CELLULAR SIGNALLING PATHWAYS INVOLVED IN MUSCLE ATROPHY IN CANCER CACHEXIA

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CELLULAR SIGNALLING PATHWAYS INVOLVED IN MUSCLE ATROPHY IN CANCER CACHEXIA: SUMMARY

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Cancer cachexia encompases severe weight loss, characterised by the debilitating atrophy of adipose and skeletal muscle mass.

Skeletal muscle proteolysis in cancer cachexia is mediated by a sulphated glycoprotein with a relative molecular mass of 24kDa, termed Proteolysis-Inducing Factor (PIF). PIF induced a significant increase in protein degradation, peaking at 4.2nM PIF ($p<0.001$), 'chymotrypsin-like' activity of the proteasome ($p<0.001$) and increased expression of components of the ATP-ubiquitin dependent proteolytic pathway. This was attenuated *in vitro* by pre-incubation with the PKC inhibitor calphostin C (100μM) and NF-κB the inhibitors SN50 (18μM), curcumin (50μM) and resveratrol (30μM), 2 hours prior to the addition of PIF.

In vivo studies found the IKK inhibitor resveratrol (1mg/kg) to be successful in attenuating protein degradation ($p<0.001$) and upregulation of ubiquitin-dependent proteolysis in MAC16 tumour bearing mice. C_2C_{12} myoblasts transfected with mutant IκBα and PKCα inserts did not elicit a PIF-induced response, suggesting the importance of the transcription factor NF-κB and PKCα involvement in PIF signal transduction.

15(*S*)-HETE acts as an intracellular mediator of PIF and exerts an effect through the activation of PKC and subsequently IKK, which phosphorylates IκBα and allows NF-κB to migrate to the nucleus. This effect was negated with the PKC inhibitor calphostin C (300nM).

A commercially produced PIF receptor antibody was raised in rabbits immunised with a peptide containing the partial N-terminal sequence of the PIF receptor. This PIF receptor antibody was successful in attenuating the PIF-induced increase in skeletal muscle catabolism and protein degradation *in vitro* at 10μg/ml ($p<0.001$) and 3.47mg/kg *in vivo* ($p<0.001$). The data suggests great potential in the development of this antibody as a therapy against cancer cachexia.

Keywords : Cachexia, Proteolysis-Inducing Factor, ATP-ubiquitin dependent proteolysis

To My Wonderful Family
& Absent Friends

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Table of Contents

Table of Figures

Abbreviations

AA	Arachidonic Acid
ADP	Adenosin-5'-phosphate
AIDS	Acquired Immunodeficiency Syndrome
AIS	Anaemia-Inducing Substance
APPR	Acute Phase Protein Response
APS	Ammonium Persulphate
AR	Adrenergic Receptor
BMR	Basal Metabolic Rate
Bq	Becquerel's
BSA	Bovine Serum Albumin
C	Celsius
cAMP	Cyclic adenosine monophosphate
CCK8	Cholecystokinin 8
CNTF	Ciliary Neurotrophic Factor
COX	Cyclooxygenase
CRE	cAMP Response Element
CRH	Corticotrophin Releasing Hormone
DEAE	Diethlaminoethyl cellulose
DMSO	Dimethlysulphoxide
DTT	Dithiothreitol
ECL	Enhanced chemoluminescence
EPA	Eicosapentaenoic Acid
FCS	Foetal Calf Serum
FFA	Free Fatty Acid
5FU	5-Flourouracil
g	Gram

