MECHANISM OF PROTEIN CATABOLISM IN SKELETAL MUSCLE DURING CANCER CACHEXIA

JWAN KHAL

Doctor of Philosophy

Aston University

September 2002

Aston University

Mechanism of protein catabolism in skeletal muscle during cancer cachexia

Jwan Khal

A thesis submitted for the degree of Doctor of Philosophy
2002

SUMMARY

Cancer cachexia is characterised by selective depletion of skeletal muscle protein reserves. The ubiquitin-proteasome proteolytic pathway has been shown to be responsible for muscle wasting in a range of cachectic conditions including cancer cachexia. To establish the importance of this pathway in muscle wasting during cancer (and sepsis), a quantitative competitive RT-PCR (QcRT-PCR) method was developed to measure the mRNA levels of the proteasome subunits C2α and C5β and the ubiquitin-conjugating enzyme $E2_{14k}$. Western blotting was also used to measure the 20S proteasome and $E2_{14k}$ protein expression. *In vivo* studies in mice bearing a cachexia-inducing murine colon adenocarcinoma (MAC16) demonstrated the effect of progressive weight loss on the mRNA and protein expression for 20S proteasome subunits, as well as the ubiquitin-conjugating enzyme, $E2_{14k}$, in gastrocnemius and pectoral muscles. QcRT-PCR measurements showed a good correlation between expression of the proteasome subunits (C2 and C5) and the $E2_{14k}$ enzyme mRNA and weight loss in gastrocnemius muscle, where expression increased with increasing weight loss followed by a decrease in expression at higher weight losses (25-27%). Similar results were obtained in pectoral muscles, but with the expression being several fold lower in comparison to that in gastrocnemius muscle, reflecting the different degrees of protein degradation in the two muscles during the process of cancer cachexia. Western blot analysis of 20S and $E2_{14k}$ protein expression followed a similar pattern with respect to weight loss as that found with mRNA. In addition, mRNA and protein expression of the 20S proteasome subunits and $E2_{14k}$ enzyme was measured in biopsies from cachectic cancer patients, which also showed a good correlation between weight loss and proteasome expression, demonstrating a progressive increase in expression of the proteasome subunits and $E2_{14k}$ mRNA and protein in cachectic patients with progressively increasing weight loss.

The effect of the cachexia-inducing tumour product PIF (proteolysis inducing factor) and 15-hydroxyeicosatetraenoic acid (15-HETE), the arachidoinic acid metabolite (thought to be the intracellular transducer of PIF action) has also been determined. Using a surrogate model system for skeletal muscle, C_2C_{12} myotubes *in vitro*, it was shown that both PIF and 15-HETE increased proteasome subunit expression (C2α and C5β) as well as the $E2_{14k}$ enzyme. This increase gene expression was attenuated by pre-incubation with EPA or the 15-lipoxygenase inhibitor CV-6504; immunoblotting also confirmed these findings. Similarly, in sepsis-induced cachexia in NMRI mice there was increased mRNA and protein expression of the 20S proteasome subunits and the $E2_{14k}$ enzyme, which was inhibited by EPA treatment. These results suggest that 15-HETE is the intracellular mediator for PIF induced protein degradation in skeletal muscle, and that elevated muscle catabolism is accomplished through upregulation of the ubiquitin-proteasome-proteolytic pathway. Furthermore, both EPA and CV-6504 have shown anti-cachectic properties, which could be used in the future for the treatment of cancer cachexia and other similar catabolic conditions.

Keywords: PIF, 15-HETE, ubiquitin-proteasome pathway, EPA, QcRT-PCR

To Mum

with love

When I touched the bough of a tree
it trembled in pain

When I held out my hand to touch the branch
the trunk started to weep
When I embraced the trunk
the soil under my feet shuddered
the rocks groaned

This time when I bent down
and collected a handful of earth
all Kurdistan screamed

Sherko Bekes

ACKNOWLEDGEMENTS

I would like to thank Professor M.J. Tisdale for giving me the opportunity to pursue this research, also my thanks to Dr Anna Hine for her technical help and support.

Many thanks to my colleagues in Cancer Biochemistry, Wolfson laboratory and animal house for their help, technical assistance and moral support.
I would like to thank Professor K. Fearon, Edinburgh Royal Infirmary, for supplying human muscle.

A very special thanks to my family especially my mother for her continued love, support, encouragement and great belief in me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine-5'-diphosphate
AIDS	Acquired immunodeficiency syndrome
AMC	Amino methyl coumarin
AMP	Adenosine 5'-monophosphate
APPs	Acute phase proteins
APPR	Acute phase protein response
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BSA	Bovine serum albumin
C2/3/5/8/9	Proteasome subunits C2/3/5/8/9
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CIAP	Calf intestinal alkaline phosphatase
COX	Cyclooxygenase
CRP	C-reactive protein
CV-6504	2,3,5-trimethyl-6-(-3-pyridylmethyl)1,4-benzoquinone
DEPC	Diethylpyrocarbonate
DHA	Docosahexanoic acid
DMEM	Dulbecco's modified Eagles medium
DMF	N,N'-diethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2 ($E2_{14k}$)	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid

FA	Fatty acid
FCS	Foetal calf serum
FFA	Free fatty acid
5'-dFUrd	Fluorinated 5' deoxy-5-fluoruouridine
g	gram
Gi	Inhibitory guanine nucleotide binding protein
G-protein	Guanine nucleotide binding protein
Gs	Stimulatory guanine nucleotide binding protein
GTP	Guanine 5'-diphosphate
h	hour
HETE (5, 12, 15)	Hydroxyeicosatetraenoic acid (5, 12 or 15)
HPETE (5, 12, 15)	Hydroperoxyeicosatetraenoic acid 5, 12 or 15
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HS	Horse serum
IFN-γ	Interferon-γ
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl β-D-thiogalactopyranoside
kg	Kilogram
kDa	Kilodaltons
l	Litres
LIF	Leukaemia inhibitory factor
LLnL	N-acetyl-L-leucinyl-L-leucinal-l-norleucinal
LMF	Lipid mobilising factor
LOX (5, 12, 15)	Lipoxygenases 5, 12 or 15
LPL	Lipoprotein lipase
kg	Kilogram
μ	Micro
M	Moles per litre
m	Milli

MAC	Murine adenocarcinoma of the colon
MAFbx	Muscle atrophy F-box
MHC	Major histocompatability complex
min	Minute
MOPS	3-[N-morpholino]-2-hydroxypropanesulphonic acid
M_r	Molecular weight
mRNA	Messenger ribonucleic acid
MuRF1	Muscle RING Finger 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
$PGE_2, H_2, D_2, F_{2\alpha}$	Prostaglandin $E_2, H_2, D_2, F_{2\alpha}$
PIF	Proteolysis inducing factor
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PSI	*N*-benzyloxycarbonyl-Ile-Glu-(*O*-t-butyl)-Ala-leucinal
PTX	Pentoxyfylline
PUFA	Polyunsaturated fatty acid
QcRT-PCR	Quantitative-competitive reverse transcription polymerase chain reaction
REE	Resting energy expenditure
rpm	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TEMED	N,N,N'N'-tetramethylethylenediamine
TNFα	Tumour necrosis factor-α
Tris	Tris (hydroxymethyl) methylamine
Tween 20	Polyoxyethylene-sorbitan
TXA_2 and B_2	Thromboxane A_2 and B_2
U	Unit

Ub	Ubiquitin
UCP (-1, -2, -3)	Uncoupling protein
UK	Urokinase
X-Gal	5-bromp-4-chloro-3-indolyl β-D-galactoside
YAH	Yoshida ascites hepatoma
ZAG	Zn-α2-glycoprotein

CHAPTER 1

Introduction

1.1 Cachexia

The word cachexia is based on the Greek word "kakos", meaning bad, and "hexis", meaning state of being. Cachexia is a complex syndrome encompassing a wide range of metabolic, hormonal and cytokine-related abnormalities that results in a wasting syndrome. It occurs in a number of disease states, including cancer, acquired immunodeficiency syndrome (AIDS), major trauma, surgery, malabsorption, and severe sepsis (Tisdale and Beck, 1990).

Weight loss in cancer is common, being seen in the majority of patients with gastric, pancreatic, lung, prostate and colon cancer (DeWys *et al.*, 1980; Nixon *et al.*, 1980). Weight loss in cancer patients is associated with shortened survival (De Wys *et al.*, 1980) and poor quality of life (Oveson *et al.*, 1993). Cachectic cancer patients have impairments of physical function and a greater risk of postoperative complications. Mortality can also result from the weight loss, which has been reported in small but significant proportions of cancer patients (Inagaki *et al.*, 1974). Weight loss in cancer patients differ from that found in simple starvation, where different mechanisms appear to be used in cancer cachexia compared to starvation. In late stage of starvation/anorexia nervosa, more than three quarters of the weight loss arises from fat and only a small amount from muscle. In contrast, in cancer cachexia, there is equal loss of both fat and muscle, thus for a given degree of weight loss, there is a more wasting of muscle in the cancer patient than in a 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 tissue, while in cancer only significant changes in liver, kidney and heart weight correspond to a marked depletion of muscle mass (Heymsfield and McManus, 1985). The spleen of cancer patients is always enlarged.

In addition to weight loss, cachexia in cancer is characterised by progressive weakness, early satiety, anorexia and nausea, anaemia, oedema, disturbances of intermediary metabolism and asthenia (Hall, 1979; Lawson *et al.*, 1982).

Warren (1932) reported that cachexia was the most commonly occurring of the various causes of death in a study of 500 cases of carcinoma. The largest proportion of deaths from cachexia occurred in carcinoma of the breast and stomach (33 and 45%, respectively), carcinoma of the bladder showed the smallest proportion of 4%, and it was suggested that cachexia could contribute to death from other causes such as infection. Although this syndrome is mainly associated with terminal cancer patients with disseminated disease, cachexia may also be present in the early stages of tumour growth, even before other signs and symptoms of malignancy appears.

Cachexia is often the principal presenting symptom of the cancer itself with up to 80% (Albrecht and Canada, 1996) or even 100% (Wigmore *et al.*, 1997b) of newly diagnosed patients have pre-existing weight loss. The frequency of weight loss varies with the type of tumour ranging from 30% in patients with favourable non-Hodgkin's lymphoma to 83-87% in patients with gastric or pancreatic cancer (De Wys *et al.*, 1980). The incidence of severe weight loss (above 10%) also varies with tumour type, being 4-10% in the low frequency group and 26-38% in the high frequency group (De Wys, 1985).

De Wys (1985) also looked at survival time of cachectic patients, his study showed that the survival time is shorter in weight loosing patients than in those without weight loss. For patients with breast cancer, colon cancer, prostate cancer and unfavourable non-Hodgkin's lymphoma the median survival is approximately twice as long in patients who had not lost weight compared with those that had. The greater the degree of weight loss the shorter the survival time. A 30% loss in body weight is invariably fatal, although the rare patient may survive up to 50% weight loss (Brennan, 1977).

Nutritional intake has been shown to be substantially reduced in weight-loosing cancer patients, with factors such as anorexia, malabsorption and alterations in energy expenditure contributing to this decline (Rich, 1987). Furthermore, reduction in food intake is a major cause of weight loss in the majority of patients. Cancer patients may frequently suffer from physical obstruction of the gastrointestinal tract, pain, depression, constipation, debility as well as the effects of treatment such as opiates, radiotherapy and chemotherapy, which may all decrease food intake. However, there are a large number of patients with advanced cancer who have no obvious clinical cause of reduced

food intake and in these cachectic patients some aspects of tumour interaction with the host must account for such anorexia.

Weight loss or gain occurs as a consequence of an imbalance between energy expenditure and energy intake. However, it has not been determined if increase in energy expenditure, a reduction in energy intake, or a combination of the two is responsible for the development of the cachectic state.

1.2 Anorexia and Cachexia

Anorexia is defined as the loss of appetite and early satiety leading to decreased food intake or relative hypophagia, i.e., energy input consistently less than energy expenditure. Anorexia is the most frequent and one of the most distressing symptoms in cancer cachexia, however it is unlikely that the weight loss in cancer arises primarily from the reduction in food intake.

Body composition of cachectic patients is different from that found in anorexic patient. During starvation, glucose utilisation by the brain is replaced by ketone bodies derived from fat. This leads to decreased gluconeogenesis from amino acids by the liver and conservation of muscle mass. Body composition analysis of anorexia nervosa patients has shown that most of the weight loss arises from fat and only a small amount from muscle (Moley *et al.*, 1987). In contrast, lung cancer patients, who had lost 30% of their pre-illness stable weight, showed an 85% decrease in total body fat and a 75% decrease in skeletal muscle protein mass (Fearon, 1992). Fearon also showed that other non-muscle body protein was conserved.

Furthermore, the measured food intake does not correspond with the degree of malnutrition, and attempts to increase dietary intake through dietary counselling or nutritional supplementation does not stop the wasting process; Evans and his colleagues (1985) have shown that the weight gain is transient and comprises fat and water and not lean body mass. A similar situation is seen in patients with HIV (Kotler *et al.*, 1990) or sepsis (Strent *et al.*, 1987), suggesting that weight loss results from complex metabolic events rather than simple nutritional insufficiency.

Both in rat and human, the loss of muscle and adipose tissue usually preceds a fall in food intake (Costa, 1963), and it has been shown that cachexia can occur without

anorexia. In some cases the anorexia may be apparent rather than real. This is usually due to loss of appetite. This explains the reason cachectic cancer patients have a decreased food intake per kilogram of their usual weight, however the food intake is not decreased when their current weight is taken into account.
These results suggest that tumour-induced weight loss occurs first, followed by a decrease in food intake, which matches the lowered body weight.

Research has shown that anorexia might be caused by a dysfunction in the hypothalamic membrane adenylate cyclase system in tumour-bearing rats, as reduced responsiveness to the inhibitory effect of neuropeptide Y and the stimulatory effect of isoprenaline was determined (Chance *et al.*, 1995); this with other studies suggested that the postsynaptic neuropeptide Y-signalling systems are altered in the hypothalamus of tumour-bearing rats. It has also been shown that enhanced brain availability of the amino acid tryptophan, a precursor of serotonin, plays a role in the pathogenesis of cancer anorexia by increasing ventromedial hypothalamic serotininergic activity.

Release of chemicals by the tumour or host immune system might also cause anorexia. Cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) act directly in the brain to produce anorexia. It has been shown that cytokines play an important role in long-term inhibition of feeding by mimicking the hypothalamic effect of excessive negative feedback signalling from leptin by persistent stimulation of anorexigenic neuropeptides such as corticotropin-releasing factor or by inhibition of the neuropeptide Y orexigenic network (Inui, 1999).

1.3 Energy Expenditure in Cancer Cachexia

If anorexia alone is not responsible for weight loss in cancer then a combination of relative anorexia and an increases energy expenditure could account for the wasting observed. In chronic starvation the energy expenditure decreases in response to a decrease in food intake. However, the metabolic response to cancer is highly variable, and in comparison with control groups, cancer patients have shown a reduced, normal, or increased energy expenditure (reviewed by Tisdale, 1997a). Many studies have been carried out to determine if the caloric intake in cancer patients is adequate for their

energy needs. De Wys *et al* (1981) showed that only 30% of cancer patients had a caloric intake that was sufficient to meet the needs for basal energy expenditure. In 40% of patients the caloric intake was greater than calculated for basal energy expenditure, but less than that required for moderate level of activity, and in 25% of patients the caloric intake was below the calculated basal energy expenditure. It has been calculated that an elevation of 12% in the metabolic rate could account for the loss of 1-2 kg of body weight per month (reviewed by Tisdale, 1997b).

Oudart *et al* (2000) investigated the energy expenditure and the body temperature in a well-characterised model of cancer cachexia (Yoshida sarcoma), and they found the resting energy expenditure (REE) to be higher in these animals in comparison to the control rats. However, the total energy expenditure did not differ between the tumour-bearing rats and controls, presumably due to the drop in activity of the cachectic tumour-bearing animals.

Tumour type appears to be a major determinant of an increased energy expenditure, for example, an elevation of REE has been described in lung cancer patients compared to control subjects, but it was not found in gastric and colorectal cancer patients (Fredix *et al.*, 1991; Jatoi *et al.*, 2001). There were no significant differences in energy intake between the cancer groups and the normal subjects. Falconer *et al* (1994) reported that patients with pancreatic cancer had an increased REE compared with that in control subjects, and this effect was found to be more pronounced in those patients with an acute-phase response. Other studies have found that cancer patients had an elevated REE compared with either weight-losing or weight-stable controls and the REE was related to an increased heart rate (Hyltander *et al.*, 1991). Thus particular changes related to the disease may be responsible for abnormalities in energy expenditure in cancer patients.

1.3.1 Carbohydrate Metabolism

The most likely explanation for an increased energy expenditure in cancer patients is due to an increased activity of the Cori cycle, whereby circulating lactate is converted into glucose by the liver. It has been shown that some tumours have a higher rate of anaerobic glycolysis resulting in an increased lactate production (reviewed by Lindsey,

1986). Glucose uptake and lactate release by human colon carcinomas have been found to exceed the peripheral tissue exchange rate by 30-fold and 43-fold, respectively (Holm *et al.*, 1995). Gluconeogenesis from lactate consumes six ATP molecules per lactate-glucose cycle and its particularly energy inefficient for the host. Tumour cells have an increased requirement for glucose as an energy source, either through an altered enzymatic activity, or due to relative hypoxia induced by a poor tumour vascularisation. Under these conditions, glucose may be the only utilisable metabolic substrate, since glycolysis is the only means of ATP production, which, does not require oxygen (reviewed by Tisdale, 1993a). Cachectic cancer patients have shown a 40% increase in hepatic glucose production compared with control subjects, however reduced levels of glucose production has been reported in patients with anorexia nervosa (Tayek, 1992).

The increase in glucose production in some cancer patients can be accounted for by an increase in Cori cycle activity. Patients with progressive cancer have been shown to have an increased glucose synthesis from lactate as well as alanine and glycerol (reviewed by Tisdale, 1997a, 2000). The Cori cycle normally accounts for 20% of glucose turnover, but it has been shown to be increased to 50% in cachectic cancer patients, accounting for the disposal of 60% of lactate produced (Holroyde *et al.*, 1975)

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. Impairment of insulin response to glucose administration has been shown to be greater in patients with cachexia. Although the number of peripheral tissue insulin receptors are not decreased, there is a decrease tissue sensitivity to insulin (reviewed by Lindsey, 1986). Cancer patients also have an increased glucose flux, which can 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 cachexia.

1.3.2 Lipid Metabolism

Loss of adipose tissue is a common factor in starvation and cancer cachexia, which is not surprising because fat comprises 90% of adult fuel reserves. Such fat is used to meet the increased metabolic demands of the host caused by the presence of the tumour.

Cachectic cancer patients showed increased glycerol and fatty acid turnover when compared with normal subjects (Shaw and Wolfe, 1987), and fasting plasma glycerol concentrations were higher, providing evidence for increasing lipolysis. Cancer patients have been shown to have an increased oxidation of fat and an increased rate of removal of infused lipids from the blood. Furthermore, increased utilisation of fatty acids as a preferred energy source has been observed even in the presence of high plasma glucose concentrations, suggesting that, in the presence of certain tumours, host tissue may increase their utilisation of fatty acids as an energy source (Waterhouse and Kemperman, 1971). Therefore, cancer patients deplete fat stores and consequently develop hypertriglyceridemia and decreased lipoprotein lipase (LPL) levels. The lower LPL levels result in failure of production of free fatty acids (FFA) and monoacylglycerol and thus, decrease adipocyte triglyceride synthesis, which depends on these fatty acids (reviewed by Puccio and Nathanson, 1997).

Since fat has a high calorific value it would be used predominantly to supply futile metabolic cycles in the host, such as the Cori cycle. In addition, some polyunsaturated fatty acids such as linoleic and arachidonic acids may be essential for tumour growth (Hussy and Tisdale, 1994). Thus mobilisation of lipids during the course of cachexia may be beneficial to both the host and the tumour and it might be an important factor contributing to tumour growth.

Several clinical studies (reviewed by Costa *et al*, 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, which 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 an individual with normal 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.

1.3.3 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 and it may also contribute to the immunologic abnormalities. Lean body mass and visceral protein depletion are characteristics of patients with cancer cachexia, and the degree of depletion may be associated with reduced survival (Nixon *et al.*, 1980). The major site of this protein loss has been observed to be the skeletal musculature (McMillan *et al.*, 1994). Studies in tumour-bearing rats showed that white or phasic muscle tends to atrophy more rapidly than red or tonic muscle (Clark and Goodlad, 1971). Peripheral muscle wasting may be due to increased muscle catabolism or decreased protein synthesis or a combination of the two.

Many studies reported increases in whole-body protein turn-over in cancer patients (Jeevanandam *et al.*, 1984), but such studies are complicated because non-skeletal muscle protein synthesis might actually increase, possibly because of hepatic production of acute phase protein (reviewed by Tisdale, 2001). Work by Lundholm *et al* (1976) showed a reduced rate of protein synthesis and an increased rate of degradation in muscle biopsy specimens from 43 newly diagnosed cancer patients with weight loss. He later showed that the net loss of protein was related to increased breakdown rather than to decrease synthesis of muscle protein (Lundholm *et al.*, 1982). However, Emery *et al* (1984) observed a decrease in muscle protein synthesis in cachectic patients, with no change in total body synthesis or degradation. In a study, where a group of weight-lossing cancer patients, in whom no changes in total-body-protein synthesis or degradation was detected, muscle protein synthesis accounted for only 8% of total body synthesis compared with 53% for healthy controls (Rennie *et al.*, 1983). However, there was a twofold increase in non-skeletal muscle protein synthesis, which may contribute to the maintenance of the total protein synthesis rate in these patients. Although the protein-synthesis rate is substantially reduced in cachexia, net loss of protein appears to be related to increased breakdown rather than decreased synthesis of muscle protein.

In the liver of cancer cachectic patients, despite low serum albumin levels, rates of albumin synthesis do not appear to be different from controls. In contrast, rates of fibrinogen synthesis are significantly elevated (reviewed by Barber *et al.*, 1999a). These

changes point to a different regulation of hepatic export protein synthesis in the cancer-bearing state. This reprioritisation of liver protein synthesis (commonly known as the acute phase protein response) occurs in trauma, infection and inflammation as well as in substantial proportions of cancer patients. About 40% of patients with pancreatic cancer exhibit an acute-phase response at diagnosis and this increases to around 80% at the time of death (Falconer *et al.*, 1995). Falconer *et al* also showed that the presence of an acute-phase protein response in pancreatic cancer patients is strongly associated with shortened survival, similar observations have been made for lung and renal cancer (Blay *et al.*, 1992; Staal-van den Brekel *et al.*, 1995). A possible explanation for the association between an acute-phase response and reduced survival is that the demand for amino acids to manufacture acute-phase proteins is met by breakdown of skeletal muscle and in the face of inadequate protein intake this leads to accelerated wasting and death.

There are also changes in the plasma amino acid profile in patients with cancer cachexia, and most studies have reported that such patients exhibit decreases in the concentrations of gluconeogenic amino acids. However, in subjects with severe malnutrition the concentration of branched amino acids in their plasma are normal or increased. Holm *et al* (1995) reported that human colon tumours have a specific requirement for serine and branched chain amino acids, i.e. valine, leucine, and isoleucine, compared with normal colon tissue. However, the two tissues had similar retention of total amino acids.

Studies have shown a circulatory factor in cachexia that initiates muscle protein degradation. In one study using a murine cachexia model, the MAC16 colon adenocarcinoma, it was shown that serum from cachectic animals was capable of increasing protein degradation in isolated gastrocnemius muscle, as measured by tyrosine release (Smith and Tisdale, 1993b). Further investigation showed that this effect was specific to the cachectic state, since serum from mice bearing the MAC13 adenocarcinoma, which did not produce weight loss, 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 appear to be mediated by catabolic factors present in the circulation.

1.4 Mediators of Cancer Cachexia

Weight loss often begins early in the course of malignant disease and correlates poorly with the size of malignant tumour. Thus, it would appear unlikely that the metabolic changes described above are the result of the metabolic demands of the tumour itself or by the body in response to the tumour.

There has been considerable research effort into elucidation of the nature of the postulated cachectic factors. The main contenders have been divided into two groups. (1) Materials with hormone-like characteristics, which results in direct catabolism of host tissue. (2) Products of host tissue, which influence host metabolism indirectly.

1.4.1 Cytokines

These belong to the latter group. It has long been appreciated that the intense antigenic stimulation of host immune system produced by cancer, resulted in production, synthesis and release of a number of cytokines. A number of studies have suggested that inappropriate release of cytokines might cause changes in host metabolism, which accompany tumour growth. In particular the cytokines TNF-α, interleukin (IL)-6, IL-1, interferon (IFN)-γ and leukaemia-inhibiting factor (LIF), produce in experimental animals some of the features seen in cancer cachexia (Matthys and Billiau, 1997). All cytokines produce a profound anorexia and cause inhibition of the clearing enzyme lipoprotein lipase (LPL), though with different potencies. It has been suggested that inhibition of LPL would prevent adipocytes from extracting fatty acids from plasma lipoproteins for storage, which would result in a net flux of lipid into the circulation. However, disruption of lipid metabolism through inhibition of LPL alone is unlikely to account for the fat cell depletion and wasting seen in cachexia, since in type I hyperlipidemia caused by an inherited deficiency in LPL, patients have normal fat stores and are not cachectic (Reviewed by Tisdale, 1997a). This fact, together with the inability of cytokines to explain all of the metabolic changes associated with cancer cachexia, has inspired investigators to search for tumour-produced catabolic factors that act directly on adipose tissue and skeletal muscle initiating the process of cachexia.

TNF-α was first identified as the mediator of anorexia/cachexia in trypanosome-infected rabbits. Both animals and human studies show that administration of TNF-α causes an initial drop in weight loss followed by the development of tachyphylaxis with repeated administration. Acute administration of TNF-α produced effects in cancer patients similar to those seen in cachexia (Starnes *et al.*, 1988). However, in chronic administration of TNF-α cachexia has not been reported as a major side effect (Bartsch *et al.*, 1989). Many more studies have been performed suggesting the function and ability of TNF-α as a factor capable of inducing cachexia. For example, in an experiment by Oliff *et al* (1987), Chinese hamster ovary cells were transfected with the gene for human TNF-α. Transplantation of such transfected cells into nude mice produced a syndrome resembling cancer cachexia with progressive wasting, anorexia and early death.

TNF-α has been shown in several studies to activate muscle protein degradation, although not all of the reports agree that TNF-α does activate protein degradation directly. In one study (Llovera *et al.*, 1993), TNF-α administration to healthy rats brought about an enhanced rate of degradation of skeletal muscle protein, even though body weight loss was not apparent in the animals. In another study (Costelli *et al.*, 1993), administration of anti-murine TNF-α immunoglobulin to rats bearing the Yoshida AH-130 ascites hepatoma led to decreases in the rates of protein degradation in the skeletal muscle, heart and liver tissues, but it had no effect on weight loss in the animals. Therefore, a direct and clear action of TNF-α has not been demonstrated by most researchers.

Production of TNF-α by skeletal muscle has been demonstrated and it has been reported to be involved in insulin resistance in cancer patients (Noguchi *et al.*, 1998). Indeed, skeletal muscle maybe an important source of both catabolic and anti-inflammatory cytokines. The balance between these two types of cytokines may well participate in the modulation of protein turnover and, therefore, muscle wasting. Furthermore, it has been suggested that other cytokines maybe involved in mediating TNF-α action in skeletal muscle and that anti-inflammatory cytokines maybe released as a counter regulatory mechanism (Alvarez *et al.*, 2002).

In addition, it has been difficult to measure TNF-α in experimental models of cachexia. Most studies have failed to show elevated levels of TNF-α in cachectic cancer patients, or have failed to associate the elevation with the development of cachexia (Maltoni *et al.*, 1997). In a study of patients with invasive breast cancer by Ming *et al* (1997) it was concluded that increased serum levels of TNF-α reflects the severity of the disease rather than the presence of cachexia; this was also concluded in gastrointestinal cancer patients (Bossola *et al.*, 2000). The inability to detect TNF-α in cachectic animal models and in patients with cancer cachexia has led to the suggestion that it acts as a paracrine/autocrine mediator rather than being the circulatory messenger in cachexia (Gelin *et al.*, 1991). Negri *et al* (2001) also concluded that central nervous system (CNS) activities of cytokines are involved in the induction of anorexia and wasting. They also concluded that the net effect of cytokines on energy balance dysregulation associated with wasting may depend on paracrine effects of their local concentrations in the brain with direct actions on specific brain target areas such as the hypothalamus.

However, elevated concentrations of TNF-α has been reported in patients with malaria and AIDS, which has been associated with death and cachexia. Thus the association of TNF-α as a mediator of cachexia is not conclusive.

Another cytokine thought to be involved in cancer cachexia is IL-1, which has been shown to have many effects similar to those of TNF-α. These similar effects include suppression of the lipoprotein lipase and enhancement of intracellular lipolysis. Administration of recombinant IL-1 was observed to induce anorexia, weight loss, hypoalbuminemia, and elevated amyloid P levels in the mouse (Moldawer *et al.*, 1988). However, there has been no reported cases in which serum IL-1 levels were found to be elevated, or any other experimental models of cachexia in which, the process of wasting was attenuated by antagonists to IL-1.

There is an abundance of data in the literature pointing to a pathophysiological role for interleukin 6 and its related cytokines such as IL-11, in cancer cachexia. Again there is a lot of controversy with respect to the role of IL-6 on muscle wasting and development of cachexia. Strassmann *et al* (1992) has given some evidence that IL-6 was involved in producing cachexia. In another study (Soda *et al.*, 1995), in which clonal variants of the

colon-26 tumour model were used, the serum concentrations of IL-6 in mice bearing a tumour clone that does not induce weight loss were lower than in mice bearing a tumour clone that does induce weight loss; however infusion of IL-6 into mice in the former group did not lead to body weight loss. These results indicate that IL-6 was not solely responsible for the induction of cachexia. Furthermore, like TNF-α, IL-6 has been found to be incapable of producing direct muscle protein degradation in an *in vitro* assay using isolated skeletal muscles (Garcia-Martinez *et al.*, 1994). Therefore, if IL-6 is responsible for muscle protein degradation that occurs during the process of cancer cachexia it must be acting indirectly.

IL-6 has also been identified as a mediator of cachexia by the growth of a uterine cervical carcinoma (Tamura *et al.*, 1995). Administration of a neutralising antibody against human IL-6 to mice, after the development of cachexia, was shown to reduce the loss of body weight and the wasting of adipose tissue.

It has been shown that in the presence of tumour burden, induced IL-6 and related cytokines are present not only in tissues adjacent to the tumour, but also at a site distant from the tumour (Burton and Murphy, 2000). Further work by these authors showed that cachexia in tumour-bearing mice developed due to systemic cytokine response induced by tumour burden, and that IL-6 like cytokines contributed independently to lipid hypercatabolism in the aetiology of cancer cachexia (Burton and Murphy, 2001).

Unlike TNF-α, increased circulatory levels of IL-6 are measurable in cancer patients. IL-6 is the main cytokine involved in the induction of hepatic acute-phase protein (APP) synthesis and elevated levels in a particular study have been reported in 39% of patients with lung cancer and an ongoing APP response (Yanagawa *et al.*, 1995).

The results of these animal and human studies strongly implicate IL-6 in the cachectic process. However, IL-6 probably does not act alone but may either induce or act in synergy with other cachectic factors.

Despite evidence that some cytokines play a role in the induction of cachexia, at least in experimental animal systems, other factors must also be involved. A study of the role of factors in the development of cachexia in nude mice bearing the human tumour

xenografts indicated that the known cytokines were only associated with four out of the eight models tested (Kajimura *et al.*, 1996). Such factors could include tumour catabolic factors, which have recently been described.

1.4.2 Tumour Catabolic Products

In addition to the cytokines, several studies have reported tumour-produced catabolic factors that act directly on skeletal muscle and adipose tissue. Such substances are present in the circulation and appear to act on the end organ in a hormone-like manner. Unlike cytokines elevated levels have been observed in cancer patients and they appear to induce weight loss by a mechanism not involving anorexia. They can be divided into two groups, those acting on adipose tissue and those acting on skeletal muscle.

1.4.2.1 Lipid Mobilising Factors (LMF)

Several studies 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 polypeptides hormones that are positively charged. Most studies provide evidence that the LMF is an acidic protein, although there appears to be variations in the molecular weight. A material termed toxohormone L was isolated from the ascitic fluid of patients with hepatoma or mice bearing sarcoma 180 (Masuno *et al.*, 1984). This substance was capable of inducing release of stored lipids from adipose tissue and was of molecular weight 70-75kDa and pH 4.7-4.8. Another group isolated a LMF of molecular weight 6kDa from the culture media of the human A375 melanoma cells lines (Taylor *et al.*, 1992).

In vivo studies in normal mice showed the LMF to produce weight loss and an 85% decrease in the weight of the fat pads compared to control mice. There was a 43% increase in serum triglycerides, which correlated with the mobilisation of stored lipids. These alterations occurred without an effect on food or water intake.

Evidence for LMF production by tumours was provided by Costa and Holland (1962), who showed that nonviable preparations of the Krebs-2 carcinoma, when injected into mice, were able to induce the early, rapid stage of fat depletion, which represented true

cachexia in this model. Furthermore, serum from mice bearing a thymic lymphoma when injected into non-tumour bearing controls produced massive fat loss (Kitada *et al.*, 1980), providing further evidence for an LMF, which was also detected in extracts of the tumour, in tissue culture medium, and in the sera of patients with adenocarcinomas of the cervix and stomach, thus suggesting that the LMF was tumour derived and circulatory.

The level of LMF in the sera of cancer patients was found to be proportional to the extent of weight loss (Groundwater *et al.*, 1990) and was reduced in those patients who showed a positive response to chemotherapy (Beck *et al.*, 1990a).The production of LMF in cancer appears to be related to the process of cachexia, since LMF is absent or present in reduced amounts in tumours that do not induce cachexia (Beck and Tisdale, 1987) and is absent from normal serum, even under conditions of starvation (Beck *et al.*, 1990b).

In the recent years LMF has been isolated first from the murine MAC16 adenocarcinoma and subsequently from the urine of cachectic patients with gastrointestinal and pancreatic cancers (Todorov *et al.*, 1998), by a combination of ion exchange, exclusion and hydrophobic interaction chromatographies, it was shown to be a 43kDa protein, homologous with the plasma protein Zn-α2-glycoprotein (ZAG) in primary sequence, electrophoretic mobility and immunoreactivity.

ZAG is a soluble protein, it has the tendency to participate zinc and has electrophoretic mobility in the region of α2 globulin. It is normally present in most body fluids including serum, sweat, saliva, cerebrospinal 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 cells lines. Hence, ZAG may participate in breast diseases including cancer (Freije *et al.*, 1991; Bundred *et al.*, 1987). There is a large homology between ZAG and class I histocompatibility antigens, which 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 manifestations of cachexia in the tumour-bearing host. Furthermore, the sequence homology existing between ZAG and LMF (Todorov *et al.*,

1998) could imply that ZAG may be involved in the induction of cachexia. Interestingly, it has been suggested that ZAG may have a role as a carrier for the lipolytic factor (McDevitt, 1996), possibly transporting conventional lipolytic hormones to adipose tissue. Previously ZAG has been found to have the role of 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.

In vivo studies confirmed the ability of LMF to cause selective loss of carcass fat with no change in body water, and a tendency to increase the non-fat mass. LMF was characterised by the ability to stimulate lipolysis directly in isolated adipocytes, as a result of stimulation of adenylate cyclase in a GTP-dependent process (Hirai *et al.*, 1998). In addition, adipocytes from cachectic mice bearing the MAC16 tumour showed a three-fold increase in lipolytic response to both isoprenaline and LMF compared with those from normal mice (Islam-Ali *et al.*, 2001). The effect appears to be due to modulation of G-protein expression, with an increase in Gαs and a decrease in Gαi, which is mediated by LMF. Thus LMF not only directly stimulates lipolysis, but also acts to sensitise adipose tissue to lipolytic stimuli.

Hirai *et al* (1998) showed that treatment of ob/ob mice with LMF, produced a specific depletion of the adipose mass together with an elevation of serum glycerol levels, as well as an increased oxygen uptake by interscapular brown adipose tissue (BAT). Pharmacological studies indicate that the β-receptor is responsible for the stimulation of oxygen consumption in BAT, and the receptor is exclusively of the β3-subtype (Howe, 1993). Induction of lipolysis in epididymal adipocytes by LMF was attenuated by the β3-adrenergic receptor blocker propranolol (Khan and Tisdale, 1999), while the biphasic effect of GTP on cAMP production by LMF in adipocyte plasma membranes suggests a receptor associated with both Gs and Gi. Only β3 and not β1-adrenergic receptor interact with Gi in adipocyte membranes (Granneman, 1995). In the light of this, Russell *et al* (2002), studied the ability of LMF to interact with the β3-adrenergic

receptor in white adipocytes and in CHO cells transfected with the human β3-adrenergic receptor. In both cases they concluded that LMF induces lipolysis through binding to a β3-adrenoreceptor.

Furthermore, the fate of the free fatty acids (FFA) and glycerol liberated from lipolysis induced by LMF is not known. These products must be catabolised and/or resynthesised in other sites to form triglyceride. It has been suggested that the uncoupling proteins (UCP-1, -2 and -3) members of the mitochondrial carrier family, may provide mechanisms for disposing of excess FFA (Samec *et al.*, 1998; Ricquier and Bouillaud, 2000). UCP-1 is expressed exclusively in BAT, a major heat producing tissue in rodents and human neonates. UCP-1 expression is stimulated by the sympathetic nervous system via β3-adrenoreceptors, being induced by cold exposure and β3-adrenoreceptor agonists, but fall on fasting (Bing *et al.*, 1997, 1998). UCP-2 and -3 are two newly described homologues of UCP-1. UCP-2 is widely expressed in most tissues including adipose tissue, muscle, heart and liver (Fleury *et al.*, 1997), while UCP-3 mRNA is preferably expressed in skeletal muscle and BAT (Boss *et al.*, 1997).
Many studies point to the fact that UCP-2 and -3 help to utilise and dispose of excess lipid. They are therefore strong candidates for removing lipolytic products generated by LMF in cancer cachexia. This hypothesis is supported by increased expression of UCP-1 in BAT, and of UCP-2 and -3 in skeletal muscle in mice bearing the MAC16-tumour, which produces LMF (Bing *et al.*, 2000). Furthermore, it has been shown by Bing *et al* (2002) that LMF upregulates UCP-1, -2 and -3 in BAT, and UCP-2 in skeletal muscle and liver, which suggests that these uncoupling proteins may serve to utilise excess lipid mobilised during fat catabolism in cancer cachexia.

Unlike the effect on adipose tissue, LMF has an anabolic effect on skeletal muscle. Thus protein synthesis was increased by 69% in soleus muscle of mice administered LMF, while there was a 26% decrease in protein degradation (Islam-Ali and Tisdale, 2001). In murine myoblasts, LMF increased protein synthesis, which was linearly related to an increase intracellular cyclic AMP, and it was attenuated by the adenylate cyclase inhibitor MDL_{12330A}. Therefore, by increasing protein synthesis in skeletal muscle, LMF may modulate the rate of loss of skeletal muscle and as such be antagonistic to tumour proteolytic factors, which are discussed below.

1.4.2.2 Proteolysis Inducing Factor (PIF)

Using bioassays to detect protein degradation, investigators have found evidence for the existence of Protein-Mobilising Factors in the sera of both animals (Smith and Tisdale, 1993b) and humans (Belizario *et al.*, 1991) with cancer cachexia. The bioactivity is capable of producing protein degradation and therefore, associated with the loss of skeletal muscle mass. Such bioactivity was later termed the proteolysis-inducing factor (PIF). This material has now been purified from a cachexia-inducing murine tumour (MAC16) and from the urine of patients with cancer cachexia by use of affinity chromatography with a monoclonal antibody derived from mice bearing the MAC16 tumour (Todorov *et al.*, 1996b).

This material from both murine and human sources appeared to be identical and was characterised as a sulphated glycoprotein of a molecular mass of 24-kDa, consisting of a central polypeptide chain of M_r 4000 with phosphate residues attached, one O-linked sulphated oligosaccharide chain of M_r 6000 and one N-linked sulphated oligosacchride chain of M_r 10000 (Todorov *et al.*, 1996a; Todorov *et al.*, 1997). The sequence of amino acids in the protein core was totally distinct from that of any reported proteins.

Although PIF was readily detected in the urine of cachectic cancer patients, irrespective of the tumour type, it was absent from the urine of cancer patients with little or no weight loss, from the urine of normal subjects or from the urine of patients with other weight-losing conditions such as major burns, multiple injuries, or surgery associated catabolism, acute pancreatitis or sleeping sickness and sepsis, suggesting that it might be confined to cachexia (Todorov *et al.*, 1996a; Cariuk *et al.*, 1997). Administration of PIF to non-tumour-bearing mice produced a state of cachexia, with rapid weight loss of about 10% of body weight over a 24 h period (Todorov *et al.*, 1996a). Weight loss occurred despite normal food and water intake, further suggesting that cachexia can occur in the absence of anorexia. Body composition analysis of treated animals showed a significant fall in non-fat carcass mass, without a change in either the water or fat compartments. Both weight loss and the loss of the non-fat carcass mass could be attenuated by pre-treatment of the mice with the mouse monoclonal antibody (Todorov *et al.*, 1996a; Cariuk *et al.*, 1997).

PIF has also been determined in human melanoma cell lines G361 (Todorov *et al.*, 1999). Human melanoma, G361, has been reported to induce severe cachexia on tumour bearing nude mice and this has previously been correlated with the expression of the cytokine leukaemia inhibitory factor (LIF) (Mori *et al.*, 1991). However, numerous studies have shown that LIF alone cannot cause the state of cachexia and that, factors other than LIF may be responsible for the cachexia induced by G361 cells. It was shown that PIF purified from G361 cells, is capable of producing loss of body weight in mice, with specific loss of the carcass non-fat mass. This was demonstrated by administration of PIF from G361 cells to female NMRI mice (20g), which lead to a pronounced depression of body weight over a 24 h period without a decrease in either food or water consumption (Todorov *et al.*, 1999). Further data has shown the expression of PIF in tumours and mesenchymal tissue of patients who have PIF detectable in their urine, and is strongly associated with weight loss in cancer patients (Cabal-Manzano *et al.*, 2001). It was concluded that tumours are the source of PIF in patients with gastrointestinal cancers, and the expression of PIF in tumours correlates directly with its presence in urine.

The relationship between PIF and acute-phase protein response has been investigated by determining the nutritional characteristics of patients with pancreatic cancer who excrete PIF in their urine (Wigmore *et al.*, 2000b). Previous studies have indicated that the acute-phase protein response is persistently raised in approximately 50 per cent of patients with pancreatic cancer and is associated with reduced protein-energy intake, increased basal metabolic rate and increased rate of weight loss. However no significant difference was found between C-reactive protein (CRP) concentration in patients who expressed PIF compared with those who did not. Therefore, the weight loss cause by PIF in cachectic patients appears to be independent of CRP concentration.

PIF has been shown to induce protein degradation in isolated gastrocnemius muscle, an effect mediated through the N- and O-linked oligosaccharide chains (Todorov *et al.*, 1996b); also in soleus muscle (Lorite *et al.*, 1997). *In vivo* studies have shown an increase (by 50%) in protein degradation and a decrease by (50%) in protein synthesis in gastrocnemius muscle, suggesting a bimodal attack on protein balance. Protein degradation *in vitro* has been associated with a significant elevation of prostaglandin E_2 (PGE_2), which may act as an intracellular messenger, since in a study by Lorite *et al*

(1997) they showed that attenuation of protein breakdown with a monoclonal antibody to PIF also inhibits the prostaglandin E_2 response.

The intermediate metabolite of the anticancer drug capecitabine, fluorinated 5'deoxy-5-fluoruouridine (5'-dFUrd) interferes with PIF biosynthesis and therefore, may have an anti-cachectic role (Hussey *et al.*, 2000). 5'-dFUrd has been shown to reverse the wasting in the colon 26-adenocarcinoma cachexia model (Tanaka *et al.*, 1990). An attempt was made to correlate the anti-cachectic activity of 5'-dFUrd with the presence of a tumour produced PIF. Treatment of animals bearing the colon 26-carcinoma and showing evidence of PIF, led to the disappearance of PIF from the tumour, serum and urine concomitant with the attenuation of the development of cachexia. The human cervical carcinoma, Yomoto, which also induced cachexia in recipient animals, showed expression of PIF in tumour, serum and urine in control mice, but it was absent in mice treated with 5'-dFUrd (Hussey *et al.*, 2000).

1.4.3 Other Putative Mediators of Cachexia

Several other factors have been postulated as possible mediators of the cachectic state. Hormones are very important in the intermediary metabolism of carbohydrates and many of them have been suggested to play a role in cancer cachexia such as, insulin, glucagon, corticotropin, adrenaline, human growth hormones and insulin-like growth factor (Puccio and Nathanson, 1997). 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. 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.

A review of the metabolic and pathophysiologic characteristics of cachexia syndrome has suggested that multiple rather than single mediators probably account for this syndrome. It may well be that tumours produce multiple active peptides or glycopeptides, which in-turn stimulate the induction of additional cytokines or lymphokines from host immune cells, and they cause abnormal hormonal responses to the metabolic stress that is posed by malignancy.

1.5 Skeletal Muscle Proteolytic Systems

Proteins in skeletal muscle, as in other mammalian tissues, undergo a continuous process of synthesis and degradation. The rate of protein turnover regulates levels of specific proteins, as well as the overall muscle protein mass during growth and atrophy. For example, increased proteolysis contributes to the muscle wasting seen with fasting and many pathological states such as sepsis, chronic renal failure, trauma and cancer. In such states, enhanced protein breakdown in skeletal muscle, the major protein reservoir in the body, is a key metabolic adaptation providing the organism with free fatty acids for energy metabolism by increased gluconeogenesis and direct oxidation. However, despite its importance in growth and human disease, the regulation of muscle proteolysis by nutritional, hormonal and mechanical factors remains poorly understood (Sugden and Fuller, 1991).

Like many mammalian tissue, skeletal muscle contains multiple proteolytic systems that are presumably responsible for the breakdown of specific proteins, the three major proteolytic systems, which operate in skeletal muscle are (Attaix and Taillandier, 1998):

1. The lysosomal proteases – The cathepsins
2. Ca^{2+}-dependent cysteine proteases – The calpains
3. Ubiquitin-proteasome-dependent pathway

However, such a division is a gross over-simplification. Conceivably, the breakdown of a particular protein can be catalysed by several proteolytic systems, which may act separately or co-ordinately.

The best-known proteolytic system is the lysosomal pathway. The major lysosomal proteinases (cathepsins B, H, L and D) do not contribute significantly to overall protein breakdown in muscle (Furuno *et al.*, 1990; Wing and Goldberg, 1993; Temparis *et al.*, 1994; Tiao *et al.*, 1994; Baracos *et al.*, 1995; Taillandier *et al.*, 1996; Voisin *et al.*, 1996). In addition, lysosomes are not involved in the degradation of myofibrillar proteins (Lowell *et al.*, 1986; Furuno *et al.*, 1990; Tiao *et al.*, 1994). This pathway is presumably responsible for the breakdown of some long-lived, membrane and endocytosed proteins (Mitch and Goldberg, 1996). Increased cathepsin activities and

mRNA levels have been reported in some, short term fasting (Wing and Goldberg, 1993), but not in all instances of muscle wasting. In general, data suggests that lysosomal proteolysis plays a minor role in muscle protein breakdown.

The best-characterised Ca^{2+}-dependent enzymes are μ-calpain and m-calpain, which are non-lysosomal proteases that differ in their affinity for Ca^{2+}. The activity of these ubiquitous cysteine proteases are regulated by Ca^{2+}, and their endogenous inhibitor, calpastatin. A third calpain p94, that is expressed abundantly only in skeletal muscle, has been characterised (Sorimachi *et al.*, 1989).

The Ca^{2+}-dependent process does not contribute significantly to overall proteolysis in muscles from control and catabolic animals (Wing and Goldberg, 1993; Tiao *et al.*, 1994; Taillandier *et al.*, 1996; Temparis *et al.*, 1994; Baracos *et al.*, 1995), except in muscular dystrophy (Turner *et al.*, 1989). There are few biological models in which significant changes in calpain activities occur, and evidence that Ca^{2+}-dependent proteinases are activated in any muscle wasting condition is lacking. The Ca^{2+}-dependent proteinases may be qualitatively important for the degradation of specific but quantitatively minor muscle proteins. They are involved in rapid removal of Z-disks and N_2 lines and degrade nebulin, titin, troponin, tropomyosin, but not myosin and actin (Goll *et al.*, 1992). Therefore, calpains play a more crucial role in comparison to cathepsins, which is probably the rate-limiting step in the breakdown of myofibrillar proteins (Solomon and Goldberg, 1996).

In the recent years it has become apparent that both lysosomal and Ca^{2+}-activated proteases do not play a major role in skeletal muscle protein breakdown, they contribute less that 15-20% of total protein breakdown in muscles from control and cachectic animals.

By contrast, recent experiments strongly suggest that the ATP-ubiquitin-dependent proteolytic pathway, which is widely believed to degrade abnormal and short-lived proteins (Rechsteiner, 1991; Hershko and Ciechanover, 1992), is the critical system responsible for the breakdown of most skeletal muscle proteins, including the long-lived contractile components (Furuno *et al.*, 1990; Wing and Goldberg, 1993). Furthermore,

the activation of the ubiquitin proteasome pathway is mainly responsible for muscle wasting that prevails in various animal models of cachexia, as well as in some human diseases (Attaix and Taillandier, 1998; Attaix *et al.*, 1994).

1.5.1 The Ubiquitin Proteasome Pathway

Ubiquitin is an abundant highly conserved 76-residue polypeptide, which seems to be present in all eukaryotic cells either free or covalently linked to other proteins. The first step in the ubiquitin-proteasome pathway is the covalent attachment of polyubiquitin chains to the degraded protein. Ubiquitin is first activated in the presence of ATP to a high-energy thiol ester intermediate by the ubiquitin-activating enzyme (E1). Following activation, ubiquitin-conjugating enzymes (E2s) transfer ubiquitin from E1 to ubiquitin protein ligases (E3s). In turn, E3s covalently bind ubiquitin to protein substrates via an isopeptide bond between the activated C-terminal residue of ubiquitin and ε-amino groups of lysine residues in protein substrate (figure 1.1), (reviewed by Hasselgren and Fischer, 1997; Attaix *et al.*, 1998). 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 (reviewed later). 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.

Degradation by the 26S proteasome results in the release of peptides and free ubiquitin, which is also energy dependent. The number of ubiquitin molecules conjugated to each protein is specific for certain proteins and also influences the rate by which the proteins are degraded.

After proteolysis, deubiquitination takes place, where the covalent bond between the ubiquitin and the protein can be cleaved by one of the ATP-independent proteases; ubiquitin is released from the polyubiquitin chain and can be reused in the proteolytic pathway.

Degradation signals are features of proteins that confer metabolic instability. Degradation signals can be active constitutively or conditionally.

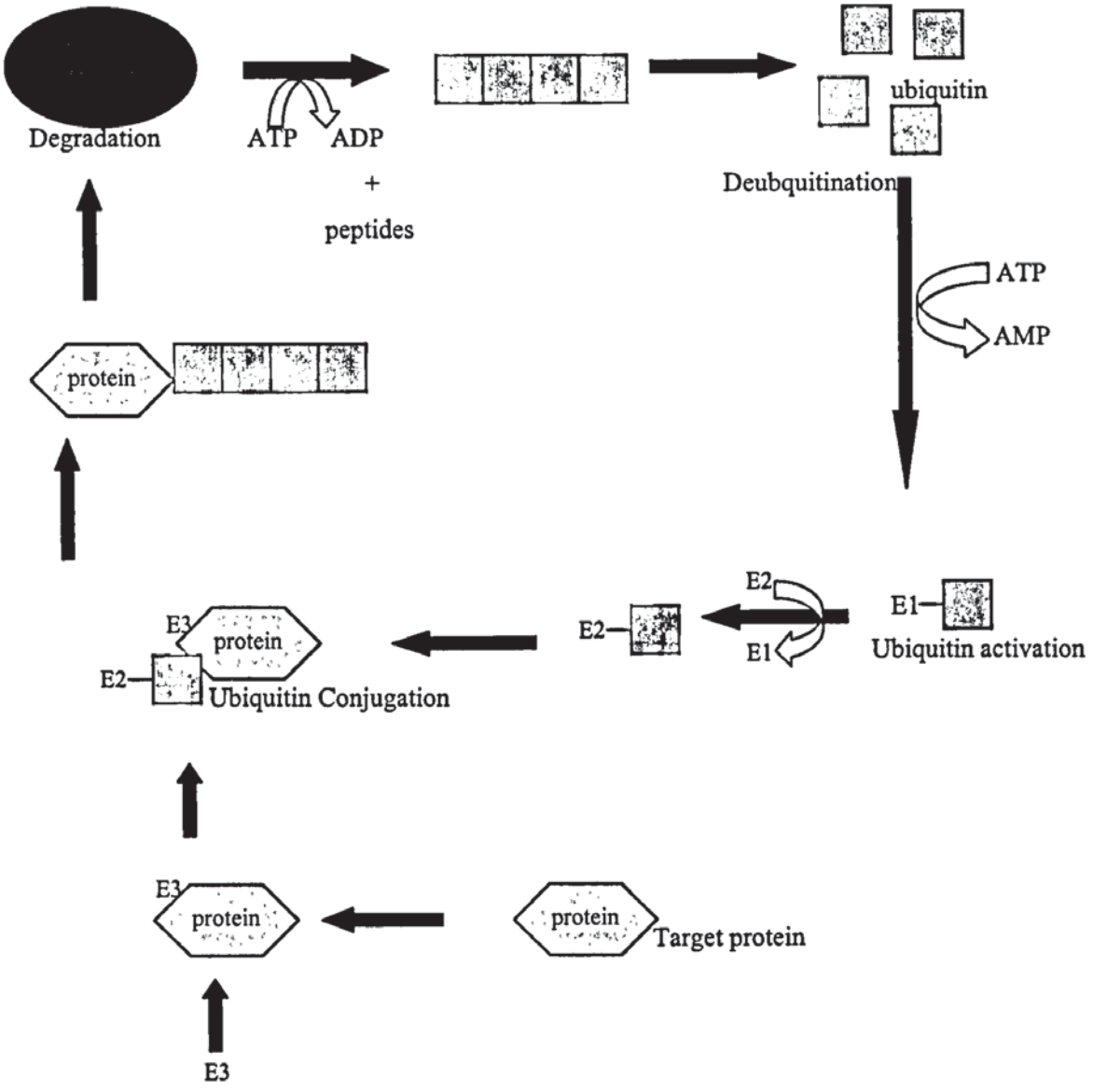


Figure 1.1. Schematic representation of the ubiquitin-proteasome proteolytic pathway; involving the E1, ubiquitin-activating enzyme, E2, ubiquitin-conjugating enzyme, E3, ubiquitin ligase.

