Second Messenger Pathways in the Action of Cachectic Factors

Bilkis Sultana Islam-Ali

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Aston University

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SUMMARY

Cachexia in cancer is characterised by progressive depletion of both adipose tissue stores and skeletal muscle mass. Two catabolic factors produced by cachexia-inducing tumours have the potential for inducing these changes in body composition: (i) proteolysis-inducing factor (PIF) which acts on skeletal muscle to induce both protein degradation and inhibit protein synthesis, (ii) lipid-mobilising factor (LMF), which has been shown to directly induce lipolysis in isolated epididymal murine white adipocytes.

Administration of LMF to mice produced a specific reduction in carcass lipid with a tendency to increase non-fat carcass mass. Treatment of murine myoblasts, myotubes and tumour cells with tumour-produced LMF, caused concentration dependent stimulation of protein synthesis, within a 24hr period. It produced an increase in intracellular cyclic AMP levels, which was linearly related to the increase in protein synthesis. The observed effect was attenuated by pretreating cells with the adenylate cyclase inhibitor, MDL_{12330A} and was additive with stimulation produced by forskolin. Both propranolol and a specific β_3 adrenergic antagonist SR59230A, significantly reduced the stimulation of protein synthesis induced by LMF. LMF also affected protein degradation *in vitro*, as demonstrated by a reduction in proteasome activity, a key component of the ubiquitin-dependent proteolytic pathway. These effects were opposite to those produced by PIF which caused both a decrease in the rate of protein synthesis and an elevation on protein breakdown when incubated *in vitro*.

Incubation of LMF with a fat cell line produced alterations in the levels of guanine-nucleotide binding proteins (G proteins). This was also evident in adipocyte plasma membranes isolated from mice bearing the tumour model of cachexia, MAC16 adenocarcinoma and from patients with cancer cachexia. Progression through the cachectic state induced an upregulation of stimulatory G proteins paralleled with a downregulation of inhibitory G proteins. These changes would contribute to the increased lipid mobilisation seen in cancer cachexia.

KEY WORDS: LIPID-MOBILISING FACTOR, PROTEIN SYNTHESIS, ADENYLATE CYCLASE, GUANINE NUCLEOTIDE BINDING PROTEINS, PROTEIN DEGRADATION.

"Proclaim!
In the name of thy Lord and Cherisher,
Who created
Created man, out of a (mere) clot of congealed Blood:
Proclaim! And thy Lord Is Most Bountiful
He Who taught (the use of) the Pen
Taught man that Which he knew not,
Nay, but man doth Transgress all bounds,
In that he looketh Upon himself as self-sufficient,
Verily, to thy Lord Is the return of All "

Qur'an: Surah Al-Alaq 96: 1-8

For you, Mum and Dad

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ABBREVIATIONS

A site	Amino acyl transfer ribonucleic acid binding site
AA	Arachidonic acid
AC	Adenylate cyclase
ADP	Adenosine-5'-diphosphate
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine 5'-monophosphate
AP-1	Activator protein-1
APPs	Acute phase proteins
APPR	Acute-phase protein response
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BAA	β-adrenergic agonist
BAT	Brown adipose tissue
BCAA	Branched chain amino acids
Bq	Becquerels
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
C2/3/8/9	Proteasome subunit C2/3/8/9
CKII	Casein kinase II
CCK	Cholecystokinin
CHO	Chinese hamster ovary
Ci	Curies
cm	centimetre
CNS	Central nervous system

CRE	Cyclic-AMP response element
CRF	Chronic renal failure
CoE	Coenzyme A
COX	Cyclo-oxygenase
CRH	Corticotrophin releasing hormone
CRP	C-reactive protein
Dbt-cAMP	Dibutyryl-cAMP
DEX	Dexamethasone
DMSO	Dimethysulphoxide
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β aminoethylether) N,N,N'N' tetraacetic acid
eEF	Eukaryotic elongation factor
EGF	Epidermal growth factor
eIF	Eukaryotic initiation factor
E-site	Ejection site
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular regulated kinase
FCS	Foetal calf serum
g	gram

GLUT-1/4	Glucose transporter-1/4
GSK	Glycogen synthase kinase
G-protein	Guanine nucleotide binding protein
GDP	Guanine 5'-diphosphate
GI	Gastrointestinal
GTP	Guanine 5'-diphosphate
h	Hour
HCR	Haemin-controlled repressor
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HSL	Hormone-sensitive lipase
IBMX	3-isobutyl-1-methyl xanthine
IFN-γ	Interferon γ
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iv	Intravenous
l	Litre
LMF	Lipid-mobilising factor
LPL	Lipoprotein lipase
kg	Kilogram
Ks	Fractional rate of protein synthesis
μ	Micro
M	Moles per litre

m	Milli
MAP-kinase	Mitogen-activated protein kinase
mAb	Monoclonal antibody
MAC	Murine adenocarcinoma of the colon
MEK	MAP kinase inhibitor
met-tRNA	Methionyl-transfer ribonucleic acid
3-MH	3-Methylhistidine
min	Minute
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide-reduced form
NF-κB	Nuclear factor κB
NSAID	Non steroidal anti-inflammatory
ODC	Ornithine carboxylase
om	Omental
PAGE	Polyacrylamide gel electrophoresis
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PCA	Perchloric acid
PEP	Phosphoenolpyruvate
PG	Prostaglandin
PHAS-1	Phosphorylated heat and acid stable protein-1
PIF	Proteolysis-inducing factor
PI	Phosphatidylinositol
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C

PLA_2	Phospholipase A_2
PLC	Phosholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulphonylfluoride
PP2A	Protein phosphatase 2A
PUFA	Polyunsaturated fatty acid
QS	Q sepharose
REE	Resting energy expenditure
RF	Releasing factor
mRNA	(messenger) ribonucleic acid
rpm	Revolutions per minute
$p90^{rsk}$	90kDa ribosomal kinase
$p70^{s6k}$	70kDa S6 protein kinase
$p85^{s6k}$	85kDa s6 protein kinase
S6	Ribosomal S6 subunit
sc	Subcutaneous
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sr	Specific radioactivity
SRE	Serum response element
SRF	Serum response factor
TAG	Triacylglycerols
TCA	Trichloroacetic acid
TEMED	N,N,N'N'-Tetramethylethylenediamine
TG	Triglycerides

TNFα	Tumour necrosis factor-α
TPA	Tetradecanoylphorbol-13-acetate
TRE	TPA response element
TPN	Total parenteral nutrition
Tris	Tris (hydroxymethy) methylamine
Tween 20	Polyoxyethylene-sorbitan
U	Unit
UTR	5'-untranslated region
Ub	Ubiquitin
WAT	White adipose tissue
YAH	Yoshida ascites hepatoma
Zn-α2-gp/ZAG	Zinc-α2-glycoprotein

CHAPTER 1

Cancer Cachexia: An Overview

1.1 Introduction

Cancer cachexia (derived from the greek words *kakos* meaning "bad" and *hexis* meaning "condition") is a complex syndrome encompassing a wide range of metabolic, hormonal and cytokine-related abnormalities that result in a wasting syndrome. It occurs in more than two thirds of patients who die with advanced cancer and, according to Warren (1932), it is responsible for the death of 22% of cancer patients. It is common in a variety of neoplastic diseases but is particularly prevalent in patients with pancreatic and gastric cancer. Patients with cachexia have an unfavourable performance status, a decreased response to chemotherapy and a shorter survival time compared with cancer patients not displaying cachexia (De Wys 1985). A 30% weight loss is frequently fatal, although a few patients may survive as much as 50% loss of body weight.

Weight loss in cancer patients differs from that found in simple starvation, where more than three-quarters of the weight is lost from body fat and only a small amount from muscle. In cancer patients weight loss arises equally from loss of muscle and fat (Cohn *et al* 1981). Thus, for a given degree of weight loss, there is more wasting of muscle in the cancer patient than in the normal subject. In addition, in cancer cachexia depletion of skeletal muscle exceeds that of visceral mass. In anorexia nervosa, the loss of visceral mass is proportional to the loss of muscular tissues, while in cancer only significant changes in liver, kidney, and heart weight correspond to marked depletion of muscle mass (Heymsfield and McManus 1985). The spleen of cancer patients is always enlarged.

1.2 Anorexia and Cachexia

Although anorexia is invariably present in the cachectic cancer patient this alone does not appear to be responsible for the loss of body weight. Thus, in both rat and man, loss of both muscle and adipose tissue precedes the fall in food intake (Costa 1977) and in malnourished cancer patients the measured 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 and its adverse influence on clinical outcome (Grosvenor *et al* 1989). Even administration of total parenteral nutrition (TPN) has failed to give long-term stabilisation of body weight (Evans *et al* 1985). A transient weight gain has been observed with TPN, but this is due to an increase in fat and water rather than skeletal muscle mass. This is exemplified by studies with the progestational agent megestrol acetate (Megace), an appetite stimulant, which has been reported to induce a weight gain of greater than 5% in 15% of the patients treated (Loprinzi *et al* 1993a). Body composition analysis, however, revealed that increases in lean body mass were not generally observed and that the weight gain arose from an increase in adipose tissue and possibly an increase in body fluid (Loprinzi *et al* 1993b).Thus cancer cachexia is a more complex aetiology than a simple calorie deficiency and its successful reversal will require detailed knowledge of the mechanisms involved (Figure 1.1).

1.3 Clinical implications of cancer cachexia/manifestations

The development of cachexia has profound implications for the cancer patient. Clinically observable manifestations of cachexia include weight loss, anorexia, weakness, muscle atrophy, easy fatigue, impaired immune function, decreased motor

and mental skills, and a decline in attention span and concentration abilities (Lindsey 1986).

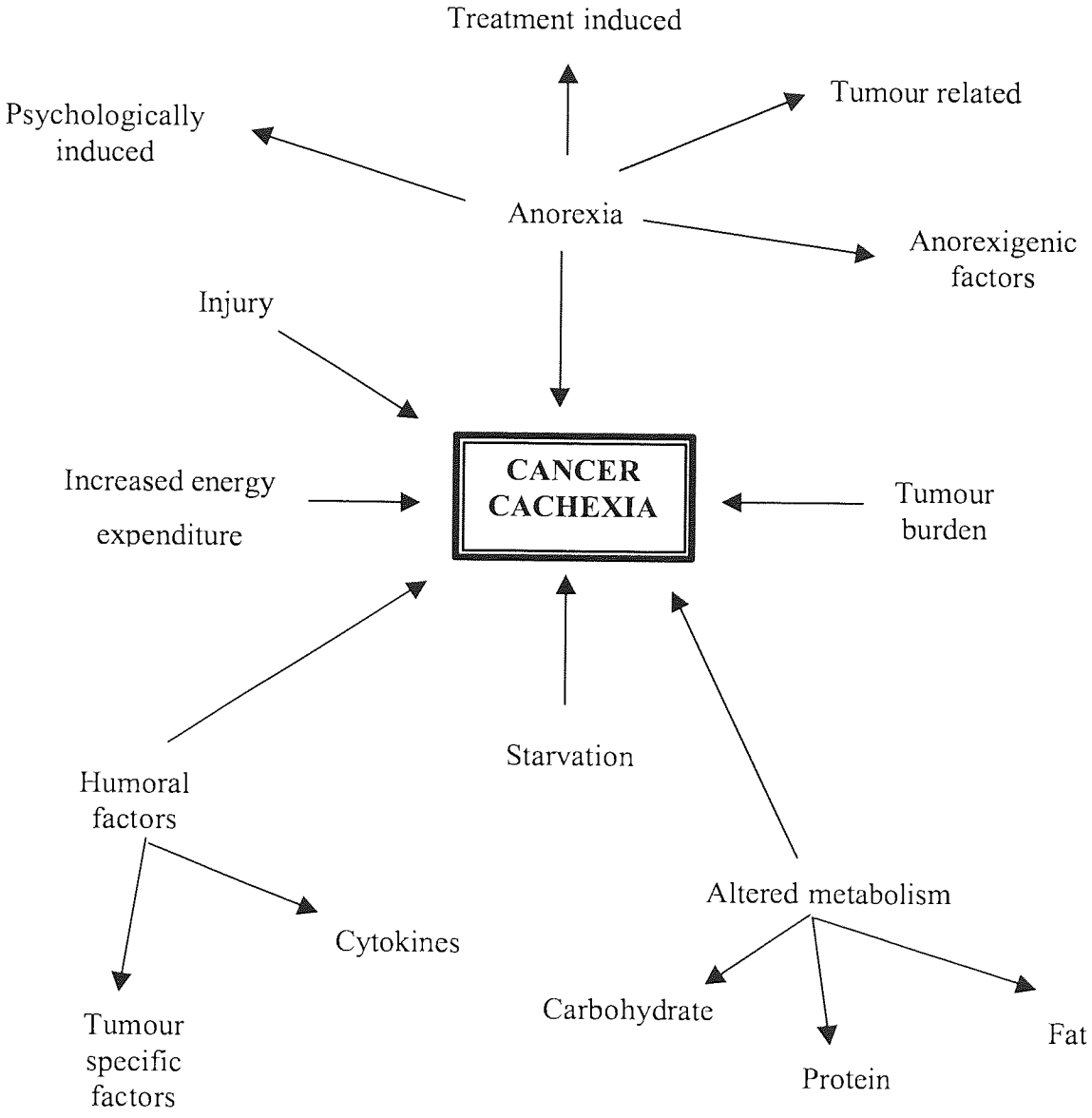


Figure 1.1. Mechanisms contributing to cancer cachexia (taken from S. Khan 1996).

Changes in appetite, food intake, and eating patterns may be noticed. In addition to weight loss, muscle wasting and loss of body fat become evident. It is the weight loss that compels patients to seek medical attention. Loss of skeletal muscle is the most important factor in the poor prognosis of the cancer patient. Its loss results not only in immobility and poor quality of life, but also in death. The cachectic individual appears physically ill as progressive asthenia and emaciation occurs. With progressive muscle wasting, there is weakness of respiratory muscles and deterioration of the individuals respiratory capacity. If cachexia is not reversed, death occurs (Lindsey 1986).

Multi-organ dysfunction is due partially to the nutritional and metabolic abnormalities which occur in these patients and is, in part independent of these phenomena. Death from malignancy does not occur by a direct, mechanical effect of the tumour. Instead, death from cancer occurs by systemic effects induced by the malignancy i.e. a type of paraneoplastic syndrome (Albrecht and Canada 1996). Sepsis associated with cancer represents the most common cause of malignancy-associated mortality in the cancer patient (Torosian 1995).

1.4 Metabolic alterations in patients with cancer cachexia

In chronic starvation, the basal metabolic rate is reduced as the body adapts to conserve tissues and energy in a low-protein, low-calorie environment (Knox *et al* 1983). However, in cancer patients there appears to be a variation in changes in energy expenditure. Tumour type appears to be the major determinant of an increased energy expenditure, e.g. an elevation in energy expenditure has been described in lung, gastric, pancreatic, sarcoma, hepatocellular cancer and acute lymphatic leukaemia (Fredrix *et al* 1991, Harvie and Campbell 2000), but only in a proportion of these patients. Metabolic

rate in weight losing patients with cancer of the colon, and oesophagus and in metastatic breast cancer and metastatic melanoma, where weight loss is not a feature, is normal. Thus while resting energy expenditure (REE) in lung cancer patients was found to be significantly increased compared with healthy controls, patients with gastric and colorectal cancer had no elevation of the REE (Fredrix *et al* 1990). There were no significant differences in energy intake between the cancer groups and the normal subjects. Falconer *et al* (1994) also reported that patients with pancreatic cancer had an increased REE relative to controls and the REE was significantly greater in patients with an acute phase response. This might explain why within a particular type of cancer REE is increased in some patients but not in others.

In some cases, hypermetabolism can be explained by loss of peripheral tissue, and a relative preservation of the metabolically more active 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.

1.4.1 Carbohydrate Metabolism.

In normal adaptation to starvation, the body uses glycogen stores in the liver and muscles to provide the obligatory energy required by the brain, leukocytes, and other tissues (Mulligan and Bloch 1998). After those stores are depleted, muscle protein initially provides the source of fuel but is gradually replaced by fat and fatty acids that are converted to ketone bodies. Ketone bodies can provide up to 95% of the fuel needed by the brain, sparing glucose and muscle protein. The cancer patient may not be able to use this adaptive mechanism. Thus, continuing glucose production and protein catabolism provides the requisite energy.

There are marked alterations in carbohydrate metabolism in the tumour-free tissues of patients with cancer, in particular the liver, which probably arises from the utilisation of glucose by the tumour as the primary energy source (Holm *et al* 1995) (Figure 1.2). The outpouring of lactic acid by some tumours leads to an increase in the conversion of lactate to glucose by the liver. This process, the Cori cycle, is energy consuming, since conversion of 2 mol of lactate to glucose requires 6 mol of ATP, while only 2 mol of ATP are recovered in the conversion of the glucose back to lactate (Tisdale 1997). This vicious cycle is detrimental, causing energy wastage and ineffective consumption of glucose. Cori-cycle activity has been found to increase from 20% in normal subjects to 50% of glucose turnover in cachectic cancer patients and accounts for the disposal of 60% of the lactate produced by the tumour (Holroyde *et al* 1975). In addition to lactate, other substrates contribute to the enhanced gluconeogenesis. Thus, glycerol (released as a consequence of adipose tissue activated lipolysis), and amino acids (released form skeletal muscle as a result of activated proteolysis), are important gluconeogenic substrates in the tumour bearing host, and contribute to the augmented hepatic glucose production, invariably associated with 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), indicative of either insulin resistance or a decreased pancreatic function. Glucose intolerance due to insulin resistance is common and may predate weight loss. Cancer patients also have an increased glucose flux, which could consume up to 40% of the ingested carbohydrate and may contribute to the weight loss (Burt and Brennan 1984). Thus, alterations in carbohydrate metabolism could contribute to the syndrome of cancer cachexia.

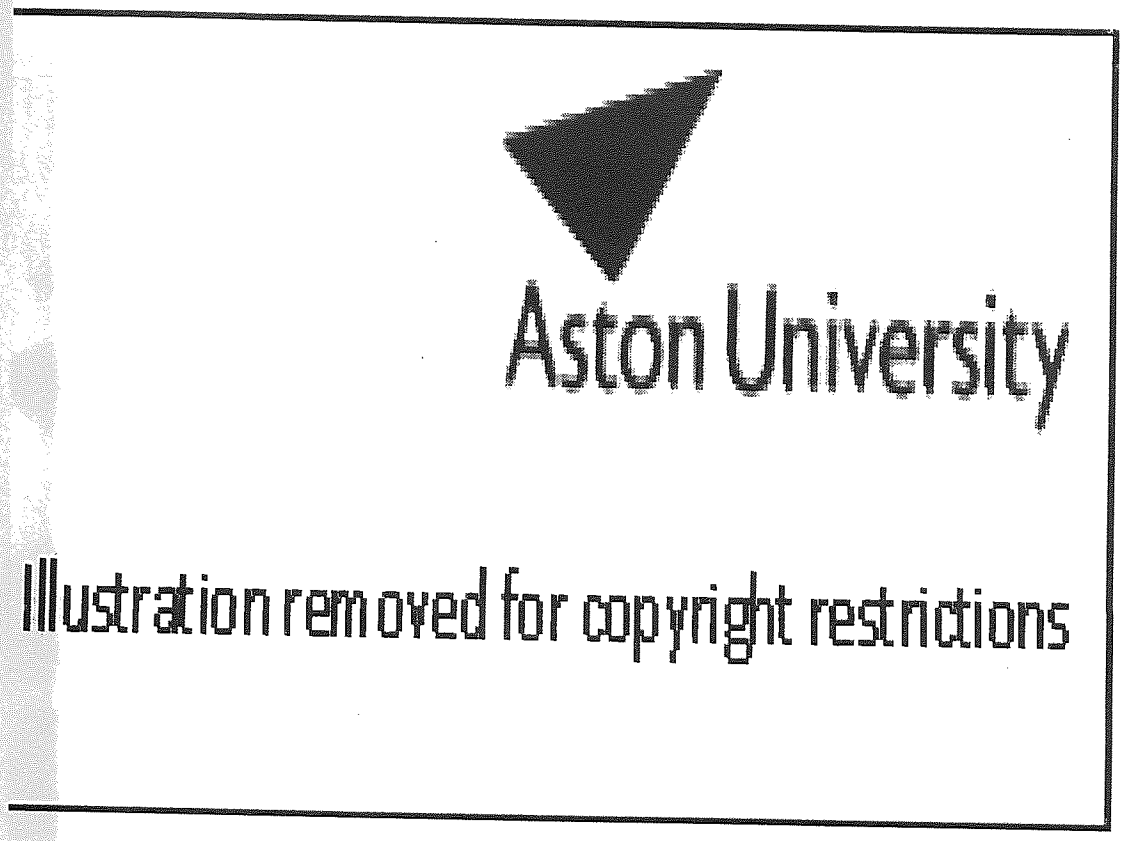


Figure 1.2. Metabolic interactions between tumour and host (taken from Argiles *et al* 1997). The three main metabolic trends associated with cancer cachexia are: (1) lactate recycling, which is asscociated with increased liver gluconeogenesis and thus Cori cycle activity; (2) lipid mobilisation, due 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.

1.4.2 Lipid Metabolism

Fat constitutes 90% of adult fuel reserves, and fat loss may account for most of the weight loss in cancer cachexia. Such fat is used to meet the increased metabolic demands on the host caused by the presence of the tumour. In addition, metabolism of the polyunsaturated fatty acid, linoleic acid, through the 12-lipoxygenase pathway may be essential in preventing apoptotic death in tumour cells (Tang *et al* 1996)

The dissolution of the fat mass is the result of three different altered processes. First there is an increase of the lipolytic activity (Thompson *et al* 1981), which results in an important release of both glycerol and fatty acids. Glycerol is directed to the liver, where it is used as a gluconeogenic substrate, while the fatty acids are used by other tissues as an alternative substrate to glucose (Figure 1.2). Second, an important decrease in the activity of lipoprotein lipase (LPL), the enzyme responsible for the cleavage of both endogenous and exogenous triacylglycerols (present in lipoproteins) into glycerol and fatty acids, occurs in white adipose tissue (Lanza-Jacoby *et al* 1984, Thompson *et al* 1981) and consequently lipid uptake is severely hampered. Finally, adipose de novo lipogenesis is also reduced in tumour-bearing states (Thompson *et al* 1981), resulting in a decreased esterification and, consequently, a decreased lipid deposition. Hyperlipaemia and hypercholesterolaemia seem to be features of cancer bearing states (Argiles *et al* 1997).

Several clinical studies (Costa 1981) have observed an increased mobilisation of fatty acids before weight loss occurs, suggesting the production of lipid-mobilising factors either by the tumour or by host tissues. These substances appear to act like polypeptide hormones, are present in the circulation, and cause catabolism of adipose tissue by the

stimulation of cyclic adenosine monophosphate (cAMP) formation (Tisdale and Beck 1991). Although normal individuals suppress lipid mobilisation with administration of glucose, there is an impaired suppression in patients with malignant diseases as well as continued oxidation of fatty acids (Edmonson 1966). Increased fatty acid oxidation in the absence of increased dietary fat intake would result in a depletion of fat stores, while increased triglyceride fatty acid cycling and gluconeogenesis from glycerol could result in an increase in metabolic rate. All of these processes, therefore, have the potential to contribute to a net loss of body weight.

1.4.3 Protein Metabolism

Although loss of adipose tissue constitutes the major proportion of the weight loss in cancer cachexia, skeletal muscle mass depletion is probably more important in the overall survival of the patient. The loss of lean and visceral protein mass has been associated with reduced survival (Nixon *et al* 1980). The major loss of this protein has been observed to be the skeletal musculature (McMillan *et al* 1994).

As previously commented, asthenia or lack of strength is one of the main characteristics of cancer cachexia and it is directly related to the muscle waste observed in cachectic states. During, fasting, muscle proteins are degraded to provide amino acids which are used for gluconeogenesis; however, during longer starvation periods, protein breakdown is decreased in order to conserve nitrogen and maintain lean body mass (Argiles *et al* 1997). This ability seems to be absent in cancer-bearing states, leading to a depletion of vital host protein both skeletal and other proteins. As commented earlier, skeletal muscle, which accounts for almost half of the whole body protein mass, is severely affected in cancer cachexia.

Both reduced rates of protein synthesis and increased rates of protein degradation have been observed in biopsies of skeletal muscle from cachectic-cancer patients (Lundholm *et al* 1976). Whereas muscle-protein synthesis is depressed, synthesis of secretory proteins, such as acute-phase reactants, by the liver is actually increased, so that there may be no change in total body-protein synthesis (Emery *et al* 1984). However, in these patients muscle-protein synthesis only accounted for 8% of total body synthesis versus 53% for healthy control subjects. The observed maintenance of the total protein synthetic rate in these patients may therefore be due to a two-fold increase in non-skeletal muscle protein synthesis such as acute-phase reactants.

The result of the enhanced proteolysis is a large release of amino acids from skeletal muscle which takes place specially as alanine and glutamine (Argiles *et al* 1997)(Figure 1.2). Alanine is mainly channelled to the liver for both gluconeogenesis and protein synthesis. Interestingly, liver fractional rates of protein synthesis are increased in tumour-bearing animals, accounting for the production of the so-called acute phase proteins, while there is a decrease in albumin synthesis, leading to hypoalbuminaemia. Glutamine is basically taken up by the tumour to sustain the energy and nitrogen demands of the growing mass. Furthermore, in wasting disorders such as in cancer, plasma concentrations of branched-chain amino acids BCAA are often increased and their turnover rates altered (Argiles and Lopez-Soriano 1990a). These amino acids (e.g. leucine, isoleucine and valine) are essential nutrients for both humans and animals, making up to 40% of the minimal daily requirements of indispensible amino acids in man (Argiles *et al* 1997).

Recently, a new proteolytic mechanism which is involved in skeletal muscle during cancer cachexia has been reported (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. The pathway responsible for the breakdown of myofibrillar proteins is the adenosine triphosphate (ATP)-ubiquitin-dependent proteolytic system, which has been shown to elevated in numerous pathological states such as sepsis and metabolic acidosis (Tisdale 2000) and after transplanatation of certain tumours such as Yoshida ascites hepatoma in rats (Llovera *et al* 1994, 1995). In this process, proteins for degradation are first conjugated with ubiquitin , which serves as a signal for degradation by a large proteolytic complex, the 26S proteasome, which requires ATP to function. In skeletal muscle of rats bearing the Yoshida ascites hepatoma, a 500% increase in expression of polyubiquitin genes was observed in relation to both pair-fed (i.e. non-tumour-bearing rats fed the same amount of food as tumour-bearing rats) and *ad-libitum* animals (Llovera *et al* 1994,1995). Thus, understanding more about this proteolytic pathway may provide important clues for the treatment of muscle wasting in cachexia.

As a result of these major changes in amino acid metabolism, plasma amino acid profiles are altered in experimental animals (Argiles and Lopez-Soriano 1990b) and humans (Fanelli *et al* 1995) during cancer-bearing states. It is observed that basically the concentrations of gluconeogenic amino acids are decreased, particularly in very cachectic tumours such as in the lung and gastrointestinal tract ones (Argiles *et al* 1997). Interestingly, the plasma concentrations of BCAA is either normal or increased, even in the presence of severe malnutrition. This finding confirms the profound differences between cancer-induced cachexia and non-cancer malnutrition, in which

both gluconeogenic and BCAA are reduced in relation to normal feeding. Another interesting finding is the elevation of concentrations of circulating free tryptophan during tumour growth (Meguid *et al* 1992), which has been suggested to be a possible marker of neoplastic disease (Fanelli *et al* 1995).

The end result of these complex changes in protein metabolism are diminution in protein mass, atrophy of skin and skeletal muscle, and hypoalbuminemia. These changes are probably reflected clinically in impaired wound healing, susceptibility to infection, asthenia, and poor performance status.

1.5. Mediators of cancer cachexia

There has been considerable research effort into the elucidation of the nature of the postulated cachectic factors. The main contenders are the cytokines, produced by the host and some tumour cells, and catabolic factors, which appear to arise from the tumour.

Although the search for the "cachectic" factor(s) started a long time ago, we are still a long way from knowing the truth. A lot of progress has been made, however, and the suggested mediators (associated with both depletion of fat stores and muscular tissue) can be divided into two categories: of tumour origin (produced and released by the neoplasm) and humoral (mainly cytokines) factors. Indeed cells of the immune system release cytokines that act on multiple target cells such as bone marrow, myocytes, hepatocytes, adipocytes, endothelial cells and neurons, where they produce a cascade of biological responses that leads to the wasting associated with cancer cachexia. Among the cytokines that have been involved in this 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). Interestingly, these cytokines share the same metabolic effects and their activity is closely inter-related, showing in many cases synergistic effects.

1.5.1 Role of cytokines in cancer cachexia

Tumour necrosis factor-α (TNF)-α was first recognised as the mediator of the anorexia-cachexia syndrome in trypanosome-infected rabbits and was given the name cachectin to indicate its role in the cachectic process (Beutler *et al* 1985). Since then, other cytokines, in particular interleukins-1 and -6, interferon-γ and leukaemia-inhibiting factor (LIF) have all been suggested to play a role in the development of cancer cachexia.

All cytokines produce a profound anorexia and weight loss. The effect on adipose tissue was thought to be mediated through an inhibition of the cleaving enzyme lipoprotein lipase (LPL), which would prevent adipocytes from extracting fatty acids from plasma lipoproteins for storage. However, disruption of lipid metabolism through inhibition of LPL alone is unlikeley to induce the massive depletion of total body fat that is the a manifestation of the cachectic process. In type 1 hyperlipidemia there are elevated serum chylomicron levels caused by an inherited deficiency of LPL yet patients are not cachectic and have normal fat stores (Tisdale 1997).Although *in vivo* studies show that muscle protein degradation is significantly increased by TNF-α (Floes *et al* 1989), the effect must be indirect, since Goldberg *et al* (1988) were unable to detect a catabolic effect after incubation of skeletal muscle with TNF-α in vitro. IL-6 (Garcia-Martinez *et al* 1994) and IL-1 (Goldberg *et al* 1988) have been shown to be incapable of inducing muscle protein degradation *in vitro*.

Although elevated levels of TNF-α have been detected in the serum of patients with infections and cachexia, the evidence for increased levels in cancer patients is controversial (Maltoni *et al 1997*). When increased serum levels of TNFα have been measurable in cancer patients, this seems to reflect the severity of the disease rather than the presence of cachexia . In a study of patients with invasive breast cancer (Ming *et al* 1997), higher serum levels of TNF-α were linked to tumour size, TNM (tumour, node, metastasis) staging and more advanced lymph node status.

Although there is much literature on the effects of TNF-α , the role in cancer cachexia is not clear. Attempts to reverse the cachexia with anti-TNF-α antibodies have produced ambiguous results. In addition, it has been difficult to correlate serum levels of TNF with the extent of cachexia in cancer patients, although it is known that cachexia is not a local effect of the tumour. Thus in patients with solid tumours and a weight loss of 8-40% no TNF-α was detected in serum samples (Socher *et al* 1988). However, in another study, serum levels were found to correlate with the extent of disease (Scaliotti *et al* 1995). Thus, the association of TNF-α as a mediator of cachexia is not conclusive.

Other cytokines already mentioned, implicated in cancer cachexia, IL-1 which stimulates TNF-α release from macrophages. Strassman *et al* (1993) suggested that IL-1 plays a role in mediating cachexia. They showed that cachexia associated with colon-26 tumours inoculated into mice could be improved by intratumoral injections of IL-1ra. Systemic administration of IL-1ra was not effective. IL-1 is thought to reduce appetite by inducing corticotropin-releasing hormone (CRH) production in the

hypothalamus, while suppressing neuropeptideY, an appetite stimulant (Dunlop and Campbell 2000).

In a study, there was some evidence that IL-6 was also involved in producing cachexia (Strassman *et al* 1992). Yasumoto *et al* (1995) administered anti-IL-6 antibodies, which reversed the weight loss. They concluded that IL-6 was a factor in producing cachexia but other factor/s must have been present.

It has been suggested that IL-6 may specifically affect muscle wasting, because an antibody to the IL-6 receptor reduced loss of the gastrocnemius muscle in mice bearing the colon 26 tumour, but did not affect the overall loss of body weight or adipose tissue (Fujita *et al* 1996).

IL-6 is the main cytokine involved in the acute-phase protein (APP) synthesis. The levels of this cytokine was elevated in patients with colon cancer and an ongoing APP response (Fearon *et al* 1991). Unfortunately, all patients in this study had lost some weight and further studies are required to determine whether IL-6 is elevated in cachectic cancer patients. Other investigators have not found any association between serum cytokine levels and anorexia/cachexia. Maltoni *et al* (1997) studied 61 cancer patients with weight loss. The serum levels of IL-1, IL-2 and TNF-α were undetectable in most serum samples. There was no correlation between serum levels and the clinical findings of cachexia. These results suggest that some other factors may be involved in the induction of cancer cachexia

Despite evidence that some cytokines play a role in the induction of cachexia, at least in experimental models, other factors must also be involved. A study of the role of factors in the development of cachexia in nude mice bearing human tumour xenografts thus indicated that the known cytokines were only associated with four out of eight models tested (Kajimura *et al* 1996). Such factors could include tumour catabolic factors which have recently been described.

1.5.2 Catabolic factors in cancer cachexia

1.5.2.1 Proteolysis Inducing Factors

Loss of skeletal muscle mass in both cancer patients (Belizario *et al* 1991) and an experimental model of cachexia in the mouse (Smith and Tisdale 1993b) has been shown to correlate with the presence in the serum of bioactivity capable of inducing protein degradation in isolated skeletal muscle. Such bioactivity has been termed the proteolysis-inducing factor (PIF). Using the MAC16 tumour, Smith and Tisdale (1993a) were able to show that serum from cachectic animals was capable of increasing protein degradation in isolated gastrocnemius muscle, as measured by tyrosine release. The effect was specific to the cachectic state, since serum from mice bearing MAC13 adenocarcinoma, which did not produce cachexia, did not increase tyrosine release. Belizario *et al* (1991) also found evidence for a circulatory skeletal muscle proteolysis-inducing factor in serum samples of cancer patients with a weight loss of greater than 10%. Thus cachexia in mice and man appears to be mediated by catabolic factors present in the circulation.

The structure of this tumour product is reported to be different from that of recognised cytokines (Todorov *et al,* 1996). PIF is a sulphated glycoprotein of Mr 24kDa,

consisting of a central polypeptide chain of Mr 4kDa with phosphate residues attached, one O-linked sulphated oligosaccharide chain of Mr 6kD containing glucosamine and one N-linked sulphated oligosacccharide chain of Mr 10kDa also containing glucosamine. Thus most of the structure of PIF resides in the carbohydrate component. The polypeptide core was shown to be phosphorylated (Todorov *et al* 1997) and showed no structural homology with other materials in the protein data base.

The carbohydrate chains in PIF were found to be responsible for biological activity and antigenic reactivity (Todorov *et al* 1997). Thus both the ability to initiate protein degradation in isolated gastronemius muscle and antibody reactivity were destroyed by incubation with enzymes capable of removing the N- and O-linked oligosaccharide chains. Using Western blotting with the MAC16 monoclonal antibody the material was found to be present in the urine of cachectic cancer patients, but absent from the urine of normal subjects, patients with weight loss due to major burns, multiple injuries or surgery-associated catabolism and sepsis, or from the urine of cancer patients with little or no weight loss. Material isolated from the urine of cancer patients was found to be chemically, immunologically and functionally identical to that found in the MAC16 tumour.

Material purified either from the MAC16 tumour or from the urine of cancer patients when administered to non-tumour bearing mice, produced a state of cachexia, with rapid weight loss of about 10% of body weight over a 24hr period, without reduction in food and water intake, suggesting that cachexia can occur in the absence of anorexia (Todorov *et al,* 1996; Cariuk *et al,* 1997). Weight loss was attenuated by pre-treatment with MAC16 monoclonal antibody, showing the specificity of the effect. Body composition analysis showed the majority of weight was lost from the skeletal muscle

mass and there was no change in body water. *In vitro* studies showed the material to be capable of inducing direct release of tyrosine from isolated gastrocnemius muscle, and this effect could also be blocked by the MAC16 monoclonal antibody.

The induction of protein degradation *in vitro* by this factor was reduced in mice pretreated with the polyunsaturated fatty acid eicosapentaenoic acid (EPA) which also inhibited the rise of PGE2 (Tisdale 1996). EPA has been shown to counteract weight loss in patients with pancreatic cancer (Wigmore *et al* 1996). In these patients there was stabilisation of protein and fat reserves accompanied by a temporary reduction in acute-phase protein production and stabilisation of resting energy expenditure It has been suggested that PGE2 is an intracellular mediator for protein degradation induced by PIF or that another metabolite of arachidonic acid formed at the same time as PGE2 is responsible (Tisdale 1998).

The body composition changes induced by PIF were similar to those observed in cancer cachexia. Thus there was no change in the weight of the heart and kidney, but an increase in the weight of 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 by PIF was attributed to the activation of the ATP-ubiquitin-dependent proteolytic pathway (the predominant pathway involved in degrading skeletal muscle protein) in animals bearing the MAC16 tumour (Lorite *et al* 1998). Increased levels of mRNA were found for the ubiquitin carrier protein and of the C9 proteasome catalytic subunit in gastrocnemius muscle. A monoclonal antibody to PIF attenuated the enhanced protein degradation in soleus muscle from mice bearing the MAC16 tumour,confirming that PIF was responsible for the loss of skeletal muscle. This suggests that PIF may enhance protein

degradation in skeletal muscle by activation of the ATP-ubiquitin-dependent proteolytic pathway.

1.5.2.2 Lipid Mobilising Factors (LMFs)

Several reports have identified tumour products capable of inducing lipolysis in adipose tissue. Most studies have isolated material that is heat stable, resistant to proteolytic enzymes, and negatively charged, thus distinguishing them from the lipolytic polypeptide hormones that are all positively charged.

Evidence that neoplastic cells are capable of elaborating a lipid mobilising substance was first provided by the injection of non-viable preperations of Krebs-2 carcinoma into Swiss mice, where a decrease in carcass fat, similar to that produced by viable preperations of these tumour cells, was observed (Costa and Holland, 1966). Serum of mice bearing a thymic lymphoma when injected in non-tumour bearing controls produced massive fat loss (Kitada *et al* 1980). This factor was also detected in extracts of the tumour, in tissue culture medium, and in the sera of cancer patients with adenocarcinomas of the cervix and stomach, suggesting that the LMF was tumour derived and circulatory.

Most studies provide evidence that the LMF is an acidic protein, although there appears to be variations in the molecular weight. A heat-stable protein of molecular weight around 5kDa was isolated from thymic lymphoma (Kitada *et al* 1981), and another heat-stable protein of molecular mass 6kDa was isolated form the conditioned medium of the A375 human melanoma cell line (Taylor *et al* 1992). A similar LMF with a molecular mass of 65kDa to 75kDa and pI 4.7, termed toxohormone-L, has been

detected in the pleural fluid of patients with ovarian tumours or hepatomas (Masuno *et al* 1984). These results all suggest that tumour cells may release a lipid mobilising substance which contributes to the loss of carcass fat in tumour-bearing animals.

Animals transplanted with the MAC16 murine colon adenocarcinoma also produce a circulatory LMF (Beck and Tisdale 1987). This material is capable of inducing weight loss in mice, which is associated with elevated plasma lipolytic activity, as measured by the release of free fatty acids and glycerol from murine epididymal adipocytes, without a reduction in food intake. The protein is heat-stable and the activity is resistant to digestion by proteolytic enzymes. Once again, the material is acidic in character, distinguishing it from the natural polypeptide hormones which are all basic. Related adenocarcinomas such as the MAC13, also display lipolytic activity, but the activity level is only one-tenth of that found in the MAC16 tumour, and the plasma levels of this material are not elevated. This suggests that mobilisation of host lipids may be a quantitative phenomenon with all tumours having the capacity to secrete LMFs, and would explain why depletion of host lipids is a function of tumour type rather than tumour burden (Spector and Burns 1987). Thus, the ability of tumours to elaborate a lipolytic factor may be essential for their growth and reproduction, in which case inhibition of such activity might be expected to lead to tumour regression. Material with identical chromatographic and molecular weight characteristics was also present in the serum of patients with clinical cancer cachexia, but absent from normal serum, even under conditions of starvation. The level of LMF in the sera of cancer patients was found to be proportional to the extent of weight loss and was reduced in those patients who showed a positive response to chemotherapy (Beck *et al* 1990)). *In vitro* studies showed inhibition of the tumour LMF by the polyunsaturated fatty acid

eicosapentaenoic acid (EPA) found in fish oil (Tisdale and Beck 1991). *In vivo* studies in mice bearing the MAC16 tumour showed EPA to completely prevent the weight loss while other fatty acids were ineffective (Tisdale and Beck 1991). In a recent clinical study, the effect of EPA was compared to that of a different fatty acid gamma linoleic acid (GLA) in cachectic patients with unresectable pancreatic cancer (Wigmore *et al,* 1996). The majority of GLA patients continued to lose weight while patients receiving EPA, who had a median weight loss of 2.9kg/mo prior to supplementation, had a median weight gain of 0.3kg/mo after 3 mo of supplementation. Thus patients treated with EPA showed stabilisation of in the rate of weight loss accompanied by a temporary reduction in APP and REE stabilisation. This suggests that it may be possible to attenuate the process of cachexia by the use of suitable inhibitors of tumour catabolic factors

Chromatographic purification of the MAC16-derived LMF produced four peaks of lipolytic activity; <20kDa, 3kDa, 1.5kDa, and <0.5kDa (Beck and Tisdale 1991), whilst purification and characterisation studies revealed LMF to be a protein with an apparent molecular weight of 40kDa which demonstrated sequence homology and identical gel migratory and antibody reactivity properties as Zn-α-2 glycoprotein (Znα2gp/ZAG) (McDevitt 1996).

ZAG is a soluble protein the name of which derives from its tendency to precipitate zinc and electrophoretic mobility in the region of the α2 globulins (Burgi and Schimd 1961, Tada *et al* 1991). It is normally present in most body fluids including serum, sweat, saliva, cerebospinal fluid, seminal plasma, milk, amniotic fluid and urine. ZAG accumulates in breast cysts, as well as in 40% of breast carcinomas, and is induced by

glucocorticoids and androgens in breast cancer cell lines. Hence ZAG may participate in breast diseases including cancer (Freije *et al* 1991, Bundred *et al* 1987). Additionally, the homology observed to exist between ZAG and class I histocompatibility antigens has lead to the postulation that ZAG may play a role in the expression of the immune response (Araki et al 1988) and so could modulate the production of cytokines and thus the manifestation of cachexia in the tumour-bearing host. Furthermore, the expression of a high level of Znα2gp mRNA could be detected in the MAC16 tumour (Todorov *et al* 1998) a finding which, allied with the sequence homology existing between ZAG and LMF, could imply that ZAG may be involved in the induction of cachexia by the MAC16 tumour. Interestingly, it has been suggested that ZAG may have a role as a carrier for the lipolytic factor (McDevitt 1996, Sanchez *et al* 1999), possible transporting conventional lipolytic hormones to adipose tissue. This role has been previously observed when ZAG was found to be a carrier protein for nephritogenic renal glycoproteins (Shibata and Miura 1982).

So far, the biological significance of ZAG is unclear and there is no conclusive evidence that ZAG itself is responsible for the induction of cachexia in tumour-bearing animals and cancer patients. Although it seems to be a likely candidate, there has been no direct information to identify it to be the lipolytic factor. If recombinant ZAG was injected into mice to demonstrate its ability to deplete fat stores, and shown to possess a direct lipolytic action in adipocytes *in vitro*, this would support a role for this agent as a lipolytic agent.

1.5.2.3 Other Putative Cachectic Factors

Several other factors have been postulated as possible mediators of the cachectic state. Due to the obvious role of hormones in intermediary metabolism of carbohydrates, their role in cachexia requires consideration. Insulin, corticotropin, adrenaline, human growth hormone, and insulin-like growth factor have all been suggested to play a role in the cachexia syndrome (Puccio and Nathanson, 1997). Infusion of hydrocortisone or cortisol, glucagon and adrealine in humans produces features of cachexia (Barber *et al* 2000a).It is well known that during early starvation decreased insulin levels and increased 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, in part, account for the fact that patients with cancer who lose weight have increased rates of glycerol and FFA turnover compared with starved healthy patients.

1.6. Model of cancer cachexia

In order to study the mechanism of protein and adipose catabolism in cancer cachexia, it is necessary to have an appropriate experimental model. Only a few experimental tumours produce cachexia, possibly because it is selected against during the process of transplantation. Throughout this study, a transplantable adenocarcinoma of the colon (MAC16) will be utilised, which was derived from prolonged administration of 1,2-dimethylhydrazine (Double *et al* 1975). The tumour is implanted subcutaneously into the flanks of NMRI mice and weight loss starts to appear 10-12 days after transplantation (Beck and Tisdale 1987). Weight loss is evident when the tumour mass comprises more than 0.3% of the total host body weight and 30% weight loss is evident when the tumour represents just 3% of the body weight. In humans, weight loss occurs

when the tumour mass represents less than 1% of the body weight and thus this model approximated well to the human situation. Other experimental models of cachexia only produce weight loss when the tumour mass represents 10-20% of the body weight and in these cases anorexia is the principal factor. In animals bearing the MAC16 tumour both carcass and muscle mass decrease in direct proportion to the weight of the tumour, and without the drop in food and water intake. This suggests that tumour-derived products may be responsible for the cachexia, and it is the nature of this catabolic factors, this study aims to elucidate.

1.7. Management of Cancer Cachexia

The ideal clinical management of cachexia would be to completely reverse this syndrome. However, such a goal is rarely achievable in the majority of these patients. The main goal of any therapeutic intervention in these patients therefore is aimed at improving the quality of life of the patient e.g. greater comfort, relief of symptoms. Current clinical management strategies include nutritional support, pharmacologic administration or a combination of these approaches. In the following sections, the roles of these interventions are discussed.

1.7.1 Nutritional Support

Nutritional support in the form of total parenteral nutritional (TPN) has failed to replete lean body mass. Furthermore, a meta-analysis of the published trials on patients receiving TPN, while undergoing chemotherapy, showed a decreased survival, a poorer tumour response, and a significant increase in infectious complications (McGeer *et al* 1990). TPN is therefore used in carefully selected cancer patients possibly in conjunction with other modalities such as megestrol acetate and anabolic steroids.

Potential indications for use includes patients who are (i) unable to maintain adequate nutrition because of tumour obstruction (which may be relieved by therapy); (ii) have complications from therapy; or (iii) preoperative for tumour resection where parenteral nutrition has been shown to decrease complications and increase survival (Muller *et al*, 1982).

When food intake is inadequate, yet the GI tract is functionally intact, enteral feedings may be a useful consideration for providing additional calories and protein. Enteral feeding is associated with fewer complications, is less expensive, and more physiologic than TPN. However, few prospective randomized trials have evaluated the use of enteral feedings in patients with cancer. Many reported trials have been flawed by poor study design and patient non-compliance (Klein 1993). The main serious risk of enteral feeding is aspiration. Other side effects involve diarrhoea or constipation, nausea, vomiting, abdominal cramps, and bloating or distension.

1.7.2 Pharmacologic Treatments

The pharmacological management of cachexia is aimed primarily at countering two of its main symptoms, anorexia and chronic nausea. It is of utmost importance to control nausea and vomiting before any pharmacological intervention is attempted for the patient's anorexia. Drugs that have been used are metoclopramide and other gastric emptying agents like domperidone and cisapride for treating chronic nausea. Corticosteroids and newer anti-emetic agents such as $5HT_3$ receptor antagonists are alternatives that may be used. Whenever nausea is not a major problem, pharmacological management of anorexia can be initiated (Vigano *et al*, 1994).

Progestational drugs such as megestrol acetate have been shown to counteract anorexia. This compound was found to improve appetite, calorific intake, and nutritional status of cancer patients in several clinical trials (Loprinzi *et al* 1990, 1993a, Gebbia *et al* 1996). Weight gains of up to 6.8kg over baseline values in 16% of the patients treated were reported. However, body composition analysis showed that the majority of patients gained weight in adipose tissue, with an increase in body fluid, which was responsible for the weight gained, with no increase in lean mass (Loprinzi *et al* 1993b). Moreover, animal studies demonstrated that the host's weight increase was associated with the doubling of the tumour's mass (Beck and Tisdale 1990). A more recent trial of megestrol acetate in patients receiving chemotherapy found an inferior response to therapy and a trend to poorer survival (Rowland *et al* 1996) Medroxyprogesterone acetate showed improvement in appetite but this effect did not result in weight gain (Downer *et al* 1993). The mechanism of action of these agents is unclear at present, although megestrol acetate has been shown to reduce serum cortisol levels (Loprinzi *et al* 1992).

Corticosteroids have also been used to increase food intake in cancer patients, and a number of uncontrolled studies have suggested that some of the symptoms in cancer patients (such as anorexia and asthenia) can be partially mitigated by corticosteroid treatment (Vigano *et al* 1994). Both dexamethasone and prednisolone have been used in different trials, and appear to act either as a result of their euphoriant activity or through the inhibition of prostaglandin metabolism. They are beneficial because they improve the quality of life of terminal cancer patients, but do not seem to have any significant effect in the reduction of mortality. In addition the use of steroids beyond a few weeks is not advisable as longer treatment periods are associated with unacceptable

side effects such as oedema, muscle weakness, dysphoria, hypokalaemia, hyperglycaemia, immune suppression (Puccio and Nathanson, 1997). They tend to be used therefore, during the preterminal phase of a patient's illness and are not suitable for early intervention.

