Taylor, R. Barraclough, M.J. Tisdale and G. Williams.
British journal of cancer 86, 612-618 (2002)

Effect of a tumour-derived lipid-mobilizing factor on glucose and lipid
metabolism *in vivo*. S.T. Russell and M.J. Tisdale. British journal of cancer, *in*
press

Abstracts

Increased gene expression of brown fat UCP1 and skeletal muscle UCP2 and UCP3 in cancer cachexic mice. C. Bing, M. Brown, P. King, S. Russell, P. Collins, M.J. Tisdale and G. Williams, Medical research society, Autumn meeting 1999.

Regulation of uncoupling protein-1 and -2 gene expression by a lipid mobilizing factor. S.T. Russell, M.J.Tisdale and C. Bing, BACR 41st Annual Scientific Meeting, Brighton, 2000.

Tumour derived factor produces up regulation of uncoupling protein-1 and -2 expression via the beta-3 Adrenoceptor. S.T. Russell, M.J. Tisdale and C. Bing. American association for cancer research, annual meeting 2001.

The role of β 3-adrenergic receptors in cancer cachexia. S.T. Russell, K Hirai and M.J. Tisdale. BACR 42nd Annual Scientific Meeting , Leeds, 2001.

INCREASED GENE EXPRESSION OF BROWN FAT UCP1 AND SKELETAL MUSCLE UCP2 AND UCP3 IN CANCER CACHEXIC MICE. ¹C. Bing, ¹M. Brown, ¹P. King, ²S. Russell, ¹P. Collins, ²M.J. Tisdale and ¹G. Williams. ¹Department of Medicine, University of Liverpool, Liverpool L69 3GA, UK., ²Biomedical Sciences Research Institute, Aston University, Birmingham B4



Aston University

Content has been removed for copyright reasons

REGULATION OF UNCOUPLING PROTEIN -1 AND -2 GENE
EXPRESSION BY A LIPID MOBILIZING FACTOR. ST Russell¹, MJ Tisdale¹
and C Bing², ¹Pharmaceutical Sciences Research Insitute, Aston University,
Birmingham B4 7ET. ²Department of Medicine, University of Liverpool,
Liverpool L69 3GA, UK.

ers in



Aston University

Content has been removed for copyright reasons

TUMOUR DERIVED FACTOR PRODUCES UP REGULATION OF UNCOUPLING PROTEIN-1 AND -2 EXPRESSION VIA THE BETA-3 ADRENOCEPTOR. ST Russell¹, MJ Tisdale¹ and C Bing², ¹Pharmaceutical Sciences Research Institute, Aston University, Birmingham B4 7ET. ²Department of Medicine, University of Liverpool, Liverpool L69 3GA, UK.

with loss



Aston University

Content has been removed for copyright reasons

THE ROLE OF β_3 -ADRENERGIC RECEPTORS IN CANCER CACHEXIA.
S.T. Russell¹, K Hirai² and M.J. Tisdale¹.¹Pharmaceutical Sciences Research
Institute, Aston University, Birmingham B4 7ET.² Department of Obstetrics and
Gynaecology, Osaka City University Medical School, Osaka 545-8585, Japan.

the



Aston University

Content has been removed for copyright reasons



Role of β_3 -adrenergic receptors in the action of a tumour lipid mobilizing factor



Aston University

Content has been removed for copyright reasons



Expression of uncoupling proteins-1, -2 and -3 mRNA is induced by an adenocarcinoma-derived lipid-mobilizing factor



Aston University

Content has been removed for copyright reasons