1.5.2 Regulation of Ubiquitin Conjugation in Skeletal Muscle

1.5.2.1 Ubiquitin Expression

Increased expression of skeletal muscle ubiquitin has been reported in several catabolic states in rodents. This adaptation reflects increased transcription. Increased ubiquitin mRNA levels were observed in both types I and II skeletal muscle fibres from starved rats, but not in liver, kidney and adipose tissue, which also undergoes significant atrophy (Medina *et al.*, 1995). However, increased expression of ubiquitin was also found in the heart (Medina *et al.*, 1995) and intestines (Samuels *et al.*, 1996) in starved rats, and skin from tumour bearing animals (Baracos *et al.*, 1995). It has been shown that high mRNA levels for ubiquitin do not systematically reflect increased protein breakdown in skeletal muscle; although ubiquitin is a heat shock protein, there was no coordinate increase in expression of other heat shock genes in the atrophying muscles from starved, denervated and cancer rats (Baracos *et al.*, 1995; Medina *et al.*, 1995).

The precise mechanisms, which result in increased ubiquitin expression, has not been elucidated. However, there is increased production of glucocorticoids in several catabolic states (Tiao *et al.*, 1996; Wing and Goldberg, 1993). Ubiquitin expression is also regulated by insulin (Larbaud *et al.*, 1996), and indirectly by several cytokines including TNF-α (Llovera *et al.*, 1996).

1.5.2.2 Ubiquitin Conjugating Enzymes (E2s)

Ubiquitin conjugating enzymes represent a superfamily of related proteins, with a molecular weight range of usually 14-35 kDa. Various E2 species have been found in the skeletal muscle, these include the 14-, 17-, and the 20-kDa species. However, only the expression of the 14-kDa E2 was found to be regulated in muscle wasting (Attaix and Taillandier, 1998). The 14-kDa E2 has two transcripts (1.8 and 1.2 kb) arising from different polyadenylation sites (Wing and Banville, 1994). The 1.8-kb transcript, which corresponds to the major mRNA species in rat muscle, is barely detectable in humans (Mansoor *et al.*, 1996) and goats (Larbaud *et al.*, 1996). The 1.2-kb transcript is tightly upregulated in various conditions of muscle atrophy in rats and humans (Mansoor *et al.*, 1996). Taillandier *et al* (1996) have shown that the increase mRNA levels of the 14-kDa

E2, which occurred in the atrophying soleus muscle from unweighted rats, entered active translation. However, it has also been shown that increased expression of the 14-kDa E2 does not always correlate with enhanced protein breakdown (Temparis *et al.*, 1994). The expression of the 14-kDa E2, has been shown to be regulated by glucocorticoids in adult rats, but not in old animals (Dardevet *et al.*, 1995). Furthermore, *in vitro* insulin and insulin-like growth factor I decrease stability of the 14-kDa E2 mRNAs.

1.5.2.3 Ubiquitin-Protein Ligases (E3s)

To date, 25 ubiquitin-ligases have been identified. There are at least six distinct E3s in mammalian cells. These enzymes play a role in the selection of substrates targeted for degradation by the ubiquitin-proteasome system (Hass and Siepmann, 1997). The 14-kDa E2 is one of the major mammalian E2s that best supports E3-dependent conjugate formation and protein breakdown (Hershko and Ciechanover, 1992). Individual E3s account for substrate specificity and also act in concert with specific E2s (Hershko and Ciechanover, 1998).

One of the known ubiquitin ligases is E3α, which regulates the ubiquitination of proteins in the so-called N-end rule pathway (Varshavsky, 1997). Proteins degraded in this pathway have a destabilising N-end consisting of one of the basic amino acids Phe, Leu, Trp, Tyr or Ile and an internal Lys to which ubiquitin is conjugated by the 14 kDa ubiquitin-conjugating enzyme $E2_{14k}$ (Varshavsky, 1997). Reports by Solomon *et al* (1998a, 1998b) suggests that a large proportion of muscle proteins are degraded in the N-end rule pathway and that this pathway may account for stimulated muscle proteolysis in sepsis and cancer. Furthermore, increased mRNA expression has been reported in skeletal muscle of septic rats (Fischer *et al.*, 2000b).

Furthermore, in an attempt to identify candidate molecular mediators of muscle atrophy, two genes were found by Bodine *et al* (2001), which encoded ubiquitin ligases that were called Muscle RING Finger 1 (MuRF1), and a Muscle Atrophy F-box (MAFbx). Over-expression of MAFbx in C_2C_{12} myotubes produced atrophy, whereas mice deficient in

either MAFbx or MuRF1 were found to be resistant to atrophy (Bodine *et al.*, 2001), implicating the involvement of ubiquitin-ligases in muscle cachexia.

1.5.2.4 Ubiquitin-Protein Conjugates

During different catabolic conditions there is an increase in ubiquitin-protein conjugates in skeletal muscle. This strongly suggests that the degradation of ubiquitin-conjugates by the 26S proteasome is rate limiting in these catabolic states. However, other studies, have given conflicting results. For example, no accumulation of ubiquitin-conjugates were observed in the muscles from septic (Tiao *et al.*, 1994) or tumour-bearing animals, even though a clear increase in ubiquitin expression occurs in such conditions (Temparis *et al.*, 1994; Tiao *et al.*, 1994). In contrast, a decrease in ubiquitin-conjugates was observed during corticosterone treatment (Auclair *et al.*, 1997). The reasons for such discrepancies are unclear, although it is possible that in some cases the changes in the levels of ubiquitin-conjugates may result from alterations in their rates of degradation. It has been observed that ubiquitylated proteins accumulate preferentially in the myofibrillar fraction, although other research has shown conflicting findings (Tiao *et al.*, 1994). Therefore, the proteins that are preferentially ubiquitylated in skeletal muscle remain to be identified. Further, it seems unlikely that the amount of free muscle ubiquitin would become rate limiting for conjugation.

1.5.3 The Proteasome

The proteasome is a multisubunit protease complex, which catalysis protein degradation. It consists of a central catalytic (20S) core and regulatory subunits.

Two large proteases have been isolated from cells, one being the 20S proteasome and the other, an ATP-activated protease with an apparent sedimentation coefficient of 26S (Rechsteiner *et al.*, 1993). 26S proteasomes are multisubunit protease complexes engaged in the turnover of short-lived proteins that regulate a variety of cellular processes including signal transduction, stress response, transcriptional control, chromosome segregation, DNA repair and cell cycle progression (Hershko and Ciechanover, 1998). The 26S protease was shown to contain the 20S proteasome

together with multiple components containing ATPases (figure 1.2) (Glickman *et al.*, 1998).

The 20S proteasomes from tissues and cells are a mixture of several subtypes. Six different subtypes have been purified from rat skeletal muscle using high-resolution anion exchange chromatography (Dahlmann *et al.*, 2001).

The 20S proteasome was found initially as a multifunctional protease complex with diverse peptidase activities, which was later demonstrated as the catalytic portion of the 26S proteasome. The molecular weight of the 20S proteasome is 700-750 kDa. The 20S proteasome is composed of a set of small subunits of 21-32 kDa, having strikingly different isoelectric points of 3-9 and being arranged in a stack of four rings that comprise a cylindrical particle (reviewed by Tanaka, 1998a). These proteasome subunits have been divided into two distinct subfamilies, α and β (Coux *et al.*, 1996). The nomenclature of the subunits of the eukaryotic proteasome is summarised in table 1.1. Seven homologous α- and β-polypeptides are assembled into each α- and β-ring, respectively, as observed by electron microscopy; and these rings are associated in the order αββα to form the cylindrical particle (figure 1.2). The primary structures of these subunits show considerably high inter-subunit homology within species, and high evolutional conservation in various eukaryotes, suggesting that they constitutes a multi-gene family and may have originated from a common ancestral gene. As was shown by X-ray crystallography 7 α- and 7 β-subunits have been found in yeast (Groll *et al.*, 1997); but in humans 7 α- and 10 β-subunits have been reported (reviewed by Tanaka, 1998a & 1998b) (table 1.1). The existence of three extra β-subunits was puzzling, because the β-ring is composed of seven subunits. Later, interferon-γ (IFN-γ), a major immunomodulatory cytokine, was found to induce replacement of three constitutively expressed β-type subunits (X, Y and Z) down-regulated in response to IFN-γ, by IFN-γ-inducible subunits (termed LMP7, LMP2 and MECL-1, respectively) having high amino acid similarity to the subunit replaced, thus producing "immunoproteasomes" responsible for production of MHC class I ligands through immunological processing of intracellular antigens (Tanaka and Kasahara, 1998).

Table 1.1: Orlowski and Wilk, 2000

Subunits of the proteasome from human and yeast

Systemic name	Yeast	Human
α1	PRS2	Iota/PRS2
α2	PRS4/Y7	C3
α3	PRS5/Y13	C9
α4	PRE6	C6/XAPC7
α5	PUP2	ZETA
α6	PRE5	C2 (PROS30)
α7	PRS1	C8
β1	PRE3	Y/delta
β1-i	_	LMP2-1
β2	PUP1	Z
β2-i	_	LMP10/MECL-1-i
β3	PUP3	C10-II
β4	PRE1	C7-I
β5	PRE2	X/MB1
β5-i	_	LMP7-i
β6	PRS3	C5
β7	PRE4	N3

The 20S proteasome exists as a latent form in cells, which is consistent with the structural finding of the α-ring, where it is found to be closed, preventing penetration of proteins into the inner surface of the β-ring on which the proteolytically active sites are located (Baumeister *et al.*, 1998). Thus, interaction between 20S core particles and regulatory complexes such as PA700 (also known as the 19S complex) or PA28 (equivalent to the 11S regulator) presumably opens the proteasome channel for entry of the protein substrate.

The regulatory complex PA700 (or the 19S) can associate with the 20S proteasome in an ATP-dependent manner to form the 26S proteasome with a molecular mass of about 2000 kDa (Baumeister *et al.*, 1998). This structure is a dumbbell-shaped particle, consisting of a centrally located, cylindrical 20S proteasome that functions as a catalytic machine, and two large terminal PA700 modules attached to the 20S core particle in opposite orientations (Figure 1.2). PA700 contains approximately 20 heterogeneous subunits of 25-110 kDa, which can be classified into two subgroups: a subgroup of at

least six ATPases, that are structurally similar and have been highly conserved during evolution, and a subgroup of over fifteen heterogeneous subunits that are structurally unrelated to the members of the ATPase family (Hilt and Wolf, 1995; Coux *et al.*, 1996).

Figure 1.2. 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).

So far, at least 6 different species of ATPases belonging to the same family have been shown to be associated with the 26S proteasome. These 6 ATPases belong to the large protein family termed AAA proteins (ATPases associated with a variety of cellular activities), which are characterised by the conserved 200 amino acid domain containing a consensus sequence for an ATP binding module. These 6 ATPases are suspected to be assembled into one ring complex that may interact with the outer α-ring of the central

20S proteasome, however this is a speculation and no experimental evidence for this theory is available yet. The role of the ATPase is to supply energy continuously for the degradation of target proteins. Presumably, the energy is utilised to unfold the proteins so that they can penetrate the channel of the α- and β-rings of the 20S proteasome. In addition, ATP is thought to be also utilised for assembly of the 26S proteasome from the 20S proteasome and PA700. However, it is still unknown why multiple homologous ATPases are present in the 26S proteasome complex.

The PA700 regulatory non-ATPase subunits seem to play a pivotal role in the function of the 26S proteasome. However, the functions of many of these non-ATPase subunits are largely unknown with the exception of two of them namely: the multi-ubiquitin receptor and the de-ubiquitinating enzyme, which are involved in trapping of ubiquitinylated target proteins (van Nocker *et al.*, 1996) and recycling of ubiquitin moieties, respectively.

PA28 or the 11S regulator was identified as a novel activator of the latent 20S proteasome (Ma *et al.*, 1992). The purified PA28 greatly stimulated multiple peptidase activities of the latent 20S proteasome. Electron microscopic investigation revealed that PA28 associates with both ends of the 20S proteasome to form a football like proteasome (Gray *et al.*, 1994) (fig 1.2). Association of PA28 with the 20S proteasome does not require ATP hydrolysis; furthermore, PA28 occupies the same sites on the 20S core particle as the regulator complex does in the case of the 26S proteasome.
cDNA cloning showed that PA28 is composed of two related proteins, named PA28α and PA28β (Ahn *et al.*, 1995), with approximately 50% amino acid sequence identity. PA28α and PA28β assemble into a heterohexameric complex with alternating α and β subunits, i.e., $PA28(\alpha\beta)_3$ (figure 1.2). Expression of PA28α and PA28β is strongly induced by IFN-γ, which suggests their involvement in the immune response (Schmidt and Kloetzel, 1997). Although the $PA28(\alpha\beta)_3$ complex greatly stimulate multiple peptidase activities of the 20S proteasome, it fails to enhance hydrolysis of large protein substrates with native or denatured structures, even though the proteins have been ubiquitinylated. Thus, PA28 does not play a central role in the initial cleavage of protein substrates. It presumably has a stimulating effect on the degradation of polypeptides of intermediate size that are generated by the 26S proteasome, implying that the 26S

proteasome and the PA28-proteasome complex may function sequentially or cooperatively.

Recently, an antibody against p45 ATPase, a subunit of the PA700 complex, was shown to immunoprecipitate not only a set of 26S proteasome components, but also PA28α and PA28β, indicating simultaneous binding of PA28 and PA700 activators to the 20S proteasome (Hendil *et al.*, 1998). Thus, a "hybrid type proteasome" might exist and may contribute to more efficient proteolysis; perhaps intact substrate proteins are recognised first by PA700 and fed into the cavity of the 20S proteasome whose cleavage ability is greatly stimulated by the PA28$(\alpha\beta)_3$ complex.

1.5.4 Biochemical Adaptations Activating Muscle Proteolysis

The conclusion that muscle wasting in various catabolic states is due primarily to the activation of the ubiquitin-proteasome pathway has received strong support from studies using selective inhibitors of the pathway. Peptide aldehydes such as MG 132 block several peptidase activities of the 20S proteasome and reduce the degradation of ubiquitin-conjugated proteins in cells (Tawa *et al.*, 1995). In muscles from normal rats, MG 132 reduces overall protein degradation (reviewed in Mitch and Goldberg, 1996). This suggests that the pathway is essential for the enhancement of proteolysis and the inhibitors of this pathway may someday be used to reduce muscle wasting in patients.

What are the critical adaptations leading to the activation of the ubiquitin-proteasome pathway in disease state? Studies, using muscle homogenates from rats with sepsis or tumour demonstrated that the rate of ubiquitin conjugation to proteins increases by a factor of two to three. Furthermore, in conditions associated with decreased muscle proteolysis, the rate of conjugation to ubiquitin is low. Therefore, activation or suppression of this pathway results from changes in the rate of ubiquitin conjugation, apparently because of alterations in the activity of E2 carrier proteins and E3 enzymes.

The increased levels of mRNAs encoding ubiquitin and proteasome subunits in all the catabolic states that have been studied suggest the activation of a common process that enhances the expression of these genes. Although the mRNAs for only few proteasome

subunits and one E2 carrier protein have been studied, it seems likely that mRNAs for most of the 25 to 30 subunits of the 26S proteasome and perhaps multiple E2 carrier proteins or E3 enzymes are co-ordinately increased (Medina *et al.*, 1995). It has been shown that this increase in mRNAs for ubiquitin and proteasome subunits is due to increased gene transcription, despite a general reduction in mRNA in the same muscles (Bailey *et al.*, 1996). Presumably, the increased muscle proteolysis in other catabolic states is also associated with an increased transcription of genes for components of the ubiquitin-proteasome pathway. Therefore, a specific transcriptional programme is available that favours muscle atrophy, but whether an increased expression of any single component of the ubiquitin-proteasome pathway causes the enhanced proteolysis is unknown.

What circulating factors trigger the enhanced protein degradation in muscle? It has been shown that in denervated or immobilised muscles, the signal originates within the inactive cells, whereas, under the conditions of fasting, acidosis, sepsis, or cancerous cachexia, hormones and cytokines trigger muscle proteolysis.

Glucocorticoids are required for the increase in muscle proteolysis, which was seen in fasting as well as in other pathological states. Wing and Goldberg (1993) reported that adrenalectomy partially prevented the increase in skeletal muscle proteolysis and totally suppressed the increase in ubiquitin mRNA levels normally seen in fasted rats. Glucocorticoids appeared essential for the activation of the ATP-dependent process, but not for the rise in lysosomal proteolysis in fasting. Glucocorticoids may also be required for increased expression of ubiquitin and 20S proteasome subunits in skeletal muscle following metabolic acidosis (Price *et al.*, 1994).

The chief factor opposing the catabolic effects of glucocorticoids is insulin. In the absence of fasting, insulin normally suppresses the breakdown of protein. Glucocorticoids can increase proteolysis when added directly to isolated muscles, but not if insulin is also present (Wing and Goldberg, 1993).

Insulin has a well-known antiproteolytic effect in muscle preparations. The hormone downregulated mRNA levels for the 14 kDa E_2 in cultured myoblasts, suggesting an inhibition of the ubiquitin proteasome pathway (Wing and Banville, 1994). Insulin-like growth factor-I (IGF-I) also has an antitiproteolytic effect; it has been shown to reduce

14-kDa E_2 mRNA levels in L6 myoblasts but showed 100-fold more potency than insulin (Wing and Bedard, 1996).

Finally, there is some evidence that hypothyroidism may result in reduced ATP-dependent proteolysis by altering the content of 20S and 26S proteasomes in skeletal muscle. Conversely, the administration of thyroid hormone stimulated ubiquitin-proteasome dependent proteolysis in rat skeletal muscle (Tawa *et al.*, 1997).

Many of the physiological responses in sepsis, cancers and burns result from the release of tumour necrosis factor and interleukins from activated macrophages and endothelial cells. Llovera *et al* (1996) demonstrated the important role of TNF-α in the activation of the ATP-ubiquitin-dependent proteolytic pathway in tumour-bearing rats. Anti-TNF treatment reverted increased expression of ubiquitin and 20S proteasome subunits in such animals. In addition, IL-6 was also shown to increase the expression of subunits of 20S (C2, C8) and 26S proteasome, as well as of cathepsins B and L in cultured C_2C_{12} myoblasts (Ebisui *et al.*, 1995).

Therefore, proteasome function in cells can be regulated by altering levels of the proteasome, proteasome regulatory proteins, or proteins of the 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). In addition to the selective degradation of individual proteins, the ubiquitin-proteasome pathway appears to be responsible for alterations in tissue size. Therefore, the complexity of this system involving over 50 gene products has hindered a systematic examination of altered expression of all components under a given condition and the exact functional consequences of such changes.

1.6 Proteolytic Pathways in Cancer Cachexia

The ubiquitin-proteasome proteolytic pathway is involved in intracellular protein degradation in a range of catabolic processes (Lecker *et al.*, 1999a), including starvation (Medina *et al.*, 1991; Wing and Goldberg, 1993), sepsis (Voisin *et al*, 1996; Hasselgren,

1999; Fischer *et al.*, 2000a), diabetes (Merforth *et al.*, 1999; Lecker *et al.*, 1999b), AIDS (Llovera *et al.*, 1998), denervation atrophy (Medina *et al.*, 1991), acidosis (Mitch *et al.*, 1994), head trauma (Mansoor *et al.*, 1996), weightlessness (Taillandier *et al.*, 1996) and cancer cachexia.

Some studies have shown evidence for the involvement of other proteolytic mechanisms in cancer cachexia, including lysosomal and calcium-dependent protein degradation, but the ubiquitin-dependent proteolysis is probably the most important mechanism of cancer-related muscle cachexia.

In a study using Yoshida sarcoma-bearing rats muscle atrophy was shown to result from activation of the ATP-ubiquitin dependent proteolytic pathway (Temparis *et al.*, 1994). In this study it was shown that only ATP depletion abolished enhanced protein breakdown in contrast with the inhibition of both lysosomal and Ca^{2+}-dependemt proteases. The inhibition of lysosomal and Ca^{2+}-activated proteases *in vitro* did not suppress increased proteolysis, and neither cathepsin B or B + L activities, nor mRNA level for cathepsin B changed in the atrophying muscle. However, increased mRNA levels for ubiquitin, 14 kDa E2, and proteasome (C8 and C9) subunits were found, which correlated with the enhancement in the energy-requiring proteolysis. The accumulation of ubiquitin conjugates was also observed in skeletal muscle of cachectic animal bearing the fast growing AH-130 Yoshida ascites hepatoma (Llovera *et al.*, 1994), where elevated ubiquitin pools were found (both free and conjugated), as a result of the tumour burden. This was associated with increased ubiquitin gene expression in muscles of tumour-bearing rats, which was over 500% in relation to non-tumour bearers. These authors further investigated the involvement of the ubiquitin-proteasome pathway in protein degradation of tumour bearing rats with respect to the lysosomal and Ca^{2+}-activated proteolytic systems. ATP-dependent proteolysis was significantly increased (150%) in muscles from tumour bearing rats. Furthermore, they showed a significant increase in ubiquitin conjugates both in gastrocnemius (42%) and in soleus (117%) muscles, which led them to conclude that energy dependent proteolysis was involved in the activation of muscle proteolysis in rats bearing Yoshida AH130 ascites hepatoma (Llovera *et al.*, 1995).

Baracos *et al* (1995) also investigated the proteolytic pathways contributing to the increase in muscle proteolysis in Yoshida rats with ascites hepatoma. Overall proteolysis in rats implanted with Yoshida ascites hepatoma (YAH) was not affected by removal of Ca^{2+} or by blocking the Ca^{2+}-dependent proteolytic system. Furthermore, inhibition of lysosomal function with methylamine reduced proteolysis (-12%) in muscles from YAH-bearing rats; and inhibition of ATP production lead to a fall of muscle proteolysis to control levels in tumour-bearing rats. Western blot analysis of YAH-bearing rat muscles showed increased levels of ubiquitin-conjugated proteins and a 27-kDa proteasome subunit. At the same time, northern hybridisation analysis showed increased levels of ubiquitin mRNA (590-880%) and mRNA for the multiple subunits of the proteasome (C2, C3, C8 and C9), which increased by 100-215%.

Cloning of the colon-26 tumour produced a cell line, termed R-1, which induced cytokine (noninterleukin-1β, interleukin-6 and tumour necrosis factor-α)-independent cachexia. Implantation of R-1 cells in mice elicited significant (20-30%) weight loss with muscle weight loss of 20-25%. Food intake was unaffected. The decrease in muscle weight reflected a decline in insoluble muscle protein that was associated with a significant increase in net protein degradation. The rate of ubiquitin conjugation of proteins was significantly elevated in muscles of cachectic mice (Lazarus *et al.*, 1999). Furthermore, the proteasome inhibitor lactacystin blocked the increase in protein breakdown but had no significant effect on proteolysis. Several markers of the ubiquitin-proteasome pathway, $E2_{14k}$ mRNA, $E2_{14k}$ protein and ubiquitin protein conjugates, were not elevated. This finding is in conflict with other works where increased levels of ubiquitin protein conjugates (Llovera *et al.*, 1994; Lorite *et al.*, 1998), and $E2_{14k}$ (Lorite *et al.*, 1998; Medina *et al.*, 1995) have been found in cachexia.

Increased expression of genes in the ubiquitin-proteasome proteolytic pathway was also shown in cachectic cancer patients (Williams *et al.*, 1999b). Tissue mRNA levels for ubiquitin, the 20S-α proteasome subunits (HC3 and HC9) and the 20S-β subunits (HC5 and HC7) were determined by dot blot analysis, which were 2 to 4 times higher in muscle from patients with cancer than in muscle from control patients. This was the first study looking at the upregulation of gene expression of the ubiquitin-proteasome pathway in cancer patients. However, similar studies have been performed in patients

with head trauma (Mansoor *et al.*, 1996), sepsis (Tiao *et al.*, 1997a) and AIDS (Llovera *et al.*, 1998), and upregulated gene expression of the ubiquitin-proteasome pathway was found in all these cases.

There is evidence suggesting that hyperinsulinaemia inhibits protein breakdown in mixed and red fibre muscles via distinct mechanisms (Larbaud *et al.*, 2001). The anti-proteolytic effect of the hormone was suppressed in the presence of inhibitors of the lysosomal and Ca^{2+}-dependent proteolytic pathways in the soleus, but not in the extensor digitorum longus (EDL) muscles. In contrast, the proteasome inhibitor MG 132 abolished the anti-proteolytic effect of insulin in the mixed-fibre EDL, but not in the red fibre soleus. Furthermore, *in vivo*, insulin downregulated the expression of ubiquitin in EDL muscle only, suggesting that insulin specifically inhibits ubiquitin-proteasome -dependent proteolysis in fast-twitch skeletal muscles.

Pharmacological studies have further proved the involvement of the ubiquitin-proteolytic pathway in muscle degradation in cachectic animals. In a study using pentoxifylline (PTX), it was shown that a daily administration of PTX prevents muscle atrophy and suppresses increased muscle breakdown in Yoshida sarcoma-bearing rats by inhibiting the activation of a nonlysosomal, Ca^{2+}-independent proteolytic pathway. PTX was shown to block the ubiquitin pathway, apparently by suppressing the enhanced expression of ubiquitin, the 14-kDa ubiquitin conjugating enzyme (E2) and the C2 20S proteasome subunit in muscle from cancer rats (Combaret *et al.*, 1999). Furthermore, mRNA levels of the ATPase subunit MSS1 of the 19S complex increased in cancer cachexia, in contrast with mRNAs of other regulatory subunits, which was suppressed by PTX, suggesting that the drug inhibited the activation of the 26S proteasome (Combaret *et al.*, 1999). The mechanism by which PTX inhibits skeletal muscle protein breakdown in tumour-bearing rats remains to be elucidated. However, PTX has been shown to prevent TNF-α transcription (Doherty *et al.*, 1991); and TNF has been reported to increase ubiquitin-dependent proteolysis, and the administration of an anti-TNF-α antibody blocked the increased expression of ubiquitin and the C8 subunit of the 20S proteasome in rats bearing the hepatoma Yoshida AH-130 (Llovera *et al.*, 1996). Therefore, the reduced expression of ubiquitin, 14-kDa E2, and subunits of

both the 20S and 26S proteasomes suggests that PTX may block the TNFα-induced activation of the ubiquitin-proteasome pathway.

Costelli and Baccino (2000) also showed cytokines such as TNFα are involved in triggering the ubiquitin-proteasome pathway. Furthermore, TNFα has been shown to cause elevation of Ca^{2+} in some cells, and it has been suggested that this may have a role in activating the Ca^{2+}-dependent proteolytic pathway, which was activated in the skeletal muscle and heart of tumour-bearing animals (Costelli *et al.*, 2001). It was suggested that this finding could raise the possibility that such activation may play a role in sparking-off the muscle protein catabolism response that characterises cancer cachexia.

In another study, skeletal muscle gene expression of components of the ubiquitin-proteasome pathway (polyubiquitin, C2α proteasome subunit, 14-kDa E2 ubiquitin conjugating protein, and ubiquitin activating protein) and the lysosomal proteolytic pathway was measured in skeletal muscle of patients with early lung cancer. Northern blotting analysis showed higher levels of mRNA for cathepsin B but not for components of the ubiquitin-proteasome pathway, in patients with cancer compared with controls (Jagoe *et al.*, 2002). Cathepsin B mRNA levels correlated with fat-free mass index and tumour stage on the studied lung cancer patients, which suggests that cathepsin B may have a role in inducing muscle wasting in the early stages of lung cancer.

Caspases might also have a role in the catabolic events in skeletal muscle involved in cancer cachexia (Belizário *et al.*, 2001). It was investigated whether caspases are involved in the proteolytic process of skeletal muscle catabolism observed in murine model of cancer cachexia (MAC16), in comparison with a related tumour (MAC13), which does not induce cachexia. Using specific peptide substrates, there was an increase of 54% in the proteolytic activity of caspase-1, 84% of caspase-8, 98% of caspase-3, 151% of caspase-6 and 177% of caspase-9, in the gastrocnemius muscle of animals bearing the MAC16 tumour, in relation to muscle from animals bearing the MAC13 tumour. It is conceivable that a small percentage of apoptotic cell death, in which proteases known as caspases, play a pivotal role, could be relevant in the muscle wasting in cancer cachexia. Activation of proteases supports the phenomenon of

apoptosis in the gastrocnemius muscle of tumour-bearing mice. However, it is not known that the caspase-like activity is related to cell death, as no evidence of DNA fragementation into nucleosomal ladder was found, which is typical of apoptosis.

Therefore, there is a vast amount of evidence implicating the ATP-ubiquitin proteasome pathway in models of cachexia. Table 1.2 (adapted from Lecker *et al.*, 1999a) summarises this evidence.

Table 1.2: Experimental observations in models of muscle wasting

Increased ATP-dependent proteolysis in isolated muscles
Increased susceptibility of proteolysis to proteasome inhibitors
Increased ubiquitin contents of muscles
Increased content of ubiquitin-protein conjugates
Increase levels of ubiquitin mRNA (total mRNA falls)
Increase mRNA levels for ubiquitin-carrier proteins (E2s)
Increased mRNA levels for proteasome subunits
Increased ubiquitin conjugation to muscle proteins

1.6.1 Mechanism of Protein Degradation by PIF

Loss of muscle mass during the process of cachexia in mice bearing the MAC16 tumour is associated with the appearance of a PIF in the circulation. Evidence that this material is responsible for the protein degrading activity in serum has been provided with the use of a monoclonal antibody to the 24kDa glycoprotein, which was capable of neutralisation of biological activity *in vitro* (Todorov *et al.*, 1996b). Furthermore, as discussed earlier, PIF appears to be responsible for wasting of skeletal muscle, and administration of PIF to non-tumour-bearing mice elicits many changes similar to those in cachexia, without a depression in food and water intake.

Other work has shown that PIF induced a decrease in weight of both gastrocnemius and soleus muscles, with no effect on the heart and kidney and an increase in weight of the liver (Lorite *et al.*, 1998). This suggests that the action of PIF is mediated predominantly on skeletal muscle.

Lorite *et al* (1998) used the cachexia-inducing murine tumour (MAC16) model, which showed a significant increase in protein degradation in isolated soleus muscle. To define which proteolytic pathway contributed to this increase, soleus muscles from mice bearing the MAC16 tumour, and non-tumour-bearing animals with administered PIF, were incubated under conditions that modify different proteolytic systems. They found that in mice bearing the MAC16 tumour, there were increase in both cathepsin B and L, and the Ca^{2+}-dependent lysosomal and the ATP-dependent pathways, which contributed to the increase proteolysis. However, in PIF injected animals, there was activation only of the ATP-ubiquitin pathway. Furthermore, a monoclonal antibody to PIF attenuated the enhanced protein degradation in soleus muscle from mice bearing the MAC16 tumour. Therefore, it confirmed that PIF is responsible for the loss of skeletal muscle in cachectic mice, and it is likely that PIF contributes directly to the activation of the ATP-ubiquitin proteolytic pathway in skeletal muscle of mice bearing the MAC16 tumour.

In another study by Lorite *et al* (2001), PIF was administered to normal mice, which produced a rapid decrease in body weight that was accompanied by increased mRNA levels for ubiquitin, the $E2_{14k}$ and the C9 proteasome subunit in gastrocnemius muscle. Immunoblotting also showed an increase in protein levels of the 20S proteasome core and the 19S regulatory subunit, suggesting activation of the ATP-ubiquitin-dependent proteolytic pathway. An increased protein catabolism was also seen in C_2C_{12} myoblasts within 24 h of PIF addition. The protein degradation increase was dose related and was inhibited by higher concentrations of PIF (Smith *et al.*, 1999). The enhanced protein degradation was attenuated by anti-PIF antibody and by the proteasome inhibitors. In addition, glycerol gradient analysis of proteasomes from PIF-treated cells showed an elevation in chymotrypsin-like activity, while Western analysis showed a dose-related increase in expression of MSSI, and ATPase, which is a regulatory subunit of the proteasome, with a dose-response curve similar to that for protein degradation. These results further confirmed that PIF acts directly on the ATP-proteasome pathway, stimulating the proteasome in muscle cells and therefore, may play an essential role in protein catabolism in cancer cachexia.

The signal mediating the response of PIF to the coordinate upregulation of ubiquitin, $E2_{14k}$ and proteasome subunits are unknown, but could involve metabolites of arachidonic acid. It was shown by Smith *et al* (1999) that PIF produces an increase

release of arachidonic acid from C_2C_{12} myoblasts, which is blocked by eicosapentaenoic acid (EPA), an inhibitor of protein degradation. The arachidonic acid metabolises to prostaglandins and hydroxyeicosatetraenoic acids (HETE) (see section 1.7), but only 15-HETE produces significant increase in protein degradation.

Prostaglandin E_2 (PGE_2) levels have previously been shown to rise in gastrocnemius muscle after incubation with serum from cachectic mice bearing the MAC16 tumour, which lead to elevation of protein degradation (Smith and Tisdale, 1993b). Induction of muscle protein degradation by PIF was also associated with a rise in muscle PGE_2 production. This appeared to be casually related to the process, as inhibition of protein degradation by PIF antibody or by pre-treatment of the mice with EPA, which has been shown to reduce protein degradation in mice bearing the MAC16 tumour (Beck *et al.*, 1991), caused an inhibition of PGE_2 production. These results suggest that PGE_2 may be the intracellular mediator of the proteolytic process induced by PIF. The role of PGE_2 in muscle protein degradation is controversial and reviewed later. Rodemann and Goldberg (1982) were the first to show that PGE_2 and arachidonic acid were able to stimulate protein degradation in isolated rat skeletal and atrial muscle. Other studies have shown $PGF_{2\alpha}$ to activate synthesis of muscle proteins (Reeds *et al.*, 1985) and arachidonate to stimulate (Palmer and Wahle, 1987) or inhibit protein synthesis (Rotman *et al.*, 1992), without affecting protein degradation. Experiments using the Yoshida ascites hepatoma (a tumour associated with a marked activation of muscle protein degradation) showed that administration of inhibitors of prostaglandin synthesis including naproxin (Strelkov *et al.*, 1989) and acetylsalicylic acid (Tessitore *et al.*, 1994) also inhibit the elevated muscle catabolism. However, the role of PGE_2 in muscle protein degradation has remained controversial (Palmer, 1990). In particular, arachidonate or PGE_2 has not been shown to affect total or myofibrillar protein degradation under a variety of conditions *in vitro* and the cyclo-oxygenase inhibitor indomethacin does not affect protein degradation in septic rats *in vivo* (Hasselgren *et al.*, 1990). Thus, the role of PGE_2 in the signal transduction pathways involved in protein degradation requires further studies.

1.7 Fatty Acids: Arachidonic Acid Metabolism

The essential fatty acids consist of two families, the ω-6 (n-6) fatty acids formed from linoleic acid, and the ω-3 (n-3) fatty acids formed from α-linolenic acid. These two fatty acid families each fulfil their own important functions in the mammalian organism.

Arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) is a C_{20} polyunsaturated fatty acid (PUFA). Arachidonic acid (AA) is stored in cell membranes. The production of AA metabolites is controlled by the rate of arachidonate release from membrane phospholipids through the action of various phospholipases.
AA is metabolised either by cyclooxygenase (COX) also known as the "cyclic pathway" or lipoxygenase (LOX) pathways /known as the "linear p pathway". The COX pathway leads to the production of prostaglandins (PGH_2, PGD_2, PGE_2 and $PGF_2\alpha$) as well as thromboxane (TxA_2 and TxB_2) and prostacyclin (see figure1.3).

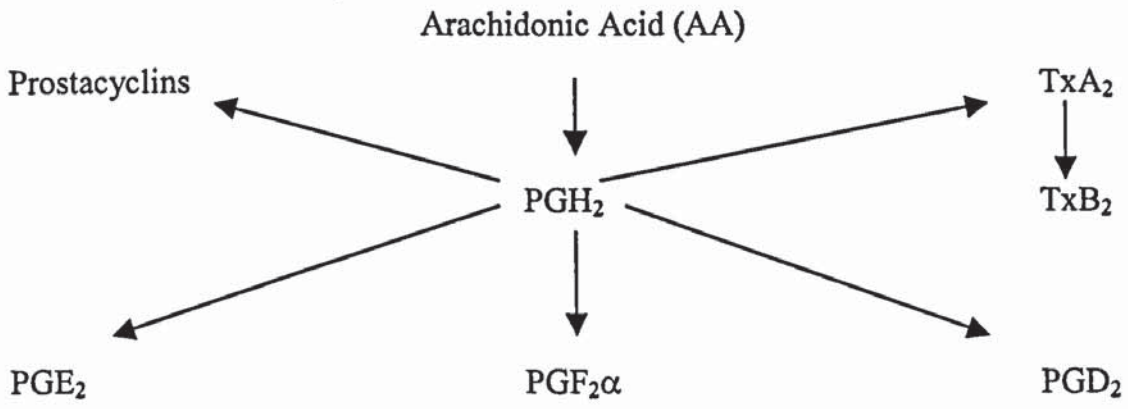


Figure 1.3. The COX pathway for arachidonic acid metabolism

The prostaglandins have various biological activities including platelet aggregation, peripheral vasodilation, and pulmonary vaso- and broncho-constriction (reviewed by Needleman *et al.*, 1986).

Lipoxygenase enzymes also metabolise AA. Oxygen is incorporated into AA by these enzymes producing hydroperoxyeicosatetraenoic acid product (HPETE). This product is then reduced forming a more stable derivative- hydroxyeicosatetraenoic acid (HETE),

which has three main isomers (5-HETE, 12-HETE and 15-HETE). However, in mammalian cells at least four types of lipoxygenase can be discriminated on the bases of the carbon atom of the substrate molecule (AA) at which oxygen is incorporated: 5-lipoxygenase, two 12-lipoxygenases (platelet type and leucocyte type) and the 15-lipoxygenases, which produce 5-HETE, 12(S)-HETE, 12(R)-HETE and 15-HETE (figure 1.4).

HETEs have various biological effects such as chemotactic mediation, intracellular calcium concentration and regulation of prostaglandins (reviewed by Spector *et al.*, 1988).

AA is a ω-6 fatty acid, synthesised from linoleic acid (also a ω-6 fatty acid) by elongation and desaturation. In a similar fashion α-linolenic acid is a precursor of 5, 8, 11, 14, 17- eicosapentaenoic acid (EPA), which is a ω-3 fatty acid. Other important precursors of 20 carbon eicosanoid products include eicosapentaenoic (ω-3) and docosahexaenoic (ω-3) acids.

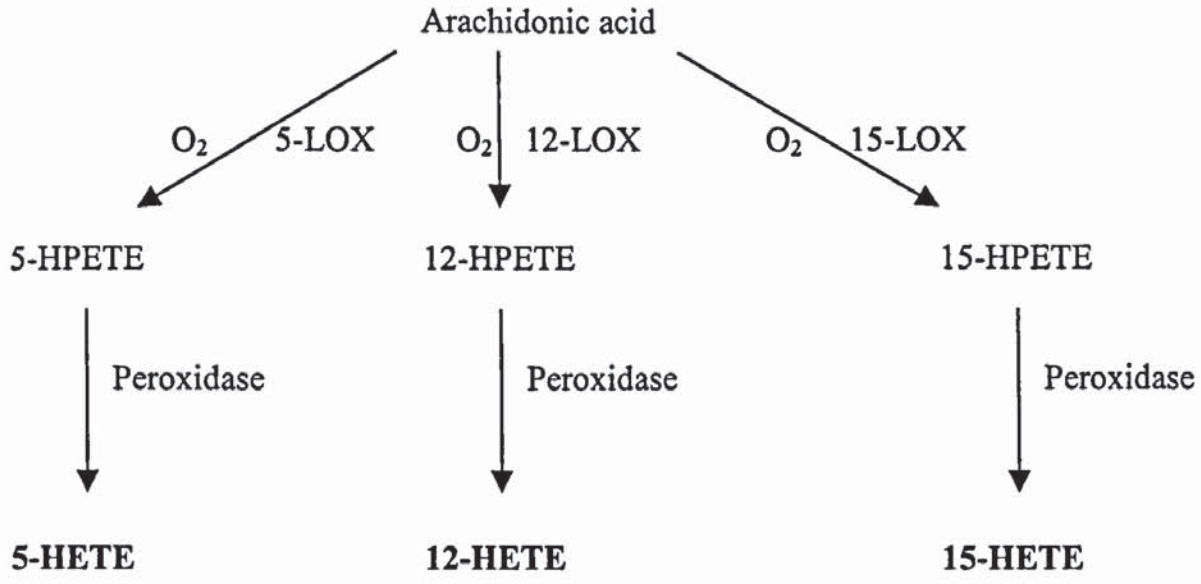


Figure1.4. Diagram to show the synthesis of the HETE isomers.

1.7.1 Polyunsaturated Fatty Acids and Cancer

Epidemiological and experimental studies provide compelling evidence that dietary fat modulate the incidence of certain types of cancer (Drasar and Irving, 1973; Hill, 1987). Data shows quality and composition of fat, not merely its amount, determines the nature of the effects (Belury *et al.*, 1993; Orengo *et al.*, 1989; Bang *et al.*, 1976).

Diets rich in ω-6 fatty acids increase the incidence of tumours in colon, pancreas and mammary gland, of animals treated with carcinogens (Belury *et al.*, 1993). By contrast, animals fed fish oil diets, that are rich in ω-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), and are low in ω-6 fatty acids, show a reduced frequency of these tumours. Orengo *et al* (1989) also reported that diets high in fish oil inhibit ultraviolet light-induced skin tumours in mice.

Eskimos, who consume diets rich in fat from marine animals, show a low incidence of cancer (Bang *et al.*, 1976). Intervention trials show a negative correlation between total ω-3/total ω-6 fatty acids and a risk for cancer mortality.

Hursting *et al* (1990) studied data from 29 countries and observed correlations between breast cancer incidence and intake of saturated and polyunsaturated fat, and inversely and non-significantly with fish ω-3 polyunsaturated fatty acids. They also observed correlations between colon cancer incidence and saturated fat, and inverse correlations with fish ω-3 PUFA. In partial support, animal studies have shown that high fat diets are tumour-promoting at both sites (Reddy *et al.*, 1980), but have tended to show that unsaturated fats are as potent in this respect as are saturated fats. Further animal studies, (Lindner, 1991; Reddy *et al.*, 1991) have shown that the ω-3 polyunsaturated fatty acids are protective, whereas the ω-6 series are promoting. Furthermore, Anti *et al* (1992) showed that ω-3 fatty acids decreased crypt cell proliferation rates (a marker of colorectal cancer link) in humans. Gonzalez *et al* (1993) showed that fish oil protects against breast carcinogenesis in a rodent model. Further evidence by Caygill *et al* (1996) suggested fish oil consumption is associated with protection against the promotional effects of animal fat in colorectal and breast cancer patients. Therefore, dietary ω-6 PUFAs promote, and ω-3 PUFAs inhibit, mammary carcinogenesis.

Furthermore, studies have also shown enhancement of growth of a transplantable mammary tumour by gamma-linolenic acid (ω-6) when administered as a 10% corn oil diet (Gobar *et al.*, 1985). Release of linolenic acid as occurs in cancer cachexia may therefore lead to a stimulation of tumour growth. Hudson *et al* (1993) studied the kinetics of cell production and cell loss in the MAC16 model to determine the effect of EPA and linolenic acid on tumour growth. Tumour stasis induced by EPA appeared to arise from an increase in the rate of cell loss from 38-71% without an effect on the rate of cell production. Addition of linolenic acid to animals administered EPA acted to increase tumour growth by reducing the cell loss factor to 45%, again without affecting the rate of cell production. Thus, PUFA appear to regulate tumour growth *in vivo* solely by affecting the rate of cell loss. This finding is important from a therapeutic standpoint, since most solid tumours tend to be populated by long-lived cells, and expansion tends to occur not by a high rate of cell production, but by a low rate of cell loss. This explains why solid tumours tend to be resistant to conventional anti-tumour drugs, which are directed towards biochemical pathways involved in cell proliferation, rather than by enhancing the rate of cell loss.

Further work by Hussey and Tisdale (1994) showed that there is a threshold dose for tumour growth stimulation by linolenic acid. The increase in tumour volume induced by linolenic acid arose from a reduction in the potential doubling time from 41 to 28 h and was effectively reversed by indomethacin. These results suggested that PUFAs may play an important role in tumour growth and may offer a potential target for the development of chemotherapeutic agents. This suggests that the effect of PUFA on tumour cell loss may be mediated by prostaglandins, and that EPA may be effective since it is also a cyclooxygenase inhibitor (McCappin *et al.*, 1987).

Previous studies (Buckman *et al.*, 1991) using a murine mammary carcinoma cell line have attributed the growth-stimulatory effect of linolenic acid *in vitro* to metabolites from the lipoxygenase pathway rather than the cyclooxygenase pathway. Further conformation of this fact was provided in a study where several lipoxygenase inhibitors were shown to inhibit the growth of tumour cells (Rose and Connolly, 1990). Lipoxygenase products are thought to act through cyclic GMP production and linolenic acid has been shown to activate guanylate cyclase (reviewed by Tisdale, 1993b).

It has been suggested that ω-3 PUFAs may act to modulate second messenger systems involved in cell signalling. Protein kinase A and C (PKA and PKC) are critical intracellular components of two second messenger systems activated by the binding of extracellular ligands to cell-surface receptors. It was reported by Moore *et al* (2001) that exposure of human breast cancer cells to physiological concentrations of EPA and DHA resulted in a strong decrease in expression of the RIα regulatory subunit of PKA and the PKC-α isozyme of PKC. This suggests that inhibition of these enzymes may be one mechanism whereby ω-3 PUFAs inhibit the growth and metastasis of oestrogen-receptor negative cancer cells.

In another study EPA was shown to competitively inhibit urokinase (UK). Urokinase is an important protease enzyme involved in carcinogenesis, as well as in the invasion and metastasis of cancer cells. Thus, regulation of UK activity is likely to be important in healthy cell metabolism. It was shown that both gamma-linolenic acid (ω-6) and EPA acted as competitive inhibitors of UK (Toit *et al.*, 1994). Therefore, EPA may regulate UK activity in tissues.

The molecular mechanisms, which underlie the effects of polyunsaturated fatty acids on cancer development are poorly understood. The complexity of homeostasis in animals makes it virtually impossible to glean the direct effect of specific dietary fatty acids on neoplasia at a molecular level. The utilisation of fatty acids and production of metabolites vary from tissue to tissue, and an overabundance of effects on cellular membranes and signalling pathways obscure many events that may take place.

Transformation represents a progressive disorder in signal transduction pathways, which regulates gene expression. Cellular phospholipids and the aracidonic acid cascade play an important role in signalling events and in transformations. Exposure to EPA or DHA produces a remodelling of cellular phospholipids so that they are substantially reduced in AA-containing species. Such changes would reduce eicosanoid synthesis, modulate signal transduction pathways and attenuate the activation of protein kinase C. Remodelling of phospholipids may affect growth factor and hormone receptors, and intercellular communication, all of which may play a role in the transformation process.

1.7.2 Effects of PUFAs on Cancer Cachexia

It has been observed that catabolism of muscle and adipose tissue in cancer cachexia can be effectively reversed *in vivo* by EPA, but not by other PUFA of either the ω-3 or ω-6 series.

Initial experiments showed that when mice bearing the MAC16 tumour were fed a diet in which the carbohydrate components were replaced by calories from fish oil, host body weight loss was significantly reduced, together with an increase in total body fat and muscle mass, although the total calorie intake and the total nitrogen intake remained constant (Tisdale and Dhesi, 1990). Since fish oil is enriched in both EPA and DHA then either one or both of the fatty acids (FA) was responsible for the anticachectic effect. However, *in vivo* administration of the pure FA to weight losing mice bearing the MAC16 adenocarcinoma showed that only EPA was capable of exerting an anticachectic and antitumour effect (Tisdale and Beck, 1991). Furthermore, in the same study other PUFAs were tested (both ω-3 and ω-6). However, only EPA inhibited the stimulatory effect of tumour lipolysis factor, therefore suggesting that inhibition of lipolysis was structurally specific, which probably arose from the interaction of EPA with a specific membrane receptor.

Induction of lipolysis by both salbutamol and ACTH was also inhibited by EPA (Tisdale and Beck, 1991). Lipolysis in adipocytes is thought to be exerted through the intracellular mediator cyclic AMP formed in response to activation of adenylate cyclase through binding of the hormone to its receptor (Butcher *et al.*, 1968). It was shown that cAMP levels in adipocytes was elevated in response to the lipolytic effects of ACTH and salbutamol, which is similar to the lipolysis induced by the tumour lipolytic factor (LMF) in adipocytes. Stimulation of adipocyte cAMP levels by the tumour lipolytic factor, ACTH and salbutamol was inhibited by EPA, suggesting that the effect is exerted at a step common to all three antagonists.

Studies have shown that the inhibitory effect of EPA on adipocyte adenylate cyclase is due to an interaction with an inhibitory guanine nucleotide binding protein (Gi). In a study, the inhibition of adenylate cyclase with EPA was shown to be GTP-dependent,

with no inhibition occurring in the absence of GTP. The concentration of GTP required for maximal inhibition of adenylate cyclase was similar to that required for other inhibitors. Further conformation that the inhibitory effect of EPA arose from an interaction with Gi rather than directly through the C unit of adenylate cyclase was provided by the ability of pertussis toxin to completely abrogate the effect, both in intact adiopcytes and in plasma membrane fractions (Tisdale, 1993b). Furthermore, stimulation of adenylate cyclase activity by forskolin, which acts directly on the enzyme without the involvement of a receptor, was also decreased in membranes of mice treated with EPA (Price and Tisdale, 1998), suggesting a direct interaction between EPA and adenylate cyclase. Pertussis toxin eliminated the inhibition of lipolysis and the stimulation of adenylate cyclase by isoprenaline and LMF in the presence of EPA, but not DHA. This suggested that the attenuation of hormonal stimulation of adenylate cyclase by EPA was due, at least in part, to Gi (Price and Tisdale, 1998).

Weight reversal in mice bearing the MAC16 tumour by EPA was associated not only with retention of adipose tissue, but also with lean body mass (Tisdale, 1996). Animals bearing the MAC16 tumour showed a decreased protein synthesis and increased protein degradation in skeletal muscle; the latter was shown to be significantly attenuated by EPA without an effect on protein synthesis (Beck and Tisdale, 1991). Serum from mice bearing the MAC16 tumour produced an increased protein degradation in isolated gastrocnemius muscle accompanied by an elevation of prostaglandin E_2 (Smith and Tisdale, 1993a). The treatment of mice with EPA inhibited the rise in muscle PGE_2 content in response to serum from cachectic mice, and also inhibited muscle protein degradation in the *in vitro* assay. Therefore, these results show that inhibition of cachexia by EPA, at least in the MAC16 model, appears to result from its capacity to interfere with tumour-produced catabolic factors acting on muscle and adipose tissue, rather than acting as a nutritional supplement.

Fish oil and EPA appear to affect a number of potential mediators of cachexia. The administration of fish oil reduces production of cytokines such as IL-6, TNF, and IL-1 in healthy subjects (Endres *et al.*, 1989; Caughey *et al.*, 1996). In one study (Wigmore *et al.*, 1997a) a reduction of production of IL-6 and TNF in patients with pancreatic cancer receiving EPA was demonstrated. Reduced concentration of C-reactive protein was also reported in pancreatic cancer patients receiving EPA. These effects on

mediators of cachexia have led to the study of the effects of fish oil and EPA on the cachectic process in cancer patients (reviewed by Barber, 2001).

In a clinical study, a group of 18 weight-losing patients with advanced pancreatic cancer were given oral fish-oil preparations containing EPA and DHA. Before treatment all patients were losing weight, after 3 mo of supplementation, patient's weight stabilised, with less than half of the patients continuing to loss weight. The patients had no change in the percentage of total body water during the study, suggesting that patients were not simply retaining fluid (Wigmore *et al.*, 1996). Very similar effects were observed in another clinical study involving 27 pancreatic cancer patients given pure EPA (Wigmore *et al.*, 2000a). Examination of the potential mediators of cachexia showed a significant decrease in the production of IL-6 in patients and insulin concentration rose. The proportion of patients excreting PIF also fell significantly and concentrations of acute-phase proteins stabilised (Barber *et al.*, 1999b). The metabolic response to feeding also was normalised in patients receiving the fish-oil enriched nutrition supplement, with a relative fall in resting energy expenditure (Barber *et al.*, 2000).

Taken together, these results suggest a profound modulation of the cachectic process. The fish-oil component of the supplement may have affected cachectic mediators, allowing a degree of normalisation of the metabolic state. This may have allowed the additional nutrients supplied to have an anabolic effect. The gain in lean tissue mass with resultant improvement in functional ability observed with the consumption of the fish-oil-enriched supplement is in marked contrast to the relative weight stability produced by fish oil or EPA alone and the lack of effect of nutritional supplement alone. Therefore, further studies are awaited to confirm these findings.

1.7.3 The Involvement of Cyclooxygenases and Lipoxygenases

The elevated levels of PGE_2 observed by Smith and Tisdale (1993b) indicates increased activation of phospholipase A_2 (PLA_2), which is involved in the release of arachidonic acid from the membrane phospholipids (as was mentioned before). Free AA can then be metabolised via cyclooxygenase enzymes to form prostaglandins. This suggests that the activation of PLA_2 may be the first step in the induction of proteolysis by PIF. However, metabolism of arachidonic acid can also occur through lipoxygenase

enzymes, resulting in the formation of HETEs. Smith *et al* (1999) showed that PIF caused a rise in both prostaglandins and HETEs but when these eicosanoids were added to C_2C_{12} myotubes only 15-HETE was capable of stimulating protein degradation, suggesting that this metabolite rather than PGE_2 is responsible for the protein degradation induced by PIF.

Few studies recently have been looking at lipoxygenase inhibitors, which have been shown to inhibit the growth of both rat (Lee and Ip, 1992) and mouse (Buckman *et al.*, 1991) mammary tumour cell and HL60 human leukaemia cells (Simon *et al.*, 1992).

Studies *in vitro* (Hussey and Tisdale, 1994; Rose and Connolly, 1990) have suggested that metabolism of PUFA through the lipoxygenase pathway may be important for stimulation of tumour growth. Metabolism through the 12-lipoxygenase pathway may be most important for tumour growth and metastasis. Thus, 12-HETE has been suggested (Liu *et al.*, 1994) as a determinant of the metastatic potential of tumour cells and may be a crucial target for intervention in metastasis. 12(S)-HETE has been shown to stimulate DNA synthesis in fetal bovine aortic endothelial cells (Setty *et al.*, 1987), an effect also demonstrated by 15-HETE; and to regulate expression of the proto-oncogenes *c-fos* and *c-myc* in rat lens epithelial cells (Lysz *et al.*, 1994), whereas 12(R)-HETE caused neovascularisation of the cornea (Masferrer *et al.*, 1991). These and other studies suggest that inhibitors of the lipoxygenase pathways may provide useful new agents to inhibit tumour growth and metastasis.

Hussey and Tisdale (1996a) investigated the effect of lipoxygenase inhibitors on established murine adenocarcinomas (MACs) in order to find the importance of PUFA metabolism through lipoxygenase pathways to tumour growth *in vivo*. Three 5-lipoxygenase inhibitors were used, namely BWA4C, BWB70C and Zileuton. Despite the similarity in action of the three agents towards the 5-lipoxygenase pathway they displayed vastly different growth-inhibitory properties to the three MAC cell lines *in vitro*, where BWA4C and BWB70C IC_{50} values were five times lower than that of Zileuton. This suggested that the inhibitory effect of the former two agents may be due to inhibition of the 12- and/or 15-lipoxygenases. Furthermore, it was concluded that the inhibitory effect of these agents on cell growth may result from an imbalance of metabolism of arachidonic acid between the 5-, 12- and 15-lipoxygenase pathways.