Cyproheptadine is an antihistamine with serotonergic properties usually used to treat allergies. Although initial clinical data suggested that it had appetite and weight-enhancing effects in both patients without cancer and patients with cancer-related cachexia, it did not prevent progressive weight loss in patients with advanced malignant disease (Kardinal *et al* 1990).

The non-steroidal antiinflammmatory drug (NSAID) ibuprofen at a dose of 400mg three times daily has been shown to reduce the levels of acute-phase proteins, IL-6 and cortisol in cachectic colorectal cancer patients (Loprinzi *et al* 1992) A new approach to treating cachexia has been to combine NSAID and megestrol acetate together (McMillan *et al* 1997). Early evidence suggests that this combination may stabilise quality of life and weight in advanced cancer patients.

Weight gain has been a recognized feature of the use of marijuana and its derivatives. A recent study by Plasse *et al* (1991) using dronabinol, the primary psychoactive substance in marijuana, have attempted to find whether some of the appetite enhancement could be dissociated from mood effects. Although the patients continued to lose weight following treatment, the rate of weight loss decreased at all doses tested, symptomatic improvement being noted in both mood and appetite. Further clinical trials are required before drawing further conclusions.

Among the compounds that counteract metabolic changes, it is interesting to consider hydrazine sulphate, a non-specific inhibitor of phosphoenolpyruvate kinase, an enzyme that drives gluconeogenesis from lactate and amino acids. A study involving small cell lung cancer indicated that hydrazine treatment improved parameters of carbohydrate metabolism and prolong survival (Chlebowski *et al* 1990). Another trial concluded that the administration of the drug reduced amino acids flux, thereby favourably influencing the metabolic abnormalities of cachexia (Tayek *et al* 1987). However, later studies have shown no effect on weight loss, with trends to worse survival and quality of life in hydrazine treated patients (Loprinzi *et al* 1994a, 1994b).

The most logical pharmacologic therapy for cachexia is an agent that promotes protein synthesis or inhibits protein breakdown. Both approaches have been investigated. Recombinant human growth hormone was shown to promote nitrogen retention in short-term studies of HIV infection and lean mass accrual in a double-blind, placebo-controlled trial (Schambelan *et al* 1996). Other anabolic agents available in clinical use are the anabolic steroids. Their application in clinical use has been impeded due to their potential for abuse for example in enhancing physical performance or to produce cosmetic effects. However, a number of trials have taken place to investigate their potential therapeutic effects. For example, Demling and DeSanti (1997) showed that oxandrolone (20mg/d) plus high protein intake and active physical therapy doubled weight gain compared with treatment program lacking the agent, in patients in recovery phase with 30% to 50% full-thickness burns. An increasing number of studies are evaluating the use of testosterone and anabolic steroids in HIV-infected patients and have shown increases in fat-free mass (Gold *et al* 1996, Bhasin *et al* 1998). β_2 agonists (i.e.clenbuterol) are potentially interesting molecules since they have very important

effects on protein metabolism in skeletal muscle favouring protein deposition. It has been described recently how β_2 agonists suppress the activation of proteolysis (through their action on the ubiquitin-dependent proteolytic system) during tumour growth (Costelli *et al* 1995a). In a similar manner, β_2 agonists are also able to suppress the increase in BCAA oxidation in skeletal muscle during cancer cachexia (Costelli *et al* 1995b). Investigations are being carried out on the usefulness of these compounds in ameliorating cancer cachexia in experimental models (Argiles *et al* 1997).

As commented on earlier, n-3 polyunsaturated fatty acids (PUFA) have been proposed active in reducing either tumour growth or the associated tissue waste, particularly adipose tissue mass (Tisdale 1993). It was shown that administration of eicosapentaenoic acid EPA to mice bearing the cachectic tumour (MAC16) resulted in reversal of tumour-induced cachexia without changes in food intake. It was thought to not only reverse the loss of adipose tissue through blocking the action of lipid-mobilising factor, but also to decrease the rate of protein degradation. It is purported that EPA can affect the production of pro-inflammatory cytokines, but also their end-organ affects (Barber *et al* 1998) possibly by influencing the activity of receptors and enzymes which have fundamental role in cellular signalling. As discussed earlier, this agent has been investigated in cancer patients in clinical trials (Wigmore *et al* 1996, 1997, Barber *et al* 1997).

Some agents in current use have anticytokine activity and can be shown to decrease protein breakdown in vitro (Kotler 2000). Some agents in current use have anticytokine properties in vitro, including appetite stimulants megestrol acetate and medroxyprogestrerone. Pentoxifylline has documented anti-TNF effects, although no significant beneficial effects have been seen in clinical studies (Combaret et al 1999,

Goldberg *et al* 1995). Administration of the glucocorticoid receptor antagonist RU38486 blunted the increased gene expression in septic rats (Tiao *et al* 1996) but did not reverse muscle hypercatabolism in rats bearing the AH-130 ascites tumour (LIovera *et al* 1996). Thalidomide has been evaluated for nutritional benefits related to its anti-inflammatory properties, which may be due, at least in part, to an increase in the degradation rate of TNF mRNA. Adjunctive use of thalidomide in HIV-infected patients receiving treatment for tuberculosis has been shown to promote weight gain (Calabrese and Fleischer 2000).

Several novel approaches to the treatment of cachexia are in preclinical, animal phases of testing including several varieties of cytokine inhibition such an antisense sequence to nuclear factor-κ, which binds to the promoter region of DNA and slows transcription of cytokine mRNA (Kawamura *et al* 1999a) and soluble TNF receptors (Eliaz *et al* 1996). Melatonin is another compound that has been found to downregulate TNF production with the possibility of efficacy in clinical situations (Kotler 2000). A diverse range of metabolic regulators are also being investigated such as insulin-sensitising agents, the hypolipidaemic agent bezafibrate (Kawamura *et al* 1999b), a novel activator of lipoprotein lipase, an aldose reductase inhibitor, Ponalrestat (Kawamura *et al* 1999c) and L- carnitine thought to affect fatty acid oxidation, are just a few agents that are currently under investigation.

A better understanding of host origin or cachexigenic factors produced by the tumour cells is required to develop new research and treatment strategies. The role of hormones and cytokines in causing this syndrome may be the focus of pharmacologic strategies in the future. In addition, future studies also need to explore the impact of various interventions on the physical well-being (appetite, weight) of patients, as well as on the

quality and quantity of life of these patients. The development of nutritional therapies aimed at increasing the preservation of skeletal body mass is vital as loss of lean mass points to a somewhat poor prognostic outcome for the cachectic individual.

It is hoped that a knowledge of the underlying mechanisms involved in host tissue catabolism by tumour-derived catabolic factors, will enable us to develop more efficacious therapeutic agents to treat cancer cachexia, in the future.

1.8. Aims and Objectives of the Study

The aim of this study is to elucidate some of the mechanisms by which cancer-derived cachectic factors, lipid-mobilising factor (LMF) and proteolysis-inducing factor (PIF), exert their effects on various tissues.

The investigation is comprised essentially of two major components. Although the themes of both are related, for simplicity, the study has been divided into two seperate results chapters. The first section (chapter 3) investigates the signalling pathways of both catabolic factors, involved in skeletal muscle protein degradation and protein synthesis, therefore contributing to the understanding of potential mechanisms involved in cancer cachexia. For this purpose, urine collected from cachectic cancer patients was purified to homogeneity to yield pure LMF and the MAC16 tumour was utilised as the source for isolating PIF. The use of a skeletal muscle cell line, C_2C_{12}, was used as a model system to study the mechanisms of these factors on muscle protein turnover. Further experiments were conducted using tumour cell lines (MAC16, MAC13), and effects on glucose utilisation were also investigated. The second part of the study (chapter 4) is focused primarily on the effects of LMF on alterations in adipocyte G-

protein expression through progression of the cachectic state. A brief insight into other potential mechanisms involved in sensitising adipocytes to lipolytic stimulation is also given.

CHAPTER 2

Materials and Methods

2.1 Animals

Pure strain NMRI mice (18-20g) were obtained from our own inbred colony. They were fed a rat and mouse breeding diet from Special Diet Services, Witham, Essex and water *ad libitum.*

Fragments of the murine adenocarcinoma (MAC16) dissected from animals with established weight loss were implanted into the flanks of male and female NMRI mice by means of trocar. Tumours were removed once palpable, approximately 14 days post transplantation, before weight loss exceeded 25% of total body weight. These were stored frozen at -20°C until used to purify the purported protein mobilising factor.

2.2. Cell Culture

2.2.1. Maintenance of cell lines

C_2C_{12} myoblasts were cultured in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin (p/s), in a humidified atmosphere of 5% CO_2 in air at 37°C. MAC 16 cells were maintained in RPMI 1640 medium containing 5% FCS. MAC13 cells were grown in the same media but in 10% FCS, 1% p/s. 3T3-L1 cells were cultured in DMEM 10% FCS in 10% CO_2 in air. It was particularly important to sub-culture these cells before reaching 100%

2.2.2. Differentiation of C_2C_{12} myoblasts to myotubes

After cells had reached confluence, differentiation was induced by changing the media surrounding the cells to DMEM containing 2% horse-serum, 1% penicillin/streptomycin. The culture medium was changed every 48hr and differentiation occurred 11-14 days post-confluence after which, cells could be used for experimentation.

2.2.3. Differentiation of 3T3-L1 fibroblasts to adipocyte cells

3T3-L1 cells were grown to confluence at 37°C in 6-well multidishes. Two days post confluence (day 0), differentiation was induced with methylisobutylxanthine IBMX (0.5mM), dexamethasone (0.25μM) and insulin (1μg/ml) in DMEM containing 10% FCS according to the method of Frost and Lane (1985). After 2 days, the IBMX and dexamethasone were removed and insulin was maintained for 2 additional days. On day 4 and thereafter, DMEM (without insulin supplementation) plus 10% FCS was replaced every 2 days. Oil Red O, a fat soluble dye was used to assess the extent of fat accumulation and therefore differentiation. Cells were used for experimentation between days 8-12 days, at which stage most cells expressed the adipocyte phenotype.

2.3 Chemicals

Affinite Reseach Products Ltd, Exeter, UK

Mouse monoclonal antibody to 20S α-subunits

Amersham International Ltd, Bucks, UK.

Anti-rabbit (donkey) IgG-HRP (HSL western blotting)

[8-^{3}H] Adenosine 3'5'-cyclic phosphate (cAMP), ammonium salt
(specific activity 888GBq/mmol)

[2-^{3}H] Adenosine 5'-monophosphate (AMP), ammonium salt
(specific activity 0.74-1.1 TBq/mmol)

[2,6-^{3}H] 2-Deoxyglucose (specific activity 1.63 TBq/mmol)

ECL Western blotting analysis system

Hybond ECL Nitrocellulose

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

Protein rainbow markers

Streptavidin-HRP

Amicon Ltd, Gloucs, UK

Filtration cells: 50ml, 200ml,400ml capacities

YM membranes, 10kDa cut-off

Microcon concentrators

Bio-Rad Laboratories Ltd, Herts, UK.

Ammonium persulphate

Bio-Rad protein reagent

Silver stain kit

Calbiochem-Novabiochem Ltd, Beeston, Nottingham, UK.

Forskolin

H8

Lactacystin

LY 294002

MDL_{12330A}

PD 98059

Rapamycin

Ro-31-8220

Wortmannin

Dako, Denmark

Rabbit anti-mouse IgG-HRP

Fisons Laboratories Supplies, Loughborough, UK.

1,2-Dichloroethane

Ethanol

Glacial acetic acid

Hydrochloric acid

Isopropanol

Methanol

Nitric acid

Optiphase Hisafe 3

Perchloric acid

Sodium carbonate

Sodium dihydrogen orthophosphate

Gelman Sciences, Northampton UK.

Acrodisc 0.2μm syringe filters

Gibco BRL Life Technologies, Paisley, Scotland, UK.

Bovine foetal calf serum (FCS)

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

Horse serum

Nunclon 60 x 15mm petri dishes

Penicillin-Streptomycin

RPMI 1640 Tissue culture medium

Trypsin/EDTA

NEN Life Science Products, Boston, ,MA, USA.

Polyclonal rabbit antisera to Gαi (AS/7) and Gαs (RM/1)

NEN Research Products, Herts, UK.

[^{3}H] 5-Methylthymidine (specific activity 3.1 TBq/mmol)

Novacastra Laboratories Ltd, Newcastle upon Tyne, UK.

Myosin heavy chain monoclonal antibody.

Oxoid, Basingstoke, Hampshire, UK.

Phosphate buffered saline tablets

Pharmacia Biotech, UK.

Resource™ ISO column 1ml

Pierce, Rockford,IL.

Colorometric PKA assay kit, SpinZyme™ format.

Super Signal™ West Dura Extended Duration Substrate

Sigma-Aldrich Co Ltd, Dorset, UK.

Acrylamide solution 30%

Adenosine 3'5'-cyclic monophosphate

Adenosine 5'-monophosphate (AMP) sodium salt

Ammonium chloride

Ammonium sulphate

Antipain

Anti-rabbit IgG-HRP (Gprotein blotting)

ATP

Bovine serum albumin (BSA)

Bromophenol Blue

Calcium chloride

Collagenase, Type II

Charcoal activated

Coomasie Brilliant Blue R250 stain

Cyclic AMP-dependent protein kinase

Cycloheximide

Cytochalasin B

Dexamethasone (lyophilised)

Dibutyryl cAMP (dbt-cAMP)

Dimethyl sulphoxide (DMSO)

Dithiothreitol (DTT)

D-glucose

Ethylene glycol-bis(β aminoethylether) N,N,N',N' tetracetic acid (EGTA)

Ethylenediaminetetraacetic acid (EDTA)

Eicosapentaenoic acid

Glycerokinase

Glycerol

Glycine

Insulin

Isoprenaline

Kodak GBX developer and replenisher

Kodak GBX fixer and replenisher

Lactate dehydrogenase

Leupeptin

Methylisobutylxanthine (IBMX)

Magenesium chloride

Magnesium sulphate

β-mercaptoethanol

α-Nicotinamide adenine dinucleotide-reduced form (NADH)

1-Nitroso-2-naphthol

5'-Nucleotidase

Oil Red O

Percoll

Phenylalanine

o-Phenylenediamine

Phenymethylsulphonylfluoride (PMSF)

Phosphoenolpyruvate

Potassium chloride

Potassium dihydrogen orthophosphate

Propranolol

Protein A

Pyruvate kinase

Sodium acetate

Sodium azide

Sodium chloride

Sodium bicarbonate

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Sucrose

N,N,N'N'-Tetramethyethylenediamine

Trichloracetic acid

Tripotassium citrate

Trizma base

Zinc sulphate

Whatman International Ltd, Maidstone, Kent, UK.

DEAE cellulose

Gifts

Affinity purified rabbit anti-rat antibody to HSL was provided by Dr. Cecilia Holm, Lund University, Sweden. Urine was provided by Dr. A. Moses and human adipose tissue by Prof. K.C.H. Fearon, Department of Surgery, Edinburgh Royal Infirmary, UK. SR 59230A was provided by Dr. L. Manara, Sanofi Winthrop, Italy.

2.4. BUFFERS

2.4.1.Affinity Purification

2.4.1.1. Anion–exchange buffer (QS1)

Tris-HCL pH 8.0	10mM
DTT	1mM
PMSF	0.5mM
EGTA	0.5mM

2.4.1.2. Elution Buffer

Glycine –HCL pH 2.5	100mM

2.4.1.3. Collecting Buffer

Tris-HCL pH 8.0	1M

2.4.2. Enzyme-Linked Immunosorbent Assay (ELISA)

2.4.2.1 Sodium carbonate/bicarbonate buffer pH 9.5

Sodium carbonate	0.1M
Sodium bicarbonate	0.1M

2.4.2.2.Sodium citrate/phosphate buffer pH 5.0

di-Sodium hydrogen orthophosphate	0.2M
Citric acid (sodium salt)	0.1M

2.4.2.3. Substrate Buffer

Phosphate/citrate buffer pH5.0	
o-Phenylenediamine (OPD)	0.04%
Hydrogen peroxide	0.012%

2.4.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.3.1 Running Buffer (10x)

Trizma base	0.25M
Glycine	1.9M
SDS	35mM

2.4.3.2. Sample buffer

Tris-HCL pH 6.8	62.5mM
SDS	0.35mM
β-Mercaptoethanol	5%
Glycerol	10%
Bromophenol blue	0.01%

Dilute 1:1 with sample.

2.4.4. Western Blotting

2.4.4.1. Blotting buffer

Trizma base	0.25M
Glycine	1.9M
SDS	50mM
For use: 10x Blotting Buffer	10%
Methanol	20%

2.4.4.2. Washing buffer

PBS + 0.5% Tween 20

2.4.4.3. Blocking solution

PBS + 0.5% Tween 20	
Marvel	5%

2.4.5 Phenylalanine stock

Phenylalanine	75mM
L-[2,6-^{3}H]-Phenylalanine	50μCi/ml

2.4.6. Lipolytic Assay

2.4.6.1. Krebs Ringer Bicarbonate Solution

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

2.4.6.2. Glycerol Assay Buffer

Triethanolamine	100mM
Magnesium sulphate	2mM
Phosphoenelpyruvate	0.4mM
α-Nicotinamide adenine dinucleotide (NADH)	0.25mM
Adenosine triphosphate	1.2mM
Pyruvate kinase	1.0 units/ml
Lactate dehydrogenase	7.0units/ml

Dissolve in deionised water and pH adjusted to 7.4 using conc HCL. The reaction was initiated with 1 unit of glycerokinase and allowed to proceed for 15 min.

2.4.7. Q Sepharose Buffer 1

Tris-HCL pH 8.0	10mM
DTT	1mM
EGTA	0.5mM
PMSF	0.5mM

2.4.8. Q Sepharose Buffer 2

Tris-HCL, pH 8.0	10mM
DTT	1mM
EGTA	0.5mM
PMSF	0.5mM
Sodium chloride	0.2mM

2.4.9. Adipocyte isolation

2.4.9.1. Sucrose Buffer pH 7.4

Sucrose	0.25M
EGTA	2mM
Tris-HCL	10mM

2.4.9.2. Concentrated Sucrose solution, pH 7.4

Sucrose	2M
EGTA	8mM
Tris-HCL	80mM

2.4.9.3. Sodium Chloride Buffer, pH 7.4

Sucrose	0.25M
EGTA	2mM
Tris-HCL	10mM
PMSF	4μM

2.4.10 Coomassie Blue Gel Stain

Coomasie brillaint blue R250 stain	0.1%
Acetic acid	10%
Methanol	25%

2.4.11. Coomassie Blue Gel Destain

Acetic acid	10%
Methanol	25%

2.4.12. 12% Resolving Gel

Distilled water	4.9ml
30% acrylamide mix	6.0ml
1.5M Tris-HCL pH8.0	3.8ml
10% SDS	0.15ml
10% ammonium sulphate	0.15ml
TEMED	0.006ml

2.4.13. 15% Resolving Gel

Distilled water	3.4ml
30% acrylamide mix	7.5ml
1.5M Tris-HCL pH 8.0	3.8ml
10% SDS	0.15ml
10% ammonium sulphate	0.15ml
TEMED	0.006ml

2.4.14. 5% Stacking Gel

Distilled water	3.4ml
30% acrylamide mix	0.83ml
1M Tris-HCL pH 6.8	0.63ml
10% SDS	0.05ml
10% ammonium sulphate	0.05ml
TEMED	0.005ml

2.4.15.Proteasome Activity Assay

2.4.15.1. Homogenising Buffer HB (used also for myosin homogenisation)

500mM Tris pH 7.5	4ml
100mM ATP (in Tris 100mM pH7.5)	2ml
50mM MgCl2	10ml
100mM DTT	2ml
dH2O	to 100ml

2.4.15.2. Fluorogenic Substrate/Substrate Buffer

N-SUCCINYL-LEU-LEU-VAL-TYR-7-AMIDO-4-	
METHYLCOUMARIN	10mg
DMSO	600µl

For use, dilute 1:100 in 100mM Tris pH8.0.

2.4.15.3. Termination Buffer

Sodium acetate pH 4.3	80mM

2.4.16. cAMP Assay

2.4.16.1. Assay buffer

HEPES pH7.5	20mM
EDTA	5mM
Isobutylmethylxanthine	100µM

2.4.16.2. Binding Protein

cAMP dependent kinase	0.02%w/v

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

2.4.16.3. Charcoal suspension

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

2.4.17. HPLC Buffers-LMF Purification

2.4.17.1. Buffer A

1) AMS	198.15g
Anhydrous sodium phosphate (dibasic)	7.1g
Deinoised water	to 1000ml
2) AMS	198.1g
sodium dihydrogen orthophosphate	7.8g
deionised water	to 1000ml

Both solutions 1) and 2) were mixed together to achieve pH 7. This was done by adding a small amount of dihydrogen to a large amount of disodium salt.

2.4.17.2. Buffer B

1) anhydrous sodium phosphate (dibasic)	7.1g
deionised water	to 1000ml
2) sodium dihydrogen phosphate (dibasic)	7.8g
deionised water	to 1000ml

pH was adjusted as for Buffer A.

2.4.18. HSL isolation and immunoblotting

2.4.18.1. Homogenising Buffer

Sucrose	0.25M
EDTA	1mM
DTT	1mM
Leupeptin	10µg/ml
Antipain	10µg/ml

2.4.19. Cathepsin Assay

2.4.19.1. Extraction Buffer

Sucrose	250mM
KCL	50mM
EDTA	1mM
K_2HPO_4	3.7mM
KH_2PO_4	6.3mM pH 7.4

2.4.19.2.Wash Buffer

Sucrose	250mM
EGTA	2mM
EDTA	2mM
Tris-HCL	20mM
pH to 7.4	

2.4.19.3. Cathepsin-L incubation buffer

Sodium acetate	30mM
Acetic acid pH5.5	60mM
EDTA	4mM

2.4.19.4 Cathepsin L termination buffer

TCA	100mM
NaOH	100mM
Sodium acetate	30mM
Acetic acid	70mM pH to 4.3

2.4.19.5. Cathepsin B incubation buffer

Potassium dihydrogen phosphate	352mM
Disodium hydrogen phosphate	48mM pH6.0
EDTA	4mM
Cysteine	8mM

2.4.19.6. Cathepsin B termination buffer

Sodium chloroacetate	100mM
Sodium acetate	30mM
Acetic acid	70mM
	pH to 4.3

2.4.20. 5' Nucleotidase buffer

Tris pH8.0	50mM
Magnesium chloride	0.18mM
AMP	0.02mM

2.5. METHODS

2.5.1 Determination of Protein Concentration

Protein concentration was determined using Bio-Rad reagent based on the method of Bradford (1976). This is a dye-binding assay which is based on the observation that the absorbance maximum for an acidic solution of Coomasie Brilliant Blue shifts from 465 to 595nm when protein binding occurs.The sample is diluted to 800µl with distilled water plus 200µl Bio-Rad solution to give a final volume of 1ml. The absorbance of the mixture is measured at 595nm in a Beckman DU-70 Spectrophotometer against a blank containing distilled water and Bio-Rad reagent only. The protein concentration is calculated according to the following formula derived from an absorption spectrum for a solution of BSA:

$$\frac{\text{Sample absorbance - Control absorbance}}{*0.053} = \text{µg of protein/µl}$$

* constant representing the gradient of the BSA calibration curve.

2.5.2 Lipid-mobilising factor (LMF) Purification

LMF was purified from the urine of cachectic cancer patients by a combination of ion exchange and hydrophobic chromatography. DEAE cellulose was equilibrated in 10ml/g of 100mM Tris-HCl for 5 min then 10mM Tris–HCl for a further 5 min. After a low speed centrifugation the supernatant was discarded. Approximately 2 litres of urine was diluted 6 times with 10mM Tri-HCl. The pre-washed DEAE cellulose was then added as a slurry and left stirring for at least an hour at 4°C. The resin was allowed to settle after which the supernatant was discarded. LMF was eluted from the

resin by washing 3 times with 0.3M NaCl. Salt was removed from the sample using an Amicon filtration cell containing a 10kDa molecular weight cut off filter against PBS and then 1.5M ammonium sulphate before being loaded on a Resource-Iso HPLC column (Section-HPLC buffers). The concentration of ammonium sulphate was reduced from 1.5M to zero in a linear gradient, with LMF eluting at approximately 0.50M ammonium sulphate. After thorough washing with PBS the activity of the factor was determined by measuring glycerol release from murine adipocytes (lipolytic assay 2.5.2). The final product was filter sterilised ready for experimental use.

2.5.2 Lipolytic Assay

This method was measured by the protocol described by McDevitt *et al* (1995). A single cell suspension of white adipocytes were prepared from finely chopped epididymal fat pads taken from male NMRI or ex-breeder male NMRI mice. Adipose tissue was excised from animals recently killed by cervical dislocation and added to pre-warmed collagenase solution (Krebs 3% BSA supplemented with 4mg/ml collagenase). Fat pads were roughly chopped with scissors, gassed with 95%O_2/ 5% CO_2 and incubated for 30min in 37°C in a shaking water bath.

Seperated adipocytes were washed three times by resuspending the cells in pre-warmed Krebs solution, allowing the cells to float to the service after each wash. Cells were resuspended in 3% Krebs/BSA ensuring a cell count of at least 1 x 10^5 cells/ml.

To measure lipolysis, 0.99ml of cells were added to 10µl of sample, gassed again at 95% CO_2/5%O_2 and incubated in a waterbath at 37°C for 2hr. Experimental controls

were prepared in the same manner, Krebs/BSA and isoprenaline used as negative and positive controls respectively.

The reaction was terminated by adding 0.5ml of 10% w/w perchloric acid to 0.5ml of incubation medium. The precipitate formed was removed by centrifugation at 13000g for 1min (Heraeus Sepatech Biofuge 13). The supernatent from each of the samples was neutralised by the addition of 40% potassium hydroxide, added slowly dropwise, and then assayed for the presence of glycerol.

Glycerol was determined by the method of Wieland (1974). An aliquot (200µl) of sample was added to 830µl of glycerol buffer. The reaction was initiated by adding 10µl glycerol kinase and the samples read at 340nm over a period of 15min. This change in OD was measured spectrophotometrically by the formation of NAD^+ from NADH. NADH absorbs maximally at 340nm so as the reaction proceeds, the absorbance decreases.

2.5.3 Measurement of the rate of protein synthesis in cell lines

MAC16 cells were seeded at $8x10^4$ cell/ml and MAC13 seeded at $4x10^4$ cells/ml in 6 well multidishes containing 2ml of medium per well. C_2C_{12} cells were seeded at $5x10^4$cells/ml.

Protein synthesis was measured by the incorporation of isotopic phenylalanine into protein as described previously by Southorn and Palmer (1990). It was measured over the final 60min of incubation by adding 10µl/ml of medium of a stock solution of phenylalanine (75µmol L-phenylalanine and 50µCi of L-[2,6-^{3}H phenylalanine/ml]. Precisely 60min later, the labelled medium was removed and the cells rinsed three times with ice-cold PBS. For MAC16 cells, which were in suspension culture, cells

were mechanically dislodged in the wells, transferred to centrifuge tubes, and subsequent steps carried in these tubes thereafter. After removing any remaining PBS, 1ml of ice-cold 0.2M PCA was added per well and the dishes stored at 4°C for 20min. The PCA was discarded and the cell monolayer solubilised in 1ml of 0.3M NaOH. After incubation for 30min at 37°C, protein was precipitated with 0.5ml 2M PCA, left on ice for 20 min then centrifuged at 3000g for 10min. The resulting supernatant was used to determine RNA content (Section 2.5.5) and the precipitate was re-dissolved in 1ml of 0.3M NaOH. An aliquot was used to determine protein concentration using Bio-rad solution. Another aliquot was mixed with scintillation fluid and amount of radioactivity incorporated measured using a 2000CA Tri-Carb liquid scintillation counter. Protein synthesis was expressed as specific radioactivity Sr disentegations per min (dpm) / μg total cellular protein.

2.5.4. Determination of RNA Concentration

RNA was determined by the method of Munro and Fleck (1966) as modified by Ashford and Pain (1986). The absorbance value of the acid supernatant fraction (Section 2.5.3) containing the RNA component was measured at two wavelengths, 232nm and 260nm. Using the following equation which corrects for any absorbance due to the presence of aromatic amino acids, the RNA content of supernatant and thus total amount per dish was calculated.

$$\text{RNA } (\mu g) = [A260.(32.9)] - [A232.(6.11)] \times \text{Volume (ml)}$$

2.5.6 Determination of cell proliferation

Cells were seeded at the following densities in 24well multidishes (1ml per well): C_2C_{12} $1x10^4$ cells/ml; MAC16 $1x10^5$ cells/ml; MAC13 $4x\ 10^4$ cells/ml.

C_2C_{12} and MAC13 cells: After cells had been seeded and incubated for 24hr in a humidified atmosphere at 37°C, LMF was added at the appropriate concentrations. On day 5 (96hr later), cells from cells were harvested, firstly by aspirating the medium. Cells were rinsed with 3 x 1ml of PBS and then incubated with 1ml trypsin/EDTA for 1-2min at 37°C. In order to dislodge the cells, the edge of the dish was tapped firmly in a horizontal direction. To ensure complete disruption of cell clumps the suspension was triturated several times with a 1ml pipette. 200µl aliquots of the suspension was taken and diluted with 9.8ml isoton and then cell number counted in a Z_1 Particle Coulter counter.

MAC16 cells (suspension culture) were grown in triplicate for each concentration of LMF. Cells were seperated by trituration with a pipette. Cell number was determined after LMF treatment, as described above.

2.5.7. Measurement of [^{3}H] methyl- thymidine uptake

Cell proliferation was determined by [^{3}H] methyl-thymidine uptake in cells, which reflected the rate of DNA synthesis by a modified protocol described by Fox *et al* 1999. C_2C_{12}, MAC16 and MAC13 cells were seeded in 6 well multidishes at the same cell densities described in the protein synthesis method. After incubation for 24hr, variable concentrations of LMF was added to the medium in triplicate wells. The isotope [^{3}H] methylthymidine was also added at this stage, 1µCi/2ml well and

incubated at 37°C for a further 48hr. Reaction was terminated by aspirating the radioactive medium and washing cells with 3x1ml ice-cold PBS. Cellular protein was precipitated with 1ml/well of 5% trichloro-acetic acid (TCA) and incubated for 1hr on ice. This was decanted and cellular material dissolved in 1ml of 0.3M NaOH at 37°C for 30min. An aliquot of the dissolved protein and DNA was taken and [^{3}H] methylthymidine uptake measured in a liquid scintillation counter.

2.5.8. Determination of glucose utilisation *in vitro*

Cells were seeded as described in the protein synthesis protocol. The culture medium was replaced with 1ml Krebs-Ringer Bicarbonate buffer and experiments were performed by the addition of 10µl/well of a solution containing [^{3}H]-deoxyglucose (0.2µCi/ml Krebs) and glucose to obtain a concentration in the medium of 0.1mM. Uptake of glucose was measured over a period of 30min. Cells were washed 3 times with ice-cold PBS, solubilised in 1ml 1M NaOH and an aliquot taken to determine the amount of radioactivity incorporated.

2.5.9. Measurement of Protein degradation *(in vitro)*

Cells were seeded at 2 x 10^4 cells/ml in 6 well multi-dishes. After 24hr, cells were radio-labelled with aqueous stock of isotopic phenylalanine for 24hr. After labelling, cells were rinsed thoroughly with 3 x1ml PBS and incubated in fresh medium without phenol red and in the presence of the catabolic factor and 1µM cycloheximide for a further 24hr. After this, an aliquot of supernant was measured for [^{3}H] phenylalanine released into the medium as a result of degradation (protein-bound radioactivity was determined as described in section 2.5.4).

2.5.9. 20S Proteasome Sample Preperation in C_2C_{12} myoblasts and myotubes

For experiments using myoblasts, cells were seeded at 4 x 10^4 cells/ml (2ml per well) in 6 well multidishes and left for 24hr to allow adherance to plastic. LMF and/or PIF was added to myoblasts and myotubes and left for a further 24hr after which medium was aspirated and cell monolayers washed with 3 x 1ml ice-cold PBS. Cells were scraped into homogenising buffer and transferred into eppendorf tubes. Cell suspensions were then subjected to 3 x 10sec pulses of sonication on ice, thereafter being centrifuged at 15000 rpm for 20min at 4°C. The resulting pellet was discarded and supernatant used for 20S activity determination and western blotting (Section 2.5.10, 2.5.18).

2.5.10. Fluorometric 20S Proteasome assay

The proteasomes chymotryptic activity was measured by following the hydrolysis of fluorometric substrate N-succinyl-LEU-LEU-VAL-TYR-7-AMIDO-4-METHYL-COUMARIN. Hydrolysis was initiated by the addition of cell homogenate (myoblasts 100µl myotubes 10µl) to 100µl substrate buffer. The reaction was allowed to proceed for 1hr on ice after which, 1ml of 80mM sodium acetate pH 4.3 was added to cease the substrate cleavage. Samples were transferred to fluorometric cuvettes and diluted with a further 2ml of sodium acetate. Fluorescence data was detected in a Perkin Elmer Luminiscence Spectrometer LS50 at 360nm excitation/460nm detection. Fluorescence activity was quantitated as arbitrary units and then corrected for protein concentration measured by the Bradford method.

2..5.11. Biological evaluation of LMF *in vivo*

To determine the biological activity of LMF, purified material from the urine of cachectic cancer patients was injected intravenously over a period of 48hr into the tail vein of NMRI male ex-breeder mice. Animals received two injections per day (8μg/100μl) morning and evening and food and water consumption was monitored throughout the experiment. Control mice received 100μl PBS. Mice were sacrificed by cervical dislocation and appropriate tissues removed quickly for further analyses. Blood samples were taken prior to sacrifice via cardiac puncture under anaesthetic.

2.5.12. Measurement of the rate of protein synthesis *in vivo*

Soleus muscles were excised and ligated as described in the *in vivo* protein degradation assay after following the injection protocol for LMF (Section 2.5.11). Muscles were placed in sterilin bijoux containing 3ml DMEM saturated in 95%O2: 5%CO2 and pre-incubated for 30min at 37°C. Muscles were rinsed with non-radioactive media and replaced with fresh media containing 20μCi of L-[4-^{3}H] phenylalanine and incubated for a further 2hr. Tissues were removed after this time and rinsed with non-radioactive medium, blotted and sonicated in 3ml of 2% $HClO_4$ until homogeneity was achieved. After centrifugation at 2800g for 15min, the supernatants were added to 1.5ml of saturated tripotassium citrate. The insoluble precipitate formed was removed by centrifugation at 2800g for 15min. An aliquot of the resulting solution was mixed with 6ml of Optiphase Hi-safe to determine intracellular free pool of L-[4-^{3}H] phenylalanine.

To determine the amount of protein bound radioactivity, the precipitate from the first centrifugation was washed 3x with 2% $HCLO_4$ (5ml) and hydrolysed in 5ml of 6M

HCl at 110°C in sealed glass tubes for 24hr. The hydrolysates were evaporated to dryness and the residues dissolved in 10ml distilled water. A 1ml sample of the solution was counted for [^{3}H] phenylalanine radioactivity. The rate of protein synthesis was calculated by dividing the specific radioactivity of protein bound [^{3}H] phenylalanine by the radioactivity present in the supernatant fraction.

3.5.13. Sample preperation from gastrocnemius muscle for myosin blotting

Animals were sacrificed by cervical dislocation after receiving twice daily injections of LMF (8µg/injection) for a 48hr period. Muscles were excised and snap frozen in liquid N_2 until processing. For use, muscles were homogenised on ice in 0.5ml HB buffer using a microhomogeniser then sonicated with 3 pulses of 15sec on ice. The sonicates were centifuged at 15000rpm for 15min at 4°C and the supernatant used for myosin western blotting (Section 2.5.18).

3.5.14. Measurement of *in vivo* protein degradation

Tyrosine release was used as a measure of degradation as it is an amino acid that is neither synthesised nor degraded in muscle. Soleus muscles recently excised from male NMRI mice administered iv LMF (Section 2.5.11) were ligated by the tendons, attached to steel supports and placed in ice-cold isotonic saline.

The muscles were placed in incubation vessels containing 3ml RPMI 1640 medium without phenol red saturated in a mixture of O_2 and CO_2 (19:1) at 37°C for 30min. Muscles were rinsed 3x with media followed by replacement with Krebs-Henseleit

buffer (3ml) supplemented with glucose (6mM), BSA (0.12% and cycloheximide (0.5mM) and incubated for a further 2hr.

Muscles were removed and 2ml of incubation buffer was deproteinised with 200µl of 30% TCA. The precipitate formed was centrifuged in a Heraeus benchtop Megafuge 1.0 at 2800g for 10min. The supernatant (2ml) was used to estimate tyrosine content (Waalkes and Udenfriend 1957). 1ml of 0.1% 1-nitroso-2-napthol in 95% ethanol and 1ml nitric acid was added to supernatants in glass centrifuge tubes. These were stoppered, mixed and placed in a shaking water bath at 55°C for 30min. After cooling, 5ml of ethylene dichloride was added and shaken to extract the unchanged 1-nitroso-2-napthol reagent. Tubes were centrifuged again at 2800g for 10min and the aqueous supernatants transferred to glass cuvettes. The fluorescence of the tyrosine derivative was measured in a Perkin Elmer Luminiscence Spectrometer LS50 (460nM excitation, 570nM emission).

3.5.15. Determination of Cathepsin B and L enzyme activities

C_2C_{12} myoblasts were seeded as described in the protein synthesis protocol. Lysosomal extracts were prepared after a 24hr incubation with LMF by removing DMEM and washing cell monolayers with PBS. Cells were detached from the wells with a cell scraper. Subsequent procedures were performed at 0-4°C.

Cells were sonicated in extraction buffer and then centrifuged at 2500g for 15min and the resulting supernatants at 20,000g for 20min in order to remove cytosolic inhibitors of cysteine proteinases. The supernatants formed were used to determine cathepsin activity.

The substrate used to determine cathepsin L activity was N-CBZ-PHE-ARG-7-amido-4-methylcoumarin. The incubation mixture contained 0.1% Brij 35 (495µl), 5µl of cell supernatant, 340mM sodium acetate, 60mM acetic acid, pH 5.5, 4mM EDTA and 8mM DTT (250µl). This was pre-incubated for 5min at 30°C before adding the substrate (5nmoles in 250µl). The mixture was incubated for 10min at 30°C and the reaction was terminated by the addition of termination buffer. The concentration of aminomethylcoumarin produced was measured in a Perkin Elmer Luminiscence Spectrometer LS50 with excitation at 360nm and emission at 460nm.

Cathepsin B activity was estimated in a similar way using Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin (2.5 nmoles into 250µl) as substrate and a slightly different incubation buffer. The reaction was carried out for 10min at 40°C and terminated with 1ml of termination buffer. One unit of enzyme activity is defined as the release of 1pmol of aminomethycoumarin from the substrate during the 10min incubation period.

3.5.16. Cyclic-AMP (cAMP) determination

C_2C_{12} cells were seeded in 6-well multidishes at a concentration of $5x10^4$cells/ml and left to incubate for 48hr. After this time, increasing concentrations of LMF were added and incubated for further 30min. Medium was removed and 0.5ml of assay buffer was added to each individual well and placed in a boiling water bath for 5min to terminate the reaction and lyse the cells. Dishes were placed on ice for 10min whereafter, cell extracts from each well were transferred to eppendorf tubes and sonicated on ice. Tubes were centrifuged at 5000rpm for 15min, the resultant supernatant being used for the assay. A volume of 50µl was taken and transferred to a fresh set of eppendorf tubes to which 50µl of diluted [^{3}H] cAMP was added (2µl of [^{3}H] cAMP diluted in

4ml assay). This was mixed thoroughly using a vortex mixture. In addition, 100µl of diluted binding protein (1:1with assay buffer) was added to each tube, mixed well and left on ice for at least 2hr to equilibrate. Charcoal suspension, 100µl was added, vortexed and left to stand for a few min. Tubes were centrifuged at 10,000rpm at 4°C for 5min. 185µl of the supernatant was removed carefully so not to disturb the charcoal pellet. This was mixed thoroughly with scinitillant fluid and the amount of unbound [^{3}H] cAMP detected in a scintillation counter.

The assay is based on competition between cAMP and [^{3}H] cAMP. Complexed cAMP was removed by adding charcoal. The remaining unbound [^{3}H] cAMP could then be measured. Thus, the more unlabelled cAMP there was present, the less likely [^{3}H] cAMP is to bind to binding protein and therefore measurements of radioactivity in supernatant would decrease.

The exact amount of cAMP produced by LMF was determined by extrapolating values from a standard curve, constructed at the same time as the experiment using known amounts of cAMP.

3.5.17. Determination of protein kinase A (PKA) activity

C_2C_{12} myoblasts were treated with LMF for 24hr and soluble fractions produced by sonication in homogenising buffer. Cell extracts were then centrifuged at 15000rpm for 20min and supernatants used for PKA activity determination, using an enzyme assay kit (Pierce). The peptide Kemptide, labelled with fluorescent dye was the substrate for the assay. The absorbance of the phosphorylated product was measured at 570nm and actual values of PKA activity were extrapolated from a standard curve constructed using exact units of PKA.

2.5.18. SDS-PAGE and Western Blotting:

2.5.18.1. Preparation of samples

Samples to be analysed were mixed 1:1 with denaturing sample buffer and heated for a suitable length of time to ensure complete denaturation (5-10min), cooled and centrifuged ready for protein electrophoresis. Molecular weight standards used were as follows: rabbit muscle myosin (M_r 205kDa), phosphorylase b (M_r 97kDa), BSA (M_r 66kDa), ovalbumin (M_r 45kDa), carbonic anhydrase (M_r 29kDa), trypsin inhibitor (M_r 20 kDa) and α lactalbumin (M_r 14kDa).

2.5.18.2. Protein separation

Protein samples were analysed electrophoretically as described by Laemmli (1970). The required amount of protein (Table 1.1) was denatured by heating with an equal volume of sample buffer, cooled and centrifuged Affinity purified PIF was separated using a 15% acrylamide gel. All other proteins were detected using 12% denaturing polyacrylamide gel (see Section 2.4.12 for gel recipes).

The running gel was poured into a pre-made gel plate apparatus (Atto Dual Mini Slab Kit) after cleaning thoroughly with 70% ethanol. The gel was overlaid with butanol water and allowed to polymerise for approximately 30 min. After removing the top layer water, the surface of the gel was cleaned several times with distilled water. The stacking gel was then poured on top of the running gel and a Teflon gel comb inserted and allowed to set for 30min.

After complete polymerisation, the gel plates were placed into the electrophoretic apparatus and the reservoirs filled with running buffer. On removal of the comb, the denatured protein samples and the molecular weight markers were loaded into the

wells. Separation was carried out at 180V until the dye front reached the base of the gel.

2.4.18.3. Western Blotting

The gel containing the electrophoresed proteins was placed into a cassette together with a piece of nitrocellulose and 3MM filter paper. This was immersed in a tank of blotting buffer between two parallel electrodes. The proteins were transferred to the nitrocellulose by applying a current passing at right angles to the gel for two hours at 80V with cooling. The remaining hydrophobic binding sites on the membrane were blocked by incubating overnight in 5% (w/v) non-fat dried milk in PBS 0.1% Tween-20 at 4°C. The membrane was then probed for the protein of interest.

2.5.18.4. Immunodetection of Western Blots

The following protocol was observed for all western blots except membranes immunoblotting for proteolysis-inducing factor (PIF), which were incubated in PBS/Tween without non-fat milk.

The blocked membrane was incubated at room temperature for 1hr with the primary antibody in 5% non-fat dried milk in PBS/Tween 0.5%. After washing 3x10min with PBS/Tween 0.10%, the membrane was incubated with the secondary antibody Horseradish Peroxidase-conjugated IgG complex for a further 1hr. The blot was washed 3x5min and then 3x15min with PBS/Tween 0.50% prior to visualisation.

The total amount of protein loaded for each gel and concentrations of antibodies used during western blotting, can be seen for the various proteins of interest in Table 1.1.

For all western blots, a parallel gel was silver stained to ensure equal loading of protein (Section 2.5.20).

Protein of Interest	[1°antibody]	[2° antibody]	Amount of protein loaded per lane (μg)
Gαs/Gαi	1:2000	1:1000	50(Gαi/5(Gαs)
HSL	1:1000	1:1000	30
20S	1:1500	1:1500	1-2
Myosin heavy chain	1:100	1:1000	5
PIF	10μg/ml	1:15000	20

Table 1.1 Antibody dilutions for the Proteins detected during Western blotting

2.5.18.5. Visualisation of Western Blots

Blots were visualised using the ECL™ Western Blotting Detection System. It is based on the detection of immobilised specific antigen, conjugated directly or indirectly with Horseradish Peroxidase (HRP)-labelled antibodies. The reaction involves luminol, which upon oxidation by HRP, emits light which can be detected by short exposure to blue-light sensitivity autoradiography film (Hyperfilm ECL).

The blots were covered completely in an equal volume of detection solution 1 with detection solution 2 for 1min, drained in Saranwrap and taped into the film cassette. The autoradiography film was then carefully placed on top of the membrane and exposed for 15sec to 20min. Films were developed by placing in a solution of Kodak Developer and Replenisher for 5min and fixed by immersing in Kodak fixer and Replenisher for 5min.

2.5.19. Coomassie Brilliant Blue Staining

For routine detection of protein on gels, protein samples were stained with Coomasie Brillaint Blue R-250, a sulphated trimethylamine dye. Staining is achieved in approximately 2 hours in the presence of the dye. Gels were destained for 2-3 hours with several changes of Destaining Solution aided by gentle agitation. Gels were fixed onto filter paper by drying under vacuum at 80°C for 2 hours.

2.5.20. Silver Staining Protocol

Gels were silver stained when 1 or <1μg of protein was to be detected. The protocol was derived from the Bio-Rad Silver Stain kit. After electrophoresis gels were fixed in 200ml of fixative 1, a solution of 40% methanol/10% acetic acid (v/v) for a minimum of 30 min. This was followed by a second fixing step involving washing twice for 15 min in 200ml of fixative 2 containing 10% ethanol/ 5% acetic acid (v/v). Gels were then immersed in 100ml of 10% oxidising solution and then washed extensively using large volumes of deionised water, repeating at least 6 times until the water remained clear. Gels were exposed to 100ml of 10% silver reagent for 20 min and then washed for a maximum of 30 sec. The gels were developed in a developing solution for approximately 30 sec or until a brown or smokey precipitate appeared. At this point the solution was poured away and replaced with fresh developer every 5 min until the desired intensity of bands was obtained. The reaction was stopped before the gels became discoloured by completely submerging the gel in 5% acetic acid (v/v).

2.5.21. Purification of Murine Proteolysis-inducing factor (PIF)

Solid MAC16 tumours dissected from mice with established cachexia were homogenised in 5ml/g of Q Sepharose buffer. The resulting homogenate was subjected to low speed bench top centrifugation (4000rpm for 20min in a Heraeus Megafuge 1.0). The pellet and top fat layer were discarded and protein content and total volume recorded. Ammonium sulphate precipitation was performed at 38% w/v added slowly with stirring at 4°C. The supernatant was then left covered with foil to equilibrate overnight.

The precipitate was removed by centrifugation at 4500rpm for 20min. The resulting solution was concentrated against PBS in an Amicon filtration cell using a membrane filter with a molecular weight cut-off of M_r 10 000. Any particulate matter present was removed by centifuging at 4500rpm for 20min. After measuring the volume and protein content, the solution was applied to an affinity column containing monoclonal antibody coupled to Protein A. The sample was circulated at a flow rate of 0.1ml/min in the cold room overnight.

The column was washed with PBS for 3 hours and the retained proteins eluted with 100mM glycine-HCl pH 2.5 at a rate of 1.0ml/min into tubes containing 0.5ml 1M Tris-HCl, pH 8.0, for neutralisation. Immunoreactive fractions were determined using an ELISA plate assay.

2.5.22. Detection of Cachectic factor by ELISA Technique

Samples to be assayed were immobilised on a 96-well polyvinylchloride assay plate by mixing 50µl of each fraction with 50µl of 0.1M carbonate-bicarbonate buffer pH 9.5 and incubating at 37°C for 2 hours or at 4°C overnight. The contents of the wells were then aspirated and washed three times with 200µl of washing buffer (PBS +

0.1%Tween 20). Non-specific sites were blocked with 200µl of blocking solution (PBS + 0.1% Tween 20 and 3% BSA) and incubated for 37°C for 30 minutes. After the blocking solution was removed from the wells and washed three times with 200µl of washing buffer, samples were incubated for 1 hour at 37°C with the monoclonal antibody (100µl of a 10µg/ml of blocking solution), whilst 100µl of blocking solution containing no antibody was added to negative control wells. The wells were washed three times before the addition of 100µl/well of protein A peroxidase conjugate diluted in blocking solution (0.25µg/ml) and incubated for a further 1 hour at 37°C, after which the substrate (0.04% O-phenylenediamine in sodium phosphate-citrate buffer pH 5.0 and 0.012% hydrogen peroxide) was added to each well. After a period of 15-30 minutes, immunoreactive cachectic material producing a yellow colouration was visible. The reaction was terminated by adding 50µl of 0.2M sulphuric acid and the absorbance determined at 492nm using a microplate reader (Anthos Labtech Instruments).