Another inhibitor of the lipoxygenase pathway is the 2,3,5-trimethyl-6-(3-pyridylmethyl)1,4-benzoquinone (CV-6504), which is a 5-lipoxygenase and thromboxane A_2 synthase inhibitor, with IC_{50} values against both enzymes of 10^{-7}M (Ohkawa *et al.*, 1991).

In a study by Hussey *et al* (1996) the anti-tumour activity of CV-6504 was demonstrated against three established murine adenocarcinomas (MAC13, MAC16 and MAC26). This anti-tumour activity of CV-6504 at low doses (10mg/kg/day) towards these tumours was reduced by the concomitant administration of pure linolenic acid. This suggested that the anti-tumour effect of CV-6504 is mediated through inhibition of the metabolism of linoleate. Higher dose levels of the drug were able to overcome the effect of linoleate suggesting a competitive effect (Hussey *et al.*, 1996).

Following administration into tumour-bearing mice CV-6504 undergoes rapid reduction by two-electron donating enzymes, such as DT-diaphorase, and the resulting hydroquinone inhibits both 5-lipoxygenase activity and lipid peroxidation as the basis of its antioxidant ability by reducing the ferric iron in the active site of the enzyme to the ferrous (resting state). Reduction of the quinone ring and subsequent conjugation yields 1- and 4-glucuronides and the corresponding sulphates. These conjugates would not be capable of inhibiting 5-lipoxygenase by the suggested mechanism. However, the glucuronide and sulphate conjugates of the CV-6504 can be metabolised to free drug by the action of β-glucuronidase and sulphatase. Hussey and Tisdale (1996b) showed that both glucuronide and sulphate conjugates were ineffective in inhibiting the growth of the MAC13 tumour *in vitro*, however, they were as effective as CV-6504 *in vivo*, when used at a concentration five times higher. Both the MAC16 and MAC13 tumour models used possessed β-glucuronidase and sulphatase at levels similar to that found in the liver. This suggests a role for these enzymes in the anti-tumour action of CV-6504.

In the same study, CV-6504 was shown to inhibit the production of 5-, 12- and 15-HETE in MAC13 tumour cells *ex vivo*. This is similar to the anti-tumour effect of EPA on MAC16 tumour as was discussed earlier (Hudson *et al.*, 1993), which also reduced the tumour concentration of 12- and 15-HETE, while the level of 5-HETE was unaffected (Rose *et al.*, 1995). Both CV-6504 1-sulphate and 4-glucuronide inhibited

formation of 5-, 12- and 15-HETE formation in MAC13 tumour cells *ex vivo* in a time dependent manner, suggesting conversion of these metabolites to free CV-6504. Inhibition of 12- and 15-HETE production occurred before inhibition of 5-HETE production (Hussey and Tisdale, 1996b). Thus suppression of 12- and/or 15-lipoxygenase pathways may be most important for inhibition of tumour growth.

In another study cell lines sensitive (MAC16, MAC13, MAC26 and Caco-2) and resistant (A549 and DU-145) to CV-6504 were used to elucidate the mechanism of the anti-tumour action of CV-6504 (Hussey and Tisdale, 1997). Pre-incubation of the sensitive cell lines with CV-6504 inhibited the conversion of arachidonic acid to 5-, 12- and 15-HETE, which further confirmed the involvement of the lipoxygenase pathway with respect to the anti-tumour effect of CV-6504. Furthermore, it was shown that CV-6504 effectively suppressing growth of the MAC16 tumour *in vivo* and preventing the accompanying cachexia.

CV-6504 was developed initially as an anti-proteinuric agent and entered phase I clinical and pharmacokinetic studies in adult volunteers and patients with chronic glomerulonephritis; where it was found that a dose of 300 mg daily in three divided doses, orally, was well tolerated, rapidly absorbed, excreted (as sulphate or glucuronide conjugate) in urine and reproducibly inhibited platelet activation and serum thromboxane B_2 formation (reviewed by Ferry *et al.*, 2000). Recently, phase II trails were undertaken after the anti-cachectic effects of this inhibitor was determined in animal models. These trials were undertaken in patients with advanced pancreatic cancer to determine: 1) if CV-6504 has anti-cancer activity in patients with pancreatic cancer, 2) its toxicity and tolerance, 3) its pharmacokinetics and pharmacodynamic effects. The results suggested that CV-6504 appeared to stabilise the disease in about 32% of patients with advanced pancreatic cancer. There was no clear relationship between stabilisation of disease by CV-6504 and its pharmacokinetics or surrogate markers of biochemical activity (Ferry *et al.*, 2000). Therefore, it was concluded that it could be combined with conventional chemotherapeutic agents in clinical trials.

1.8 Aims and Objectives of this Study

This study focused on the mechanism of protein loss observed in cancer cachexia and the involvement of the ATP-ubiquitin-proteasome pathway in muscle catabolism.
The aims of this study were:

1. To design and set up a competitive quantitative reverse transcription-polymerase chain reaction (Qc-RT-PCR) for measuring the expression of the 20S proteasome subunits C2 (α6) and C5 (β6), also the E2-14k ubiquitin-conjugating enzyme.

2. Examine the effect of PIF and 15-HETE on the expression of the proteasome subunits (C2 and C5) and the $E2_{14k}$ enzyme; and to look at the role of EPA and CV-6504 on such effects. The skeletal muscle cell line C_2C_{12} was used as a model system to study the mechanisms of these factors on muscle protein turnover.

3. To investigate the expression of the proteasome subunits and the $E2_{14k}$ enzyme in the gastrocnemius and pectoral muscles of murine adenocarcinoma model MAC16 with respect to different % weight loss.

4. Examine the expression of the proteasome and the $E2_{14k}$ enzyme from muscle biopsies of cachectic cancer patients, and to determine the relationship between the extent of weight loss and expression of the ubiquitin-proteasome proteolytic pathway.

5. To look at the involvement of the ATP-ubiquitin-proteasome pathway in cachexia of sepsis using a murine model. Furthermore, to examine the effect of EPA on this effect.

There is increased expression of the ubiquitin-proteasome proteolytic pathway with attenuation by EPA in cancer cachexia.

CHAPTER 2

Materials and Methods

2. 1 Materials

Affinite Research products Ltd, Exeter, UK

Mouse monoclonal antibody to 20s α-subunit

Ambion Ltd, Cambridgeshire, UK

DNAse/DNAse removing reagent

RNA storage buffer

Amersham International Ltd, Bucks, UK

ATP

ECL Western blotting analysis system

Hybond ECL Nitrocellulose membrane

Protein rainbow marker

Taq polymerase

dNTPs

X-Gal

$Pd(N)_6$ Random Hexamer 5' Phosphate

Bio-Rad Laboratories LTD, Herts, UK

Ammonium persulphate

Bio-Rad protein reagent

Boehringer Mannheim, USA

Agarose gel DNA extraction Kit

Dako, Denmark

Rabbit anti-mouse IgG-HRP

Gibco BRL Life Technologies, Paisley, Scotland, UK

Agarose

Bovine foetal calf serum (FCS)

DH5α cells (ready competent)

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

Horse serum (HS)

Penicillin-Streptomycin

Tissue culture flasks

Trypsin

MBI Fermentus, Sunderland, UK

Klenow fragment

MWG Biotech, Ebersberg, Germany

Oligonucleotide primers

New England Biolabs, Hertfordshire, UK

Hind III

Ligase

Nde I

Sma I

Xba I

Xho I

Oxoid, Basingstoke, Hampshire, UK

Agar

Bacto-tryptone

Bacto-yeast extract

Phosphate buffered saline tablets

Promega, Southampton, UK

Calf Intestinal Alkaline Phosphatase (CIAP)

100 bp DNA ladder

1 kb DNA ladder

Ethidium Bromide

IPTG

Magnesium chloride (PCR grade)

M-MLV Reverse transcriptase

Recombinant RNAsin Inhibitor

Reverse transcriptase buffer

T7 *in vitro* Transcription kit

T4 polynucleotide kinase

Wizard™ mini prep

Wizard™ maxi prep

Roche, Switzerland

PCR Buffer (without $MgCl_2$)

Scotia Pharmaceuticals, Stirling, UK

Eicosapentaenoic Acid (for *in vivo* experiments)

Sigma, Aldrich

Acrylamide solution 30%

Ampicillin

ATP

Boric acid

Bovine Serum Albumin (BSA)

Bromophenol Blue

Calcium chloride

Chloroform (molecular biology grade)

Coomasie Brilliant Blue R250 stain

Diethylpyrocarbonate (DEPC)

Dithiothreitol (DTT)

Ethanol (molecular biology grade)

Ethylendiaminetetracetic acid

Formaldehyde

Formamide

Glycerol

Glycine

Isopropanol (molecular biology grade)

Kodak GBX developer and replenisher

Kodak GBX fixer and replenisher

Magnesium chloride

Methanol

3-[N-morpholino]-2-hydroxypropanesulphonic acid (MOPS), pH 7.0

N-Succinyl-Leu-Leu-Val-Tyr-Amino Methyl Coumarin

Sodium acetate

Sodium chloride

Sodium hydroxide

Spin columns

TEMED

Tri Reagent

Trizma base

Gifts

Human rectus abdominin muscle biopsies were provided by Prof. K.C.H. Fearon, Department of Surgery, Edinburgh Royal Infirmary, UK.

Rabbit anti mouse $E2_{14k}$ antisera was donated by Dr S Wing, McGill University, Montreal, Canada.

2.2 Medium

2.2.1 L.B. Broth

Bacto-tryptone	10g/l (1%)
Bacto-yeast extract	5g/l (0.5%)
NaCl	10g/l (1%)

At a pH of 7.5

2.2.2 L.B. Agar

Bacto-tryptone	10g/l (1%)
Bacto-yeast extract	5g/l (0.5%)
NaCl	10g/l (1%)
Bacto-agar	15g (1.5%)

At a pH of 7.5

All media were sterilised by autoclaving at 121°C.

Antibiotics filter sterilised and added to the medium (cooled down to about 40°C), as required

Antibiotic	Stock Solution	Working Concentrations
Ampercillin	50mg/ml	50µg/µl
Kanamycin	10mg/ml	30µg/µl

2.3 Buffers

2.3.1 Molecular Biology Buffers

2.3.1.1 TBE 10X

Tris base	107.80g/l
Boric acid	~55g/l
Disodium EDTA.$_2H_2O$	7.44g/l

At a pH of 8.3

2.3.1.2 TE Buffer

Tris/HCl (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

2.3.1.3 $CaCl_2$ (50mM)

$CaCl_2$

Distilled H_2O

2.3.1.4 Agarose gel Loading Buffer (DNA)

Bromophenol blue	0. 25%
Xylene cynol	0. 25%
Glycerol in H_2O	30%

2.3.1.5 Agarose gel loading buffer (RNA)

Glycerol	50%
EDTA	1mM
Bromophenol blue	0.4%

2.3.1.6 RNA sample buffer

Deionised formamide	10ml
37% Formaldehyde	3.5ml
Mops 5X buffer	2ml

2.3.1.7 MOPS 5X buffer

3-[N-morphplino]-2-hydroxypropanesulfonic acid (MOPS) pH 7.0	0.2M
Sodium acetate	0.05M
EDTA (pH 8.0)	0.005M

2.3.1.8 DEPC-treated water (RNA)

Diethyl pyrocarbonate (DEPC) was added to deionised water at a final concentration of 0.1%. The water was incubated at 37 °C overnight and then autoclaved at 121°C.

2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Analysis Buffers

2.3.2.1 Running buffer (10x)

Trizma base	25mM
Glycine	190mM
SDS	35mM

2.3.2.2 Sample buffer

Tris-HCl pH 6.8	125mM
SDS	4%
β-Mercaptoethanol	2%
Glycerol	10%
Bromophenol blue	0.006%

2.3.2.3 Blotting buffer

Trizma base	25mM
Glycine	190mM
SDS	50mM
For use: 10x Blotting buffer	10%
Methanol	20%

2.3.2.4 Wash buffer

PBS + 0.5% Tween 20

2.3.2.5 Blocking solution

PBS + 0.1% Tween 20	
Marvel	5%

2.3.2.6 Homogenising Buffer

Tris pH 7.5	500mM
ATP	100mM
$MgCl_2$	50mM
DTT	50mM

2.3.2.7 Coomassie Blue Gel Stain

Coomassie brilliant blue R250 stain	0.1%
Acetic Acid	10%
Methanol	25%

2.3.2.8 Coomassie Blue Gel Destain

Acetic acid	10%
Methanol	25%

2.3.2.9 Polyacrylamide Gels

Reagents	12% Resolving Gel (ml)	Stacking Gel (ml)
Distilled water	6.6	2.8
30% acrylamide solution	8	0.66
Tris (1.5M pH 8.8)	5	0
Tris (1.0M pH 6.8)	0	0.5
10% SDS	0.2	0.04
10% ammonium phosphate	0.2	0.04
TEMED	0.008	0.004

2.3.3 Proteasome activity assay buffer

2.3.3.1 Fluorogenic substrate buffer

N-Succinyl-Leu-Leu-Tyr-7-Amido-4-methylcoumarin	10mg
DMSO	600µl

For use: dilute 1:100 in 100mM Tris pH 8.0.

2.4 Methods

Animals

Pure strain NMRI mice were obtained from our own inbred colony and were fed a rat and mouse breeding diet (Special Diet Services, Witham, UK). Fragments of the murine adenocarcinoma (MAC16), excised from donor animals with established weight loss, were implanted into the flanks of NMRI mice by means of a trochar (Beck and Tisdale, 1987). The animals were killed after weight loss has reached 5-25% starting weight, which took approximately10-12 days after transplantation

Tissue culture

The C_2C_{12} mouse myoblast cell line was grown in tissue culture flasks (Nunc) containing Dulbecco's modified Eagle medium (DMEM) supplemented with 12% Foetal Calf Serum (FCS), 1% non-essential amino acids and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C.

These cells were passaged after confluency every 3-4 days, where they were washed with sterile PBS and incubated with 3ml sterile 1% Trypsin for few minutes to disrupt the monolayer. An appropriate amount of DMEM was then added to neutralise the solution, then the cells were sub-cultured into sterile flasks and incubated as before.

Myotube formation

C_2C_{12} myoblasts were transformed into myotubes after they reached confluency by incubating them in DMEM containing 2% horse serum and 1% penicillin-streptomycin. The culture medium was changed every 48 h and differentiation occurred 11-14 days post-confluence, producing multinucleated myotubes (Black and Olsen, 1998), which could be used for experimentations.

2.4.1 Molecular Biology

2.4.1.1 DNA preparation

Promega Wizard™ mini or maxi preps were used to prepare plasmid DNA from overnight cultures of bacteria clones. Manufacturer's instructions were followed for all plasmid preparations unless stated otherwise.

2.4.1.2 RNA preparation

RNA was isolated from C_2C_{12} cells and muscle using Sigma Tri Reagent. Again manufacturers instructions were followed unless stated otherwise.

Yields of RNA were determined by optical absorbance at 260nm, and quality of RNA was assessed by electrophoresis on 1% agarose gels containing 0.67% formaldehyde or 2% agarose gels in TBE buffer.

2.4.1.3 Ethanol Precipitation

One-tenth volume of 3M tri-sodium acetate (pH 5.5), and two times volume of ice-cold absolute ethanol were added to the DNA and mixed by vortexing. The mixture was incubated at -20°C for 15 minutes before the DNA was pelleted by centrifugation for 15-20 minutes at 14000r.p.m. in a microfuge tube. The liquid was removed and the pellet was dried by evaporation. The DNA pellet was then resuspended in water.

2.4.1.4 Gel Electrophoresis

1X TBE buffer was used to make all agarose gels. Agarose gels were made using agarose (Gibco BRL, Paisley), 1% and 2%, and SeaPlaque agarose (FMC, Rockland, USA) 2%, which was used when DNA fragments needed to be purified from the gels. The gels were stained with ethidium bromide solution (1mg/ml solution in 1X TBE) at a 1:25 dilution, then electrophoresed for about 1.5 hours at 10 volts per cm length of gel in 1X TBE.

Gels were visualised under UV light using transilluminator, or the UVP transilluminator photographic system.

2.4.1.5 DNA purification from agarose gels

Desired DNA bands were excised from the SeaPlaque gels and placed in a spin filter obtained from Promega's Wizard™ mini purification kit. The spin filters were placed in a 1.5 ml eppendorf tubes and centrifuged at 6000r.p.m. for 5 minutes at room temperature to obtain the pure DNA.

2.4.1.6 Restriction enzyme digests

Restriction digests were carried out as instructed by the manufacturer. In general three units of enzyme was used to digest 1 μg of DNA in a 1 X buffer (specific for each

enzyme, supplied by the manufacturer) and incubated at 37 °C for 1.5 hours unless otherwise stated.

2.4.1.7 DNA "blunt ending" reaction

Blunt ending reactions were carried out in 1X PCR buffer with the addition of 1µl of 1mM dNTPs, 1µl of 10mM ATP, 0.5 units of the Klenow fragment of *E. coli* DNA polymerase, and 0.5 units of T4 polynucleotide kinase. This mixture was incubated at 37 °C for 30 minutes. The enzymes were then inactivated by heating to 65 °C for 20 minutes.

2.4.1.8 Removal of DNA 5' phosphates

DNA digested with a restriction enzyme was further suspended in 1X buffer (appropriate for each restriction enzyme used), 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was used per pmol of DNA ends. Reactions were incubated at 37 °C for 1 hour. The CIAP was then removed from the DNA using the Agarose Gel DNA Extraction Kit as instructed by the manufacturer.

2.4.1.9 Ligation reactions

Reactions were carried out using 50ng cut/dephosphorylated vector with 3X molar excess of insert in the presence of 1 mM ATP, 1 X ligase buffer and 1 unit ligase, in a 20 µl final reaction.

Self-ligations were also carried out as a control for all ligations. All ligations were then incubated at 16 °C overnight.

2.4.1.10 Preparation of competent *E. coli* cells

E.coli DH5α were streaked onto an agar plate and incubated overnight at 37 °C. A single colony was then inoculated into a small amount of LB medium (2.2) and incubated at 37 °C overnight to grow the cells. 500 µl of this culture was then inoculated into 50 ml of LB media and incubated at 37 °C for 2-2.5 hours until the A_{600} was between 0.3 and 0.5. The cells were then pelleted by centrifugation (4000 r.p.m, 5 min, 4 °C). The supernatant was then discarded and the pellet was resuspended in 6 ml of ice cold 50 mM $CaCl_2$. The cells were left on ice for 10 minutes. Afterwards the cells were pelleted as before and resuspended in 6 ml ice cold $CaCl_2$. Again the cells were

left on ice for 10 minutes before being pelleted and resuspended in 1.2 ml ice cold $CaCl_2$. The cells were then placed on ice for 20 minutes.

2.4.1.11 Transformation of competent DH5α cells

DNA (from ligations) was added to 100 µl of competent DH5α cells in a chilled microfuge tube and mixed before leaving it on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 37 °C and immediately put back on ice for 2 minutes. 500 µl of LB was added to the cells and incubated at 37 °C for 40 minutes. Afterwards, a 200 µl aliquot of the transformed cells were spread on LB agar plates containing ampicillin or kanamycin. With respect to the plasmids with blue/white colour selection, the 200 µl transformed cells were added to 50 µl 2% X-Gal (2% 5-bromo-4-chloro-3-3indol-b-D-galactoside in DMF) and 10 µl of 100mM IPTG before spreading them on the appropriate plate. The plates were incubated overnight at 37 °C. Control plates also accompanied all transformations where untransformed cells were also plated onto LB agar containing ampicillin or kanamycin.

2.4.1.12 Colony screening

Colonies were screened by PCR. The colonies were picked from the plate using a sterile pipette tip and smeared on the bottom of a small PCR eppendorf and subsequently on a LB plate containing the selection antibiotics. 25 µl of the PCR mixture (below) was added to each tube and placed in a Primus 25/96 Thermocycler for the PCR to take place.

A denaturation step was used for these colonies of an additional 3 minutes at 95 °C prior to cycling in-order to lyse the bacterial cells.

2.4.1.13 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 25 µl or 50 µl reaction mixtures of the following;

1X	PCR buffer (without $MgCl_2$)
20 pmol	Forward Primer
20 pmol	Reverse Primer
10 mM	NTPs (each)
2.5 mM	$MgCl_2$
1 unit/50 µl	Taq polymerase

PCR reactions were carried out in a Primus 25/96 Thermocycler, using a standard PCR cycle of 30 cycles with the following programme;

Denaturing	95 °C for 1 minute
Annealing	58 °C for 1 minute
Elongation	72 °C for 2 minutes

2.4.1.14 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Reverse Transcription (RT)

Initially the RNA was denatured at 95 °C for five minutes before adding 1 μg of random hexamer to 2μg of the RNA. The RNA hexamer (primer) and the template RNA were heated up to 70 °C in the Thermocycler for 5 minutes and immediately cooled on ice to allow primer annealing to occur. Then to this primer-RNA mixture the following was added:

5 X	Reverse transcriptase (RT) buffer
10 mM	dNTPs (each)
1 unit	Reverse transcriptase
1 unit	RNAsin

Reverse transcriptions were performed in 25 μl reactions, incubated for one hour at 37 °C in the Thermocycler.

PCR

PCR reactions were carried out in 50 μl reaction mixture of;

1 X	PCR buffer (without $MgCl_2$)
10 or 20 pmol	Forward Primer
10 or 20 pmol	Reverse primer
2.5 - 3.5 mM	$MgCl_2$
1 unit	Taq polymerase

The reactions were then carried out in the Thermocycler using the previous PCR programme (2.4.1.13).

2.4.1.15 *In vitro* Transcription

RNA was made from plasmid DNA using T7 *in vitro* transcription kit (Promega). Manufacturers instructions were followed unless stated otherwise.

2.4.2 SDS PAGE and Western Blotting

2.4.2.1 Sample preparation

From C_2C_{12} myotubes – Cells were washed twice in PBS and scraped in approximately 1ml homogenising buffer (2.3.2.6). Samples were then sonicated for three pulses of 15 sec, with 10sec intervals and centrifuged at 15,000 r.p.m for 10 minutes.

From Muscle - Protein samples were prepared from MAC16 gastrocnemius or pectoral muscle, or human rectus abdominus biopsies. The muscles were homogenised in ice-cold homogenising buffer (2.3.2.6), then sonicated and centrifuged as above.

The supernatant from the samples were then removed and protein concentrations were measured.

2.4.2.2 Determination of Protein Concentration

Protein concentrations were determined spectrophotometrically using commercially available Bio-Rad reagent; manufactures instructions were followed. Coomassie blue stain was used to show the accuracy of this protein measurement (see below), where 5µg of the protein was electrophoresed on a polyacylamide gel (see below) and then stained with 1% coomassie blue (2.3.2.7) for one hour, before it was de-stained with coomasie blue de-stain solution (2.3.2.8).

The samples were mixed with denaturing sample buffer (2.3.2.2) (1:1) and then heated at 95°C for 5-15 min to completely denature the protein samples, which were then cooled and centrifuged ready for protein electrophoresis.

2.4.2.3 Gel preparation

12% Acrylamide gels were prepared as described (2.3.2.9), and poured into pre-made gel plate apparatus after thorough cleaning with 70% ethanol. The gel was overlaid with saturated butanol and allowed to polymerise for approximately one hour. Afterwards,

the butanol was removed by thorough rinsing of the gel surface with distilled water and the stacking gel (2.3.2.9) was then poured on top of the resolving gel and a gel comb was inserted. The gel was allowed to polymerise for at least 1 hour.

After complete polymerisation, the gels were used to run the protein samples, which were loaded into the wells along with 'Rainbow' molecular weight markers. Separations were carried out at 180V for approximately 45 minutes. Parallel gels were also electrophoresed and then stained in coomassie blue to ensure equal loading. Where gels were stained for one hour in 1% coomassie blue (2.3.2.7), and then de-stained changing the solution 3-4 times with coomasie blue de-stain solution (2.3.2.8).

2.4.2.4 Western Blotting

The electrophoresed protein gel was then transferred to a nitrocellulose membrane using a closed cassette system and a current was applied for 2 hours at 80V with cooling. After transfer the membranes were blocked overnight in blocking solution (2.3.2.5) at 4°C.

The blocked membrane was then incubated with the appropriate primary antibody (table 2.1) diluted in wash buffer (2.3.2.4) for 1 hour at room temperature. This was followed by washing for 1 hour with 0.1% PBS-Tween (2.3.2.4) at room temperature with agitation and changing the wash buffer every 15 min. The membrane was then incubated for 1 hour at room temperature with the appropriate secondary antibody Horseradish Peroxidase conjugated IgG complex, diluted 1:2000 in wash buffer. The membrane was washed again for a further 1 hour with agitation, again changing the buffer every 15 minutes.

Antisera	Origin	1° antibody	2°antibody
20S	Mouse monoclonal	1:1500	1:1500
$E2_{14k}$	Rabbit polyclonal	1:1000	1:1500

Table 2.1 Antibody dilutions for the proteins detected during Western Blotting

2.4.2.5 Visualisation of Western Blots

Blots were visualised using ECL™ Western Blotting Detection System. The system is based upon the detection of immobilised specific antigen, conjugated directly or indirectly with Horseradish Peroxidase (HRP), which oxidises luminol, resulting in light emission, detected by a blue light sensitive autoradiography film (Hyperfilm ECL). The blots were completely covered in an equal volume of detection solution 1 and 2 for 1 min then drained and covered in Saranwrap then placed in the film cassette. The autoradiography film was then exposed to the membrane in the cassette for 15 sec to 15 min. Films were developed using Kodak Developer and Replenisher for 5 min and fixed by immersing in Kodak fixer and Replenisher for 5 min.

2.4.3 Fluorometric Proteasome Assay

The proteasome chymotrypsin-like activity was measured by following the hydrolysis of the fluorometric substrate N-succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methyl-coumarin. This substrate was prepared and 100µl were added to 10µl protein sample, which were prepared as described (2.4.2.1). A duplicate set of samples were included to which 10µM lactocystin was added. The reaction was allowed to proceed for 1 hour.
Fluorescence of the substrate was measured using a Perkin Elmer Luminescence Spectrometer LS50 at 360nm excitation/460nm emission. Values were adjusted for equal protein concentrations and minus a reaction blank.

2.4.4 Developmentof murine sepsis model

An overnight culture of *Staphylococcus aureus* (NCTC 6571) was grown in Mueller Hinton broth (MHB) at 37°C with vigorous shaking for 18 h. From this culture 1 ml was inoculated into a sterile pre-warmed MHB and grown at 37°C with shaking until the OD470nm reached 1.0. An aliquot of 0.1ml of this culture was added to 0.9ml of sterile saline (0.9%w/v) to give a final cell concentration of 10^7 colony forming units per ml. A volume of 0.1ml was the inoculum for the thigh sepsis model (Gudmundsson and Erlendsdottir, 1999).
Male albino MF-1 (weight 35g +/- 5%) were used. Each mouse was chemically anaesthetised with Hypnorm. After 5 minutes, the right thigh of each mouse was shaved and 0.1ml of the bacterial suspension was injected into the thigh muscle.

CHAPTER 3

Designing and Setting up Quantitative Competitive Reverse Transcription-Polymerase Chain Reaction (QcRT-PCR) to measure Gene Expression

3.1 Introduction

The analysis of gene expression patterns is becoming increasingly important to our knowledge and understanding of complex biological processes and their role in disease pathology at the molecular level. The aim is to determine the roles of specific genes in regulatory biology and pathogenesis by understanding how their function and alteration in expression contribute to disease.

Traditional methods for studying gene expression have significant limitations and therefore, decreased use as the demand for rapid, quantitative and robust methods for the simultaneous evaluation of multiple genes increases.

Several techniques have been used over the years for the analysis of gene expression. Northern blots and RNase protection assays are the oldest and most well-established techniques. However, these techniques are labour intensive. For example, northern blots require large amounts of RNA and are time consuming with respect to both personnel time and the time to obtain a result. In addition, northern blots use radioactive labels and enable the analysis of relatively few samples simultaneously. RNase protection assays require less RNA and are more sensitive than northern blots but cannot be used for analysing large numbers of samples (reviewed by Snider *et al.*, 2001). In addition, RNase protection assays are time intensive and require the use of radioactive labels, which most modern laboratories try to pursue experimental techniques that do not require the use of radioactive labels.

Another widely used technique for analysing gene expression is the DNA microarray, which is used to measure the expression of tens of thousands of genes in parallel. These arrays have been used to identify disease subtypes with distinct clinical outcomes, as was recently reported for diffuse large B-cell lymphoma (Alizadeh *et al*, 2000).

However, microarray hybridisation has many limitations such as large amounts of RNA is required, it is time consuming and labour intensive.
Thus, there has been need for quantitative methods that are more robust, easy to perform and economical in its RNA requirement.

PCR techniques have become the preferred method for most nucleic acid analysis projects. They have several advantages over the northern blots and RNase protection assays; for example, PCR is extremely sensitive, being performed successfully on samples as small as a single copy of template. The process is simple and is amendable to the rapid analysis of large numbers of samples. However, PCR does have limitations, such as its sensitivity to inhibitors that can be present in the starting sample. Although PCR is regarded as a powerful qualitative detection technique, there are several inherent factors that have limited its use as a quantitative technique such as: the plateau of product accumulation and variable reaction efficiency across different samples in the analysis. In an effort to overcome these two limitations, reverse transcription and PCR have been combined (RT-PCR) and used for quantitating mRNA (Becker-André and Hahlbrock, 1989; Cross, 1995).
Generally, a quantitative RT-PCR method consists of four steps: generation of internal standards, RT-PCR, detection of products and data analysis.

The initial step in RT-PCR is the production of a single-strand complementary DNA copy (cDNA) of the RNA through the action of a retroviral enzyme, reverse transcriptase. An oligonucleotide primer is required to initiate cDNA synthesis. The primer anneals to the RNA, and the cDNA is extended towards the 5' end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase.
The reverse transcription (RT) step is the source of most of the variability in quantitative RT-PCR experiment. The reverse transcriptase enzyme is sensitive to salts, alcohols or phenol. The signal output variability is a central issue in quantitative RT-PCR (QRT-PCR). It cannot be assumed that different reactions have the same RT efficiency.
Following the RT reaction, the cDNA is amplified by PCR, which is usually carried out using an aliquot of the RT reaction as the template for the reaction.

Quantitative competitive RT-PCR (QcRT-PCR) assay is based on competetive co-amplification of a specific target sequence together with known concentrations of an internal standard in one reaction tube. The internal standard has to share primer recognition sites with the specific template, both specific template and internal standard must be PCR-amplified with the same efficiency and it must be possible to analyse the PCR amplified products of specific template and internal standard separately. Quantitation can then be performed by comparing the PCR signals of the specific template with the PCR signal obtained with known concentrations of the internal standard (competitor).

Internal standards for competitive PCR or RT-PCR are DNA or RNA fragments. mRNA levels of various genes were quantitated by Laville *et al* (1996), using cDNA competitors to investigate the regulation of various muscle enzymes by insulin. However, it is generally accepted that DNA fragments are not an optimal choice for competitors in QRT-PCR because they do not compensate for the inherent variability in the RT step.

Endogenous RNA standards or internal standards, actin (Coenen Schimke *et al.*, 1999) or G3PDH (Horikoshi and Sakakibara, 2000) and heterologous synthetic RNAs have both been used as amplification standards (Auboeuf and Vidal, 1997). Endogenous internal standards have problems of widely differing abundance, different amplification primers and their expression is sensitive to some experimental treatments. Heterologous external standard RNAs are an improvement over endogenous standard RNAs because their levels can be controlled, but they are not homologous to the sequence of interest and are likely to have differing amplification efficiencies.

Homologous synthetic RNA standards have also been used, these can be defined as an *in vitro*-transcribed synthetic RNA that shares the same primer binding sites as the native RNA and has the same intervening sequence except for a small insertion, deletion or mutation to facilitate differentiation from the native signal during quantification (reviewed by Kendall and Riley, 2000). Homologous RNA standards are the most suitable for two reasons: 1) an RNA standard (as opposed to DNA) must be used to control for variability during the RT step; and 2) a homologous RNA standard is most likely to have the same or very similar RT and PCR efficiencies.

Homologous RNA standards are generally created from the entire native gene, or a portion of it, cloned into a plasmid containing an RNA polymerase promoter suitable for an *in vitro* transcription. A small deletion or insertion or a mutation is made in the standard so that the native and standard amplification products can be differentiated by size on an electrophoresis gel. It has been shown that the smaller the alteration, the more similar the amplification characteristics of the standard (McCulloch *et al*, 1995; Cottrez *et al*, 1994). This can be explained by the fact that amplification efficiency is related to the length and secondary structure of the RNA and ensuing product. A longer product requires greater processivity of the enzyme, therefore compromising efficiency. Thus, a balance must be found with deletion/addition style standards, where the products are easily discriminated, but are as similar as possible to ensure equal efficiencies.

The simplest construction procedure is to use a composite primer containing two specific target sequences at a predetermined distance from each other (thus resulting in a deletion) and the second primer specific for the opposite strand (Celi *et al.*, 1993; Riedy *et al.*, 1995). The amplification of the internal or competitor templates with such primers results in a PCR product shorter than the wild-type template and can thus easily be identified. Furthermore, elongated internal standards can be constructed using a "looped oligo" method by amplifying cDNA with a primer containing a non-templated nucleotide insertion between template sequences (Sarkar and Bolander 1994).

Alternatively, a mutation introduced into the cloned gene by site-directed mutagenesis, can be used to create (or eliminate) a unique restriction enzyme site in the standard (Becker-André and Hahlbrock, 1989). Gilliland *et al* (1990) also used site directed mutagenesis to engineer target cDNA containing a unique restriction site. After the RT-PCR, the reaction products are endonuclease-digested and then resolved by gel electrophoresis. The signal of the two digested standard bands are summed and compared to the native signal (or vice versa if the native restriction enzyme site is eliminated in the standard).

Once the homogenous standard is constructed, it is usually loaded on a gel, separated excised and extracted. They can then be used directly in a competitive PCR assay (Taylor *et al.*, 1998), but more often; they are cloned into plasmids, preferably by the help of additionally introduced restriction sites (Porcher *et al.*, 1992).

Competitive RT-PCR is the most common approach in quantitative RT-RCR. In competitive RT-RCR, a dilution series of the standard RNA is co-amplified with equal amounts of total RNA (therefore equal amounts of native RNA) (figure 3.1). The standard competes with the native for primers and enzyme, thus reducing the signal of the native when the standard is in excess. As the amount of standard increases, the native signal decreases (Becker-André and Hahlbrock, 1989; and Gilliland *et al*, 1990) (figure 3.1).

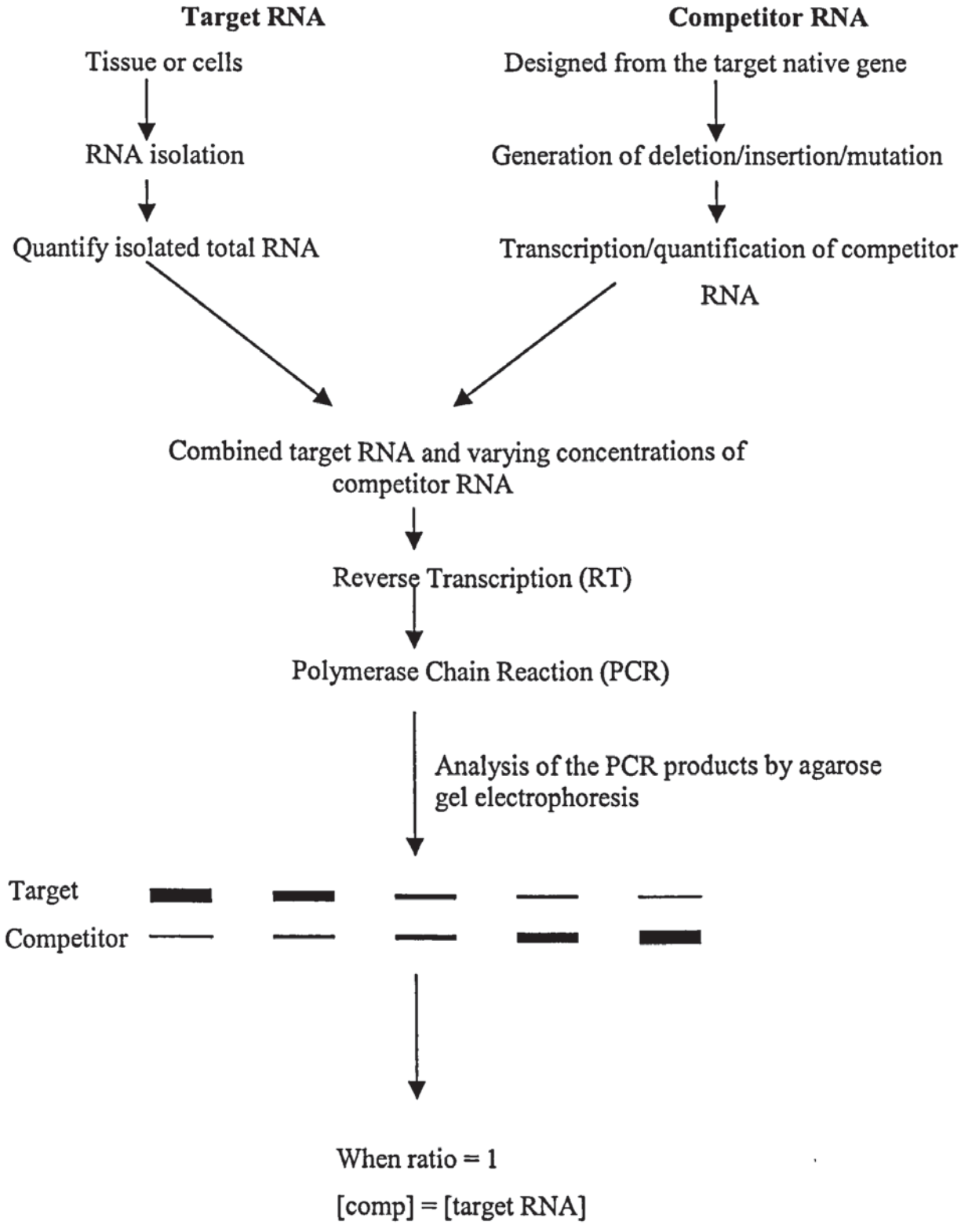
Accuracy of quantitative competitive RT-PCR can be potentiated by simultaneous determination of a gene of interest and a reporter gene in one reaction tube. The expression of the titrated gene of interest is then calculated with reference to the titrated level of the reporter gene.

The PCR products of different sizes are usually separated by conventional gel electrophoresis, capillary gel electrophoresis or HPLC. The easiest and most commonly used method is to use gel electrophoresis for separation of the PCR products and to visualise the DNA bands by ethidium bromide or equivalent fluorescence.
The respective ratios of unknown wild-type template and known competitive standard concentrations are then used to generate a diagram that can be utilised to quantitate precisely the amount of wild-type or target template.

This technique is exquisitely sensitive, allowing analysis of gene expression from very small amounts of RNA. Moreover, this approach can be conducted on a large number of samples and or many different genes in the same experiment.

These theories were used to develop this technique (QcRT-PCR) in our laboratory to measure gene expression of some of the components involved in ubiquitin-proteasome pathway, namely the 20S proteasome subunits C2 and C5 as well as the ubiquitin conjugating enzyme ($E2_{14k}$).

Figure 3.1. Schematic representation of the technical steps in QcRT-PCR



3.2 Competitor Construction

Competitor genes for the proteasome subunits C2, C5 and the $E2_{14k}$ ubiquitin conjugation enzyme were constructed by using composite primers as described above.

3.2.1 $E2_{14k}$-ubiquitin conjugating enzyme

The rat sequence of this gene was obtained from the GenBank (see appendix I), and primers were constructed according to this gene sequence.
The forward and reverse primers were constructed for amplifying the $E2_{14k}$ gene by PCR, which gave a product of 395-bp. The sequence and position of the primers are:

Forward primer: 68- 5'CTC ATG CGG GAT TTC AAG CG'3 -87
Reverse primer: 468- 5'CTC TTC TCA TAC TCC CGT TTG'3 -443

In-order to make the $E2_{14k}$ competitor for the quantitation procedure, a third primer was designed which produced a 284-bp product, this is 111-bp shorter than the native $E2_{14k}$ gene described above. The sequence and position of this primer is:

468- 5'CTC TTC TCA TAC TCC CGT TTG CAT CGG TTC TGC AGG ATG TC3' -312

Therefore, this competitor primer is designed to have the original E2 reverse primer attached to it, which will produce a loop in the DNA during PCR amplification process as shown below. The DNA loop is 111-bp long, therefore the product (competitor) is 111-bp shorter than the native gene.

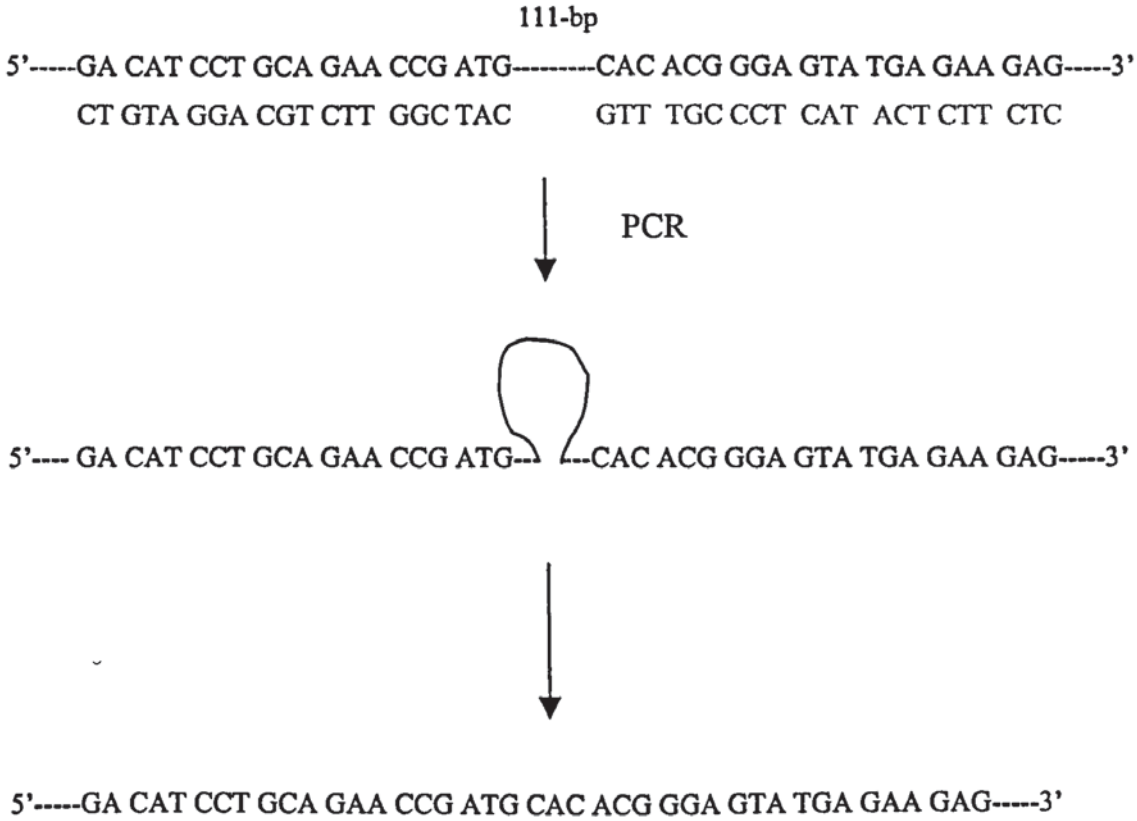


Table 3.1

Properties of the $E2_{14k}$ ubiquitin conjugating enzyme primers:

Properties	E2 Forward	E2 Reverse	E2 competitor
Length	17-bp	18-bp	41-bp
GC content	52.9%	55.6%	51.2%
Tm	52.8°C	56.0°C	74.4°C

3.2.2 C2 20S Proteasome α subunit

The mouse sequence of this gene was obtained from Dr. Simon Wing, Canada, and primers were designed according to this sequence (see appendix I).

Forward and reverse oligonucleotides were constructed in-order to amplify this gene by PCR, these primers produced a 385-bp product. The properties of these primers are shown in table 3.2 and their sequence and positions are:

Forward primer: 176- 5'CGC ACG CAG TGC TGG TTG CAC3' -187

Reverse Primer: 552- 5'GTA CGA GCT GAT TGA GAA CGG3'-531

The competitor primer was designed to produce a product 76-bp shorter than the wild-type C2 gene, this was also constructed using the same composite primer PCR method. Therefore, the competitor product was 309-bp long.

Competitor primer:
552- 5'GTA CGA GCT GAT TGA GAA CGG CAT AAC CAG CAA TGA GCA GCC3' -435

Table 3.2
Properties of the C2 proteasome gene primers:

Properties	C2 Forward	C2 Reverse	C2 Competitor
Length	20-bp	21-bp	42-bp
GC content	55%	52.4%	52.4%
Tm	59.4°C	59.8°C	>75°C

3.2.3 C5 Proteasome β subunit

The mouse gene sequence of the C5 gene was also kingly provided by Dr Simon Wing. Primers were designed with respect to this sequence (see appendix I).
The product produced with the C5 forward and reverse primers was 414-bp long, the position and sequence of these primers are:

Forward primer: 101- 5'TCA ACG GAG GTA CTG TAT TGG3' -121
Reverse primer: 514- 5'GCA TGG CAC TTG CTG AGC C3' -496

The third primer was also designed, 117-bp upstream of the reverse primer producing a competitor product 297-bp long; its sequence and position are:

514- 5'GCA TGG CAC TTG CTG AGC CGC ATG GCA CTT GCT GAG CC3' –358

Table 3.3

Properties of the C5 proteasome gene oligonucleotides:

Properties	C5 Forward	C5 Reverse	C5 competitor
Length	21-bp	19-bp	39-bp
GC content	47.6%	63.2%	59%
Tm	58.2°C	57.9°C	>75°C

3.3 Cloning of the competitor DNAs

Forward and competitor primers for the E2, C2 and C5 genes (3.2.1, 3.2.2 and 3.2.3 respectively) were used in a RT-PCR reaction (2.4.1.14) to make the corresponding competitor genes, using RNA extracted (2.4.1.2) from C_2C_{12} myoblasts (2.4) as the template.

The competitor products obtained from the PCR reactions were separated by electrophoresis on a 2% agarose gel (2.4.1.4). The product was then cut from the gel (2.4.1.5) and cloned into pET30a vector by carrying out a standard blunt ending reaction (2.4.1.7). The blunt ended product was then ligated into the vector (2.4.1.9).

The vector was blunt-end digested with *Sma* I restriction enzyme before the terminal phosphate residues were hydrolysed (2.4.1.8) to prevent self-ligation. Subsequently the ligated vector was used to transform DH5α competent cells (2.4.1.11), the cells were then plated on kanamycin containing LB agar and incubated at 37°C overnight. Table 3.4 shows an example of the transformation results obtained.

Table 3.4 Example of a typical transformation result

	Number of colonies
Competitor ligation	8
Self ligation	2
Cut pET30a	0
Uncut pET30a	136

Several of the white colonies obtained from the ligation plate were screened by PCR (2.4.1.13) using the forward and competitor primers with respect to each gene, to identify positive clones. After amplification of the colonies, the PCR products were subjected to agarose gel electrophoresis (2.4.1.4) and visualised under UV light.

Plasmid DNA from few of the positive clones of each gene was prepared using Wizard™ Mini-prep (2.4.1.1), each DNA sample was then further examined by restriction digest analysis (2.4.1.6), where two restriction enzymes were used in each case to cut the pET30a plasmid flanking the cloned DNA, which were absent from the cloned competitor DNA. Table 3.5 shows the restriction enzymes used for the double digestion of each competitor gene.

Table 3.5 Restriction enzymes.

Competitor gene	**Restriction enzymes**
E2	*Xba* I and *Xho* I
C2	*Hind* III and *Xba* I
C5	*Nde* I and *Xho* I

The competitor DNA of each gene was also sequenced for absolute assurance of their identity. The sequencing was carried out by MWG biotech.

3.4 Production of the Competitor RNA

In vitro transcription was used to make competitor RNA from each of the competitor DNA clones prepared (2.4.1.15). The RNA produced was then aliquoted and stored at -70°C.

3.5 RT-PCR Optimisation

Optimisation of RT-PCR was carried out for each gene described individually ($E2_{14k}$, C2, C5). Initial RT-PCR of each gene produced a lot of products of different size to the desired product due to non-specific amplification. To produce best yield and also cleaner product of each of these genes (wild-type and competitor) optimisation of the RT-PCR was carried out where, the $MgCl_2$ concentration, dNTP concentrations, amount of primers, *Taq* DNA polymerase and primer annealing temperatures were investigated and altered in-order to find the optimised reaction conditions.

3.6 Optimisation of the Competitor Concentration

Each competitor RNA was quantified precisely by measuring the optical density at 260nm. The concentration of the competitor RNA produced from the *in vitro* transcription method was very high and it had to be diluted. Therefore, for each competitor, ten-fold serial dilutions were prepared and were used in RT-PCR to find out the initial optimal dilution series needed for performing the competitive RT-PCR method. Each reaction contained a specific concentration of the competitor RNA (from the ten-fold dilution series) and 0.25µg target RNA, which was also measured spectrophotometriclly at 260nm obtaining the precise concentration. After obtaining the optimal 10-fold dilution series, another RT-PCR was performed to find the optimal 2-fold dilution series needed for quantitating the target RNA (Figure 3.2).

This optimal 2-fold serial dilution of the competitor (E2, C2 or C5) was used with respect to each specific target RNA to quantitate the target gene. In each competitive PCR experiment, six 2-fold serial dilutions of the competitor was used, and these particular dilutions used for any particular RNA sample was selected to span the expected concentration of the target RNA in the sample. Therefore, each competitive

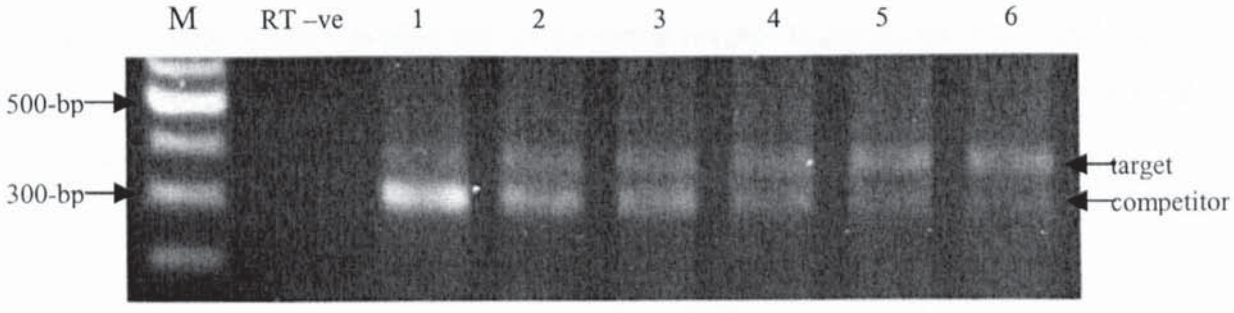
RT-PCR experiment consisted of 6 reactions, each containing 0.25μg total RNA and one of the dilutions of competitor RNA (E2, C2 or C5). The RT-PCR was performed as discussed in the method section (2.4.1.14).

Control reactions containing all components except the reverse transcriptase were carried out in each experiment to show that the RNA (both competitor and target) had no DNA contamination (RT –ve); also controls in which RNA or cDNA template was omitted to show the presence of no PCR contaminations (PCR –ve), figures 3.2, 3.3 and 3.4.

3.7 Competitive RT-PCR analysis

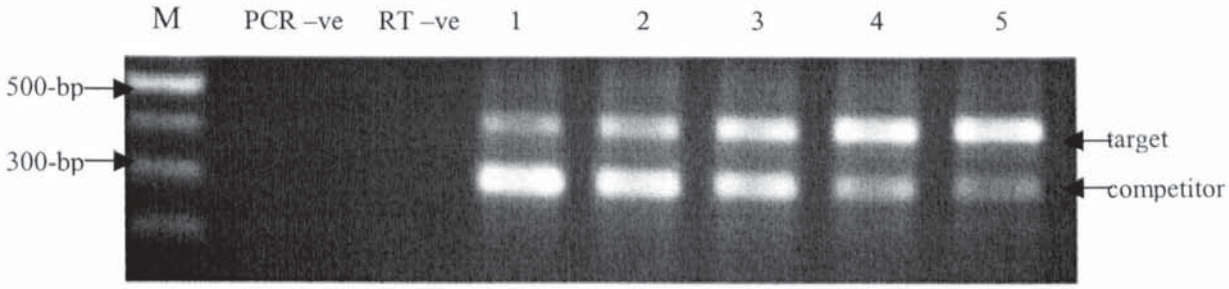
For analysis of results, 15μl (40%) of each PCR was subjected to 2% (w/v) agarose gel electrophoresis in the presence of 0.5μg/ml ethidium bromide to separate the amplified competitor and target DNAs. Ethidium bromide-stained bands were visualised on a transilluminator and photographed. Figures 3.2, 3.3 and 3.4 show the dilution series and the product formation from competitive RT-PCR reactions of C2 20S, C5 20S proteasome subunit and $E2_{14k}$ genes respectively.

Figure 3.2 QcRT-PCR of the C2 20S proteasome subunit gene. 40% of total PCR reactions were examined by electrophoresis in a 2% agarose gel.



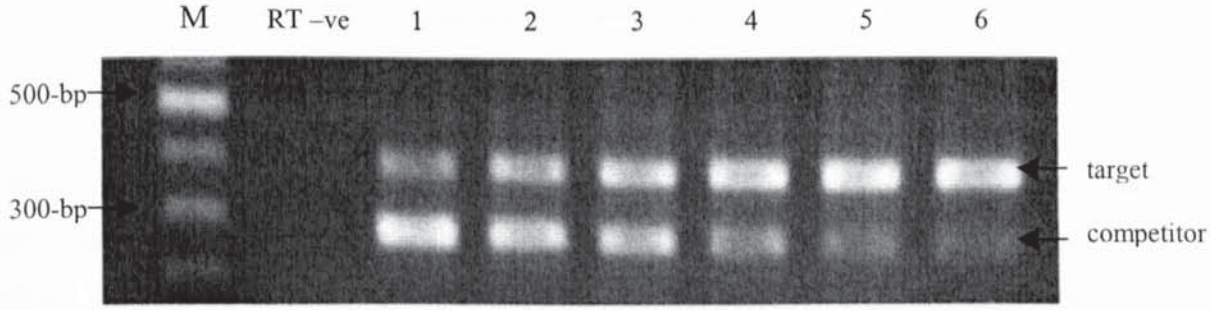
Lanes; M: 100-bp DNA marker, RT –ve: negative reverse transcription reaction, lanes 1-6: each contain 0.25μg target RNA and a specific amount of the C2 competitor RNA ranging from 0.1563pg to 5pg in 2-fold dilutions.

Figure 3.3 QcRT-PCR of the C5 20S proteasome subunit gene, PCRs (40% of total reaction) were examined in a 2% agarose gel by electrophoresis.



Lanes; M: 100-bp DNA ladder, PCR-ve: negative PCR reaction, RT-ve: negative reverse transcription reaction, lanes 1-6: each contain 0.25μg target RNA and a specific amount of the C5 competitor RNA ranging from 0.00781-0.25ng in 2-fold dilutions.

Figure 3.4 QcRT-PCR of the $E2_{14k}$ ubiquitin conjugation enzyme gene. PCRs (40% of total reactions) were examined by electrophoresis in a 2% agarose gel.