2.5.23. Isolation of Murine Adipocyte Plasma Membranes

Epididymal fat pads were isolated from male NMRI mice recently killed by cervical dislocation. Each pair of fat pads were placed immediately into 1ml of Krebs Ringer Bicarbonate solution supplemented with 3% BSA and 4mg/ml collagenase enzyme, pre-warmed to 37°C. The tissue was then thoroughly chopped with scissors and the resulting cell suspension gassed with 95%O_2/5%CO_2 vortexed and subject to digestion carried at 37°C with constant shaking for 30min.

Isolated adipocytes were washed three times with sucrose buffer and re-suspended in a small volume. Cells were then sheared under force by aspirating rapidly through a

Swinny filter at least 10 times to create a cell suspension and centrifuged for 5min at 1000rpm in a Heraeus Sepatech Megafuge 1.0. The resulting fat layer on the surface was removed carefully, and the remaining cell homogenate transferred to Beckman ultracentrifuge microtubes. The samples were then centrifuged in a Beckman ultracentrifuge for 1 hour at 19000rpm at 4°C, which yielded a pellet and supernatant. The adipocyte membranes were contained within the pellet; these were separated from the presence of other cell organelles on a percoll gradient. The pellet was resuspended in 50-100µl of sucrose buffer and then added to a mixture of percoll, sucrose buffer and concentrated sucrose buffer in the proportions of 7:32:1 respectively and centrifuged at 13000rpm for 30min at 4°C.The diffuse top layer of the percoll gradient containing the membrane fraction was carefully removed using a small pipette tip and transferred to a fresh tube. The membranes were washed three times with sodium chloride buffer, centrifuging at 13000rpm for 2min at 4°C between each wash. Washed membranes were suspended in plasma membrane buffer, adjusted to a protein concentration of 1-2mg/ml, snap frozen in liquid nitrogen and stored at -70°C until further use.

2.5.24. Isolation of human adipocytes

Human adipose tissue was removed under general anaesthesia and transported immediately to the laboratory. Tissue was roughly chopped into small pieces and digested as described for murine adipocyte isolation.

3.5.25. Sample preparation and immunoblotting of hormone sensitive lipase (HSL)

3T3-L1 were plated into 6 well multi-dishes at 4 x 10^4 cells/ml and left to grow to confluence. Cells were then subjected to hormonal treatment to promote differentiation into adipocytes (Section 2.2.3). In order to confirm that all cells had undergone differentiation, the wells were stained with Oil Red O, a fat soluble dye, which stains oil droplets within differentiated cells which are observable under the microscope (Section 2.2.3).

LMF was added to differentiated fat cells at the desired concentration and left to incubate for 24hr. Reaction was stopped by removing incubation media and washing cells with 3 x 1ml ice-cold PBS.

All samples were homogenised into HB buffer at 4°C, sonicated on ice 3 x 10sec pulses. Fat depleted infranatants from cells were prepared by centrifugation at 100000g in a Beckman Ultracentrifuge for 1hr. Infranatants were analysed by western blotting for the presence of HSL. All samples were measured for protein content using the method of Bradford then denatured in sample buffer and stored at -20°C until western blotting analysis.

2.5.26. Assay for 5' -nucleotidase

5'-Nucleotidase is found almost exclusively in the plasma membrane. It is measured using the method of Avruch and Wallach (1970). This enzyme catalyses the conversion of 5' AMP to adenosine.

50µl of the membrane sample to be assayed was added to a microfuge tube containing 1ml of incubation buffer (see materials section). 1µCi [2-H^3] AMP (1µl) tracer was added to each sample vessel (a second blank sample was included which contained incubation buffer and tracer alone). Samples were mixed and then incubated at 37°C in a shaking water bath. To terminate the reaction, 0.2ml of 0.25M zinc sulphate was added. 0.2ml 0.25M barium hydroxide was added to precipitate any remaining unhydrolysed AMP. Samples were centrifuged at 13000rpm for 1 min and 0.7ml transferred to scintillation vials containing 10ml of Optiphase Hisafe3 scintillation fluid for counting. A higher dpm reading compared to blank correlated with the presence of the enzyme and therefore plasma membranes.

CHAPTER 3

Second messenger pathways in the action of cancer cachectic factors

3.1 Introduction

Skeletal muscle is the largest tissue of the human body. Its mass comprises approximately 40-45% of the total body weight and constitutes the largest single element of the protein-bound nitrogen pool in the mammalian organism. Its main function is to provide power for locomotion in addition to having an important role in metabolism; it is a major repository of protein (± 50% of total body protein) and free amino acids in the body. It also serves as a source of precursors for glucose (gluconeogenesis) and essential protein synthesis in other tissues (Mann and Truswell 1998).

Skeletal muscle growth is regulated by two processes, hyperplasia, an increase in cell number and DNA content and secondly, hypertrophy, a stimulation of protein per DNA unit within that tissue. The factors that regulate these processes are diverse, however, in mature skeletal muscle, growth is primarily related to the rate of turnover of the constituent muscle protein, although proliferation is still evident in certain situations such as muscle trauma (Grounds 1991)

As muscle forms during development, the protein composition of the tissue is defined. It incorporates not only actomyosin myofibrillar proteins which are required for muscular contraction and constitute approximately 60% of skeletal muscle protein, but also the sarcoplasmic proteins required for cytoskeletal and enzymatic processes within the myofibre (Morrison 1995).

The mass of mature of skeletal muscle fibre is therefore determined by the relative rates of synthesis and degradation i.e. protein turnover. This is a cyclical process, which must be regulated co-ordinately to maintain equilibrium. During muscle hypertrophy, synthesis exceeds breakdown whereas during muscle loss (atrophy) degradation takes precedence over synthesis. In certain disease states e.g. fasting where amino acids are required for gluconeogenesis or in cancer cachexia, this equilibrium is disrupted and a net loss of protein ensues.

Protein synthesis rates in mammalian skeletal muscle are sensitive to both the hormonal and nutritional status of the animal and the synthesis rate of this tissue represents 30-50% of whole body protein synthesis at developmental maturity (Waterlow 1984). Thus, targetting of this tissue for implementing strategies to increase protein synthesis and/or reduce protein degradation have obvious potential clinical application in conditions already mentioned such as muscle atrophy or cancer cachexia, and also agricultural application in the formation of a leaner carcass.

The primary focus of this study is to elucidate the signalling pathways involved in modulating protein turnover in muscle by tumour-derived catabolic factors, lipid-mobilising factor (LMF) and proteolysis-inducing factor (PIF). A brief synopsis of these agents was given in the previous introductory section. In order to appreciate the complexity of their proposed effects on protein turnover, an in-depth understanding of the molecular basis of processes such as protein synthesis and degradation are required. Thus, the following sections will review the molecular/biochemical processes and current understanding of the signal transduction pathways, which regulate protein synthesis and degradation in skeletal muscle.

3.2. PROTEIN SYNTHESIS

3.2.1 Translation

It has become evident in recent years that mRNA translation represents an important control point in gene expression (Jansen *et al* 1995). This process is conveniently divided into three stages: initiation, elongation and termination. Each of these aspects of translation is mediated by regulatory factors termed initiation (eukaryotic initiation factors eIF), elongation (elongation factors eF) and releasing factors (eRF) respectively. Initiation seems to be the major locus of regulation, modulation of the activities of eIFs by phosphorylation has received considerable attention. It is less clear what role the control of elongation plays in the regulation of translation, although the activities of elongation factors can also be modulated by phosphorylation.

3.2.1.1 Mechanism of Initiation

Initiation of protein synthesis involves the sequential binding of first the 40S and then the 60S ribosomal subunit to a messenger RNA molecule. The process can be divided into three stages (Figure 3.1): association of initiator tRNA (Met-tRNAi) and several initiation factors with the 40S ribosomal subunit to form the 43S preinitiation complex; 2) the binding of this complex to mRNA and 3) the addition of the 60S ribosomal subunit to assemble an 80S ribosome at the initiation codon, ready to commence translation of the coding sequence. This last step requires the prior release of the initiation factors bound to the 40S ribosomal subunit during the earlier stages; these factors are then recycled to catalyze further initiation events.

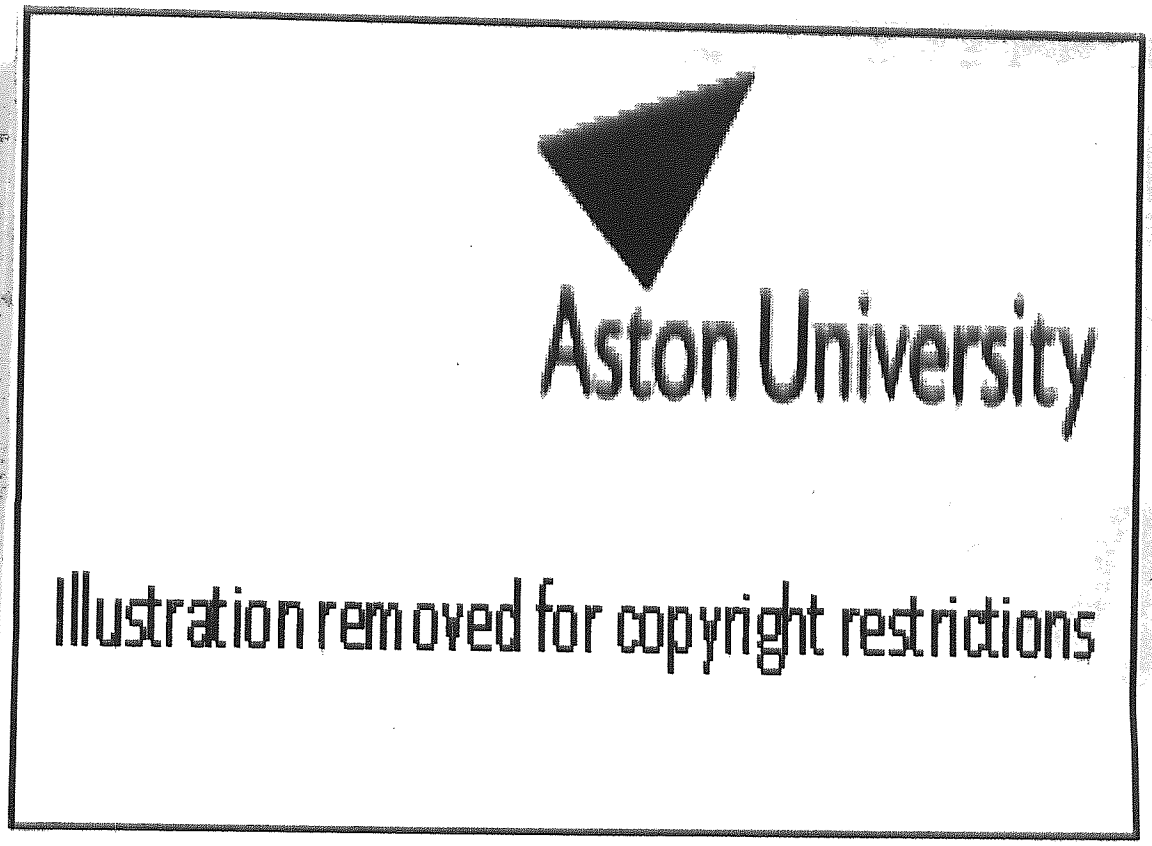


Figure 3.1. Mechanism of Initiation of Protein Synthesis (taken from Pain 1996).

3.2.1.1.1 Stage1

80S ribosomes dissociate and 40S subunits are captured for initiation by binding eIF1A and eIF3. Under intracellular conditions the equilibrium between free, or native, ribosomal subunits, and 80S couples is strongly weighted towards the latter. These factors are thought to bind newly dissociated 40S ribosomal subunits and to delay reassociation with 60S subunits for long enough to permit their recruitment for initiation. eIF3 (a multimeric complex of MW 500-750kDa) has long been known to stabilise the 43S preinitiation complexes *in vitro*, and also be essential for binding of these complexes to mRNA (Pain 1996). The initiator tRNA (Met-tRNAi) then interacts with GTP and eIF2 (a complex of 3 polypeptide chains, α, β γ) to form a complex. It would appear eIF2 remains associated throughout the initiation cycle. The continuity of the initiation events requires a guanine nucleotide exchange factor, eIF-2B, which catalyses the reaction required to recycle the eIF2 released from initiation complexes as an eIF2.GDP complex to the eIF2.GTP form capable of recruiting a new molecule of initiator tRNA.

3.2.1.1.2 Stage 2

Eukaryotic mRNAs have a unique structure at their 5' terminus which is termed the cap. This structure is a 7-methylguanosine moiety attached via a 5'-5' phosphodiester link. It aids in the stability of the structure and has a strong stimulatory effect on translation of mRNA.

The 43S initiation complex binds to mRNA at the 5' terminal cap structure and then migrates towards the AUG initiation codon. The initial binding involves the factors eIF-4E (or eIF-4α), eIF4G and eIF4A, which assemble the 5'-end of mRNA, creating conditions that allow the melting of intramolecular secondary structures within the

mRNA that would otherwise prevent the binding of the 43S preinitiation complex. In the vast majority of cases, 5' AUG codon is utilized for initiation.

3.2.1.1.3 Stage 3

When the preinitiation complex stops at the initiation codon, the GTP molecule introduced as part of the eIF2 complex is hydrolyzed to GDP and this powers the ejection of the initiation factors bound to the 40S ribosomal subunit. The initiation factor eIF-5 is involved in this process. The release of these factors permits the association of a native 60S ribosomal subunit to reconstitute an 80S ribosome at the initiation codon poised to commence the elongation stage of translation.

3.2.1.2 Alternate initiation schemes

There are two alternate methods to get ribosomes to an initiating codon (Pain 1996). The first of these is reinitiation. By definition, these mRNAs must be polycistronic, having at least two open reading frames (ORFs). The suggestion is that the first initiation event occurs as explained earlier. After completion of the polypeptide chain, the 40S unit continues to scan or move down the mRNA, a certain percent of 40S subunits are lost at the termination step or during subsequent scanning. At the same point, a new ternary complex must be acquired, both to serve as the initiator tRNA and to locate the next initiating codon, thus providing all the components necessary for AUG selection and subsequent subunit joining to take place. The second rare initiation event is internal initiation, often characterized by cap-independent initiation. Simply, the 43S complex binds to a portion of the mRNA distant from the 5' end and then scans, if necessary, to locate the initiating AUG codon. This process is being further investigated.

3.2.2 Elongation

Each ribosome has three sites of association with a transfer tRNA molecule. These sites are termed A (aminoacyl) site, the P (peptidyl) site and the E (exit) site, each of which receive each tRNA in successive steps of the elongation cycle (Karp 1998)

In the complete 80S ribosome formed during the process of initiation, the A site is free. Before the second aminoacyl-tRNA can effectively bind to the mRNA in the A site, it must combine with the protein elongation factor 1 (eEF1) linked to GTP. This complex then allows the aminoacyl-tRNA to enter the A site with the release of eEF1-GDP and phosphate.

The α amino group of the new aminoacyl-tRNA in the A site carries out a nucleophilic attack on the esterified carboxyl group of the peptidyl-tRNA occupying the P site. This reaction is catalyzed by peptidyl transferase, of the 60s ribosomal subunit. Because the amino acid on the amino-acyl tRNA is already "activated" no further energy source is required for this reaction. The reaction results in attachment of the growing peptide chain to the tRNA in the A site.

Upon removal of the peptidyl moiety from the P site, the discharged tRNA quickly dissociates from the P site. Elongation factor 2 (eEF2) and GTP are responsible for the translocation of the newly formed peptidyl-tRNA at the A site into the P site and the transfer of the deacylated tRNA from the P site into the E site. The GTP required for eEF2 is hydrolyzed to GDP and phosphate during the translocation process. The translation of the newly formed petidyl-tRNA and its corresponding codon into the P site then frees the A site for another cycle of aminoacyl-tRNA codon recognition and

elongation. The whole process requires the hydrolysis of four high-energy phosphate bonds, two ATP molecules to ADP and two GTP molecules to GDP.

3.2.3 Termination

A chain terminating UAG, UAA or UGA codon in the ribosomal A site does not promote the binding of any tRNA species. Instead, another non-ribosomal protein, release factor (RF) binds to the ribosome as an RF-GTP complex. The peptide-tRNA ester linkage is then cleaved through the action of peptidyl transferase, acting at this stage as a hydrolase. The completed peptide is released from its carrier tRNA and from the ribosome. Dissociation of the RF from the ribosome requires hydrolysis of the GTP molecule. This probably results in a change in RF conformation and the dissociation of the ribosome into 40S and 60S subunits to then re-enter the protein synthetic cycle at the initiation stage.

3.2.4 Regulation of translation

There are two distinct types of translational control: 1) general control of overall translation affecting the bulk of mRNAs in the cell and 2) selective regulation of specific mRNAs or subsets of mRNAs; the latter may arise by virtue of structural features of these mRNAs (especially secondary structure in the 5' terminal region) or by mRNA-binding proteins. These transacting factors may either facilitate initiation factor binding or prevent their interaction (Bardocz and Hesketh 1989).

3.2.4.1 Initiation

Translation is recognised as an important site of regulation of gene expression, with the initiation stage as the most commonly observed target for physiological control, as previously discussed. Modulation of initiation can influence both the overall, global,

rate of protein synthesis (quantitative protein synthesis) and the relative rates of synthesis of different proteins (qualitative protein regulation); frequently, controls at these two levels are superimposed. Two particular steps of the initiation pathway appear to be subject to physiological regulation; 1) the binding of Met-tRNAi to the 40S ribosomal subunit, mediated by eIF2, 2) the initial binding of the 43S preinitiation complex to the 5' end of mRNA mediated by eIF4E and associated factors.

An increasing number of trans-acting factors, commonly proteins associated with mRNA in the messenger ribonucleoprotein particles, are now being recognised as possible modulators of translation.

3.2.4.1.1.Regulation and eIF-2 activity

It has been observed that numerous physiological conditions that serve to inhibit the initiation step of protein synthesis, has been a result of a decrease in the activity of eIF2 and thus impairs the formation of 43S preinitiation complexes (Hershey JW 1991, Proud CG 1992). The site of control of eIF2 activity has been identified as the recycling step involving the guanine nucleotide exchange factor eIF2B. Two mechanisms regulate this step. The first is phosphorylation of α-subunit of eIF2 at a single serine residue, Ser51 which results in increased affinity of eIF2 for eIF2B. However, the complex fails to carry out guanine nucleotide exchange. This then decreases the concentration of eIF2B available to recycle even non-phosphorylated eIF.2GDP. The second mechanism discovered more recently involves direct regulation of eIF2B activity independent of changes in eIF2 phosphorylation.

Phosphorylation of the α-subunit of eIF2 is one of the best characterized mechanisms of down regulating protein synthesis in higher eukaryotes in response to various stress conditions. Three distinct protein kinases regulate protein synthesis in eukaryotic cells by phosphorylation of the α subunit of eIF2 on serine 51. There are two mammalian eIF2α kinases: the double stranded RNA-dependent kinase (PKR) and heme-regulated inhibitor kinase (HRI) and yeast GCN2. These will be now be considered more closely.

The role of changes in the activity of eIF2B in regulating peptide-chain initiation emerged from work in the reticulocyte lysate system (Thomas *et al* 1984). His work demonstrated that haem deficiency lead to the activation of a protein kinase, HRI (haem-regulated inhibitor) which phosphorylated α-subunits on eIF2. It was discovered later that increased phosphorylation of eIF2α seen in these reticulocyte lysates led to inhibition of eIF2 activity due to block in the eIF2B mediated recycling step. The reason being that phosphorylated eIF2 had a higher affinity for eIF2B than non-phosphorylated; eIF2αP acts to inhibit it competitively and therefore inhibiting the activity of eIF2B. This mechanism is rendered particularly effective by the molar ratio of eIF2/eIF2B; the higher the ratio the more sensitive the system becomes to the level of phosphorylation of eIF2α.

The eIF2α kinase, PKR, protein kinase activated by double–stranded RNA demonstrated the above phenomena (Samuel CE 1993). This is markedly induced in many cell types by interferon α and β and is thought to play a role in its anti-viral effect. Upon viral infection, the kinase is activated and severely inhibits translation by increased eIF2α phosphorylation and blocking recycling of eIF2B. This is deleterious to the individual cell but prevents the utilisation of its translational apparatus for the production of viral replication within the cell population as a whole.

Another well characterized eIF2α kinase GCN2 is a *Saccharomyces cerevisiae* protein of 182kDa which is activated during chronic amino acid starvation (Hinnebusch 1994). Phosphorylation of eIF2α by GCN2 kinase paradoxically mediated increased translation of mRNA encoding transcription factor GCN4. The physiological significance of this is that GCN4 can promote expression of genes encoding a number of enzymes involved in *de novo* amino acid synthesis; thus the ability to switch on its synthesis provides a mechanism for the yeast cells to compensate for their nutritional deficiencies. In simplistic terms, in the absence of amino acids, GCN4 expression increases without an increase in mRNA content. Once amino acids rise, GCN4 expression is decreased again with no apparent change in mRNA levels.

This unique response depends on four short upstream open reading frames (uORFs) in the region of GCN4 mRNA (Hinnebusch 1994). Initiation begins in the usual cap-dependent manner and the ribosomes scan until they initiate at either ORF-1 or ORF-2. Following termination, the 40S subunit continues scanning in a 5'-3' direction. However, in the absence of the ternary complex (eIF-2.GTP.Met-tRNA$_i$) recognition of future AUG codons is not possible. Therefore, to reinitiate protein synthesis, the scanning 40S subunit must acquire a new ternary complex and when it does so, is ready to reinitiate. Under high levels of amino acids, the ternary complex is acquired quickly and a second round of initiation occurs at ORF-3 or ORF-4. If this happens, the scanning 40S subunit either falls off the mRNA or does not recover in time and consequently bypasses the AUG codons for GCN4 ORF. On the other hand, in the case of amino acid starvation, the scanning ribosome bypasses ORF-3 and ORF-4 but recovers a ternary complex in time to initiate protein synthesis at the initiating AUG for GCN4.

The key element in this scheme is regulating the rapidity of how a ternary complex might reassociate with the scanning ribosome. It is believed that GCN2, a protein kinase with a His-tRNA synthetase-like carboxy terminus (Krupitza and Thireos 1990) "senses" uncharged tRNAs and this activates its activity. This causes the phosphorylation of eIF2α which results in a decrease in the activity of eIF2B and therefore the pool size of the ternary complex is diminished.

Decreased eIF-2B activity is seen in extracts from serum depleted cells and this is restored following provision of fresh serum (Montine and Henshaw 1989). Henshaw and colleagues showed that serum treatment quickly lead to decreased phosphorylation of eIF-2α and activation of eIF2B, which slightly preceded the stimulation of protein synthesis.

In contrast, there are a number of situations where alterations in eIF2B activity can not be accounted for by changes in the phosphorylation of eIF2α usually because no changes are observed. In skeletal muscle of intact rats, starvation and diabetes resulted in a fall of eIF2B activity without alteration in the level of phosphorylation of eIF2α (Kimball and Jefferson 1988, 1991). In serum starved Swiss 3T3 cells, eIF2B activity increased after treatment with serum, insulin , epidermal growth factor or phorbol esters but again no significant changes in eIF2α phosphorylation (Welsh and Proud 1992). This strongly suggests that eIF2B activity can be regulated by other mechanisms. These will now be considered.

3.2.4.1.2 Direct regulation of eIF2B activity

Mechanisms involving allosteric regulation have been described in some reports. The activity of purified mammalian eIF2B has been shown to be modulated by NAD+ and related molecules. NAD+ and NADP+ inhibit eIF2B mediated nucleotide exchange,

while their reduced forms counter this effect (Oldfield and Proud 1992). However, other researchers conflict with this data. Results from *in vitro* do not correlate *in vivo* (Kimball *et al* 1996). Physiological importance of allosteric regulation by polyamines and glucose-6-phosphate is also unclear at present (Trachsel 1996).

Dholakia and Wahba (1988) showed that purified reticulocyte eIF2B could be phosphorylated by casein kinase-2. (CK-2) an enzyme whose role in cellular regulation remains unclear. They reported that phosphorylation of CK-2 caused a five-fold activation of the exchange activity of eIF2B. Treatment of fibroblasts, or Chinese hamster ovary cells over expressing the insulin receptor, in the presence of insulin increased the eIF2B activity by down regulating the phosphorylation of eIF2B (Welsh and Proud 1992). It was later suggested that it may be glycogen synthase-3 (GSK-3), an insulin regulated enzyme, which plays a key role in the control of glycogen metabolism and probably transcription. This was further confirmed by adding purified GSK-3 to eIF2B which did phosphorylate the target subunit of eIF2B.

3.2.4.1.3. mRNA binding to ribosomes

The binding of the 40S subunit to mRNA involves several initiation factors and has the potential for controlling both the overall rate of translation and the relative rates of utilisation of different mRNA molecules in response to physiological signals (Morley 1996). At the extreme 5'- end of all cytoplasmic mRNAs is a modified guanosine moiety (7-methylguanosine), termed the 5' cap. The mRNA scanning process which aligns the ribosome with the initiation codon, is thought to involve the binding of various proteins to the cap structure. It is an energy-dependent process in which the energy is derived from ATP hydrolysis. Three initiation factors, eIF4A, eIF4B and eIF4F are required for mRNA binding. eIF-4A, which in mammals exists as two

different, but very similar gene products, is an RNA-dependent ATPase, and in conjuction with eIF4B exhibits RNA helicase activity. eIF-4F is a 3-subunit complex that is composed of : 1) eIF-4A; 2) eIF-4E, a 24kDa polypeptide that specifically interacts with the cap structure. It is activated by release from its binding protein, 4E-BP1; 3) eIF-4G (p220/eIF-4γ) subunit. This is a scaffolding protein which mediates binding of the 40S ribosomal subunit to the mRNA in a cap-dependent manner. It increases the binding of eIF-4E to the cap structure and brings the 40S ribosome subunit to the mRNA through its affinity for eIF-3. eIF-4F is twenty times more effective as a helicase as compared to its eIF-4A catalytic unit in its free form. eIF-4F also exhibits sequence-non specific RNA binding activity. These activities support the notion that eIF-4F functions together with eIF-4B in the unwinding of the mRNA 5' secondary structure to effect efficient ribosome binding.

Secondary structure in the 5' untranslated region of an mRNA impedes the translation probably by interfering with the scanning mechanism by which the 40S subunit locates the initiation codon. Secondary structure has to be "unwound" to permit scanning which is achieved by eIF4A (as part of the eIF-4F complex) which possesses ATP-dependent RNA helicase activity. It is thought that once the eIF-4E and eIF-4G have been released from the cap by eIF-4B, then further, free eIF-4A can independently bind to the mRNA to facilitate further unwinding.

3.2.4.1.4. Regulation of initiation factors involved in binding the 43S preinitiation complex

Two features of eIF-4E render it an ideal candidate as a key role player in regulation of translation and cell growth. First, it is present in limiting amounts relative to other initiation factors (0.01-0.20 molecules per ribosome as compared to 0.5-3.0 molecules

per ribosome for other initiation factors). Secondly, there is a strong correlation between the phosphorylation state of eIF4E and translation rates in the cell. For example, during heat shock (Duncan and Hershey 1989) translation is reduced concomitantly with a reduction in eIF4E phosphorylation. Lastly, eIF-4E is phosphorylated in response to treatment of cells with numerous growth factors and mitogens (Rhoads 1991). These observations were biologically significant as phosphorylation appeared to enhance eIF-4E activity since only phosphorylated eIF-4E was present in the 48S mRNA ribosomal complex.

In general growth inhibitory conditions lead to de-phosphorylation of eIF-4E and a decrease in total protein synthesis while growth stimulatory conditions coincide with eIF4E phosphorylation and an increase in protein synthesis (Kleijn *et al* 1998). However, there are a few exceptions whereby eIF4E dephosphorylation coincided with increased protein synthesis. Increased eIF4F formation in spite of decreased eIF4E phosphorylation, might explain this result but it appears that translation can be regulated by a mechanism other than phosphorylation.

Growth factors, mitogens, hormones and cytokines induce eIF4E phosphorylation (Sonenburg 1994) which include various peptide growth factors e.g. EGF, PDGF, TNFα; hormones such as insulin and differentiation promoting factor NGF, and angiotensin II which is a hormone that acts on vascular smooth muscle cells and causes hypertrophy. Differentiation of PC12 with NGF was due to increased eIF4E phosphorylation but in another report P19 cells underwent differentiation in the absence of eIF4E phosphorylation so the data in this area is conflicting (Frederickson *et al* 1992, Kleijn *et al* 1995).

Phosphorylation of eIF4E is increased after mitogenic stimulation of human T cells by phytohemagglutinin or of porcine peripheral blood mononuclear cells by either phorbol ester or concanavalin A, concomitant with an increase in the rate of protein synthesis (Morley *et al* 1993). Fertilisation of sea urchin eggs and starfish oocytes results in a dramatic increase in translation rates coincident with an increased phosphorylation of eIF4E. (Akkaraju *et al* 1991).

The role of eIF4E during viral infections and during differentiation of cells, is unclear. Dephosphorylation of eIF4E has been described after infection with influenza, adenoviruses, encephalomyocarditis virus (EMCV) or poliovirus and is one way to shut off host protein synthesis. However, eIF4E dephosphorylation and host protein synthesis shut off was only linked after infection with adenovirus (Feigenblum and Schneider 1993). A role for eIF4E dephosphorylation by okadoic acid had no effect on the course of infection of picorn viruses (Kleijn *et al* 1998). This area is subject to further analysis.

3.2.4.1.5. Regulation of eIF-4E binding protein, 4E-BP1

This protein binds to amino acid residues in eIF-4E that are also bound by eIF-4G to form the eIF4F complex (Kliejn *et al* 1998). The shared binding motif of 4E-BP1 and eIF4G results in competition for eIF4E. Binding of this to eIF4E and phosphorylation of eIF4E are incompletely understood. Immunoprecipitation of 4E-BP1 from CHO cell lysates showed that eIF4E bound to 4E-BP1 was phosphorylated to the same extent as total eIF4E, indicating that phosphorylated eIF4E can bind to 4E-BP1(Hynn and Proud 1996). A wide variety of mitogens and stress conditions can influence the phosphorylation state of 4E-BP1 (Kleijn *et al* 1998) which suggest that regulation of eIF4E activity by 4E-BP1 binding is common and important mechanism in translation

initiation. Overall, the change in 4E-BP1 phosphorylation was consistent with changes in the rate of protein synthesis. However, data from a concomitant increase in protein synthesis has only been reported in a few cases, e.g. in insulin-stimulated myeloid progenitor cells (Mendez *et al* 1996). The net effect of eIF4E activation on protein synthesis under various conditions such as growth factor stimulation, cellular stress or viral infections is only partially understood. It does appear however that in light of all the evidence regulation of eIF-4E activity is a delicate balance between eIF4E. 4E-BP1 complex formation and phosphorylation of eIF4E.

Under numerous conditions, eIF4B has been shown to be phosphorylated and there is an excellent correlation between the level of protein synthetic activity and eIF4B phosphorylation, with the highest levels associated with the most extensively phosphorylated eIF4B (Merrick 1992). In a similar manner, eIF4F is also correlated with enhanced protein synthetic activity. It has been demonstrated *in vitro* that the fully phosphorylated eIF4F is approximately five times more active than unphosphorylated eIF4F. Levels of eIF4A of which there are two isoenzymes expressed in a tissue specific manner, are not known to be regulated within specific tissues (Merrick 1992)

3.2.4.2. Elongation factors

Although not as well characterized as the circumstances that lead to the covalent modification of initiation factors, eEF1 and eEF2 appear to undergo post-translational modification that regulates their activity that correlates well with changes in protein synthetic rate.

To reiterate, peptide chain elongation require two elongation factors, named above. eEF1 consists of α, β, γ and δ subunits. It mediates the attachment of the aminoacyl-

tRNAs to the ribosome during peptide chain elongation while eEF2 , a monomeric protein which also binds ATP , is required for the translocation step of elongation during which the ribosome moves relative to the mRNA and the peptidyl-tRNA migrates from the A- to the P- site of the ribosome. The regulation of these factors will now be considered.

Experiments carried out by Venama *et al* (1991) found that eEF1 activity was enhanced by phosphorylation *in vivo* with phorbol ester or *in vitro* by phosphorylation with protein kinase C. The predominant phosphorylation in this instance was on β and δ subunit.

eEF2 can also be phosphorylated. The sites of phosphorylation appear to be the threonine residues at positions 56 and 58 in the mature protein. The original report citing it as a major substrate for calcium/calmodulin dependent protein kinase III (Nairn and Palfrey 1987), is a protein kinase whose activity can be regulated by phosphorylation. Phosphorylation results in complete inactivation of eEF2, apparently by inhibiting its ability to bind to the ribosome, an interaction that also involves regions within the GTP-binding domain. Phosphorylation has been shown to inactivate eEF2 in the translation of endogenous mRNA in the reticulocyte lysate (Redpath and Proud 1993).The level of phosphorylated eEF2 can also be regulated by the level of phosphatases, in particular the 2A phosphatase type, whose activity is induced by treatment with phorbol ester TPA (Gschwendt *et al* 1989).

The role and relevance of eEF2 phosphorylation to the physiological control of translation still remain obscure and requires further investigation.

3.2.5. Regulation of S6 phosphorylation

The protein that has been studied most extensively is the 40S ribosomal protein S6, which is located in the area of the 40S subunit that is implicated in mRNA binding. S6 is regulated by phosphorylation on serine and threonine residues; the two kinases that phosphorylate the S6 protein are the 70kDa S6 protein kinase (p70s6k) and the ribosomal protein S6 kinase p90rsk, 70 and 90 refers to the approximate MW values. Phosphorylation of S6 by p70s6k can be induced by a number of mitogens and seems to be significant for regulation of translation initiation. Ribosomal subunits containing phosphorylated S6 are recruited preferentially into polysomes compared with non-phosphorylated subunits. Translation of mRNAs containing a 5'-terminal oligopyrimidine tract (TOP) is induced specifically by S6 phosphorylation (Jefferies *et al* 1994); TOP mRNAs encode for elongation factors and ribosomal proteins.

At the molecular level, phosphorylation of S6 may influence protein synthesis in cells moving from Go to G1 phase in serum-stimulated HeLa cells or Swiss 3T3 cells (Nielson *et al* 1981). In these cells, inactive 80S ribosomes moved rapidly into actively translating polysomes in parallel with increased levels of S6 phosphorylation. In immature *Xenopus laevis* oocytes, S6 was shown to be almost completely in the dephosphorylated state, but as the oocytes completed maturation, S6 became maximally phosphorylated. The accompanied maturation was a two- to fourfold increase in the rate of protein synthesis (Wasserman *et al* 1982).

Other studies have shown that the initiation of protein synthesis was enhanced by serum, epidermal growth factor (EGF), prostaglandin PGF2α and insulin (Parker *et al* 1985, Pierre *et al* 1986); all caused phosphorylation of S6. The ribosomes containing highly phosphorylated forms of S6 were found to have a selective advantage in entering

polysomes (Thomas *et al* 1982). These findings strongly suggest that phosphorylation of S6 is involved in upregulation of protein synthesis.

3.2.6. Transcriptional control of protein synthesis

Most, if not all, signal transduction pathways ultimately impinge on gene transcription and alter the expression of genes in response to extracellular and intracellular ones. This usually involves changes in the capacity of the cell to react to its environment, thus changes in gene expression play a role in a vast number of cell responses (the regulators of gene transcription, transcription factors, acutely control gene expression).

Extracellular stimuli generate signals that lead to long-term changes in cell behaviour by changing programmes of gene expression. The activation of immediate-early genes such as c-fos occurs within minutes of receipt of a signal. In turn, over a period of hours to days, transcription factors encoded by immediate-early genes affect secondary and tertiary genes collectively called "late" genes, whose products perform tasks in cell division, differentiation and other activities. To activate or repress transcription, transcription factors must be located in the nucleus, bind DNA and interact with the basal transcription apparatus.

There are three general mechansims by which information at the cell surface can be conveyed to the nucleus in order to effect transcription (Edwards 1994), all of which involve phosphorylation. 1) activation and translation of cytoplasmic kinases to the nucleus, leading to changes in transcription factor function e.g. MAP kinase route. 2) Direct activation of latent cytoplasmic transcription factors by phosphorylation e.g. Stat 91. 3) release of transcription factor from cytoplasmic anchor proteins, allowing it to translocate to the nucleus, bind target sequences and therefore activate gene expression e.g.NF-κB.

The important group of signal-regulated transcription factors are the BZip proteins, so named because of their conserved basic (B) and leucine zipper (Zip) domains that are required for DNA binding and dimerization respectively (Vinson *et al* 1989). The leucine zipper consensus contains a leucine amino acid every 7 residues along the polypeptide chain of the transcription factor. It is thought that the leucines allow the transcription factors to form heterodimers and homodimers. Dimerisation is thought to occur before the factors bind to the DNA. One example of the leucine zipper structure is the formation of Jun-Jun homodimers and Jun-fos heterodimers. These sequence specifc factors have a modular structure consisting of distinct and separable DNA binding, dimerisation and transcriptional activation domains (Lamb and McKnight 1991).

The most studied members of this superfamily are the AP-1 (Jun/fos) and CREB/ATF proteins that control gene expression by binding to the TPA (12-0-tetradecanoylphorbol-13-acetate) response element (TRE) and cyclic AMP (cAMP) response element (CRE) respectively (Karin 1992). These factors will be discussed to serve as a paradigm in illustrating the signalling pathways that can alter gene expression.

AP-1 has been one of the most extensively studied owing to two of its components being oncogenic. Molecular cloning has revealed that AP-1 consists of a collection of structurally related transcription factors, which belong to the Jun and fos families; these associate to form a variety of homo- and heterodimers, all of which recognise TRE (Angel and Karin 1991). Like all members of the BZip family, the AP-1 components must dimerise prior to DNA binding. The Jun proteins bind DNA as either homodimers or Jun-Jun heterodimers, whereas Fos proteins must heterodimerise with one of the Jun

proteins since they cannot form stable Fos-Fos homo-or heterodimers (Angel and Karin 1991). Among the Jun proteins, c-Jun is the most potent transcriptional activator, either as a homodimer or in combination with Fos (Chiu *et al* 1989). Owing to their increased stability, the Jun-Fos dimers exhibit more DNA –binding activity and *trans*-activation capability than the corresponding Jun-Jun dimers. All of these dimers are thought to contribute to AP-1 activity and participate to varying extents in its regulation by extracellular stimuli (Angel and Karin 1991). AP-1 is regarded as a nuclear messenger that mediates the actions of signal transaction pathways stimulated by growth factors, hormones, cytokines and neurotransmitters most of which are initiated with the activation of either tyrosine kinases or phospholipid turnover (Cantley *et al* 1991).

In common with AP-1, CRE- binding activity is due to a number of closely related proteins (Hai *et al* 1991). The family of CRE-binding proteins currently consists of at least eight members, of which only CREB has been established as a mediator of cAMP action (Karin 1992). The exact function of other CRE-binding proteins, also known as ATFs (activating transcription factors) is not known. AP-1 complexes bind to CRE, albeit with lower affinity in comparison to their interaction with TRE.

Whilst AP-1 is activated in response to growth stimulants and stress, elevation of cAMP induces a distinct set of transcriptional regulators. Agonist induced synthesis of cAMP causes dissociation of the regulatory subunit of cyclic AMP-dependent protein kinase and the release of its catalytic domain which largely translocates to the nucleus. A number of genes are stimulated by elevation of cAMP and share a responsive element related to the AP-1 (Brindle and Montminy 1992). Several proteins bind to this cyclic AMP response element (CRE) and the first to be identified was CREB.

Phosphorylation of CREB at Ser133 is required for activation (Gonzalez and Montminy 1989). The basis of this activation is shown to be phosphorylation-dependent binding to a transcriptional accesory factor termed CREB binding protein (CBP) which directly interacts with TFIIB, a central component of the transcriptional machinery (Chrivia *et al* 1993). CREB contains other potential phosphorylation sites which may modulate its activity, although Ser133 is the dominant regulatory element.

Another cAMP-responsive transcription factor that also binds to CREs, CREM, is subject to more complex regulation (Foulkes 1992). The CREM gene is differentially spliced generating several distinct proteins some of which are antagonistic such as CREM. Like CREB, CREM is a phosphoprotein and is phosphorylated at Ser117 by cAMP-dependent protein kinase. This site is also targeted by other protein kinases such as $p70^{S6}$ kinase and Ca^{2+}/calmodulin-dependent protein kinase (deGroot *et al* 1994). CREM may therefore integrate multiple signals impinging on CRE-contaning genes.

CREs are also responsive to expression of adenoviral proteins, specifically E1A protein, which interacts with and activates a transcription factor termed ATF2. Like c-Jun, ATF requires phosphorylation by stress-activated protein kinases (SAPKs) for activation (at Thr69 and Thr71) and binding of the kinase occurs in a region distal to the phosphorylation site (Livingston *et al* 1995).

In general, the CRE–binding proteins do not bind TREs (Karin and Smeal 1992). However, several of them can form heterodimers with the Jun/Fos proteins that can bind both types of elements e.g c-Jun-ATF2 heterodimers bind to CREs with higher affinity than the TREs. This overlapping specificity of the AP-1 and CREB/ATF

proteins is likely to play a role in their ability to either antagonise or synergise with each other.

3.2.6.1. Signals that regulate AP-1 and CREB/ATF

3.2.6.1.1. AP-1

In addition to TPA, AP-1 activity is induced by a variety of polypeptide hormones, growth factors, cytokines, and neurotransmitters (Angel and Karin 1991). These agents activate signalling pathways that are initiated with either stimulation of membrane-associated tyrosine kinases or phospholipid turnover, the latter giving rise to increased PKC activity (Cantley 1991). In addition, AP-1 activity is elevated in cells that express a variety of transforming oncogenes, whose products act as constitutively activated intermediates in the signal transduction pathway that transmits information from cell-surface tyrosine kinases to the nucleus. Such oncogene products include v-Src, Ha-Ras and v-Raf (Foulkes *et al* 1992). These events lead to increased Ras activity, which plays a pivotal role in the activation of downstream threonine-specific protein kinases, such as Raf-1 and the ERKs (Boulton *et al* 1991).

These signalling pathways mainly affect AP-1 activity at two levels: transcriptional and post-translational. Firstly, transcription of the fos genes is very low and in most non-stimulate cells, is induced in response to a variety of extracellular stimuli. Most rapid induction is exhibited by *c-fos* which occurs through transcription of serum response element (SRE) which is recognised by several different factors of which the major ones are p67SRF and p62TCF (Hipskind 1991). It is not clear how the activities of these constitutiveley expressed proteins are stimulated by extracellular signals. Induction of fos transcription results in increased synthesis of Fos proteins, which combine with pre-

existing Jun proteins to form stable heterodimers and thereby increasing level of AP-1 binding activity.

Phosphorylation of c-Jun occurs on five sites, two within its amino-terminal activation domain and three that are clustered next to its carboxy terminal DNA binding domain (Boyle *et al* 1991). The kinase that phosphorylates the amino-terminal sites of c-Jun remains to be identified although it has been suggested that these sites are phosphorylated by the 42kDa and 44kDa ERK (MAP) kinases, but this remains to be substantiated.

3.2.6.1.2. CREB

In comparison to the AP-1 proteins, expression of the CREB/ATF proteins so far appears to be constitutive and relatively unresponsive to extracellular stimuli. Therefore a major level of control affecting these proteins is post-translational (Karin 1992). CREB contains three phosphorylation sites in its amino-terminal half, of which only the function of Ser133 is known (Gonzalez *et al* 1989). This residue is phosphorylated by PKA both *in vitro* and *in vivo*, resulting in ten to twenty fold increase in CREBs transcriptional activity (Gonzalez and Montminy 1989). Phosphorylation does not detectably alter the DNA-binding affinity of CREB and therefore it only affects the trans-activation domain by altering its conformation so that it can better interact with the transcriptional machinery. As yet, no function has been established for the other phosphoacceptor sites of CREB Ser121 and Ser156.

3.2.7. Regulation of protein synthesis in skeletal muscle

3.2.7.1. Role of Prostaglandins

Studies have suggested that stimulation of protein synthesis by insulin and the glucocorticoid-induced reduction in rates of protein synthesis were all associated with concomitant changes in PGF2α production (Reeds and Palmer 1983, 1984). They demonstrated that the addition of insulin (8ng/ml) *in vitro* to muscles from fasted rabbits increased protein synthesis by over 80%, a value similar to that found in muscles from fed donors. The addition of the prostaglandin inhibitors indomethacin or meclofenemate completely blocked this effect of insulin. Muscles from the fasted rabbits released less prostaglandin PGF2α into the medium. The presence of insulin increased prostaglandin release whilst indomethacin and meclofenemate reduced it. In another study by the same authors (1989), forelimb digit extensor muscles were taken from fed rabbits and incubated in the absence or presence of dexamethasone (100nM). The presence of dexamethasone decreased the rate of protein synthesis with a concomitant reduction in the release of prostaglandins. The proposed mechanism involves hydrolysis of membrane phospholipids by the activation of phospholipase A_2 (PLA_2) which causes release of arachidonic acid (ARA) and production of PGF2α. Similar results were obtained *in vivo* whereby in fasted rats (18hr), administration of prostaglandin inhibitors prevented restoration of protein synthesis rate in comparison to the fed state (McNurlan *et al* 1987). In a different study using starved rats infused with insulin, there was a dose-dependent rise in certain organs receiving insulin compared to controls infused with diluent. Indomethacin blocked the rise in protein synthesis and blocked the increase in plasma prostaglandins (Reeds *et al* 1985). These results show

that an involvement of AA metabolism in the action of insulin on protein synthesis is physiologically relevant.

The addition of insulin to L6 skeletal muscle myoblasts stimulated translation at 1nM or below (Palmer and Bain 1989). The cyclooxygenase (COX) enzyme inhibitor, indomethacin, known to have low specificity and inhibit PLA2, was used in initial studies, but was not very selective. Another study using ibuprofen, a more selective COX inhibitor, confirmed the role of this pathway in protein synthesis (Thompson *et al* 1995).

3.2.7.2. Role of S6 phosphorylation and Mitogen Activated Protein (MAP) kinase

Many studies in various cell types have concentrated on phosphorylation of the 40S ribosomal protein S6 as it correlates with increased rates of translation as mentioned earlier on. S6 phosphorylation is effected by through two distinct S6 kinase families; the 70kDa and 85kDa kinases (p70s6k) and the 90kDa (p90rsk) S6 kinases. The pathway activating p90rsk involves MAP kinases whilst kinases activating p70s6k are unknown. Palmer and Bain (1989) showed that the addition of insulin to L6 myoblasts stimulated protein synthesis affecting both transcription and translation at concentrations within physiological range i.e <1nM. However, Thompson and co-workers (1995) demonstrated that concentrations up to 100nM were required to activate the MAP kinase. Thus there was no correlation between the ability of insulin to stimulate MAP kinase and translation. In addition, rapamycin which specifically inhibits p70s6k, whilst having no effect on MAP kinase, completely prevented the stimulation of translation by 1nM insulin in L6 cells. This result was reflected in another study whereby rapamycin also partially blocked the stimulation of protein synthesis by insulin in C_2C_{12} skeletal muscle cell line (Palmer *et al* 1997). This

evidence does suggest that p70s6k is responsible for the majority of the increase in S6 phosphorylation.

In another experiment using epitrochlearis muscle from fasted rats *in vitro*, wortmannin and LY294002, which are phosphatidylinositol 3-kinase (PtIns 3-kinase) inhibitors, abolished the stimulation of translation by 30nM insulin or Insulin-like-Growth factor-1 (IGF-1). Once again, the stimulation of translation by both these factors was attenuated in the presence of rapamycin with no observed activation of MAP kinase (Dardavet *et al 1996*).

As discussed earlier, the activity of the mRNA cap-binding protein, eIF-4E is thought to be rate limiting for initiation. This is controlled by the translational regulator, phosphorylated heat- and acid-stable protein-1 (PHAS-1, previously described as 4E-BP1). In the non-phosphorylated form, PHAS-1 binds tightly to eIF-4E and prevents translation. When PHAS-1 is phosphorylated following insulin treatment, eIF-4E dissociates from PHAS-1 and is able to participate in the initiation of translation (Pause *et al* 1996). PHAS-1 has been shown to be a substrate for MAP kinase *in vitro* unlike Azpiazu *et al* (1996) who showed that in skeletal muscle, activation of PHAS-1 by insulin involved MAP kinase-independent and rapamycin sensitive pathways.

3.2.7.3. The role of Protein kinase C (PKC)

Insulin stimulated protein synthesis in L6 myoblasts; ibuprofen, an inhibitor of cyclo-oxygenase failed to block the effects of insulin, whereas a second cyclo-oxygenase inhibitor, indomethacin had only a partial inhibitory effect (Thompson *et al* 1993a). The protein kinase C (PKC) inhibitor Ro-31-8220 totally blocked the effect of insulin.

Since indomethacin is also recognised to inhibit phospholipase A2 (Kaplan *et al* 1978), this suggests that insulin acts on protein synthesis in myoblasts by arachidonate activation of PKC; perhaps one or more isoforms of PKC may mediate insulin action. Immunoblotting of total L6 cell extracts showed the presence of PKC α, δ, μ, ϵ and probably ι and ζ. Other isoforms were not detected (Thompson *et al* 1997). If ARA and PKC are both involved in the increase in transcription elicited by insulin, one possible mechanism involves the activation of one or more isoforms of PKC by ARA (Thompson *et al* 1997). Preincubation of L6 cells and L8 cells (Hong *et al* 1995) with TPA to downregulate PKC, showed the loss of α, δ and ε. Thus this result implicates a PKC isoform which is ARA-sensitive and resistant to downregulation by TPA i.e. PKC ζ.

3.2.7.4. Role of Phospholipase D (PLD)

Phospholipid metabolism is a major target of TPA action, in particular activation of PLD via PKC, producing phosphatidic acid is well documented (Kiss 1990). In L6 skeletal muscle cells, it was demonstrated that TPA and vasopressin stimulated both protein synthesis and a PLD that degrades phosphatidylcholine (Thompson *et al* 1994). Furthermore, incubation of L6 cells with exogenous PLD mimicked the effects of TPA and vasopressin on protein synthesis, implying a link between the two events. Interestingly however, TPA increased protein synthesis during short term (90min) incubations, which were neither blocked by the transcription inhibitor Actinomycin D, nor accompanied by increases in RNA, implying an effect on translation. In contrast, the synthesis response to vasopressin was detected solely over longer term (6hr) incubations, where increases in RNA content and sensitivity to actinomycin D were observed, demonstrating effects on transcription.

One explanation of why TPA and vasopressin produce such temporally different effects on protein synthesis involves the hydrolysis by PLD of phospholipids other than phosphatidylcholine with the production of different phosphatidic acid (PA) species that either directly or indirectly manipulate translation and transcription. It is now clear that phosphatidylethanolamine (PE) is also a substrate for PLD. In another study with the same workers (Thompson *et al* 1997) the same agents when added to prelabelled L6 myoblasts elicited an increase in [^{14}C] release suggesting activation of phospholipase D activity. Release of this both intra- and extracellularly, was inhibited by the protein kinase C (PKC) inhibitor Ro-31-8220 and PKC downregulation by preincubation with TPA. When protein synthesis was examined, the stimulation of translation by TPA and transcription by vasopressin were inhibited by Ro-31-8220. In contrast, downregulation of PKC inhibited the synthesis response to TPA but not vasopressin. Furthermore, following downregulation, the effect of vasopressin was still blocked by PKC inhibitors Ro-31-8220 and bisindolylmaleimide suggesting that the effect involves a PKC resistant to downregulation. Analysis of PKC isoforms in L6 cells showed that downregulation removed both cytosolic (α) and membrane-bound (ε and δ) isoforms. Thus, elevation of PLD activity induced by TPA and vasopressin and stimulation of translation by TPA involves PKC α, ϵ and/or δ. In contrast, the increased transcription elicited by vasopressin involves μ, ι and/or ζ. Hence, although phospholipase D may be linked to an increase in translation elicited by TPA, it is not involved in the stimulation of transcription by vasopressin.

In view of the finding that vasopressin stimulates ARA release from these cells (Thompson and Palmer 1998) the ARA sensitive PKC ζ appears a plausible candidate to mediate this response. Thus, it is possible that vasopressin and insulin may stimulate

transcription in L6 cells through very similar mechanisms (Thompson and Palmer 1998).

3.2.7.5. Role of cyclic AMP and protein kinase A (PKA)

This pathway which involves cyclic AMP and cAMP-dependent protein kinase (Protein kinase A, PKA) has received considerably less attention.

The role of cAMP as a second messenger in the stimulation of protein synthesis and the potential involvement of mitogen activated protein (MAP) kinase in this response was studied in L6 myoblasts (Thompson *et al* 1996). Dibutyryl cAMP (dbt-cAMP) permeates the plasma membrane and is metabolised in the cell to generate cAMP. When added to L6 myoblasts, it increased protein synthesis at 90min and 6hr in a concentration dependent manner. The response at 90min were probably mediated by increased translation as it was not blocked by actinomycin; effects at 6hr were accompanied by increases in RNA content implying a transcriptional component. Cell extracts from the cells immunoblotted with MAP kinase antibody showed a stimulation of 42/44kDa isoforms of MAP kinase. Responses to forskolin, another potent activator of adenylate cyclase, were similar to that of dbt-cAMP. Insulin (1nM) and vasopressin (100nM) also increased protein synthesis. These responses were additive to those of 500μM dbt-cAMP. A synergistic response in terms of MAP kinase activation was observed in the presence of dbt-cAMP, insulin alone had no effect. This data implies that cAMP stimulates protein synthesis in L6 cells and suggests a role for MAP kinase in this event. The above responses were not inhibited by rapamycin, an agent that has been shown to completely inhibit the activation of p70s6k (whilst having no effect on other kinases such as p90rsk or MAP kinase). This suggests that this pathway is not involved in eliciting this response in L6 myoblasts. It was then proposed that a potential

pathway linking cAMP to the phosphorylation of S6 may involve the MAP kinase, through the p90rsk pathway. This contrasts with a study in CCL39 hamster fibroblasts where elevation of cAMP activated p70s6k (Kahan *et al* 1992). In addition to a possible involvement of S6 phosphorylation, cAMP activation of MAP kinase may play a role in the stimulation of translation through others mechanisms. These include activation of initiation factors such as eIF-4E and eIF-2B.

The data also implied that cAMP may play a part in the regulation of transcription. This may involve the cAMP-responsive element (CRE)-binding proteins. These factors are phosphorylated by PKA leading to stimulation of transactivating potential (Gonzalez and Montminy 1989). This remains to be investigated.

Furthermore, cAMP and its analogue dibutyryl-cAMP, were shown to stimulate both cAMP-dependent kinase activity and S6 phosphorylation in bovine anterior pituitary gland (Barden and Labrie 1973), and in rat skeletal muscle, cAMP stimulated the rate of protein synthesis (Lewis *et al* 1982). Purified PKA has been shown to phosphorylate several initiation factors and elongation factor-2-kinase (Redpath and Proud 1994). A cAMP-dependent kinase was shown to directly phosphorylate hepatic ribosomal S6 protein in both whole ribosomes and 40S ribosomal subunits (Wettenhall and Cohen 1982). Both PKA and elevated levels of intracellular cAMP have been shown to stimulate ribosomal protein S6 phosphorylation which in turn may promote the recruitment of mRNA to increase the number of polysomes (Gressner and Van de Leur 1980).

3.2.8. β-adrenergic agonists and hypertrophy of skeletal muscle

A large aspect of this study involves characterising the anabolic effect of LMF (possibly via a β-receptor) on protein turnover. Therefore consideration will be given to the effects of β-agonists on skeletal muscle, which are well known to potentiate muscle accretion.

β-adrenergic agonists have been fed to avian and mammalian species to modulate growth. Such treatment produces an increased rate of gain, decreased food consumption, increased skeletal muscle and decreased fat accretion (Bell *et al* 1998). There is good evidence for the inhibition of adipose tissue lipogenesis and stimulation of lipolysis, as well as stimulation of skeletal muscle protein synthesis and inhibition of protein degradation. The degree to which each of these effects is observed varies considerably between experiments. Some of the diversity may be explained by species and/or tissue differences in distribution of β adrenergic receptor subtypes and the pharmacodynamics of the agonist.

3.2.8.1. β-adrenergic receptor (BAR) subtypes

BAR receptors are present in essentially all mammalian tissues and cell types, although less is known about avian species. BAR are stimulated by the adrenal medullary hormone, adrenaline and by the sympathetic neurotransmitter, noradrenaline (Yang and McElligott 1989). Noradrenaline in the plasma can also act as a hormone. Adrenaline and noradrenaline are both synthesised from tyrosine, with adrenaline being the methylation product of noradrenaline. They are both equally potent at β_1 adrenoceptors, whereas adrenaline is much more potent than noradrenaline at β_2 adrenoceptors. With the development of various high affinity and differing selectivity of inhibitors, it has

been demonstrated that β_1 and β_2 adrenoceptors have differing pharmacological properties (Stiles *et al* 1984). An interest in thermogenesis and brown fat function in the rat as a possible mechanism for obesity in the 1980s had led to the discovery of the β_3 adrenergic receptor. The initial evidence was from pharmacological approaches using tissue function and ligand binding (Arch and Kaumann 1993), and followed by isolation of a clone for β_3 (Strosberg 1992). The β_3 receptor is also present in white adipose tissue.

3.2.8.2. Post-receptor events

In the absence of solid contradictory evidence, the signal transduction sequence for β-adrenergic agonist action is presumed to be similar to that in other tissues. It is clear that β agonists bind to β receptors, stimulate increased cyclic AMP and the activation of cyclic AMP-dependent protein kinase (Bowman and Nott 1969). However, subsequent events leading to the regulation of protein turnover are not well studied in muscle. Phosphorylation of two intermediate filament proteins desmin and vimentin was observed in avian skeletal muscle cells in culture (Gard and Lazarides 1982). Post-receptor events were examined in other tissues. The phosphorylation of at least 13 proteins in rat cardiac ventricular cells and the dephosphorylation of a single protein of MW 21kDa is stimulated by isoprenaline (Blackshear *et al* 1984). Three of the proteins were identified as troponin I, C-protein and phospholamban, the modulator of the sarcoplasmic reticulum calcium-dependent pump ATPase.

3.2.8.3. Muscle effects: mechanisms

Experiments *in vitro* have indicated stimulation of protein synthesis and/or diminution of protein degradation in skeletal muscle preparations. However, some experiments do

not indicate such effects (Kim and Sainz 1992). Studies on clonal cell lines may or may not reflect skeletal muscle function *in vivo*. In spite of negative data on muscle, there is evidence of an increase in protein synthesis and/or decrease in protein degradation *in vivo* for various domestic species fed BAR agonists (Kim and Sainz 1992, Koohmaraie *et al* 1991). It has been demonstrated that effects on protein degradative enzymes, usually result in the involvement of the calpain system, but in some cases, catheptic enzymes are implicated (Bechet *et al* 1990). Overall, it appears that oral administration of BAR agonists has the potential to stimulate skeletal muscle hypertrophy through both an increase in protein synthesis and a decrease in protein degradation; the mechanism depends most likely on the species, the breed, the agonist and the nutritional status of the animal.

Because BAR are distributed in most tissues, there could be many other mechanisms to produce or enhance the observed growth effect *in vivo*. Blood flow to various tissues is increased by BAR agonists so that delivery of substrates and/or removal of metabolites may be increased (Eisemann *et al* 1988). Increased amino acid delivery and uptake may increase muscle protein synthesis whereas increased removal of non esterified fatty acids may increase lipolysis and remove toxic detergent effects of fatty acids; many endocrine glands can be modulated by BAR agonists (Yang and McElligott 1989). Given the breadth of possibilities for the receptor microheterogeneity (within and across species) for BAR subtype distribution, for diverse agonist structures and for species-specific pharmacodynamic processing, it is expected that there will be much variation in the response to BAR agonists *in vivo*.

3.3. PROTEIN DEGRADATION

Skeletal muscle contains several proteolytic systems which may be responsible for the degradation of specific classes of proteins. These include the lysosomal proteases (cathepsins and other hydrolases) (Bird *et al* 1990), calcium-dependent proteases (calpains)(Zeman *et al* 1985) and cytosolic, ATP-ubiquitin (Ub)-dependent pathway (Fagan *et al* 1987).

3.3.1. The Lysosomal System

Lysosomes are a large ubiquitous family of related cytoplasmic single membranes vesicular structures, which contain a variety of hydrolytic enzymes capable of degrading cellular macromolecules (nucleic acids, proteins, polysaccharides and lipids). Cathepsins, the lysosomal enzymes, are classified both functionally and according to the amino acid structure at their active sites and are most active at low pH. Although found in all tissues, their concentration is particularly high in kidney, spleen, liver and macrophages. By contrast, skeletal muscle contains few lysosomes. The major lysosomal proteinases (cathepsin B,H, L and D) do not appear to contribute significantly to overall breakdown; purified cathepsins B, D, H and L degraded purified actin and myosin to a limited degree (Bird *et al* 1980). Lowell and colleagues (1986a) concluded that lysosomes were not involved in the degradation of myofibrillar proteins. Furthermore, studies with inhibitors of lysosomal proteinases failed to prevent the increased release of 3-methylhistidine release from either perfused muscle or starved rats (Lowell *et al* 1986b) or denervated soleus muscle *in vitro* (Furuno *et al* 1990). Although these findings imply that lysosomal proteinases themselves are not involved in the breakdown of myofibrillar proteins, it does not rule out the possibility that other proteolytic pathways might use the lysosome at some point in the degradative process.

For example, an increase in ubiquitin-protein conjugates in the myofibrillar fraction and the presence of ubiqutinated proteins in the lysosome has been observed (Wing *et al* 1995). In a different study using a cell line containing a mutant thermolabile ubiquitin-activating enzyme, E1, demonstrated that this enzyme was necessary for the stress induced lysosomal degradation of proteins (Gropper *et al* 1991). The possibilty that there may be a link between the ATP-ubiquitin-dependent pathway and the lysosome with regard to the degradation of myofibrillar proteins in skeletal muscle remains a possibility and requires further investigation.

3.3.2. Calcium-activated proteinases

Calpain, the most typical cytosolic calcium dependent cysteine proteinase has been studied extensively, since its ubiquitous expression suggests an indispensible physiological function as one of the cellular receptors of calcium ions. Two molecular species, μ- and m- calpains, have been identified (Murachi 1983, Pontremoli and Melloni 1986). Both are heterodimers consisting of a homologous catalytic 80kDa subunit and the identical regulatory 30kDa subunit made up of distinct domains. The largest subunit is responsible for protease activity and Ca^{2+} dependency and can be structurally divided into four domains. The second domain (II) is a cysteine protease domain, and the fourth domain (IV) is a Ca^{2+}-binding domain. The functions of the first and third domains (I and III) are not clear at present. The differing species are distinguished by the Ca^{2+} concentration required for *in vitro* activity: μ-calpain requires μM levels of Ca^{2+}, while m-calpain requires mM levels.

Some calpain homologues in mammals have been found to be predominantly expressed in a limited number of organs in contrast with the ubiquitous expression of the

"conventional" μ and m calpains (Sorimachi *et al* 1997). These "tissue-specific" calpains, such as skeletal-muscle-specific p94 (also called nCL-1 or calpain 3), whose exact function is unknown and stomach-specific nCL-2 and nCL2' are probably closely related to the specific functions of the organs in which they are predominantly expressed.

The activity of these ubiquitous enzymes is regulated not only by Ca^{2+} but also by their endogenous inhibitor, calpastatin (Pontremoli *et al* 1992). This is a collective name given to a family of calpain-specific endogenous inhibitor proteins that are widely distributed among mammalian and avian cells. Both calpain and calpastatin co-exist in tissue-homogenates (Murachi 1983, Pontremoli and Melloni 1986). Calpastatin possess four inhibitory domains that contain a consensus sequence (TIPPEYR) which in itself is able to inhibit calpain specifically. It can be phosphorylated by calcium-independent protein kinase present in muscle cells. Interestingly, the dephosphorylated form of calpastatin shows greater inhibitory efficiency against μ-calpain, whereas the phosphorylated form shows maximal inhibition versus m-calpain. As both forms of calpastatin are present in muscle, this might explain how two calpain isoforms may be regulated by a single inhibitor (Pontremoli *et al* 1992).

Involvement of calpains in the initiation of protein degradation by cleavage of a limited number of sites has been suggested, thus enabling access of other proteases to the actomyosin molecule (Goll *et al* 1992). In intact muscles *in vitro*, calpain inhibitors failed to prevent myofibrillar protein breakdown (Lowell *et al* 1986a), whilst the elevation of the intracellular calcium concentration by a variety of agents failed to stimulate myofibrillar breakdown (Goodman 1987). However, it has been suggested

that they are involved in the initiation of MF protein degradation by cleaving a limited number of specific sites (Goll *et al* 1992). In addition, calpains appear to be critically involved in post-mortem meat tenderisation by degrading proteins, such as titin and nebulin (Koomaraie 1994). Furthermore, calpains initiate digestion of individual myofibrillar proteins, including desmin, filamen, c-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin and vinculin (Huang and Forsberg 1998, and Goll *et al* 1992) but do not degrade α-actin and α-actinin or myosin heavy chain (Koohmaraie, 1994). In fact, there are few biological examples in which significant changes in calpain activities occur (Illian and Forsberg 1992) and evidence that Ca^{2+}-dependent proteinases are activated in muscle wasting condition is lacking (Attaix and Taillandier 1998). There is now growing evidence that calpains do not play a major role in general turnover of intracellular protein, but are involved in limited proteolysis of some specific target proteins, possibly involved in the early stages of MF proteolysis. They appear to be qualitatively important in the degradation of specific, but quantitatively minor muscle proteins already mentioned.

In recent years, it has become apparent that both lysosomal and Ca^{2+} -activated proteases do not play a major role in this tissue; they contribute less than 15-20% of total protein breakdown in muscles from control and cachetic animals, and are not responsible for the breakdown of myofibrillar proteins (Furuno *et al* 1990, Lowell *et al* 1986a).

3.3.3. The ATP-ubiquitin-dependent pathway

Skeletal muscle also contains the soluble, ATP-ubiquitin (Ub)-dependent proteolytic system (Fagan *et al* 1987). This pathway degrades cytosolic and nuclear proteins via a mechanism that is centered in a multicatalytic proteinase complex called the 26S proteasome (Kisselev *et al* 1998). Substrate proteins are targeted for degradation by the addition of multiple monomers of ubiquitin, a 76 amino acid polypeptide, to specific residues in a multi-step reaction requiring three classes of enzymes called E1, E2 and E3 (Figure 3.2). Initially, an ubiquitin-activating enzyme (E1) activates the carboxyl terminal glycine residue on ubiquitin to a high energy thiol ester intermediate (Haas and Rose 1982)). Next, activated ubiquitin is transferred to a family of ubiquitin carrier proteins E2 enzymes, also known as ubiquitin-conjugating enzymes (Jentsch 1992). The final step involves ligation of the ubiquitin to amino groups on protein substrates either directly by E2 or in the presence of an Ub-protein ligase (E3) (Haas and Siepmann 1997). E3s play a role in the selection of proteins for conjugation and catalyse the formation of polyubiquitin chains on the target protein, which are recognised as a degradation signal by the 26S proteasome. It should be noted that E2s can directly transfer ubiquitin to some protein substrates (typically basic proteins such as histones) in the absence of E3 (Attaix *et al* 1998a). However, in this case, most of the conjugates formed are monoubiquitylated proteins, which are usually not targeted for degradation. The first ubiquitin molecule is usually bound to the substrate by an isopeptide bond between the C-terminal glycine of ubiquitin and an ϵ-NH2 group of a lysine residue of the substrate (Busch 1984). The polyubiquitin chain is formed in multiple cycles of this reaction by addition of another ubiquitin molecule to the lysine at position 48 of the previously already conjugated ubiquitin.

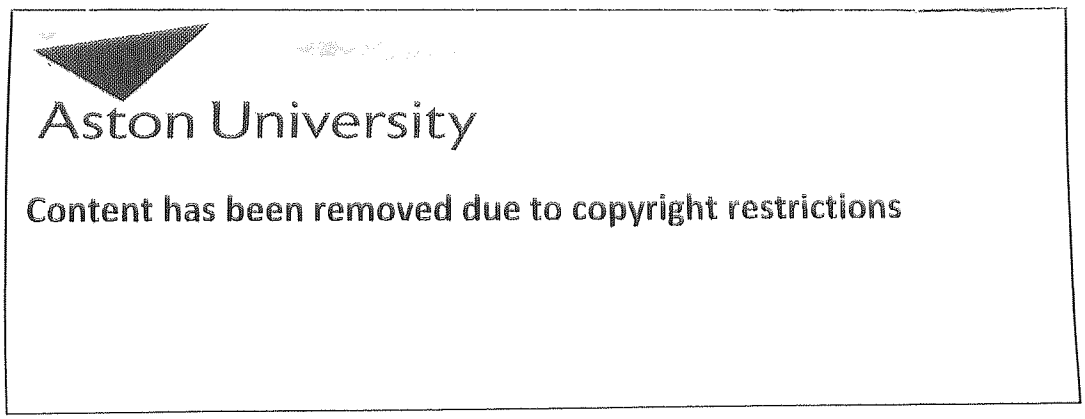


Figure 3.2. The sequence of reactions involved in ubiquitin (Ub) conjugation and subsequent ATP-dependent proteasome-mediated protein degradation (taken from Argiles and Lopez-Soriano 1996). E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub protein ligase.

Release of ubiquitin from the isopeptide linkage with the lysine residue is performed by isopeptidases called ubiquitin terminal hydrolases (UCH) (Shaeffer and Cohen 1996). Their function is probably important not only in recycling ubiquitin monomers after substrate degradation but also in the recovery of poorly or incorrectly ubiquitinated proteins.

Recent studies suggest that the deubquitination of proteins, removal of ubiquitin from substrates, is also important for the regulation of protein breakdown rates (Kornitzer and Ciechanover 2000). Changing the rate of ubiquitin removal from the protein may influence the probability of the ubiquitinated substrate being recognised by the 26S proteasome complex. Thus, deubiquitination may be as important for the regulation of the ubiquitin pathway as the E1, E2 and E3 enzymes.

The second step in the ubiquitin-proteasome pathway is the degradation of polyubiquintinated proteins by the 26S proteasome complex (Coux *et al* 1996). The assembly of this complex is energy-requiring and has a molecular weight of 100 to 1500kDa. It is formed by the assembly of three components, the 20S proteasome and two 19S molecules. The 20S proteasome is the "catalytic core" of the 26S proteasome and the structure and function of which will be explained in greater detail.

3.3.3.1. The 20S and 26S Proteasome complexes

The most intricate component of the ubiquitin-dependent proteolytic pathway, with over 30 distinct subunits, is the proteasome. Proteins marked for degradation by Ub are digested to small peptides within the 20S proteasome particle. The 600kDa particle is a major cell constituent, comprising up to 1% of cellular proteins. The 20S particle is a barrel-shaped structure of four stacked rings, each composed of seven subunits surrounding a central cavity (Coux *et al* 1996, Lowe *et al* 1995). The 20S subunits are

classified as α and β subunits on the basis of their homology to a simpler version of the proteasome found in the archaebacterium *Thermoplasma acidophilum*. Electron microscopy images and X-ray crystallographic structures of the 20S particles from *T. acidophilum* and *Saccharomyces cerevisiae* reveal a cylindrical structure made up of 7α7β7β7α rings with a narrow channel running through the centre of the structure. Proteins are hydrolysed inside the cylinder by proteolytic sites localised on β subunits (see Figure 3.3 for structure of proteasome).

The mechansim by which peptide bonds are cleaved in the proteasome is unique. Proteasomes do not fit the standard classification of proteolytic enzymes according to their active sites (e.g.serine, cysteine, acidic or metalloproteases) (Epstein 1996). The sequences of their β subunits are not homologous to those of known enzymes and the pattern of sensitivity to various inhibitors differs from that of any known protease family. X-ray diffraction studies, and mutagenesis of different amino acids in the proteasome, have uncovered a new type of proteolytic mechanism (Lowe *et al* 1995). The active site nucleophile of the proteasome is the hydroxyl group of a threonine at the amino terminus of the β subunit. This novel mechanism has allowed for selective inhibitors of the proteasome to be synthesised. For eg. lactacystin, a natural product of Streptomyces was discovered to inhibit intracellular protein degradation by reacting selectively with this terminal threonine (Bogyo *et al* 1997).

The mammalian proteasome contains at least three active sites that differ in their specificity for different types of bonds (Vinitsky *et al* 1994). One activity cleaves preferentially after basic amino acids, others after large hydrophobic amino acids and after small, neutral amino acids or after acidic residues. These activities which function together to catalyse the complete digestion of protein seem to be associated with

different β subunits. The α chains although catalytically inactive, play an essential role in stabilising the two β rings, as well as a role in binding the 19S regulatory cap complex.

Although the 20S proteasome can be activated *in vitro*, it may never function as an isolated enzyme in cells but rather only when bound to regulatory proteins (DeMartino and Slaughter 1999). These proteins appear to mediate proteasome function, in particular, by overcoming the restrictions of proteasome structure on substrate access and therefore can be considered as proteasome activators. Because the protein forms complexes with different proteins, it appears to be the centre of a modular system in which different regulatory proteins confer unique catalytic and regulatory properties on the proteasome.

The role of the proteasome in the ubiquitin pathway is mediated by the regulatory protein known as 19S regulatory particle (also called PA700 or 19S cap) (Attaix *et al* 1999)(Figure 3.3). It is a molecule of 700kDa, 20-subunit complex that binds to one or both of the terminal rings of the proteasome (Adams *et al* 1998). Binding of the 19S cap to the proteasome greatly enhances the ability of the proteasome to degrade both ubiquitinated proteins and non-ubiquitinated peptides. The degradation of the former but not the latter, requires continuous ATP hydrolysis beyond that required for complex formation (DeMartino and Slaughter 1999).

The 19S regulator is thought to carry out a number of different biochemical functions (Kornitzer and Ciechanover 2000). First, it can recognise polyubiquitated substrates. Second, it is predicted to have an isopeptidase activity to cleave the polyubiquitin chains in to Ub monomers, which are then recycled.

Figure 3.3. Schematic representation of the 20S proteasome, PA28 particle and 26S proteasome. α and β denotes the α and β subunits of the 20S proteasome, respectively (taken from Attaix *et al* 1998).

Ub is not degraded by the proteasome. Third, binding of the 19S complex to either or both ends of the 20S particle is thought to open the narrow pores at the ends of the 20S structure.

Finally proteins must be unfolded to allow entry into the 20S chamber. Therefore, the 19S regulator is thought to have reverse chaperone activity to denature substrates and translocate the unfolded polypeptides to the proteolytic compartment of the 20S particle.

A recent discovery in S.cerevisie has revealed that that the 19S regulator can be dissociated into sub-complexes called the base and the lid (Glickman *et al* 1998). This has provided new insights into the structure and function of the 19S particle.

The 19S regulator base consists of six ATPases (Rpn1-6) that all belong to the ATPases-associated-with-different-cellular-activities (AAA) family and the non-ATPases Rpn2(S1), Rpn1(S2) and Rpn10(S5a) (Glickman *et al* 1998). It is thought to interact with the 20S particle causing a narrow pore to open, which allows access into the inner compartment of the 20S complex. It functions to unfold proteins before translocating them into the 20S core for proteolysis. Little is known about the structure of the 19S regulator lid but it is thought to consist of 8 non-ATPases subunits and is assumed that is essential for the polyubiquitin substrate processing prior to degradation. The lid is homologous to cop-signalsome complexes; the functional significance of this homology is unclear (Kornitzer and Ciechanover 2000).

PA28 or the 11S regulator is another activator of the 20S proteasome. PA28, free of the proteasome, has been revealed to be a ring-shaped particle, and forms conical caps by its association with both ends of the 20S proteasome (Dubiel *et al* 1992). Association of PA28 with the proteasome does not require ATP-hydrolysis in contrast to the ATP-

dependent assembly of the 26S proteasome (DeMartino and Slaughter 1999). It consists of two 28kDa subunits α and β that are 50% identical in primary structure. The subunits form a ring-shaped molecule of about 180kDa whose exact quarternery structure is unclear but is likely to be either heterohexameric or heteroheptameric. Capping of proteasomes by PA28 stimulates the hydrolysis of oligopeptide substrates but not the hydrolysis of large proteins. PA28 like 19S regulator probably activates the proteasome by opening the channel at the terminal rings thereby increasing access of substrates to the catalytic sites. The inability of PA28 to activate hydrolysis of large proteins probably reflects its inability to unfold and/or translocate these substrates in an ATP-dependent manner. The physiological role is not known. Several lines of evidence suggest that one function is to regulate the proteasomes production of antigenic peptides for presentation by class I molecules, a process known to involve the ubiquitin-proteasome pathway. However, the exact function of the PA28-20S complex in the ubiquitin pathway is presently unclear.

3.3.3.2. Regulation of the proteasome system

Proteasome function in cells can be regulated by altering levels of the proteasome, proteasome regulatory proteins or proteins of ubiquitin conjugation system. Thus, cells may adjust their capacity for ubiquitin/proteasome-dependent proteolysis by changing levels of proteins that participate in substrate selection and/or in substrate degradation (DeMartino and Slaughter 1999). As this particular study has concentrated on proteasomal effects, a brief overview of how the proteasome can be regulated will be considered.

Degradation of specific substrates of the ubiquitin pathway is often regulated. Regulation can be achieved via several mechanisms; the substrate could be modified so

as to be recognised or not recognised by the appropriate E3 complex; or the activity of specific E2/E3 complexes toward certain substrates, or classes of substrates could be modulated (Kornitzer and Ciechanover 2000).

Furthermore, the proteasome itself can be subject to regulation. The enhanced proteolysis seen in various catabolic conditions is suppressed in ATP-depleted muscles for e.g. in denervation, fasting, cancer, acidosis and sepsis (Attaix *et al* 1998). This indirectly suggests that the proteasome is involved in the breakdown of muscle proteins. In other experiments using specific inhibitors of the proteasome (e.g. peptidyl aldehydes and/or lactacystin) lead to identical interpretations. MG-132 (CBZ-leu-leu-leucinal), a potent inhibitor of the branched chain amino acid-preferring, peptidylglutamyl peptide hydrolase and chymotryptic activities of the proteasome (Coux *et al* 1996), was reported to block the activation of the ubiquitin-proteasome pathway in acidosis (Bailey *et al* 1996), diabetes (Price *et al* 1996), denervation and sepsis following administration of thyroid hormones (Tawa *et al* 1997). Hobler and co-workers (1998) observed that lactacystin also blocks increased total and myofibrillar protein breakdown in rats.

The second piece of evidence that the proteasome is involved in the breakdown of muscle proteins, is based on increased expression of 20S proteasome subunits that occurs in atrophying muscles such as in head trauma (Mansoor *et al* 1996) and sepsis (Tiao *et al* 1997). Enhanced mRNA levels have been consistent only in muscles exhibiting high rates of protein breakdown (Dardevet *et al* 1995, Temparis *et al* 1994). The enhanced expression of the 20S proteasome subunits correlated with increased abundance of the 27kDa subunit in cancer (Baracos *et al* 1995). Bailey and colleagues (1996) observed an increase in transcribed C3 proteasome subunit mRNA in acidosis.

Furthermore, analysis of subunit C9 mRNA was shown to be increased in abundance and also undergo active translation in simulated weightlessness (Taillandier *et al* 1996). In some studies, parallel changes in mRNA encoding all the subunits of the proteasome that were measured, strongly suggested increased synthesis of the 20S particle (Medina *et al* 1995).Thus, it is apparent that there is a transcriptional programme favouring increased expression of 20S subunits in muscle atrophy (Mitch and Goldberg 1996).

There is growing evidence that changes in levels of subunit mRNAs of 19S complex that binds to the 20S proteasome to form 26S proteasome occurs in various proteolytic conditions in skeletal muscle (Attaix *et al* 1998). However, the expression of ATPase and non-ATPase subunits of the 19S complex is regulated independently of the expression of 20S proteasome subunits. Dawson *et al* (1995) observed that in abdominal intersegmental muscles of *Maduca sexta*, there was a dramatic increase in the expression of the ATPase subunit MS73 during programmed cell death. Western blot analyses showed increases in several ATPases including MS73, MSS1 and mts2 but not TBP1. This corresponded with large changes in the peptidase activities of the 26S proteasome. In another study conducted by Attaix *et al* (1997) there was an increase in the expression of some subunits of the 19S complex in atrophying muscles from hind-limb suspended and tumour-bearing rats. Increased mRNA levels for two ATPases (MSS1 and P45) and two non-ATPases (P112 and P31) subunit were observed in unweighted muscles. By contrast, only the mRNA for MSS1 prevailed in the muscles from tumour bearing rats. Thus, it appears that expression of 19S subunits is dependent on a given catabolic condition.

The 11S regulator (or PA28) can associate with the 20S proteasome and stimulates its peptidase activities, without affecting the breakdown of proteins (Dubiel *et al* 1992). Bovine PA28 modulated the peptidase activities of the lobster muscle proteasome (Mykles 1996). A small increase in the mRNA levels of α and β was noted in unweighted muscles (Attaix *et al* 1997). It is purported that PA28 plays a role either in the final cleavages of peptides arising from protein breakdown and/or the breakdown of some abundant endogenous peptides present in muscle e.g. anserine and carnosine (Attaix and Taillandier 1998). The significance of these results remains unclear; in other catabolic states e.g. cancer, no change in mRNA in 11S subunits was reported (Attaix *et al* 1997).

3.3.3.3. Role of ATP – ubiquitin system in myofibrillar (MF) protein breakdown

An increasing body of evidence implicates the ATP-ubiquitin pathway in the degradation of long-lived MF proteins (Tiao *et al* 1994, 1997). For example, increased mRNA levels for components of this pathway have been detected in skeletal muscle in various animal models where muscle wasting occurs. These include fasting, glucocorticoid administration, denervation, acidosis, sepsis and cancer (Attaix and Taillandier 1998). An increase in ubiquitin-protein conjugates has also been reported in several of the above situations. These ubiquitinated proteins were found in the MF fraction, predominantly located at the Z-discs of myofibrils and conjugated proteins were degraded by the proteasome (Hilenski *et al* 1992, Riley *et al* 1988).

Other studies performed using specific inhibitors of the proteasome, further support the role of the ATP-ubiquitin pathway in MF breakdown. Tawa *et al* 1997 showed that using peptide aldehyde inhibitors of the proteasome such as LLN, N-acetyl-leucyl-

leucyl-norleucinal and MG132 suppressed proteolysis in incubated rat skeletal muscle. These agents inhibited non-lysosomal protein breakdown by up to 50% which was rapidly reversed upon removal of the inhibitor. These agents had more pronounced effects on proteolysis in atrophying muscles than in controls. In denervated soleus undergoing atrophy, the increase in ATP-dependent proteolysis was reduced by 70% by MG132. Similar results were obtained by Thompson and colleagues (1999) in C_2C_{12} myotubes. Incubation of these cells with MG132 prevented both the basal release of 3-methylhistidine (3-MH, is post-translationally modified amino acid and metabolically stable, which is a marker of long lived MF proteins) and the increased release into medium in response to dexamethasone (>95% in C_2C_{12} myotubes).

Although there seems to be strong evidence supporting a role for the ATP-ubiquitin pathway in the degradation of MF proteins, several observations suggest that even if the role of the ubiquitin system is the major factor controlling proteolysis, it is unlikely to be a universal mechanism in all cases where MF degradation is changed. For e.g. in contrast to all muscle wasting conditions seen so far examined in rodents, where protein breakdown and mRNAs for components of the ubiquitin system both increase, there is no alteration in gene expression of components of the ubiquitin-protein proteolytic pathway in either dystrophin-deficient muscles from mice or Duchenne muscluar dystrophy (DMD) patients (Combaret *et al* 1996). One explanation for this finding is that the activity of pre-existing components may be altered by covalent modification, but it is also possible that this particular pathological condition involves a non ATP-ubiquitin-dependent proteolytic pathway.

Several other anomalous situations have been reported that further complicate ubiquitin-dependent proteolysis. At least two proteins with a rapid rate of turnover, ornithine decarboxylase (Murakami *et al* 1992) and c-Jun (Jariel-Encontre *et al* 1995) are degraded by the 26S proteasome in ubiquitin-independent manner. Targeting of ornithine decarboxylase to the 26S proteasome depends on its association with a small protein called antizyme. This suggests that either (i) at least some proteins possess intrinsic motifs or other signals which facilitate direct recognition and breakdown by the 26S proteasome or that (ii) there may be a sub species of the 26S complex which does not recognise ubiquitinated proteins but co-purifies with the ubiquitination specific 26S proteasome. It is not clear if either of these situations could apply to any of the long-lived MF proteins.

3.3.3.4. Ubiquitin-proteasome proteolysis in pathological states

Medina *et al* (1995) showed that denervation in the rat (caused by cutting the sciatic nerve) resulted in rapid atrophy of the soleus muscle causing a 30% reduction in muscle weight in only a few days. The atrophy was accompanied by enhanced protein degradation with no change in synthesis. This proteolysis was markedly inhibited by blocking ATP production but not affected by other treatments which prevented lysosomal and Ca^{2+}-dependent proteolysis.

A prominent feature of several types of cancer is cachexia. This profound loss of tissue mass in cancer cannot be explained simply by anorexia, as highlighted in the introductory section. Tumour-bearing rats showed a greater loss of muscle protein and a higher rate of proteolysis than rats fed on equal amount of calories (Baracos *et al* 1995). Implantation of the rapidly growing Yoshida sarcoma into the leg muscle of rats

produced atrophy of adjacent muscles, presumably because the tumour releases locally active catabolic factor (Temparis *et al* 1994). The muscle atrophy was due to an increase in the rates of protein degradation compared to rates measured in muscles of the contralateral leg. Studies with inhibitors of proteolytic pathways indicated there was an increased activity of the ATP-dependent process and there was an increase in the content of ubiquitin mRNAs. In tumour-bearing MAC16 mice possessing weight loss Lorite *et al* (1998) observed activation of cathepsin B and L and ATP and Ca^{2+}-dependent pathways in soleus muscles excised from these animals. In PIF injected animals there was an activation of the ATP-dependent pathway only.

Medina *et al* (1995) showed that food deprivation (known to cause a rapid increase in muscle protein breakdown) resulted in an increase in ATP-dependent proteolysis that paralelled an increase in ubiquitin conjugates and polyubiquitin mRNA in rat skeletal muscle after only 24hr starvation. Refeeding resulted in a reversal of the increased gene expression only 24hr later. Fasting also increases the mRNA encoding at least one ubiquitin-conjugating enzyme, $E2_{14K}$ (Wing and Goldberg 1993).

In dietary protein deficiency, the metabolic adaptations that take place are directed towards conserving proteins. Similarly, protein malnutrition is an e.g. of a metabolic state where important molecular mechanisms are activated and inactivated to conserve protein (in contrast to that found during food deprivation) and especially essential amino acids. Tawa *et al* (1992) found that protein breakdown in skeletal muscle was decreased during dietary protein deficiency. Following a study in which animals were subjected to 72hr protein deprivation, they concluded that both the lysosomal and the ATP-dependent proteolytic pathways were involved.

Tiao *et al* (1994) tested the role of different intracellular proteolytic pathways in sepsis-induced muscle proteolysis. Muscle breakdown rose in rats within 6hrs after injection with endotoxin, live bacteria or after puncture of the caecum to produce peritonitis and fulminant sepsis. These increases were associated not only with an increase in ATP-dependent proteolysis but also increases in ubiquitin conjugates and ubiquitin gene expression in the affected muscles. The other proteolytic pathways (lysosomal and Ca^{2+} dependent) showed no change in activity during sepsis. Presumably, this response provides the infected organism with a source of amino acids for energy metabolism and synthesis of new proteins e.g. acute phase reactant proteins.

Patients with burns or other traumatic injuries experience a similar rapid loss of body protein primarily due to enhanced proteolysis in muscle (Mansoor *et al* 1996). When a thermal injury is applied to the back of rats, the ATP-dependent degradative process is stimulated in leg muscles, there is as much a seven-fold increase in the degradation of myofibrillar proteins and an increase in the level of ubiquitin mRNA (Fang *et al* 1995).

Metabolic acidosis is often associated with conditions in which there is a marked negative nitrogen balance associated with increases in muscle proteolysis and oxidation of branched chain amino acids (Bailey *et al* 1996). This is the case with chronic renal failure, where the patients are subject to extensive body wastage, which can be reverted by the administration of sodium bicarbonate to compensate for the acidosis. Metabolic acidosis in rats caused an increase of 2.5 to 4-fold in the levels of mRNAs for ubiquitin and two subunits of the 20S proteasome specifically in muscle (Mitch *et al* 1994). Similar findings were also made in muscles of acidotic rats with acute or chronic renal

failure, both of which stimulate protein degradation (May *et al* 1987), without changes in protein synthesis.

Inactivity can also promote muscle atrophy. This phenomenon is often seen in immobilised patients and those after prolonged bed rest and most dramatically in astronauts (Lecker *et al* 1999). This atrophy has been modelled in hind limb-suspended rats (Thomason and Booth 1990) and in rats with denervated limbs (Furuno and Goldberg 1986). Muscle atrophy has also been measured in rats exposed to microgravity -spaceflight (Caizzo *et al* 1996). As in other types of atrophy the ubiquitin-proteasome pathway appears responsible for the increased protein breakdown.

3.3.4. Aim of study

This study examines the effect of LMF on protein synthesis and degradation in C_2C_{12} murine myoblasts and in soleus muscle of mice, and attempts to elucidate the role of cAMP in this process. Glucose utilisation studies in the presence of LMF and the mechanistic pathways elicited by PIF on protein turnover will also be examined. A brief consideration is given to the combined effects of both these factors in C_2C_{12} cells *in vitro*.

3.4. RESULTS

3.4.1. Effect of LMF on protein synthesis

Lipid-mobilising factor (LMF) was isolated from the urine of cachectic cancer patients. After a serial purification process involving both ion exchange and hydrophobic chromatography a 43kDa protein representing LMF (Todorov *et al* 1998) was detected after staining with coomassie blue (Figure 3.4.1). This final product was used in both the *in vivo* and *in vitro* work. In order to check the lipolytic activity of newly purified LMF, 1μg/μl of the material was subjected to the lipolytic assay. LMF was able to stimulate lipolysis in isolated murine epididymal adipocytes, determined by glycerol release (Figure 3.4.2). The validity of the assay was established using isoprenaline as a positive control (0.1μM). LMF lost its ability to induce protein synthesis after losing its lipolytic activity, usually after a period of 2-3 weeks at 4°C.

When C_2C_{12} cells were incubated for 24hr in the presence of LMF, the rate of protein synthesis was increased in a dose-dependent manner for concentrations ranging from 11.6nM up to 580nM. In both myoblasts and myotubes maximal effects were achieved at 580nM, 42% increase in myoblasts and 38% in myotubes (Figure 3.4.3 and Figure 3.4.4). These results were not due to a stimulation of transport of [^{3}H] Phe as no changes were observed in the levels of radioactivity in intracellular pools. Incubation of C_2C_{12} myoblasts with LMF for shorter incubation periods, 90min and 6hr, did not affect the rate of protein synthesis (Figure 3.4.5).

A similar dose-response curve was obtained for LMF stimulating protein synthesis in MAC16 cells, again reaching a peak at 580nM LMF, very similar to that obtained in C_2C_{12} cells (Figure 3.4.6). Interestingly, no effect was demonstrated in MAC13 cells (Figure 3.4.7).

In order to confirm the specific effect of LMF on protein synthesis, experiments were conducted to determine cell number (Figure 3.4.8) and [^{3}H] thymidine incorporation (Figure 3.4.9) into cell lines. Cells were exposed to increasing concentrations of LMF but no effect was shown on either of these parameters.

Numerous inhibitors were selected to elucidate the second messenger pathways involved in the increase of protein synthesis in C_2C_{12} cells (Table 3.1). No effect on LMF stimulation was seen in the presence of Ro-31-8220, a protein kinase C inhibitor. The stimulatory effect of LMF on protein synthesis in C_2C_{12} myoblasts was attenuated by the adenylate cyclase inhibitor MDL $_{12330A}$ and by the the cyclic AMP dependent protein kinase inhibitor H8 although the latter did not reach significance. To further investigate the possible role of cAMP in these responses, two other agents were utilised. Forskolin and dibutyryl-cAMP (dbt-cAMP) both have the ability to elevate intracellular cAMP; dibutyryl-cAMP permeates the plasma membrane and is metabolised in the cell to generate cAMP whilst forskolin has a direct action at the level of the catalytic subunit. The addition of these agents to C_2C_{12} myoblasts for 24hr alone elicited an increase in protein synthesis, confirming their role in this process. The effects of forskolin (25μM) and dbt-cAMP (1μM) were additive to LMF but the latter was not significant. These results taken together suggest a strong role for cAMP as a mediator of LMF induced protein synthesis.

Protein synthesis in C_2C_{12} cells was inhibited by a polyclonal antibody to zinc-alpha-2-glycoprotein ZAG (10μg/ml) at concentrations of LMF of 406nM and 580nM (Figure 3.4.10). The non-specific β receptor blocker, propranolol (10μM) also attenuated the effect at the same concentrations of LMF (Figure 3.4.11). The β_3-receptor antagonist SR 59230A also reduced LMF stimulation of protein synthesis (Figure 3.4.12). The attenuation was most pronounced at 10^{-5}M with the response decreased down to base level. This suggests that stimulation of protein synthesis induced by LMF may act through a β_3 receptor.

To examine the possibility that the induction of protein synthesis by LMF may be attributed to a mechanism involving MAP kinase or related pathways, various inhibitors were used (Table 3.2). There was no effect on LMF induced protein synthesis by inhibitors of p70S6 kinase (rapamycin), mitogen activated kinase (PD 98059) and phophatidylinositide-3-OH kinase (wortmannin or LY 294002).

Incubation of C_2C_{12} cells with increasing concentrations of LMF for 30min produced a dose-related increase in cAMP levels with the maximal amount being produced at concentrations between 400 and 580nM LMF (Figure 3.4.13). This result demonstrates the direct effect that LMF has on cAMP accumulation, and further supports the possible role of this agent as an intracellular mediator of LMF. The increase in cAMP was linearly related to the increase in protein synthesis observed after 24hr (r^2 =0.97 p=0.004), which suggests a strong correlation between these two separate effects (Figure 3.4.14). The PKA activity in C_2C_{12} myoblasts was also investigated (Figure 3.4.15). An increase in PKA activity occurred reaching a peak at 58nM

with no further increase in activity with higher concentrations until 580nM where a slight elevation in activity was observed, which was statistically significant.

To determine whether LMF purified from the urine of cachectic cancer patients was capable of elevating protein synthesis in skeletal muscle *in vivo,* material was injected intravenously into the tail vein of male NMRI ex-breeder mice (Figure 3.4.16). Injections of 8μg were given over 48hr, two per day, morning and evening; monitoring of body weights and food/water intake prior to each injection was done throughout throughout the experiment. A parallel group of mice were treated with equal volumes of PBS. On the third day, mice were sacrificed by cervical dislocation. There was a progressive decrease in body weight by mice administered LMF in comparison to mice receiving PBS, reaching significance after 24hr. There was no difference in food and water intake between the two groups (Figure 3.4.17). The average weight of the mice on initiation of the experiment was 44.50g and was 41.33g after 48hr treatment with LMF. Despite this overall loss of body weight which was primarily fat, soleus muscles taken from LMF treated mice showed a marked increase in protein synthesis (69% $p=0.006$) determined by an *in vitro* assay system (Figure 3.4.18). This mirrored results of immunoblots probed for myosin levels taken from the cytosolic extracts of these muscles excised from LMF treated mice (Figure 3.4.19). Densitometric analysis showed a significant increase in myosin after treatment with LMF (46 ±9%) compared with animals receiving PBS.

SDS-PAGE gel of purified LMF after HPLC visualised by coomassie stain

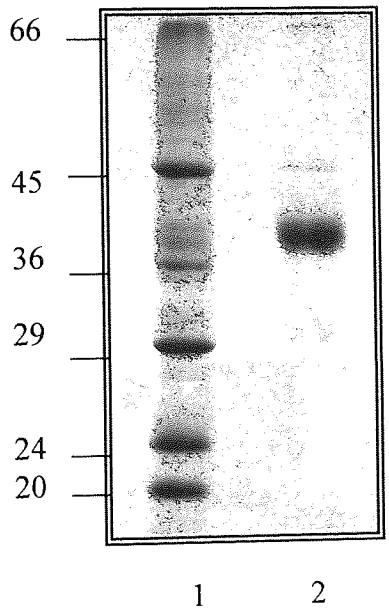


Figure 3.4.1. Coomassie stain of purified LMF isolated from cachectic patient urine. Lane 1= SDS 7 molecular weight marker. Lane 2 = LMF (5μg). Numbers represent approximate MW.