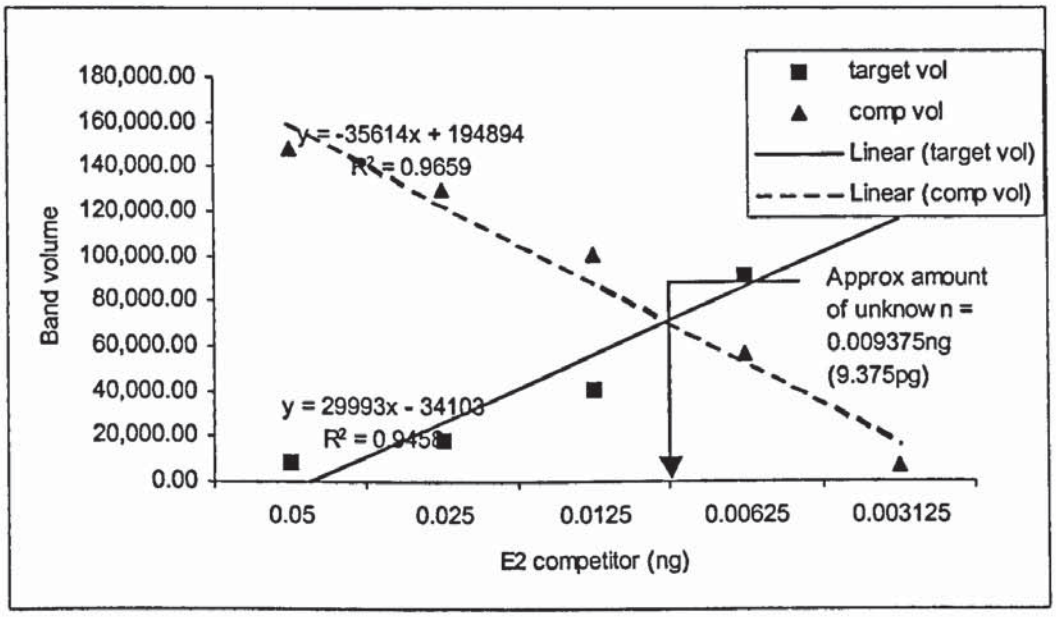


Lanes; M: 100-bp DNA ladder, RT-ve: negative reverse transcription reaction, lanes 1-6: each contain 0.25μg target RNA and a specific amount of the E2 competitor RNA ranging from 3.125pg to 50pg in 2-fold dilutions.

The intensity and volume of the bands were quantified using a Phoretix photo-imager programme. The volumes of the competitor and target bands were plotted against the known serial dilution of the competitor used in the experiment. The molar amount of specific mRNA corresponded to the molar amount of competitor when the ratio of competitor to target was 1, and this value was expressed per 0.25μg target RNA based

on the precise optical density measurements of the RNA made in each experiment (see above). Figure 3.5 shows an example of a typical graph plotted to quantify the target gene.

Figure 3.5 Volume of the target E2 gene band against the E2 competitor DNA band



The exquisite sensitivity of RT-PCR makes it possible to detect mRNAs of extremely rare abundance, mRNAs in small numbers of cells or in small amounts of tissue, and mRNAs expressed in mixed-cell populations. Furthermore, RT-PCR requires only a few hours to perform.

Although RT-PCR has many advantages over RNA blot methods, it can be difficult to obtain quantitative information. This is due to the exponential nature of the PCR amplification, where small variations in amplification efficiency result in dramatic changes in product yields. In addition, the amount of product generated decreases during later stages of the reaction due to consumption of necessary components and generation of inhibitors. These characteristics of PCR can obscure differences in the initial amounts of target sequences during the course of amplification. However, these problems can be overcome in QcRT-PCR by the use of internal controls sharing primer

binding sites and high internal sequence similarity (Hayward *et al.*, 1998; and Morrison and Gannon, 1994), which were used in this case.

Therefore, using competitive RT-PCR, it is possible to obtain quantitative information of mRNAs comparable to RNA blot analysis. The ability to perform quantitative analysis of mRNAs by competitive RT-PCR will undoubtedly expand the use and applications of PCR.

CHAPTER 4

The Effect of EPA and CV-6504 on PIF and 15-HETE treated C_2C_{12} Myotubes

4.1 Introduction

Many studies have demonstrated the direct effect of the catabolic factor PIF (proteolysis inducing factor) in protein degradation seen in cancer cachexia and its attenuation by the polyunsaturated fatty acid, EPA (Lorite *et al.*, 1997). However, the mechanisms involved are not yet clear. Previous studies (Lorite *et al.*, 1998) have shown that the ATP-ubiquitin-dependent proteolytic pathway is responsible for the loss of skeletal muscle in cancer cachexia.

Smith *et al* (1999) found 50-90% increase in the rate of protein degradation in C_2C_{12} myoblasts after addition of PIF, which was completely abolished by pre-treatment with eicosapentaenoic acid (EPA). They also observed that induction of protein degradation *in vitro* by PIF was associated with the release of arachidonic acid, which was also attenuated by EPA. Furthermore, a rise in PGE_2, $PGF_{2\alpha}$, 5-, 12- and 15-hydroxyeicosatetraenoic acid (HETE) was caused by PIF. When these metabolites were added to C_2C_{12} myoblasts, no significant stimulation was observed by PGE_2, $PGF_{2\alpha}$, 5-HETE or 12-HETE. However, 15-HETE produced a dose-dependent stimulation of protein degradation with a bell-shaped profile similar to that produced by PIF and with a maximal 2-fold stimulation at a concentration of 30 nM (Smith *et al.*, 1999). These results suggested that the intracellular signal for protein degradation induced by PIF is 15-HETE. This could explain why the 5-lipoxygenase inhibitor 2,3,5-trimethyl-6-(-3-pyridylmethyl)1,4-benzoquinone (CV-6504) (Hussey and Tisdale, 1997), was capable of attenuating the development of cachexia in mice bearing the MAC16 tumour (Hussy *et al*, 1996).

CV-6504 has been shown to have an anti-tumour and anti-cachectic function. In a study by Hussey and Tisdale (1997) CV-6504 effectively suppressed growth of the MAC16 tumour *in vivo* and prevented the accompanying cachexia, when administered daily at a dose of 10mg/kg. Furthermore, CV-6504 was shown to inhibit the 5-, 12- and 15-

lipoxygenase pathways in MAC13 tumour (Hussey and Tisdale, 1996b), as well as in MAC13, MAC16, MAC26 and Caco-2 cells (Hussey and Tisdale, 1997). Despite the fact that CV-6504 is a 5-lipoxygenase inhibitor (Ohkawa *et al.*, 1991), it is capable of profound inhibition of both the 12- and 15-lipoxygenase pathways. This role of the CV-6504 on these lipoxygenase pathways i.e. inhibition of 5-, 12- and 15-HETE formation is similar to that of EPA observed on the MAC16 tumour (Hudson *et al.*, 1993; Rose *et al.*, 1995).

Therefore, it is possible that both EPA and CV-6504 exert their anti-cachectic effect via a common pathway involving the arachidonic acid metabolite 15-HETE. Furthermore, CV-6504 could also inhibit the effect of PIF on protein catabolism.

The ATP-ubiquitin-proteasome pathway is believed to be responsible for the breakdown of the intracellular proteins in skeletal muscle (Lecker *et al.*, 1999a) and the importance of this pathway has been demonstrated in cancer cachexia (Baracos *et al.*, 1995; Temparis *et al.*, 1994). Increased mRNA levels of the $E2_{14k}$ ubiquitin conjugating enzyme and the two of the proteasome subunits (C8 and C9) was detected in Yoshida sarcoma-bearing rats with muscle atrophy (Temparis *et al.*, 1994). Baracos *et al* (1995) also found an increase in proteasome mRNA subunits (C2, C3, C8 and C9) and increased levels of ubiquitin mRNA in Yoshida ascites hepatoma. Furthermore, the importance of the proteasome pathway in cancer-induced muscle catabolism in cancer patients has recently been demonstrated (Williams *et al.*, 1999b) by a 2-4 fold increase in tissue levels of mRNA for ubiquitin and 20S proteasome subunits in rectus abdominus muscle of cancer patients compared with patients with benign diseases.

The ubiquitin-proteasome pathway has also been shown to play a major role in protein degradation occurring in skeletal muscle of mice bearing the MAC16 tumour (Lorite *et al.*, 1998; Whitehouse *et al.*, 2001), where increased levels of ubiquitin-conjugated proteins and increased expression of mRNA for the $E2_{14k}$ ubiquitin-conjugating protein, as well as the C9 proteasome subunit, in gastrocnemius muscle from cachectic mice bearing the MAC16 tumour were reported (Lorite *et al.*, 1998). Increased gene expression of proteasomal subunits has been suggested to be crucial for enhanced protein catabolism in skeletal muscle, and has been used as a marker for looking at the upregulation and the downregulation of the proteasome proteolytic pathway.

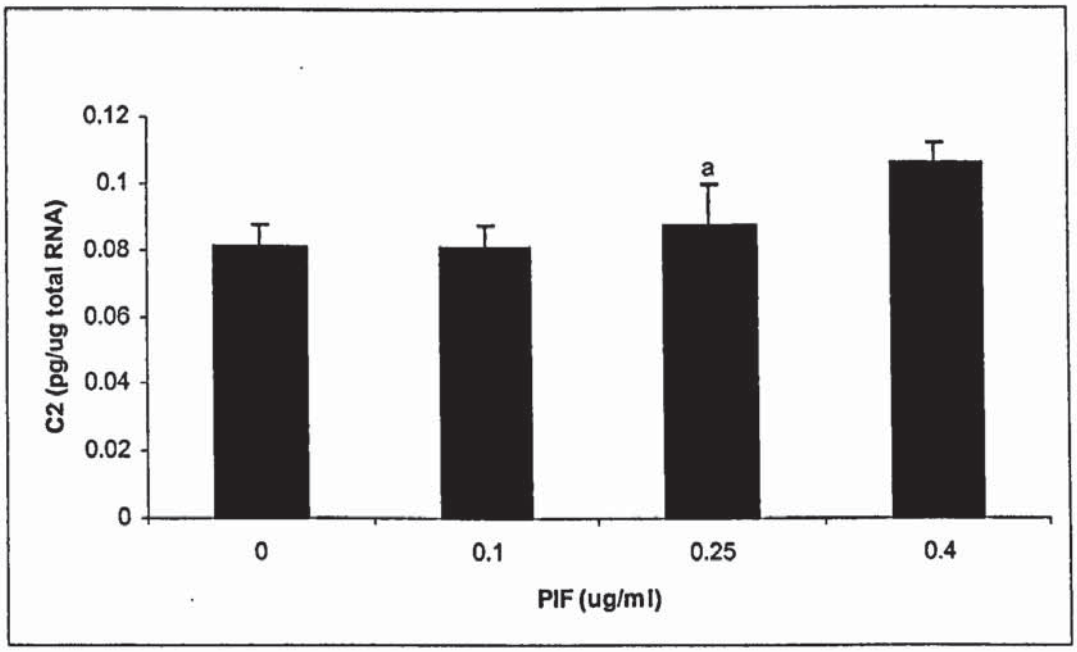
One of the aims of this project was to investigate the effect of PIF and 15-HETE on the ubiquitin-proteasome pathway. Furthermore, to examine the effect of EPA and CV-6504 upon the effects implicated by PIF and 15-HETE. To do this, competitive quantitative RT-PCR (QcRT-PCR) was set up (Chapter 3) to quantitatively investigate the expression of the C2 and C5 subunits of the 20S proteasome as well as the $E2_{14k}$ ubiquitin-conjugating enzyme gene. An *in vitro* muscle model was used for this purpose. The mus muscular C311 myoblast cell line – C_2C_{12} was differentiated into myotubes representative of mature differentiated muscle and used for all *in vitro* experiments.

4.2 Results and Discussion

4.2.1 Effect of PIF

To determine the effect of PIF *in vitro*, C_2C_{12} myotubes were treated with three different concentrations of PIF (0.1μg/ml, 0.25μg/ml and 0.4μg/ml) for 24 hours. RNA was then extracted from the cells (2.4.1.2) and was used as the target template in the QcRT-PCR technique (3.6 and 3.7). Figure 4.2.1.1 shows the expression of the C2 20S proteasome subunit. The C2 expression seems to be stimulated in the presence of PIF with the maximal activity (approx. 31%) at 0.4μg/ml PIF.

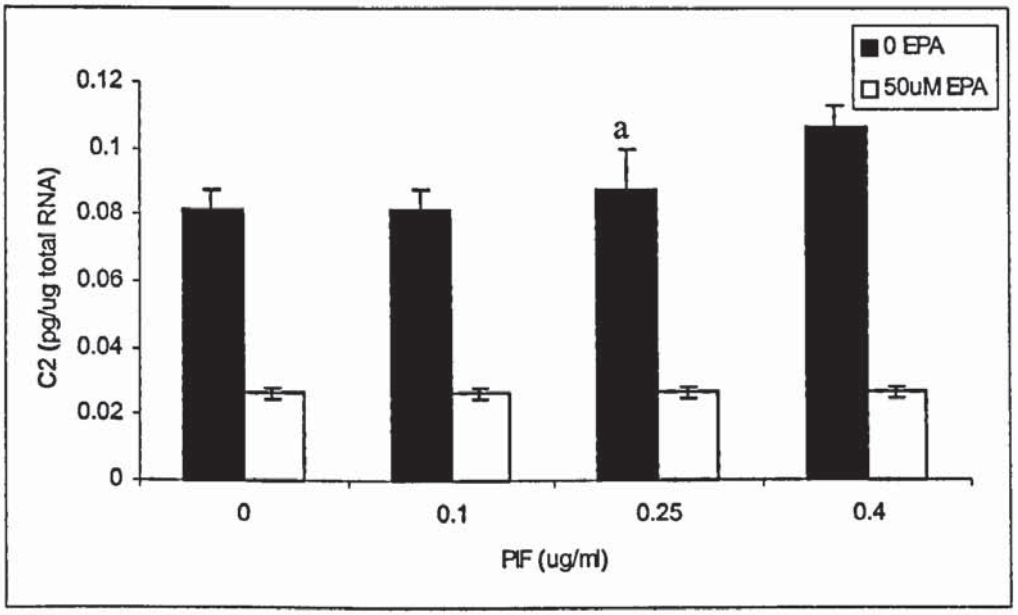
Fig 4.2.1.1 Effect of PIF on C2 20S proteasome subunit expression in C_2C_{12} myotubes



The experiment was repeated 4 times. This figure is a representative showing mean ± SEM where n=2. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$.

In a parallel experiment, C_2C_{12} myoblasts were treated with 50μM EPA for 3 h prior to the 24 h PIF treatment. Fig 4.2.1.2 shows attenuation of the C2 expression by EPA at all concentrations of PIF, where, figure 4.2.1.1 is re-plotted to show the effect of the EPA.

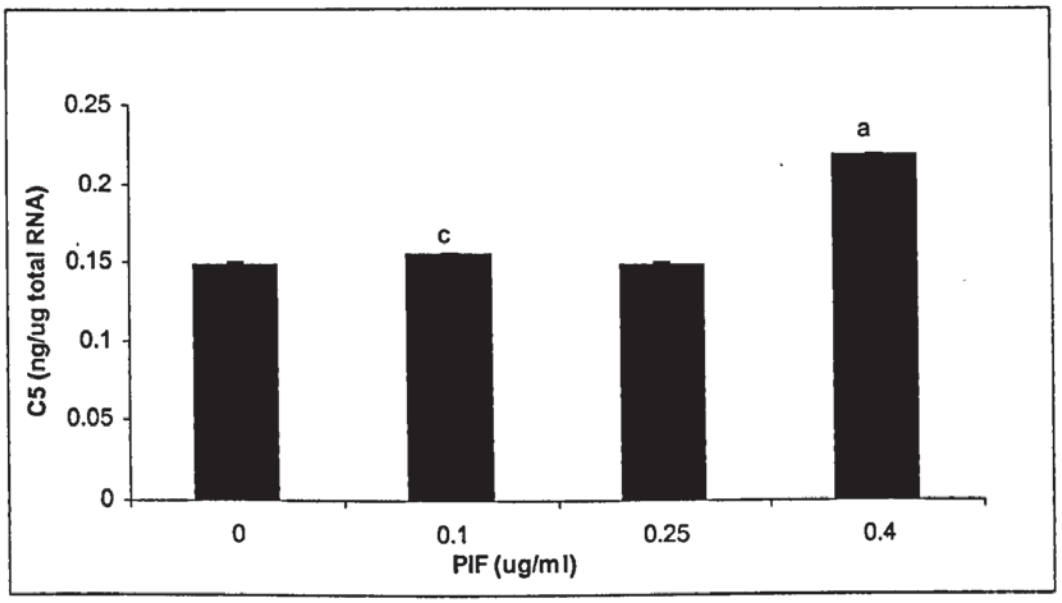
Fig 4.2.1.2 The effect of PIF and EPA on the expression of C2 20S proteasome subunit in C_2C_{12} myotubes



The experiment was repeated 3 times, this figure is a representative, where values represent the mean ± SEM where n=2. Difference from control value was determined by one-way ANOVA with Tukey's test, where a = $p<0.05$.

The expression of the β6-20S proteasome subunit (C5) was also measured in the same RNA samples isolated from C_2C_{12} myotubes treated with PIF and with PIF and EPA as discussed above. Figure 4.2.1.3 shows the effect of different concentrations of PIF on the expression of C5 gene. The highest expression was observed at 0.4μg/ml PIF concentration (approx. 47%).

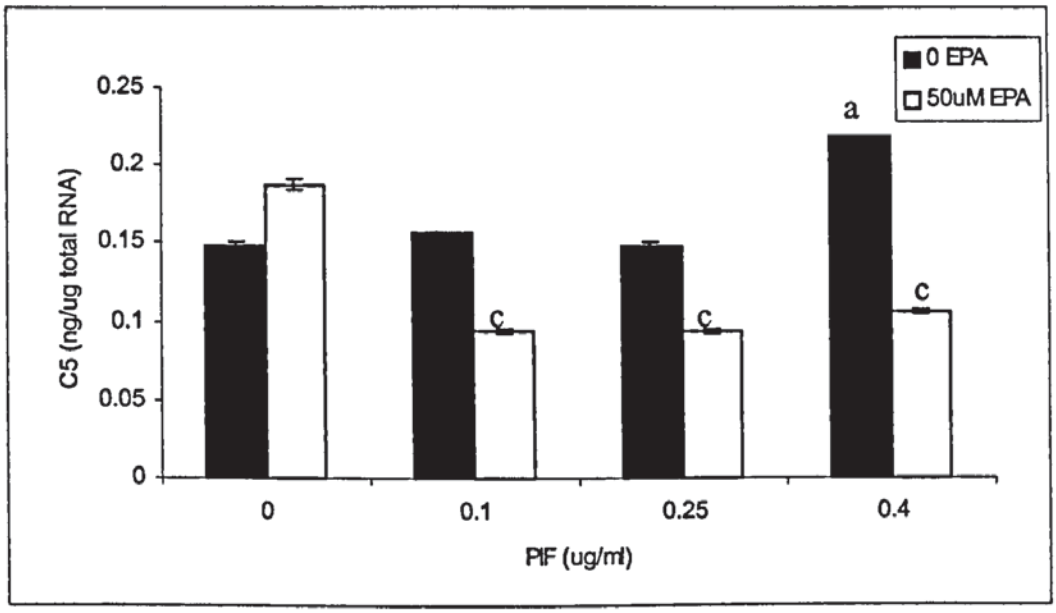
Fig 4.2.1.3 Effect of PIF on the C5-20S proteasome subunit expression in C_2C_{12} myotubes



This experiment was repeated 4 times. This figure is a representative showing mean ± SEM where n=2. Statistical analysis performed using one-way ANOVA with Tukey's test. Differences from control values are indicated by a = $p<0.05$ and c = $p<0.001$.

Figure 4.2.1.4 shows the effect of EPA on the enhanced expression of C5 subunit with respect to the PIF effect (figure 4.2.1.3 is re-plotted to show the effect of EPA). The expression is seen to be attenuated to a level below that of the control.

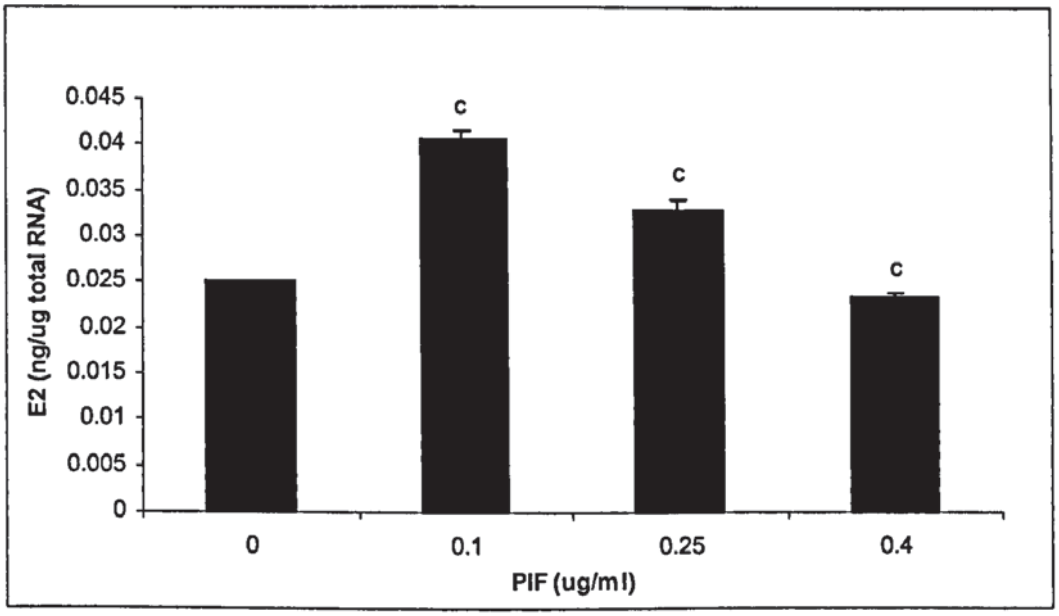
Fig 4.2.1.4 Effect of PIF and EPA on the expression of the C5 20S proteasome subunit in C_2C_{12} myotubes



The experiment was repeated 3 times, this figure is a representative, where results shown as mean ± SEM where n=2. Statistical analysis using one-way ANOVA was performed where a = $p<0.05$ and c = $p<0.001$, in comparison to control.

The expression of the ubiquitin-conjugating enzyme $E2_{14k}$ was then measured in these samples. Figure 4.2.1.5 shows the $E2_{14k}$ expression in the C_2C_{12} myotubes treated with PIF. PIF at 0.1ug/ml concentration produced the peak increase in $E2_{14k}$ gene expression (approx. 63%), and the response is a bell-shaped dose response curve typically seen with respect to the effect of PIF on protein degradation (Smith *et al.*, 1999; Whitehouse, PhD thesis).

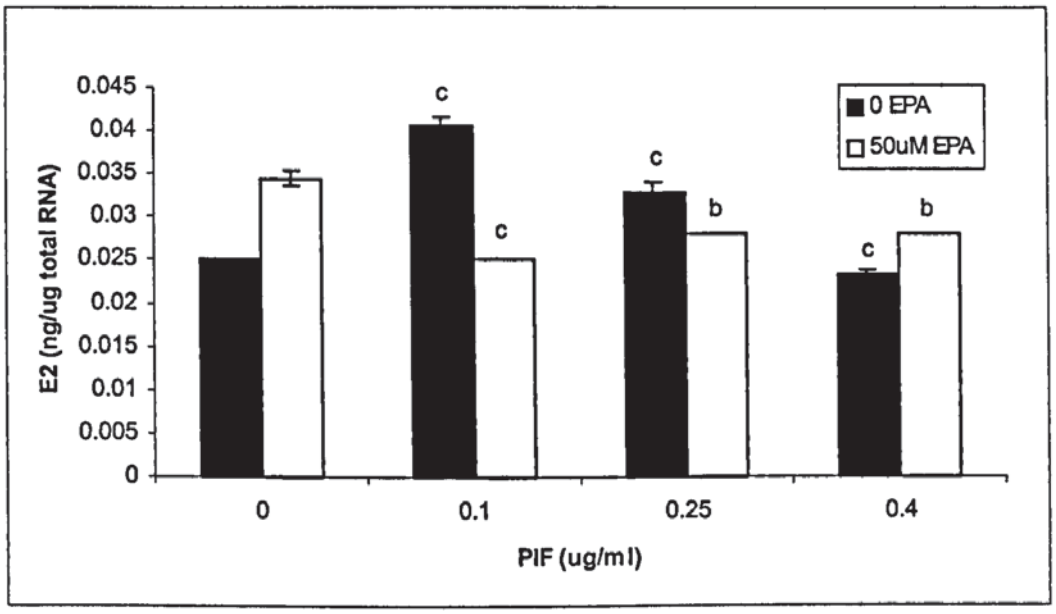
Fig 4.2.1.5 The effect of PIF on the expression of the $E2_{14k}$ gene in C_2C_{12} myotubes



The experiment was repeated 4 times. This figure is a representative showing mean ± SEM where n=2. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby c = $p<0.001$.

Again EPA attenuated the effect of PIF on the $E2_{14k}$ expression (figure 4.2.1.6), however, the effect is not as profound as seen previously with respect to the C2 (4.2.1.2) and C5 (4.2.1.4).

Fig 4.2.1.6 Effect of PIF and EPA on the expression of the $E2_{14k}$ gene in C_2C_{12} myotubes

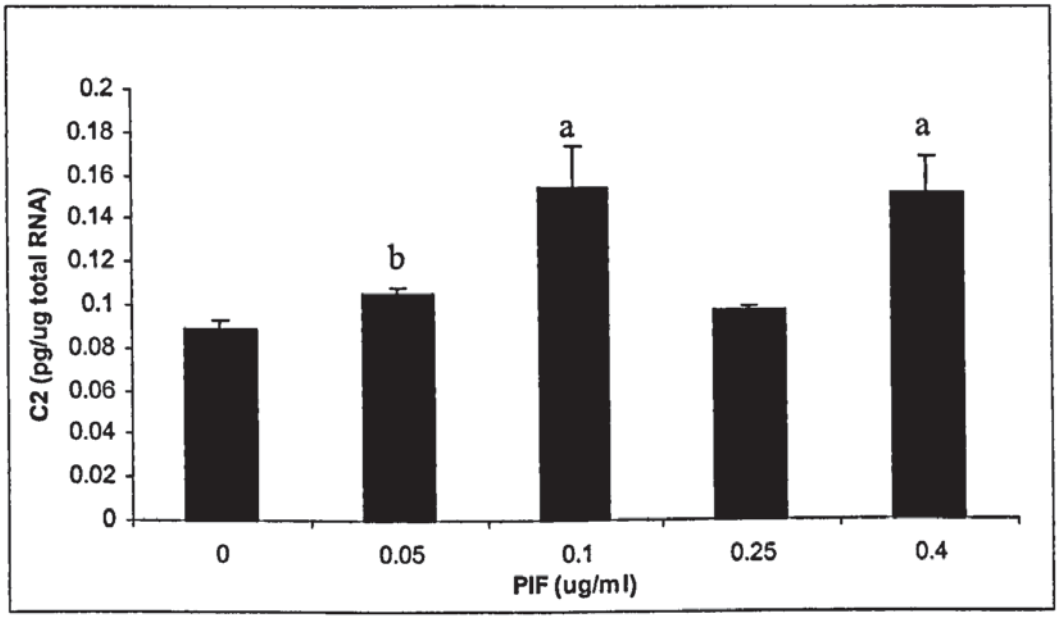


The experiment was repeated 3 times. This figure is a representative showing mean ± SEM where n=2. Statistical analysis performed using one-way ANOVA with Tukey's test. Differences from control values as indicated by b = $p<0.01$, c = $p<0.001$. Figure 4.2.1.5 was re-plotted to show the effect of EPA.

These results are similar to those found by Smith *et al* (1999) where protein degradation was shown to be increasing in C_2C_{12} myoblasts with increasing concentrations of PIF, whereas EPA completely abolished the increase in protein degradation. However, the protein degradation observed by Smith *et al* (1999) in the C_2C_{12} myoblasts increased by 50-90% in response to PIF in comparison with 31% and 47% increase gene expression shown by the C2 and C5 20S proteasome subunits respectively. Measuring the gene expression of the proteasome subunits by QcRT-PCR is at the level of transcription since RNA levels are measured in contrast to the measure of protein by Smith *et al* (1999). Therefore, the upregulation of the ubiquitin-proteasome pathway might occur much earlier. To determine this, C_2C_{12} myoblasts were treated with four different concentrations of PIF (0.05μg/ml, 0.1μg/ml, 0.25μg/ml and 0.4μg/ml) for 4 h, and the mRNA levels of C2, C5 and $E2_{14k}$ genes were measured.

Figure 4.2.1.7 shows the expression of the C2 proteasome subunit in C_2C_{12} myotubes incubated with PIF for 4 hours. The C2 expression is shown to increase in the presence of PIF with a maximum stimulatory peak seen at 0.1μg/ml PIF (approx. 74%).

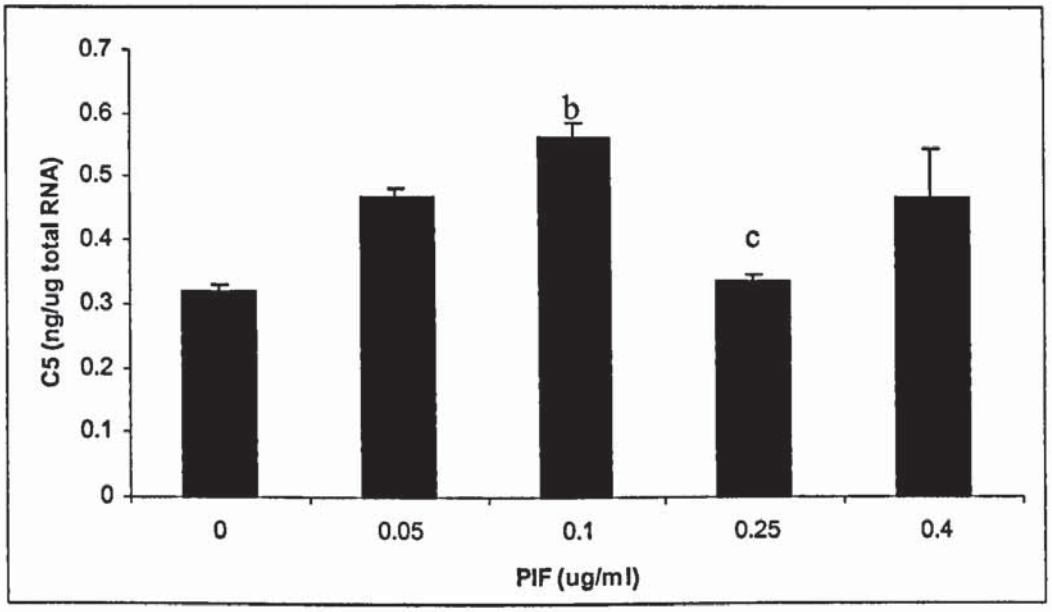
Fig 4.2.1.7 Expression of C2-20s proteasome subunit in C_2C_{12} myotubes incubated with PIF for 4 hours



Results shown are the mean ± SEM where n=4. Difference from control value was determined by one-way ANOVA with Student-Tukey's test, where a = $p<0.05$ and b = $p<0.01$.

Therefore, lower concentrations of PIF (0.1μg/ml) had a bigger effect (73%) on the expression of the C2 20S proteasome subunit when the C_2C_{12} myotubes were incubated for 4 h in comparison with the 24 h incubation, which only produced a 31% increase in C2 expression at 0.4μg/ml of PIF. This effect was also similar with respect to the proteasome C5 subunit expression with a bell-shaped dose response where, the peak up-regulation of this gene was reached when PIF concentration was 0.1μg/ml producing approximately 76% increase in C5 expression.

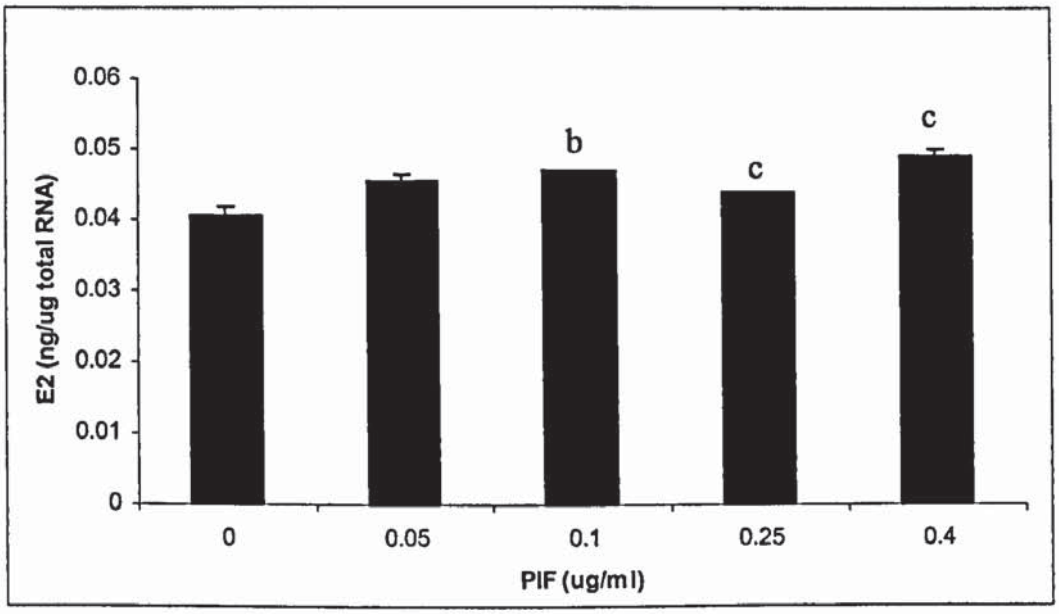
Fig 4.2.1.8 Expression of C5 20S proteasome subunit in C_2C_{12} myotubes treated with PIF for 4 hours



Values represent the mean ± SEM where n=4. Difference from control value was determined by one-way ANOVA with Tukey's test, where b = $p<0.01$ and c = $p<0.001$.

The $E2_{14k}$ expression was also measured in these C_2C_{12} myotube RNA samples and results are shown in figure 4.2.1.9. The maximal peak of stimulatory expression (approx. 21%) was at 0.4μg/ml PIF, which was much lower than that seen in the previous experiment (approx. 63%) at 0.1μg/ml PIF, where the C_2C_{12} myotubes were incubated with PIF for 24 h (figure 4.2.1.5). This suggests that the proteasome is stimulated much sooner than the ubiquitin-conjugating enzyme ($E2_{14k}$). This mechanism needs further investigation, since $E2_{14k}$ is upstream of the proteasome in the ubiquitin-proteasome pathway and it has been suggested that activation of this enzyme leads to the protein degradation by the proteasome (Attaix *et al.*, 1998). Therefore, in theory $E2_{14k}$ should be expressed earlier than the proteasome in response to a stimulatory effect.

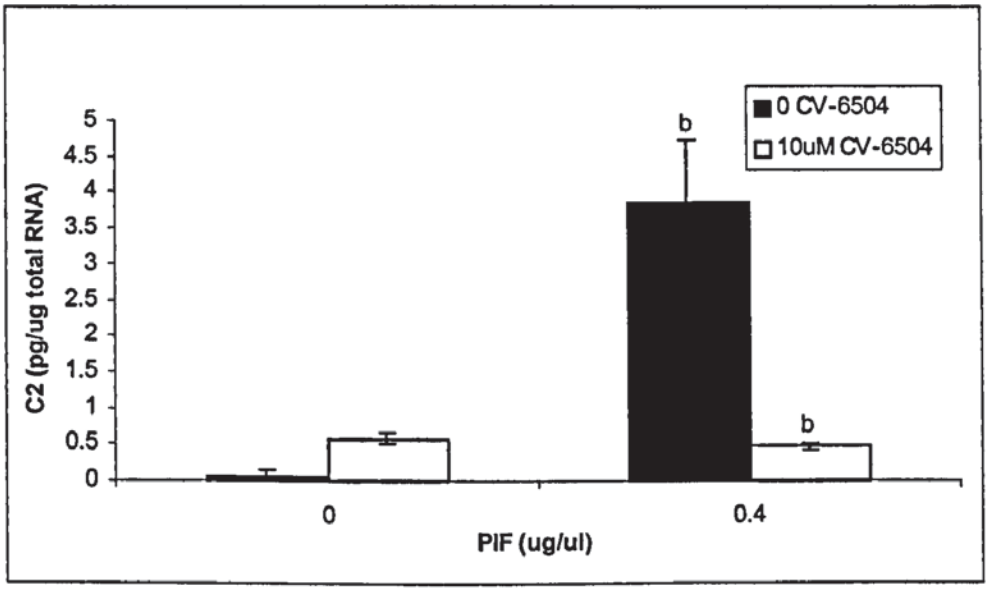
Fig 4.2.1.9 Expression of the $E2_{14k}$ in C_2C_{12} myotubes treated with PIF for 4 hours



Results shown are the mean ± SEM where n=4. Difference from control value was determined by one-way ANOVA with Student-Tukey's test, where b = $p<0.01$ and c = $p<0.001$.

The effect of CV-6504 was also investigated, where C_2C_{12} myotubes were pre-treated with 10μM CV-6504 for 3 h followed by 0.4 μg/ml PIF treatment for 4 h. Figure 4.2.1.10 shows the effect of CV-6504 on the expression of C2 20S proteasome subunit with and without PIF. The enhanced C2 expression with PIF is attenuated by the 5-lipoxygenase inhibitor CV-6504 (by approx. 88%).

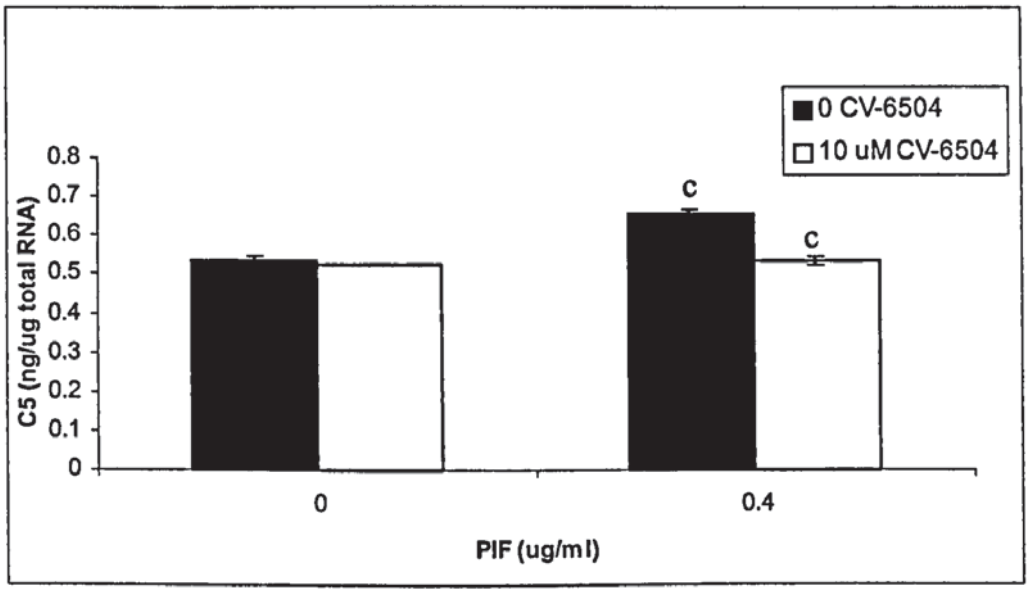
Fig 4.2.1.10 Effect of PIF and CV-6504 on the expression of the C2 20S proteasome subunit in C_2C_{12} myotubes



Results shown are the mean ± SEM where n=3. Differences from control value was determined by one-way ANOVA with Student-Tukey's t-test, where b = $p<0.01$.

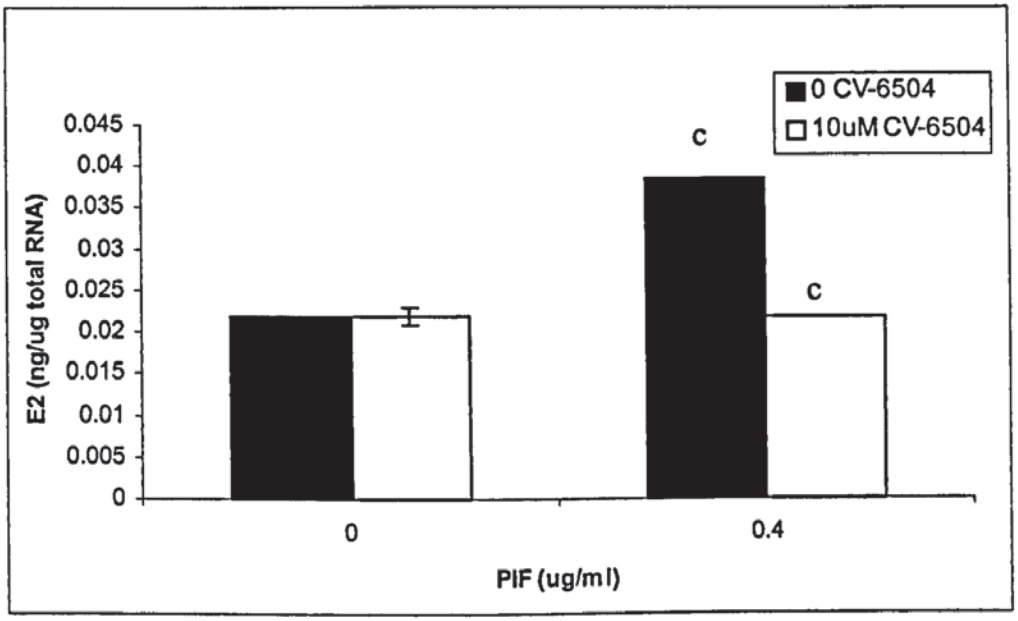
Figure 4.2.1.11 shows the effect of CV-6504 on the expression of the C5 proteasome subunit with and without PIF (0.4μg/ml). This figure shows that CV-6504 attenuates the effect of PIF to control levels. This is also seen in fig 4.2.1.12, where the $E2_{14k}$ expression is increased with 0.4μg/ml PIF (approx. 76%), and this increased expression was attenuated with pre-incubation of the C_2C_{12} myotubes with 10μM CV-6504, to that of control levels.

Fig 4.2.1.11 Effect of PIF and CV-6504 on the expression of the C5 20S proteasome subunit in C_2C_{12} myotubes



Results are shown as mean ± SEM where n=3 and statistical analysis was performed using one-way ANOVA with Student-Tukey's test, whereby c = $p<0.001$.

Fig 4.2.1.12 The effect of PIF and CV-6504 on the expression of $E2_{14k}$ in C_2C_{12} myotubes

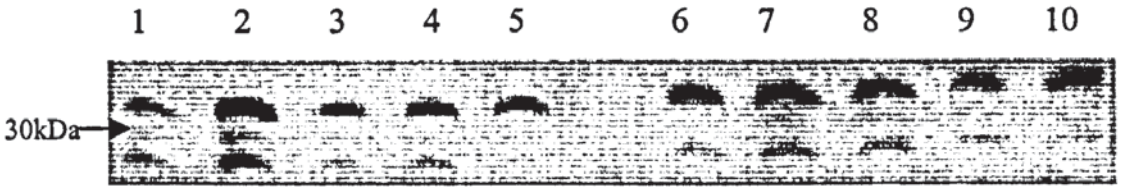


Results are shown as mean ± SEM where n=3 and statistical analysis was performed using one-way ANOVA with Student-Tukey's test, whereby c = $p<0.001$.

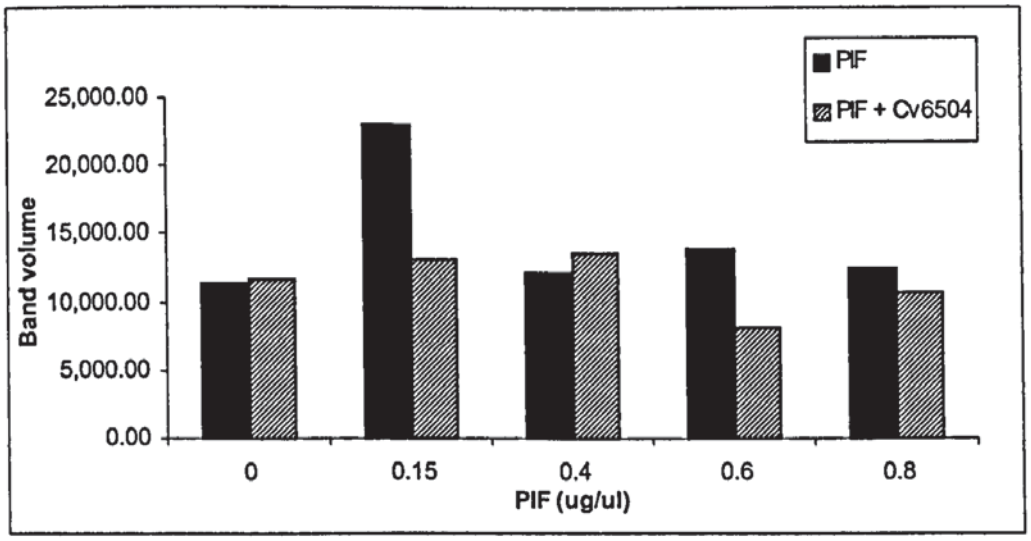
Furthermore, Western blotting was performed to measure both the proteasome 20S α-subunits and $E2_{14k}$ components of the ubiquitin-proteasome pathway in C_2C_{12} myotubes treated with PIF and CV-6504. Figure 4.2.1.13 shows the expression of the 20S α-subunits, detected with MCP-231, a murine monoclonal antibody, increasing over the concentration range of PIF (0.15μg/ml-0.8μg/ml)*, with a maximum stimulation of 104% at 0.15μg/ml PIF. This increased expression was attenuated by 92% when the myotubes were pre-incubated with 10μM CV-6504.

* The concentration of PIF was decided by preliminary experiments, which indicated the best concentration range for PIF activity, with respect to a particular PIF purified.

Fig 4.2.1.13 Western and densitometric analysis demonstrating the effect of PIF and CV-6504 on the 20S α-subunits in C_2C_{12} myotubes



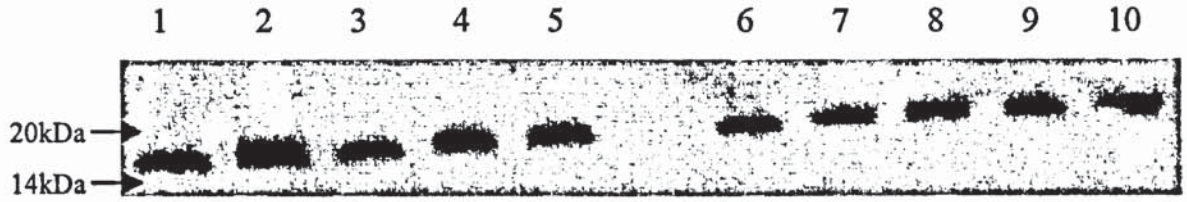
1	0μg/ml PIF	6	0μg/ml PIF + 10μM CV-6504
2	0.15μg/ml PIF	7	0.15μg/ml PIF + 10μM CV-6504
3	0.4μg/ml PIF	8	0.4μg/ml PIF + 10μM CV-6504
4	0.6μg/ml PIF	9	0.6μg/ml PIF + 10μM CV-6504
5	0.8μg/ml PIF	10	0.8μg/ml PIF + 10μM CV-6504



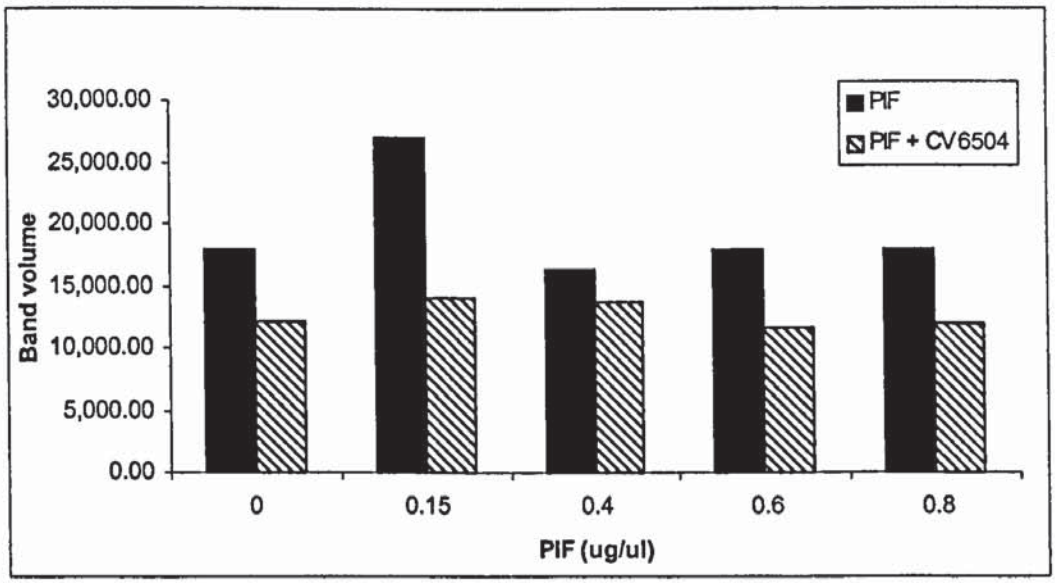
The volume of the 30kDa band was measured using Phoretix photo-imager programme.

Immunoblotting for the ubiquitin-conjugating enzyme-$E2_{14k}$ was also performed using the same protein samples described above. Figure 4.2.1.14 shows a similar effect for the $E2_{14k}$ expression, where PIF stimulated the expression of $E2_{14k}$ by 51% at 0.15μg/ml PIF. CV-6504 attenuated this expression by 35%, which was below the control levels.

Fig 4.2.1.14 Immunoblot and densitometric analysis of $E2_{14k}$ isolated from C_2C_{12} myotubes treated with PIF and CV-6504



1	0μg/ml PIF	6	0μg/ml PIF + 10μM CV-6504
2	0.15μg/ml PIF	7	0.15μg/ml PIF + 10μM CV-6504
3	0.4μg/ml PIF	8	0.4μg/ml PIF + 10μM CV-6504
4	0.6μg/ml PIF	9	0.6μg/ml PIF + 10μM CV-6504
5	0.8μg/ml PIF	10	0.8μg/ml PIF + 10μM CV-6504



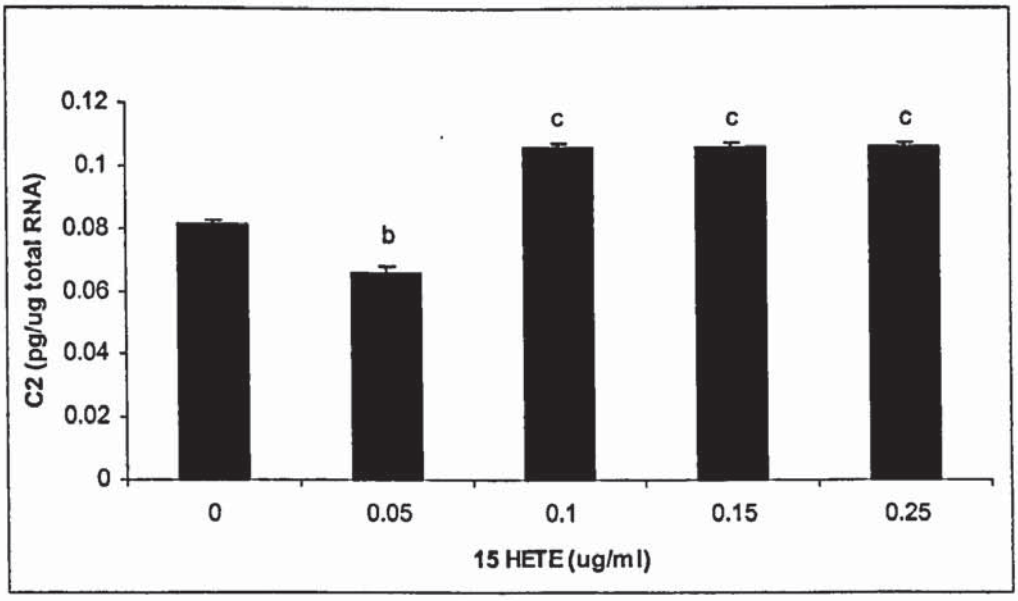
Volume of the band was measured using Phoretix photo-imager programme.

4.2.2 Effect of 15-HETE

To examine the effect of 15-HETE on the ubiquitin-proteasome pathway, C_2C_{12} myotubes were treated with varying concentrations of 15-HETE (0.05μg/ml, 0.1μg/ml, 0.15μg/ml and 0.25μg/ml) and incubated for 24 h. Total RNA was extracted from the cells (2.4.1.2) and expression of $E2_{14k}$ ubiquitin-conjugating enzyme and the 20S proteasome subunits C2 and C5 were measured by QcRT-PCR (3.6 and 3.7).

Figure 4.2.2.1 shows the expression of the C2 20S proteasome subunit, which significantly increased with 15-HETE at a concentration of 0.1μg/ml (to approx. 31%), and this increased expression prolonged with 0.15μg/ml and 0.25μg/ml 15-HETE.

Fig 4.2.2.1 Effect of 15-HETE on the expression of the C2 20S proteasome subunit in C_2C_{12} myotubes

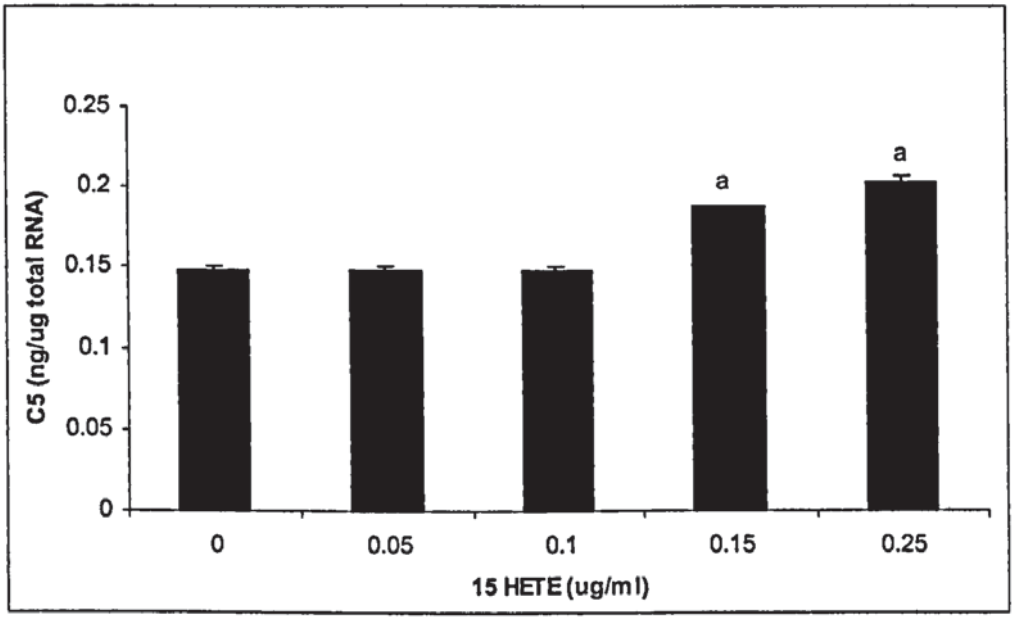


The experiment was repeated 4 times and this figure is a representative showing mean ± SEM where n=3. Statistical analysis performed using one-way ANOVA with Student's t-test comparing values to control, where b = $p<0.01$ and c = $p<0.001$.