Effect of purified LMF and isoprenaline on the induction of lipolysis in isolated epididymal adipocytes

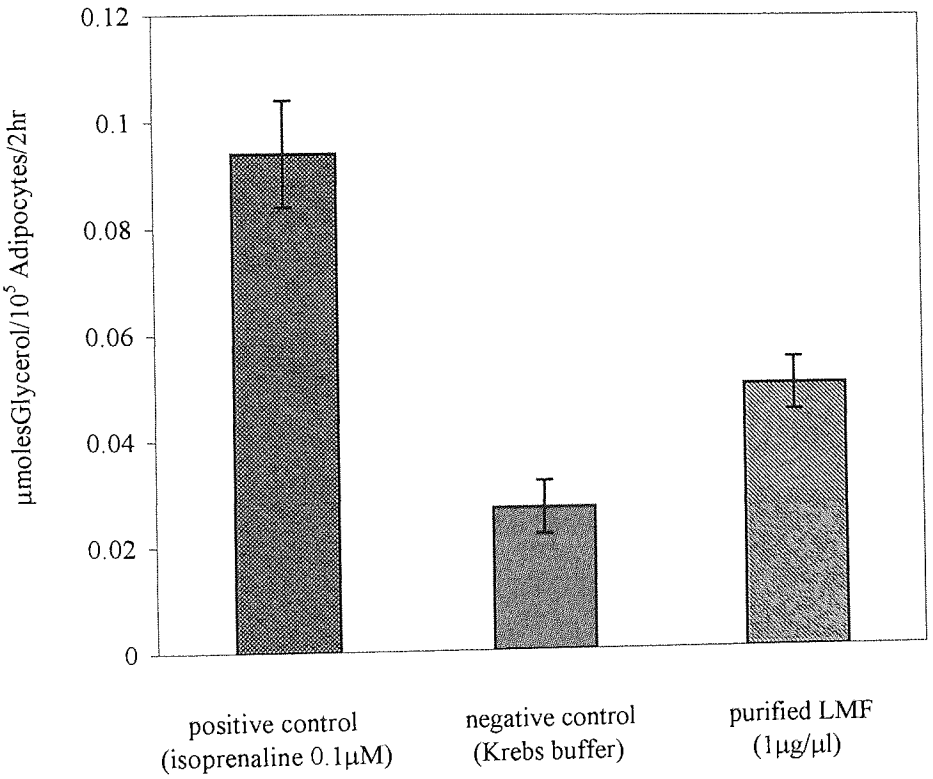


Figure 3.4.2. Murine epididymal white adipocytes were incubated in the presence of purified LMF (1µg/µl) and positive control (isoprenaline 0.1µM) for 2hr after. Lipolytic activity was determined by measuring glycerol released. Results expressed as mean ± SEM where n=2.

Effect of LMF on protein synthesis in C_2C_{12} myoblasts after a 24hr incubation period

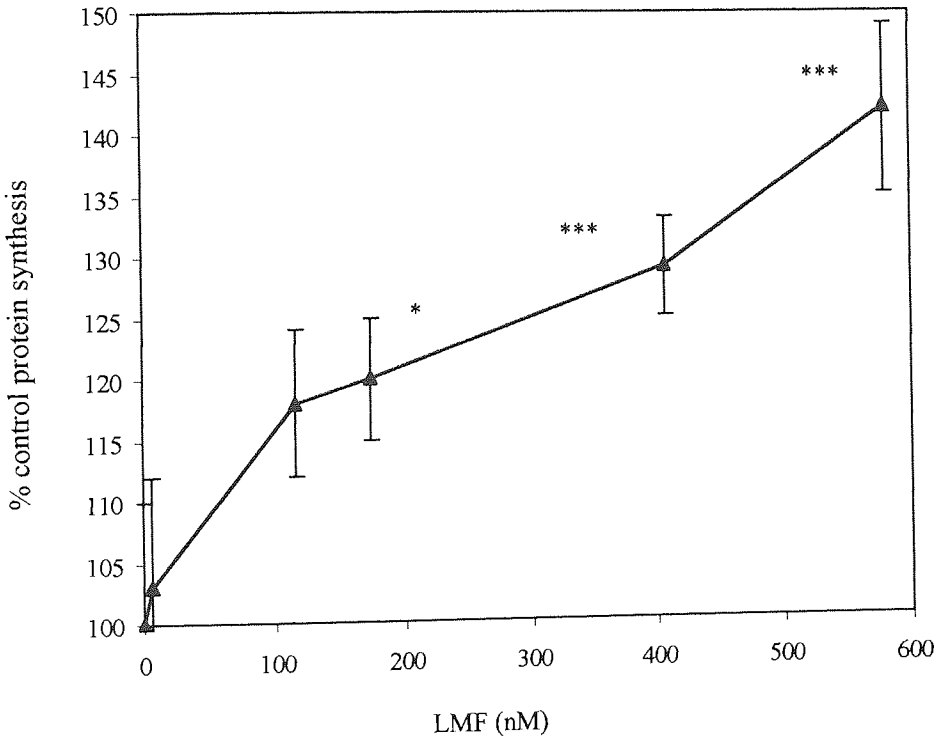


Figure 3.4.3. Effect of LMF on C_2C_{12} myoblasts. Results expressed as mean ± SEM where n=3. Experiments were repeated out at least 2 times. Statistical test performed using one-way ANOVA with Student-Newman- Keuls test whereby * = $p<0.05$, *** = $p<0.01$

Effect of LMF on protein synthesis in C_2C_{12} myotubes after a 24hr incubation period

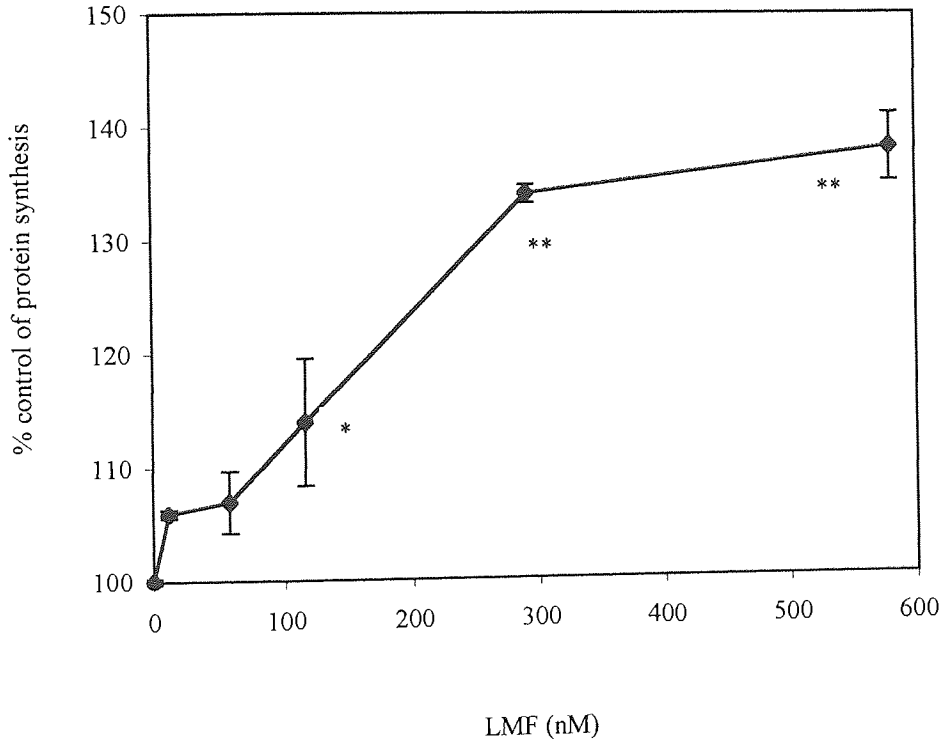


Figure 3.4.4. Effect of LMF on protein synthesis in differentiated myotubes. C_2C_{12} myoblasts were fused by incubating cells in the presence of horseserum (Section 2.2.2). Values represent mean ± SEM where n = 3. Experiments were repeated at least 2 times. Statistical tests using one-way ANOVA with Student-Newman-Keuls test whereby * = $p<0.05$, ** = $p<0.01$

Effect of LMF on protein synthesis in C_2C_{12} myoblasts after 6hr and 90min incubation periods

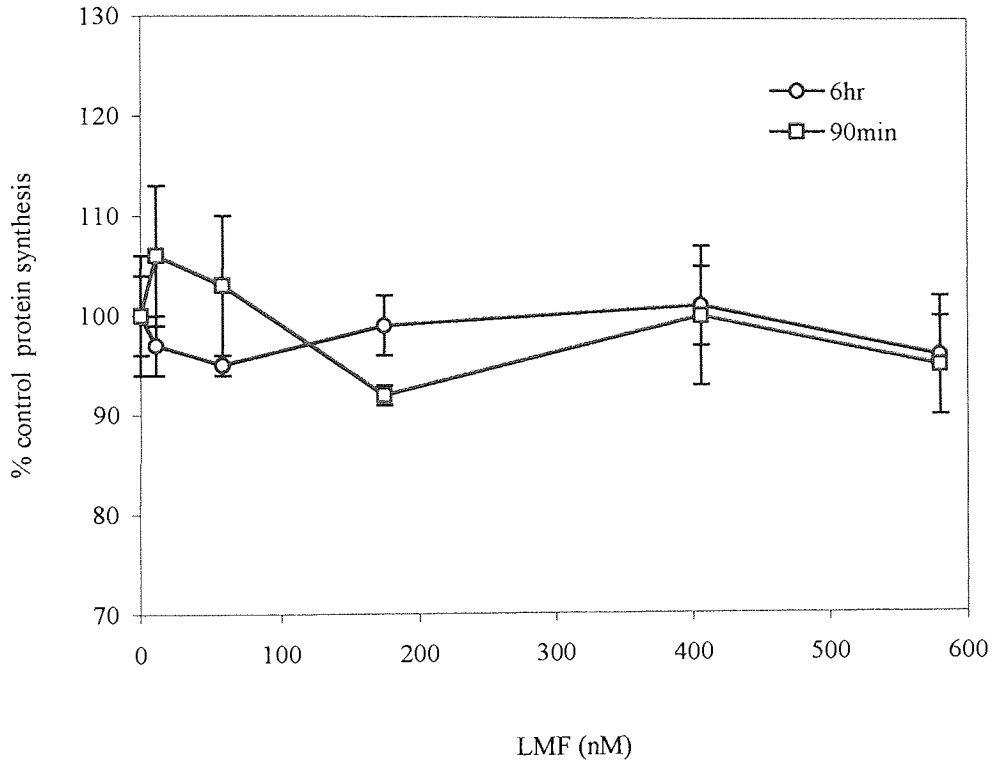


Figure 3.4.5. Effect of LMF on protein synthesis in C_2C_{12} myoblasts during a 6hr and 90min incubation period. Values are the mean ± SEM where n = 3. Experiments were repeated 3 times.

Effect of LMF on protein synthesis in MAC16 cells after a 24hr incubation period

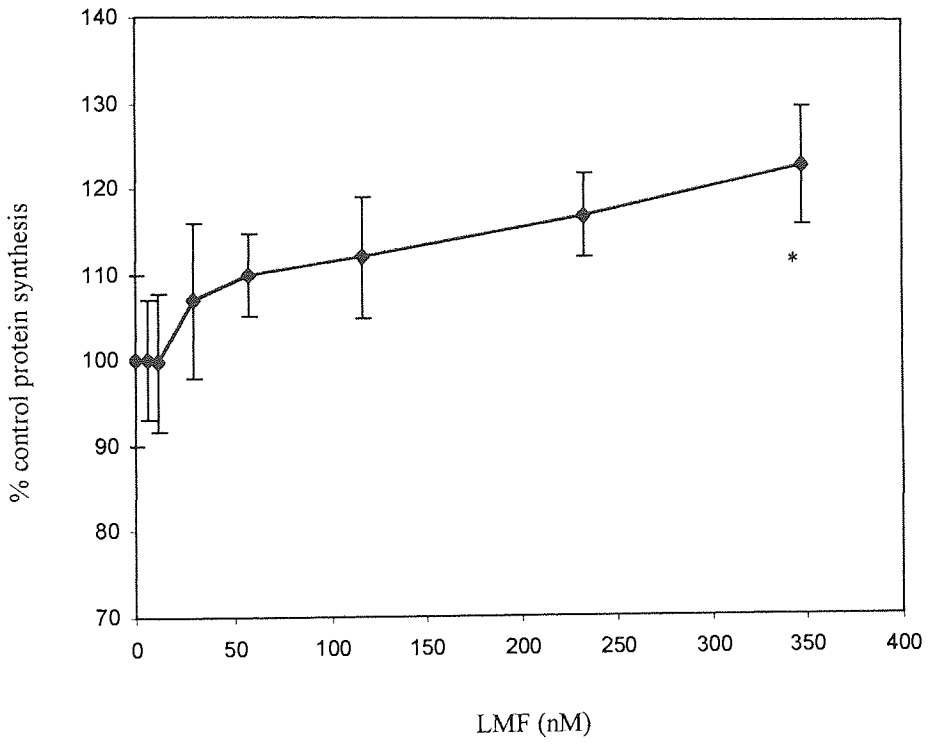


Figure 3.4.6. Effect of LMF on MAC16 tumour cell line incubated with the lipolytic factor for 24hr. Results represent the mean ± SEM where n = 3. Experiments were conducted 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test, whereby * = $p < 0.05$.

Effect of LMF on protein synthesis in MAC13 cells after a 24hr incubation period

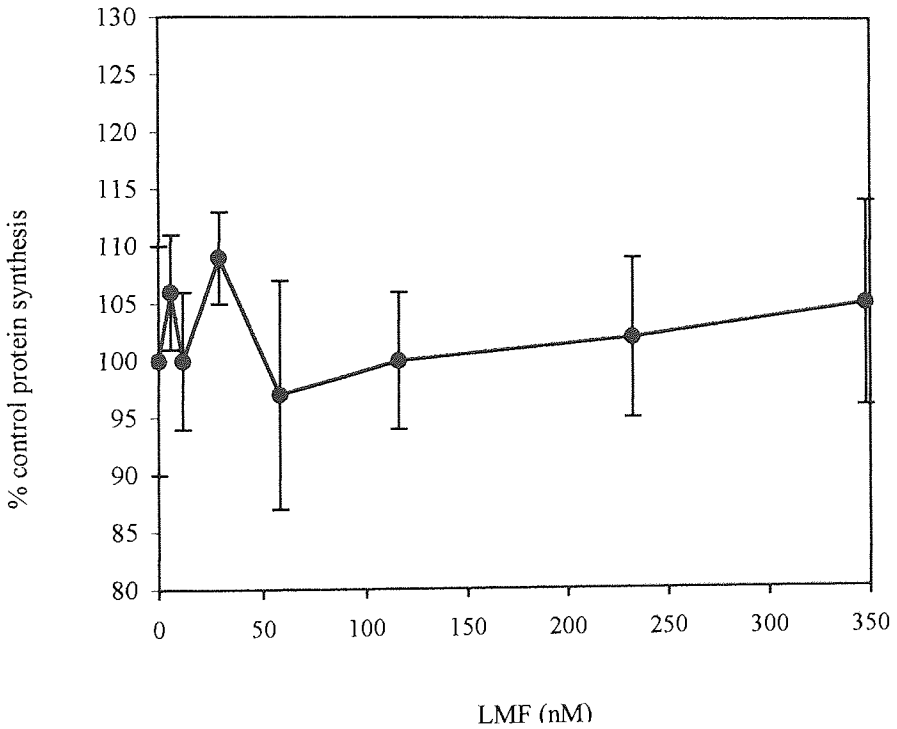


Figure 3.4.7. Effect of LMF on MAC13 tumour cells. Results shown represent the mean ± SEM where n = 3. Experiment was repeated 3 times.

Effect of LMF on cell proliferation in three different cell lines

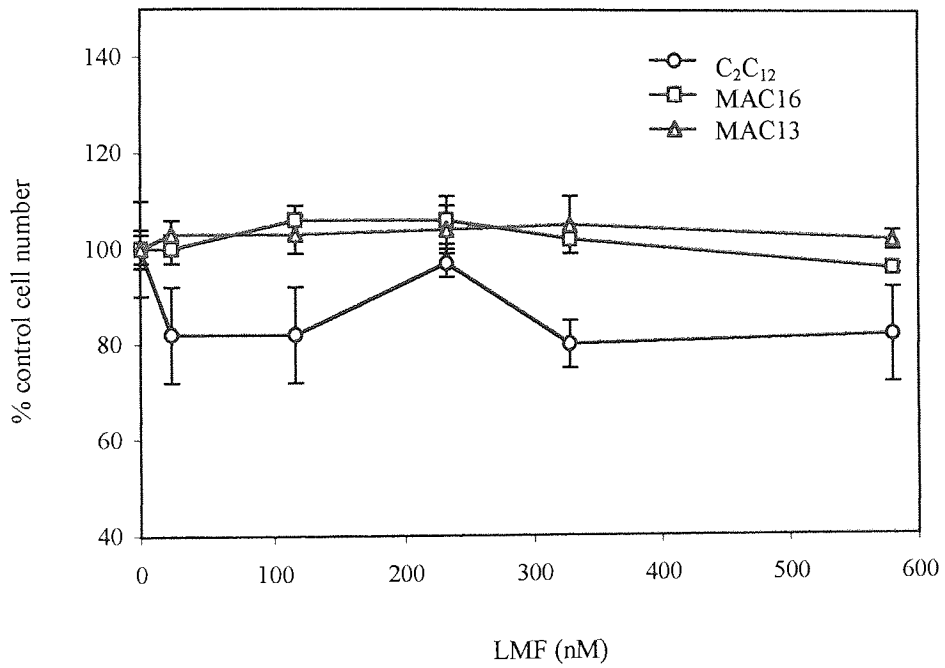


Figure 3.4.8. The effect of LMF on cell proliferation in C_2C_{12}, MAC16 and MAC13 cells. Cells were seeded on day 1 and counted on day 5. Results show the mean ± SEM n=4. Experiments were conducted 3 times.

Effect of LMF on [^{3}H] methyl-thymidine uptake in three different cell lines

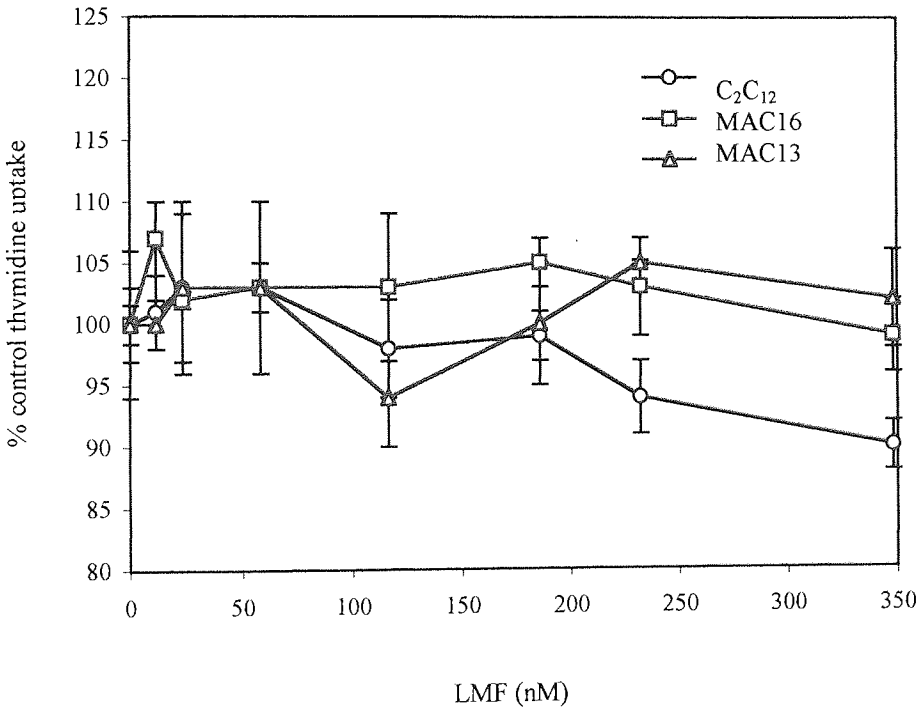


Figure 3.4.9. Effect of LMF on [^{3}H] methyl-thymidine uptake in C_2C_{12}, MAC16 and MAC13 cells. Cells were incubated with the radiolabel for 48hr. Values represent mean ± SEM where n = 3. Experiment was carried out 3 times.

The effects of various treatments on LMF induced protein synthesis in C_2C_{12} myoblasts

TREATMENT	% CONTROL PROTEIN SYNTHESIS	DIFFERENCE (COMPARED TO CONTROL / INHIBITOR CONTROL)	SIGNIFICANCE (P)
LMF 580nM	136 ± 6.9	36	P<0.001
Ro-31-8220	94 ± 6.7	-	NS
Ro-31-8220 + LMF	129 ± 5.6	35	NS
MDL $_{12330A}$ 20μM	96 ± 10.1	-	NS
MDL$_{12330A}$ + LMF	113 ± 14.3	17	P<0.05
H8 10μM	104 ± 3.2	-	NS
H8 10μM + LMF	125 ± 2.2	21	NS
Forskolin 25μM	158 ± 7.4	-	P<0.001
Forskolin 25μM + LMF	178 ± 4.7	20	P<0.001
Dbt cAMP 1mM	125 ± 4.3	-	P<0.01
Dbt cAMP 1mM + LMF	148 ± 3.1	23	NS

Table 3.1. Cells were incubated in the presence of LMF for 24hr. All inhibitors were added 1 hr prior to LMF. Results represent the mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test comparing (i) LMF or treatment to control (ii) LMF + treatment to LMF alone.

The effect of polyclonal antibody to zinc-alpha-2 glycoprotein (Znα2gp) on LMF induced protein synthesis in C_2C_{12} myoblasts

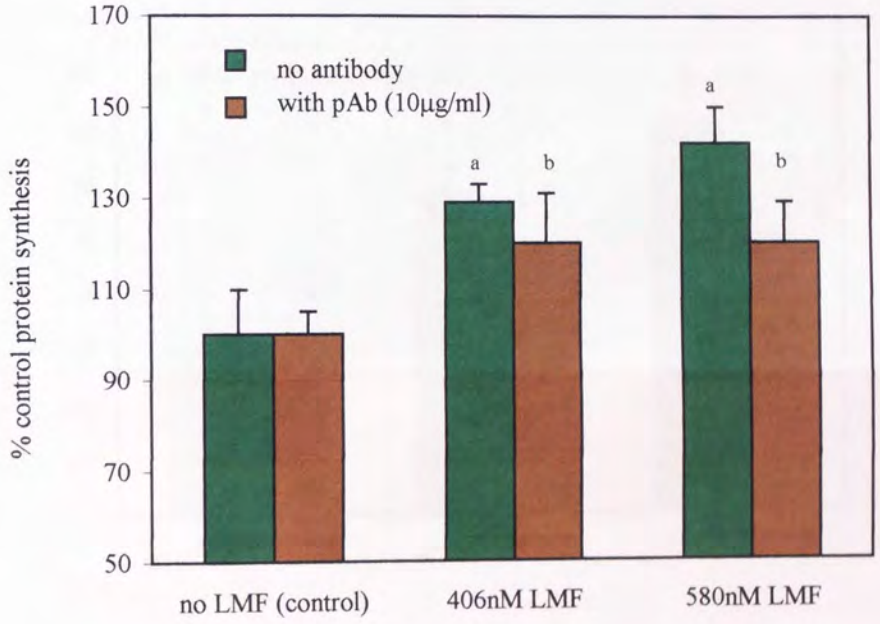


Figure 3.4.10. Cells were incubated in the presence of LMF for 24hr. Zinc-α2-gp polyclonal antibody (pAb) was added to cells 1hr prior to the addition of LMF. Bar chart shown represents the mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test. This is comparing (i) LMF 406nM/580nM to control (a = $p<0.01$) (ii) LMF 406nM/580nM + pAb to LMF 406nM/580nM alone (b = $p<0.05$).

Effect of a non-specific β-receptor antagonist propranolol on LMF induced protein synthesis in C_2C_{12} myoblasts

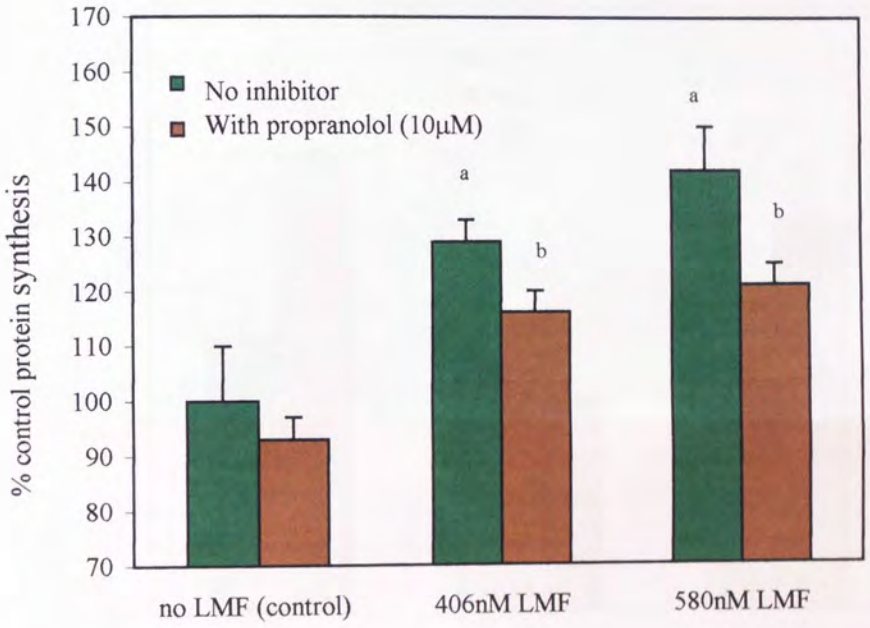


Figure 3.4.11. Cells were incubated in the presence of LMF for 24hr. Propranolol was added to cells 1hr prior to LMF. Bar charts represent mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test comparing: (i) LMF 406nM/580nM to control (a = $p<0.01$) and (ii) LMF 406nM/580nM + propranolol to LMF 406nM/580nM alone (b = $p<0.05$).

Effect of specific β_3-receptor antagonist SR 59230A on LMF induced protein synthesis in C_2C_{12} myoblasts

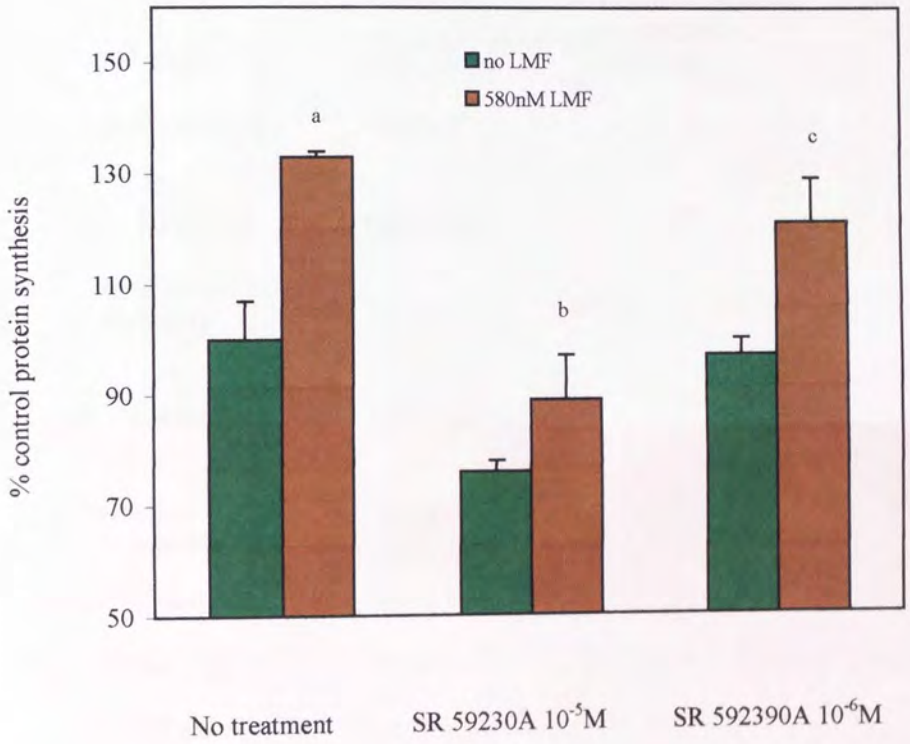


Figure 3.4.12. Cells were incubated in the presence of LMF for 24hr. SR 59230A was dissolved in DMSO and added 1hr prior to the addition of LMF. Bar charts represent mean ± SEM where n=3. Experiment was carried out 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test. This is comparing: (i) LMF 580nM to control (a = $p<0.001$) (ii) LMF 580nM + inhibitor 10^{-5}M to LMF 580nM alone (b = $p<0.001$) and (iii) LMF 580nM + inhibitor 10^{-6}M to LMF 580nM alone (c = $p<0.01$).

The effect of potential inhibitors on LMF induced protein synthesis in C_2C_{12} myoblasts

Treatment	% control protein synthesis	Difference (compared to control or inhibitor control)	P value (inhibitor vs. control)
LMF 406nM	132 ±8.1	32	-
LY294002 (0.05μM)	89 ±3.27	-	NS
LMF + LY294002	128 ±10.22	39	-
Wortmannin (0.02μM)	77 ±10.42	-	P<0.05
LMF + Wortmannin	107 ±5.86	30	-
Rapamycin (0.5ng/ml)	62 ±7.49	-	p<0.001
LMF + Rapamycin	93 ±2.00	31	-
PD 98059 (0.625μM)	92 ±4.93	-	NS
LMF + PD98059	129 ±4.06	37	-

Table 3.2. Cells were incubated in the presence of LMF for 24hr. Inhibitors were added to wells 1hr prior to the addition of LMF. Results are expressed as mean ± SEM where n=3. Experiment was repeated 3 times. Statistical significance determined by Student-Newman-Keuls test.

Effect of LMF on levels of intracellular cAMP in C_2C_{12} myoblasts

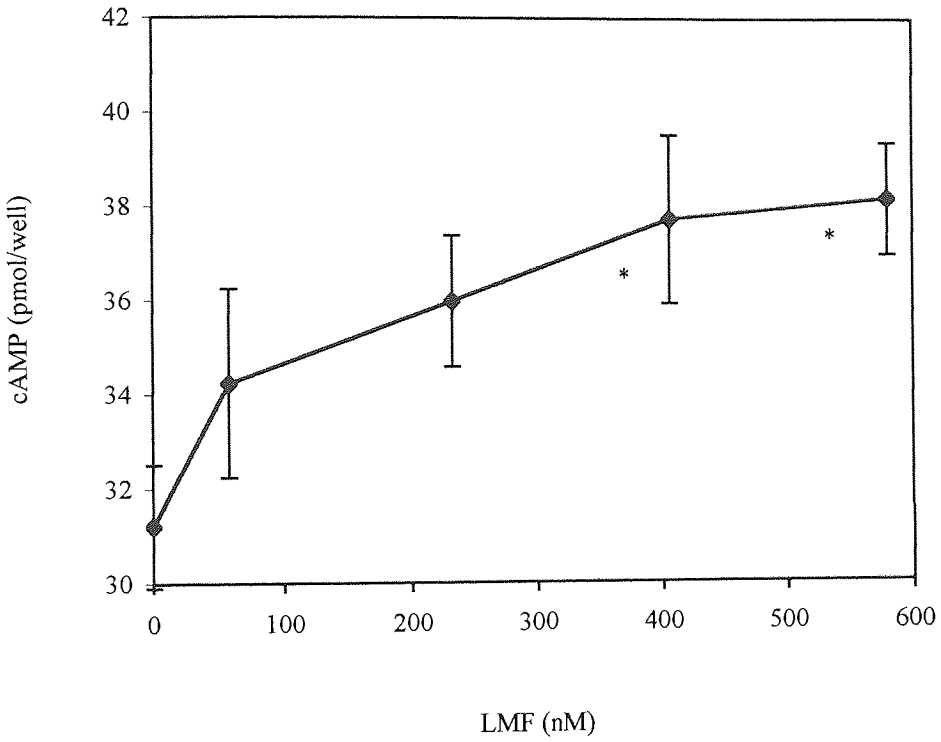


Figure 3.4.13. Cyclic AMP was determined by binding protein assay (Section 2.4.16). Each point represents the mean ± SEM where n=4. Experiment was repeated at least 2 times. Exact amounts of cAMP was determined by extrapolating from a standard curve. Statistical analysis using one-way ANOVA with Student-Newman-Keuls test comparing treated to control whereby * = $p<0.05$.

Graph showing correlation between % control in protein synthesis and cAMP content in C_2C_{12} myoblasts

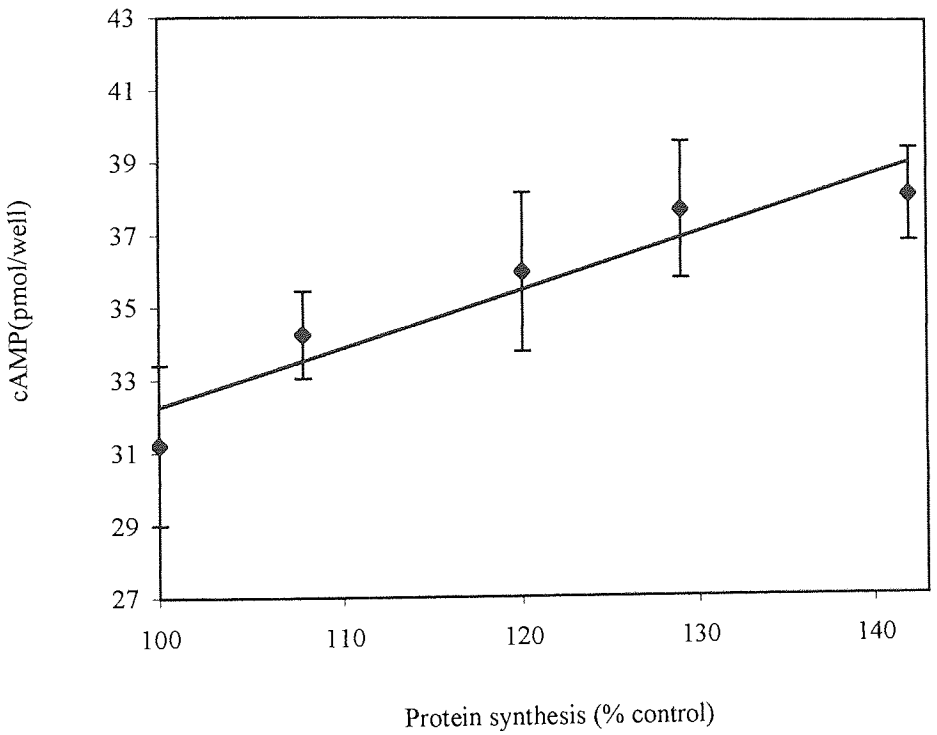


Figure 3.4.14. Relationship between the increase in protein synthesis in C_2C_{12} myoblasts, (24hr incubation with LMF) and the intracellular concentration of cyclic AMP (determined after pulsing with LMF for 30min). r^2 value = 0.953, p=0.0044. Experiment was repeated 3 times.

Effect of LMF on PKA activity in C_2C_{12} myoblasts

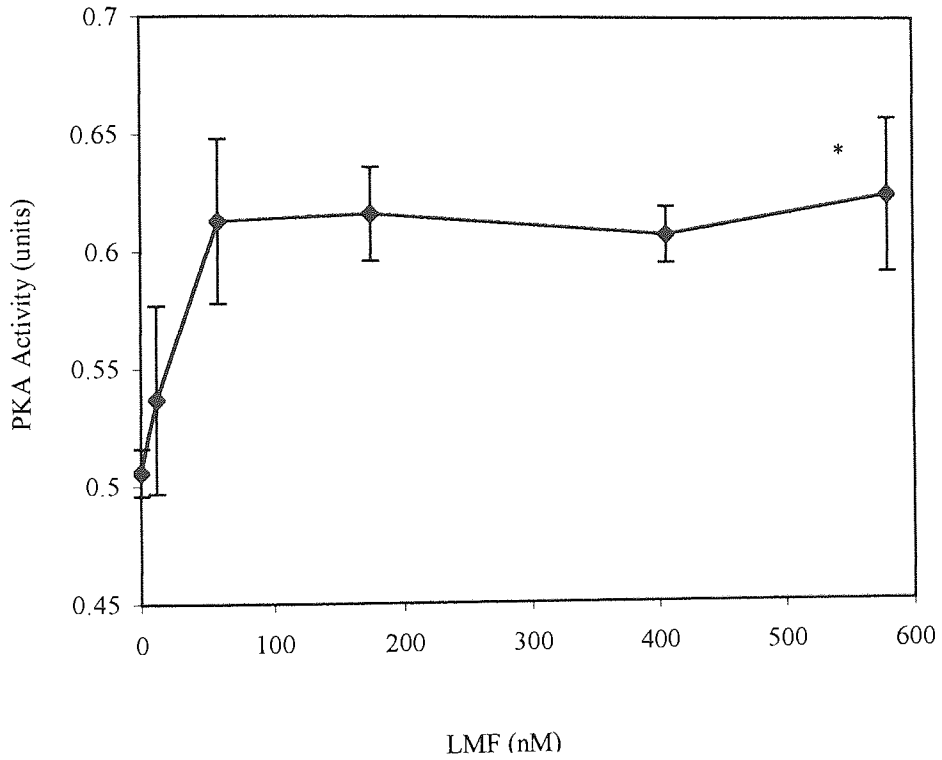


Figure 3.4.15. PKA activity was determined using an enzyme assay kit based on colorimetric analysis. Values of activity were extrapolated from a standard curve constructed at the same time of the experiment. Each point represents the mean ± SEM where n = 4. Experiments were repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test, comparing experimental values to control.

The effect of intravenously administered LMF on weight loss in exbreeder NMRI mice

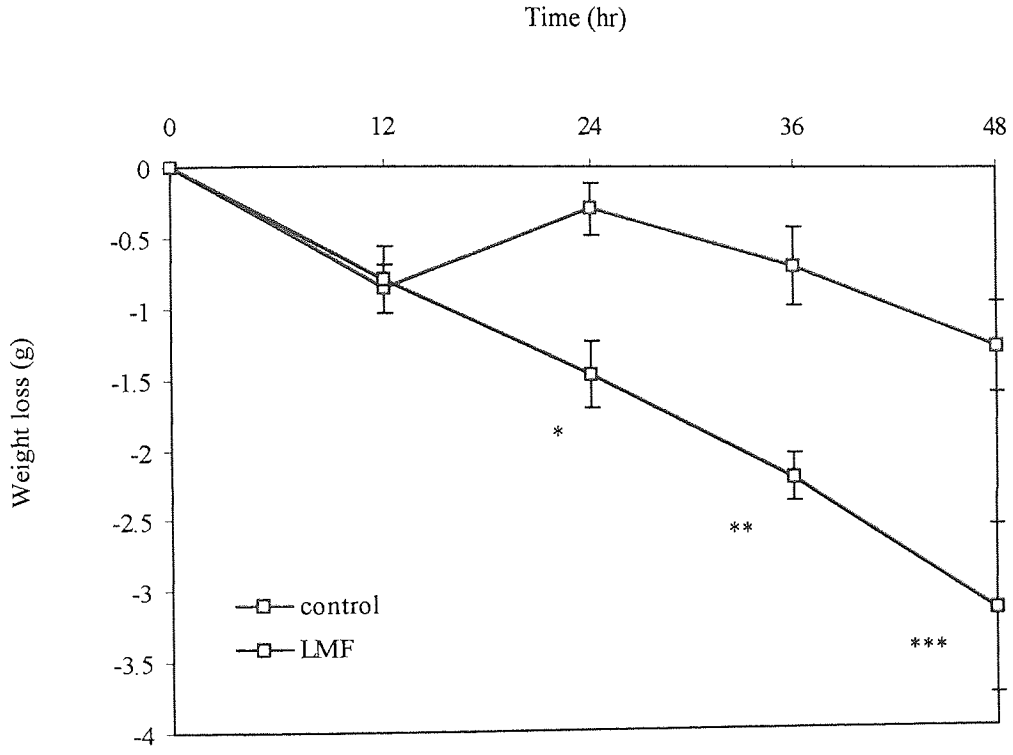
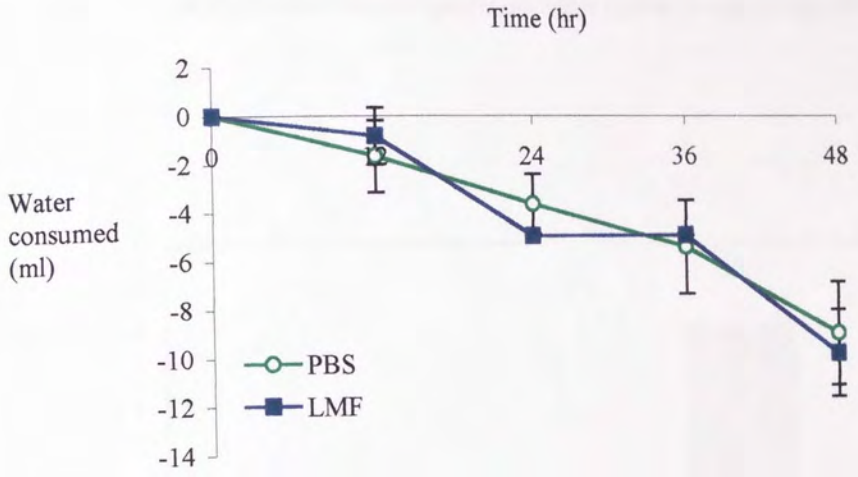


Figure 3.4.16. Effect of LMF (8μg/100μl bd) and PBS (control) on loss of total body weight over a 48hr period. Injections were administered into the tail vein of male NMRI mice. Results are expressed as mean ± SEM where n=5. The experiment was repeated 2 times. Statistical analysis performed using Student's unpaired t-test, LMF vs control, where * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Food and Water consumption in NMRI mice injected with intravenous LMF and PBS

A



B

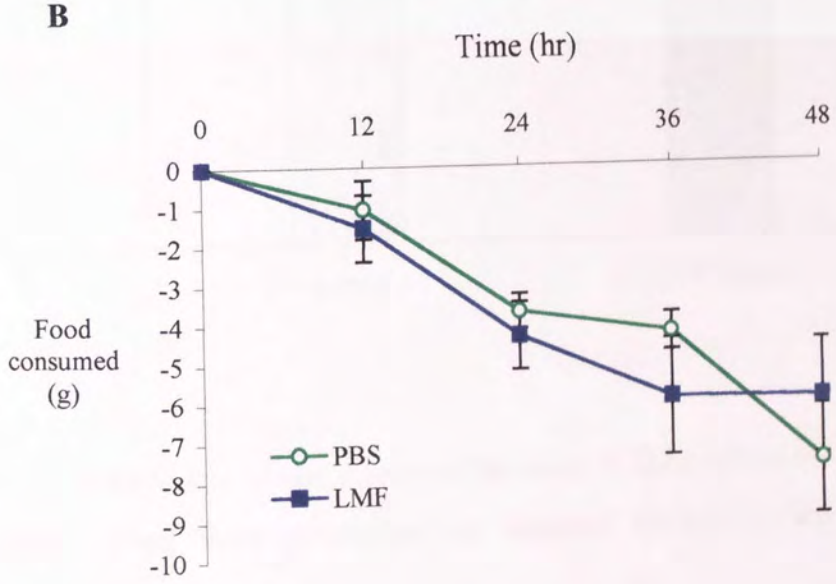


Figure 3.4.17. Graphs showing water (A) and food (B) consumption in NMRI mice injected with LMF (8µg/100µl bd) and PBS (100µL bd). Results represent the mean ± SEM where n=5.

Effect of LMF on protein synthesis in soleus muscle of exbreeder male NMRI mice in comparison with animals receiving PBS.

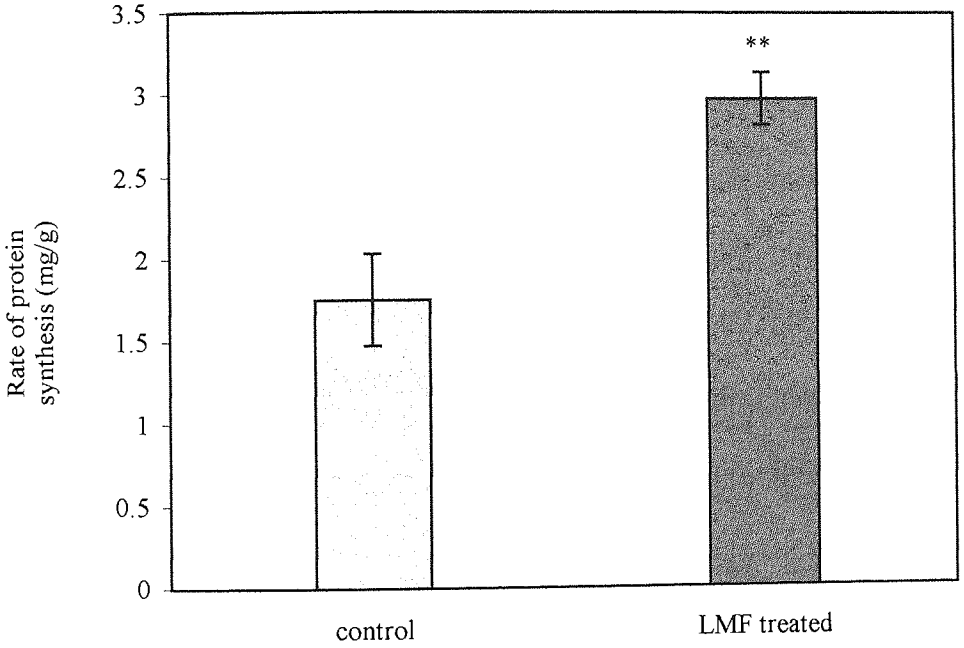
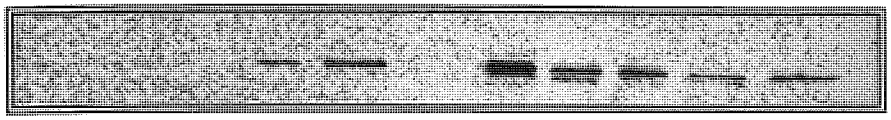


Figure 3.4.18. Bar charts represent the mean ± SEM where n= 6. Differences from control values were determined by unpaired Student's t-test where **=$p<0.01$. Experiment was repeated twice.

Densitometric analysis of the effects of LMF and PBS on heavy chain myosin content of soleus muscle of NMRI mice



1 2 3 4 5 6 7 8 9 10

Lanes: 1-5 = PBS; 6-10 = LMF

Treatment	Densitometric values (peak intensity)	% control
PBS	121.625 ± 14.6	100 ± 12
LMF	177.094 ± 15.93	146 ± 9 (*)

Figure 3.4.19. Western blot of cytosolic supernatants isolated from LMF/PBS treated NMRI mice. Blots were detected using monoclonal antibody to myosin heavy chain. Results shown are the mean ± SEM where n=5. Experiment was conducted twice. Statistical analysis using Student's t-test where * = $p<0.05$.

3.4.2. The effect of LMF on glucose utilisation

The effect of LMF on glucose utilisation was investigated by observing 2-deoxyglucose uptake in cell lines. LMF caused a concentration dependent increase in 2-deoxyglucose uptake in C_2C_{12} myoblasts (Figure 3.4.20) and MAC16 cells (Figure 3.4.21), after 24hr, paralleling protein synthesis curves. Maximum uptake was at a concentration of 580nM. However, no change was seen in C_2C_{12} myoblasts incubated with LMF for 90min.

To ascertain the type of receptor interaction involved in the stimulation of glucose uptake, the β_3 receptor antagonist SR 59230A was used (Table 3.3). Interestingly, the inhibitor itself increased 2-deoxyglucose uptake. When it was incubated together with LMF, a further increase over that of inhibitor alone was measured.

Cytochalasin B (1μM) was used to determine whether glucose transporters were involved in stimulating glucose utilisation. This had no effect on 2-deoxyglucose uptake in C_2C_{12} cells, indicating that glucose transport does not occur via this pathway.

In an *in vivo* experiment carried out by Steve Russell, LMF was injected i.v. (8μg bd) over 48hr. Serum glucose levels fell to 57% ± 9.52 in comparison to PBS control group (result not shown). This correlated well with the *in vitro* data, which showed that there was an increase in glucose uptake in C_2C_{12} muscle cells in the presence of LMF. This might explain, in part, why there is a drop in glucose levels after treating mice with LMF.

Effect of LMF on [^{3}H] 2-deoxyglucose uptake in C_2C_{12} myoblasts after 24hr and 90min incubation periods

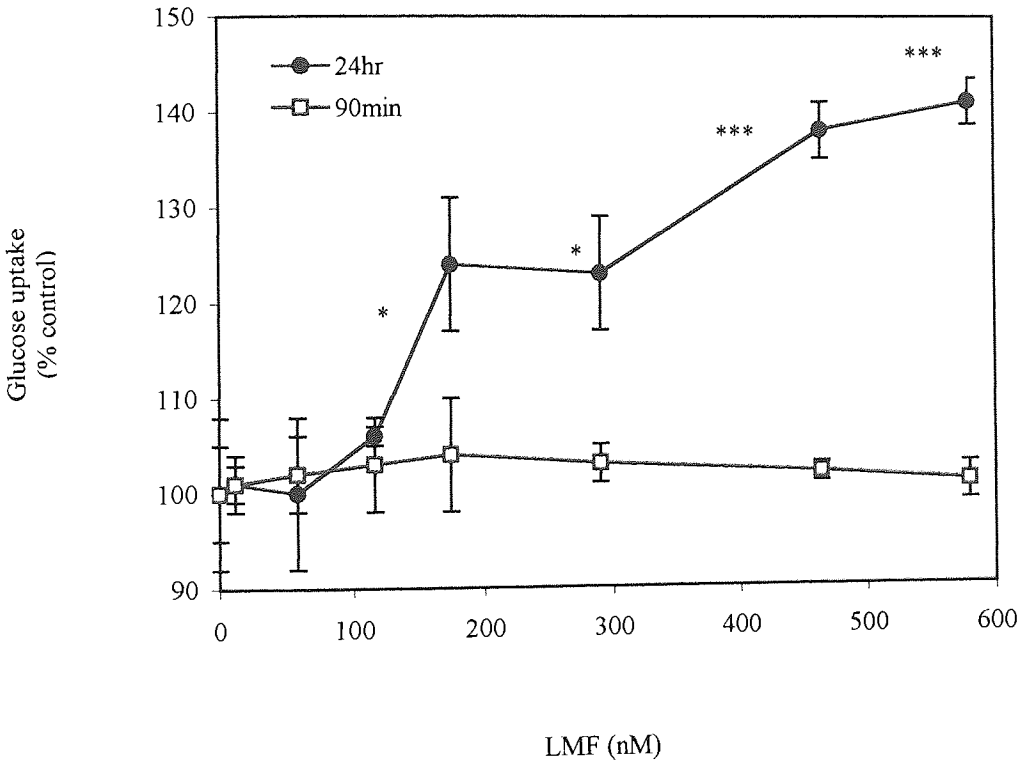


Figure 3.4.20. Results represent the mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test comparing treatment to control whereby * = $p<0.05$, *** = $p<0.001$.

Effect of LMF on [^{3}H] 2-deoxyglucose uptake in MAC16 cells after a 24hr incubation period

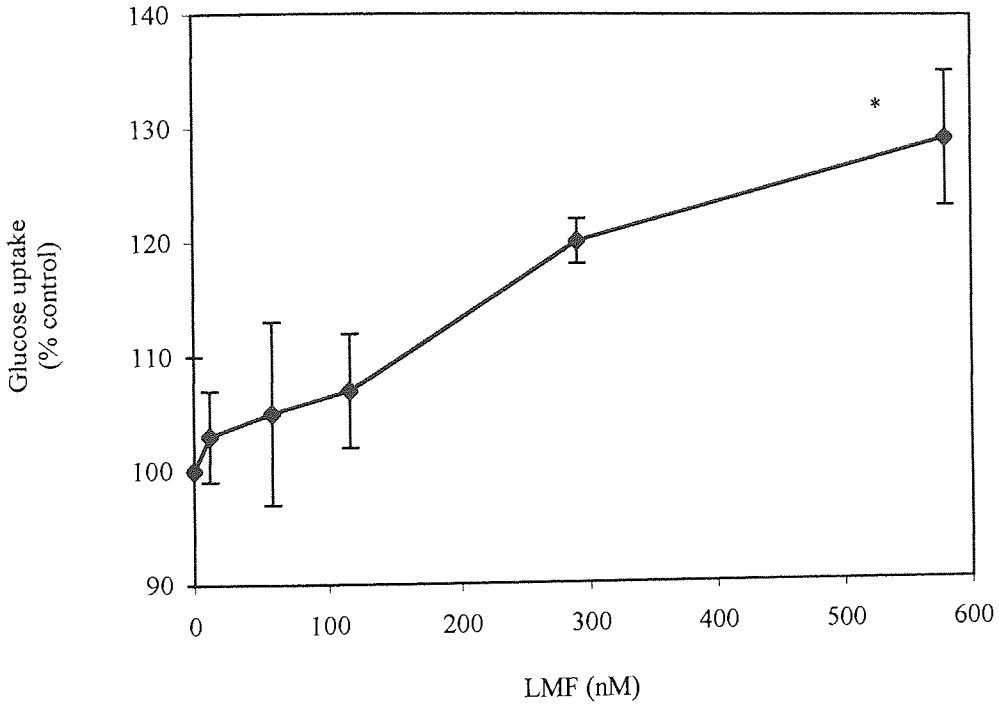


Figure 3.4.21. Results represent the mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis using Student-Newman-Keuls test, comparing treated to control, whereby * =$p<0.05$.

The effects of various treatments on LMF induced [^{3}H] 2-deoxyglucose uptake.

Treatment	% control 2-DG uptake	Difference from control/inhibitor control	P value (<)
LMF 870nM	170 ±3.3	70	0.001
SR 59230A 10^{-5}M	138 ±10.4	-	0.001
SR 59230A 10^{-5}M + LMF	232 ±7.1	94	0.001
SR 59230A 10^{-6}M	120 ±9.3	-	0.05
SR 59230A 10^{-6}M + LMF	191 ±5.6	71	0.01
Cytochalasin B	65 ±5.1	-	0.001
Cytochalasin B + LMF	125± 7.4	60	0.001

Table 3.3. Cells were incubated in the presence of LMF for 24hr. Inhibitors were added 1hr prior to LMF. Protein was precipitated and [^{3}H]-deoxyglucose uptake was detected in a scintillation counter. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test comparing (i) treatment to control (ii) treatment + LMF to LMF alone.

3.4.3. The effect of LMF on protein degradation

In addition to causing an effect on protein synthesis, it appears that LMF has a distinct effect on protein degradation. This was measured by release of radiolabelled [^{3}H] phenylalanine into the surrounding culture medium in the presence of cycloheximide (to block protein synthesis). This value was then expressed as a rate by taking into account protein bound radioactivity. In C_2C_{12} myoblasts incubated with increasing concentrations of LMF for a period of 24hr, a dose-dependent decrease in spontaneous protein degradation was observed (Figure 3.4.22). These experiments were done in the presence of cycloheximide (1μM), a protein synthesis inhibitor, showing that this is a separate effect of LMF.

This result was reflected *in vivo* when NMRI ex-breeeder mice administered LMF (8μg b.d) i.v (Figure 3.4.23). Soleus muscles excised from these animals showed a 26% decrease in protein degradation, exhibited as reduced release of tyrosine, an amino acid used as a marker for total protein breakdown because once it is degraded, it is not re-synthesised in to protein.

Numerous pathways are involved in protein degradation but the most significant involved in skeletal muscle breakdown is the ATP-ubiquitin pathway, the proteasome being an essential component of this (Fagan *et al* 1987). To determine the role of this pathway in LMF induced protein degradation, the proteasomes's chymotrypsin-like activity was measured by following the hydrolysis of the fluorometric substrate succinyl LLVY-MCA. In C_2C_{12} myoblasts and myotubes, a dose-related decrease in enzyme activity was illustrated (Figure 3.4.24 and Figure 3.4.25), maximally reduced at 580nM.

Measurements were made of levels of the subunits which comprise the proteasome unit. This was done by western blotting soluble extracts of LMF treated cells using a murine monoclonal antibody to the 20S proteasome (MCP231) which detects strongly 3 α-subunits. A marked decrease in the expression of these units was observed in both C_2C_{12} myoblasts and myotubes, as LMF concentration increased (Figure 3.4.26 and Figure 3.4.27). This correlated well with the functional decrease in enzyme activity.

It has been reported that β agonists affect the levels of lysosomal cathepsins in muscle (Bechet *et al* 1990). As postulated earlier, LMF may exert its effect by acting through this receptor subtype so cathepsin activity was further investigated. C_2C_{12} myoblasts were incubated for 24hr in the presence of increasing concentrations of LMF and then subjected to fluorometric assays for cathepsins B and L. LMF had no effect on either enzyme activities (Figure 3.4.28).

Effect of LMF on protein degradation in C_2C_{12} myoblasts

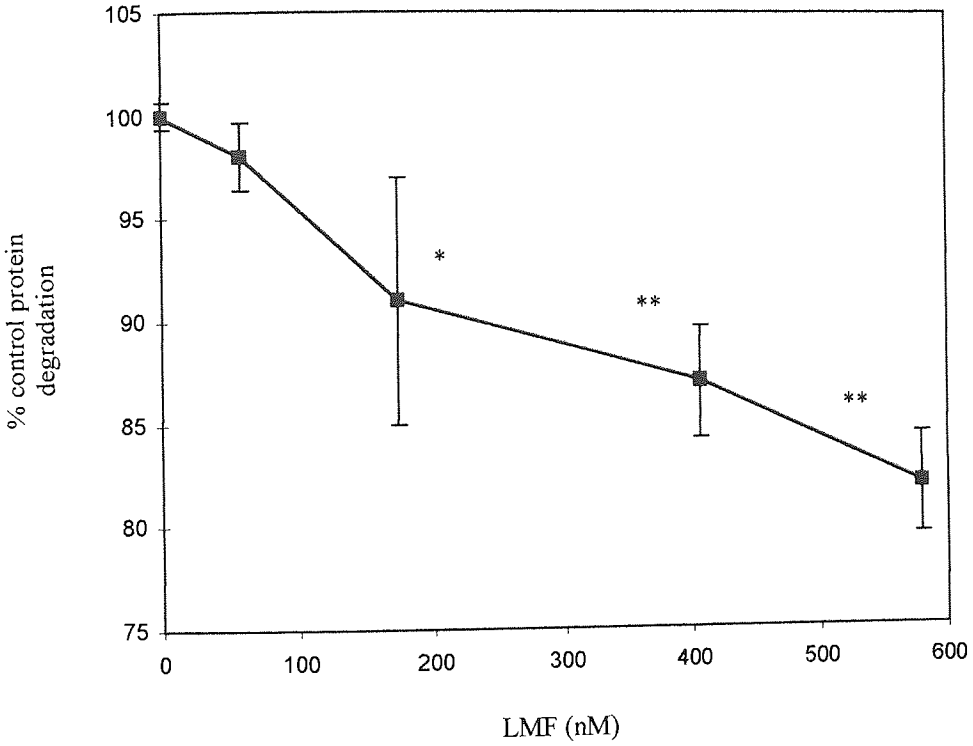


Figure 3.4.22. Protein degradation was measured in the presence of cycloheximide (1μM) and LMF for 24hr. Each experiment was repeated 3 times. Values represent the mean ± SEM where n=3. Statistical analysis using one-way ANOVA with Student-Newman-Keuls test whereby * = $p<0.05$ and ** = $p<0.01$ comparing treatment to control.

The effect of LMF on protein degradation in soleus muscle of exbreeder male NMRI mice

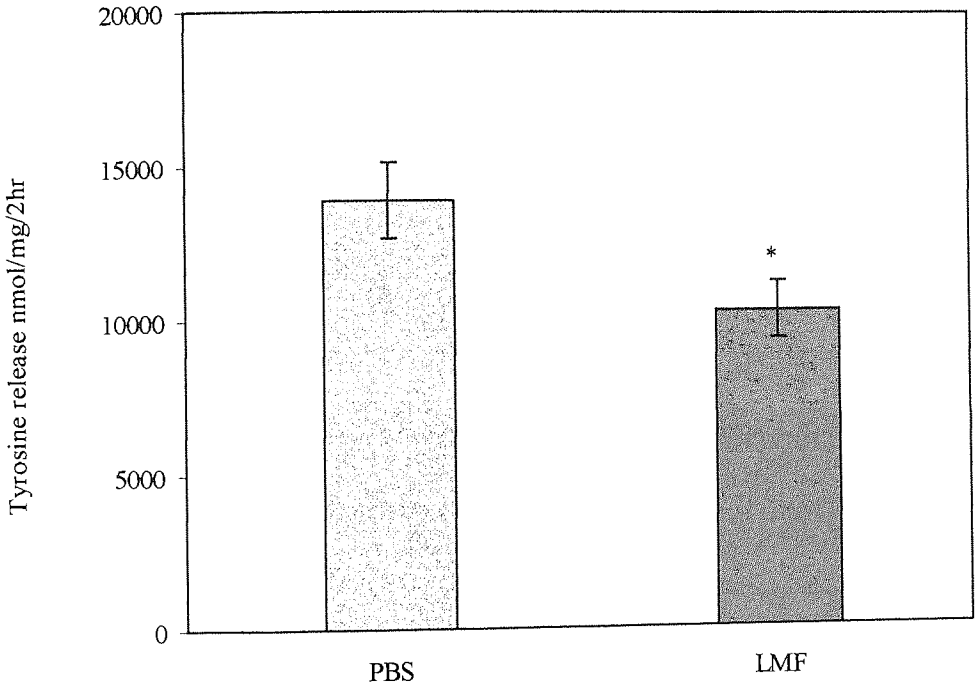


Figure 3.4.23. Control animals received 100µl PBS. Results shown are the mean ± SEM where n=6. Experiment was repeated twice. Difference from control value was determined by unpaired Student's t-test where * = $p<0.05$.

Effect of LMF on chymotryptic activity of soluble extracts of C_2C_{12} myoblasts after a 24hr incubation period

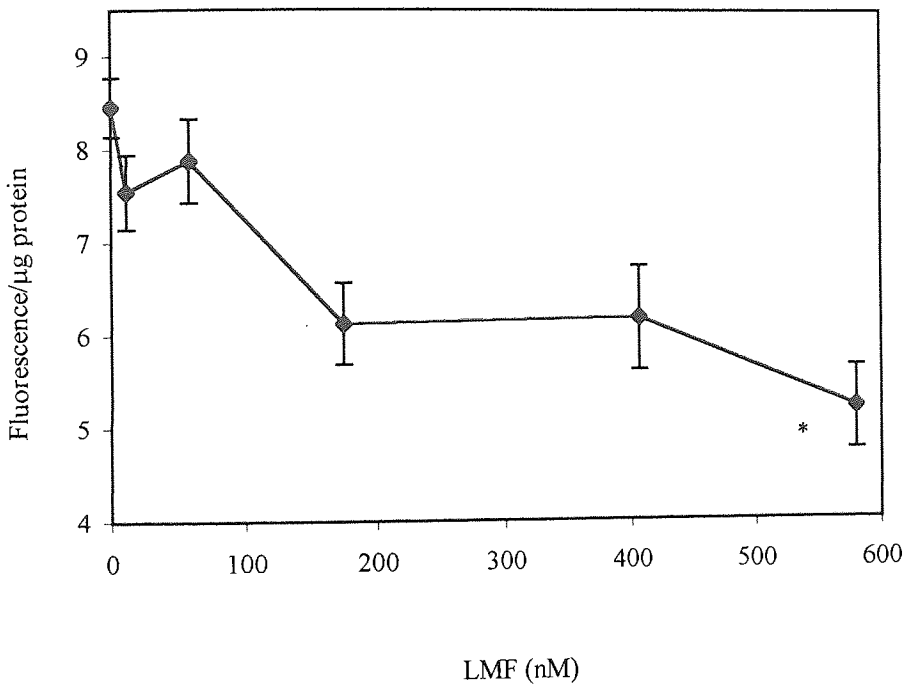


Figure 3.4.24. Fluorogenic substrate used was suc LLVYMCA. Fluorescence was measured at 360nm excitation and 460nm emission in a Perkin Luminiscence Spectrophotometer LS50. Results shown as mean ± SEM where n=2 and the experiment repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test whereby *=$p<0.05$, in comparison to control.

Effect of LMF on chymotrypsin activity of soluble extracts of C_2C_{12} myotubes after a 24hr incubation period.

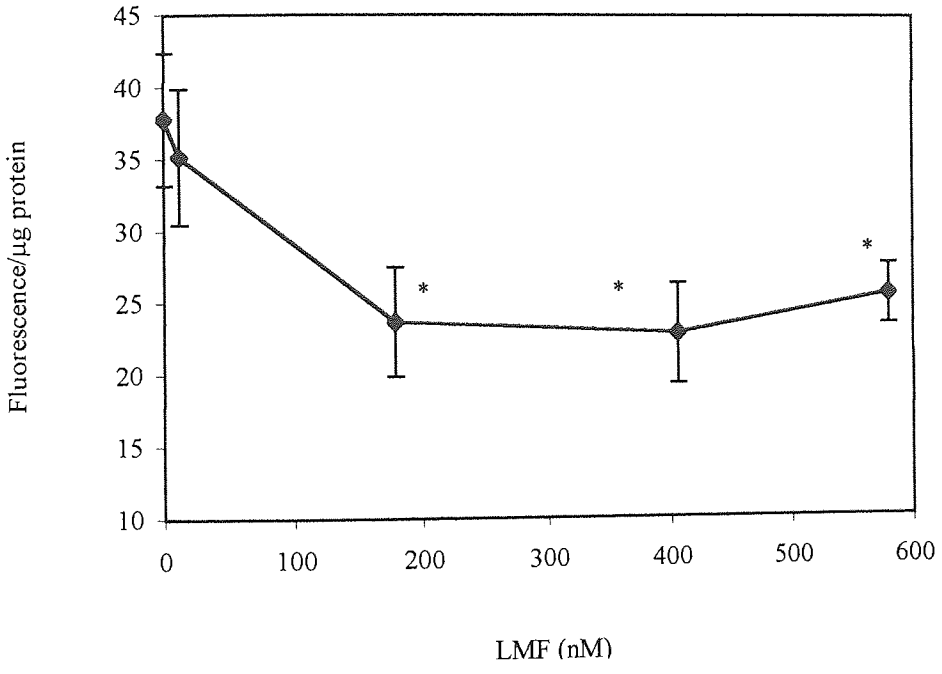
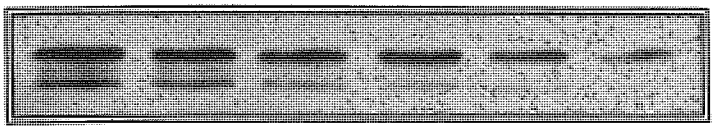


Figure 3.4.25. Fluorescence was measured at 360nm excitation and 460nm emission in a Perkin Elmer Luminiscence Spectrometer LS50. Results shown represent mean ± SEM where n=2. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test. Differences from control values as indicated by * = $p<0.05$.

Densitometric analysis of 20S subunit expression in C_2C_{12} myoblasts treated with increasing concentrations of LMF



1 2 3 4 5 6

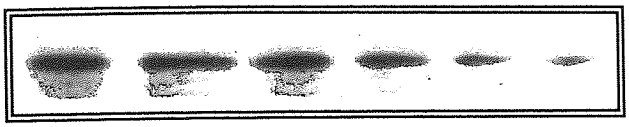
Lanes

1= control 2= 11.6nM LMF 3= 58nM 4= 174nM 5= 406nM 6= 580nM

LMF (nM)	Densitometric (peak intensity)	% control
1	449.34	100
2	399.68	89
3	339.29	76
4	231.6	52
5	210.48	47
6	157.56	35

Figure 3.4.26. Western blot of cytosolic extracts isolated from myoblasts treated with increasing concentrations of LMF for 24hr. Blots were detected using monoclonal antibody to 20S α-subunits (MCP231).

Immunoblot of 20S isolated from C_2C_{12} myotubes after treatment with increasing concentrations of LMF



1 2 3 4 5 6

Lanes

1 = control, 2 = 11.6nM LMF, 3 = 58nM, 4 = 174nM, 5 = 406nM, 6 = 580nM.

LMF (nM)	Densitometric values (peak intensity)	% control
1	277.02	100
2	293.31	105
3	292.86	106
4	222.52	80
5	123.9	45
6	57.21	21

Figure 3.4.27. Western blotting of cytosolic extracts of myotubes treated with LMF for 24hr, immunodetected with monoclonal antibody to 20S α-subunits (MCP231).

Effect of LMF on Cathepsin B an L activity in C_2C_{12} myoblasts after a 24hr incubation period

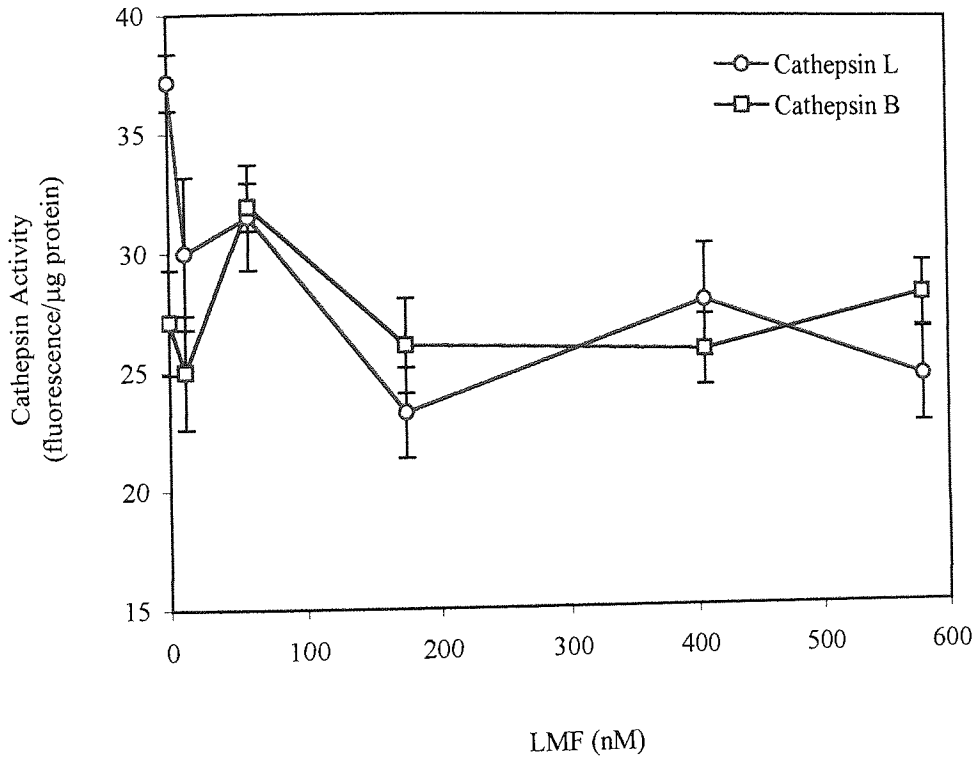


Figure 3.4.28. Fluorescence was measured at 360nm excitation and 460nm detection in a Perkin Elmer Luminiscence Spectrometer LS50. Results shown are the mean ± SEM where n = 3. Experiment was repeated 3 times.

3.4.4. The effect of proteolysis-inducing factor (PIF) on protein turnover

An immunoreactive material was isolated from the MAC16 adenocarcinoma by means of affinity chromatography using a monoclonal antibody. Western blotting analysis yielded a band posessing a molecular weight of 24kDa, corresponding to the proteolytic factor, PIF (McDevitt *et al* 1995) (Figure 3.4.29).

PIF exerted a significant alteration in the rate of protein synthesis in C_2C_{12} myoblasts opposite to that of LMF. After a 90-min incubation in the presence of the proteolysis-inducing factor (PIF), inhibition of protein synthesis was detected with concentrations ranging from 2.1nM up to 12.6nM (Figure 3.4.30) A maximal decrease in protein synthesis of 17% was achieved at 6.3nM PIF. Incubation of C_2C_{12} myoblasts with PIF for longer periods of time also induced a dose-related decrease in protein synthesis. When C_2C_{12} myoblasts were incubated with PIF for 6hr, a decrease in protein synthesis was observed which was maximal at 8.4nM (Figure 3.4.31). Note that these effects are opposite to that obtained for the lipid-mobilising factor (LMF), which increased the rate of protein synthesis at a longer incubation period (24hr).

The depression in protein synthesis rate induced by PIF was shown to be specific for the proteolytic factor as the inhibition was attenuated by pre-treating the cells with the PIF monoclonal antibody (10μg/ml) (Figure 3.4.32). The inhibition was reversed back to control level.

The effect of PIF on RNA accretion was also investigated. After 90min, PIF elicit no detectable change in levels of total RNA (Figure 3.4.33). After a 6hr incubation, there was a significant decrease in the amount of RNA, the maximal decrease achieved at

8.4nM PIF (Figure 3.4.34).

In addition to decreasing protein synthesis, PIF also caused an increase in protein degradation, 24hr after its addition, once again, an effect opposite to that of LMF (Figure 3.4.35). This was maximal at 8.4nM, the same concentration at which the greatest inhibition of protein synthesis was observed after a 6hr incubation.

To determine whether the increase in protein degradation was due to the activation of the ATP-ubiquitin pathway, cytosolic extracts of C_2C_{12} myoblasts and myotubes previously exposed to PIF, were subject to a fluorometric assay to assess the chymotrypsin like enzyme activity of the 20S proteasome. In myoblasts, there was a steady increase in enzyme activity as PIF concentration increased (Figure 3.4.36). This was reflected in western blots performed on soluble extracts taken from C_2C_{12} cells probed with 20S antibody (Figure 3.4.37). This showed a marked increase in the expression of proteasome α-subunits, with increasing concentrations of PIF up to 10.5nM. It was noticed, however, that data from the *in vitro* protein degradation assay did not completely correspond with the enzyme and western blotting results; in the degradation assay, protein breakdown tapered off after 8.3nM whilst enzyme activity and α subunit expression increased up to 10.5nM. In C_2C_{12} myotubes, there was a biphasic increase in chymotrypsin activity with peaks occurring at 1.05 and 8.3nM (Figure 3.4.36). Again, this was mirrored by western blot analysis of α-subunits, which mimicked a similar profile of expression to that of enzyme activity (Figure 3.4.38).

Immunoblot of PIF isolated from murine MAC16 adenocarcinoma

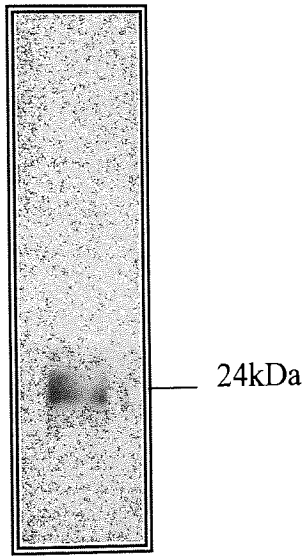


Figure 3.4.29. Western blot of PIF isolated from homogenised tumours and subject to affinity purification detected using PIF monoclonal antibody. 20μg of protein was loaded.

Effect of PIF on protein synthesis in C_2C_{12} myoblasts after a 90min incubation

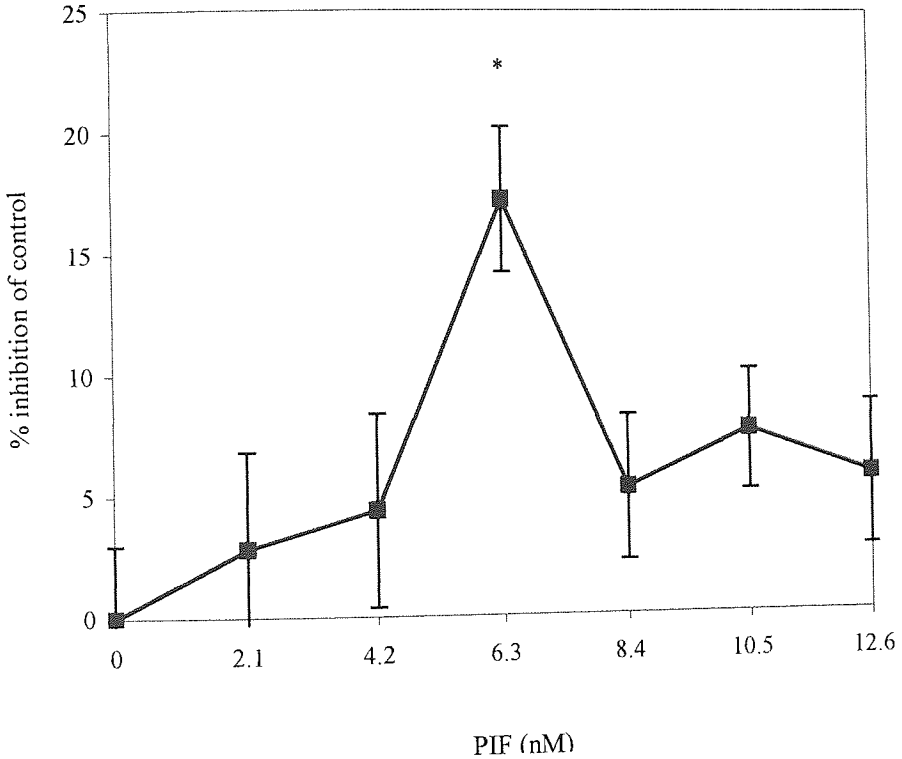


Figure 3.4.30. Dose response curve showing the effect of PIF on protein synthesis in C_2C_{12} myoblasts over a 90min incubation period. Values are represented as a mean ± SEM where n=3 and the experiment was repeated 3 times. Statistical analysis was performed using one-way ANOVA with Student-Newman-Keuls test whereby * = $p<0.05$.

Effect of PIF on protein synthesis in C_2C_{12} myoblasts after a 6hr incubation period

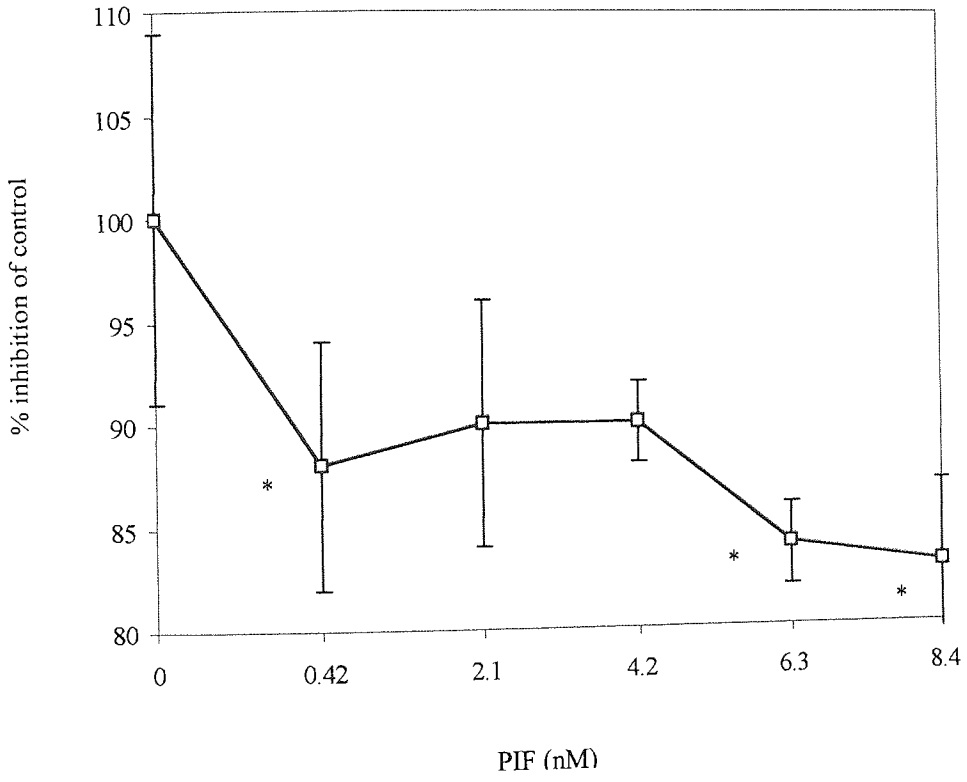


Figure 3.4.31. Effect of PIF on protein synthesis in C_2C_{12} ,myoblasts over a 6hr incubation period. Values represent the mean ±SEM where n=3. Each experiment was repeated 3 times. Statistical analysis using one-way ANOVA with Student-Newman-Keuls test as a post test whereby * = $p<0.05$

Effect of monoclonal antibody on PIF-induced inhibition of protein synthesis in C_2C_{12} myoblasts

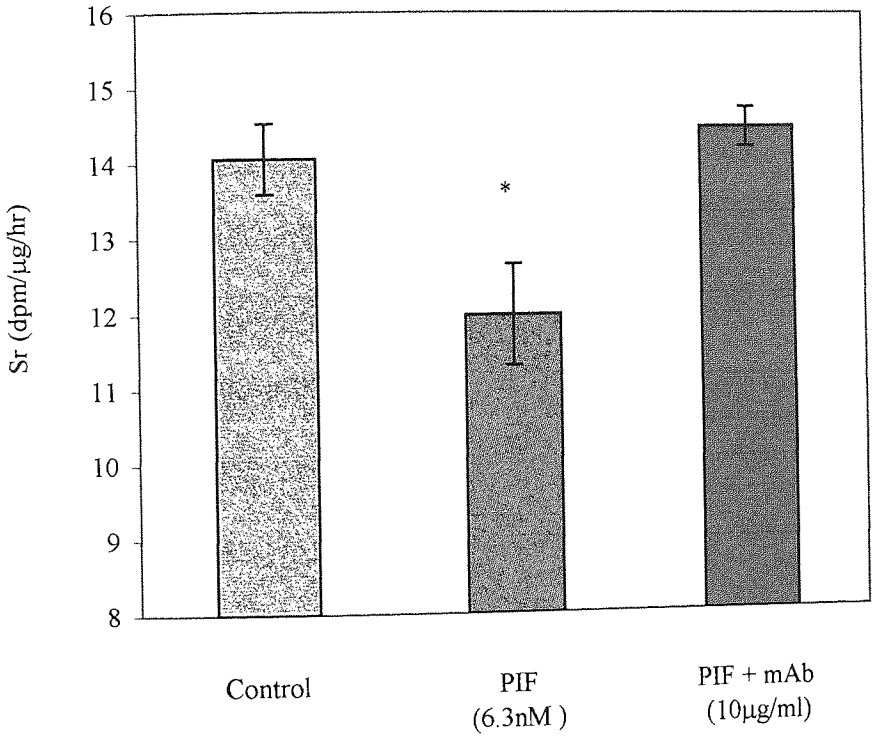


Figure 3.4.32. Effect of monoclonal antibody to PIF induced inhibition of protein synthesis in C_2C_{12} myoblasts. Cells were pre-treated with antibody 1hr beforehand prior to adding the PIF for a further 90min. Samples represent mean ± SEM where n = 4. Experiment was repeated 2 times. Statistical analysis performed using unpaired t test, whereby * = $p<0.05$.

Effect of PIF on RNA accretion in C_2C_{12} myoblasts after a 90min incubation period

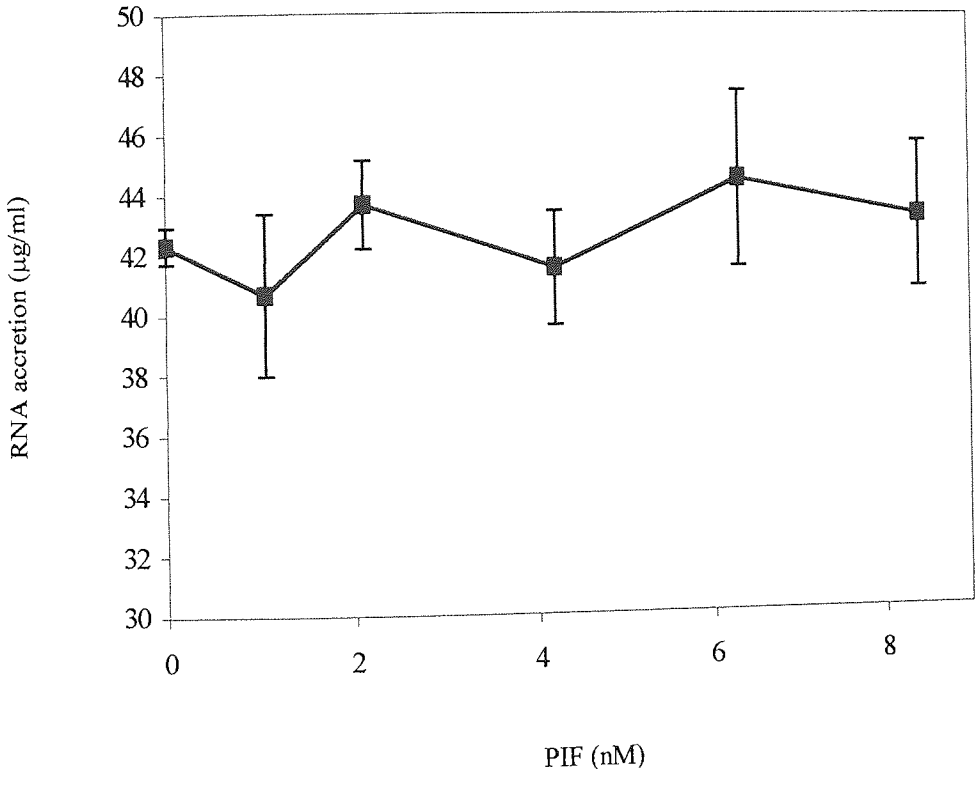


Figure 3.4.33. Effect of PIF on RNA accretion in C_2C_{12} myoblasts after 90min incubation. Each point represents the mean ± SEM of n=3 and each experiment was repeated least 2 times.

Effect of PIF on RNA accretion during after a 6hr incubation period

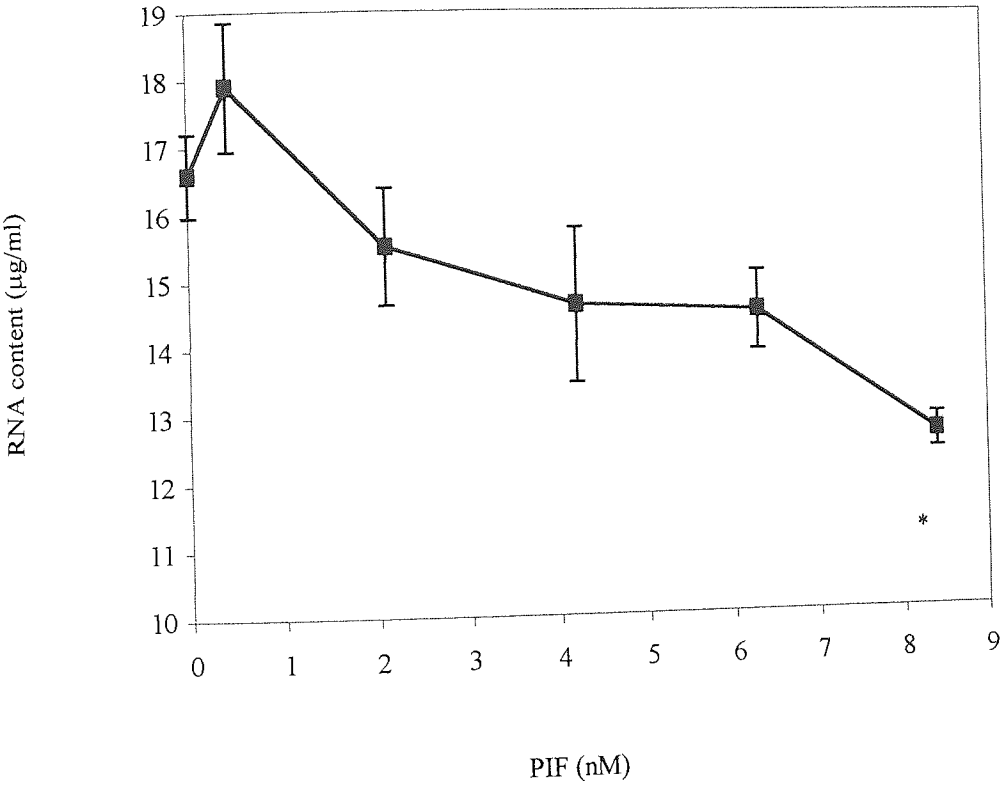


Figure 3.4.34. Effect of PIF on RNA accretion in C_2C_{12} myoblasts over a 6hr period. Values represent mean ± SEM where n=3. Each experiment was carried out 3 times. Statistical analysis was performed using one-way ANOVA with Student-Newman-Keuls test as a post test whereby * = $p < 0.05$

Effect of PIF on protein degradation in C_2C_{12} myoblasts

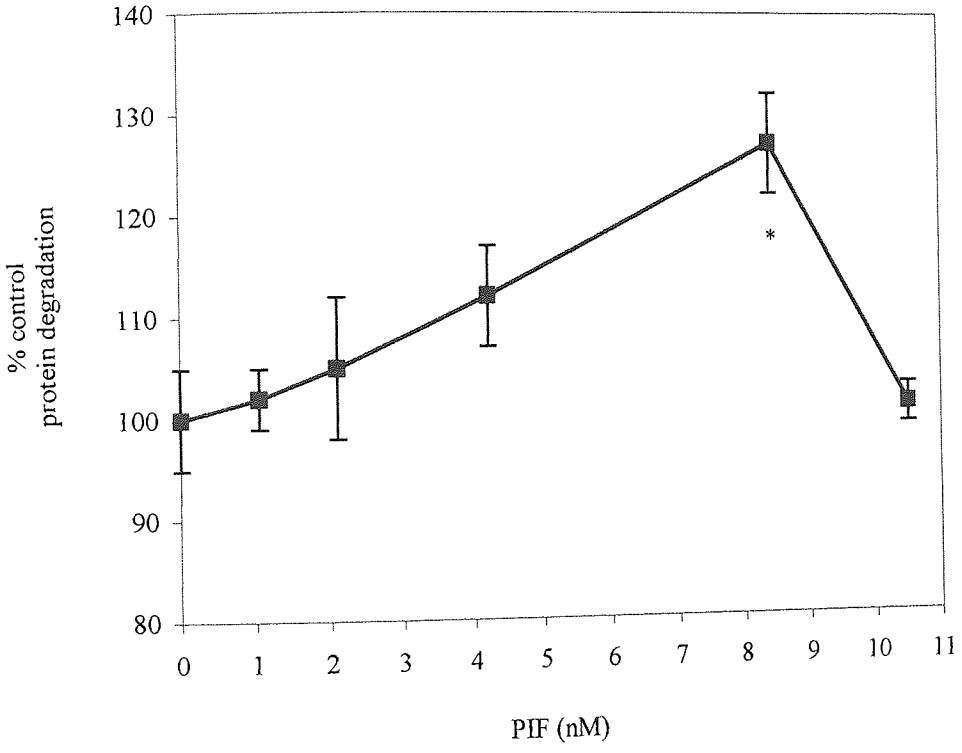


Figure 3.4.35. Effect of PIF on protein degradation *in vitro.* Values shown represent the mean ± SEM where n = 3. Experiment was repeated 3 times. Statistical analysis performed using one-way ANOVA with Student-Newman-Keuls test whereby * = $p < 0.05$

Effect of PIF on chymotryptic activity in C_2C_{12} myoblasts and myotubes

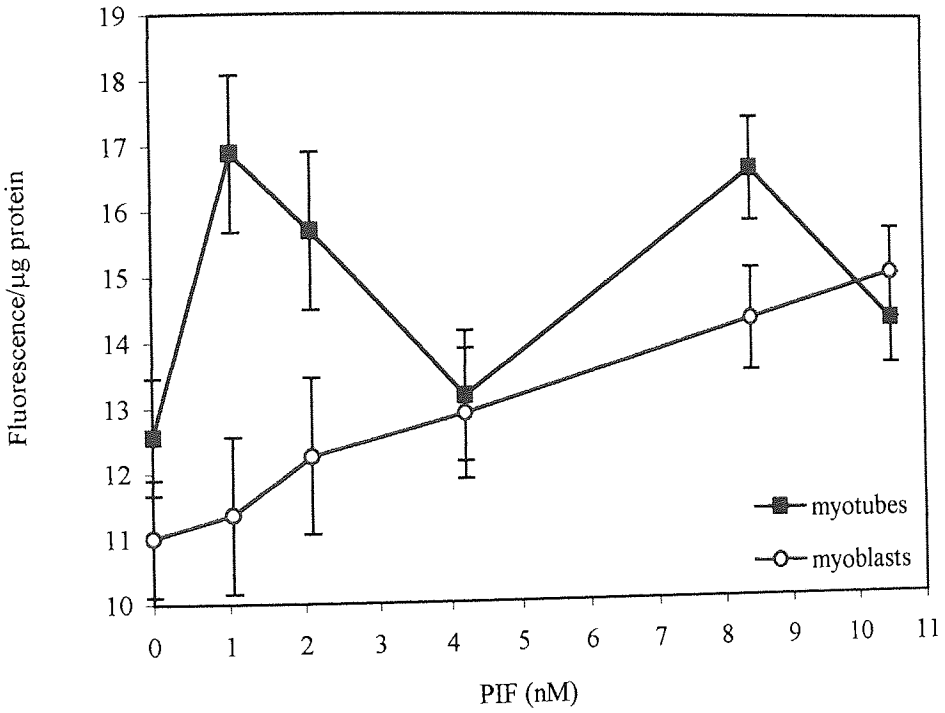
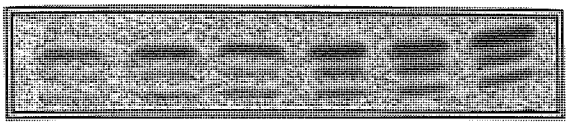


Figure 3.4.36. Fluorescence was measured in at 360nm excitation and 460nm emission in Perkin Elmer Luminiscence Spectrometer LS50. Results represent the mean ± SEM where n=2. Experiment was repeated 2 times.

Densitometric analysis of 20S isolated from C_2C_{12} myoblasts after treatment with increasing concentrations of PIF



1 2 3 4 5 6

Lane No.	Densitometric values (peak intensity)	% control
1	355.25	100
2	376.57	107
3	362.36	102
4	408.54	115
5	476.04	134
6	618.14	174

Figure 3.4.37. Western blot of cytosolic extracts treated with PIF. Lane 1 = control, 2 = 1.05nM PIF, 3 = 2.10nM, 4 = 4.2nM, 5 = 8.3nM, 6 = 10.5nM. Blots were detected using monoclonal antibody to 20S α-subunits (MCP231).

Densitometric analysis of 20S isolated from C_2C_{12} myotubes treated with increasing concentrations of PIF

1 2 3 4 5 6

Lane No.	Densitometric values (peak intensity)	% control
1	192.3	100
2	218.69	114
3	343.09	178
4	159.19	83
5	299.18	156
6	329.44	171

Figure 3.4.38. Western blots of cytosolic extracts of cells treated with increasing concentrations of PIF. Blots detetected using MCP231 α-subunit monoclonal antibody. Lanes 1= control, 2 = 1.05 nM PIF, 3 = 2.10nM, 4 = 4.2nM, 5 = 8.4nM, 6 = 10.5nM.

3.4.5. Experiments investigating the combined effects of PIF and LMF.

Experiments were conducted to investigate what would happen if both catabolic factors, PIF and LMF were incubated together.

When 580nM LMF was added to an increasing amount of PIF in C_2C_{12} myoblasts, overall protein degradation was attenuated, which was statistically significant at 2, 5 and 8.3nM PIF (Figure 3.4.39). Parallel effects were observed on chymotrypsin activity in myoblasts and myotubes whereby LMF reduced the increase in enzyme activity caused by PIF (Figure 3.4.40 and Figure 3.4.41). On western blotting of α subunits of the proteasome, addition of LMF (580nM) to varied concentrations of PIF, completely attenuated the increased expression of the 20S α-subunits, caused by PIF alone (Figure 3.4.42).

Effect of LMF on PIF induced protein degradation in C_2C_{12} myoblasts

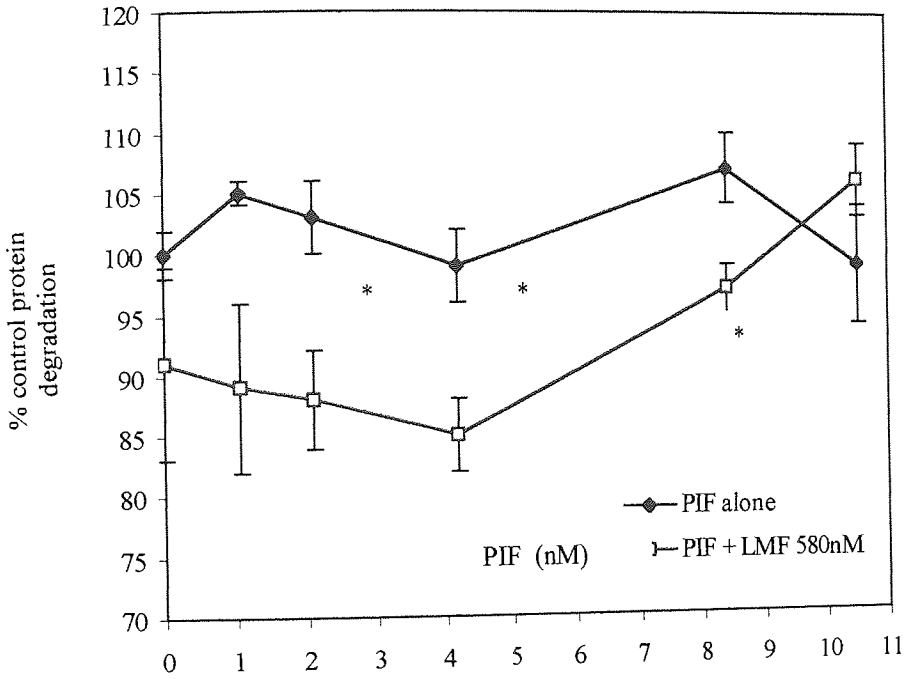


Figure 3.4.39. Results represent the mean ± SEM where n=3. Experiment was repeated 3 times. Statistical analysis performed using Student-Newman-Keuls test comparing values to control. *=$p<0.05$.

Effect of LMF and PIF on chymotryptic activity in C_2C_{12} myoblasts

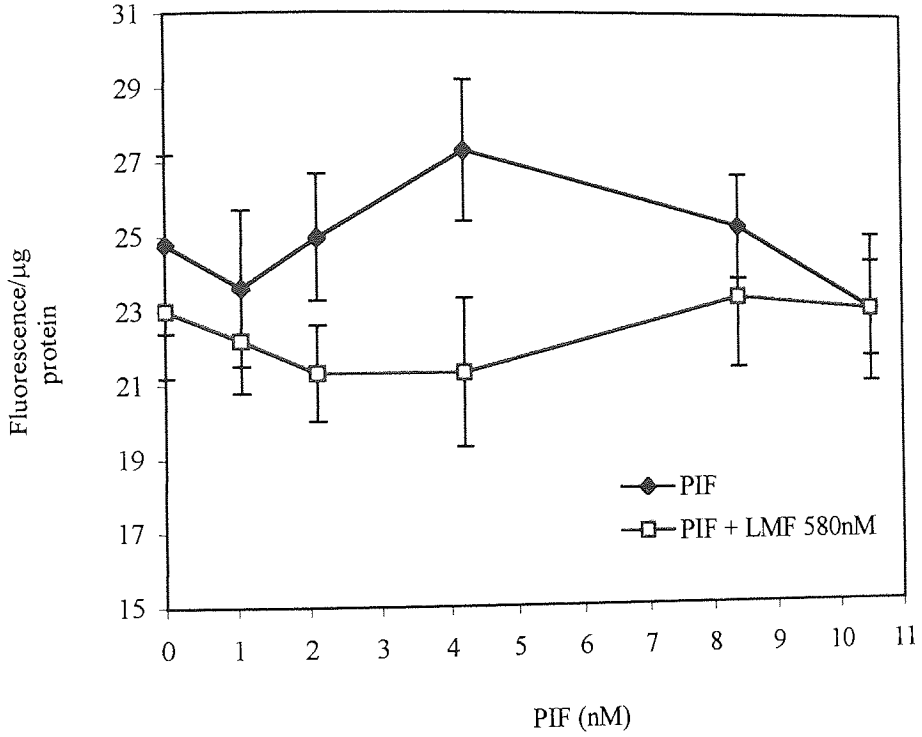


Figure 3.4.40. Fluorescence was detected at 360nm excitation and 460nm emission in a Perkin Luminiscence Spectrometer LS50. LMF was added 1hr prior to PIF. Results shown represent then mean ± SEM where n=2.