Figure 4.2.2.2 shows the expression of C5 20S proteasome in the same RNA samples extracted from C_2C_{12} 15-HETE treated myotubes. Stimulation of the C5 gene

expression was shown with 0.15μg/ml and 0.25μg/ml 15-HETE producing 26% and 37% increased expression, respectively.

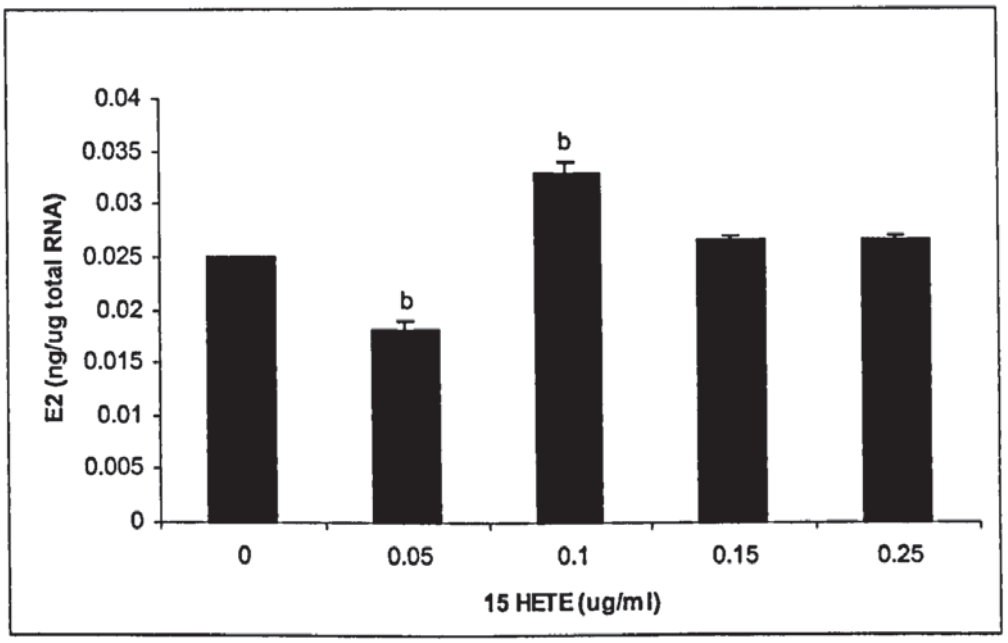
Fig 4.2.2.2 Effect of 15-HETE on the expression of C5 20S proteasome subunit in C_2C_{12} myotubes



Results represent the mean ± SEM where n=2 in an experiment. The experiment was repeated 4 times. Statistical analysis was performed using Student's t-test comparing values to control, a = $p < 0.05$.

Expression of the $E2_{14k}$ ubiquitin-proteasome enzyme was also measured by the QcRT-PCR. 15-HETE increased the $E2_{14k}$ expression in the C_2C_{12} myotubes and the peak stimulation was seen at a concentration of 0.1μg/ml 15-HETE (to approx. 31%). This is shown in figure 4.2.2.3.

Fig 4.2.2.3 Effect of 15-HETE on the expression of $E2_{14k}$ in C_2C_{12} myotubes

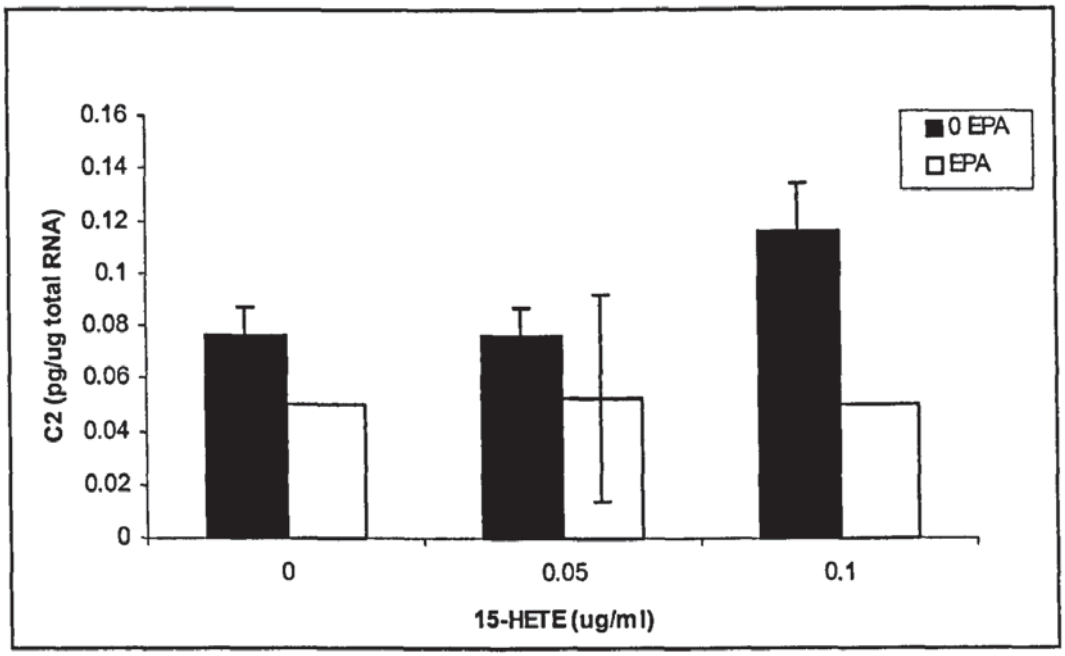


The experiment was repeated 4 times. This figure is a representative showing mean ± SEM where n=2. Statistical analysis performed using one-way ANOVA with Tukey's test, whereby b = $p < 0.01$.

The effect of EPA on the stimulation by 15-HETE was also investigated, where C_2C_{12} myotubes were treated with 50μM EPA for 3 hours prior to treatment of 15-HETE. The concentration of 15-HETE was decided by preliminary experiments, which demonstrated the greatest effect upon proteasome C2 and C5 subunits and $E2_{14k}$ activity.

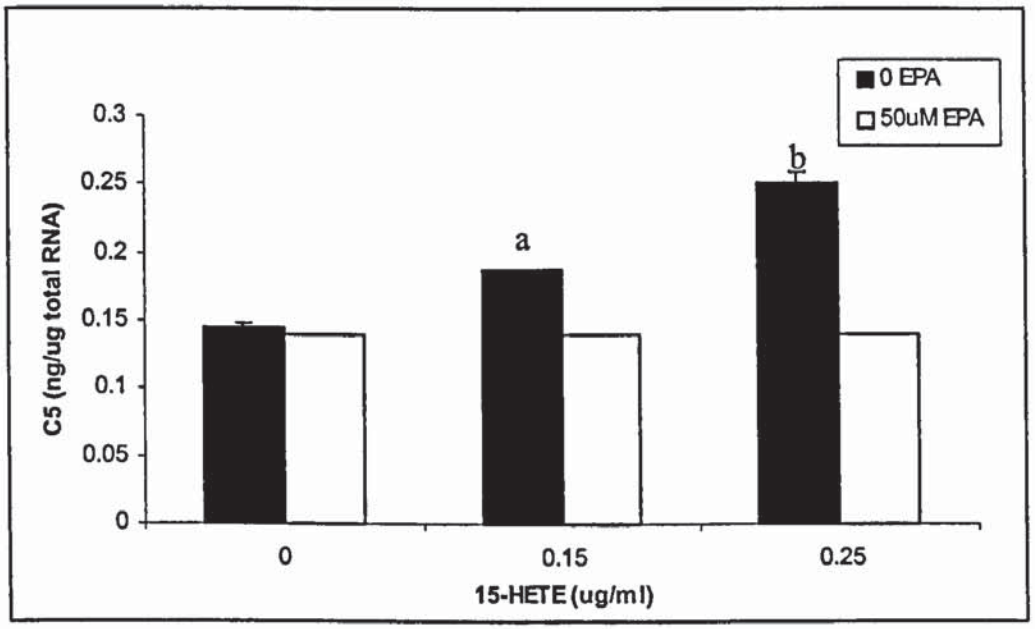
EPA attenuated the effect of 15-HETE on the C2 and C5 20S proteasome subunits as well as the $E2_{14k}$ enzyme to below that of the control levels. These results are shown in figure 4.2.2.4, figure 4.2.2.5 and figure 4.2.2.6, respectively.

Fig 4.2.2.4 Effect of 15-HETE and EPA on the expression of the C2 20S proteasome subunit in C_2C_{12} myotubes



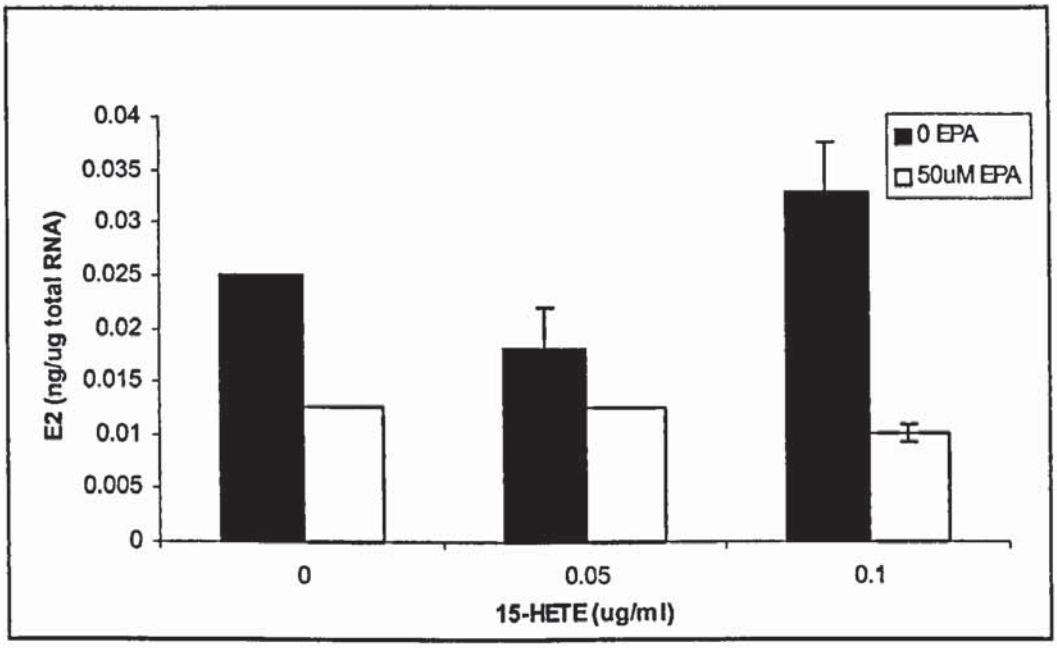
Results shown represent the mean ± SEM where n=2.

Fig 4.2.2.5 Effect of 15-HETE and EPA on the expression of C5 20S proteasome subunit in C_2C_{12} myotubes



Results shown represent the mean ± SEM where n=2. Statistical analysis was performed using Student's t-test comparing values to control, whereby a = $p< 0.05$ and b = $p< 0.01$.

Fig 4.2.2.6 Effect of 15-HETE and EPA on the expression of $E2_{14k}$ in C_2C_{12} myotubes

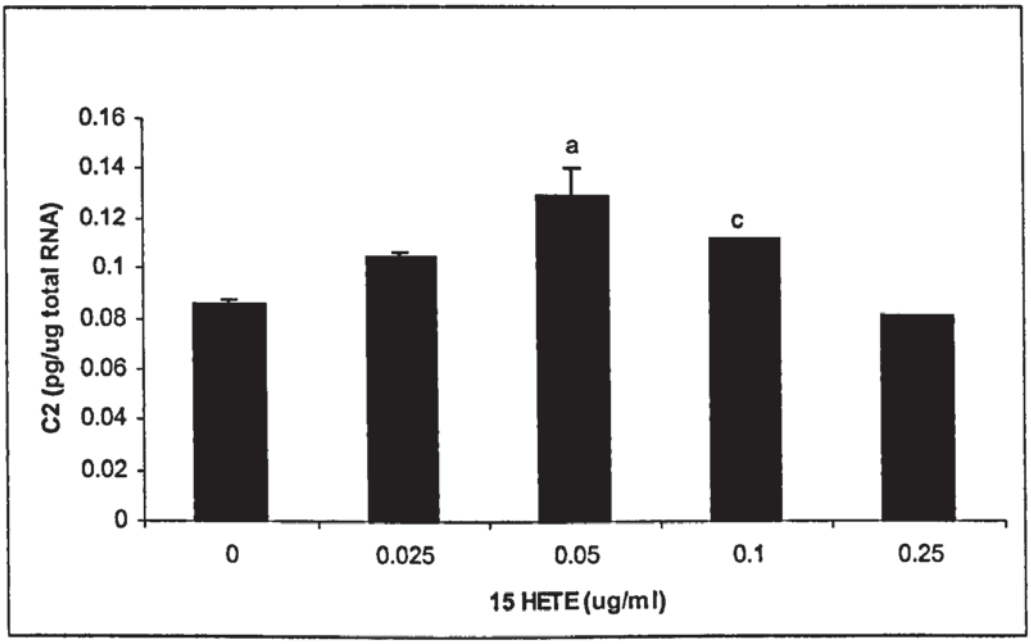


Results shown represent the mean ± SEM where n=2.

Furthermore, in the light of the previous observation, where PIF had a greater effect on the expression of the proteasome and the ubiquitin-conjugating enzyme after 4 hour incubation compared to 24 h incubation typically used; it was thought that 15-HETE could have the same effect. For this purpose, C_2C_{12} myotubes were treated for 4 h with varying concentrations of 15-HETE (0.025μg/ml, 0.05μg/ml, 0.1μg/ml and 0.25μg/ml).

Figure 4.2.2.7 shows the expression of C2 20S proteasome subunit in C_2C_{12} myotubes treated with 15-HETE for 4 hours, which produced a typical bell-shaped dose-response curve similar to that produced by PIF (4.2.1.7) and reported by Smith *et al* (1999). The maximum stimulation was approximately 51% at 0.05μg/ml 15-HETE which is significantly higher than previously seen with respect to the 24 h incubation (31% at 0.1μg/ml 15-HETE).

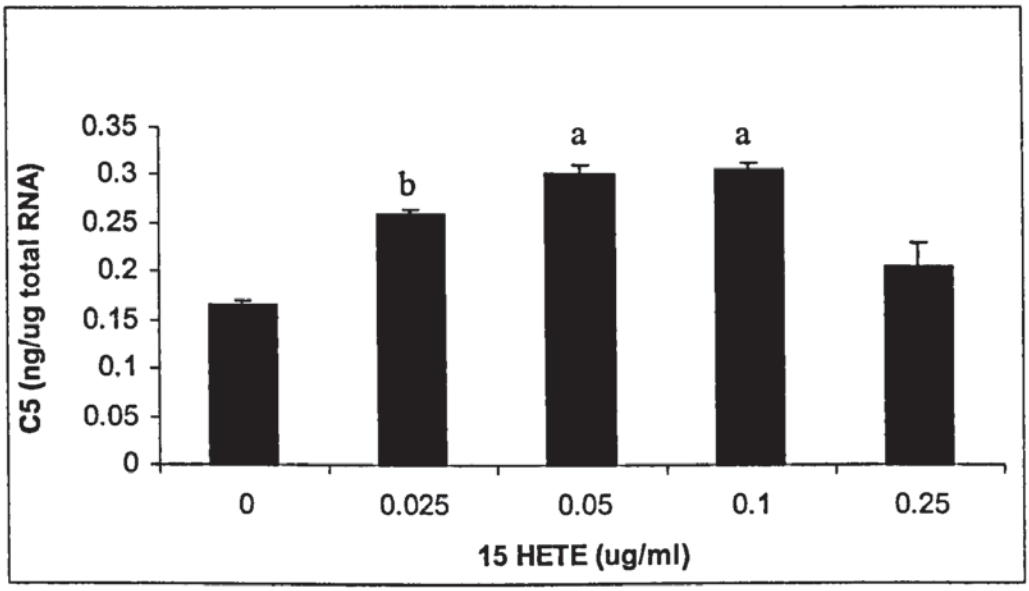
Fig 4.2.2.7 Expression of C2 proteasome subunit in C_2C_{12} myotubes treated with 15-HETE for 4 hours



Results represent the mean ± SEM where n=3. Statistical analysis was determined by using Tukey's post-test whereby, a = $p<0.05$ and c = $p<0.001$.

A lower concentration of 15-HETE (0.1μg/ml) produced maximum stimulation of the C5 proteasome subunit expression (approx. 85%) (figure 4.2.2.8), which is much higher than was seen previously with longer incubation period (approx. 26%), figure 4.2.2.2. Furthermore, the pattern of stimulation is very different in this case, even a small amount of 15-HETE (0.025μg/ml) stimulated expression of the C5 gene and the higher concentration of 15-HETE (0.25μg/ml) reduced the C5 gene expression, this is opposite to what was seen above (4.2.2.2), where 0.25μg/ml gave the maximal expression of C5 proteasome gene (approx. 37%). Again the picture seen in figure 4.2.2.8 is a typical bell-shaped response that has been reported before.

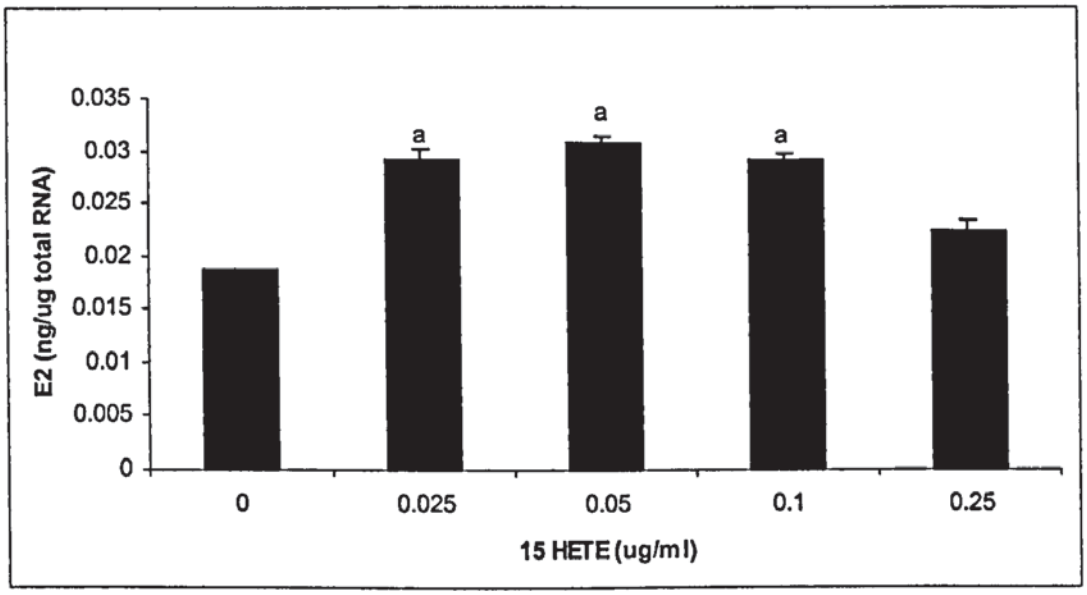
Fig 4.2.2.8 Expression of the C5 20S proteasome subunit in C_2C_{12} myotubes treated with 15-HETE for 4 hours



Results shown are mean ± SEM where n=3. Statistical analysis was performed using one-way ANOVA with Student-Tukey's test, comparing values to control where, a = $p<0.05$ and b = $p<0.01$.

Figure 4.2.2.9 shows that the expression of the $E2_{14k}$ ubiquitin-conjugating enzyme, which is maximum at 0.05μg/ml 15-HETE in C_2C_{12} myotubes treated for 4 hours. This significant increase was approximately 64% with respect to that observed in control untreated myotubes. This stimulation was much higher than observed in the previous experiment (24 h treatment), where 0.1μg/ml 15-HETE produced the maximum stimulation of the $E2_{14k}$ expression of approximately 31%.

Fig 4.2.2.9 Expression of the $E2_{14k}$ in C_2C_{12} myotubes treated with 15-HETE for 4 hours



Results represent the mean + SEM where n=3. Statistical analysis using one way ANOVA was performed comparing values to control, a = $p< 0.05$.

These results demonstrate that both PIF and 15-HETE can increase the expression of the proteasome subunits (C2 and C5) and the ubiquitin-conjugating enzyme ($E2_{14k}$) at the level of transcription in a concentration dependent bell-shaped response. Furthermore, it is evident that EPA can effectively attenuate the increased expression of these subunits *in vitro*. The 5-lipoxygenase inhibitor, CV-6504, also showed an attenuating effect on PIF.

A possible explanation of these results is that 15-HETE acts as the intracellular mediator for the stimulation of protein degradation by PIF, which exerts its effect by stimulating the expression of the proteasome and the other components of ubiquitin-proteasome pathway. Therefore, in the presence of the CV-6504 the production of 15-HETE is inhibited so preventing the stimulatory effect of PIF on the ubiquitin-proteasome pathway. Furthermore, the inhibitory effect of EPA on both 15-HETE and PIF suggests that EPA acts downstream of 15-HETE generation, which in-turn inhibits

the transcription of the proteasome subunits as well as the $E2_{14k}$ enzyme, i.e. inhibition of the ubiquitin-proteasome pathway.

The mechanism by which 15-HETE induces the expression of the proteasome pathway is not known, and further work is required to investigate this, which in-turn could explain the mechanism by which EPA inhibits the effect of 15-HETE. Furthermore, in view of the increased stimulation of proteasome mRNA at shorter time points, lower incubation periods (1 to 2 h) should be tested with both PIF and 15-HETE to find out the maximum *in vitro* effect of both PIF and 15-HETE on the level of mRNA expression of the proteasome subunits.

CHAPTER 5

Expression of the Ubiquitin-Proteasome Pathway in Skeletal Muscle of Cancer Cachectic Mice

5.1 Introduction

There are three major proteolytic pathways involved in the intracellular degradation of proteins, the lysosomal pathway, Ca^{2+} activated proteinases and the ubiquitin-proteasome system. Lysosomal proteolysis plays a minor role in skeletal muscle protein breakdown (Attaix and Taillandier, 1998); however, the Ca^{2+}-dependent proteases may play an important role in the degradation of specific but minor proteins. In contrast the ATP-ubiquitin-dependent pathway is believed to be responsible for the breakdown of the bulk of the intracellular proteins in skeletal muscle (Lecker *et al.*, 1999a), which has been demonstrated in starvation (Wing and Goldberg, 1993), sepsis (Voisin *et al.*, 1996), metabolic acidosis (Mitch *et al.*, 1994), denervation atrophy (Medina *et al.*, 1995) head trauma (Mansoor *et al.*, 1996) and cancer cachexia, both in animal models (Temparis *et al.*, 1994; Lorite *et al.*, 1998; Whitehouse *et al.*, 2001) and cancer patients (Attaix and Taillandier, 1998).

In the cachexia-inducing Yoshida AH-130 ascites tumour in mice, it was shown that the ubiquitin-proteasome pathway was predominantly upregulated in contrast to the other proteoytic pathways (Llovera *et al.*, 1994), which may be responsible for the muscle proteolysis and wastage observed in this animal tumour model. This study reported over 500% increase in muscle ubiquitin gene expression in tumour-bearing animals compared with non-tumour bearers. Increased mRNA levels for ubiquitin, the 14 kDa ubiquitin-conjugating protein E2, and the C8 and C9 proteasome subunits have also been reported in skeletal muscle of mice bearing Yoshida sarcoma (Temparis *et al.*, 1994), which suggested that the activation of the ATP-ubiquitin-dependent proteolytic pathway was mainly responsible for muscle atrophy in Yoshida sarcoma-bearing rats. Similar effects were also found in muscles from cachectic mice bearing the C-26 tumour (Fujita *et al.*, 1996). An increase in the ubiquitin-proteasome proteolytic system was also supported by the finding of increased levels of ubiquitin-conjugated proteins in skeletal muscle of rats bearing the Yoshida ascites hepatoma (Baracos *et al.*, 1995), they

also showed increased levels of ubiquitin mRNA and mRNA of multiple subunits of the proteasome (C2, C3, C8 and C9). However, liver, kidney, heart and brain showed no weight loss and no change in these mRNA species.

Another *in vivo* experimental model for studying mechanisms of muscle wasting in cancer cachexia is the murine MAC16 colon adenocarcinoma model, in which weight loss occurs with small tumour burden (less than 0.1% of host weight), and without a reduction of food and water intake (Beck and Tisdale, 1987), allowing a study of the metabolic components of cachexia. Lorite *et al* (1998) showed increased levels of ubiquitin-conjugated proteins as well as increased mRNA levels of the C9 proteasome subunit in gastrocnemius muscles of mice bearing the MAC16 tumour, thus supporting the involvement of the ATP-ubiquitin-dependent proteolytic system in protein catabolism in this tumour-bearing animal model. Furthermore, increased mRNA levels of the $E2_{14k}$ ubiquitin conjugating enzyme was also demonstrated, providing further evidence for the involvement of the ubiquitin-proteolytic pathway, since $E2_{14k}$ has been suggested to be rate limiting for ubiquitin conjugation (Wing and Banville, 1994).

The aim of this study was to determine the effect of progressive weight loss on the expression of mRNA for proteasome subunits C2 and C5, as well as the ubiquitin-conjugating enzyme $E2_{14k}$, in gastrocnemius and pectoral muscles of the MAC16 colon adenocarcinoma model. For this purpose quantitative competitive RT-PCR was used. Immunoblotting was also used to measure protein levels of proteasome subunits and $E2_{14k}$ protein to determine if altered mRNA expression reflects changes in protein expression.

5.2 Results and Discussion

5.2.1 Effect on Gastrocnemius Muscle

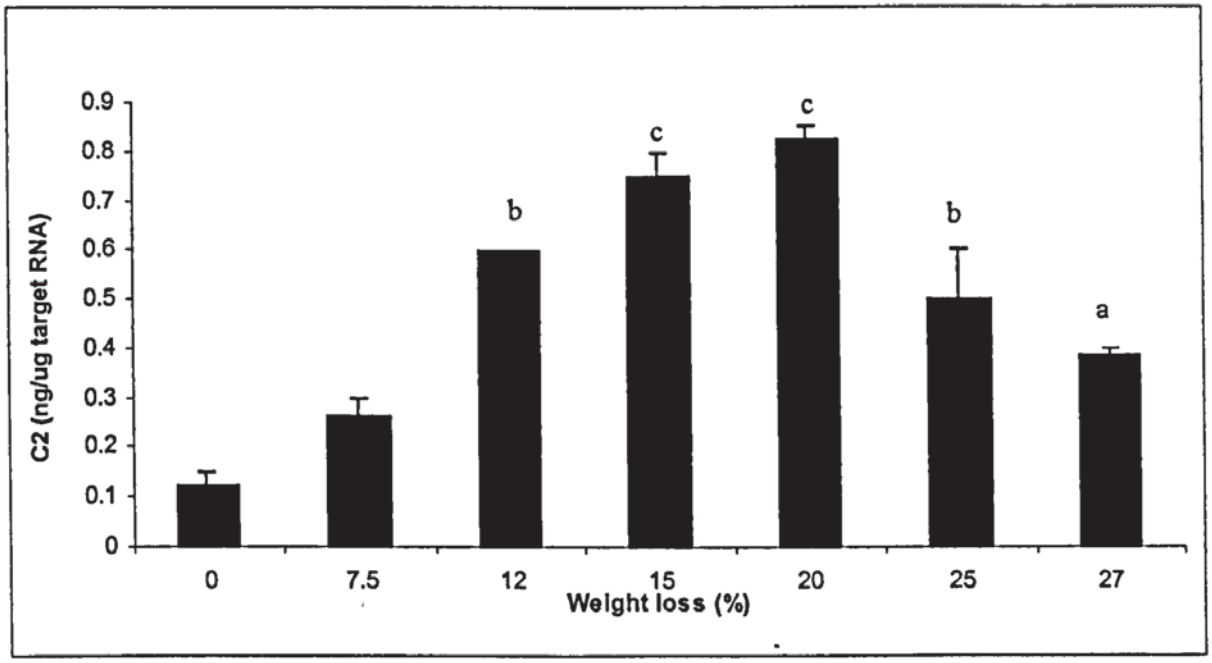
NMRI mice were transplanted with cachexia-inducing tumour MAC16, by means of a trocar into the flank. Once weight loss was established (between 5-27%) the animals were terminated and both the gastrocnemius and pectoral muscles were isolated and immediately frozen in liquid nitrogen, and stored at -70°C before further analysis. Total RNA was extracted from the muscles (2.4.1.2) and used in the RT-PCR quantitation (see chapter 3 for methodology).

The effect of increasing weight loss on the level of expression of C2 and C5 20S proteasome subunit mRNAs as well as the $E2_{14k}$ mRNA in gastrocnemius muscle was examined using QcRT-PCR. Figure 5.2.1.1 quantitates the expression of the C2 mRNA, which shows a significant increase in C2 mRNA with increasing weight loss from 12%, reaching a maximum at 15-20% weight loss (of approx 600% and 660%, respectively). The level of C2 expression decreased with weight losses at 25-27%, although the levels were still significantly increased compared with the control (animals without weight loss); figure 5.2.1.1.

The C5 proteasome subunit was also measured in the same samples and its expression was significantly increased at all weight losses (figure 5.2.1.2). The maximum increase in the level of expression was again at 20% weight loss with approximately 212% above baseline, which was less pronounced than C2 mRNA expression. Furthermore, C5 expression did not decrease in the 25-27% weight loss as was seen with respect to the C2 gene expression.

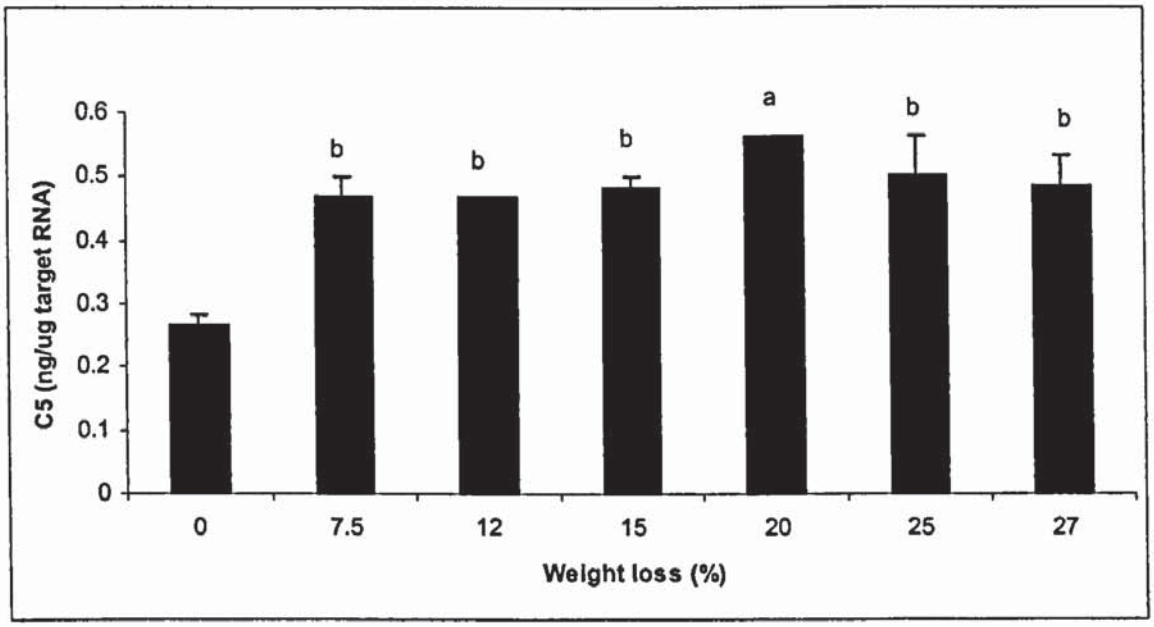
The effect of progressive weight loss on the expression of the ubiquitin-conjugating enzyme mRNA $E2_{14k}$, followed a similar pattern to that observed with the proteasome subunits (figure 5.2.1.3). E2 expression significantly increased in gastrocnemius muscle of mice with 12% weight loss and reached a maximum at 20% weight loss (of approx. 90%). Again the E2 expression decreased with higher weight losses 25-27%.

Fig 5.2.1.1 Expression of the C2 20S proteasome subunit in MAC16 gastrocnemius muscle with different extents of weight loss



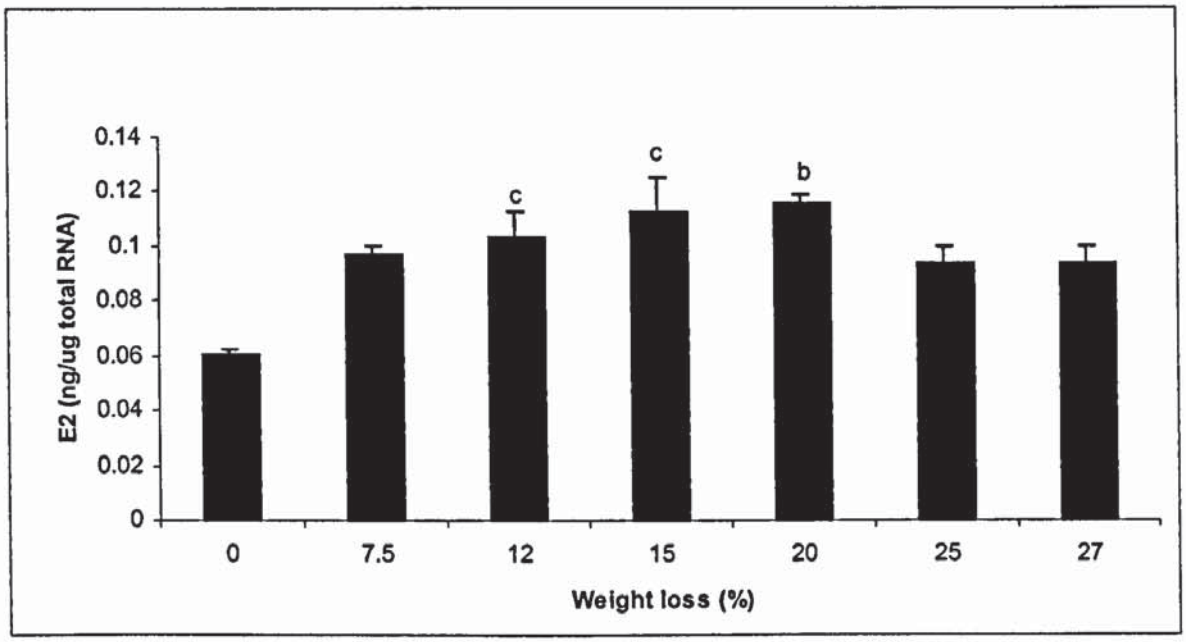
Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$, b = $p<0.01$ and c = $p<0.001$.

Fig 5.2.1.2 Expression of the C5 proteasome subunit in MAC16 gastrocnemius muscle with different extents of weight loss



Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$ and b = $p<0.01$.

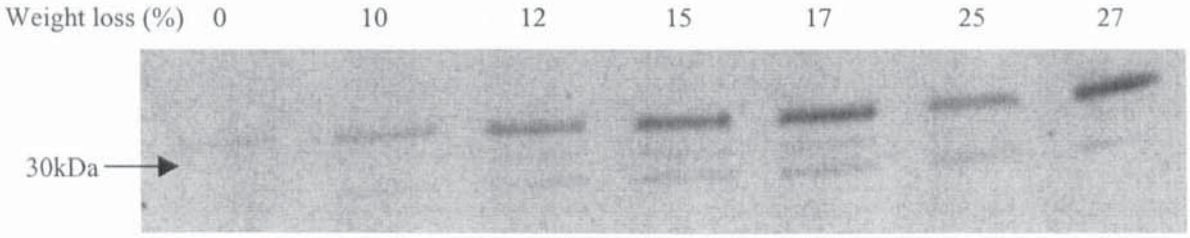
Fig 5.2.1.3 Expression of the ubiquitin-conjugating enzyme $E2_{14k}$ in MAC16 gastrocnemius muscle with different extents of weight loss

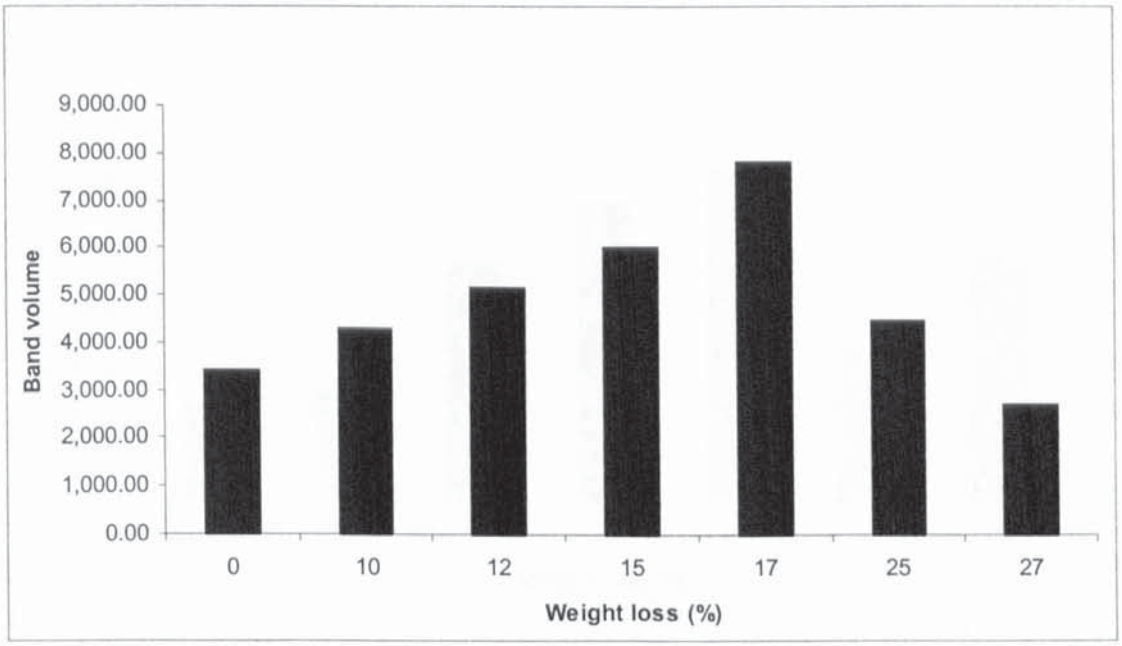


Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby b = $p<0.01$ and c = $p<0.001$.

The effect of increasing weight loss in gastrocnemius muscle of mice bearing the MAC16 tumour on 20S α-subunit protein levels were also detected by Western blotting (figure 5.2.1.4). Although the monoclonal antibody to the 20S proteasome reacts with 6 different α-type subunits, only three bands were apparent at approximate M_r 29, 32 and 35kDa (Whitehouse *et al.*, 2001). Figure 5.2.1.4 shows the 20S proteasome immunoblot, which indicates a good correlation with the mRNA expression of the proteasome subunits especially with the C2 mRNA expression (figure 5.2.1.1), showing a progressive increase of the 20S proteasome protein expression with increasing weight loss reaching a maximum stimulation of approximately 228% above baseline, then decrease in 20S protein expression with weight losses at 25% and 27%.

Fig 5.2.1.4 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of 20S α-subunits in gastrocnemius muscle of mice bearing the MAC16 tumour

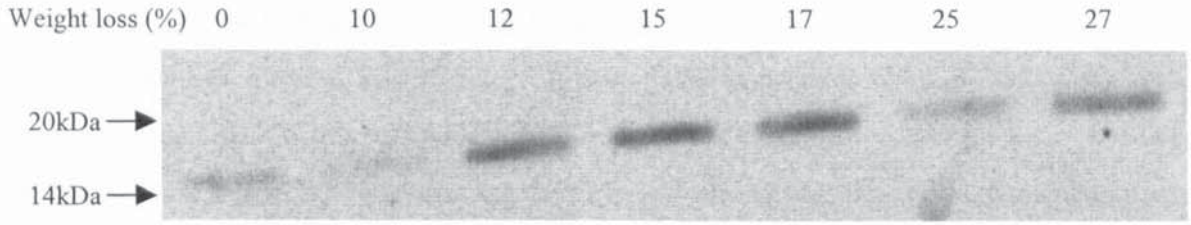


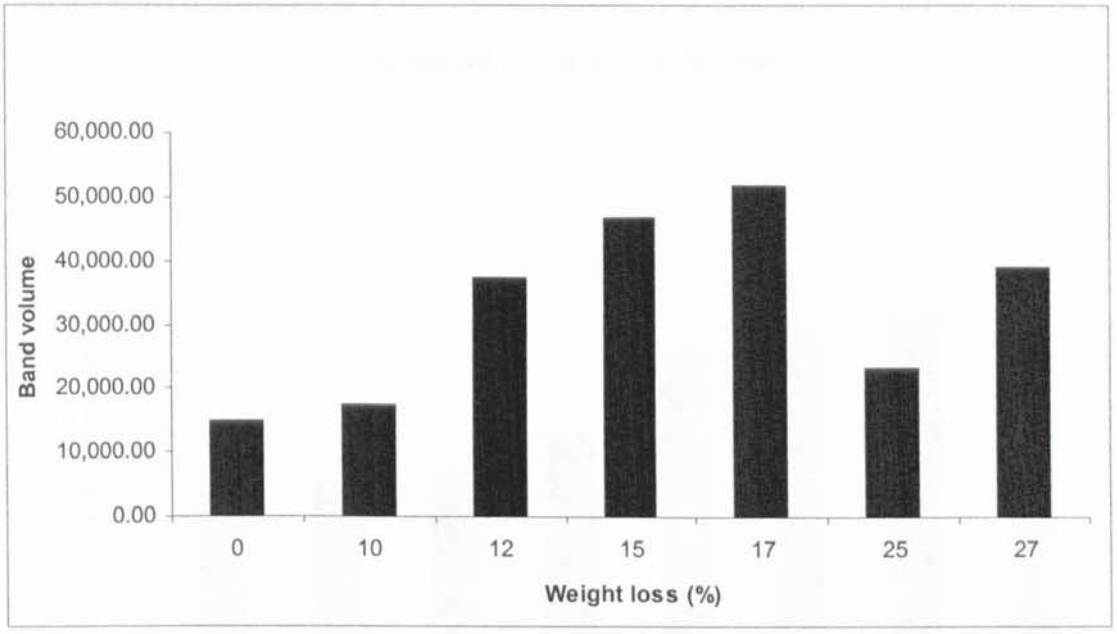


The volume of the 30kDa band was measured using Phoretix photo-imager programme.

The expression of $E2_{14k}$ protein was also determined by Western blotting (figure 5.2.1.5), which followed a similar pattern reaching a maximal expression at 15-17% weight loss (of approx. 213-245%). This pattern also correlates with the $E2_{14k}$ mRNA expression seen in figure 5.2.1.3.

Fig 5.2.1.5 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of $E2_{14k}$ in gastrocnemius muscle of mice bearing the MAC16 tumour



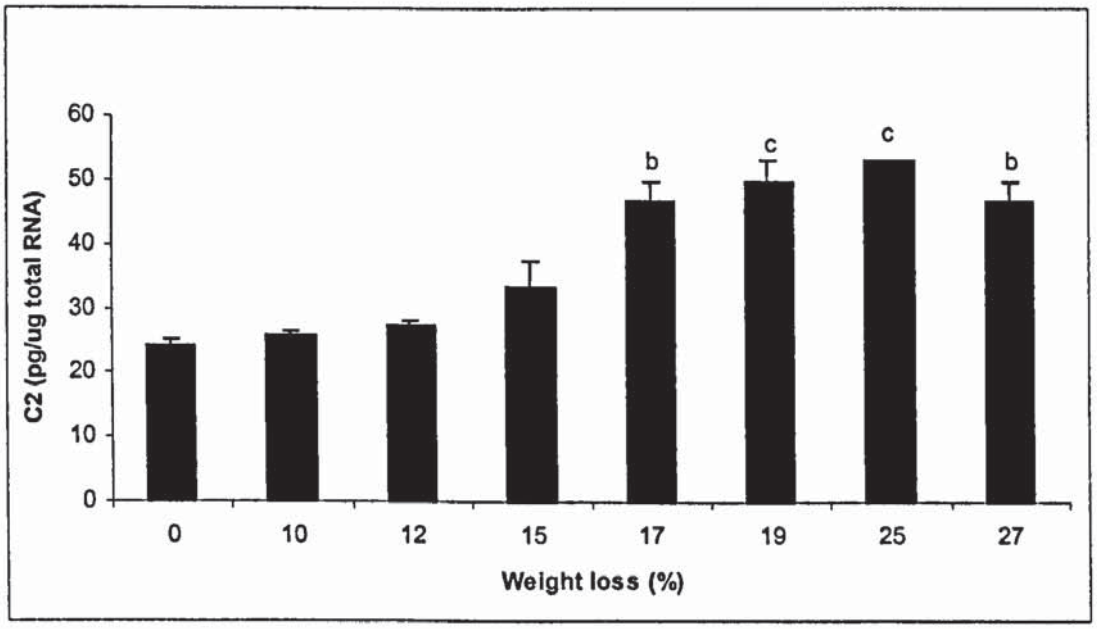


Volume of the band was measured using Phoretix photo-imager programme.

5.2.2 Effect on Pectoral Muscle

The effect of increasing weight loss on mRNA expression of C2 and C5 proteasome subunits and $E2_{14k}$ ubiquitin-conjugating enzyme in pectoral muscle was also investigated using QcRT-PCR, which followed a different expression pattern from that in gastrocnemius muscle. Figure 5.2.2.1 shows the expression of the C2 20S proteasome subunit, which shows increase level of expression with increasing weight loss, reaching maximum at 25% weight loss (of approx. 219%), which was less pronounced than that in gastrocnemius muscle.

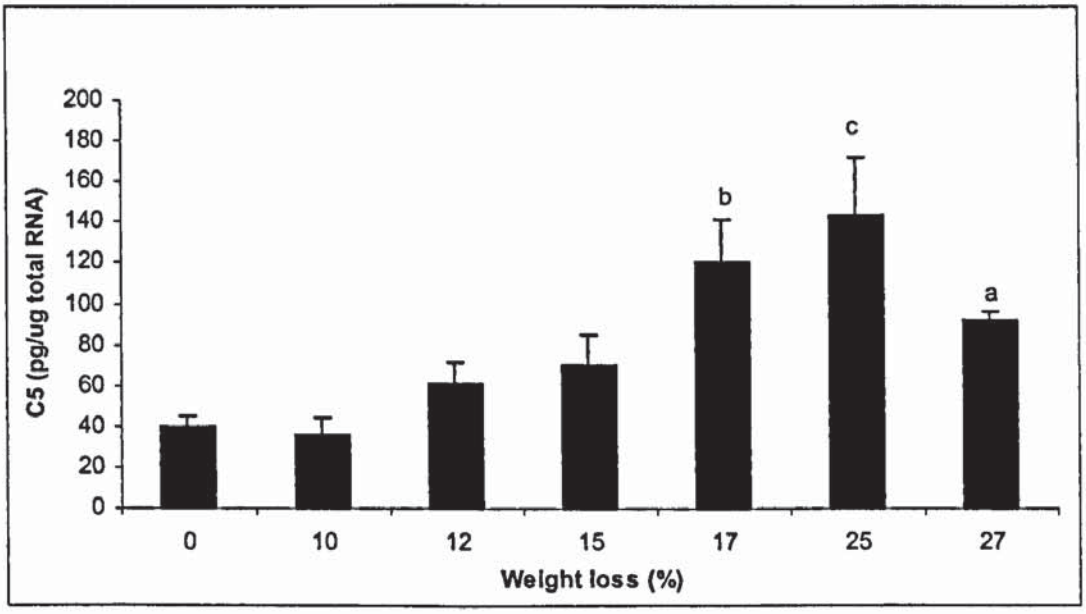
Fig 5.2.2.1 Expression of C2 proteasome subunit in MAC16 pectoral muscle with different extents of weight loss



Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with Tukey's test was performed, where, b = $p<0.01$ and c = $p<0.001$.

Figure 5.2.2.2 shows the expression of the C5 proteasome gene, where again expression is significantly increased when weight loss reached 17%, and the maximum C5 expression was at 25% weight loss (of approx. 359%), and expression decreased at 27% weight loss producing a similar bell-shaped pattern.

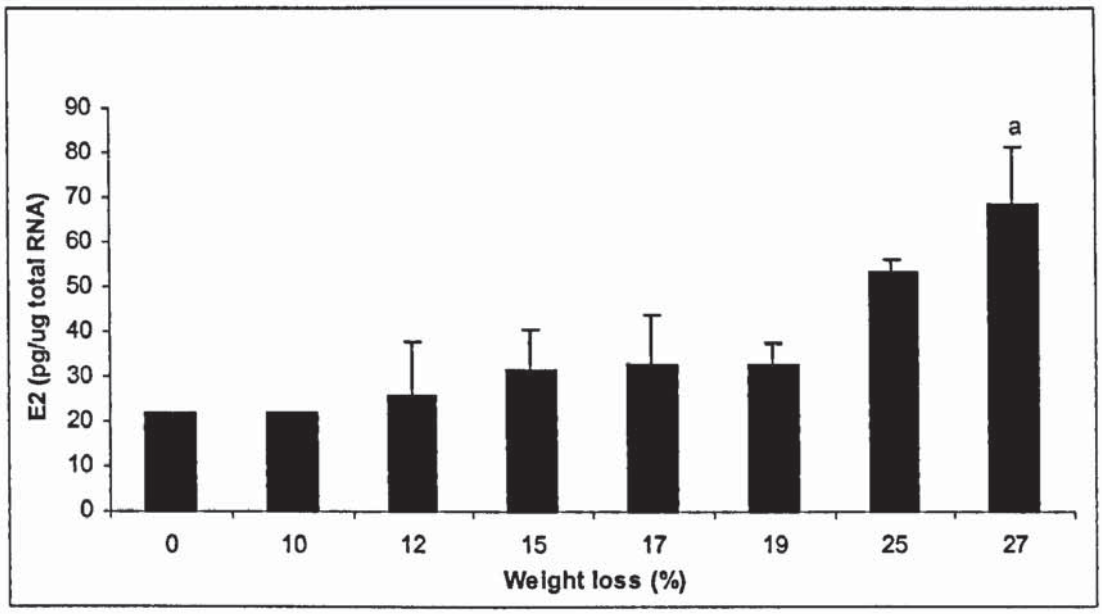
Fig 5.2.2.2 Expression of the C5 proteasome subunit in MAC16 tumour model pectoral muscles with different extents of weight loss



Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with Tukey's test was performed, where, a = $p<0.05$, b = $p<0.01$ and c = $p<0.001$.

$E2_{14k}$ expression was also measures in pectoral muscle of mice bearing the MAC16 tumour. Figure 5.2.2.3 shows that expression of E2 mRNA, which increased with increasing weight loss but the increase only reached significance at 27% weight loss, which showed the highest stimulation of the E2 expression of approximately 314% above the baseline.

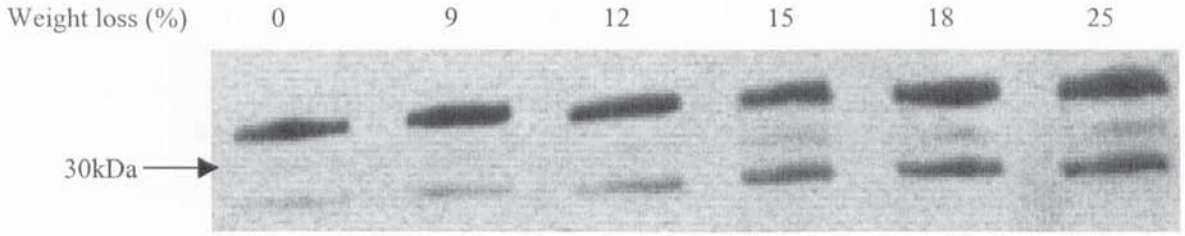
Fig 5.2.2.3 Expression of $E2_{14k}$ mRNA in MAC16 bearing mouse pectoral muscle with different extents of weight loss

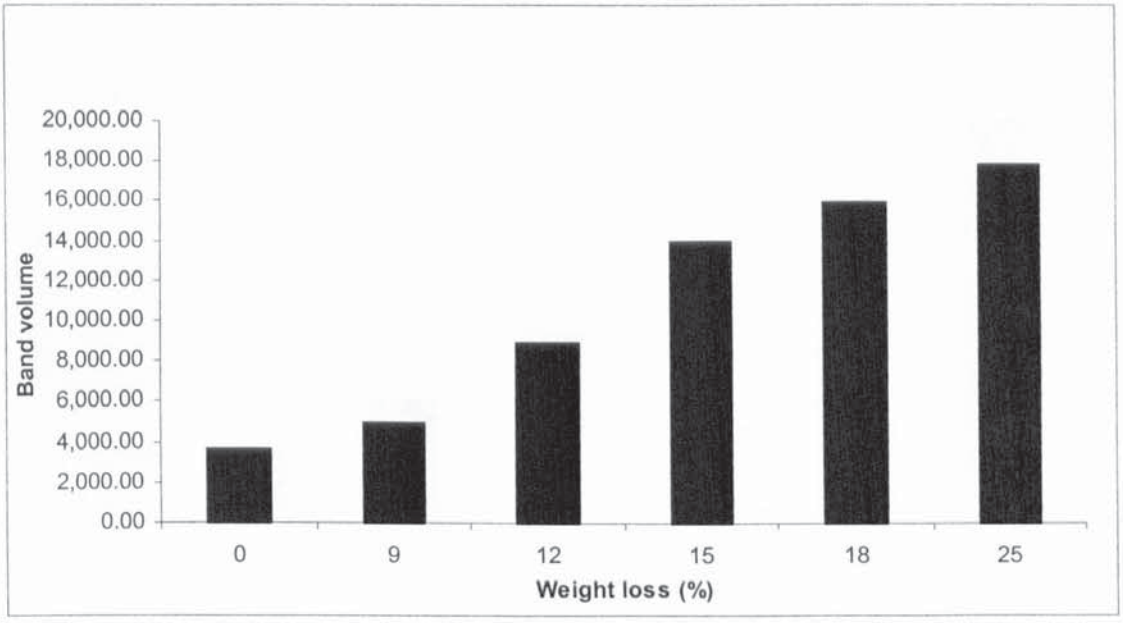


Results shown are the mean ± SEM where n=2. The experiment was repeated twice. Statistical analysis using one-way ANOVA with Student-Tukey's test was performed, where, a = $p<0.05$.

Protein was also extracted from the pectoral muscles of mice bearing the MAC16 tumour and used to measure the expression of the 20S proteasome α-subunits and the $E2_{14k}$ protein. Figure 5.2.2.4 shows the Western blot of the 20S proteasome α-subunits. There is a good correlation with the C2 and C5 expression, showing an increase of 20S proteasome α-subunits with increasing weight loss and reaching maximal at 25% weight loss of approximately 494% above the baseline.