Effect of LMF and PIF on chymotryptic activity in C_2C_{12} myotubes

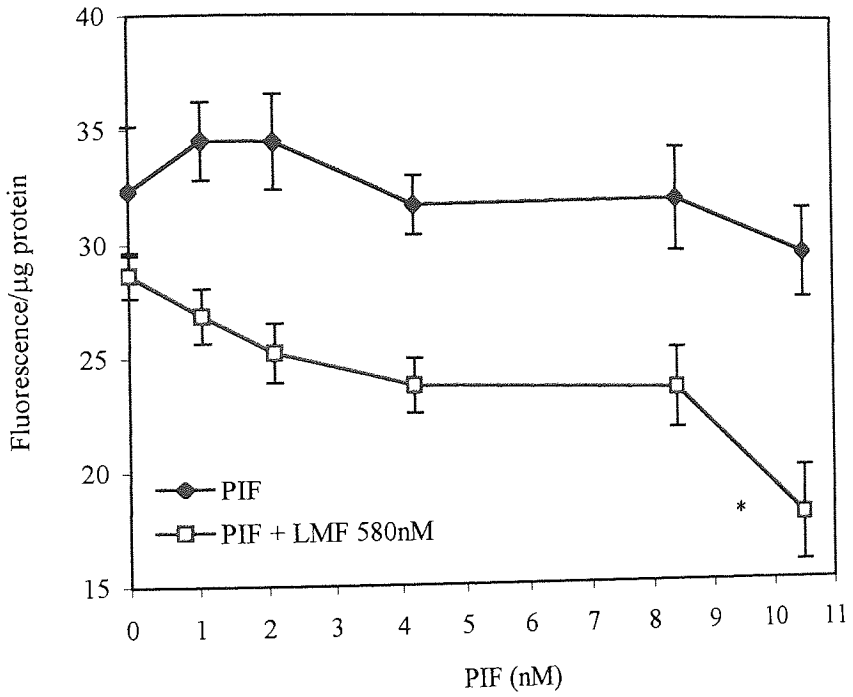
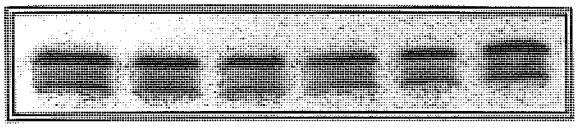


Figure 3.4.41. Fluorescence detected at 360nm excitation and 460nm emission in a Perkin Elmer Luminiscence Spectrometer LS50. LMF was added 1hr prior to PIF and left to incubate for 24hr. Results shown are mean ± SEM where n=2. Experiment was repeated 2 times. Statistical analysis performed using Student's t-test comparing PIF to PIF + LMF whereby * = $p<0.05$.

Immunoblot of 20S isolated from C_2C_{12} myoblasts treated with increasing concentrations of PIF and LMF (580nM)



1 2 3 4 5 6

Lane No.	Densitometric values (peak intensity)	% control
1	363.35	100
2	406.05	112
3	362.83	100
4	357.40	98
5	365.33	101
6	380.38	105

Figure 3.4.42. Western blot of cytosolic extracts of cells after treatment with PIF and LMF. Blots were detected using monoclonal antibody to 20 S α-subunits. Lanes 1 = control, 2 = 1.05nM PIF, 3 = 2.1nM 4 = 4.2nM, 5 = 8.4nM, 6=10.5nM (lanes 2-6 were in the presence of 580nM LMF).

3.5. DISCUSSION

3.5.1. Effect of LMF on protein synthesis

In this study, cancer cachexia has been attributed to the production of tumour-derived protein factors, PIF (proteolysis-inducing factor) and LMF (lipid-mobilising factor). PIF has been shown to increase protein catabolism in skeletal muscle and also inhibition of protein synthesis (Lorite *et al* 1997). LMF induces lipolysis in isolated murine white adipocytes associated with stimulation of adenylate cyclase (Khan and Tisdale 1999), and has a direct lipid–mobilising effect on lipid stores when injected in to mice (Hirai *et al* 1998). However, in this present study, a paradoxical "anti-cachectic" effect of LMF on skeletal muscle is described.

Administration of LMF to ex-breeder NMRI mice over an 89hr period produced a decrease in weight without a change in food and water intake (Hirai *et al* 1998). Body composition analysis showed a 42% reduction in carcass lipid when compared with controls. Interestingly however, there was nearly a 25% increase in non-fat mass, although this did not reach statistical significance. To investigate this further, an *in vitro* assay system was employed whereby a skeletal muscle cell line,C_2C_{12}, was selected as a model to study this effect.

This cell line was developed by Yaffe and Saxel (1977), and is derived from the satellite cell population of the thigh muscle of a C3H two month old mouse. It is an immortalised cell line, which is not only responsive to the effects of various hormones and growth factors, but also cells undergo fusion to form myotubes, which reflects similar biochemical and histological characteristics *in vivo*. This cell line was also used

to elucidate the signal transduction mechanisms involved in eliciting the response to LMF in muscle, which will be discussed later.

When LMF was added to C_2C_{12} myoblasts for a 90min and 6hr period, no change in the rate of protein synthesis was noted. However, when myotubes and myoblasts were incubated for a 24hr period, a marked increase in protein synthesis was observed. This implies that LMF was effecting transcription as opposed to translation, where an effect at shorter time spans would have been evident. This effect was a result of a direct action on the protein synthetic machinery as no change in cell proliferation or [^{3}H] methyl-thymidine uptake was noted, suggesting there was a hypertrophic response to the tumour-derived product. It would appear therefore that LMF increases the width and size of skeletal muscle fibres. Indeed, *in vivo* data show similar results. Rehfeldt *et al* (1994) showed that clenbuterol induced hypertrophy occured with no replication of DNA in rat muscle fibres. The muscle growth in response to β agonist treatment appears to be true muscle hypertrophy, in contrast with other types of muscle growth, i.e. compensatory hypertrophy, in which satellite cell division precedes protein accumulation (Salleo *et al* 1980). The muscle DNA concentration decreased in cimaterol-treated lambs, but the total DNA was not altered (Kim *et al* 1987).

To demonstrate the same effect *in vivo*, ex-breeder male mice were injected with LMF for 48hr. Soleus muscle was selected as a tissue to study muscle protein synthesis because in contrast with gastrocnemius, the muscle could be dissected out and ligated with ease, and was also thin enough to permit oxygenation and uptake of substances by diffusion, in a surrounding medium, with has ready access to muscle cells. Protein synthesis was determined by measuring uptake of radiolabelled phenylalanine

introduced into the media surrounding the ligated muscles. A statistically significant increase in protein synthesis was observed in LMF treated mice. This result was further corroborated by blotting cytosolic extracts of muscle from LMF treated animal, which showed an increase in myosin heavy chain expression, compared with control animals. This corresponds with the experiment conducted by Hirai and colleagues (1998) who showed an increase in overall non-fat mass after LMF treatment in mice.

In light of the above results, it is proposed that LMF acts by an unknown mechanism to increase gene expression/transcription of muscle proteins present in skeletal muscle. The enhancement of myofibrillar protein synthesis by the BAA ractopamine, may have arisen from an effect on the transcription of genes encoding myofibrillar proteins (Adeola *et al* 1992). In a different study (Helferich *et al* 1990), using the same agent, it was observed that feeding of pigs with this stimulated a 55% increase in the rate of semimembranosus muscle alpha-actin, a protein that makes up 23% of myofibrillar proteins, which was thought to be a result of enhanced expression of genes coded for myofibrillar proteins. Other workers have reported an increase in protein synthesis allied with an abundance of mRNA for muscle specific proteins (Grant *et al* 1993, Smith *et al* 1989).

In order to investigate the nature of the receptor interaction involved in this response, selected receptor antagonists were utilised. Khan (1996) purported a role for the β_3-adrenoceptor in the mobilisation of host lipid stores associated with cachexia. The lack of receptor desensitisation, or down-regulation, in adipocytes isolated from mice which had undergone long term exposure to LMF allied with the knowledge that unlike β_1 and β_2, β_3 adrenoceptors may lack the structural determinants involved in agonist-induced

receptor desensitisation (Giacobino, 1995), lead to this assumption. Furthermore, in another study, the lipolytic response of LMF was reversed by the non-specific β-adrenergic antagonist propranolol and was postulated to act via the β_3 receptor on adipocytes (Khan and Tisdale 1999, Price 1997). It was possible therefore that LMF may exert its protein synthetic effect via this receptor subtype present on muscle.

The β-adrenergic antagonists, propranolol and SR59230A were chosen. Propranolol is a non-specific β-receptor antagonist effecting both β_1 and β_2 equally *in vitro* and blocks the recently discovered β_3 subtype (Murphy *et al* 1993). SR59230A is a specific β_3 receptor antagonist which has been reported by Nisoli *et al* (1996) to have a ten-fold selectivity for β_3 than β_1. As postulated, the enhanced effect on protein synthesis produced by LMF was attenuated by both these agents, suggesting that the action of LMF may be mediated by this receptor sub-type.

β adrenoceptors have been identified and shown to be predominantly of the β_2 subtype (Kim and Sainz 1992). However, in an autoradiographic study (Kim *et al* 1991) has suggested that skeletal muscles have the β_3 adrenoceptor subtype in addition to β_2 receptors; the potential β_3 adrenoceptor subtype was observed in rat soleus muscles which are composed mostly of slow-twitch oxidative muscle subtype. In plantaris and gastrocnemius muscles, the potential β_3 receptors were found in areas where oxidative muscle fibres were distributed. In support of this finding, β_3 adrenoceptor mRNA has been found in rat soleus muscles (Emorine *et al* 1989). In a more recent study, the presence of the β_3 receptor was recently identified in skeletal muscle, by using a monoclonal antibody Mab72c that recognised an epitope of the native β_3-adrenoceptor

(Chamberlain *et al* 1999). They also suggested the possibility of a further atypical β receptor, perhaps a β_4 receptor present on skeletal muscle.

Skeletal muscle hypertrophy has also been linked to fibre-type muscle composition, oxidative capacity and density of β-adrenoceptors (Maltin *et al* 1989). Larger increases in muscle mass have been described in those muscles that include larger number of fast-twitch (FT) fibres (either oxidative or oxidative-glycolytic). The density of β_3 subtype adrenoceptors is more abundant in ST (slow-twitch) muscles than in FT. Therefore soleus muscles contain predominantly β_3 subtype while gastrocnemius and plantaris have a higher density of β_2, although β_3 receptors are also found in areas where oxidative muscle fibres are involved. Muscle hypertrophy does not seem to be related to β adrenoceptor density. In fact, a larger amount of receptors has been described in muscles where hypertrophy is less relevant (Mersmann, 1998). In this study, soleus muscle was used as a model to examine protein synthesis. It would be interesting therefore to see the effect on iv administered LMF on other muscle subtypes such as gastrocnemius.

There are numerous reports describing the anabolic effects produced by β-adrenergic agonists on muscle tissue in various species. A 10-20% increase in muscle weight was observed after treating rats with the β agonist clenbuterol for only 1-2 weeks (Emery *et al* 1984). Lambs fed cimaterol for approximately two months showed a 25-30% increase in the weights of several muscles compared to lambs fed a control diet (Beermann *et al* 1986). The gastrocnemius muscle was reported to increase as much as 40% in weight (Kim *et al* 1987). A study examining fractional synthesis rates of various muscle fractions ractopamine-fed pigs showed a stimulation of myofibrillar

protein synthesis. In a more recent study, cimaterol was infused into the hindlimbs of six young steers, allowing for arteriovenous measurements to be undertaken to monitor uptake of amino acids (Byrem *et al* 1998). The workers concluded that increases in skeletal muscle protein accretion was due to a direct action of cimaterol on muscle BAA and not from indirect effects of BAA for e.g. on endocrine status. Cepero (2000) and co-workers reported a significant increase in muscle hypertrophy, defined as an increase in muscle to body mass ratio, in skeletal muscles taken from rats after treatment with salbutamol, i.e. soleus, gastrocnemius and plantaris in a dose dependent manner.

Other β adrenergic agonists have been shown to lead to increased muscle protein synthesis, accompanied or followed by decreased protein degradation (Bell *et al* 1998), through a cyclic AMP-dependent pathway. The increase in protein synthesis produced by LMF in C_2C_{12} myoblasts was attenuated by the adenylate cyclase inhibitor, MDL_{12330A} and the cyclic AMP dependent protein kinase (PKA) inhibitor H8, although the latter did not reach significance. Although H8, did not reverse the LMF induced protein synthesis, PKA enzyme activity was shown to be increased in the presence of LMF. The reason for this discrepancy cannot be explained. Perhaps the concentration of inhibitor used was not sufficient to inhibit total enzyme activity; a positive control was not included to fully confirm that the H8 inhibited the enzyme *in vitro*. The increase in protein synthesis was linearly related to increases in cyclic AMP as determined by competitive binding assay. Furthermore, the potent activators of cAMP, forskolin and dibutyryl cAMP produced effects additive to that produced by LMF alone; These results suggest the role played by cyclic AMP and protein kinase A as intracellular mediators of LMF action. This correlates well with a study conducted in

female Wistar rates whereby clenbuterol was administered s/c giving rise to increased cyclic AMP levels. The effects of the agent on both muscle growth and protein synthesis were abolished by giving simulataneous intraperitineal injections of propranolol (Maclennan and Edwards 1989).

It seems probable that LMF exerts its effect on skeletal muscle protein synthesis by a direct interaction of the factor on muscle. However, the mechanisms leading from an increase in cyclic AMP to increased protein synthesis have not been fully elucidated. The pathways leading from cAMP and PKA to changes in protein synthesis are incompletely understood.

Protein S6 of the small (40S) ribosomal subunit is the major phosphoprotein of mammalian ribosomes. S6 can be phosphorylated by cyclic AMP-dependent protein kinase (Wettenhall and Cohen 1982). Although PKA can directly phosphorylate S6, there are two distict S6 kinase families; the 70kDa ($p70^{s6k}$) and 90kDa ($p90^{rsk}$) (Sturgill and Wu 1991). The signalling cascade regulating p70s6k is poorly understood, but it can be stimulated by cAMP (Kahan *et al* 1992). In contrast, $p90^{rsk}$ is phosphorylated and activated by mitogen-activated protein (MAP) kinase (Davis 1993). Furthermore, MAP kinase has been shown to translocate from the cytoplasm to the nucleus and influence transcriptional events through the regulation of transcription factors. In both COS-7 (Faure *et al* 1994) cells and in rat phaeochromocytoma PC12 cells (Frodin *et al* 1995) cAMP has been shown to activate MAP kinase. Thus it is a possibility that cAMP might alter transcription rates via a mechanism involving MAP kinase. Kimball *et al* (1998) showed that stimulation of protein synthesis in L6 cells by insulin was blocked by inhibitors of $p70^{rsk}$ (rapamycin) and MAP kinase (PD 98059) as well as

inhibitors of phosphatidylinositide-3-OH kinase (wortmannin). However, none of these agents had any effect on LMF stimulation of protein synthesis in C_2C_{12} myoblasts, suggesting that an alternative pathway was involved.

As eluded to earlier, LMF may activate the transcriptional process and hence increase protein synthesis. Cyclic AMP mediates the hormonal stimulation of a variety of eukaryotic genes through a conserved cAMP response element (CRE) (Montminy *et al* 1986). Since all of the known cellular effects of cAMP occur via the catalytic subunit of cyclic AMP-dependent protein kinase, PKA, it appears likely that this enzyme mediates the phosphorylation of factors that are critical for transcriptional response. These cyclic AMP-responsive element binding proteins (CREB/ATF) are phosphorylated by PKA leading to stimulation of the transactivation potential (Gonzalez and Montminy 1989, de Groot *et al* 1993). It was demonstrated that phosphorylation of CREB at Ser-133 is induced 6-fold *in vivo*, following treatment of PC12 cells with forskolin (Gonzalez and Montminy 1989). No such induction was observed in the PKA deficient PC12 cell line A126-1B2. They showed that CREB was phosphorylated *in vivo* at Ser-133 in response to forskolin and that phosphorylation of Ser-133 was critical to the activation of gene transcription by cAMP. Hence, these CRE-binding proteins may be involved in LMF stimulation of protein synthesis.

LMF has been isolated from MAC16 tumours in previous experiments (Todorov *et al* 1998). The study provided strong evidence that LMF is identical to human zinc-α2-glycoprotein (ZAG), a protein closely related to antigens of the major histocompatibility complex (MHC) in amino acid sequence and in domain structure. The molecular weight, presence of carbohydrate, amino acid sequence, antigenicity,

and expression of biological activity of ZAG mirrored that of LMF. The polyclonal antisera to ZAG was able to neutralise *in vitro* lipolysis by human isolated LMF which was apparent in this study where LMF stimulated protein synthesis in C_2C_{12} was attenuated in the presence of the ZAG antibody. This information together with evidence that the amino acid sequence of LMF is homologous to that of ZAG, suggest that LMF is indeed ZAG (Hirai *et al* 1998).

ZAG has been reported to be present in blood plasma and in secretions such as saliva, sweat and seminal plasma. In addition, experimental tumours (MAC16, B16) that deplete carcass lipids, have shown expression on the ZAG mRNA. This suggests that there may be an overproduction of ZAG by certain tumours. Indeed, other studies have shown elevated levels of this protein in specific tumour types, e.g. in a comparative analysis in mammary tissues from women with different diseases revealed enhanced expression of ZAG gene in benign breast lesions and a variable expression in breast cancers (Freije *et al* 1991). A significant association has also been observed between ZAG levels and the histological grade of breast tumours, with higher levels found in well-differentiated tumours than in moderately or poorly differentiated ones (Diez-Itza *et al* 1993). Analogously, ZAG levels are much higher in benign prostatic hyperplasia than in adenocarcinoma of the prostrate; the latter is characterised by dedifferentiation and a loss of secretory activity (Frenette *et al* 1987).

These findings suggest that ZAG may possess a pathological role in disease states. Up until now, no direct evidence has been provided to suggest a link between ZAG and the pathogenesis of cancer cachexia. Further experiments need to be conducted using ZAG to demonstrate if it possesses lipolytic activity when injected into mice, and to ascertain

if it has a direct lipid-mobilising effect when incubated *in vitro* in the presence of isolated adipocytes.

In addition to increasing synthesis in C_2C_{12} cells, LMF enhanced protein synthesis in MAC16 cells without an effect on DNA synthesis or cell number. Interestingly, no effect was observed in the MAC13 cell line, a tumour that does not produce cachexia. MAC13 cells were not affected by LMF possibly because the cells were simply unresponsive, did not possess the desired receptor for interaction, or maybe because the cells were less sensitive than MAC16 cells, which would themselves be producing a certain amount of the factor. The MAC13 tumour has been shown to display some lipolytic activity but the activity level is one tenth of that found in the MAC16 tumour and plasma levels of this material were not elevated (Beck and Tisdale 1997). This suggests that low levels of the lipolytic factor is produced by these tumour cells so conducting a dose-response curve utilising higher concentrations of LMF, may elicit a positive response on protein synthesis. This remains to be investigated.

In summary, protein synthesis was stimulated by LMF not only in skeletal muscle but cultures ex vivo and in C_2C_{12} myotubes and MAC16 cells. It was not determined what protein fractions were synthesised in increased amounts in each cell type. This would be the next level of further investigation.

As deduced from this study, it is postulated that LMF is produced by the tumour as a mechanism of increasing its own bulk and therefore its invasive potential. PIF, the protein catabolic factor, is produced by cachectic tumours and when injected in a purified form, causes a 10% weight loss in mice (Lorite *et al* 1997). LMF in converse

to this, increases muscle mass. Thus, in cachectic tumours, LMF is postulated to be produced by the tumour to attenuate the degree and rapidity of weight loss. It is proposed that LMF is synthesised in cells to function as a potential growth factor for the tumour which would also serve as a self-regulating mechanism which would somehow control the extent of cachexia. These suggestions are purely hypothetical and future experiments may yield some of the answers.

3.5.2. Glucose uptake studies

LMF stimulated 2-deoxygluocose uptake in C_2C_{12} myoblasts which suggests that it facilitates glucose utilisation. This was observed only after a long incubation period (24hr) suggesting that the effect might involve protein synthesis. Administration of LMF to mice produced a decrease in blood glucose which was reported previously (Hirai *et al* 1998). Interesting to note, the other catabolic factor, proteolysis-inducing factor, PIF, produced an opposite effect in C_2C_{12} myoblasts, causing a decrease in glucose uptake, which was reflected *in vivo*, where, skeletal and diaphragm muscle glucose consumption was significantly reduced. However, PIF, like LMF, also produced a depression in blood glucose *in vivo* (Hussey *et al* 1999). This depression of glucose by both factors was also observed in mice bearing MAC16 tumour which was unrelated to production of insulin-like growth factor I or insulin like growth factor II (Mcdevitt and Tisdale 1992). Glucose uptake studies were only conducted *in vitro*, in muscle cells. It is possible that LMF causes depression of glucose uptake *in vivo* due to over-utilisation by gastrocnemius muscle, as reflected in the *in vitro* experiment with C_2C_{12} cells. This would require further investigation. For completeness, it would be interesting to conduct future experiments to investigate if the same glucose stimulatory effect by LMF is evident *in vivo*, for example in gastrocnemius muscle.

Furthermore, LMF stimulated glucose uptake in MAC16 tumour cells. Warburg (1936) demonstrated abnormal glucose metabolism in neoplastic tissue *in vitro*. Compared to normal hepatic tissue, rapidly growing, poorly differentiated hepatic tumours showed increased glycolysis and lactate production despite the abundance of oxygen, resulting in high rates of glucose consumption. The mechanism of this increased glucose consumption probably depended on the type of tissue and may be due to increased glucose transport through the cell membrane (Hatanaka 1974) or an enhanced capacity for glycolysis because of increased activity of the key glycolytic enzymes (Weber *et al* 1977).

In lung tumours, there was a high rate of uptake of the glucose analogue ^{18}FDG (fluoro-2-deoxy-D-glucose) (Nolop *et al* 1987). In tumour-bearing animals, circulating glucose levels decreased with tumour burden even though endogenous glucose production was increased and blood lactate levels were increased (Inculet *et al* 1987). Increased glucose utilisation and lactate production occurs also in peripheral tissues and are predominant metabolic changes observed in cancer cachexia (Albert *et al* 1986). The abnormal metabolic effect occurs not only occurs across limb-bearing soft tissue sarcoma in humans (Norton *et al* 1980) but also in tissues that are not in close proximity to the tumour (Burt *et al* 1983).

Sufficient glucose uptake is essential for transformed cells, transport being mediated by a family of highly related membrane proteins, which allow bidirectional movement of glucose down its chemical gradient either into or out of cells, in an energy-independent way (Muckler 1994). The distribution of these transporters (Gluts) vary amongst tissues but have been reported to be altered in malignant cells (Binder *et al* 1997). In this study, the glucose transportor inhibitor, cytochalasin B, had no effect on LMF

stimulated glucose uptake, suggesting an alternative mechanism of action. No effect on glucose uptake was observed after 1hr, suggesting that the effect involves protein synthesis perhaps of glycolytic enzymes (Weber *et al* 1977). The β_3 adrenergic receptor antagonist was utilised to ascertain the receptor interaction involved. An unexpected increase in glucose uptake was observed by the inhibitor alone, which has not been reported elsewhere, suggesting that the compound itself possesses an intrinsic glucose stimulatory effect.

Li and Adrian (1999) have also reported that pancreatic cancer cells produce a bioactive factor which stimulates glucose uptake and utilisation in murine myoblasts. Although the reported Mr (5kDa) of this factor is much lower than that of LMF, in seperate experiments (unpublished) it has been shown that LMF undergoes tryptic cleavage to yield a bioactive fragment of comparable molecular weight.

It is possible therefore that LMF, in addition to its other effects, facilitates the uptake of glucose into cells, therefore aiding it to maintain its substrate demand. This may be another reason for its production by tumours.

3.5.3. Effect of LMF on protein degradation

In addition to stimulation of protein synthesis, LMF also attenuates protein catabolism in skeletal muscle. The ubiquitin-proteasome system is considered to be the major pathway for selective protein breakdown in muscle, while lysosomal proteolysis plays a minor role (Attaix and Taillander, 1998). Although Belchet and colleagues (1990) showed reduction of cathepsin activities by cimaterol *in vitro*, there was no effect of LMF on the lysosomal proteolytic enzymes cathepsins B and L. However, there was

significant inhibition of proteasome catalytic activity, through a decreased expression of the proteasome α-type subunits. Although LMF induced a decrease in protein degradation *in vivo* measured by reduced tyrosine release into surrounding media, in future experiments it would be interesting to determine the effects on proteasome activity and expression in cytosolic extracts of muscles dissected from LMF treated animals, to confirm if the same mechanism of inhibition prevails *in vivo*.

There have been a few studies that have reported changes in components of the ATP-dependent pathway by pharmacological agents. Clenbuterol, a well known β-adrenergic agonist prevented skeletal muscle wasting in AH-130-bearing rats by restoring protein degradation rates close to control values (Costelli *et al* 1995). This normalisation was thought to occur through a decrease of the hyperactivation of the ATP-ubiquitin-dependent pathway, by suppressing the expression of polyubiquitin genes. Another agent shown to perturb components of the ATP-ubiquitin dependent pathway is pentoxifylline (PTX), a xanthine derivative, widely used in humans as a haemorheological agent, able to inhibit tumour necrosis factor transcription (Combaret *et al* 1999). Daily administration of PTX prevented atrophy and suppressed increased protein breakdown in Yoshida sarcoma-bearing rats. It was shown to block the ubiquitin pathway, by suppressing the enhanced expression of ubiquitin, E2 enzyme and the C2 20S proteasome subunit in muscle from the cancer rats. The 19S complex and 11S regulator associate with the 20S proteasome and regulate its peptidase activities. The mRNA levels of for the ATPase subunit MSS1 of the 19S complex increased in cancer cachexia, in contrast with mRNAs of other regulatory subunits. This adaptation was suppressed by PTX, suggesting that the drug inhibited the activation of the 26S proteasome. In subsequent studies, the effect of LMF on 19S

expression or indeed other components of the ubiquitin-proteasome pathway is worth investigating.

LMF may exert its effect on protein degradation via a receptor sub-type identical to that through which protein synthesis is stimulated, although there is no evidence to substantiate this. Numerous studies investigating the effects of β-agonists on skeletal muscle have reported a decrease in protein degradation and/or increase in protein synthesis as a mechanism of overall increase in total protein mass. Experiments conducted with LMF demonstrate that it affects both of these distinct processes. Other agents have been shown to effect skeletal muscle degradation. Administration of a β-adrenergic agonist, $L_{644,969}$, to wether lambs (Koohmaraie *et al* 1991), increased muscle mass through hypertrophy as a result of an increase in muscle protein and reduced degradation of muscle proteins. Furthermore, a study looking at the effects of various β-agonists on protein turnover in isolated chick skeletal muscle and atrial muscle (Rogers and Fagan 1991) observed that cimaterol, clenbuterol and isoprenaline significantly decreased the release of tyrosine release by 8-11%. This effect was seen to be dose-dependent. Administration of metaproterenol (orciprenaline) to young rats induced an anabolic response in muscle, apparently mediated by a reduction in muscle protein breakdown with minor changes in protein synthesis (Martinez *et al* 1991).

These studies, allied with information on β-agonists and LMF affecting protein synthesis, perhaps provide indirect evidence, supporting a role for a possible β-adrenergic mediated effect by LMF on skeletal muscle, affecting not only skeletal muscle protein synthesis, but also to attenuate protein degradation. However, further

investigations has to be undertaken to determine if LMF induced protein degradation is a β-adrenergic effect.

3.5.4. The effects of PIF and PIF+LMF on protein turnover

The results presented demonstrate that PIF is capable of increasing protein degradation in addition to depressing protein synthesis in C_2C_{12} myoblasts, effects that are directly opposite to those induced by LMF. The result of these two combined actions would serve to increase the overall rate of protein turnover allowing protein loss to ensue. These results mirror those obtained by previous workers (Lorite *et al* 1997), who demonstrated that when PIF was injected *in vivo*, there was a pronounced decrease in body weight, approximately 10%, over a 24hr period. The major contribution to the decrease was a reduction in predominantly lean body mass. The decrease in body mass was accounted for by a 50% increase in protein degradation concomitant with a 50% decrease in protein synthesis in gastrocnemius muscle.

It is possible that PIF exerts it action by influencing both transcription and/or translation. The results suggest that it may be effecting both these systems: inhibition of synthesis may result in its ability to block translation due to effects seen at 90min. It may also influence the transcriptional process as is evident from changes seen after a 6hr incubation period with the factor, as well as the decrease in RNA accretion seen after 6hr. It is thought that long term effects would involve changes in the number of translation factors and ribosomes whilst short-term effects would involve changes in the activity due to phosphorylation events. The effect produced is specific to PIF, since it was completely abolished in the presence of monoclonal antibody to the factor.

The hormone insulin is known to stimulate protein synthesis involving activation of mRNA translation (Proud and Denton 1997, Combettes-Souverain and Issad 1998, Moule and Denton 1997). To investigate the effect of PIF in the presence of insulin, experiments were conducted using the C_2C_{12} cell line. Lorite (1997) pretreated cells with insulin for 90min. This prevented any effect induced by the material. In addition, all concentrations of insulin used (0.1nM and 10nM), significantly increased the rates of protein synthesis in the presence of PIF at a concentration which maximally suppressed protein synthesis in the absence of the hormone. This strongly suggests the possibility that PIF affects the translational process.

Insulin regulates several translation factors usually as a consequence of changes in their phosphorylation states (Proud and Denton 1997). The initiation factor eIF4E binds to the cap structure at the 5' end of the mRNA and mediates assembly of an initiation complex termed eIF4F. Assembly of this complex can be regulated by eIF4E-binding proteins (4E-BPs), which inhibit eIF4F complex assembly. Insulin induces phosphorylation of the 4E-BPs, resulting in the alleviation of the inhibition. This regulatory mechanism is thought to be especially important for the control of translation of specific mRNAs whose 5'-untranslated regions (5'-UTRs) are rich in secondary structure. Translation by insulin also appears to involve the rapamycin-sensitive signalling pathway, which leads to activation of the 70kDa ribosomal protein S6 kinase (p70 S6 kinase) and the phosphorylation of the ribosomal protein S6. Overall stimulation of translation involves the activation of eIF2B, required for all initiation events. Peptide-chain elongation can also be activated by insulin, and this is associated with the dephosphorylation and activation of elongation factor eEF2, probably as a consequence of the insulin-induced reduction in eEF2 kinase activity. Thus, multiple signalling pathways acting on different steps in translation are involved

in the activation of this process by insulin and lead both to general activation of translation and the selective regulation of specific mRNAs. It is feasible to suggest therefore that insulin reverses PIF induced inhibition of protein synthesis via one or more of these pathways. This warrants further investigation. In order to confirm the effect of PIF on transcription, northern blotting analyses of possible target skeletal muscle proteins mRNA inhibited in the presence of PIF, would substantiate this.

The mechanism of protein degradation induced by PIF can be attributed to the activation of the ATP-ubiquitin pathway as demonstrated by increased chymotrypsin activity of the 20S proteasome in the presence of PIF. The components of the 20S, α-type subunits, were also upregulated suggesting that PIF effects mRNA gene transcription of these units. However, the reason for the bimodal increase in expression of α-subunits and enzyme activity observed in C_2C_{12} myotubes, cannot be explained. In another study conducted to determine the proteolytic pathway activated in skeletal muscle after administration of PIF administered iv to NMRI mice (Lorite *et al* 1998), also showed that the ATP-dependent pathway was activated in soleus muscles excised from these animals. However, the researchers were unable to determine if this pathway was ubiquitin dependent. The *in vitro* data obtained from C_2C_{12} myoblasts however, supports the role of this pathway in inducing skeletal muscle proteolysis. This corresponds with the literature as this is the most significant pathway for protein degradation of skeletal muscle protein (Attaix and Taillander 1998).

When LMF was added to an increasing amount of PIF, the activation of the chymotrypsin like activity by PIF was attenuated, which was also shown by a normalisation of band intensity in western blotted 20S proteasome subunits.

It seems therefore that LMF reverses the effects of PIF by acting through identical mechanisms but in opposite ways. This appears somewhat unusual. The question most significant is why a tumour would produce two catabolic factors possessing identically opposite effects? The possible reasons for this is purely speculative. Both of these proteins serve to potentiate the weight loss in cachexia, in this way they perpetuate each others effects. However, at a mechanistic level their effects appear to antagonise each other.

In cancer patients and animal models of cachexia, it is probable that there is an unknown complex interplay of these catabolic factors and possibly even other factors known to induce weight loss for e.g. cytokines, the end result of which would be to allow overall weight loss to occur. The loss of skeletal muscle determines the prognostic outcome for a cachectic patient so PIF would be the most important factor in terms of mortality in cachectic cancer patients. Because LMF mobilises fat stores liberating fatty acids, this would provide energy for the tumour, but would result in the diminution of host stores consequently causing weight loss to occur. Furthermore, LMF was shown to potentiate the uptake of glucose in tumours. Perhaps therefore, the primarily role of this factor is to provide for the energy demands of the tumour. Additionally however, it stimulates protein synthesis concomitant with a reduction in degradation. *In vivo,* these secondary effects of LMF would serve to attenuate any weight loss. It is proposed that as protein catabolism in cachexia appears to be due to an upregulation of proteasome expression and it is apparent that LMF effects are antagonistic to PIF, this may function as a means of modulating the rate of loss of skeletal muscle mass that occurs in cancer cachexia. Further experiments are required to substantiate these statements.

Other points to be borne in mind is the nature of these experiments. These studies have been carried out in a controlled environment in isolated muscle cells, very different to the *in vivo* situation. In the *in vivo* state there will be a complicated array of many other factors, which may or may not have a bearing on the observed results. Nonetheless, it would be impossible to take into account the influence of these factors in an *in vitro* assay system. Another point to be considered are the limitations of the model system, C_2C_{12} cell line, used to study the effects of the factors on both protein synthesis and degradation. The first relates to the fact that these cells are immortalised and therefore presumably reflect an abnormality of the normal growth control mechanisms, which regulate their activity. The second factor applicable to myoblasts is the suitability of measuring of protein synthesis, which would primarily reflect cell cycle progression and mitosis compared with synthesis of structural proteins within differentiated, non-proliferating skeletal muscle. In an attempt to overcome this problem, the effects of the agents were examined in differentiated, non proliferating C_2C_{12} myotubes, and also *in vivo* experiments.

CHAPTER 4

G-protein modulation by a lipid-mobilising factor

4.1 Introduction

A murine model of cancer cachexia, the MAC16 adenocarcinoma, produced the loss of both muscle and adipose in recipient animals (Beck and Tisdale 1987). The loss in weight of mice bearing this tumour, was closely correlated with a rise in serum level of a lipid-mobilising factor produced by the tumour (McDevitt *et al* 1995). Lipid-mobilising activity obtained from the MAC16 tumour when injected into mice was capable of inducing weight loss, confirming that it has a potential role in inducing cachexia. Further purification and characterisation of LMF from the tumour extract yielded a 40kDa protein capable of inducing lipolysis via an increase in cAMP (Tisdale and Beck 1991).

It has been postulated that LMF induced lipolysis may occur via the modulation of membrane associated heterotrimeric guanine nucleotide binding proteins (G proteins) (Price 1997, Khan 1996). Because G proteins are obligate intermediaries in the signal transduction process, factors affecting their content could influence cell signalling and therefore the response to lipolytic stimuli elicit by LMF. The G protein content of adipocytes with altered physiology has previously been measured, e.g. concentrations of Gαi1 and Gαi2 are more than two-fold greater in adipocytes from obese versus normal-weight subjects (Kaartinen *et al* 1994). Endurance exercises increase Gαs, Gαi1 and Gαi3 but decreases Gαi2 in adipose plasma membranes compared to controls (Nieto 1996).

The primary aim of this study was to attempt to elucidate some of the mechanisms by which LMF induces its effect in adipose tissue, using both *in vitro* and *in vivo* model systems. A brief overview of the individual components involved in the lipolytic cascade is given below.

4.2. G proteins and transmembrane signalling

Members of a family of guanine nucleotide binding regulatory proteins (G proteins) are responsible for transmission of information from many membrane-bound receptors to their intracellular effectors. They play a pivotal role in signal transduction of a wide variety of plasma membrane located receptors for hormones, auto- and paracrine hormonal factors and light. These signal transducing systems are composed out of at least three membrane-associated components, the hormone-specific receptor, the G-protein and an effector system, e.g. an intracellular signal forming enzyme or an ion channel. The best studied of these pathways are those for dual (stimulatory and inhibitory) regulation of the rate of cAMP synthesis (e.g. the β-adrenergic and muscarinic agonists and for light stimulated hydrolysis of cGMP in retinal rods and cones).

The heterotrimeric G proteins consist of three non-identical polypeptide subunits named α, β and γ. The nomenclature is based simply on the fact that the α-subunit is the largest (39-46kDa) of the polypeptides in the complex, followed by the β (35-37kDa) and the γ (8-10kDa) subunits. Four classes of G proteins designated Gs, Gi, Go and Gt have been described based on functional and structural criteria (Graziona and Gilman 1987).

4.2.1 α subunits

Mammals have over 20 different G protein α subunits (16 gene products, some with alternative spliced isoforms) (Simon *et al* 1990). The proteins can be divided into four major classes according to their similarity of amino acid sequences that ranges from 56%-95% identity. With the exception of G proteins found in sensory organs (such as αt, αgust or αolf), and a few types of α subunits that are predominantly expressed in haematopoietic cells (α16) or neural cells (αo), most α subunits are widely expressed. Individual cells contain at least 4 or 5 types of α subunits (Neer 1995). The α-subunit of Gs, the G protein responsible for stimulation of adenylate cyclase, is expressed as four distinct polypeptides with predicted molecular masses ranging from 44200 to 45700Da although these proteins migrate on SDS gels as two distinct bands with apparent molecular weights of 45000 and 52000Da. These variants arise because of alternative splicing of a single precursor mRNA (Hepler and Gilman 1992).

All α subunits are themselves enzymes i.e. they possess intrinsic GTPase activity at varying rates; they will hydrolyse the terminal phosphate of bound GTP to yield bound GDP and free inorganic phosphate (Pi) (Morgan 1991). In some cases, α subunits possess specific residues that can be modified by bacterial toxins. Cholera toxin catalyses the transfer of the ADP-ribose moiety of NAD to a specific arginine residue in certain α-subunits. This irreversible covalent modification locks the enzyme in the active, GTP-bound state (Hardie, 1991), thus potentiating Gαs function and increasing cAMP levels. Similarly, pertussis toxin ADP-ribosylates those α-subunits that possess a specific cysteine residue near the carboxy terminus. Modification of α by cholera toxin constitutively activates these proteins (by inhibiting their GTPase activity),

whereas modification by pertussis toxin prevents receptor mediated activation of G proteins. Thus these toxins serve as invaluable tools in identifying many G protein mediated responses. Although none of the G protein subunits contains regions that might obviously associate with the lipid bilayer, the heterodimer is bound to the plasma membrane. This is apparently due to the fact that γsubunits are prenylated and at least some α-subunits (those of the Gαi subfamily) are myristoylated. These lipid modifications serve to anchor the subunits to the membrane (perhaps by increasing the affinity of protein-protein interactions) and they also increase the affinity of α for βγ.

4.2.2. βγ subunits

The five known mammalian β subunits are between 53% and 90% identical to one another (Watson *et al* 1994). In contrast, the six γ subunits are much more different from each other than are the β subunits or α subunits (Cali *et al* 1992). Five different β subunits and at least six γ subunits could produce 30 different combinations. However, not all the possible pairs can form. So far, there appears to be no difference in the ability of reconstituted βγ pairs to activate effectors or interact with α subunits except for $\beta_1\gamma_1$ which is sometimes much less active (Clapham and Neer 1993). However, this dimer is only found in retina, so this selectively does not help answer questions about the specific function of βγ subunits in other cells.

Eight of the 18 known subunits of G proteins have been identified in mature adipocytes by immunoblot analysis: Gαi1, Gαi2, Gαi3, Gαo, Gq/11α, G13α and the long and short isoforms of Gαs (Denis-Henriot *et al* 1996).

4.3. G protein activation

The basal or inactive state of an individual G protein is the oligomer with GDP bound to the α-subunit (GDP.αβγ) (Figure 4.1.1); the rate dissociation of GDP from the complex is extremely slow. Interaction of the G protein with an appropriate agonist-receptor complex promotes the dissociation of GDP and permits binding of the more prevalent cellular guanine nucleotide, GTP. This interaction between GTP and the G protein's α-subunit is believed to promote dissociation of αGTP from a complex of the β and γ subunits. It is in this dissociated state that the G protein is thought to modulate the status of a given effector such as adenylate cyclase.

Figure 4.1.1. The regulatory cycle of heterotrimeric G proteins (taken from Neer 1995).

Slow hydrolysis of bound GTP by GTPase intrinsic to the α subunit leads to reassociation of the oligomer and cessation of the signal. G protein signalling is therefore controlled by three inter-dependent association-dissociation cycles. This ultimately leads to the amplification of the signal because a single agonist-occupied receptor is capable of activating multiple G proteins and the activation of the signal is, in part, determined by the lifetime of the GTP-bound form of the α subunit. This subunit dissociation model focuses attention therefore, on the interactions of activated G proteins α subunits with relevant effectors.

It has been discovered that the βγ subunit can positively regulate effectors (Logothetis *et al* 1987) where it was discovered that the βγ subunits could activate the muscarinic K+ channel. Subsequently, the βγ subunit has been shown to be a positive regulator of a large number of effectors including adenylate cyclase, phosholipase A2, phosphoinositide 3-kinase (PI3-kinase) and β-adrenergic receptor kinase (Clapham and Neer 1993).

4.4. Lipolysis and adrenoceptors in adipose tissue

Lipolysis refers to the process by which triacylgycerol (TAG) molecules are hydrolysed to free fatty acids (FFA) and glycerol. Catecholamines and hormones such as glucagon and ACTH induce fat cell lipolysis as a consequence of stimulation of adenylate cyclase (AC) activity (Lafontan and Berlan, 1993).Binding of catecholamines to the β-adrenoreceptors activates adenylate cyclase via a stimulatory G protein (Gs). The proposed signal transduction cascade implicated in the stimulation of lipolysis by LMF is thought to be via a pathway similar to that of catecholamines (See Figure 4.1.2).

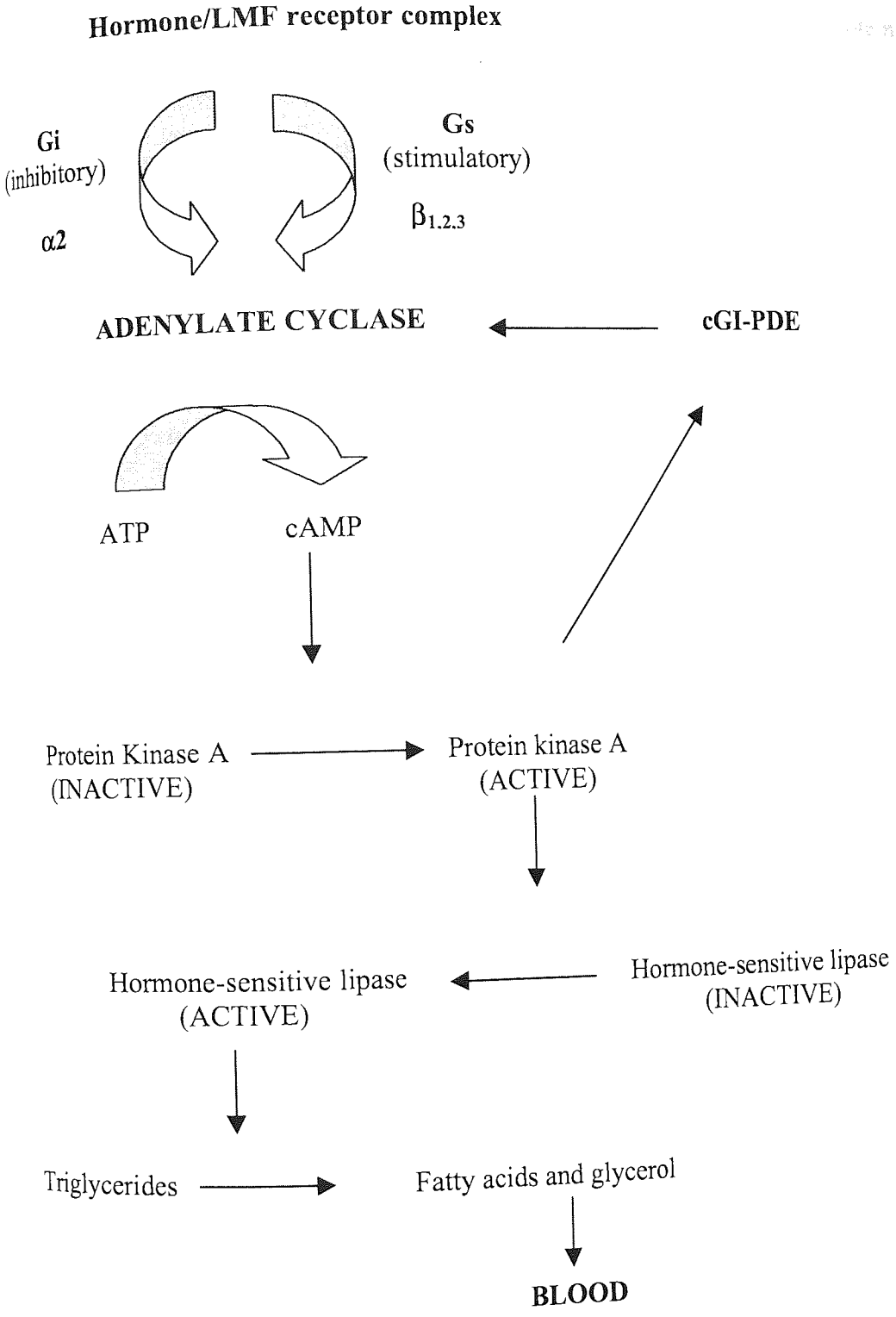


Figure 4.1.2. Activation of lipolysis via adrenoreceptor-coupled systems (Adapted from Vance and Vance 1996).

After LMF has bound to it's receptor, cAMP is activated via Gs protein. This catalyses the conversion of ATP to cAMP. cAMP binds the regulatory subunit of protein kinase A, releasing the active catalytic subunit. Active protein kinase A in turn phosphorylates the hormone-sensitive lipase, which translocates to the triacylglycerol droplet and begins to hydrolyse the stored lipid. The same signal may affect an inhibitory G protein (Gi), which inhibits the activity of adenylate cyclase. PKA also phosphorylates and activates the cGMP-inhibited cAMP phoshodiesterase (cGI-PDE), providing a feedback system to lower intracellular cAMP. Disappearance of cAMP eventually causes the cAMP to dissociate from the regulatory subunit of protein kinase A, which then inactivates the catalytic subunit by reassociation. In the absence of continued phosphorylation, dephosphorylation inactivates the hormone-sensitive lipase (Vance and Vance, 1996).

As mentioned, the β-adrenergic agonists stimulate the lipolytic pathway. Conversely, an anti-lipolytic signal is transduced by the α_2 adrenergic agonists; the α_1-adrenergic agonist are involved in a seperate pathway not linked to lipolysis regulation, involved in the control of glycogenolysis and lactate production (Hepler amd Gilman 1992). Although lipolysis is the observed outcome of catecholamine stimulation, it is the steady state result of competition between two opposing pathways triggered by the same signal. Thus the presence of the inhibitory α_2 receptor provides the cell with the opportunity for dual regulation of cyclase activity.

The catecholamines mediate their actions via five adrenergic receptor subtypes: β_1, β_2, β_3, α_1 and α_2 (Lafontan *et al* 1997). These adrenergic receptors are members of the superfamily of G-protein-coupled receptors that are characterized by 7 transmembrane

spans of 20-28 hydrophobic amino acids (with the intervening hydrophilic loops alternating their exposure intra-and extracellularly), an extracellular amino terminus with glycosylation sites and an intracellular carboxyl terminus, that is palmitoylated for stabilisation of the protein in the membrane. The extracellular surface is critical for ligand binding while the intracellular surface is involved in G-protein recognition, as discussed earlier.

It is now well established that three β adrenoceptor subtypes (β_1, β_2 and β_3) co-exist in fat cells of various lab animals (Arch and Wilson 1992). In man however, the presence of a biologically active β_3 adrenoceptor has been controversial. Some studies have not convincingly demonstrated the functional β_3 receptor. However, the mRNA of β_3 has been detected in adipose, gall bladder and colon (Krief *et al* 1993). Furthermore, the existence of lipolytically functional β_3 BAR, which co-exist with β_1 and β_2 have been demonstrated in human isolated fat cells (Lonnqvist *et al* 1993).

The biological significance of having 3 β receptors in adipocytes is unclear but suggests that each receptor subtype may serve a different signalling role. All 3 receptors couple Gαs and activate adenylate cyclase but only β_3 receptors have been shown to interact with Gαi in adipocyte membranes, suggesting a differential cross-talk of these receptors with inhibitory receptors. It has been suggested that β_1 receptors which are more sensitive to catecholamines and desensitise rapidly, mediate acute effects of low level catecholamine stimulation while β_3 receptors which require higher levels of catecholamines to become activated and are less sensitive to desensitisation, deliver a more sustained signal (Arch and Wilson 1996).

It is of interest to note that the β_3 adrenoceptor is the principal receptor mediating catecholamine stimulated thermogenesis is brown adipose tissue (BAT), a tissue which oxidises NEFA and produces heat which could be involved in the metabolism of excess fat (Trayhurn 1996). The essential function of BAT (so-named because of its colour) is the expenditure of fatty-acid-derived energy for maintenance of the organisms thermal stability in contrast with WAT which stores and releases energy in the form of free fatty acids.

A new lipolytic pathway has recently been reported by Lafontan and co-workers (2000). A putative lipolytic effect of natriuretic peptides was investigated on adipose tissue. Atrial natriuretic peptide (ANP) receptors have been described on rodent adipocytes and expression of ANP mRNA found in human adipose tissue. It was shown that ANP stimulated lipolysis as much as isoprenaline, a non-selective β-AR agonist on human fat cells. This raises questions about the physiological and/or pathological role of this new pathway.

4.5. Intracellular cAMP

Adenylate cyclase is the enzyme component of a protein triad that also comprises membrane bound G proteins and hormone receptors. This 120kDa protein actually represents a diverse family of at least 9 isoenzymes; the isoform present in the adipocyte is unknown. Adenylate cyclase is characterized by two cytoplasmic domains and two hydrophobic stretches with 6 transmembrane domains each (Hanoune *et al* 1997). In adipose tissue, adenylate cyclase is stimulated or inhibited by subunits of G proteins (Gαs, Gαi, βγ). In the absence of stimulation, cyclase is inactive. Cyclase activity increases as membrane fluidity increases. Thus agents that alter membrane lipid

properties are likely to influence cyclase activity. Cyclic AMP is degraded via phosphodiesterase (PDE). The isoform present in adipocytes is PDE3B and is phosphorylated by insulin, adenosine, and catecholamines (Degerman *et al* 1997). Phosphorylation on Ser302 is speculated to activate adipocyte PDE3B by relieving inhibition on the catalytic domain of the enzyme.

4.6. Regulation of Hormone-sensitive lipase (HSL)

Hormone-sensitive lipase is an 82 to 88kDa protein found in adipose tissue of mammalian species (Holm *et al* 1989). This enzyme catalyses the rate limiting step, the hydrolysis of triacylglycerol and subsequently to monoacylglycerol. Another enzyme, hormone-insensitive monoacylglycerol lipase, catalyses the final hydrolysis of the monoacylglycerols. HSL activity can be regulated by reversible phosphorylation.

Phosphorylation of partially purified HSL by PKA, lead to activation of the lipase activity *in vitro* (Huttunen *et al* 1990). Subsequent phosphopeptide mapping and phosphoamino acid analysis suggested that HSL was phosphorylated on a single serine residue, named the regulatory site, which was later identified as Ser563 (Stralfors and Belfrage 1985). However, more recent data, has challenged this view on the short-term regulation of HSL. The finding that mutation of the "regulatory" serine did not abolish PKA-induced activation of HSL lead to the identification of two novel PKA sites, Ser-659 and Ser-660, responsible for *in vitro* activation of HSL (Anthonsen *et al* 1998). The role of phosphorylation of Ser-563 remains elusive.

Other kinases are able to phosphorylate HSL. Glycogen synthase-kinase-4, Ca^{2+}/calmodulin–dependent kinase II and AMP-activated protein kinase (AMPK) phosphorylate Ser-565 *in vitro*, i.e. 2 residues C-terminal to Ser-563, without any direct

effect on enzyme activity (Holm *et al* 2000). HSL is dephosphorylated by protein phosphatases 2A and 2C; the isoenzymes have significant activity against HSL and each isoenzyme has greater activity at Ser-565 than Ser-563. The dephosphorylation of the novel PKA sites, Ser-659 and Ser-660, remains to be investigated.

Discrepancy exists between HSL activity in the purified form compared to the cell. Phosphorylation of purified HSL leads to minor increases in activity, whereas phosphorylation of HSL in intact cells can cause up to 50-fold increase in lipolysis (Holm *et al* 2000). This discrepancy may reflect compartmentation of HSL in intact cells. Eagan *et al* (1992) demonstrated that HSL is in the aqueous supernatant phase of homogenised rat epididymal adipocytes when cells are lipolytically inactive, but is bound to the fat cake when cells are lipolytically stimulated. This suggests that HSL is transloclated from the cytosol to the lipid droplet upon hormone stimulation of adipocytes. Translocation may be facilitated by a family of lipid droplet–associated proteins called perilipins. These proteins are heavily phosphorylated by protein kinase A and dephosphorylated by insulin. Immunogold-labelling shows these to be on or within the limiting phospholipid monolayer that surrounds the triglyceride droplet (Blanchet-Mackie *et al* 1995). The coordinated phosphorylation of both HSL and perilipins suggest that the latter may serve to increase HSL access to substrate either by acting as a docking protein for activated HSL, or by facilitating substrate exposure.

Existing adipocyte-like cell lines such as 3T3-L1 and 3T3-F442A and BFC-1 and other primary cultures have been used to investigate the effect of different nutritional and hormonal factors on HSL expression. For example, in isolated rat adipocytes in culture, dexamethasone increased HSL mRNA 4-fold, whereas adrenaline and growth hormone had no effect (Slavin *et al* 1994). Glucose deprivation has been shown to reduce HSL

mRNA levels in 3T3-F442A cells (Raclot 1998). However, results from other studies give differing results, which may be attributed to differences in experimental systems. Nevertheless, agents that affect gene expression, offer a means of long term regulation of HSL.

4.7. Aim of study

The present study seeks to examine the sensitivity to lipolytic stimuli of adipose tissue from mice bearing an experimental model of cancer cachexia (MAC16), as well as alterations in adipocyte G-protein expression during the development of cachexia, both in mice and man and the role of LMF in this process.

4. 8. RESULTS

In order to examine the possibility that G protein modulation might contribute to increased lipid catabolism in the cachectic state, the expression of both the stimulatory (Gαs) and inhibitory (Gαi) G-proteins was investigated.

5' nucleotidase is an enzyme which catalyses the formation of adenosine from adenosine –5-monophosphate, found exclusively in cell membranes. Its activity was measured as a marker of membrane isolation after the purification protocol (Figure 4.8.1). Adipocyte plasma membranes isolated from male NMRI mice bearing the MAC16 tumour and possessing varying degrees of weight loss, were resolved on SDS-PAGE and blotted onto nitro-cellulose. This revealed a marked alteration in the expression of G proteins, with progression of the cachectic state. Specific antibodies were used to probe for Gαs and Gαi with bands being quantified densitometrically and expressed as % control in arbitrary units.

Plasma membranes express two forms of Gαs (46 and 54kDa) derived by alternative splicing of the gene. Immunoblotting of membranes with Gαs antibody detected a significant increase in the expression of Gαs, reaching a maximum at 10% weight loss, giving a 147% increase in band density compared to control non-tumour bearing animals (Figure 4.8.2). The expression of both subunit forms thereafter, decreased returning to baseline at 20% weight loss. Conversely, the 40kDa Gαi band density decreased as cachexia progressed reaching its maximum at 10% weight loss, a reduction of 35% compared to control, which returned to normal levels with further host wasting (Figure 4.8.3).

It can therefore be observed that there is concomitant increase in Gαs which mirrors the decrease in Gαi, with progression through the cachectic state. The ratio of Gαs to Gαi was calculated, which showed a marked increase as weight loss increased reaching a peak at 10% weight loss, with an overall increase nearly four-fold above control (Table 4.1). Thereafter, the ratio decreased and returned to levels comparable to that of control.

To determine the effect of LMF on G protein expression in plasma membranes *in vivo* ex-breeder NMRI mice were injected i.v with LMF 8μg b.d for 48hr. Epididymal fat pads were excised immediately and plasma membranes were isolated. Gαs bands immunoblotted from the membranes of these animals showed a significant elevation in the amounts of these subunits, 134% increase in comparison to control mice. Gαi band density decreased only slightly but the overall Gαs/Gαi ratio remained twice that of control, comparable to the ratio achieved for 15% weight loss in MAC16 mice (Table 4.1).

Treatment of mice bearing the MAC16 colon adenocarcinoma with 10% weight loss with eicasopentanoiec acid (EPA 0.5g/kg), attenuated the weight loss; MAC16 control mice exhibited weight loss of 77.6% ± 0.66 of the original body weight in comparison to EPA dosed MAC16 mice which lost 86.2% ± 0.64. Analysis of plasma membranes showed that the Gαs band density was reduced to 77% of control whilst Gαi remained the same. The overall Gαs/Gαi ratio of 0.66, was below that of control.

A similar pattern of G protein expression was noted in non-weight losing and weight-losing colonic cancer patients. Omental and subcutaneous adipose tissues were excised under anesthetic and transported to the laboratory. Immunoblotting of these plasma membranes from the cachectic patient revealed a significant increase in the levels of Gαs for both adipose tissue types with a directly opposite effect in Gαi occurring in membranes isolated from the non-weight losing patient (Figure 4.8.4 and 4.8.5). The Gαs/Gαi ratio was increased to values similar to that obtained in mice bearing MAC16 tumours (Table 4.1). These results in isolation, however, do not substantiate LMF's role in modulating these effects.

In order to determine whether LMF does cause a direct effect on G protein expression, an *in vitro* assay system was employed. Pre-adipocyte cell lines 3T3-L1 were subjected to hormonal treatment to promote fat accumulation. After a process of differentiation lasting between 8-10 days, 3T3-L1 cells were treated with increasing concentrations of LMF, ranging from 11.6nM up to 580nM and incubated for 24hr.

Plasma membranes were isolated and separated by SDS-PAGE. Immunodetection of Gαs and Gαi yielded a very similar profile to that of earlier experiments. The expression of Gαs subunits increased progressively, reaching maximum increase of 53% at 580nM LMF (Figure 4.8.6). Gαi decreased dose-dependently with a maximal reduction of 37% (580nM) (Figure 4.8.7). The Gαs/Gαi ratio increased as LMF concentration increased with the most marked effect occurring at 174 and 580nM (Table 4.2).

When 3T3-L1 cells were pre-treated with 50μM EPA, the upregulation of Gαs and downregulation of Gαi was completely attenuated (Figure 4.8.8, 4.8.9). This was reflected in the Gαs/Gαi ratio which remained near control levels (Table 4.2). These results confirm that LMF has the ability to directly modulate G proteins, causing a downregulation of Gαi expression allied with an increase in Gαs expression. This also suggests a possible mechanism by which EPA exerts its inhibitory effect on LMF and therefore reverse the potentiation of lipolysis associated with LMF.

The expression of the rate-limiting enzyme hormone-sensitive lipase (HSL) was investigated in 3T3-L1 cells. At lower concentrations of LMF (11-58nM) HSL expression was increased in cytosolic extracts isolated from these cells incubated with LMF for 24hr (Figure 4.8.10). At higher concentrations, expression levels remained near control levels. These results demonstrate that LMF modifies not only G protein expression but also affects HSL gene expression. These effects together would serve to favour maximum lipolysis induced by LMF.

Experiment to detect the presence of plasma membranes after purification protocol by measuring 5' nucleotidase enzyme activity

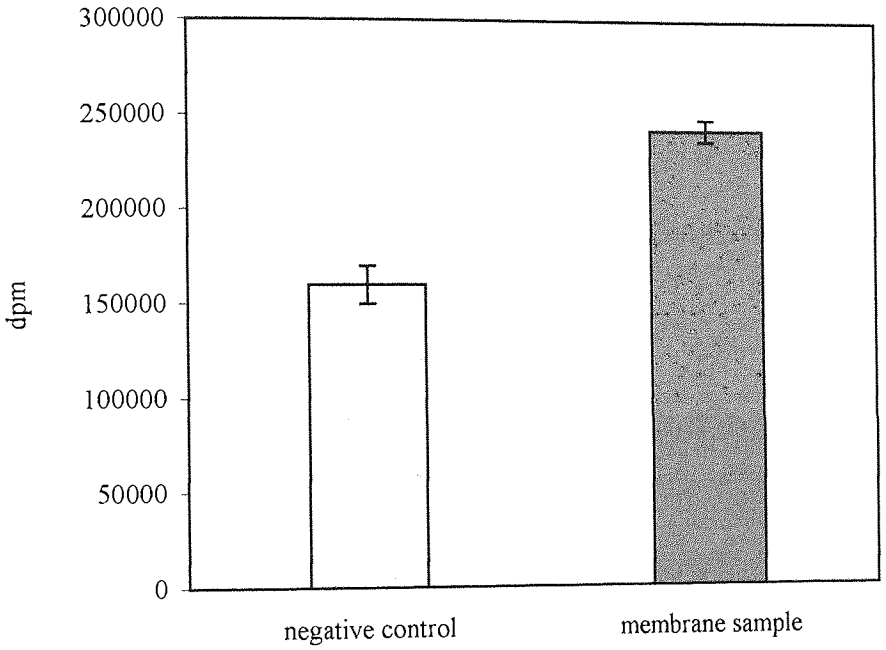


Figure 4.8.1. After purification, 50μl of the final sample was subjected to this assay in order to confirm that plasma membranes had been isolated. 5' nucleotidase is present exclusively in membranes and catalyses the formation of [^{3}H] adenosine from [^{3}H] adenosine 5' monophosphate which can be detected in a scintillation counter.

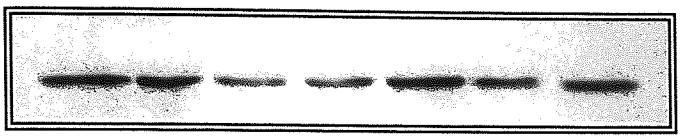
Immunodetection of Gαs present in Adipocyte Plasma Membranes Isolated from Cachectic MAC16 Tumour-bearing Mice

1 2 3 4 5 6 7

Lane No.	Densitometric values (peak intensity)	% control
1	144.49	100
2	281.27	195
3	356.41	247
4	271.74	188
5	128.94	89
6	338.2	234
7	111.81	77

Figure 4.8.2. Western blot of adipocyte plasma membranes were isolated from normal NMRI mice (lane 1) and mice bearing MAC16 tumour who exhibited 5% (lane 2) 10% (lane 3) 15% (lane 4) 20% (lane 5) weight loss, and from mice normal NMRI mice injected with LMF (8μg/bd) (lane 6), and MAC16 mice with 10% weight loss treated with EPA 0.5g/kg (lane 7). Blots were detected using polyclonal anti-Gαs antibody. 5μg of protein was loaded for each lane

Immundetection of Gαi isolated from Adipocyte membranes excised from Cachectic MAC 16 Tumour-bearing mice



1 2 3 4 5 6 7

Lane No.	Densitometric values (peak intensity)	% control
1	173.51	100
2	164.00	95
3	113.44	65
4	124.37	72
5	177.75	102
6	151.76	87
7	169.48	98

Figure 4.8.3. Western blot of adipocyte plasma membranes isolated from normal NMRI mice (lane 1) and mice bearing the MAC16 tumour who exhibited 5% (lane 2) 10% (lane 3) 15% (lane 4) 20% (lane 5) weight loss and normal NMRI mice injected with LMF 8μg/bd (lane 6). MAC16 mice with 10% weight loss treated with EPA 0.5g/kg (lane 7 were also investigated. Blots were detected using polyclonal anti-Gαi1/2 antibody. 50μg protein was loaded in each lane.

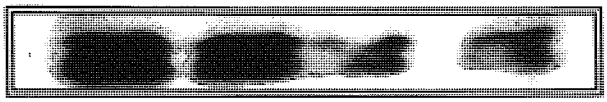
Immunodetection of Gαs isolated from Human Omental and Subcutaneous Adipose tissue

1 2 3 4

Lane No.	Tissue type	Densitometrc values (peak intensity)	% control
1	non-cachectic s/c	105.48	100
3	cachectic s/c	330.71	313
2	non-cachectic omental	212.73	100
4	cachectic omental	299.23	141

Figure 4.8.4. Western blot of adipocyte plasma membranes were isolated from human adipose tissue recently dissected from patients under anaesthetic. Non cachectic subcutaneous adipose (lane 1) non-cachectic omental adipose (lane 2) cachectic subcutaneous adipose (lane 3) cachectic omental adipose (lane 4). Peak intensity was used to determine the amount of G protein present. Blots were detected using polyclonal anti-Gαs antibody. 5μg protein was loaded per lane.

Immunodetection of Gαi isolated from Human Omental and Subcutaneous Adipose tissue



1 2 3 4

Lane No.	Tissue type	Densitometric values (peak intensity)	% control
1	non-cachectic s/c	326.7	100
3	cachectic s/c	281.9	86
2	non-cachectic omental	404.74	100
4	cachectic omental	232.73	57

Figure 4.8.5. Western blot of adipocyte plasma membranes isolated from recently dissected adipose tissue under anaesthetic and transported to laboratory on ice. Non cachectic subcutaneous adipose (lane 1), non cachectic omental adipose (lane 2),cachectic subcutaneous adipose(lane 3) cachectic omental adipose (lane 4). Blots was detected using polyclonal anti-Gαi1/2 antibody. 50μg of protein was loaded per lane.

Gαs/Gαi ratio in adipocyte plasma membranes isolated from mice bearing MAC 16 tumour or administered LMF

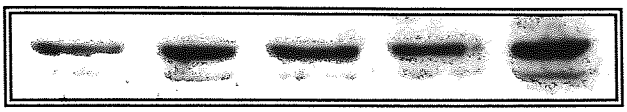
Weight loss or treatment	Gαs/Gαi ratio
0	0.83
5	1.72
10	3.14
15	2.18
20	0.73
LMF	2.23
MAC16 + EPA	0.66

Gαs/Gαi ratio in subcutaneous (s.c) and omental (om) adipocyte membranes from cachectic and non-cachectic subjects

Subject	Gαs/Gαi ratio
Non-cachectic (sc)	0.32
Cachectic (sc)	1.17
Non-cachectic (om)	0.53
Cachectic (om)	1.29

Table 4.1. Immunoblots for values obtained in the above tables are illustrated in Figures 4.8.2. 4.8.3, 4.8.4, 4.8.5. The values obtained to calculate the ratio of Gαs/Gαi was determined as peak intensity using Gel Works Intermediate.

Immunodetection of Gαs present in 3T3-L1 Adipocyte Cell line Treated with Increasing Concentrations of LMF

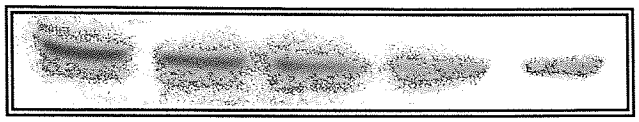


1 2 3 4 5

Lane No.	Densitometric values (peak intensity)	% control value
1	163.05	100
2	230.32	141
3	211.78	130
4	203.72	125
5	250.12	153

Figure 4.8.6. Western blot of adipocyte plasma membranes isolated from 3T3-L1 adipocyte cell line. LMF was added to cells at increasing concentrations; control (lane 1), 11.6nM (lane 2), 58nM (lane 3), 174nM (lane 4) and 580nM (lane 5). Blots were detected using polyclonal anti-Gαs antibody. 30μg of protein was loaded per lane.

Immunodetection of Gαi present in 3T3-L1 adipocytes treated with increasing concentrations of LMF

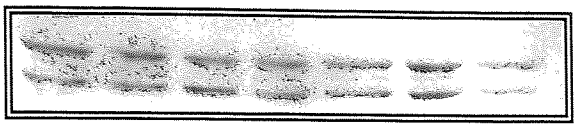


1 2 3 4 5

Lane No.	Densitometric values (peak intensity)	% control
1	218.11	100
2	223.59	103
3	217.15	100
4	171.73	79
5	136.52	63

Figure 4.8.7 Western blot of adipocyte plasma membranes isolated from 3T3-L1 cells. Cells were differentiated using various hormones until fat accumulation occurred. Cells were then treated with doses of LMF, control (lane 1), 11.6nM (lane 2) 58nM (lane3), 174nM (lane 4) and 580nM (lane 5). Blots were detected using polyclonal anti-Gαi1/2 antibody. 30μg protein was loaded per lane.

Immunodetection of Gαs isolated from 3T3L1 adipocytes pretreated with EPA then increasing concentrations of LMF

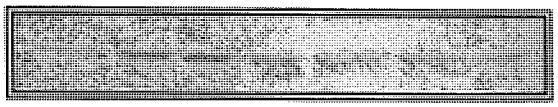


1 2 3 4 5 6 7

Lane No.	Densitometric values (peak intensity)	% control
1	196.65	100
2	211.38	107
3	211.16	107
4	192.92	98
5	187.27	95
6	216.58	110
7	125.40	64

Figure 4.8.8. Western blot of adipocyte cell line 3T3-L1 membranes, pre-treated with 50μM EPA then various doses of LMF; control (lane 1) 11.6nM (lane 2), 58nM (lane 3), 174nM (lane 4), 406nM (lane 5) 580nM (lane 6), EPA alone (lane 7). Blots were detected using polyclonal anti-Gαs antibody. 30μg of protein was loaded per lane.

Immunodetection of Gαi isolated from 3T3-L1 adipocyte membranes pretreated with EPA then increasing concentrations of LMF



1 2 3 4 5 6 7

Lane No.	Densitometric values (peak intensity)	% control
1	151.43	100
2	173.52	114
3	171.32	113
4	137.50	91
5	116.96	77
6	131.25	87
7	148.97	98

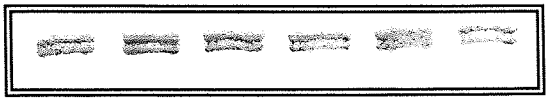
Figure 4.8.9. Western blot of adipocyte cell line 3T3-L1 membranes, pretreated for 2hr with 50μM EPA prior to adding LMF at various doses: control (lane 1), 11.6nM (lane 2), 58nM (lane 3), 174nM (lane 4), 406nM (lane 5) 580nM (lane 6). Blots were detected using polyclonal anti-Gαi1/2 antibody. 30μg protein was loaded per lane.

Gαs/Gαi ratio in adipocyte membranes isolated from 3T3-L1 cells in the absence and presence 50μM EPA

LMF(nM)	Gαs/Gαi **without EPA**	Gαs/Gαi **With EPA**
0	1.00	1.00
11.6	1.37	0.94
58	1.30	0.94
174	1.58	1.08
406	-	1.23
580	2.43	1.26

Table 4.2. Values depicted in this table are illustrated in Figures 4.8.6, 4.8.7, 4.8.8, 4.8.9 The peak intensity values obtained to calculate the ratio of Gαs/Gαi was determined by Gel Works Intermediate. All values have been normalised to a control value of 1.00.

Immunoblot of HSL isolated from 3T3-L1 adipocytes treated with increasing doses of LMF



1 2 3 4 5 6

Lane No.	Densitometric values (peak intensity)	% control
1	139.86	100
2	217.35	155
3	195.43	140
4	158.81	114
5	161.88	116
6	132.79	95

Figure 4.8.10. Western blot of adipocyte cell line 3T3-L1 membranes treated with LMF then immunodetected with anti-rat HSL antibody (cross-reacts with mouse HSL); control (Lane 1), 11.6nM (lane 2), 58nM (lane 3), 174nM (lane 4), 406nM (lane 5), 580nM (lane 6). 30μg of protein was loaded per lane.

4.9. DISCUSSION

Weight loss in mice bearing the MAC16 tumour was closely associated with a rise in the serum level of a lipid-mobilising factor produced by the tumour (Groundwater *et al* 1995). A linear relationship was observed between both the serum and urinary lipid-mobilising activity and weight loss in cancer patients. Lipid-mobilising activity obtained from the MAC16 tumour when injected into mice was capable of inducing weight loss, confirming that it has the potential to induce cachexia (Beck *et al* 1990). The first preliminary studies carried out on this factor demonstrated its ability to activate the membrane bound enzyme adenylate cyclase and the possible involvement of G-proteins in the mediation of lipolysis and its attenuation by fatty acids (Adamson, 1992). Furthermore, the induction of lipolysis and the formation of cAMP by both isoprenaline and LMF were shown to be upregulated in epididymal fat pads from cachectic mice bearing the MAC16 tumour compared with non-tumour bearing mice (Khan 1996). This increased response suggests that long term alterations may have been targeted towards membrane associated events, which would include receptors, G-proteins and/or adenylate cyclase (AC). Preliminary analysis using forskolin (which binds with a high affinity to the complex of Gsα and adenylate cyclase, bypassing receptors) tended to rule out either availability or activity of adenylate cyclase in the plasma membranes of MAC16 tumour-bearing mice, since cAMP production was higher in membranes from the non tumour-bearing mice. This suggests therefore that the upregulated response in adipose tissue from cachectic animals is due to modification of receptor number or guanine nucleotide-binding protein expression.

The G proteins in the adenylate cyclase pathway consist of Gs and Gi families that stimulate and inhibit adenylate cyclase respectively (Levitsky 1987). Adipocyte plasma

membranes contain two Gs α-subunits and two Gi α-subunits, but no Go (Hinsch *et al* 1988). A number of stimuli have been shown to be able to modify G-protein expression in adipose tissue. Immunoblot analysis of adipocyte membranes revealed that lowering serum growth hormone *in vivo* resulted in at least a 3-fold increase in the levels of α-subunit of Gi2, but had no effect on the α subunits of Gi1 and Gi3 nor on the 42 and 45kDa forms of the α subunit of Gs (Doris *et al* 1994). Gasic (1999) and colleagues showed that prolonged treatment of adipocytes with tumour necrosis factor-α (TNFα) stimulated lipolysis. This was a result of decreased concentration of α-subunits of all three Gi subtypes without a change in Gs proteins or β-subunits. Furthermore, prolonged incubation of rat adipocytes with PIA, a non-metabolizable A1 adenosine receptor agonist, lead to down-regulation each of the three subtypes of Gi (Green *et al* 1990). Another similar study showed that treatment of fat cells with the α_2 agonist, UK 14304 for 4 days, caused Gi1 and Gi2 downregulation to about 15% of the control value with Gi3 being decreased to 30% control (Gasic and Green 1995). Fat cells isolated from obese subjects demonstrated decreased sensitivity in the control of lipolysis to hormones which function via interaction with Gi. Levels were substantially lower in relation to overall protein concentration in fat-cell membranes derived from obese subjects compared with those of normal subjects (Ohisalo and Milligan 1989). The androgen testosterone has also been observed to influence G protein expression in fat cells, with depletion by castration inducing a down regulation of both Gαs and Gαi1 and Giα2 and testosterone replacement restoring Gαs expression (Dieudonne *et al* 1993). A concurrent up-regulation of the expression of Gαi 1, Gαi2 and Gαi3, but not Gαs, has been noted in adipocytes isolated from hypothyroid rats (Milligan and Saggerson 1990), indicating an increased response to anti-lipolytic hormones such as

adenosine, and correlating with the gain in body weight often observed in individuals exhibiting hypothyroidism. Thus in addition to agonist mediated receptor expression, adipocyte response to lipolytic stimuli also depends upon the expression and conformation of G-proteins.

To date there have been no measurements of G-protein expression in adipose tissue in cancer cachexia. This study reports alterations in adipocyte G-proteins with the development of the cachectic state in both mice bearing the cachexia inducing MAC16 tumour and in a patient with cancer cachexia. These changes consisted of essentially an increase in membrane Gαs expression allied with a reduction in Gαi expression. The maximal change was observed in mice bearing the MAC16 tumour when weight loss had reached 10%. This correlates with maximal production of LMF by the tumour (Groundwater *et al* 1990). All of these changes would favour the mobilisation of lipid stores from adipocytes and hence facilitate host tissue catabolism.

It is well established through *in vivo* and *in vitro* investigations that there are regional variations in the lipolytic activity of human adipose tissue (Arner 1995). The rate of lipolysis is low in the subcutaneous femoral/gluteal region, intermediate in the subcutaneous abdominal region and high in the visceral i.e. omental region. The differences in non-obese subjects between the subcutaneous and visceral fat depots may be explained by site variations in the function of receptors for insulin, cathecholamines and adenosine. The lipolytic β_1 and β_2 and β_3 adrenoceptors are most active in the visceral cells. The antilipolytic insulin receptors, α_2 adrenoceptors and adenosine receptors are most active in the subcutaneous fat cells. The results obtained in the present study demonstrated differences in the G protein expression from human fat

dissected from omental and subcutaneous regions. Basal G protein expression of Gαs and Gαi from the non-cachectic cancer patient showed an increased Gαs/Gαi ratio in omental compared to subcutaneous fat. Thus, omental tissue might have greater potential in perpetuating the lipolytic process. This corresponds with the increased lipolysis observed by Arner (1995) in omental tissue. This was also evident in omental tissue obtained from the cachectic patient, which showed a greater ratio compared to subcutaneous tissue. The elevation of Gαs/Gαi observed in both subcutaneous and omental adipose tissue from the cachectic subject would help facilitate the lipolytic cascade by making it more sensitive to lipolytic signals and therefore potentiate host weight loss.

To ascertain whether the observed changes were a result of a direct effect of LMF on plasma membranes, an *in vitro* assay system was utilised. The 3T3-L1 cell line is a suitable model system to study the lipolysis pathway because upon differentiation, it acquires morphologic and metabolic characteristics of adipocytes (Green and Kehinde 1974). This is a well-established cell line that is used extensively in studying adipocyte biology. These cells typically grow in a culture medium until they reach confluence at which time they are induced to differentiate by hormonal treatment. In addition, 3T3-L1 cells have been shown to possess three functional BAR subtypes (Shimizu *et al* 1996). When LMF was added to these cells, it was shown to modify plasma G protein expression in a similar manner to that observed in MAC16 epididymal plasma membranes and human tissue. This is evidence that LMF modulates G proteins in plasma membranes in a direct manner.

Khan (1996) examined the potentiated response of adipocytes from mice bearing the MAC16 tumour to both adrenergic and LMF stimulation. It was suggested that some of the observed changes in lipolytic stimuli were attributable not only to short term changes, but may be a result of additional long-term alterations, possibly at the level of mRNA expression. A good candidate for this is the expression of hormone sensitive lipase (HSL), a key enzyme and rate limiting step in the mediation of lipolysis. More appropriately, Thompson (1993b) and colleagues postulated that one of the mechanisms by which fat is lost in cancer patients is through increased expression of mRNA for HSL. They compared the total enzymatic activity and relative mRNA levels for HSL, lipoprotein lipase and fatty acid synthase, in adipose tissue of control and cancer patients. They found that there was no significant changes in the latter two enzyme, but there was a significant two-fold increase in HSL mRNA, which was probably responsible for the two-fold increase in serum triacylglycerol levels and free fatty acid levels observed in cancer patients. Thus, this enzyme would be an ideal target for LMF to exert its effect. Indeed as predicted, when 3T3-L1 cells were exposed to increasing amounts of LMF, there was an elevation of HSL expression at low concentrations as observed from western blotting of cellular supernatants.

Increased HSL expression is a well-reported mechanism by which fat cells become more sensitive to lipolytic stimuli. The elevated lipolytic response to cathecholamines after testosterone treatment was mediated by an up-regulation of β-adrenergic receptor density and several post-receptor events including a possible up-regulation of adenylate cyclase activity and an up-regulation of PKA/HSL activity with apparent lack of any changes in the expression of G-protein α-subunits (Xu *et al* 1993). In primary cultures treated with adrenaline, glucagon, growth hormone and dexamethasone, only the latter

agent resulted in an increase in HSL mRNA by approximately 4-fold, although all of hormones caused increased HSL activity (Slavin *et al* 1994). They concluded that much regulation of HSL was likely due to reversible phosphorylation/dephosphorylation, although some regulation at the mRNA level is possible in response to dexamethasone. Another study which observed HSL expression and activity in relation to lipolysis in human fat cells, showed that HSL protein and mRNA expressions were major determinants of the maximum lipolytic capacity (Large *et al* 1998). Furthermore, Sztalryd and Kraemer (1994) reported an increase in HSL activity after 3 days of fasting in rats that was paralleled by increases in HSL protein and HSL mRNA. Conversely, Large *et al* (1999) showed in obese subjects, that there was a decreased expression HSL in subcutaneous fat cells causing decreased enzyme function and impaired lipolytic capacity of adipocytes. It was suggested that the impaired expression of the HSL gene might at least in part explain the enzyme defect. These studies taken together illustrate the importance of this enzyme as a possible target for regulating lipolysis. Certainly, the upregulation of HSL provides a novel mechanism for the depletion of lipid from adipose tissue in cancer patients at the level of increased expression of mRNA of this lipolytic regulatory enzyme. The factors that modify HSL gene expression are largely unknown. Consequently, the molecular mechanisms underlying the effect of LMF on HSL expression are speculative. It may be a result of cAMP-dependant modulation of HSL gene expression, analogous to cAMP-mediated muscle protein transcription, elicited by LMF as described previously. However, this remains to be investigated.

The polyunsaturated fatty acid, eicosapentaenoic acid (EPA) was able to inhibit the stimulation of lipolysis in murine adipocytes in response to a lipid-mobilising factor

produced by a cachexia-inducing murine adenocarcinoma (Beck and Tisdale 1991). This effect was structurally specific since other related fatty acids of both the (n-3) and the (n-6) series were ineffective as inhibitors of the lipolytic process. An attenuation in weight loss was observed in patients with unresectable pancreatic cancer who received dietary supplementation with EPA (Wigmore *et al* 1996, Barber *et al* 2000b), with preservation of adipose stores and a downregulation of acute-phase response (Wigmore *et al* 1997). The mechanism by which this agent exerts its effect is currently being investigated by other members of the research group, however, some work has been carried out in this area. Price and Tisdale (1998) showed that EPA inhibited the elevation in intracellular cyclic AMP induced in intact adipocytes by incubation with tumour LMF, arising from a direct inhibition of adenylate cyclase activity. The effect was thought to be mediated in part, through Gαi, because pertussis toxin blocked both the inhibition of lipolysis by EPA as well as the inhibition of the stimulation of adenylate cyclase by the tumour LMF. In the present study, EPA was given to weight-losing MAC 16 mice for 48hr . This short term administration of EPA markedly altered the content of G proteins present in the adipocyte plasma membranes by restoring the LMF induced changes in Gαs and Gαi. This was also evident in the 3T3-L1 cells which also showed a restoration in the Gαs/Gαi ratio comparable to control levels. Hence it seems probable therefore that in addition to preventing AC activation and stimulating Gαi it is possible that EPA acts the level of the gene, effecting G protein mRNA transcription. This hypothesis could be further confirmed by northern blot analysis. It follows therefore that if EPA modulates G protein expression both in MAC16 animals and in 3T3-L1 cells, a similar mechanism may account for the stabilisation of adipose tissue stores observed in pancreatic cancer patients (Wigmore *et al* 1996). This requires further investigation.

The results taken together suggest that this tumour-derived lipolytic factor acts to sensitise adipose tissue to lipolytic stimuli, by modulating both G protein and HSL expression. EPA appears to attenuate the effect of LMF on G proteins, which is known to preserve adipose tissue, possibly via a mechanism involving gene transcription.

CHAPTER 5

Final Discussion and Conclusions

Cancer cachexia is a syndrome characterised by loss of adipose tissue and skeletal muscle mass. In this study it has been postulated to be the result of the action of two tumour-derived factors, lipid-mobilising factor (LMF) and proteolysis-inducing factor (PIF). The primary aim of this study was to elucidate the second messenger pathways involved in the action of these catabolic factors.

The main objectives were (i) to establish the signalling mechanisms involved in effects elicited by LMF and PIF and (ii) to examine the role of LMF in modulating adipocyte membranes as a mechanism of enhancing lipid depletion in adipose tissue.

These factors have been shown to be capable of inducing loss of both lean body mass (PIF) and depletion of lipid stores (LMF), both *in vitro* and *in vivo*. Administration of purified PIF to mice produces weight loss with depletion of skeletal muscle but without an effect on adipose tissue mass (Todorov *et al* 1996). In contrast LMF produces a specific reduction in carcass lipid, with a tendency to increase the non-fat carcass mass (Hirai *et al* 1998). Thus, the net effects of both of these factors would be to potentiate weight loss in the cancer bearing host.

β-agonists stimulate skeletal muscle hypertrophy in animals (Mersman 1995) and some have been reported to increase protein synthesis in multinucleated muscle cells in culture (Anderson *et al* 1990, Grant *et al* 1990). The mechanism requires an increase in cyclic AMP, although the changes leading from cyclic AMP to changes in protein synthesis are incompletely understood. LMF stimulated protein synthesis involving a cAMP dependent mechanism, as evident from a linear increase in cAMP in relation to

elevated protein synthesis. The cyclic AMP-dependent protein kinase, protein kinase A (PKA), activity was also increased in the presence of LMF, suggesting it to be part of the same pathway, despite the lack of inhibition by the H8 inhibitor at 10μM. It is possible that higher concentrations of H8 were required for the total inhibition of PKA in this system and this requires further investigation.

Due to the delayed response of LMF affecting protein synthesis (24hr), this lead to the assumption that the factor was acting by some mechanism, affecting gene transcription. Transcriptional regulation following stimulation of adenylate cyclase can be mediated by the family of cyclic AMP-response element (CRE)-binding proteins (Habener 1990). These proteins have been shown to be phosphorylated by PKA with increasing cAMP (de Groot *et al* 1993). It is postulated that these factors may be involved in LMF stimulation of protein synthesis.

Interestingly, LMF stimulated protein synthesis in MAC16 cells but not in MAC13 cells, the non-cachexigenic tumour. It is this observation together with the evidence of its effect on facilitating increased glucose utilisation, that gives a clue as to the possible function of this agent, in the *in vivo* setting. LMF may be a potential growth factor for the tumour, providing it with a mechanism to increase overall tumour size without affecting cellular proliferation and aiding it to concurrently satisfy its energy demand through increased glucose uptake. The MAC13 tumour cell line was unresponsive, possibly because it did not possess the desired receptor subtype, or the concentration maximum was too low to provide growth stimulus for these cells. This is subject to further analysis.

Induction of lipolysis by LMF in epididymal adipocytes was attenuated by the β-adrenergic receptor (β-AR) antagonist propranolol (Khan and Tisdale 1999), suggesting that the action may be mediated through a β-AR. The lack of receptor desensitisation in adipocytes isolated from mice which have undergone long term exposure to LMF lead Khan (1996) to postulate a role for the β_3 adrenoceptor in host mobilisation of fat depots, since this particular receptor subtype is resistant to desensitisation (most likely because it lacks sequences for phosphorylation by specific kinases present in β_1 and β_2 (Stosberg and Pietri-Rouxel 1996)). This information was corroborated by work conducted by Price (1997), who provided evidence that the β_3 adrenergic receptor antagonist, SR 59230A attenuated induction of lipolysis in isolated adipocytes. In this study, the β_3 antagonist was able to abrogate the stimulatory effect of LMF on protein synthesis in murine myoblasts, suggesting a role for this receptor subtype in the mediation of this effect.

In addition to stimulation of protein synthesis, LMF also attenuates protein catabolism, both *in vitro* and *in vivo*. This is through depression of the ATP-ubiquitin pathway, as illustrated by a decrease in enzyme activity of the chymotrypsin like activity of the 20S subunit, an important component of this pathway, although this was not determined in muscles excised from LMF treated mice. Immunoblotting of 20S subunits in cytosol from C_2C_{12} extracts further substantiated this, by showing a decreased expression of these proteins. It may be assumed therefore that LMF affects transcription of these proteins by an unknown mechanism, which as yet has not been established. PIF on the other hand, demonstrated effects directly opposite to that induced by LMF. PIF decreased protein synthesis, by effecting both translation and transcription, unlike LMF which had an effect on transcription only. The monoclonal antibody to PIF reversed the

effect, implying the effect to be PIF specific. The exact mechanisms by which PIF inhibits protein synthesis remains unknown. Additionally, it increased skeletal muscle breakdown via an activation of the ATP-ubiquitin pathway. Thus, while PIF would serve to increase overall protein turnover, the combined effects of LMF, would be converse to this. Taken together, LMF and PIF appear to act in manner that is antagonistic to each other. This certainly seemed to be the case in an experiment conducted in C_2C_{12} in the presence of both the factors; the upregulated activation of chymotrypsin activity caused by PIF alone, was attenuated with a single high dose of LMF. In other words, LMF attenuates the proteolytic affect of PIF. Unfortunately, there is no data on the relative levels of LMF and PIF *in vivo* in cachectic animals or humans, to be able to quantify and therefore explain the results *in vivo*. However, in the MAC16 murine cachexia model, LMF is maximally elevated at 15% weight loss and thereafter declines (Groundwater *et al* 1990). In this model, a decrease in protein synthesis and an increase in protein degradation is not seen until the weight loss exceeds 16% (Smith and Tisdale 1993). This suggests that either PIF production is not apparent at low weight loss or that LMF attenuates the action of PIF. This can be determined by administering pure factors to mice.

The increased responsiveness to LMF in adipocytes isolated from cachectic mice *in vitro* (Khan 1996), implies an up-regulation of either receptor numbers or changes in G protein expression. Immunoblotting of adipocyte plasma membranes isolated from MAC16 bearing mice, a human cachectic patient and 3T3-L1 adipocyte cell exposed to LMF, indeed revealed changes in G protein expression. There was an upregulation of $G\alpha s$ expression and a reciprocal decrease in $G\alpha i$, which was attenuated in the fat cell line by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA). In addition to this, HSL expression was enhanced in 3T3-L1 cells by low concentrations of LMF, a result

which correlated to a study conducted in cancer patients, where mRNA levels of HSL were increased two-fold in comparison to normal controls (Thompson *et al* 1993). These modifications suggest that this tumour-derived lipolytic factor acts to sensitise adipose tissue to lipolytic stimuli, and the effect is attenuated by EPA, which is known to preserve adipose tissue in cachexia (Wigmore *et al* 1996, Barber *et al* 2000b).

Cachexia inducing tumours have been shown to induce profound metabolic changes, whereby the invading tumour invokes responses that produces a catabolic state whereby mobilised substrates such as lipid are utilised by the tumour to support its metabolic requirements. Thus, LMF produced by the tumour might alter the expression of membrane G proteins, probably by affecting mRNA transcription, which would further accelerate this process. This might account for the altered changes observed in NMRI mice bearing the MAC16 tumour and cachectic cancer patient. So, not only does the factor mobilise fatty acids from fat stores in adipocytes, it increases Gαsand HSL expression which sensitises the adipocyte to lipolytic signals, such that the actions of LMF upon the adipocyte are enhanced.

Overall, the findings of this study are very interesting. However, some of the presented data is somewhat unexpected and perplexing. For instance, the reason why two structurally and functionally different catabolic factors would possess identically opposite biological effects with LMF possessing an " anti-cachectic" effect ? Indeed, it is these results which are the most important in this study because it adds a more complex picture to an already complicated aetiology of cancer cachexia. This investigation has been unable to shed light on this particular matter, one can only postulate. Nevertheless, the evidence is compelling, LMF has a strong stimulatory effect on protein synthesis, but also attenuates protein catabolism, effects that are

directly opposite to those induced by PIF. The function of these combined effects would be that they act antagonistically to one another. It is envisaged that in the cachectic state, these two factors operate an unknown self-regulatory balancing mechanism, whereby the effects of one of the factors may predominate, allowing its catalytic activity to ensue. For example, in the situation observed in the MAC16 model where the "PIF-like effects" become apparent only after 16% weight loss. Also bearing in mind that an array of other factors are known to cause weight loss such as the cytokines, a complex interplay, possibly even "cross-talk" (a known phenomenon in the *in vivo* setting) of all this factors may exist, makes this hypothesis more imaginable. Thus, in the cachectic individual there may be a mileu of circulating factors such as LMF and PIF, favouring catabolism in host tissues.

So how can this information benefit us scientifically? Elucidation of the signalling pathways elicited by these factors and subsequent events regulating protein synthesis and degradation clearly enhances current understanding of the basic mechanisms underlying these processes. Furthermore, knowledge of how these events are manipulated by these agents may provide potential in fields as diverse as medicine and agriculture. For example, it should allow more effective control of cachexia and increase animal productivity and product quality (protein/fat ratio in meat) in commercial species. For instance, LMF may have a potential role in this area due to its stimulatory effect on protein synthesis and lipolytic ability. Of course the other obvious indication of the factor would be in the treatment of obesity.

Obesity is a multigenic and multifactorial disease. Present pharmacotherapy, with the exception of Orlistat (recently approved gastrointestinal lipase inhibitor), are either catecholinergic or serotonergic CNS-active anorectic agents. Several of these drugs

have a high abuse potential and may lead to dependency. In addition, on cessation of treatment, weight is in general, rapidly regained, so new therapeutic strategies to treat obesity is a subject of widespread research interest.

Another possible indication for LMF might be in the treatment of type II diabetes which is largely associated with obesity. Thus LMF would be able to mediate the loss of adipose tissue with a concomitant decrease in blood glucose as illustrated by a decrease in blood glucose in NMRI mice injected with LMF and glucose uptake studies in an *in vitro* muscle cell line.

It is interesting to note, that in recent studies, a missense mutation in the coding region of the β_3 adrenoceptor gene resulting in the substitution of tryptophan to arginine at codon 64(Trp64Arg), has been correlated to subjects possessing a greater capacity to gain weight, earlier onset of diabetes and higher waist-to-hip ratios (WHRs) compared to wild-type subjects (Hoffstedt *et al* 1999). However, it is unlikely that a single amino acid change would be sufficient to explain the onset of this disease, although it may certainly contribute to its aggravation. This information allied with knowledge that the primary role of β_3 is to mediate lipolysis (Umekawa *et al* 1999), does bring to light, a possible target for combating obesity and in fact, β_3 agonists are under development as anti-obesity agents (Van der Ploeg 2000). The author explained that β_3 agonists would raise cAMP levels in brown and white adipose tissue, leading to activation of hormone-sensitive lipase resulting in increased fatty acid oxidation and increased thermogenesis by activation of UCP in brown adipose tissue. However long term increases in metabolic rate following chronic treatment with β_3 agonists are yet to be determined in humans.

LMFs ability to mobilise fat stores, to stimulate skeletal muscle protein accumulation, and its proposed action via the β_3 pathway, would appear therefore to be the perfect drug. On the other hand, its effect on muscle protein degradation, and the possibility of side effects such as those associated with already established BAR acting agents e.g. β_2 agonists possessing side effects such as tachycardia, tremor, have yet to be established with chronic treatment of LMF in humans and other β_3 agonists.

Knowledge of the mechanism of cancer cachexia will present fresh opportunities for the pharmacological intervention in the treatment of cachexia. Such treatments would be expected to benefit patients not only in an improved quality of life, but also extended survival time. The relationship between tumour growth and cachexia is not known, but it is possible that catabolism of adipose tissue and skeletal muscle could provide the tumour with essential fatty acids and amino acids vital to tumour growth and metabolism. Understanding the nature of tumour catabolic factors and their action on host tissues may provide insight into the functioning of solid tumours.

It is far too early to propose a use for LMF in humans. There are many more experiments that need to be carried out to ascertain basic fundamental information such as receptor identification and cloning, biological significance, physical structure etc. However, it is a pleasant thought to imagine that a biological agent possessing these qualities currently exists. It will be very interesting to see how the LMF story unfolds in the years to come. Could it be the next anti-obesity drug of the future or even anabolic agent for use in livestock? The prospect for such research to expand is certainly an exciting one, the outcome is eagerly awaited.

CHAPTER 6

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APPENDIX

Publications

Effect of a tumour-produced lipid-mobilising factor on protein synthesis and degradation. Islam-Ali BS & Tisdale MJ. British Journal of Cancer. *In press.*

Modulation of adipocyte G-protein expression in cancer cachexia by a lipid-mobilising factor (LMF).Islam-Ali BS, Khan S, Price SA & Tisdale MJ. *In press.*

Abstracts

Effect of a tumour produced lipid-mobilising factor on adipose tissue and skeletal muscle. Islam-Ali BS & Tisdale MJ. BACR 41st Annual Scientific Meeting, Brighton, 2000.

Effect of a tumour produced lipid-mobilising factor on skeletal and adipose tissue. Islam-Ali BS & Tisdale MJ. EACR 16th Meeting, Halkidiki, Greece, 2000.

Effect of a tumour produced lipid-mobilising factor on adipose tissue and skeletal muscle. Islam-Ali BS, Tisdale MJ. Pharmaceutical Sciences Institute Aston University Birmingham, B4 7ET.