Fig 5.2.2.4 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of 20S α-subunits in pectoral muscle of mice bearing the MAC16 tumour

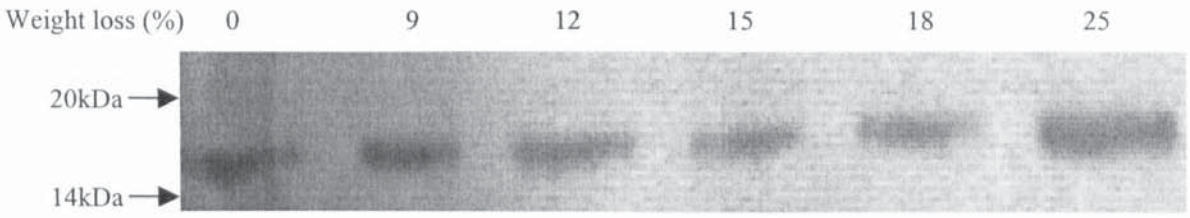


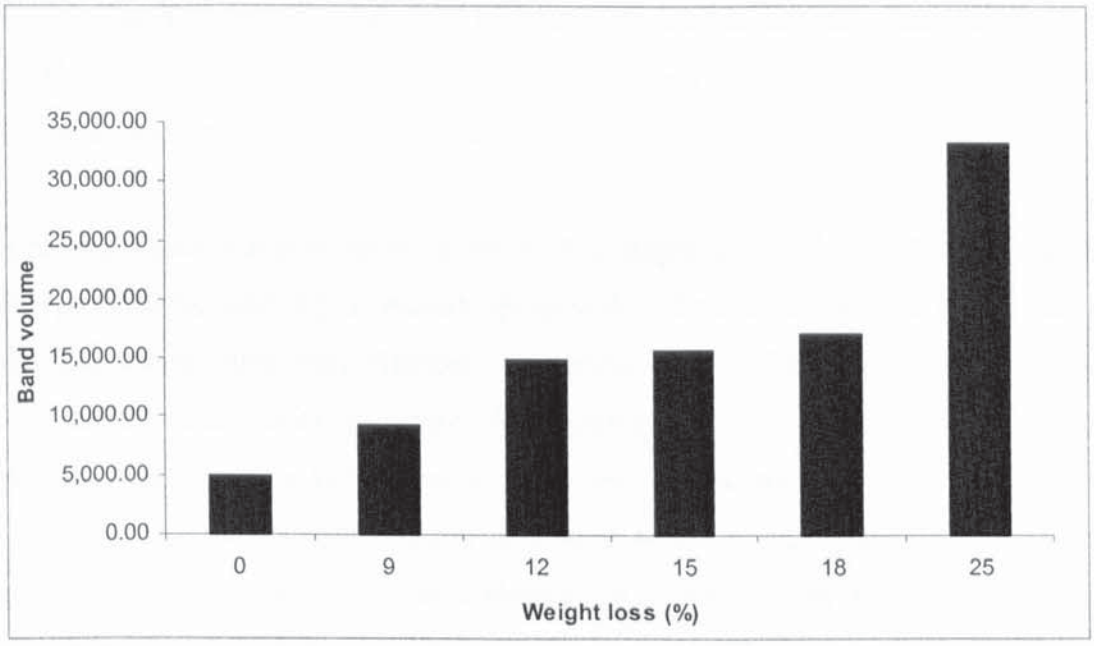


The volume of the 30kDa band was measured using Phoretix photo-imager programme.

Western blotting was also performed to look at the expression of $E2_{14k}$ in the same muscle samples. Figure 5.2.2.5 shows a similar pattern seen with that of $E2_{14k}$ mRNA in figure 5.2.2.3, where expression steadily increases with increasing weight loss reaching maximum expression at 25% weight loss of approximately 339% above the baseline.

Fig 5.2.2.5 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of $E2_{14k}$ in pectoral muscle of mice bearing the MAC16 tumour





Volume of the band was measured using Phoretix photo-imager programme.

The ubiquitin-proteasome pathway is believed to be responsible for the breakdown of bulk intracellular proteins in skeletal muscle in many physiological conditions including cancer cachexia (Lecker *et al.*, 1999a). Furthermore, skeletal muscle protein breakdown in cancer cachexia has been suggested to require increased gene expression of proteasome subunits (Temparis *et al.*, 1994). Therefore, in this study the expression of the C2-α and C5-β proteasome subunits have been measured by QcRT-PCR. In addition expression of $E2_{14k}$ mRNA levels were also measured, as a possibly rate-limiting component in ubiquitin conjugation of the substrate (Wing and Banville, 1994). Protein expression of both the 20S proteasome α-subunits and $E2_{14k}$ were also determined by

immunoblotting, since it has been suggested that in various cells elevated concentrations of mRNA and proteasome subunits were not found to be accompanied by increased concentrations of proteasomes (Kanayama *et al.*, 1991; Shimbara *et al.*, 1992).

The present study has shown a good correlation between weight loss and expression of these genes in both gastrocnemius and pectoral muscles. In all cases, the level of expression seems to be upregulated with increasing weight loss in both types of muscle. In pectoral muscle the upregulation of these genes were not as profound as that found in the gastrocnemius muscle and the pattern of increase was different to that in gastrocnemius muscle with respect to both the proteasome and $E2_{14k}$ expression, where expression is shown to be only significant with higher weight losses (above 17%) and to continue increasing with increasing weight loss.

In contrast, in gastrocnemius muscle the levels of expression of both mRNA and protein of the proteasome and E2 increased up to weight loss of 20%, and decreased with weight loss greater than 20%. Recently Whitehouse *et al* (2001) showed in the MAC16 tumour model, that the chymotrypsin-like activity of the proteasome increases with increasing weight loss up to 15% weight loss and then decreases. However, it has been reported that the total body nitrogen decreases with increasing weight loss in mice bearing the MAC16 tumour, which showed to increase with higher weight losses (>25%) (Smith and Tisdale, 1993a). de Blaauiv *et al* (1997) also reported that the levels of muscle catabolism were higher at small tumour burdens and decreased as the tumour size increased. Furthermore, it has been shown that the activity of the serum factor in MAC16 tumours, which increased protein degradation, increased with increasing weight loss up to 20%, and decreased with weight loss above 20% (Smith and Tisdale, 1993b). This factor was later found to be the proteolysis-inducing factor (PIF) (Todorov *et al.*, 1996a). PIF has been shown to be responsible for the loss of skeletal muscle in cachectic mice (Lorite *et al.*, 1998) and the changes in expression of the proteasome subunits and E2 with changes in serum levels of PIF suggests that PIF is responsible for the upregulation of the ubiquitin-proteasome pathway in mice bearing the MAC16 tumour. The mechanism by which PIF exerts this effect is yet to be determined, and further work is required to investigate this.

CHAPTER 6

Expression of the Ubiquitin-Proteasome Pathway in Skeletal Muscle of Cancer Cachectic Patients

6.1 Introduction

Although cancer cachexia reflects depletion of both adipose and muscle tissue, muscle atrophy is particularly important as a prognostic factor in the overall survival of the patient with cancer. Muscle wasting severely influences the quality of life in patients with cancer. For example, when respiratory muscles are involved, pulmonary complications including pneumonia from aspirations, are common. In addition, there is evidence that the response to chemotherapy is impaired in patients with cachexia (van Eys, 1985), and it has been estimated that almost one third of deaths in patients with cancer are related to muscle cachexia (Warren, 1932).

The cancer related catabolic response in skeletal muscle is primarily caused by stimulated protein breakdown, which is regulated by multiple proteolytic pathways, including lysosomal, Ca^{2+}-dependent, and ubiquitin-proteasome-dependent mechanism (Attaix and Taillandier, 1998). Recent studies in tumour-bearing rats and mice suggest that muscle proteolysis in cancer is regulated mainly by the ubiquitin-proteasome pathway and is associated with the upregulated expression of several genes in the pathway (Llovera *et al.*, 1996; Baracos *et al.*, 1995, Temparis *et al.*, 1994; Lorite *et al.*, 1998). Furthermore, few studies have also been performed examining the ubiquitin-proteasome pathway in human muscle tissue, such as in head trauma patients (Mansoor *et al.*, 1996), sepsis (Tiao *et al.*, 1997a), AIDS (Llovera *et al.*, 1998), cancer (Williams *et al.*, 1999b), Cushing's syndrome (Ralliére *et al.*, 1997) and muscular dystrophy (Combaret *et al.*, 1996). In head trauma patients, sepsis patients, AIDS patients and cancer patients increased gene expression of the ubiquitin-proteasome pathway was reported. However, no change of gene expression was seen in Cushing's syndrome and muscular dystrophy patients.

Williams *et al* (1999b) performed the first study in human cancer patients, where they used dot blot analysis to measure the mRNA levels of ubiquitin and four of the

proteasome subunits (HC3, HC5, HC7 and HC9). The study showed mRNA levels of both the ubiquitin and the 20S proteasome subunits to be 2-4 times higher in muscle from patients with gastrointestinal cancer than in muscle from control patients. However, there were only 6 cancer and 6 control subjects in this study and further evidence is required to prove the theory that muscle cachexia in cancer patients is mainly regulated by the ubiquitin-proteasome pathway, especially since Jagoe *et al* (2002) reported increased gene expression of the lysosomal protease cathepsin B in the skeletal muscle of patients with early lung cancer and no change in the mRNA levels of the components of the ubiquitin-proteasome pathway ($E2_{14k}$, polyubiquitin and C2α proteasome subunit). However, the majority of patients studied in this case did not have advanced or extensive cancer, and most had no clear abnormalities in several commonly used nutritional indices, including upper limb muscle size, strength and weight loss. Therefore, these patients had few signs of cachexia. It was further concluded in this study that ubiquitin-proteasome pathway is not activated in early lung cancer and it is likely to be activated in advanced disease. Furthermore, the results reported in this study suggested a role for cathepsin B in the initiation of muscle wasting in early lung cancer (Jagoe *et al.*, 2002). In contrast, Bossola *et al* (2001) showed levels of ubiquitin mRNA to be higher in muscles of gastric cancer patients in comparison to controls, and the expression of ubiquitin mRNA was seen to be influenced by the tumour stage, being higher in stage III and IV patients than in those with stage I or II cancer.

Therefore, further insight is needed into the involvement of the ubiquitin-proteasome pathway in human cancer cachexia and its correlation with the results of the animal studies so far reported.

In this study, the aim was to further investigate the role of the ubiquitin-proteasome pathway in muscle catabolism in cachectic cancer patients; for this purpose skeletal muscle (rectus abdominis) biopsies were obtained from 28 patients of which, some were control with non-cancerous conditions, some had cancer but no weight loss was reported (control), and the rest were cachectic cancer patients with various percentages of weight loss. The level of proteasome subunit gene expression (C2-α6 and C5-β6) was obtained quantitatively using QcRT-PCR for each muscle biopsy sample.

Immunoblotting was also used to measure protein levels of proteasome subunits to determine if altered mRNA expression reflects changes in protein expression.

6.2 Results and Discussion

The biopsy specimen obtained from the patients was immediately frozen in liquid nitrogen and stored at -70°C until use. Total RNA was then extracted from each muscle and used in QcRT-PCR (see chapter 3) to measure the level of expression of the 20S proteasome subunits C2, C5 and the $E2_{14k}$ ubiquitin-conjugating enzyme.

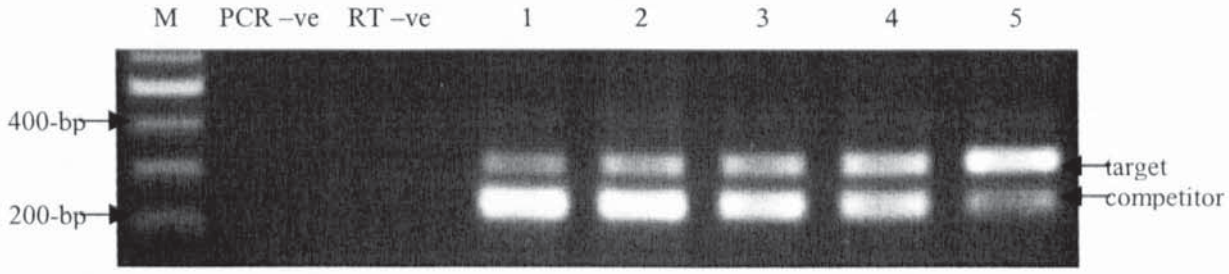
As described in chapter 3, the mouse or rat gene sequence was used to select the oligonucleotides for each of the genes mentioned and for producing the subsequent competitor. Although in each case the oligonuleotide sequences selected were at least 80% complementary to the human gene, only the human C5 proteasome gene was amplified by the original oligonucleotides.

A different forward primer was selected for the C2 proteasome gene, which was 100% complementary to both mouse and human gene sequences, which in turn amplified both target (human) and competitor (mouse) sequences (Figure 6.1), producing the direct product competition described in chapter 3 and amplifying the product sizes 316-bp (target) and 240-bp (competitor). The sequence and position of this new C2 forward primer is:

235- 5'CAT GTT GAC AAC CAT ATT GGT3' –551

This primer is 21-bp long, having a 38.1% GC content and 54°C Tm.

Fig 6.1 QcRT-PCR of the C2 20S proteasome gene with amplified target gene (human) and competitor (mouse) gene. 40% of total PCR reactions were examined by electrophoresis in a 2% agarose gel.



Lanes; M: 100-bp DNA ladder, PCR-ve: negative PCR reaction, RT-ve: negative reverse transcription reaction, lanes 1-5: each contains 0.25μg target RNA (human) and a specific amount of the C2 competitor RNA (mouse) ranging from 0.1563pg to 5pg.

New primers were also selected for the $E2_{14k}$ gene, in-order to amplify both the human (target) and the rat (competitor) sequences. Although these primers were highly complementary to both species genes, the products produced were minor and could not be compared to each other in a direct competition, therefore could not be used in QcRT-PCR for quantitating gene expression.

Therefore, only the expression of the proteasome subunits C2 and C5 were measured in the human samples, and Table 6.1 shows information of each patient used in this study. The expression of both C2 and C5 were measured and expressed quantitively, which is shown in table 6.2.

Table 6.1 Details of patients used in this study.

Subject	Age	Sex	Wt (kg)	wt loss (%)	Condition	Operation
99010	61	f	44.35	0	Liver cyst	1 open deroofing
99014	72	m	74	0	Inguinal hernia	1 mesh repair
99016	70	m	67.8	0	Gallstones	1 lap chloe + liver cyst
99019	69	m	93.2	0	Gallstones	1 cholecystactomy
99021	62	m	72.5	0	Gallstones	1 lap chloe
99023	78	f	64.55	0	Gallstones	1 lap chloe
99024	60	m	101.6	0	Gallstones	1 lap chloe
99025	86	m	60	0	Inguinal hernia	1 repair
99027	72	m	77.6	0	Gallstones	1 lap chloe
99028	66	m	96.2	0	Gallstones	1 lap chloe
99037	77	m	63	0	Rectal cancer	Anterior resection
99038	82	f	74.5	0	Caecal carcinoma	Right hemicolectomy
99031	81	m	68	0	Caecal carcinoma	Anterior resection
99033	78	f	56.75	0	Rectal cancer	Anterior resection
99035	81	m	69	0	Caecum carcinoma	Anterior resection
99039	82	f	66.7	3.7	Caecal and transial small tumours	Right hemicolectomy
99029	63	f	70.1	4	Adenocarcinoma - pancreas	2 whipple
99032	73	m	71.5	6	Adenocarcinoma - pancreas	Right hemicolectomy
99001	62	m	73	8	Adenocarcinoma - pancreas	2 bypass
99007	72	f	62.7	10	Adenocarcinoma - pancreas	2 whipple
99034	75	m	71	11	Transverse colon adenocarcinoma	Right hemicolectomy
99018	83	m	75	14.5	Adenocarcinoma - pancreas	2 bypass
99012	71	m	62.3	16	Adenocarcinoma - pancreas	2 biliery bypass
99015	81	m	48	17	Adenocarcinoma - pancreas	2 whipple
99022	58	m	77.5	18.4	Adenocarcinoma - pancreas	3 bypass
33036	72	f	50.9	19	carcinoma of caecum	hemadectomy
99026	64	m	51.3	26.5	Adenocarcinoma - pancreas	3 bypass
99020	62	m	72.65	34	Adenocarcinoma - pancreas	3 bypass

Table 6.2 Quantitated expression of the proteasome subunits C2 and C5.

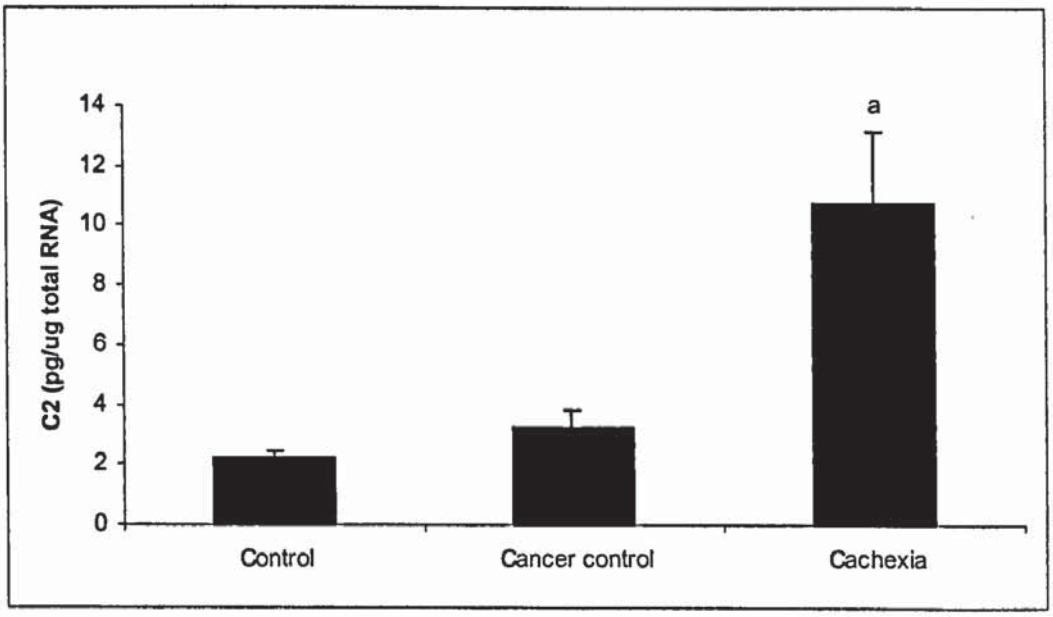
Subject	weight loss (%)	C2 (pg/ug total RNA)	C5 (pg/ug total RNA)
99010	0	1.563	3.515
99014	0	2.5	6.24
99016	0	/	3.711
99019	0	2.656	/
99021	0	2.813	2.73
99023	0	/	3.516
99024	0	2.813	1.953
99025	0	2.344	/
99027	0	1.563	5.469
99028	0	1.563	7.813
99037	0	4.375	6.25
99038	0	4.375	/
99031	0	2.5	5.48
99033	0	2.03	/
99035	0	/	3.676
99039	3.7	4.375	10.938
99029	4	3.75	14.063
99032	6	3.72	6.24
99001	8	2.5	6.25
99007	10	2.5	10.938
99034	11	5	20.303
99018	14.5	17.5	12.52
99012	16	22.5	18.75
99015	17	20	21.875
99022	18.4	22.5	25
33036	19	22.5	23.46
99026	26.5	8.75	10.96
99020	34	4.06	6.25

The details from tables 6.1 and 6.2 have been simplified in table 6.3, which shows average parameter details of the patients as well as the quantitated gene expression of both C2 and C5 proteasome subunits in control, cancer control and cachectic cancer patients. Figure 6.2.1 shows the mRNA expression of the C2 proteasome subunit as a representative of data from table 6.3 in all three groups, which shows a significant increase of C2 expression in cachectic cancer patients with respect to the control non-cancerous patients (of approx. 373%). Furthermore, there was a small increase in C2 gene expression in cancer control patients (patients with cancer but not cachexia) compared to the non-cancerous control, but this increase was not significant.

Table 6.3 Simplified data obtained from table 6.1 and 6.2, showing the average parameters expressed as mean ± SEM, and the statistical analysis of C2 and C5 proteasome gene expression using one-way ANOVA with student's Tukey's test, where * represent comparison to control and ** to cancer control.

	Control	Cancer control	Cachexia
Age (years)	69.6 ± 2.557	79.8 ± 0.97	70.6 ± 2.305
m/f	8/2	3/2	9/4
weight (kg)	75.2± 5.605	66.3 ± 3	65.6 ± 2.738
weight loss (%)	0	0	14.47 ± 2.457
n	10	5	13
C2 (pg/µg total RNA)	2.227 ± 0.2019	3.23 ± 0.6167	10.743 ± 2.405 ($p<0.05$*)
C5 (pg/µg total RNA)	4.230 ± 0.628	5.136 ± 0.762	14.427 ± 1.867 ($p<0.001$*, 0.05**)

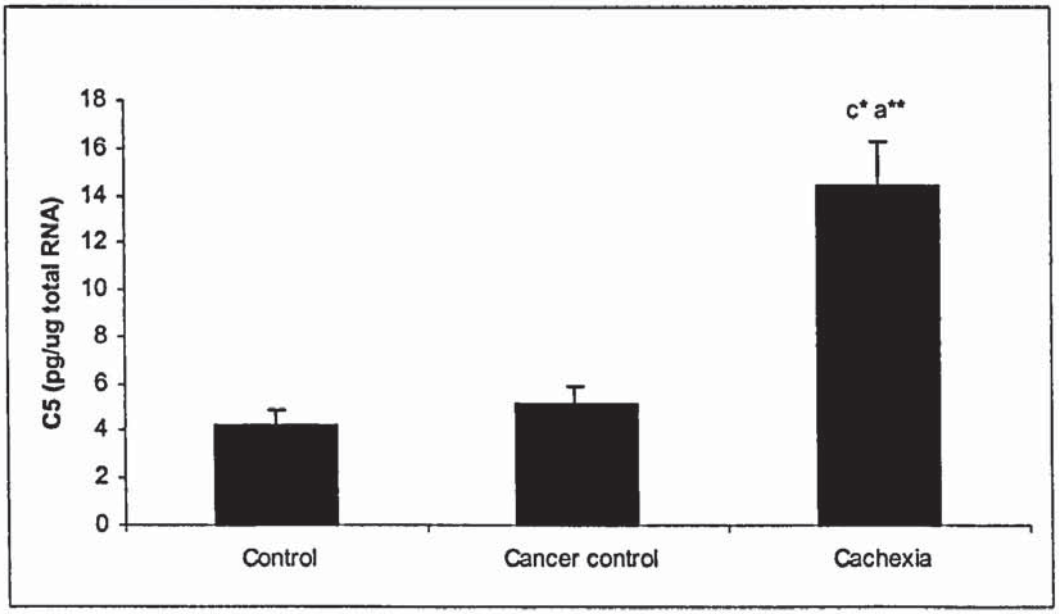
Fig 6.2.1 Expression of the C2 20S proteasome subunit in biopsies of rectus abdominis muscle of control, cancer control and cachectic cancer patients



Results shown are the mean ± SEM where n=10 in control group, 5 in cancer control group and 13 in cachexia group. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$.

Figure 6.2.2 similarly shows the C5 proteasome subunit expression (the data is obtained from table 6.3). Again it shows a significant increase of C5 expression in cachectic cancer muscle compared to both non-cancerous control (of approx. 241%) and cancer control (of approx. 181%) patients. However, the increased expression in C5 gene in cancer-control to non-cancer control is not significant ($p>0.05$).

Figure 6.2.2 Expression of the C5 proteasome subunit in biopsies of rectus abdominis muscle of control, cancer control and cachectic cancer patients



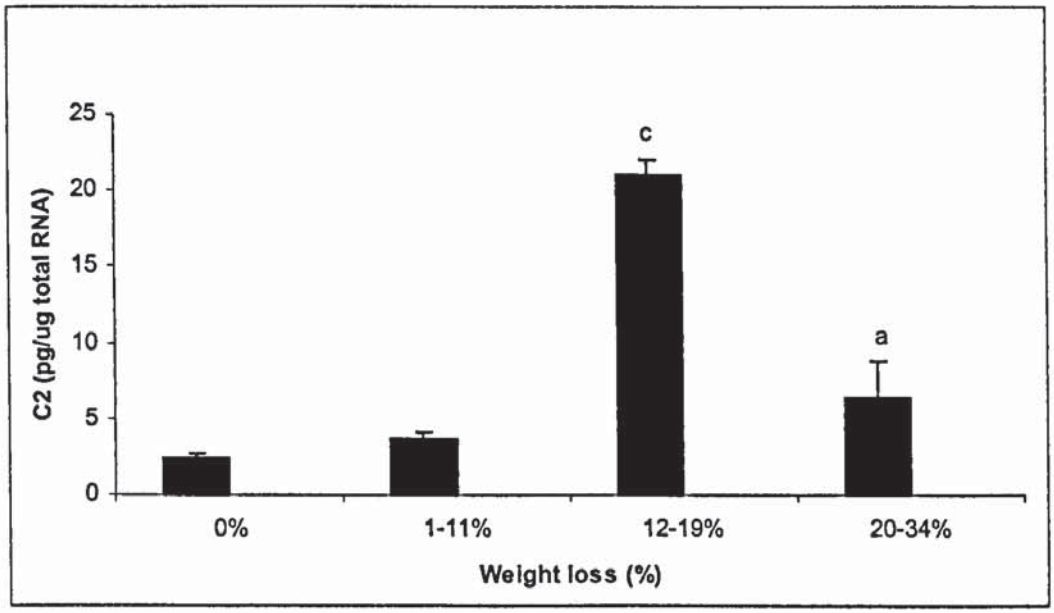
Results shown are the mean ± SEM where n=10 in control group, 5 in cancer control group and 13 in cachexia group. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$ and c = $p<0.001$; * represent comparison to control and ** to cancer control.

These results suggest that the expression of the proteasome subunits (C2 and C5) increases in cachectic cancer patients in comparison to non-cancer and noncachectic cancer patients, which suggests that the activity of the ubiquitin-proteasome pathway is upregulated in cachectic cancer patients. These results also correlate with that found in

rats with cancer cachexia (Temparis *et al.*, 1994) and cancer patients (Williams *et al.*, 1999).

Furthermore, table 6.2 shows that in general, the level of expression of both C2 and C5 20S proteasome genes increase with increasing % weight loss of the patients, showing a similar pattern found in MAC16 tumours (chapter 5) where, it seems that the higher the % of weight loss the higher the level of proteasome subunit expression up to 19% weight loss, and in patients with weight losses above 19%, (26.5% and 34%) there seems to be a downregulation of proteasome expression. The data from the cachectic patients shown in table 6.2 are placed into 3 weight losing groups (1-11%, 12-19% and 20-34% weight loss). Figure 6.2.3 shows the C2 expression changes in these groups, which, shows only a small non-significant increase (of approx.16%) in group 1-11% weight loss, approximately 477% increase in group 12-19% weight loss ($p<0.001$) and then the expression is downregulated in group 20-34% weight loss, which is again significant with respect to the control group.

Fig 6.2.3 Expression of the C2 20S proteasome subunit in rectus abdominis biopsies of Cachectic Cancer patients with different weight losses

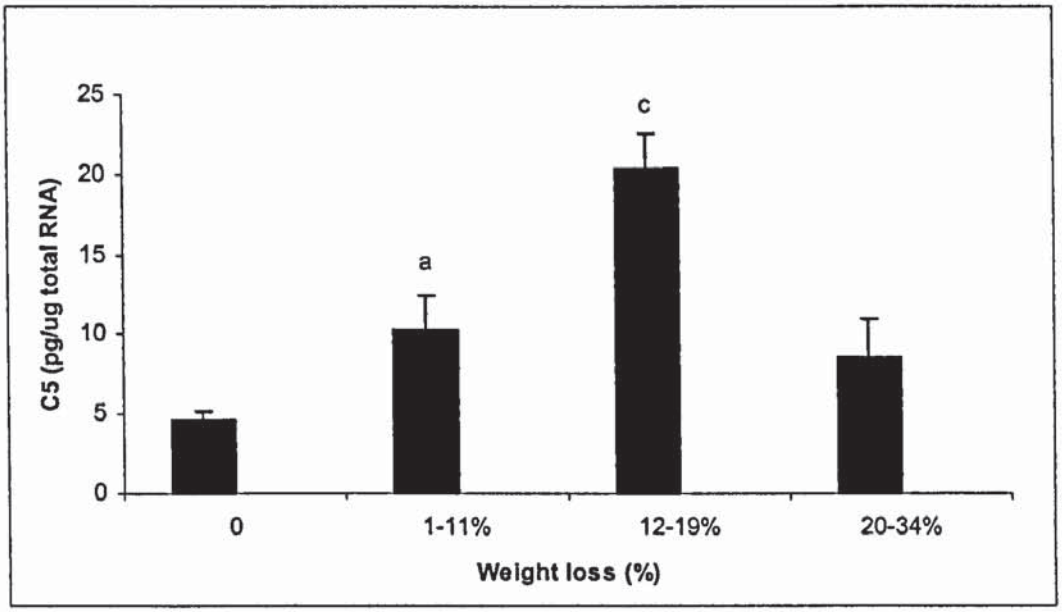


Results shown are the mean ± SEM where n=12 in control group, 6 in 1-11% weight loss group, 5 in 12-19% weight loss group and 2 in 20-34% weight loss group.

Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$ and c = $p<0.001$.

Similarly, figure 6.2.4 shows the C5 20S proteasome gene expression, where expression is significantly increased in groups 1-11% and 12-19% weight loss compared to controls by approximately 124% and 344%, respectively. Again the gene expression is downregulated in the higher weight loss group (20-34%).

Fig 6.2.4 Expression of the C5 20S proteasome subunit in rectus abdominis biopsies of Cancer Cachectic patients with different weight losses

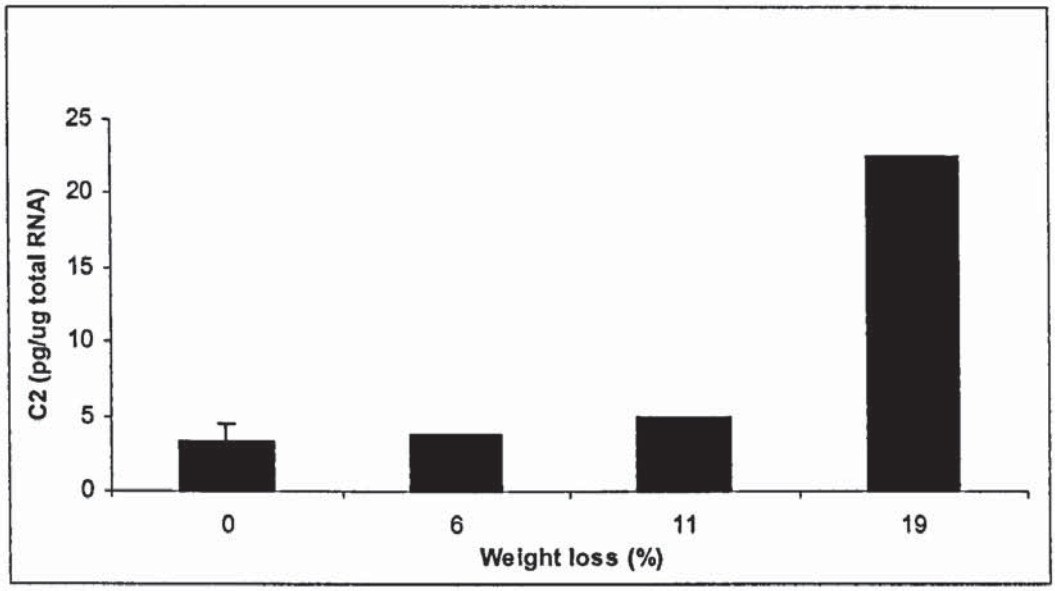


Results shown are the mean ± SEM where n=11 in control group, 6 in 1-11% weight loss group, 5 in 12-19% weight loss group and 2 in 20-34% weight loss group. Statistical analysis using one-way ANOVA with student-Tukey's test was performed, whereby a = $p<0.05$ and c = $p<0.001$.

As a representation of these results, figures 6.2.5 and 6.2.6 show the expression of C2 and C5 proteasome subunits respectively, in 3 cachectic cancer patients with different weight losses compared with the average of 3 cancer-control patients. The C2 proteasome expression only increased by approximately 10% in the rectus abdominis muscle of the patient with 6% weight loss, which increased to 48% in the patient with

11% weight loss and further increased to 565% in the patient with 19% weight loss (figure 6.2.5).

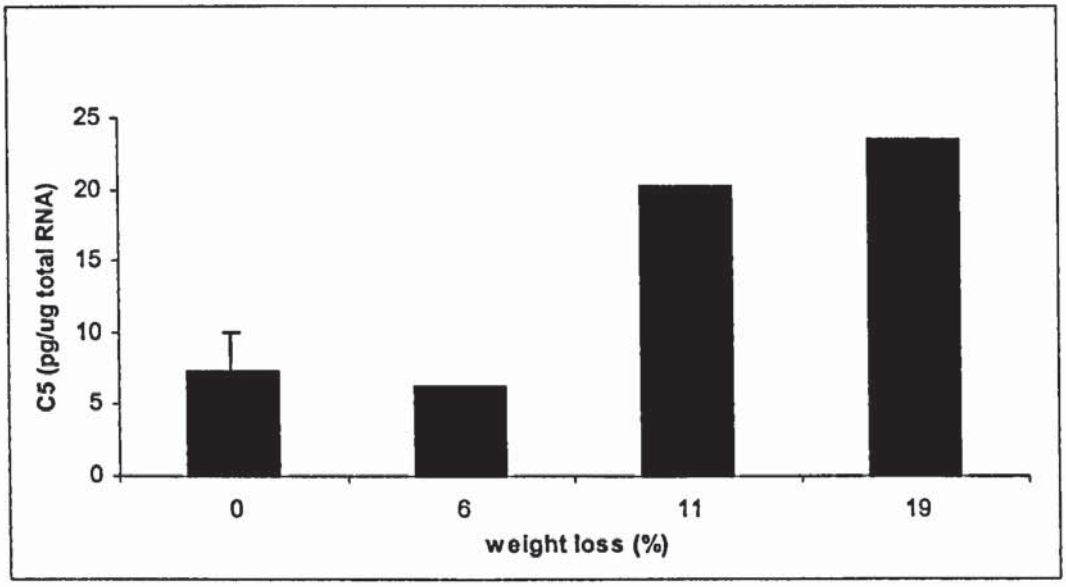
Fig 6.2.5 Expression of C2 20S proteasome subunit in rectus abdominis muscle of Cachectic Cancer patients with different extents of weight loss



Control (weight loss 0%) result represent mean ± SEM where n=3.

The pattern is similar with respect to the C5 proteasome gene expression, seen in figure 6.2.6, where the C5 proteasome subunit expression increases with increasing extent of weight loss, however, with 6% weight loss no increased C5 expression was observed, which was due to a high expression in one of the control subjects. The cachectic cancer patient with 11% weight loss showed an approximately 181% increase in the level of C5 gene expression, which increased further to approximately 225% in the rectus abdominis muscle of the patient with 19% weight loss (figure 6.2.6).

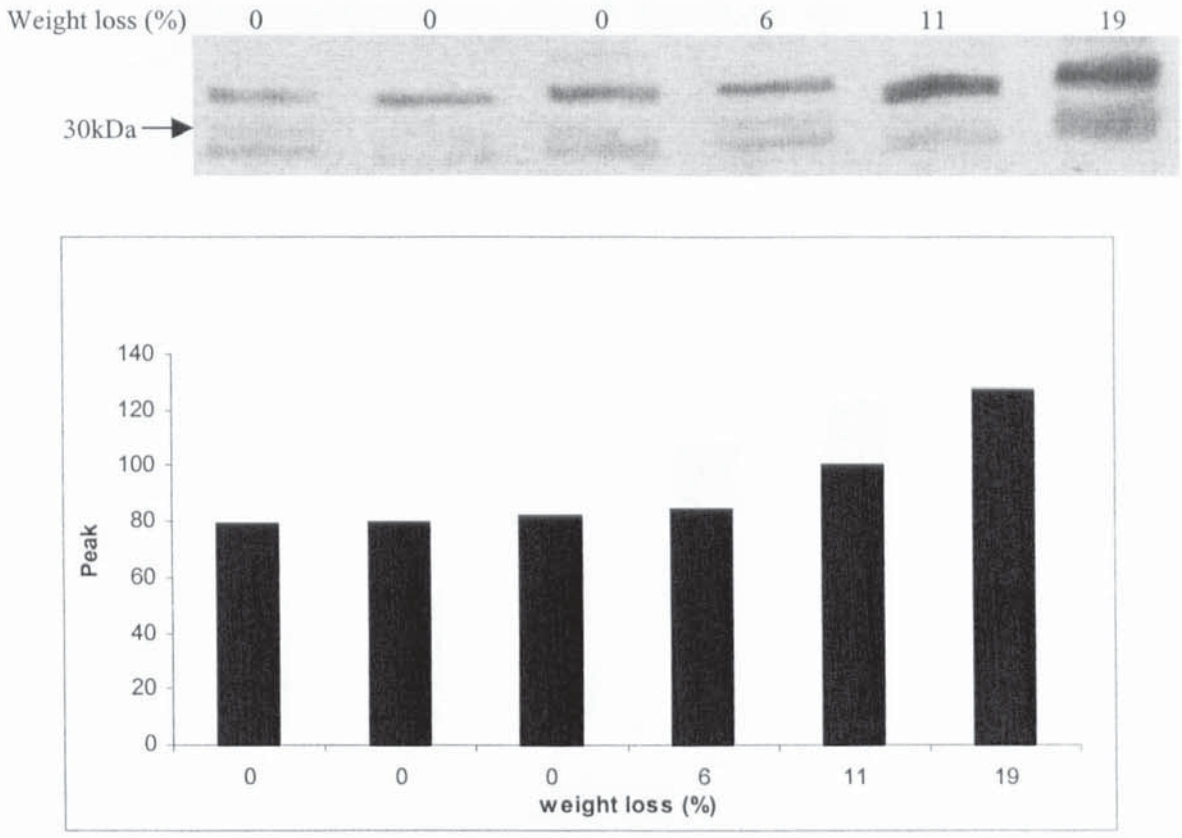
Fig 6.2.6 Expression of C5 20S proteasome subunit in rectus abdominis muscle of Cachectic Cancer patients with different extents of weight loss



Control (weight loss 0%) result represent mean ± SEM where n=3.

20S α-subunit protein levels were also investigated in the same samples using immunoblotting. The monoclonal antibody to the 20S proteasome reacts with 6 different α-type subunits, only three bands were apparent at approximate M_r 29, 32 and 35kDa (Whitehouse *et al.*, 2001). Figure 6.2.7 shows the 20S proteasome immunoblot, which indicates a progressive increase of the protein expression in the patients with increasing weight loss.

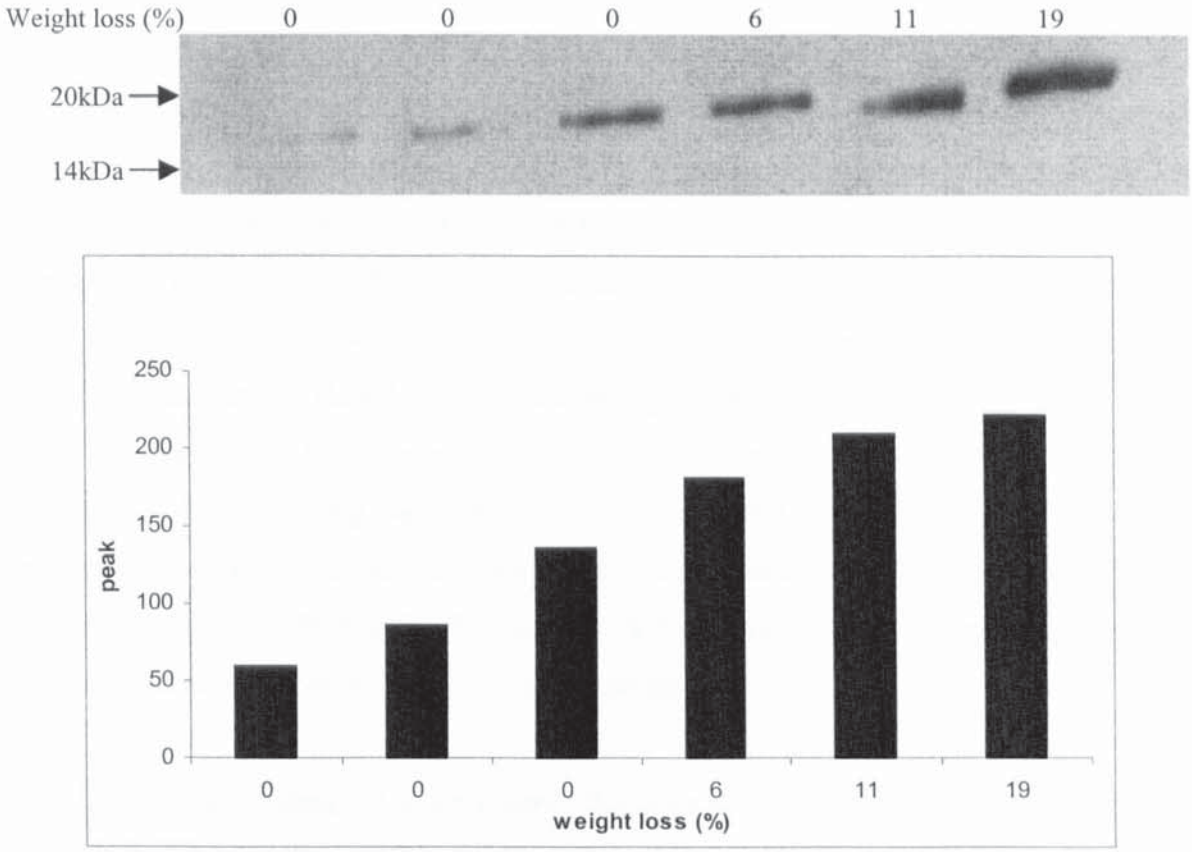
Fig 6.2.7 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of 20S proteasome α-subunits in rectus abdominis muscle of cachectic cancer patients



The volume of the 30kDa band was measured using Phoretix photo-imager programme.

The protein expression of $E2_{14k}$ was also determined by immunoblotting (figure 6.2.8), which shows a similar pattern to that of 20S α-subunit proteasome expression (figure 6.2.7). This indicated, that the E2 protein level is upregulated in cachectic cancer patients, which might also be upregulated at the transcription stage.

Fig 6.2.8 Western and densitometric analysis demonstrating the effect of different extents of weight loss on the expression of $E2_{14k}$ ubiquitin-conjugating enzyme in rectus abdominis muscle of cachectic cancer patients



Volume of the band was measured using Phoretix photo-imager programme.

Immunoblotting was performed for all the patient biopsy samples listed in tables 6.1 and 6.2, figures 6.2.7 and 6.2.8 show a representative of all the immunoblotts performed.

These results suggest that muscle cachexia in cancer patients may at least in part be regulated by the ubiquitin-proteasome pathway, which is similar to those in previous reports in which upregulated gene expression of several components of the ubiquitin-proteasome pathway was found in muscle of human (Williams *et al.*, 1999b), rats and mice with experimental tumours (Llovera *et al.*, 1996; Baracos *et al.*, 1995; Temparis *et al.*, 1994; Lorite *et al.*, 1998).

In this study the expression of C2-α and C5-β 20S proteasome subunits have been measured by QcRT-PCR. In addition, protein expression of the 20S proteasome α-subunits has also been determined by immunoblotting, since increased mRNA levels for

the 20S proteasome subunits do not necessarily mean that the protein levels are elevated or that the function of the proteins is increased (Kanayama *et al.*, 1991; Shimbara *et al.*, 1992).

Results here have shown a good correlation between weight loss and expression of the proteasome subunit genes, which correlates with the increased mRNA levels seen in gastric cancer patients (Bossola *et al.*, 2001). The level of expression of both mRNA and protein levels seem to be upregulated with increasing weight loss of the cancer patients up to about 19-20% weight loss, and decreased with weight loss greater than 20%. These results also correlated with that found in mice bearing the MAC16 tumour (shown in chapter 5). Based on the theories suggested in chapter 5 with respect to the MAC16 tumour that, PIF is responsible for the upregulation of the ubiquitin-proteasome pathway in mice bearing the MAC16 tumour and the fact that PIF also has been determined in human urine (Todorov *et al.*, 1996a) and human melanoma cell lines (Todorov *et al.*, 1999) it is likely that PIF is responsible for the upregulation of the ubiquitin-proteasome pathway in cachectic cancer patients.

The human $E2_{14k}$ competitor gene has also been constructed using oligonucleotides selected from the human E2 gene sequence (HSU39318) (see appendix I). However, due to time difficulties the competitor gene has not been cloned yet and therefore, this work needs to be continued.

Furthermore, due to the small human muscle biopsies received, in some cases the RNA extracted was not sufficient to perform all the experiments. Most of the muscle biopsies were prepared over 2 years ago and have been stored at -70°C until its use recently, and it has been noted that these muscles had less total RNA than muscles received much later from which RNA was extracted immediately. Furthermore, RNA from the older muscles seemed to be degraded in comparison to the RNA from newer muscles, producing few anomalous results, which is seen in table 6.2; this pointed to the fact that the activity of the proteasome mRNA is degraded with longer storage of the muscles.
Despite the limitations described, the present results are important because they suggest that the catabolic response to cancer cachexia may be regulated at the gene level in human muscle tissue, and the results provide further support for the role of the

ubiquitin-proteasome pathway in the regulation of muscle proteolysis during cancer cachexia. Another important implication of the findings in this study is that changes in the ubiquitin-proteasome proteolytic pathway noted previously during cancer cachexia in mice and rats (Llovera *et al.*, 1996; Baracos *et al.*, 1995; Temparis *et al.*, 1994; Lorite *et al.*, 1998) reflect changes that occur in patients with cancer cachexia. Thus, the experimental models used should be valid to explore mediators and mechanisms of the cancer cachectic-induced activation of the ubiquitin-proteasome system and, more importantly, to test different therapeutic interventions aimed at reducing the catabolic response to cancer cachexia.

CHAPTER 7

The Effect of EPA in an *in vivo* Model of Sepsis

7.1 Introduction

Sepsis is a major cause of death throughout the world. Sepsis is associated with a very large range of disorders, such as profound hemodynamic and nutritional derangements, organ failure and multiple metabolic alterations with increased energy substrate turnover, altered hormonal pattern, and intensive protein catabolism. One of the most striking metabolic consequences of sepsis is the catabolic response in skeletal muscle resulting in muscle wasting and fatigue. Studies have shown that muscle catabolism during sepsis is mainly caused by increased protein degradation, in particular myofibrillar protein degradation, although reduced protein synthesis and inhibited amino acid uptake contribute to the negative protein balance in muscle (Clawes *et al.*, 1983; Hasselgren *et al.*, 1986).

One consequence of muscle catabolism is peripheral release of amino acids. A large proportion of amino acids are taken up by the liver, providing support for acute-phase protein synthesis and gluconeogenesis (Sax *et al.*, 1988). Other amino acids, in particular glutamine, are utilised by cells in the immune system (Newsholme and Parry Billings, 1992) and by enterocytes (Windmueller and Spaeth, 1980). Thus, the catabolic response in skeletal muscle may be beneficial to the organism, at least during the early phase of sepsis, providing important substrates for other cells and tissues.

During prolonged and severe sepsis, however, muscle breakdown results in muscle atrophy, weakness and fatigue that may adversely affect the outcome in these patients by impairing the ability to ambulate and by affecting pulmonary function, secondary to weakness of respiratory muscles (Reid and MacGowan, 1998).

In septic patients, muscle wasting occurs progressively (Leverve *et al.*, 1984), and increased skeletal muscle proteolysis contributes to this loss (O'Donnel *et al.*, 1976; Clawes *et al.*, 1983). The myofibrillar proteins actin and myosin constitute the bulk of muscle protein, and there is evidence suggesting that these contractile proteins are

particularly sensitive to the effect of sepsis (Hasselgren *et al.*, 1989). As discussed in much detail earlier, intracellular protein breakdown in skeletal muscle is regulated by several proteolytic pathways, the most common ones involved are the lysosomal proteases, Ca^{2+}-dependent cystein proteases and ATP-ubiquitin-proteasome pathway (Attaix and Taillandier, 1998). A relatively large number of studies have provided evidence that the ubiquitin-proteasome pathway accounts for a major portion of muscle protein breakdown during sepsis (Tiao *et al.*, 1996; Tiao *et al.*, 1994). By using specific blockers of the individual proteolytic pathways, it was shown that muscle catabolism during sepsis in rats is almost exclusively caused by an increase in nonlysosomal and Ca^{2+}-independent, energy-dependent protein breakdown (Tiao *et al.*, 1994).

Voisin *et al* (1996) also reported a major role for the ubiquitin-proteasome pathway in skeletal muscle protein degradation in sepsis, where they found increased mRNA levels for ubiquitin and subunits of the 20S proteasome, which paralleled the increased rate of protein catabolism in acute-septic rat models. In addition, increased mRNA levels for the 14-kDa ubiquitin-conjugating enzyme E2 was also found in chronic sepsis as well as mRNA for cathepsin B and m-calpain, which suggested that the activation of lysosomal and Ca^{2+}-dependent proteolysis may be important in the chronic phase (Voisin *et al.*, 1996). In fact some studies have suggested that myofilaments are released from the sarcomere by a calcium-calpain-dependent mechanism before they are ubiquitinated and degraded by the proteasome (Williams *et al.*, 1999a; Dahlmenn *et al.*, 1986). Furthermore, it has been shown that the calcium antagonist dantrolene effectively blocks sepsis-induced muscle proteolysis (Hotchkiss and Karl, 1994; Fischer *et al.*, 2001), and was later shown to block the sepsis-induced increase in the gene expression of ubiquitin, E3α, and the 20S proteasome subunit C3 (Wray *et al.*, 2002).

Other studies have also reported elevated mRNA levels for ubiquitin and several proteasome subunits in skeletal muscles of septic rats (Tiao *et al.*, 1997b) and humans (Tiao *et al.*, 1997a). $E2_{14k}$ has also been measured in skeletal muscle from septic rats where, sepsis resulted in a 70% increase in the $E2_{14k}$ transcript in the fast-twitch extensor digitorum longus muscle, whereas no changes were seen in the slow-twitch soleus muscle (Hobler *et al.*, 1999). Increased expression of ubiquitin-ligase E3α along

with $E2_{14k}$ in skeletal muscle of septic rats has also been reported (Fischer *et al.*, 2000b).

Metabolic inhibitors have also been used to study the role of ubiquitin-proteasome pathway in sepsis. For example, the proteasome inhibitor *N*-benzyloxycarbonyl-Ile-Glu-(*O-t*-butyl)-Ala-leucinal (PSI) inhibited sepsis-induced increase in total and myofibrillar muscle protein breakdown in rats *in vivo* (Fischer *et al.*, 2000a). In addition, proteasome inhibitors N-acetyl-L-leucinyl-L-leucinal-l-norleucinal (LLnL) and lactacystein also caused inhibition of protein breakdown in an *in vitro* model of rat septic muscle (Hobler *et al.*, 1998). These studies further support the concept that the ubiquitin-proteasome pathway plays a central role in sepsis-induced muscle proteolysis.

It has been demonstrated here and elsewhere, that the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), can at least in part attenuate protein catabolism in cancer cachexia, due to its effects of attenuating the elevated proteasome activity and expression (discussed in earlier chapters). Furthermore, EPA was shown to have similar effects in acute starvation (Whitehouse and Tisdale, 2001). It was shown that pre-treatment of mice with EPA prior to fasting attenuated total protein breakdown in muscle, as well as increase in chymotrypsin-like activity of the proteasome, the increased expression of the 20S proteasome α-subunits, the 19S regulator, p42 and the ubiquitin-conjugating enzyme $E2_{14k}$ (Whitehouse and Tisdale, 2001). The effect was shown to be specific to EPA since it was not shown with the related n-3 fatty acid DHA or the n-6 fatty acid linoleic acid. These results suggested that protein catabolism in cancer cachexia and starvation is mediated by a common pathway, which is inhibited by EPA, possibly as a result of inhibition of the formation of arachidonic acid metabolite 15-hydroxyeicosatetraenoic acid (15-HETE).

Since there is evidence that the ubiquitin-proteasome-proteolytic pathway is elevated in sepsis, it is possible that EPA could also have an attenuating effect on the protein catabolism in sepsis. In this study, the effect of EPA on proteasome function and expression was investigated, as well as the ubiquitin-conjugating enzyme $E2_{14k}$ in septic mice.

7.2 Results and Discussion

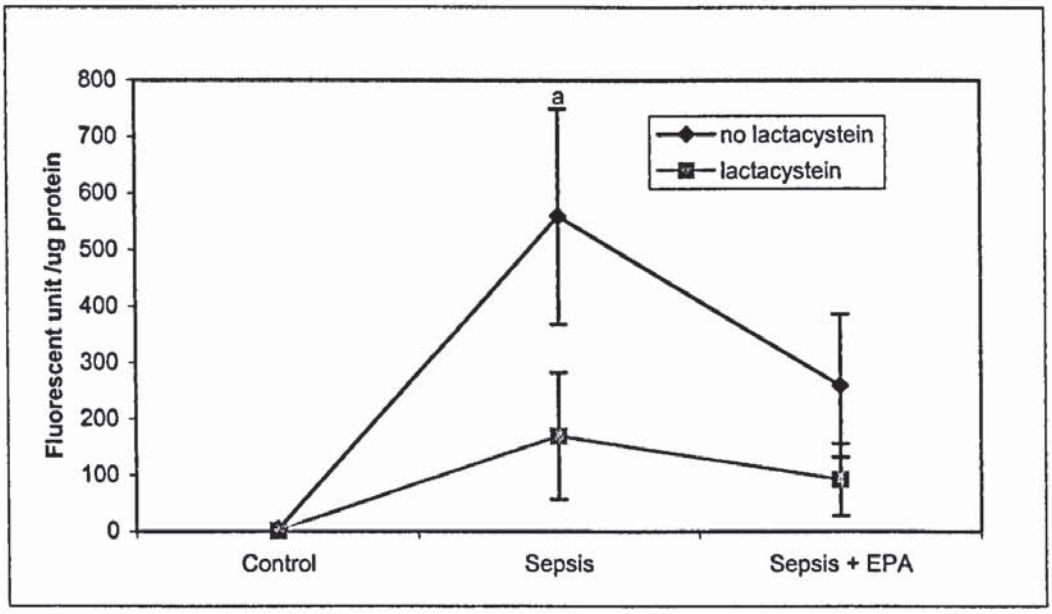
Sepsis was induced in male albino MF-1 mice (2.4.4), these experiments were performed on three groups of mice: control, sepsis and sepsis treated with EPA. Gastrocnemius muscles were isolated and immediately frozen in liquid nitrogen, and stored at -70°C before further analysis.

The activity and function of the ubiquitin-proteasome pathway was investigated in soluble extracts of the isolated muscles. First, the chymotrypsin-like activity of the proteasome was investigated.

The eukaryotic proteasome is a multicatalytic protease characterised by three activities against short synthetic peptides. These are the "chymotrypsin-like" activity, "trypsin-like" activity and the "peptidyl-glutamyl peptide bond-hydrolysing" activity. The chymotrypsin-like activity represents the most dominant catalytic activity of the proteasome and found to be rate-limiting in proteasome-dependent protein degradation (Kisselev *et al.*, 1999), and is most widely used as a marker of functional proteasome activity. Suc-LLVY-AMC is a fluorogenic peptide substrate of the "chymotrypsin-like" enzyme activity. Once cleaved, the fluorescent AMC (amino methyl coumarin) is released, which is proportional to the level of proteasome activity. The specific proteasome inhibitor lactacystin is usually used to accompany this assay, to differentiate any fluorescence from non-proteasomal sources.

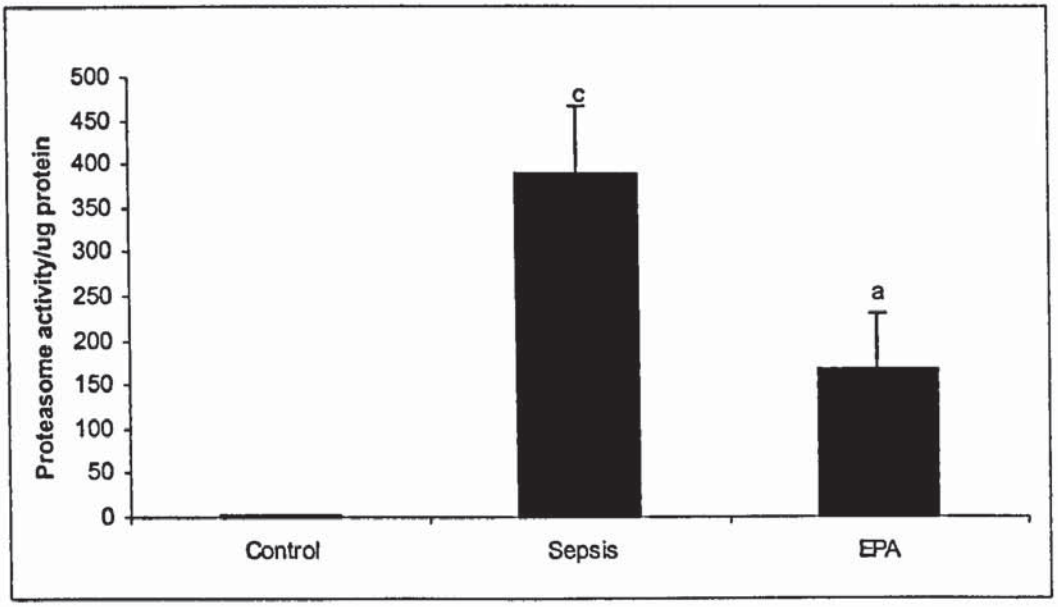
Figure 7.2.1 shows the "chymotrypsin-like" enzyme activity of the proteasome in gastrocnemius muscles of control mice, septic mice and septic mice treated with 0.5g/kg EPA. This figure shows that the proteasome activity is much higher in gastrocnemius muscle of septic muscle in comparison to control non-septic mice muscles. The increase activity shown is approximately 129-fold in septic muscle, which decreases by 57% in the EPA treated septic muscle. When 10μM lactacystein was included, the increased proteasome activity seen in septic muscle was highly reduced indicating that the elevated activity was due to increased proteasome activity. This is further demonstrated in figure 7.2.2, which shows the proteasome activity of the described muscles.

Fig 7.2.1 Chymotrypsin-like activity in soluble extracts of gastrocnemius muscle of control and septic mice (with and without EPA)



Fluorescence was measured at 360nm excitation and 460nm emission in a Perkin Elmer Luminescence Spectrometer LS50. Results shown represent mean ± SEM where n=12. Statistical analysis performed using one-way ANOVA with Tukey's test. Differences from control values are indicated by a = $p<0.05$.

Fig 7.2.2 Proteasome (Chymotrypsin-like) activity in soluble extracts of gastrocnemius muscle of control and septic mice (with and without EPA)

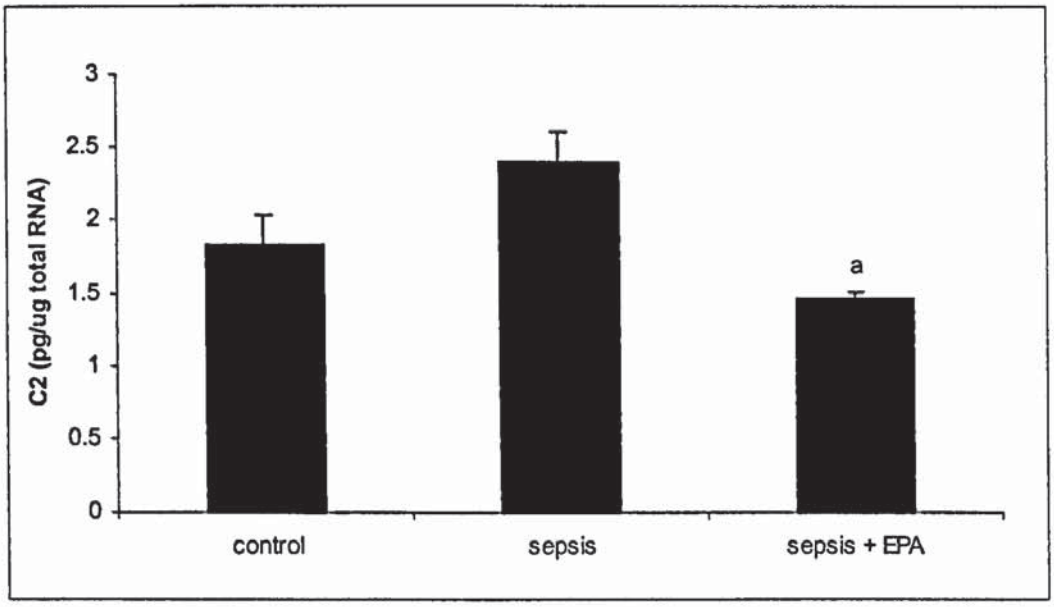


Results shown represent mean ± SEM where n=12. Statistical analysis performed using one-way ANOVA with Tukey's test. Differences from control values are indicated by a = $p<0.05$ and c = $p<0.001$.

This figure shows a considerable reduction of proteasome activity in septic muscle when treated with 0.5g/kg EPA, however, higher concentrations of EPA might further reduce the proteasome activity and in-turn protein catabolism in the skeletal muscle of septic mice.

In addition, total RNA was extracted (2.4.1.2) from a portion of the gastrocnemius muscles of the 3 groups of mice described above and was used to measure the gene expression of the 20S proteasome subunits C2-α and C5-β, as well as the ubiquitin-conjugating enzyme $E2_{14k}$ by means of QcRT-PCR (chapter 3). Figure 7.2.3 shows the level of expression of the C2 20S proteasome subunit, which has increased in the gastrocnemius muscle of septic mice (by approx. 31%), and EPA attenuated this increased expression by approximately 39% to below that of the control (figure 7.2.3).

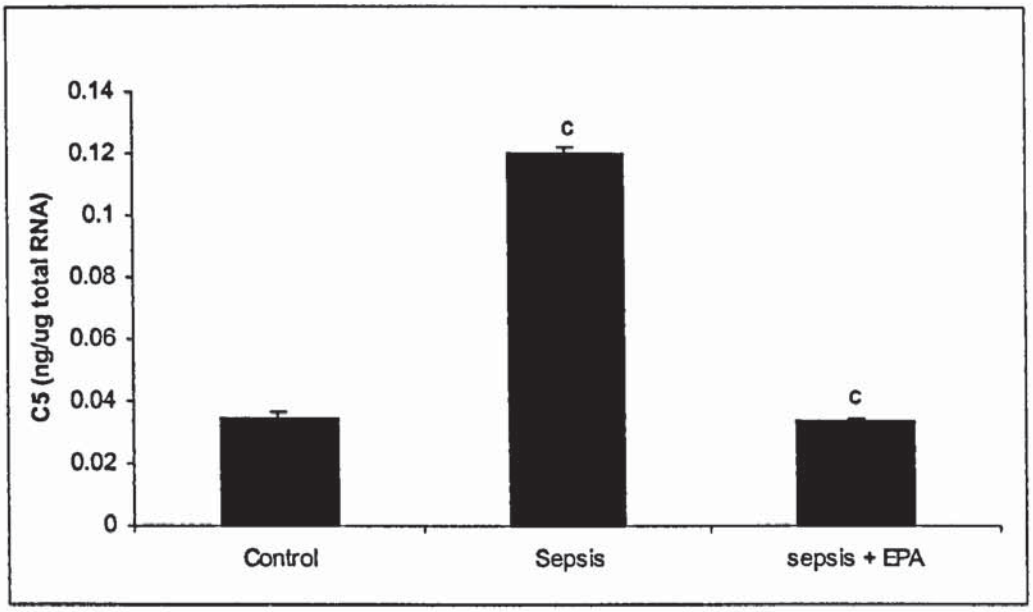
Fig 7.2.3 Effect of EPA on the expression of the C2 20S proteasome α-subunit in gastrocnemius muscle of septic mice



The experiment was repeated 5 times. This figure shows a representative where mean ± SEM and n=3. Statistical analysis performed using one-way ANOVA with Tukey's test comparing values to control, whereby a = $p<0.05$.

The expression of the C5 20S proteasome β-subunit was also measured by QcRT-PCR (figure 7.2.4), which significantly increased in septic mouse gastrocnemius muscle (by approx. 247%) in comparison to that of non-septic muscle. Furthermore, EPA significantly attenuated the increased expression of C5 in septic muscle to below baseline levels.

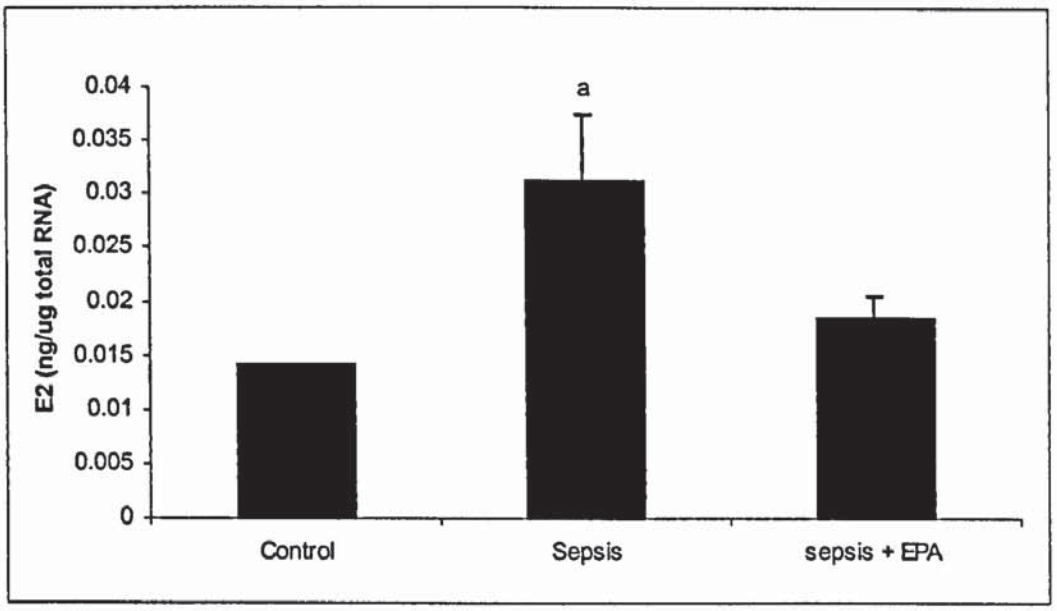
Fig 7.2.4 Effect of EPA on the expression of C5 20S proteasome subunit in gastrocnemius muscle of septic mice



The experiment was repeated 5 times. This figure is a representative showing mean ± SEM where n=3. Statistical analysis performed using one-way ANOVA with Tukey's test comparing values to control, whereby c = $p<0.001$.

Figure 7.2.5 shows the quantitative expression of the ubiquitin-conjugating enzyme $E2_{14k}$ in the same muscle samples described. The level of expression increased by approximately 112% in septic muscle in comparison to control, which was attenuated by 0.5g/kg EPA to almost control levels (figure 7.2.5).

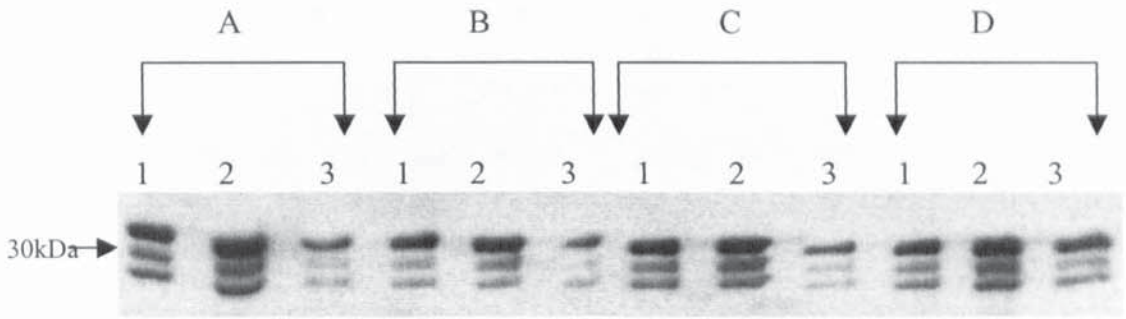
Fig 7.2.5 Effect of EPA on the expression of $E2_{14k}$ in gastrocnemius muscle of septic mice



The experiment was repeated 5 times. This figure is a representative showing mean ± SEM where n=3. Statistical analysis performed using one-way ANOVA with Tukey's test comparing values to control, whereby a = $p<0.05$.

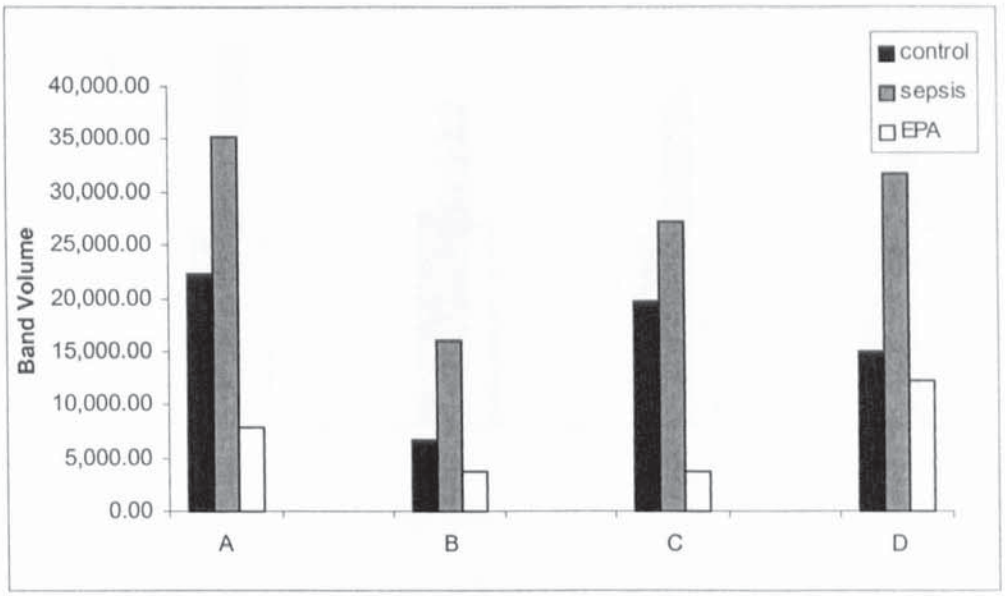
Immunoblotting was also performed to measure the protein expression of both proteasome 20S α-subunits and the $E2_{14k}$ by Western blotting, to determine if increased mRNA expression of these subunits was accompanied by increased protein levels. Figure 7.2.6 shows the immunoblotting and densitometric data of the 20S proteasome α-subunits in the soluble extracts of the gastrocnemius muscle of control and septic mice muscles (in the presence and absence of EPA). Four groups were studied as seen in figure 7.2.6. This shows an increase 20S proteasome expression in muscle from septic mice in comparison to that of control, which is attenuated to below baseline with EPA treatment in all 4 study groups.

Fig 7.2.6 Western and densitometric analysis demonstrating the effect of EPA on the expression of 20S proteasome α-subunits in gastrocnemius muscle of septic mice



A-D represents the study groups.

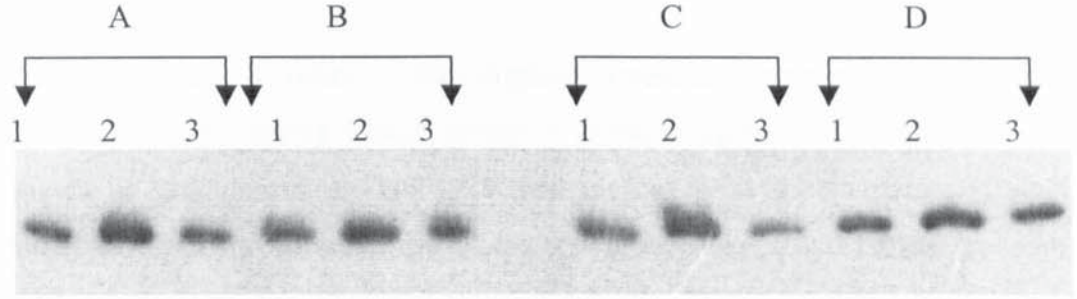
1. Control
2. Sepsis
3. Sepsis + 0.5g/kg EPA



The volume of the 30kDa band was measured using Phoretix photo-imager programme.

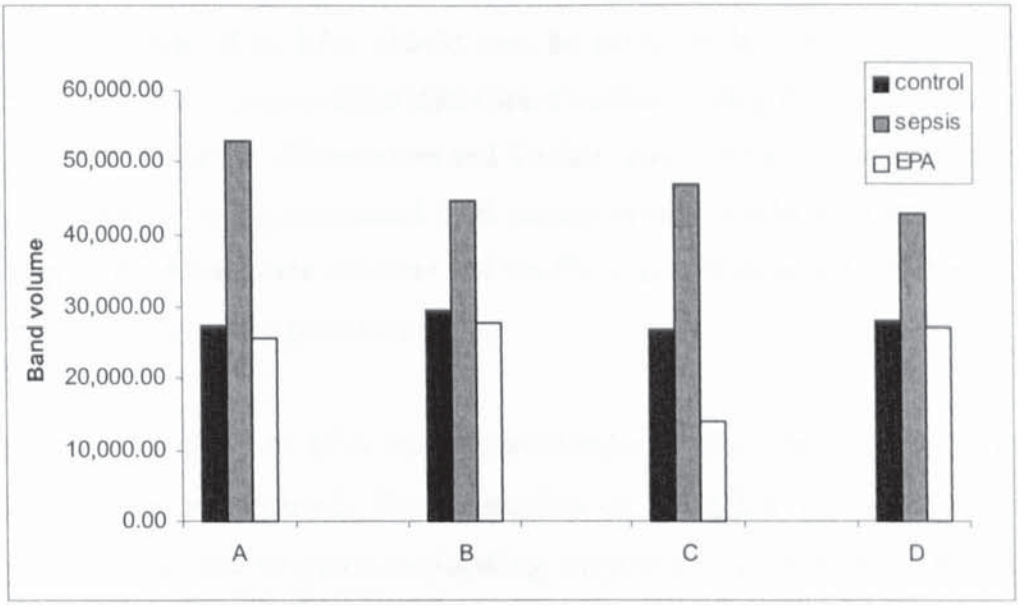
A similar expression pattern is shown in the Western blotting performed to look at the expression of the ubiquitin-conjugating enzyme $E2_{14k}$, figure 7.2.7.

Fig 7.2.7 Western and densitometric analysis demonstrating the effect of EPA on the expression of $E2_{14k}$ in gastrocnemius muscle of septic mice



A-D represents the study groups.

1. Control
2. Sepsis
3. Sepsis + 0.5g/kg EPA



Volume of the band was measured using Phoretix photo-imager programme.

Several experiments have shown that muscle breakdown during sepsis is associated with upregulated expression and activity of the ubiquitin-proteasome-proteolytic pathway (Tiao *et al.* 1994; Tiao *et al.*, 1997a), similar to a number of other catabolic conditions such as AIDS (Llovera *et al.*, 1998), denervation (Medina *et al.*, 1991), fasting (Wing and Goldberg, 1993), metabolic acidosis (Mitch *et al.*, 1994) and cancer

cachexia (Temparis *et al.*, 1994; Baracos *et al.*, 1994; Williams *et al.*, 1999b). This suggests that there may be a common activation pathway for enhanced activation of the ubiquitin-proteasome pathway in these conditions.

For most catabolic conditions the signals regulating proteasome expression are unknown. However, during cancer cachexia protein degradation in skeletal muscle is stimulated by the tumour product 'PIF', which has been shown here and elsewhere (Lorite *et al.*, 1998) to increase the expression of both $E2_{14k}$ and proteasome subunits. Furthermore, it has been shown that the catabolic actions of PIF can be completely attenuated by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), both *in vitro* (Smith *et al.*, 1999; Chapter 4) and *in vivo* (Beck *et al.*, 1991). Again, it has been suggested here and elsewhere (Smith *et al.*, 1999) that the arachidonic acid metabolite 15-hydroxyeicosatetraenoic acid (15-HETE) may act as a secondary messenger for upregulation of the degradative pathways caused by PIF and that EPA is attenuating the production of 15-HETE. Therefore, if the 15-HETE system is common to other catabolic conditions, then EPA should also be effective in down-regulating muscle protein catabolism in systems other than cancer cachexia. This theory was proved to be viable in acute starvation (Whitehouse and Tisdale, 2001), where pre-treatment of mice with EPA prior to fasting attenuated total protein breakdown in muscle, increased the expression of the proteasome subunits and the $E2_{14k}$ as well as increased chymotrypsin-like enzyme activity of the proteasome.

In this study, the effect of EPA on the sepsis-induced ubiquitin-proteasome pathway upregulation was investigated. The expression of the C2-α and C5-β proteasome subunits as well as the ubiquitin-conjugating enzyme ($E2_{14k}$) have been measured by QcRT-PCR. In addition, protein expression of both the 20S proteasome α-subunits and $E2_{14k}$ were determined by immunoblotting, since it has been suggested that in various cells elevated concentrations of mRNA and proteasome subunits were not found to be accompanied by increased concentrations of proteasomes (Kanayama *et al.*, 1991; Shimbara *et al.*, 1992). Chymotrypsin-like activity of the proteasome was also determined by measuring the fluorogenic substrate activity of Suc-LLVY-MCA in the presence or absence of the proteasome inhibitor lactacystin.

The results showed an increase in the mRNA expression of the proteasome subunits C2 and C5 as well as the $E2_{14k}$ in gastrocnemius muscle from septic mice which, was attenuated by pre-treatment with EPA to baseline level. This was accompanied by an increase in protein expression of both the proteasome α-subunits and the $E2_{14k}$, determined by Western blotting, which again was attenuated by EPA. Similar results were seen with respect to the chymotrypsin-like activity of the proteasome. These results suggest that protein catabolism in sepsis and cancer cachexia is mediated by a common pathway, which is inhibited by EPA, possibly by inhibiting the formation of 15-HETE.

CHAPTER 8

Final Discussion and Conclusions

Cancer cachexia is a complex syndrome, which includes anorexia, asthenia, anaemia, and progressive weight loss. The prognosis of cancer patients with cachexia is poor; weight loss is an important prognostic factor in determining the overall survival of cancer patients (Shamberger, 1984). Tissue wasting is particularly evident in adipose tissue and skeletal muscle (Tisdale, 1991, 1993a). However, it is visceral protein and lean body mass depletion that have a worse prognostic impact (Nixon *et al.*, 1980). Increased protein breakdown contributes to muscle wasting in several tumour-bearing animal models (Tessitore *et al.*, 1987; Lopes *et al.*, 1989; Smith and Tisdale, 1993a) and in some cancer patients (Lundholm *et al.*, 1976; Jeevanandam *et al.*, 1984; O'Keefe *et al.*, 1990).

Like all mammalian tissues, skeletal muscle contains multiple proteolytic systems. The best know system is the lysosomal pathway, which involves the cathepsins B, L, H and D. Increased cathepsin activities have been reported in muscles from cancer patients (Lundholm *et al.*, 1976). However, lysosomes account for a minor part of increased skeletal muscle proteolysis in many catabolic states and are not involved in the breakdown of myofibrillar proteins (Lowell *et al.*, 1986; Furuno *et al.*, 1990). Skeletal muscle also contains the Ca^{2+}-dependent proteases, which like lysosomal proteases do not play an important role in the degradation of the contractile proteins actin and myosin (Lowell *et al.*, 1986; Furuno *et al.*, 1990). By contrast, the third proteolytic system in skeletal muscle, ATP-ubiquitin-proteasome proteolytic pathway, which is widely believed to degrade abnormal and short lived proteins (Rechsteiner, 1991; Hershko and Ciechanover, 1992), is the critical system responsible for the breakdown of most skeletal muscle proteins, including the long-lived contractile components (Furuno *et al.*, 1990; Wing and Goldberg, 1993). In addition few studies have suggested Ca^{2+}-calpain dependent pathway has a specific role in releasing myofilaments from the sarcomere before they are ubiquitinated and degraded by the proteasome (Williams *et al.*, 1999a; Dahlmenn *et al.*, 1986).

Recent studies in rats and mice with experimental tumours (Temparis *et al.*, 1994; Llovera *et al.*, 1994; Baracos *et al.*, 1995; Lorite *et al.*, 1998) and humans (Williams *et al.*, 1999b) suggest that muscle proteolysis in cancer is regulated mainly by the ubiquitin-proteasome pathway and is associated with the upregulated expression of several genes in this pathway.

In this study quantitative-competitive RT-PCR was set up to measure the expression of the C2α and C5β 20S proteasome subunits and the ubiquitin-conjugating enzyme $E2_{14k}$, to further investigate the involvement of the ubiquitin-proteasome pathway in muscle proteolysis in cancer cachectic state.

Results in this study showed increased mRNA and protein expression of the proteasome subunits C2 and C5 and the ubiquitin-conjugating enzyme $E2_{14k}$ in gastrocnemius muscle of mice bearing the MAC16 colon adenocarcinoma, which increased with increasing weight loss up to 20% weight loss. Furthermore, the expression of these genes decreased in cachectic mice with greater weight loss than 20%. Chymotrypsin-like activity showed similar results in mice bearing the MAC16 tumour (Whitehouse *et al.*, 2001), where the proteasome chymotrypsin-like activity increased with increasing weight loss up to 15% weight loss and then decreased. However, in pectoral muscle of mice bearing the MAC16 tumour, the mRNA and protein expression of the C2 and C5 proteasome subunits and the $E2_{14k}$ enzyme was elevated at higher weight losses and the expression was several fold lower than seen in gastrocnemius muscles, this reflects the different degrees of protein degradation in the two muscles during the process of cancer cachexia, and could be explained by the fact that atrophied gastrocnemius muscle is specifically sensitive to the ubiquitin-proteasome proteolytic pathway (Ikemoto *et al.*, 2001).

A tumour factor has been recently isolated and purified from both a cachexia-inducing murine tumour (MAC16) and from the urine of patients with cancer cachexia (Todorov *et al.*, 1996a; Cariuk *et al.*, 1997). It was shown to be a sulphated glycoprotein of 24kDa molecular weight and was called proteolysis-inducing factor (PIF). This material produces a state of cachexia when administered to non-tumour bearing mice with

specific depletion of the non-fat carcass mass (Todorov *et al.*, 1996a) and produced direct proteolysis in isolated skeletal muscle (Lorite *et al.*, 1997).

Using an *in vitro* model, results here showed that PIF increased the expression of the proteasome and the ubiquitin-conjugating enzyme $E2_{14k}$. Furthermore, the arachidonic acid metabolite 15-HETE also increased the expression of the proteasome and the $E2_{14k}$, which was attenuated, together with the effect of PIF, by EPA. Previous studies showed that induction of protein degradation *in vitro* by PIF is associated with the release of arachidonic acid, which was also attenuated by EPA (Smith *et al.*, 1999). Furthermore, results here showed that the increased expression of the proteasome and $E2_{14k}$ by PIF was attenuated by the 5-lipoxygenase inhibitor CV-6504. CV-6504 was previously shown to be capable of attenuating the development of cachexia in mice bearing the MAC16 tumour (Hussy *et al*, 1996). Together these results suggest that 15-HETE is the intracellular signal for protein degradation induced by PIF; furthermore, it is likely that both EPA and CV-6504 have a downregulating effect on the function of 15-HETE working downstream of it, reducing the expression of the proteasome and other components of the ubiquitin-proteasome pathway (e.g. $E2_{14k}$) therefore, reducing proteolysis.

It has been shown that PIF activity in MAC16 tumours increases protein degradation, and its activity increased with increasing weight loss up to 20%, and decreased with weight loss above 20% (Smith and Tisdale, 1993b). Furthermore, PIF has been shown to be responsible for the loss of skeletal muscle in cachectic mice (Lorite *et al.*, 1998) and the changes in expression of the proteasome subunits and E2 with changes in serum levels of PIF suggests that PIF is responsible for the upregulation of the ubiquitin-proteasome pathway in mice bearing the MAC16 tumour. This might be also the case in cachectic cancer patients since the mRNA and protein expression of the C2 and C5 proteasome subunits and the ubiquitin-conjugating enzyme $E2_{14k}$ was upregulated in cancer cachectic patients and their expression increased with increasing weight loss up to that of about 20% weight loss, similar to that seen in MAC16 tumour-bearing mice. Furthermore PIF has also been isolated and purified from urine of cachectic cancer patients (Todorov *et al.*, 1996a). Therefore, it is likely that PIF is responsible for the upregulation of the ubiquitin-proteasome pathway in cachectic cancer patients. It is likely that PIF concentration and activity might increase with progression of the disease and growth of the tumour, and as the activity of the PIF increases it further upregulates

the ubiquitin-proteasome proteolytic pathway, causing loss of more skeletal muscle protein and so skeletal mass and in-turn increase in weight loss. This may explains the results shown in this study where the expression of the components of the ubiquitin-proteasome pathway increased with increasing weight loss of both tumour-induced mice and cachectic cancer patients.

The results presented here also show that the ubiquitin-proteasome pathway is upregulated in septic animals, which is also attenuated by EPA, demonstrating that EPA attenuates upregulation of proteasome subunits and proteasome activity, as well as the ubiquitin-conjugating enzyme involved in the ubiquitin-proteasome-proteolytic pathway. This could be explained by the fact that EPA can affect the production of 15-HETE from arachidonic acid via the LOX pathway, showing an anti-cachectic effect in this condition. Therefore, this suggests that protein catabolism in sepsis and cancer cachexia is mediated through a common pathway. This indicated that EPA can have similar anti-cachectic effects in other biological conditions by affecting the production of 15-HETE. This has been recently demonstrated in acutely starved mice (Whitehouse and Tisdale, 2001). Furthermore, the fact that the 5-lipoxygenase inhibitor CV-6504 attenuated proteasome function and expression with dynamics comparable to EPA, suggests that EPA is anti-cachectic partly through interference with the arachidonic cascade having the ability to prevent 15-HETE production. However, additional studies are required to fully understand the mechanism by which EPA modulates the activity of the proteasome pathway.

There is already a large amount of clinical, biochemical and epidemiological evidence showing the anti-tumour and anti-cachectic properties of EPA. It has been shown that cachectic cancer patients taking EPA supplements gain lean tissue mass resulting in improvement of their functional ability and relative weight stability (Barber, 2001), suggesting the possible potential benefits of EPA in the treatment of cachexia which might eventually lead to nutritional therapy that could improve functional ability, quality of life and survival of millions of patients.

CHAPTER 9

References

Ahn JY, Tanahashi N, Akiyama K, Hisamatsu H, Noda C, Tanaka K, Chung CH, Shimbara N, Willy PJ, Mott, JD, Slaughter C and DeMartino GN (1995) Primary structures of two homologous subunits of PA28, a γ-interferon-inducible protein activator of the 20S proteasome. FEBS Lett, 366: 37-42.

Albrecht JT and Canada TW (1996) Cachexia and anorexia in malignancy. Heamatol and Oncology, Clinics of North America, 10: 791-800.

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, BoldrickJC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Lu L, Lewis DB, Robert T, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO and Staudt LM (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature, 403: 503-511.

Alvarez B, Quinn LS, Basquets S, Lopez-Soriano FJ and Argiles JM (2002) TNF-α modulate cytokine receptors in C2C12 myotubes. Cancer Lett, 175: 181-185.

Anti M, Marra G and Amaleo F (1992) Effect of n-3 fatty acids on rectal mucosal proliferation in subjects at risk for colon cancer. Gastroenterology, 103: 883-891.

Araki T, Gejyo F, Takagaki K, Haupt H, Gerhard Schwick H, Burgi W, Marti T, Schaller J, Rickli E, Brossmer R, Atkinson PH, Putnam FW and Schmid K (1988) Complete amino acid sequence of human plasma Znα2 glycoprotein and its homology to hitocompatibility antigens. Proc Natl Acad Sci USA, 85: 679-683.

Attaix D, Aurousseau E, Combaret L, Kee A, Larbaud D, Ralliére C, Souweine B, Taillender D and Tilignac T (1998) Ubiquitin-proteasome-dependent proteolysis in skeletal muscle. Reprod Nutr Dev, 38: 153-165.

Attaix D and Taillandier D (1998) The critical role of the ubiquitin-Proteasome pathway in muscle wasting in comparison to lysosomal and Ca^{2+}-dependent systems. Adv Mol Cell Biol, 27: 235-266.

Attaix D, Taillandier D, Temparis S, Larbaud D, Aurousseau E, Combaret L and Voision L (1994) Regulation of ATP-ubiquitin dependent proteolysis in muscle wasting. Reprod Nutr Dev, 34: 583-597.

Auboeuf D and Vidal H (1997) The Use of the Reverse-Transcription Polymerase Chain Reaction to Investigate the in vivo Regulation of Gene Expression in Small Tissue Samples. Analytical Biochem, 245: 141-148.

Auclair D, Garrel DR, Zerouala AC and Ferland JH (1997) Activation of the ubiquitin pathway in rat skeletal muscle by catabolic doses of glucocorticoids. Am J Physiol, 272: C1007-C1016.

Bailey JL, Wang X, England BK, Price SR, Ding X and Mitch WE (1996) The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent, ubiquitin-proteasome pathwyay. J Clin Inves, 97: 1447-1453.

Bang Ho, Dyerberg J and Hijorne N (1976) The composition of food consumed by Greenland Eskimos. Acta Med Scand, 200: 69-73.

Baracos VE, DeVivo C, Hoyle DHR and Goldberg AL (1995) Activation of the ATP-ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. Am J Physiol, 268: E996-E1006.

Barber MD (2001) Cancer cachexia and its treatment with fish-oil-enriched nutritional supplementation. Nutrition, 17: 751-755.

Barber MD, McMillan DC, Preston T, Ross JA and Fearon KCH (2000) The metabolic response to feeding in weight-losing pancreatic patients and its modulation by a fish oil-enriched nutritional supplement. Clin Sci, 98: 389-399.

Barber MD, Ross JA and Fearon KCH (1999a) Cancer cachexia. Surg Oncol, 8: 133-141.

Barber MD, Ross JA, Preston T, Shenkin A and Fearon KCH (1999b) Fish oil-enriches nutritional supplement attenuates progression of the acute phase response in weight-losing patients with advanced pancreatic cancer. J Nutr, 129: 1120-1125.

Bartsch HH, Pfizenmaier K, Schroeder M and Nagel GA (1989) Intralesional application of recombinant human tumour necrosis factor alpha induces local tumour regression in patients with advanced malignancies. Eur J Cancer Clin Oncol, 25: 287-291.

Baumeister W, Walz J, Zühl F and Seemüller E (1998) The proteasome: paradigm of a self-compartmentalizing protease. Cell, 92: 367-380.

Beck SA, Groundwater P, Barton C and Tisdale MJ (1990a) Alterations in serum lipolytic activity of cancer patients with response to therapy. Br J Cancer, 62: 822-825.

Beck SA, Mulligan HD and Tisdale MJ (1990b) Lipolytic factors associated with murine and human cancer cachexia. J Natl Cancer Inst, 82: 1922-1926.

Beck SA, Smith KL and Tisdale MJ (1991) Anticachectic and antitumor effect of eicosapentanoic acid and its effect on protein turnover. Cancer Res, 51: 6089-6093.

Beck SA and Tisdale MJ (1987) Production of the lipolytic and proteolytic factors by a murine tumor-producing cachexia in the host. Cancer Res, 47: 5919-5923.

Becker-André M and Hahlbrock (1989) Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acid Res, 17: 9437-9446.

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

Belizário JE, Lorite MJ and Tisdale MJ (2001) Cleavage of caspases-1, -3, -6, -8 and -9 substrates by proteases in skeletal muscles from mice undergoing cancer cachexia. Br J Cancer, 84: 1135-1140.

Belury MH, Locniskar M and Fischer SM (1993) Modulation of phorbol-ester associated events in epidermal cells by linoleate and arachidonate. Lipids, 28: 407-413.

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

Bing C, Hopkins D, Wang Q, Frankish HM, Buckingham R and Williams G (1998) Thermogenic effects of BRL-35135 is independent of hypothalamic neuropeptide Y. Peptides, 19: 167-170.

Bing C, Pickavance L, Wang Q, Frankish HM, Trayhurn P and Williams G (1997) Role of hypothalamic neuropeptide Y neurones in the defective thermogenic response to acute cold exposure in fatty Zucker rats. Neuroscience, 80: 277-284.

Bing C, Russell ST, Beckett EE, Collins P, Taylor S, Barraclough R, Tisdale MJ and Williams G (2002) Expression of uncoupling proteins-1, -2 and -3 mRNA is induced by an adenocarcinoma-derived lipid mobilising factor. Br J Cancer, 86: 612-618.

Black BL and Olsen EN. (1998) Annu Rev Cell Dev Biol 14: 167

Blay YL, Negrier S, Combaret V, Attali S and Goillot E (1992) Serum level of interleukin-6 as a prognostic factor in metastatic renal cell carcinoma. Cancer Res, 52: 3317-3322.

Bodine SC, Latres E, Baumhueter S, Lai VKM, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan AQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD and Glass DJ (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. Science, 294: 1704-1707.

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

Bossola M, Muscaritoli M, Bellantone R, Pacelli F, Cascino A, Sgadari A, Battaglia F, Piccioni E, Scambia G, Doglietto GB and Fanelli FR (2000) Serum tumour necrosis factor-α levels in cancer patients are discontinuous and correlate with weight loss. Euro J Clin Invest, 30: 1107-1112.

Bossola M, Muscaritoli M, Costelli P, Bellantone R, Pacelli F, Busquets S, Argiles J, Lopez-Spranio FJ, Civello IM, Baccino FM, Fanelli FR and Doglietto GB (2001) Increased muscle ubiquitin mRNA levels in gastric cancer patients. Am J Physiol Regulatory Integrative Comp Physiol, 280: R1518-1523.

Brennan MF (1977) Uncomplicated starvation versus cancer cachexia. Cancer Res, 37: 2359-2364.

Buckman DK, Hubbard NE and Erickson KL (1991) Eicosanoids and linolate-enhanced growth of mouse mammary tumour cells. Prostaglandins Leukotrienes and Essential Fatty Acids, 44: 177-184.

Bundred NJ, Miller WR and Walker RA (1987) An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, Zinc alpha-2 glycoprotein. Histopathology, 11: 603-610.

Burt ME and Brennan MF (1984) Nutritional support of the patient with esophageal cancer. Semin Oncol, 11: 127.

Burton BE and Murphy TF (2000) Constitutive expression of IL-6 like cytokines in normal bone marrow: implications for myeloma physiology. Cytokine, 12: 1537-1545.

Burton BE and Murphy TF (2001) Cancer cachexia is mediated in part by the induction of IL-6-like cytokines from the spleen. Cytokine, 16: 251-257.

Butcher RN, Baird CE and Sutherland EW (1968) Effects of lipolytic and antilipolytic substanced on adenosine 3', 5'- monophosphate levels in isolated fat cells. J Biol Chem, 243: 1705-1712.

Cabal-Manzano R, Bhargava P, Torres-Duarte A, Marshall J Bhargava P and Wainer IW (2001) Proteolysis-inducing factor is expressed in tumours of patients with gastrointestinal cancers and correlates with weight loss. Br J Cancer, 84: 1599-1601.

Cariuk P, Lorite MJ, Todovov PT, Field WN, Wigmore SJ and Tisdale MJ (1997) Induction of cachexia in mice by a product isolated from the urine of cachectic cancer patients. Br J Cancer, 76: 606-613.

Caughey GE, Mantzioris E, Gibson RA, Cleland LG and James MJ (1996) The effect on human tumour necrosis factor α and interleukin 1β production of diets enriched in n-3 fatty acids from vegetable or fish oil. Am J Clin Nutr, 63: 116-122.

Caygill CPJ, Charlett A and Hill MJ (1996) Fat, fish, Fish oil and cancer. Br J Cancer, 74: 159-164.

Celi FS, Zenilman MF and Shuldiner AR (1993) A rapid and versatile method to synthesise internal standards for competitive PCR. Nucleic Acid Res, 21: 1047.

Chance WT, Balasubramaniam A, Borchero M and Fischer JE (1995) Refractory hypothalamic adenylate cyclase in anorectic tumour bearing rats. Implications for NPY-induced feeding. Brain Res, 691: 180-184.

Clark CM and Goodlad GAJ (1971) Depletion of proteins of phasic and tonic muscles in tumour-bearing rats. Eur J Cancer, 7: 3-9.

Clawes GHA, George BC, Villee CA and Saravis CA (1983) Muscle proteolysis induced by a circulating peptide in patients with sepsis or trauma. N Engl J Med, 308: 545-552.

Coenen Schmke JM, Ljungqvist OK, Sarker G, Conover CA and Sreekumaran Nair K (1999) A quantitative PCR measurement of messenger RNA expression of IGF-I, IGF-II and IGFBP-5 in human skeletal muscle. Growth Hormone & IGF Research, 9: 179-186.

Combaret L, Ralloére C, Taillndier D, Tanaka K and Attaix D (1999) Manipulation of the ubiquitin-proteasome pathway in cachexia: pentoxifylline suppresses the activation of 20S and 26S proteasomes in muscles from tumour bearing rats. Mol Biol Rep, 26: 95-101.

Combaret L, Taillandier D, Voisin L, Samueles SE, Boespflug-Tanguy O and Attaix D (1996) No alteration in gene expression of components of the ubiquitin-proteasome proteolytic pathway in dystrophin-deficient muscle. FEBS Lett, 393: 292-296.

Costa G (1963) Cachexia, the metabolic component of neoplastic disease. Prog Exp Tumour Res, 3: 321-369.

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

Costa G and Holland JF (1962) Effects of Krebs-2 carcinoma on the lipid metabolism of male Swiss mice. Cancer Res, 22: 1081-1083.

Costelli P and Baccino FM (2000) Cancer cachexia: from experimental models to patient management. Curr Op Clin Nutr Metab Care, 3: 177-181.

Costelli P, Carbo N, Tessitore L, Bagby GJ, Lopez-Soriano FJ, Argiles JM and Baccino FM (1993) Tumor necrosis factor-α mediates changes in tissue protein turnover in a rat cancer cachexia model. J Clin Invest, 92: 2783-2789.

Costelli P, Tullio RD, Baccino FM and Melloni E (2001) Activation of Ca2+-dependent proteolysis in skeletal muscle and heart in cancer ataxia. Br J Cancer, 84: 946-950.

Cottrez F, Auriault C, Capron A and Groux H (1994) Quantitative PCR: validation of the use of a multispecific internal control. Nucleic Acid Res, 22: 2712-2713.

Coux O, Tanaka K and Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem, 65: 801-847.

Cross NCP (1995) Quantitative PCR techniques and applications. Br J Heamatol, 89: 693-697.

Dahlmann B, Ruppert T, kloetzel PM and Kuehn L (2001) Subtypes pf 20S proteasomes from skeletal muscle. Biochimie, 83; 295-299.

Dahlmann B, Rutschmann M and Reinauer H (1986) Effect of starvation or treatment with corticosterone on the amount of easily releasable myofilaments in rat skeletal muscle. Biochem J, 234: 659-664.

Dardevet D, Sornet C, Taillandier D, Savary I, Attiax D and Grizard J (1995) Sensitivity and protein turnover response to glucocorticoids are different in skeletal muscle from adult and old rats. Lack of regulation of the ubiquitin-proteasome proteolytic pathway in aging. J Clin Invest, 96: 2113-2119.

de Blaauiv I, Heeneman S, Deutz NEP and Meyenfeldt MF (1997) Increased whole-body protein and glutamine turnover in advanced cancer is not matched by an increased muscle protein and glutamine turnover. J Surg Res, 68: 44-55.

DeMartino GN and Slaughter CA (1999) The proteasome, a novel protease regulated by multiple mechanism. J Biol Chem, 274: 22123-22126.

De Wys WD (1985) Management of cancer cachexia. Semin Oncol, 12: 452-460.

De Wys WD, Costa G and Aenkin R (1981) Clinical parameters related to anorexia. Cancer Treat Rep, 65: 49-52.

De Wys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, Cohen MH, Douglass HO, Engstorm PF, Ezdinli EZ, Hortonn J, Johonson GJ, Moertel CG, Oken MM, Perlia C, Rosenbaum C, Silverstein MN, Skeel RT, Sponzo RW and Tormey DC (1980) Prognostic effect of weight loss prior to chemotherapy in cancer patients. Am J Medicine, 69: 491-497.

Doherty GM, Jensen JC, Alexander HR, Buresh CM and Norton JA (1991) Pentoxifylline suppression of tumour necrosis factor gene transcription. Surgery, 110: 192-198.

Drasar BS and Irving D (1973) Environmental factors and cancer of the colon and breast. Br J Cancer, 27: 167-172.

Ebisui C, Tsujinaka T, Morimoto T, Kan K, Iijima S, Yono M, Kominami E, Tanaka K and Monden M (1995) Interleukin-6 induces proteolysis by activation intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. Clinical Sci, 89: 431-439.

Edmonson JH (1966) Fatty acid mobilisation and glucose metabolism in patients with cancer. Cancer, 19: 277-280.

Emery PW, Edwards RH, Rennie MJ, Souhami RL and Halliday D (1984) Protein synthesis in muscle measured in vivo in cachectic patients with cancer. Br Med J, 289: 584-586.

Endres S, Ghorbani R, Kelly VE, Georgiles K, Lonnemann G, Van Der Meer JWM, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaefer EJ, Wolef SM and Dinarello CA (1989) The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumour-necrosis factor by mononuclear cells. N Engl J Med, 320: 265-271.

Evans WK, Makuch R, Clamon GH, Feld R, Weiner RS, Moran E, Blum R, Shephard FA, Jeejeebhoy KN and De Wys WD (1985) Limited impact of total parenteral nutrition

of nutritional status during treatment for small cell lung cancer. Cancer Res, 45: 3347-3355.

Falconer JS, Fearon KC, Plester CE, Ross JA and Carter DC (1994) Cytokines, the acute-phase response, and resting energy expenditure in cachectic patients with pancreatic cancer. Ann Surg, 219: 325-313.

Falconer JS, Fearon KCH, Ross JA, Elton R, Wigmore SJ, Garden OJ and Carter Dc (1995) Acute-phase protein response and survival duration of patients with pancreatic cancer. Cancer, 75: 2077-2082.

Fearon KCH (1992) The mechanism and treatment of weight loss in cancer. Proc Nutr Soc, 51: 251-265.

Ferry DR, Deakin M, Baddeley J, Daryanani S, Bramhall S, Anderson DA, Wakelam MJO, Doran J, Pemberton G, Young AM, Buckels J and Kerr DJ (2000) A phase II study of the 5-lipoxygenase inhibitor, CV6504, in advanced pancreatic cancer: correlation of clinical data with pharmacokinetic and pharmacodynamic endpoints. Ann Oncol, 11: 1165-1170.

Fischer D, Gang G, Pritts T and Hasselgren PO (2000a) Sepsis induced muscle proteolysis is prevented by a proteasome inhibitor *in vivo*. Biochem Biophys Res Commun, 270: 215-221.

Fischer D, Sun X, Gang G, Pritts T and Hasselgren PO (2000b) The gene expression of ubiquitin-ligase E3α is upregulated in skeletal muscle during sepsis in rats-potential role of glucocorticoids. Biochem Biophys Res Commun, 267: 504-508.

Fischer DR, Sun X, Williams AB, Gang G, Pritt TA, James JH, Molloy M, Fischer JE, Paul RJ and Hasselgren PO (2001) Dantrolene reduced serum TNFα and corticosterone levels and muscle calcium, calpain gene expression, and protein breakdown in septic rats. Shock, 15: 200.

Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levis-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D and Wardenm CH (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nature Genet, 15: 269-272.

Fredix EW, Soeters PB, Wouters EF, Deerenberg IM, von Meyerfeldt MF and Saris WH (1991) Effect of different tumour types on resting energy expenditure. Cancer Res, 51: 6138-6141.

Freije JO, Fueyo A, Uria J and Lopez-Otin C (1991) Human Zn-α2-glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissue. FEBS lett, 290: 247-249.

Fujita J, Tsujinaka T, Yano M, Ebisui C, Saito H, Katsume A, Akamatsu K, Ohsugi Y, Shiozaki H and Monden (1996) Anti-interleukin-6 receptor antibody prevents muscle atrophy in colon-26 adenocarcinoma-bearing mice with modulation of lysosomal and ATP-ubiquitin-dependent proteolytic pathways. Int J Cancer, 68: 637-643.

Furuno K, Goodman MN and Goldberg AL (1990) Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. J Biol Chem, 265: 8550-8557.

Garcia-Martinez C, Lopez-Soranio HJ and Argiles JM (1994) Interleukin-6 does not activate protein breakdown in rat skeletal muscle. Cancer Lett, 76: 1-7.

Gelin J, Moldawer LL, Lonnroth C, Sherry B, Chizzonite R and Lundholm K (1991) Role of endogenous tumor necrosis factor α and interleukin I for experimental tumor growth and development of cancer cachexia. cancer Res 51: 415-421.

Gilliland G, Perrin S, Blanchard K and Bunn HF (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87: 2725-2729.

Glickman MH, Rubin DM, Fried AV and Finley D (1998) The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mo Cell Biol, 18: 3149-3162.

Gobar H, Hillyard LA and Abraham S (1985) Effect of dietary fat on growth kinetics of transplantable mammary adenocarcinoma in BALB/c mice. J Natl Cancer Instit, 74: 1299-1305.

Goll DE, Thompson VF, Taylor RG and Zalewska T (1992) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? Bioessays, 14: 549-556.

Gonzalez MJ, Schemmel RA, Dugan L, Gray JI and Welsch CW (1993) Dietary fish oil inhibits human breast carcinoma growth: a function of increased lipid peroxidase. Lipids, 28: 827-832.

Granneman JG (1995) why do adipocytes make the 3-adrenergic receptor? Cell Sig, 7: 9-15.

Gray CW, Slaughter CA and DeMartino GN (1994) PA28 activator protein forms regulatory caps on proteasome stacked rings. J Mol Biol, 236: 7-15.

Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD and Huber R (1997) Structure of 20s proteasome from yeast at 2.4 Å resolution. Nature, 386: 463-471.

Groundwater P, Beck SA, Barton C, Adamson C, Ferrier IN and Tisdale MJ (1990) Alterations of serum and urinary lipolytic activity with weight loss in cachectic cancer patients. Br J Cancer, 62: 816-821.

Gudmundsson S and Erlendsdottir H (1999) Murine thigh model. In O. Zak and M. A. Sande (eds), Handbook of animal models of infection. Academic Press, San Diego, California, USA. 137-144.

Hass AL and Siepmann TJ (1997) Pathways of ubiquitin-conjugation. FASEB J, 11: 1257-1268.

Hall JC (1979) The cachexia of cancer. Biomedicine, 30: 287-291.

Hasselgren PO (1999) Role of the ubiquitin proteasome pathway in sepsis-induced muscle catabolism. Mol Biol Rep, 26: 71-76.

Hasselgren PO and Fischer J (1997) The ubiquitin-proteasome pathway. Review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. Ann Surg, 225: 307-316.

Hasselgren PO, James JH, Benson DW, Hall-Angerås M, Angerås U, HiyamaDT, Li S and Fischer JE (1989) Total and myofibrillar protein breakdown in different types of rat skeletal muscle: effects of sepsis and regulation by insulin. Metabolism, 38: 634-640.

Hasselgren PO, Talamini M, James JH and Fischer JE (1986) Protein metabolism in different types of skeletal muscle during early and late sepsis in rats. Arch Surg, 121; 918-923.

Hasselgren PO, Zamir O, James J and Fischer JE (1990) Prostaglandin E_2 does not regulate total or myofibrillar protein breakdown in incubated skeletal muscle from normal or septic rats. Biochem J, 270: 45-50.

Hayward AL, Oefner PJ, Sabatini S, Kainer DB, Hinojos CA and Doris PA (1998) Modeling and analysis of competitive RT-PCR. Nucleic Acid Res, 26: 2511-2518.

Hendil KB, Khan S and Tanaka K (1998) Simultaneous binding of PA28 and PA700 activators to 20S proteasomes. Biochem J, 332: 749-754.

Hershko A and Ciechanover A (1992) The ubiquitin system for protein degradation. Annu Rev Biochem, 61: 761-807.

Hershko A and Ciechanover (1998) The ubiquitin system. Ann Rev Biochem, 67: 425-479.

Heymsfield SB and McManus CB (1985) Tissue components of weight loss in cancer patients. Cancer, 55: 2238.

Hill MJ (1987) Dietary fat and human cancer. Anticancer Res, 7: 281-293.

Hilt W and Wolf DH (1996) Proteasome: destruction as a programme. Trends Biochem Sci, 21: 96-102.

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

Hobler SC, Tiao G, Fischer JE, Monaco J and Hasselgren PO (1998) Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. Am J Physiol, 274: R30-R37.

Hobler SC, Wang JJ, Williams AB, Melandri F, Xiaoyan S, Fischer JE and Hasselgren PO (1999) Sepsis is associated with increased ubiquitin-conjugating enzyme $E2_{14k}$ mRNA in skeletal muscle. Am J Physiol, 276: R468-R473.

Holm E, Hagmuller E, Staedt U, Schlickeiser G, Gunther HJ, Lewelling H, Tokus M and Kollmar HB (1995). Substrate balances across colonic carcinomas in humans. Cancer Res, 55: 1373-1378.

Holroyde CP, Gabuzda TG, Putnam RC, Paul P and Reichard GA (1975) Altered glucose metabolism in metastatic carcinoma. Cancer Res, 35: 3710-3714.

Horikishi T and Sakakibara M (2000) Quantification of relative mRNA expression in the rat brain using simple RT-PCR and ethidium bromide staining. J Neuroscience Methods, 99: 45-51.

Hotchkiss RS and Karl IE (1994) Dantrolene ameliorates the metabolic hallmarks of sepsis in rats and improves survival in a mouse model of endotoxemia. Proc Natl Acad Sci USA, 91: 3039-3043.

Howe R (1993) β3-Adrenergic agonists. Drugs Future, 18: 529-549.

Hudson EA, Beck SA and Tisdale MJ (1993) Kinetics of the inhibition of tumour growth in mice by eicosapentaenoic acid-reversal by linolenic acid. Biochem Pharmacol, 45: 2189-2194.

Hursting SD, Thornquist M and Henderson MM (1990) Types of dietary fat and the incident of cancer at five sites. Prev Medm, 19: 242-253.

Hussey HJ, Bibby MC and Tisdale MJ (1996) Novel anti-tumour activity of 2,3,5-trimethyl-6-(3-pyridylmethyl)-1,4-benzoquinone (CV-6504) against established murine adenocarcinomas (MAC). Br J Cancer, 73: 1187-1192.

Hussey HJ and Tisdale MJ (1994) Effect of polyunsaturated fatty acids on the growth of murine colon adenocarcinoma *in vitro* and *in vivo*. Br J Cancer, 70: 6-10.

Hussey HJ and Tisdale MJ (1996a) Inhibition of tumour growth by lipoxygenase inhibitors. Br J Cancer, 74: 683-687.

Hussey HJ and Tisdale MJ (1996b) Metabolism and pharmacokinetics of the anti-tumour agent 2,3,5-trimethyl-6-(3-pyridylmethyl)1, 4-benzoquinone (CV-6504). Br J Cancer, 74: 1349-1353.

Hussey HJ and Tisdale MJ (1997) Mechanism of the anti-tumour effect of 2,3,5-trimethyl-6-(3-pyridylmethyl)-1,4-bezoquinone (CV-6504). Br J Cancer, 75: 845-849.

Hussey HJ, Todorov PT, Field WN, Inagaki N, Ishitsuka H and Tisdale MJ (2000) Effect of a fluorinated pyrimidine on cachexia and tumour growth in murine cachexia models: relationship with proteolysis inducing factor. Br J Cancer, 83: 56-62.

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

Ikemoto M, Nikawa T, Takeda S, Watanabe C, Kitano T, Baldwin KM, Izumi R, Nonaka I, Tawatari T, Teshima S, Rokutan K and Kishi K (2001) Space shuttle flight (STS-90) enhances degradation of rat myosin heavy chain in association with activation of ubiquitin-proteasome pathway. FASEB, 15: 1279-121281.

Inagaki J, Rodriguez V and Bodey GP (1974) Cause of death in cancer patients. Cancer, 33: 568-573.

Inui A (1999) Cancer anorexia-cachexia syndrome: are neuropeptides the key? Cancer Res, 59: 4493-?.

Islam-Ali B, Khan S, Price SA and Tisdale MJ (2001) Modulation of adipocyte G-protein expression in cancer cachexia by a lipid-mobilizing factor (LMF). BrJ Cancer, 85: 758-763.

Islam-Ali BS and Tisdale MJ (2001) Effect of tumour-produced lipid-mobilizing factor on protein synthesis and degradation. Br J Cancer, 84: 1648-1655.

Jagoe RT, Redfern CPF, Roberts RG, Gibson GJ and Goodship THJ (2002) Skeletal muscle mRNA levels for cathepsin B, but not components of the ubiquitin-proteasome pathway , are increased in patients with lung cancer referred for thoracotomy. Clin Sci, 102: 353-361.

Jatoi A, Daly BDT, Hughes VA, Dallal GE, Kehayias J and Roubenoff R (2001) Do patients with nonmetastatic non-small cell lung cancer demonstrate altered resting energy expenditure. Ann Thorac Surg, 72: 348-351.

Jeevanandam M, Lowry SF, Horowitz GD and Brennan MF (1984) Cancer cachexia and protein metabolism. Lancet, 1: 1423-1426.

Kajimura N, Iseki H, Tanaka R, Ohue C, Otsubo K, Gyoutoku M, Sasaki K, Akiyama Y and Yamaguchi K (1996) Toxohormones responsible for cancer cachexia syndrome in nude mice bearing human cancer cell lines. Cancer Chemother Pharmacol, 38 (suppl): S48-52.

Kanayama H, Tanaka K, Aki M, Kagawa S, Miaji H, Satoh M, Okada F, Sato S, Shimbara N and Ichihara A (1991) Changes in expression of proteasome and ubiquitin genes in human renal cancer cells. Cancer Res, 51: 6677-6685.

Kendall LV and Riley LK (2000) Quantitative competitive PCR. Contemporary Topics, 39: 47.

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

Kisselev F, Akopian TN, Castillo V and Goldberg AL (1999) Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. Mol Cell, 4: 395-402.

Kitada S, Hays EF and Mead JF (1980) A lipid mobilising factor in serum of tumor-bearing mice. Lipids, 15: 168-174.

Kotler DP, Tiemey AR, Culpepper-Morgan JA, Wang J and Pierson RN (1990) Effect of home total parenteral nutrition on body composition in patients with acquired immunodeficiency syndrome. JPEN, 14: 454-?.

Larbaud D, Balage M, Taillandier D, Combaret L, Grizard J and Attaix D (2001) Differential regulation of the lysosomal, Ca^{2+}-dependent and ubiquitin/proteasome-dependent proteolytic pathways in fast-twitch and slow-twitch rat muscle following hyperinsulinaemia. Clin Sci, 101: 551-558.

Larbaud D, Debras E, Taillandier D, Samules SE, Temparis S, Champredon C, Grizard J and Attaix D (1996) Euglycemic hyperinsulinemia and hyperaminoacidemia decrease skeletal muscle ubiquitin mRNA in goats. Am J Physiol, 271: E505-E512.

Laville M, Auboeuf D, Khalfallah N, Vega N, Riou JP and Vidal H (1996) Acute Regulation by Insulin of Phosphatidylinositol-3-kinase, Rad, Glat 4, and Lipoprotein Lipase mRNA Levels in Human Muscle. J Clin Invest, 98: 43-49.

Lawson DH, Richmond A, Nixon DW and Rudman D (1982) Metabolic approaches to cancer cachexia. Annual Rev Nutrition, 2: 277-301.

Lazarus DD, Destree AT, Mazzola LM, McCormack TA, Dick LR, Xu B, Huang JQ, Pierce JW, Read MA, Coggins MB, Solomon V, Goldberg AL, Brand SJ and Elliot PJ (1999) A new model of cancer cachexia: contribution of the ubiquitin-proteasome pathway. Am J physiol, 277: E332-E342.

Lecker SH, Solomon V, Mitch WE and Goldberg AL (1999a) Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J Nutr 129: 227S-237S.

Lecker SH, Solomon V, Price RS, Kwon YT, Mitch WE and Goldberg AL (1999b) Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in the muscle of diabetic rats. J Clin Invest, 104; 1411-1420.

Lee P-PH and Ip MM (1992) Regulation of proliferation of rat mammary tumour cells by inhibitors of cyclooxygenase and lipoxygenase. Prostaglandins Leukotrienes Fatty Acid, 45: 21-31.

Leverve X, Guignier M, Carpentier F, Serre JC and Caravel JP (1984) Effect of parenteral nutrition on muscle amino acid output and 3-methyl-histidine excretion in septic patients. Metabolism, 33: 471-477.

Lidnsey AM (1986) Cancer cachexia: effects of the disease and its treatment. Semin Oncol Nursing, 2: 19-29.

Lindner MA (1991) A fish oil diet inhibits colon cancer in mice. Nutr Cancer, 15: 1-11.

Liu B, Marnett LJ, Chaudhary A, Ji C, Blair IA, Johnson CR, Diglio CA and Honn KV (1994) Biosynthesis of 12(S)-hydroxyeicosatetraenoic acid by B16 amelanoic melanoma cells is a determinant of their metastatic potential. Lab Invest, 70: 314-323.

Llovera M, Garcia-Martinez C, Agell N, Marzabal M, Lopez-Soriano FJ and Argiles JM (1994) Ubiquitin gene expression in increased in skeletal muscle of tumour-bearing rats. FEBS Lett, 338: 311-318.

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

Llovera M, Carbo N, Garcia-Martinez C, Costelli P, Tessitore L, Baccino FM, Agell N, Bugby GJ, Lopez-Soriano FJ and Argiles JM (1996) Anti-TNF treatment reverts increased muscle ubiquitin gene expression in tumour-bearing rats. Biochem Biophys Res Commun, 221: 653-655.

Llovera M, Garcia-Martinez C, Agell N and Lopez-Soriano FJ (1998) Ubiquitin and Proteasome gene expression is associated in skeletal muscle of slim AIDS patients. Int J Mol Med, 2: 69-73.

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

Lopes MN, Black P, Ashford AJ and Pain VM (1989) Protein metabolism in the tumour-bearing mouse. Biochem J, 364: 713-719.

Lorite MJ, Cariuk P and Tisdale MJ (1997) Induction of muscle protein degradation by a tumour factor. Br J Cancer, 76: 1035-1040.

Lorite MJ, Smith HJ, Arnold JA, Morris A, Thompson MG and Tisdale MJ (2001) Activation of ATP-ubiquitin-dependent proteolysis in skeletal musce in vivo and murine myoblasts in vitro by a proteolysis-inducing factor (PIF). Br J Cancer, 85: 297-302.

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

Lowell BB, Ruderman NB and Goodman MN (1986) Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. Biochem J, 234: 237-240.

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

Lundholm K, Bennegard K, Eden E, Svanrnger G, Emery PW and Rennie MJ (1982) Efflux of 3-methylhistidine from the leg in cancer patients who experience weight loss. Cancer Res, 42: 4807-4811.

Lysz TW, Arora JK, Lin C and Zelenka PS (1994) 12(S) Hydroxyeicosatetraenoic acid regulates DNA synthesis and protooncogene expression induced by epidermal growth factor and insulin in rat lens epithelium. Cell Growth Different, 5: 1069-1076.

Ma CP, Slaughter CA and DeMartino GN (1992) Identification, purification and characterization of a protein activator (PA28) of the 20S proteasome (macropain). J Biol Chem, 267: 10515-10523.

Maltoni M, Fabbri L, Nanni O, Scarpi E, Pezzi L and Flamini S (1997) Serum levels of tumour necrosis factor alpha and other cytokines do not correlate with weight loss and anorexia in cancer patients. Support Care Cancer, 5: 130-135.

Mansoor O, Beaufrere B, Boirie Y, Ralliere C, Taillandier D, Anrousseau E, Schoeffler P, Arnal M and Attaix D (1996) Increased mRNA levels for components of the lysosomal, Ca^{2+}-activated, and ATP-ubiquitin dependent proteolytic pathways in skeletal muscle from head trauma patients. Proc Natl Acad Sci USA, 93: 2714-2718.

Masferrer JL, Rimarachin JA, Gerritsen ME, Falck JR, Yadagiri P, Dunn MW and Laniado-Schwartzman M (1991) 12(R)-Hydroxyeicosatrienoic acid, a potent chemotactic and angiogenic factor produced by the cornea. Exp Eye Res, 52: 417-424.

Masuno H, Yashimura H, Ogawa N and Okuda H (1984) Isolation of a lipolytic factor (Toxohormone-L) from ascutes fluid of patients with hepatoma and its effect on feeding behaviour. Eur J Cancer Clin Oncol, 20: 1177-1185.

Matthys P and Billiau A (1997) Cytokines and cachexia. Nutrition, 13: 763-770.

McCappin SC, Vandongen R and Croft KD (1987) The effect of dietary eicosapentaenoic acid on arachidonic acid incorporation and metabolism in rat leukocytes. Prostaglandins, 34: 505-517.

McCulloch RK, Chong CS and Hurley DM (1995) An evaluation of competitor type and size for use in the determination of mRNA by competitive PCR. PCR Methods Appl 4: 219-226.

McDevitt T (1996) Purification and characterisation of two cancer cachexia factors. PhD thesis, Aston University.

McMillan DC, Preston T, Watson WS, Simpson JM, Fearon KC, Shenkin A, Burns HJG and Mcardle CS (1994) Relationship between weight loss, reduction of body cell mass and inflammatory response in patients with cancer. Br J Surg, 81: 1011-1014.

Medina R, Wing SS, Hass A and Goldberg AL (1991) Activation of the ATP-ubiquitin dependent proteolytic system in skeletal muscle during fasting and denervation atrophy. Biomed Biochem Acta, 50: 347-356.

Medina R, Wing SS and Goldberg AL (1995) Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy. Biochem J, 307: 631-637.

Merforth S, Osmers A and Dalhmann B (1999) Alterations of proteasome activities in skeletal muscle tissue of diabetic rats. Mol Biol Rep, 26: 83-87.

Ming S, Chen S, Chen WJ, Eng HL and Chou FF (1997) Serum concentration of tumor nectrosis factor in patients with breast cancer. Breast Cancer Res Treat, 43: 211-215.

Mitch WE and Goldberg AL (1996) Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. N Engl J Med, 335: 1897-1905.

Mitch WE, Medina R, Grieber S, May RC, England BK, Price SR, Bailey JL and Goldberg AL (1994) Metabolic acidosis stimulates muscle protein degradation by activating the adenosine triphosphate-dependent pathway involving ubiquitin and proteasomes. J Clin Invest, 93: 2127-2133.

Moldawer LL, Andersson C, Gelin J and Lundholm KG (1988) Regulation of food intake and hepatic protein synthesis by recombinant derived cytokines. Am J Physiol, 254: G450-G460.

Moley JF, Aamodt R, Rumble W, Kaye W and Norton JA (1987) Body cell mass in cancer bearing and anorexia patients. JPEN, 11, 219.

Moore NG, Wang-Johanning F, Chang PL and Johanning GK (2001) Omega-3 fatty acids decrease protein kinase expression in human breast cancer cells. Breast Cancer Res Teat, 67: 279-283.

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

Morrison C and Gannon F (1994) The impact of the PCR plateau phase on quantitative PCR. Biochemica and Biophysica Acta, 1219: 493-498.

Needleman P, Turk J, Kacschik BA, Morrison AR and Lefkowith JB (1986) Arachidonic acid metabolism. Ann Rev Biochem, 55: 69-102.

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

Newsholme EA and Parry Billings M (1992) Properties of glutamine release from muscle and its importance for the immune system. J Parenter Ent Nutr, 14: 63S-67S.

Nixon DW, Heymsfield SB, Cohen AE, Jutner MH, Ausley J, Lawson DH and Rudman D (1980) Protein-calorie under nutrition in hospitalised cancer patients. Am J Medicine, 68: 683-690.

Noguchi Y, Yoshikawa T, Marat D, Doi C, Makino T, Fukuzawa K, Tsuburaya A, Satoh S, Ito T and Mitsuse S (1998) Insulin resistance in cancer patients is associated with enhanced tumor necrosis factor expression in skeletal muscle. Biochem Biophys Res Commun, 253: 887-892.

O'Donnel TF, Clawes GHA and Blackburn GL (1976) Proteolysis associated with a deficit of peripheral energy fuel substrates in septic man. Ann Surg, 80: 192-200.

Ohkawa S, Terao S, Terashita Z, Shibouta Y and Nishihana K (1991) Dual inhibition of thromboxane A2 synthase and 5-lipoxygease with scavenging activity of active oxygen species. Synthesis of a novel series of (3-pyridylmethyl) benzoquinone derivatives. J Med Chem, 34; 267-276.

O'Keefe SJD, Ogden J, Ramjee G and Rund J (1990) Contribution of elevated protein turnover and anorexia to cachexia in patients with hepatocellular carcinoma. Cancer Res, 50: 1226-1230.

Oliff A, Defo-Jones D, Boyer M, Martinez D, Kiefer D, Vuocolo G, et al (1987) Tumors secreting human TNF/cachectin induce cachexia in mice. Cell, 50: 555-563.

Orengo IF, Black HS, Kettler AH and Wolf JE (1989) Influences of dietary menhaden oil upon carcinogenesis and various cutaneous responses to ultraviolet radiation. Photochem Photobiol, 49: 71-77.

Orlowski M and Wilk S (2000) Catalytic activities of the 20S proteasome, a multicatalytic Proteinase complex. Arch Biochem Biophys, 383: 1-16.

Oudart H, Malan A, Maho Y le and Geloen A (2000) Day-night pattern of energy expenditure and body temperature in cachectic tumour bearing rats. Br J Cancer, 83: 1055-1060.

Oveson L, Hannibal J and Motenson EL (1993) the interrelationship of weight loss, dietary intake, and quality of life in ambulatory patients with cancer of the lung, breast, and ovary. Nutrition and Cancer, 19: 159-167.

Palmer RM (1990) Prostaglandins and the control of muscle protein synthesis and degradation. Prostag Leukotr Essential Fatty acids, 39: 95-104.

Palmer RM and Wahle KWJ (1987) Protein synthesis and degradation in isolated muscle. Biochem J, 242: 615-618.

Porcher C, Malinge MC, Picat C and Grandechamp B (1992) A simplified method for determination of specific DNA or RNA copy number using quantitative PCR and an automatic DNA sequencer. Biotechniques, 13: 106-113.

Price SR, England BK, Bailey JL, Van Vreede K and Mitch WE (1994) Acidosis and glucocorticoids concomitantly increase ubiquitin and proteasome subunit mRNA levels in rat muscles. 1m J Physiol, 267: C955-C960.

Price SA and Tisdale MJ (1998) Mechanism of inhibition of a tumour lipid-mobilizing factor by eicosapaenoic acid. Cancer Res, 58: 4827-4831.

Puccio M and Nathanson L (1997) The cancer cachexia syndrome. Semin Oncol, 24: 277-287.

Ralliére C, Tauveron I, Taillandier D, Guy L, Boiteux JP, Giraud B, Attaix D and Thieblot P (1997) Glucocorticoids do not regulate the expression of genes in skeletal muscle from Cushing syndrome patients. J Clin Endocrinol Metab, 82: 3161-3164.

Rechsteiner M (1991) Natural substrates of the ubiquitin proteolytic pathway. Cell, 66: 615-618.

Rechsteiner M, Hoffman L and Dubiel W (1993) The multicatalytic and 26S proteases. J Biol Chem, 268: 6065-6068.

Reddy BS, Burrill C and Rigotty J (1991) Effect of a diet high in omega-3 and omega-6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. Cancer Res. 51: 487-491.

Reddy BS, Cohen LA, McCoy GD, Hill P, Weisburger JH and Wynder ET (1980) Nutrition and its relationship to cancer. Adv Cancer Res, 32: 237-245.

Reeds P, Hay S, Glennie R, Mackie W and Garlick P (1985) The effect of indomethacin on the stimulation of protein synthesis by insulin in young postabsorptive rats. Biochem J, 227: 255-261.

Reid WD and MacGowan NA (1998) Respiratory muscle injury in animal models and humans. Molecular Cell Biochem, 179: 63-80.

Rennie MJ, Edwards RHT, Emergy PW, Haidey D, Lundholm K and Millward DJ (1983) Depressed protein synthesis is the dominant characteristic of muscle wasting and cachexia. Clin Physiol, 3: 387-398.

Rich AJ (1987) Nutritional status in cancer. Anticancer Res, 7: 271-280.

Ricquier D and Bouillaud F (2000) The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. Biochem J, 345: 161-179.

Riedy MC, Timm EA and Stewart CC (1995) Quantitative RT-PCR for measuring gene expression. Biotechniques , 18: 70-76.

Rodemann HP and Goldberg AL (1982) Arachidonic acid, prostaglandin E_2 and $F_{2\alpha}$, influence rates of protein turnover in skeletal and cardiac muscle. J Biol Chem, 257: 1632-1638.

Rofe AM, Bourgeosiscs CS, Coyle P, Taylor A and Abdi EA (1994) Altered insulin response to glucose in weight-loosing cancer patients. Anticancer Res, 14: 647-650.

Rose DP and Connolly JM (1990) Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. Cancer Res, 50: 7139-7144.

Rose DP, Connolly JM, Rayburn J and Coleman M (1995) Influence of diets containing eicosapentaenoic or decosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. J Natl Cancer Inst, 87: 587-592.

Rotman E, Brostrom MA and Brostrom CO (1992) Inhibition of protein synthesis in intact mammalian cells by arachidonic acid. Biochem J, 282: 487-494.

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

Samec S, Seydoux J and Dulloo AG (1998) Role of UCP homologs in skeletal muscle and brown adipose tissue – mediators of thermogenesis or regulators of lipids as fuel substrate. FASEB J, 12: 715-724.

Samules SE, Taillandier D, Aurousseau E, Cherel Y, Le Maho Y, Arnal M and Ayyaix D (1996) Gastrointestinal tract protein synthesis and mRNA levels for proteolytic systems in adult fasted rats. Am J Physiol, 271: E232-E238.

Sarkar G and Bolander ME (1994) The "looped oligo" method for generating reference molecules for quantitative PCR. Biotechniques, 17: 864-866.

Sax HC, Talamini MA, Hasselgren PO, Rosenblum L, Ogle CK and Fischer JE (1988) Increased synthesis of secreted hepatic proteins during abdominal sepsis. J Surg Res, 44: 109-106.

Schmidt M and Kloetzel PM (1997) FASEB J, 11: 1235-1243.

Setty BNY, Graeber JE and Stuart MJ (1987) The mitogenic effect of 15- and 12-hydroxyeicosatetraenoic acids on endothelial cells maybe mediated via diacylglycerol kinase inhibition. J Biol Chem, 262: 17613-17622.

Shamberger RJ (1984) Cancer cachexia. In Nutrition and Cancer, p. 353. plenum Press: London.

Shaw JH and Wolfe RR (1987) Fatty acid and glycerol kinetics in septic patients and in patients with gastrointestinal cancer. Ann Surg, 205: 368-376.

Shibata S and Miura K (1982) Nephritogenic glucoprotein IX. Plasma Znα2 glycoprotein as a second source of nephritogenic glycoprotein in urine. Nephron, 31(2): 170-176.

Shimbara N, Orino E, Sone S, Ogura T, Takashina M, Shono M, Tanura T and Ichihara A (1992) Regulation of gene expression of proteasomes (multi-protease complexes) during growth and differentiation of human hematopoitic cells. J Biol Chem, 267: 18100-18109.

Simon A, Najid A, Chulia AJ, Delage C and Riagaud M (1992) Inhibition of lipoxygenase activity and HL60 leukemic cell proliferation by ursolic acid isolated from heather flowers (*Calluna vulgaris*). Biochem Biophys Acta, 1125: 68-72.

Smith HJ, Lorite MJ and Tisdale MJ (1999) Effect of a cancer cachectic factor on protein synthesis/degradation in murine C_2C_{12} myoblasts – modulation by eicosapentoic acid. Cancer Res, 59: 5507-5513.

Smith KL and Tisdale MJ (1993a) Increased protein degradation and decreased protein synthesis in skeletal muscle during cancer cachexia. Br J Cancer, 67: 680-685.

Smith KL and Tisdale MJ (1993b) Mechanism of muscle protein degradation in cancer cachexia. Br J Cancer, 68: 314-318.

Snider JV, Wechser MA and Lossos IS (2001) Human disease characterization: real-time quantitative PCR analysis of gene expression. Drug Discovery Today, 6: 1062-1067.

Soda K, Kawakami M, Kashii K and Miyata M (1995) Manifestations of cancer cachexia induced by colon 26 adenocarcinoma are not fully ascribable to interleukin-6. Int J Cancer, 62: 332-36.

Solomon V and Goldberg AL (1996) Importance of the ATP-ubiquitin-Proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. J Biol Chem, 271: 26690-26697.

Solomon V, Baraccos V, Sarraf P and Goldberg AL (1998a) Rates of ubiquitin conjugation increase when muscle atrophy, largely through activation of the N-rule pathway. Proc Natl Acad Sci USA, 95: 12602-12607.

Solomon V, Lecker SH and Goldberg AL (1998b) The N-rule pathway catalyses a major fraction of the protein degradation in skeletal muscle. J Biol Chem, 273: 25216-25222.

Sorimachi H, Imajoh-Ohmi S, Emori Y, Kawasaki H, Ohno S, Minami Y and Suzuki K (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and mu type: specific expression of the mRNA in skeletal muscle. J Biol Chem, 264: 20106-20111.

Spector AA, Gordon JA and Moore SA (1988) Hydroxyeicosatetraenoic acids. Prog Lipid Res, 27: 271-323.

Staal-van den Brekel AJ, Dentener MA, Schols AMWJ, Buurman WA and Wouters EFM (1995) Increased resting energy expenditure and weight loss are related to a systemic inflammatory response in lung cancer patients. J Clin Oncol, 13: 2600-2605.

Starnes HFJ, Warren RS, Jeevanandum M, Gabrilove JL, Larchian W, Oettgen HF, and Brennan MF (1988) Tumour necrosis factor and the acute metabolic response to tissue injury in man. J Clin Invest, 82: 1321-1325.

Strassmann G, Fong M, Kenny Js and Jacob CO (1992) Evidence for the involvement of interleukin 6 in experimental cancer ataxia. J Clin Invest, 89: 1681-1684.

Strelkov AB, Fields ALA and Baracos VE (1989) Effects of systemic inhibition of prostaglandin production on protein metabolism in tumour-bearing rats. Am J Physiol, 257: C261-C269.

Streat SJ, Beddol AH and Hill GL (1987) Aggressive nutritional support does not prevent protein loss despite fat gain in septic intensive care patients. J Trauma, 27: 262.

Sugden PH and Fuller SJ (1991) Regulation of protein turnover in skeletal and cardiac muscle. Biochem J, 273: 21-37.

Taillandier D, Aurousseau E, Meynial-Denis D, Bechet D, Ferrara M, Cottin P, Ducastaing A, Bigard X, Guezennec CY and Schmid HP (1996) Coordinate activation of lysosomal, Ca^{2+}-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. Biochem J, 316: 65-72.

Tamura S, Fajimoto-Ouchi K, Mori K, Endo M, Matsumoto T, Eda H, Tanaka Y, Ishitsuka H, Tokita H and Yamaguchi K (1995) Invelvement of human interleukin 6 in experimental cachexia induced by a human uterine carcinoma xenograft. Clin Cancer Res, 1: 1353-1358.

Tanaka K (1998a) Molecular biology of the proteasome. Biochem Biophys Res Commun, 247: 537-541.

Tanaka K (1998b) Proteasomes: structure and biology. J Biochem, 123: 195-204.

Tanaka Y, Eda H, Fujimoto K, Tanaka T, Ishikawa T and Ishitsuka H (1990) Anticachectic activity of 5'-deoxy-5-fluotouridine in a murine tumour cachexia model, colon 26 adenocarcinoma. Cancer Res, 50: 4528-4532.

Tanaka K and Kasahara M (1998) Immunol Rev, 163:

Tawa NE, Odessey R and Goldberg AL (1995) Peptide aldehyde inhibitors of the proteasome reduce the accelerated proteolysis in atrophying skeletal muscles. Surg Forum, 46: 12-15.

Tawa NE, Odessey R and Goldberg AL (1997) Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. J Clin Invest 100: 197-203.

Tayek JA (1992) A review of cancer cachexia and abnormal glucose metabolism in humans with cancer. J Am Coll Nutr, 11: 445-456.

Taylor DD, Gercel-Taylor C, Jenis LJ and Devereux DF (1992) Identification of a human tumour derived lipolysis-promoting factor. Cancer Res, 52: 829-834.

Taylor SL, Plat-Higgins A, Rudland PS, Winstanley JHR and Barraclough R (1998) Cytoplasm C staining of e-erbB-2 is not associated with the presence of detectable c-erbB-2 mRNA in breast cancer specimen. Int J Cancer, 76: 459-463.

Temparis S, Asensi M, Taillandier D, Aurousseua E, Karbaud D, Obled A, Béchet D, Ferrara M, Estrela JM and Attaix D (1994) Increased ATP-ubiquitin-dependent proteolysis in skeletal muscles of tumour bearing rats. Cancer Res, 54: 5568-5573.

Tessitore L, Bonnelli G and Baccino FM (1987) Early development of protein metabolic perturbations in the liver and skeletal muscle of tumour-bearing rats. Biochem J, 241: 153-158.

Tessitore L, Costelli P and Baccino FM (1994) Pharmacological interference with tissue hypercatabolism in tumour-bearing rats. Biochem J, 299: 71-78.

Tiao G, Fagan JM, Roegner V, Leiberman M, Wing JJ, Fischer JE and Hasselgren PO (1996) Energy-ubiquitin-dependent muscle proteolysis during sepsis is regulated by glucocorticoids. J Clin Invest, 97: 339-348.

Tiao G, Fagan JM, Samuels N, James JH, Hudson K, Lieberman M, Fischer JE and Hasselgren PO (1994) Sepsis stimulates nonlysosomal, energy dependent proteolysis and increases ubiquitin mRNA levels in rat skeletal muscle. J Clin Invest, 94: 2255-2264.

Tiao G, Hobler S, Meyer TA, Luchette FA, Fischer JE and Hasselgren PO (1997a) Sepsis is associated with increased mRNAs of the ubiquitin proteasome proteolytic pathway in human skeletal muscle. J Clin Invest, 99: 163-168.

Tiao G, Lieberman M Fischer JE and Hasselgren PO (1997b) Intracellular regulation of protein degradation during sepsis is different in fast- and slow-twitch muscle. Am J Physiol, 272: R849-R856.

Tisdale MJ (1991) Cancer cachexia. Br J Cancer, 63: 337-342.

Tisdale MJ (1993a) Cancer cachexia. Anti-Cancer Drugs, 4: 115-125.

Tisdale MJ (1993b) Mechanism of lipid mobilization associated with cancer cachexia: interaction between the polyunsaturated fatty acid, eicosapentaenoic acid, and inhibitory guanine nucleotide-regulatory protein. Prostaglandins Leukotrienes and Fatty Acids, 48: 105-109.

Tisdale MJ (1996) Inhibition of lipolysis and muscle protein degradation by EPA in cancer cachexia. Suppl to Nutr, 12: S31-S33.

Tisdale MJ (1997a) Cancer cachexia: Metabolic Alterations and Clinical Manifestations. Nutrition, 13: 1-7.

Tisdale MJ (1997b) Biology of cachexia. J Nat Cancer Instit, 89: 1763-1773.

Tisdale MJ (2000) Metabolic abnormalities in cachexia and anorexia. Nutrition, 16: 1013-1014.

Tisdale MJ (2001) Cancer anorexia and cachexia. Nutrition, 17: 438-442.

Tisdale MJ and Beck SA (1990) Cancer Cachexia. International J Pancreatology, 7: 141-150.

Tisdale MJ and Beck SA (1991) Inhibition of tumour-induced lipolysis in vitro and cachexia and tumour growth in vivo by eicosapentaenoic acid. Biochem Pharmacol, 41: 103-107.

Tisdale MJ and Dhesi JK (1990) Inhibition of weight loss by ω-3 fatty acids in an experimental ataxia model. Cancer Res, 50: 5022-5026.

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

Todorov PT, McDevitt TM, Cariuk P, Coles B, Deacon M and Tisdale MJ (1996b) Induction of muscle protein degradation and weight loss by a tumour product. Cancer Res, 56: 1256-1261.

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

Todorov PT, Field WN and Tisdale MJ (1999) Role of proteolysis-inducing factor (PIF) in ataxia induced by a human melanoma (G361). Br J Cancer, 80: 1734-1737.

Todorov PT, McDevitt TM, Meyer DJ, Ueyama I, Ohkubo I and Tisdale MJ (1998) Purification and characterization of a tumour lipid mobilizing factor. Cancer Res, 58: 2353-2358.

Toit PJ, Aswegen CH and Plessis DJ (1994) The effect of gamma-linolenic acid and eicosapentaenoic acid on urokinase activity. Prostaglandins Leukotrienes Essent Fatty Acids, 51: 121-124.

Turner PR, Westwood T, Regen CM and Steinhardt RA (1988) Increased protein degradation results from elevated free calcium levels found from mdx mice. Nature, 335: 735-738.

van Eys J (1985) Nutrition and cancer: physiological interrelationships. Annu Rev Nutr, 5: 435-61.

van Nocker S, Deveraux Q, Rechsteiner M and Vierstra R (1996) *Arabidopsis* MBP1 gene encodes a conserved ubiquitin recognition component of the 26S proteasome. Proc Natl Acad Sci USA, 93: 856-860.

Varshavsky A (1997) The ubiquitin system. TIBS, 22: 338-387.

Voisin L, Breuille D, Combaret L, Pouyet C, Taillandier D, Aurousseau E, Obled C and Attaix D (1996) Muscle wasting in a rat model of long lasting sepsis results from the activation of lysosomal, calcium activated and ubiquitin proteasome proteolytic pathways. J Clin Invest, 97: 1610-1617.

Warren S (1932) The immediate causes of death in cancer. Am J Med Sci, 184: 610-615.

Waterhouse C and Kemperman JH (1971) Carbohydrate metabolism in subjects with cancer. Cancer Res, 31: 1273-1278.

Whitehouse AS (2001) Muscle catabolism in cancer and its attenuation by eicosapentaenoic acid. PhD thesis, Aston University.

Whitehouse AS, Smith HJ, Drake JL and Tisdale MJ (2001) Mechanism of attenuation of skeletal muscle protein catabolism in cancer cachexia by eicosapentaenoic acid. Cancer Res, 61; 3604-3609.

Whitehouse AS and Tisdale MJ (2001) Downregulation of ubiquitin-proteasome proteolysis by eicosapentaenoic acid in acute starvation. Biochem Biophys Res Commun, 285: 598-602.

Wigmore SJ, Barber MD, Ross JA, Tisdale MJ and Fearon KCH (2000a) Effect of oral eicosapentaenoic acid on weight loss in patients with pancreatic cancer. Nutr Cancer, 36: 177.

Wigmore SJ, Fearon KCH, Maingay JP and Ross JA (1997a) Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci, 92: 215-221.

Wigmore SJ, Plester CE, Richardson RA and Fearon KCH (1997b) Changes in nutritional status associated with unrespectable pancreatic cancer. Br J Cancer, 75: 106-109.

Wigmore SJ, Ross JA, Falconer JS, et al (1996) The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer. Nutrition, 12 (suppl), S27-S30.

Wigmore SJ, Todorov PT, Barber MD, Ross JA, Tisdale MJ and Fearon CH (2000b) Characteristics of patients with pancreatic cancer expressing a novel cancer cachectic factor. Br J Surg, 87: 53-58.

Williams B, de Courten-Myers GM, Fischer JE, Luo G, Sun X and Hasselgren PO (1999a) Sepsis stimulates release of myofilaments in skeletal muscle by a calcium-dependent mechanism. FASEB J, 13: 1435-1443.

Williams A, Sun X, Fischer JF and Hasselgren PO (1999b) The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. Surgery, 126: 744-750.

Windmueller HG and Spaeth AE (1980) Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. J Biol Chem, 255: 107-112.

Wing SS and Banville D (1994) 14-kDa ubiquitin-conjugating enzyme: structure of the rat gene and regulation upon fasting and by insulin. Am J Physiol, 267: E39-E48.

Wing SS and Bederd N (1996) Insulin-like growth factor I stimulates degradation od a mRNA transcript encoding the 14 kDa ubiquitin-conjugating enzyme. Biochem J, 319: 455-461.

Wing SS and Goldberg AL (1993) Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. Am J Physiol, 264: E668-E676.

Wray CJ, Sun X, Gang GI and Hasselgren PO (2002) Dantrolene downregulates the gene expression and activity of the ubiquitin-proteasome proteolytic pathway in septic skeletal muscle. J Surg Res, 104: 82-87.

Yanagawa H, Sone S, Talcahishi Y, et al. (1995) serum levels of interleukin 6 in patients with lung cancer. Br J Cancer, 71: 1095-1098.

APPENDIX I

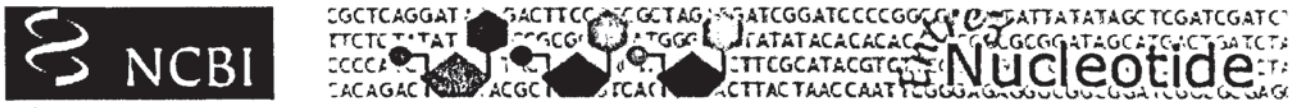
- Rattus norvegicus ubiquitin conjugating enzyme, RNA sequence.
- Mus musculus proteasome subunit C2 mRNA sequence.
- Mus musculus proteasome subunit C5 mRNA sequence.
- Human E2 ubiquitin conjugating enzyme mRNA sequence.

1: M62388. Rattus norvegicus...[gi:207554] Links

```
LOCUS       RATUCE                   923 bp    mRNA    linear   ROD 26-AUG-1994
DEFINITION  Rattus norvegicus ubiquitin conjugating enzyme mRNA, complete cds.
ACCESSION   M62388
VERSION     M62388.1  GI:207554
KEYWORDS    ubiquitin carrier protein; ubiquitin conjugating enzyme.
SOURCE      Rattus norvegicus (Norway rat)
  ORGANISM  Rattus norvegicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
            Rattus.
REFERENCE   1  (bases 1 to 923)
  AUTHORS   Wing,S.S. and Banville,D.
  TITLE     14-kDa ubiquitin-conjugating enzyme: structure of the rat gene and
            regulation upon fasting and by insulin
  JOURNAL   Am. J. Physiol. 267 (1 Pt 1), E39-E48 (1994)
  MEDLINE   94324482
   PUBMED   8048511
REFERENCE   2  (bases 1 to 923)
  AUTHORS   Wing,S.S., Dumas,F. and Banville,D.
  TITLE     A rabbit reticulocyte ubiquitin carrier protein that supports
            ubiquitin-dependent proteolysis (E214k) is homologous to the yeast
            DNA repair gene RAD6
  JOURNAL   J. Biol. Chem. 267 (10), 6495-6501 (1992)
  MEDLINE   92202189
   PUBMED   1313008
REFERENCE   3  (bases 1 to 923)
  AUTHORS   Wing,S.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-FEB-1992) Simon S. Wing, Molecular Biology Sector,
            Biotechnology Research Institute, National Research Council of
            Canada, Montreal, Quebec, Canada
COMMENT     Original source text: Rattus norvegicus cDNA to mRNA.
FEATURES             Location/Qualifiers
     source          1..923
                     /organism="Rattus norvegicus"
                     /db_xref="taxon:10116"
                     /tissue_type="liver"
     gene            1..923
                     /gene="ubiquitin conjugating-protein"
     5'UTR           1..43
                     /gene="ubiquitin conjugating-protein"
     CDS             44..502
                     /gene="ubiquitin conjugating-protein"
                     /codon_start=1
                     /product="ubiquitin conjugating-protein"
                     /protein_id="AAA21087.1"
                     /db_xref="GI:207555"
                     /translation="MSTPARRRLMRDFKRLQEDPPVGVSGAPSENNIMQWNAVIFGPE
                     GTPFEDGTFKLVIEFSEEYPNKPPTVRFLSKMFHPNVYADGSICLDILQNRWSPTYDV
                     SSILTSIQSLLDEPNPNSPANSQAAQLYQENKREYEKRVSAIVEQSWNDS"
     3'UTR           503..923
```

```
                     /gene="ubiquitin conjugating-protein"
BASE COUNT      270 a    176 c    196 g    281 t
ORIGIN
        1 gggactcttt tctttcagac tgaccgcggg gcagctgcgg agcatgtcga ccccggcccg
       61 taggaggctc atgcgggatt tcaagcgatt gcaagaggac ccacctgtgg gggtcagtgg
      121 tgcaccatct gaaaacaaca tcatgcagtg gaacgcagtt atatttggac cagaagggac
      181 acccttgaa gatggtactt ttaaactagt aatagaattt tctgaagaat atccaaataa
      241 accaccaacc gttaggtttt tatccaaaat gtttcatcca aatgtgtatg ctgacggcag
      301 catatgctta gacatcctgc agaaccgatg gagccccacg tacgacgtct cctccatctt
      361 aacttcaatt cagtctctgt tggatgagcc gaatccaaac agtccggcca atagccaagc
      421 agcacagctt tatcaggaaa acaaacggga gtatgagaag agggtttcgg ccattgttga
      481 gcagagctgg aatgactcat aatagacacc tggtctgtcc acctttccat cgtcgttgtg
      541 tataatttac ctcccagtag gaaggctagc aagttttaag tgccacgggt tttaaggatt
      601 ctgcagagaa aaaagaaaaa gtccttcagt ttagaaccta caaaagcttg tgtatcttga
      661 ttaatgtact ttttattgca tggtgtgaac taagttattg ctgcataaat ttgtaatata
      721 tcctgtttgt atttttttcc aagtgtataa tgttggtgtg gagttttcat gacagaatat
      781 acacattttg taaatctgta ctttttttcaa atattgaatg ccttattttt gaattcttta
      841 gatttttaaa ttggagaaaa gcacttaaag ttttttatat atgaatatta catgtaaagc
      901 tgttaaaata cataacttca gtg
```

Revised: July 5, 2002.



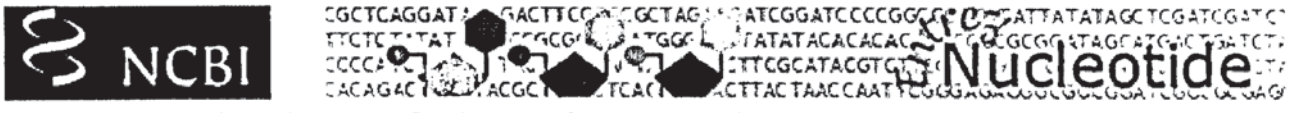
1: AF060088. Mus musculus prot...[gi:5757652] Links

```
LOCUS       AF060088                1121 bp    mRNA    linear   ROD 23-AUG-1999
DEFINITION  Mus musculus proteasome subunit C2 (Psma1) mRNA, complete cds.
ACCESSION   AF060088
VERSION     AF060088.1  GI:5757652
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1121)
  AUTHORS   Elenich,L.A., Nandi,D., Kent,A.E., McCluskey,T.S., Cruz,M.,
            Iyer,M.N., Woodward,E.C., Conn,C.W., Ochoa,A.L., Ginsburg,D.B. and
            Monaco,J.J.
  TITLE     The complete primary structure of mouse 20S proteasomes
  JOURNAL   Immunogenetics 49 (10), 835-842 (1999)
  MEDLINE   99367391
   PUBMED   10436176
REFERENCE   2  (bases 1 to 1121)
  AUTHORS   Elenich,L.A. and Monaco,J.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-APR-1998) Molecular Genetics ML0524, University of
            Cincinnati, 231 Bethesda Ave., Cincinnati, OH 45267-0524, USA
FEATURES             Location/Qualifiers
     source          1..1121
                     /organism="Mus musculus"
                     /strain="B10.A"
                     /db_xref="taxon:10090"
                     /cell_type="macrophage"
     gene            1..1121
                     /gene="Psma1"
     CDS             38..829
                     /gene="Psma1"
                     /codon_start=1
                     /product="proteasome subunit C2"
                     /protein_id="AAD50533.1"
                     /db_xref="GI:5757653"
                     /translation="MFRNQYDNDVTVWSPQGRIHQIEYAMEAVKQGSATVGLKSKTHA
                     VLVALKRAQSELAAHQKKILHVDNHIGISIAGLTADARLLCNFMRQECLDSRFVFDRP
                     LPVSRLVSLIGSKTQIPTQRYGRRPYGVGLLIAGYDDMGPHIFQTCPSANYFDCRAMS
                     IGARSQSARTYLERHMSEFMECNLDELVKHGLRALRETLPAEQDLTTKNVSIGIVGKD
                     LEFTIYDDDDVSPFLDGLEERPQRKAQPSQAAEEPAEKADEPMEH"
BASE COUNT      327 a    223 c    258 g    313 t
ORIGIN
        1 gtccgctgct gggtcaggtt gtcgctgcgc cggagctatg tttcgaaacc agtatgacaa
       61 tgatgtcact gtttggagcc ctcagggcag gattcatcaa attgaatatg caatggaagc
      121 tgttaagcaa ggttcagcaa cagttggtct aaaatcaaaa acgcacgcag tgctggttgc
      181 actgaagaga gcacagtcag agcttgccgc tcaccagaag aaaattctcc atgttgacaa
      241 ccatattggt atctcaattg cgggtctaac tgctgatgcc agactgttat gcaactttat
      301 gcgccaggag tgtttggatt ccagatttgt gtttgacaga ccacttcctg tgtctcgtct
      361 tgtgtctcta attggaagca aaacccagat cccaacacag cgatatggcc ggaggccgta
      421 tggtgttggg ctgctcattg ctggttatga tgatatgggc cctcatattt tccaaacctg
```

```
 481 tccatctgct aactattttg actgcagagc tatgtctatt ggagcccgtt ctcaatcagc
 541 tcgtacttac ctggagagac atatgtctga atttatggag tgcaatttgg atgaactggt
 601 taaacatggt ctgcgtgcct taagagaaac actccctgca gagcaggacc tgaccacaaa
 661 gaatgtttcc attggaatcg ttggtaaaga cttggaattt acaatctacg atgatgatga
 721 tgtatctcca ttcctggatg gtcttgaaga aagaccacag agaaaagcac agccttcaca
 781 ggctgctgag gaacctgcag aaaaagccga tgaaccaatg gaacattaag tgataaaggt
 841 tacaaggaca taagaatgca gggacataca ctggcgacaa taatctgtat tttaaaccaa
 901 cagctgcagt gtattgggtg gtatgtttta gaaatcagtc cagatgtaag ttttctctaa
 961 gcaacttcac agaaaccata taatggggtg cattttcttt gaaagggtat acataatcat
1021 tttctaggaa gtataggtat ctataccaat gtttttatat gaagaaaata agtgtctttg
1081 cagttttaaa gacaactgtg aaataaaatt gtttcaccac c
```

Revised: July 5, 2002.

Dec 13 2002 14:41:17



1: U60824. Mus musculus prot...[gi:1698583] Links

```
LOCUS       MMU60824                  812 bp    mRNA    linear   ROD 03-DEC-1996
DEFINITION  Mus musculus proteasome beta-subunit C5 (Psmb1) mRNA, allele B,
            complete cds.
ACCESSION   U60824
VERSION     U60824.1  GI:1698583
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (sites)
  AUTHORS   Savioz,A., Houghton,I. and Davies,R.W.
  TITLE     Cloning and sequencing of a murine cDNA encoding the proteasome
            component C5
  JOURNAL   DNA Seq. 5 (5), 307-309 (1995)
  MEDLINE   96063022
   PUBMED   7579585
REFERENCE   2  (bases 1 to 812)
  AUTHORS   Trachtulec,Z., Forejt,J., Vincek,V. and Klein,J.
  TITLE     Tight linkage of Tbp and Psmb1 genes in mouse and human
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 812)
  AUTHORS   Trachtulec,Z., Forejt,J., Vincek,V. and Klein,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JUN-1996) Microbiology, University of Miami, 1600 NW
            10 Ave, Miami, FL 33136, USA
FEATURES             Location/Qualifiers
     source          1..812
                     /organism="Mus musculus"
                     /strain="C57BL/10J"
                     /sub_species="domesticus"
                     /db_xref="taxon:10090"
                     /chromosome="17"
                     /tissue_type="testes"
                     /note="maps between D17Ph4e and Tbp"
     gene            1..812
                     /gene="Psmb1"
     CDS             30..752
                     /gene="Psmb1"
                     /function="involved in ATP-dependent protein degradation"
                     /note="allele B"
                     /codon_start=1
                     /product="proteasome beta-subunit C5"
                     /protein_id="AAB37251.1"
                     /db_xref="GI:1698584"
                     /translation="MLSTAAYRDVERELGMGPHGSAGPVQLRFSPYAFNGGTVLAIAG
                     EDFSIVASDTRLSEGFSIHTRDSPKCYKLTDKTVIGCSGFHGDCLTLTKIIEARLKMY
                     KHSNNKAMTTGAIAAMLSTILYSRRFFPYYVYNIIGGLDEEGKGAVYSFDPVGSYQRD
                     SFKAGGSASAMLQPLLDNQVGFKNMQNVEHVPLTLDRAMRLVKDVFISAAERDVYTGD
                     ALRICIVTKEGIREETVPLRKD"
     variation       593
```

```
                     /gene="Psmb1"
                     /note="located in C3H/J strains"
                     /citation=[1]
                     /replace="g"
     variation       754
                     /gene="Psmb1"
                     /note="located in C3H/J strains"
                     /citation=[1]
                     /replace="g"
     variation       783
                     /gene="Psmb1"
                     /note="absent in C3H/J strains"
                     /citation=[1]
                     /replace=""
     polyA_site      812
                     /gene="Psmb1"
                     /note="7 A nucleotides"
BASE COUNT      212 a    182 c    219 g    199 t
ORIGIN
        1 ttgccgtgag acagcaggag ttaggcgcga tgctttccac tgctgcttac cgagacgtgg
       61 aacgagagct gggaatgggg cctcacggct ccgccggccc tgtgcagctg cggttttcgc
      121 cttatgcctt caacggaggt actgtattgg caattgctgg agaagatttt tccatcgtcg
      181 cttcagacac tcgattgagt gaaggatttt caattcatac ccgagatagc cccaaatgct
      241 acaaactgac agacaagaca gtaattggct gcagtggttt ccatggagat tgtctcactt
      301 tgacaaagat tattgaagca agattaaaga tgtacaagca ttccaataac aaggccatga
      361 caacgggggc gattgctgca atgctgtcta ccatcctgta ctcacggcgc ttcttccctt
      421 actatgttta caacatcatt ggaggacttg atgaagaagg aaagggagct gtgtacagct
      481 ttgacccagt gggctcttac cagagagact ctttcaaggc gggaggctca gcaagtgcca
      541 tgctgcagcc tctgctcgac aaccaggttg gcttcaaaaa tatgcagaat gtagagcacg
      601 tccccctgac gctggacaga gccatgaggc tggtgaaaga tgtcttcatt tctgcagccg
      661 agagggatgt gtatactgga gatgctctca ggatctgcat cgtgaccaaa gagggcatca
      721 gggaggaaac tgttcccctg cggaaagact gacatgcggg tcatatattg gagctgtggc
      781 acaagtttgt tttattaaaa agaatgtaaa gt
//
```

Revised: July 5, 2002.

☐ 1: U39318. Human E2 ubiquiti...[gi:1145690] Links

```
LOCUS       HSU39318                  724 bp    mRNA    linear   PRI 07-MAR-1996
DEFINITION  Human E2 ubiquitin conjugating enzyme UbcH5C (UBCH5C) mRNA,
            complete cds.
ACCESSION   U39318
VERSION     U39318.1  GI:1145690
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 724)
  AUTHORS   Jensen,J.P., Bates,P.W., Yang,M., Vierstra,R.D. and Weissman,A.M.
  TITLE     Identification of a family of closely related human ubiquitin
            conjugating enzymes
  JOURNAL   J. Biol. Chem. 270 (51), 30408-30414 (1995)
  MEDLINE   96107191
   PUBMED   8530467
REFERENCE   2  (bases 1 to 724)
  AUTHORS   Jensen,J.P., Bates,P.W., Yang,M., Vierstra,R.D. and Weissman,A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-OCT-1995) Allan M. Weissman, Laboratory of Immune
            Cell Biology, NCI/NIH, Bldg. 10 Rm. 1B34, 9000 Rockville Pike,
            Bethesda, MD 20892-1152, USA
FEATURES             Location/Qualifiers
     source          1..724
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /cell_type="peripheral blood lymphocyte"
     gene            1..724
                     /gene="UBCH5C"
     CDS             46..489
                     /gene="UBCH5C"
                     /function="E2 ubiquitin conjugating enzyme; functions with
                     E6-AP in ubiquitin conjugation"
                     /note="Transcript is widely expressed. Related to S.
                     Cerevisiae UBC4 and UBC5. Closely related to human
                     UbcH5(A) and to UbcH5B"
                     /codon_start=1
                     /product="UbcH5C"
                     /protein_id="AAA91461.1"
                     /db_xref="GI:1145691"
                     /translation="MALKRINKELSDLARDPPAQCSAGPVGDDMFHWQATIMGPNDSP
                     YQGGVFFLTIHFPTDYPFKPPKVAFTTRIYHPNINSNGSICLDILRSQWSPALTISKV
                     LLSICSLLCDPNPDDPLVPEIARIYKTDRDKYNRISREWTQKYAM"
BASE COUNT      214 a    160 c    131 g    219 t
ORIGIN
        1 gagcatacac cgagagagtg aggagccaga cgacaagcac acactatggc gctgaaacgg
       61 attaataagg aacttagtga tttggcccgt gaccctccag cacaatgttc tgcaggtcca
      121 gttggggatg atatgtttca ttggcaagcc acaattatgg gacctaatga cagcccatat
      181 caaggcggtg tattcttttt gacaattcat tttcctacag actacccctt caaaccacct
      241 aaggttgcat ttacaacaag aatttatcat ccaaatatta acagtaatgg cagcatttgt
```

```
     301 ctcgatattc taagatcaca gtggtcgcct gctttaacaa tttctaaagt tcttttatcc
     361 atttgttcac tgctatgtga tccaaaccca gatgaccccc tagtgccaga gattgcacgg
     421 atctataaaa cagacagaga taagtacaac agaatatctc gggaatggac tcagaagtat
     481 gccatgtgat gctaccttaa agtcagaata acctgcatta tagctggaat aaactttaaa
     541 ttactgttcc ttttttgatt ttcttatccg gctgctcccc tgatcagacc tcatcttttt
     601 taattttatt ttttgtttac ctccctccat tcattcacat gctcatctga gaagacttaa
     661 gttcttccag ctttgacaat aactgctttt agaaactgta aagtagttac aagagaacag
     721 ttgg
//
```

Revised: July 5, 2002.

APPENDIX II

Publications

Expression of the ubiquitin-proteasome pathway in skeletal muscle and weight loss in a murine cachexia model. J. Khal, A.V. Hine and M.J. Tisdale. British Journal of Cancer. *Submitted.*

Increased gene and protein expression of proteasome subunits in skeletal muscle of cancer patients with weight losses. Jwan Khal, Anna V. Hine, Kenneth C.H. Fearon and Michael J. Tisdale. *In preparation.*

Abstracts

Quantitative RT-PCR to investigate the expression of the proteasome subunits in cancer cachectic patients. Khal J, Hine AV and Tisdale MJ. EACR 17th meeting. Granada, Spain, 2002.

Quantitative RT-PCR to investigate the expression of the proteasome subunits in cancer cachectic patients. **Khal J, Hine AV and Tisdale MJ. Pharmaceutical Sciences Research Institute, Aston University, Birmingham, B4 7ET, UK.**

Cancer-induced cachexia is a common manifestation observed in patients with malignancies. The ubiquitin-proteasome proteolytic pathway has been shown to be responsible for muscle wasting in a range of cachectic conditions including cancer cachexia in mice.

To establish the importance of this pathway in human cancer cachexia a quantitative competitive RT-PCR method was developed to measure the proteasome subunits, C2 and C5 mRNA from human gastrocnemius muscle biopsy samples from patients with and without cancer cachexia. The main advantage of the method relies on the use, for each target sequence, of an internal competitor construct similar to the relevant target, but 70-100bp shorter in size. Accuracy of quantitative competitive RT-PCR is potentiated by simultaneous determination of the target gene (C2 or C5) and the shorter competitor gene in one reaction tube. The expression of the titrated target gene is then calculated with reference to the titrated level of the competitor gene.

The weight loss of the cachectic patients ranged from 5-19%. Quantitating the C2 and C5 proteasome subunits in these samples showed increase expression in the cachectic patients compared to the non-cachectic patients. The C5 gene expression increased by about 2 fold in cancer cachectic patients (cachectic 13.21 $\pm$ 2.33 pg/ug total RNA, non-cachctic 6.63 $\pm$ 1.15 pg/ug total RNA; p= 0.02), whereas the C2 gene expression increased by 3 fold in the cancer cachectic patients. (cachectic 9.69 $\pm$ 0.67 ng/ug total RNA, non-cachectic 3.01 $\pm$ 0.12 ng/ug total RNA; $p<0.0001$). Furthermore, the level of increase gene expression was dependent on the amount of weight loss of the patients. A cachectic patient with 6% weight loss had a 1.1 fold increase in C5 gene expression and 1.5 fold increase in C2 gene expression, a patient with 11% weight loss had a 1.6 fold increase in C5 gene expression and 2.5 fold increase in C2 gene expression, whereas a patient with 19% weight loss had a 6.4 fold increase in C5 gene expression and 4 fold increase in C2 gene expression. These results suggest that accelerated muscle proteolysis and muscle wasting in cachectic cancer patients results primarily from activation of the ATP-dependent ubiquitin-proteasome pathway, and the higher the percentage of weight loss in the patients the higher the level of activation of the ubiquitin-proteasome pathway and so the higher the level of muscle proteolysis.