GDP	Guanosine diphosphate
GC	Glucocorticoid
GTP	Guanosine triphosphate
GU	Glucose Uptake
h	Hour
HCAP	Human Cachexia Associated Protein
HETE	Hydroxyeicosatetraenoic Acid
HPLC	High Performance Liquid Chromatography
HSL	Hormone Sensitive Lipase
IFNγ	Interferon-gamma
IκB	Inhibitory Kappa B
Iκκ	Inhibitory Kappa Kinase
IL	Interleukin
l	Litre
LIF	Leukaemia Inhibitory Factor
LMF	Lipid Mobilising Factor
LPL	Lipoprotein Lipase
kDa	Kilo Dalton
Kg	Kilogram
μ	Micro
M	Mole
m	Milli
MAC	Murine Adenocarcinoma
min	Minute
MoAb	Monoclonal Antibody
mRNA	Messenger Ribonucleic Acid
n	Nano
NAD+	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide (reduced form)
NEFA	Non-Esterified Fatty Acid
NF-κB	Nuclear Factor Kappa B
NPY	Neuropeptide Y
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PIF	Proteolysis Inducing Factor
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptor
PUFA	Polyunsaturated Fatty Acid
REE	Resting Energy Expenditure
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
TEMED	N,N,N,N,'-Tetra-methyl-ethylenediamine
TNF-α	Tumour Necrosis Factor- alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TPN	Total Parenteral Nutrition
Tris	Tris (hydroxymethyl) methylamine
Tween 20	Polyoxyethylene sorbitan
U	Unit
UCP	Uncoupling protein
ZAG	Zinc-α2-Glycoprotein

Introduction

1.1 Cancer and weight loss

Weight loss, asthenia, anorexia and chronic nausea are some of the most common symptomatic problems affecting patients with advanced cancer. Pre-chemotherapy studies suggest weight loss and its associated metabolic abnormalities to account for 25% of cancer deaths (Warren (1932)).

Cancer related weight loss and poor nutrition results in reduced quality of life with an impaired response to chemotherapy. Weight loss of <5% worsens patient prognosis (Tisdale (1993)). The frequency of weight loss varies according to tumour type from 30% incidence in patients with favourable non Hodgkin's lymphoma to 90% in patients with gastric or pancreatic cancer (De Wys *et al* (1980)). Death occurs at a weight loss of 30% (Brennan (1977)).

Cancer related weight loss may be a result of anorexia, cachexia or both.

1.2 Cancer Anorexia

Anorexia encompasses loss of appetite, early satiety and decreased food intake. Anorexia is frequently associated with cancer and contributes to the poor nutritional status of cancer patients. Anorexia is apparent in 50% of newly diagnosed cancer patients (Grosvenor *et al* (1989)). The resulting weight loss significantly increases morbidity and mortality.

Patients with cancer often report taste abnormalities. A study involving untreated cancer patients reported elevated thresholds for sweet and bitter tastes (Abrasov (1961)). There is also evidence of increased sweet and decreased bitter recognition, accompanied by an overall decrease in appetite (De Wys (1974)). Carson *et al* (1977) proposed cancer and the extent of metastasisis to be related to taste abnormality.

Food aversion also contributes to cancer anorexia as a learned response when consumed prior to drug treatments, whereby previously accepted foods are rejected (Bernstein *et al* (1982)).

Anorexia is a classic symptom of severe depression. Evidence now suggests mood and food intake are related. Feelings of malaise are often accompanied by a reduction in food intake and a lack of appetite (Holland *et al* (1977)). Psychological causes of anorexia are often accompanied by physical causes such as ulceration and infection of the mouth, nausea and vomiting (Samson *et al* (2001)).

Hormones and centrally produced neuropeptides have been identified as potent regulators of appetite. Leptin controls production and release of the anorexigenic neuropeptide α- melancyte stimulating hormone (α-MSH) and the product of hypothalamic proopiomelanocortin (POMC) gene transcription and translation. Leptin also acts on orexigenic peptides such as orexin, neuropeptide Y (NPY) and agouti gene- related peptide (AGRP) (Wisse (2001)).

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α stimulate the central proopiomelanocortin (POMC) system. The POMC gene product is the anorexigenic α-melanocyte stimulating hormone (α-MSH) which activates melanocortin receptors MC3R and MC4R which are expressed in the hypothalamus and other brain regions and result in anorexia (Samson *et al* (2001)). The melanocortin receptor antagonist SHU9119 was successful in blocking the anorexigenic effects of α-MSH (Wisse (2001)).

Heisle *et al* (2002) demonstrated that serotonin also activates the POMC neurons in the arcuate nucleus (ARC) which is an anorexigenic pathway, leading to the inhibition of NPY neurons.

Anorexia in tumour bearing animals results from a dysfunctional hypothalamic adenylate cyclase system, and impaired NPY response. NPY is a potent orexigenic peptide but responsiveness to NPY is reduced in tumour bearing animals. NPY levels remain elevated in fasting animals suggesting that post-synaptic NPY signalling systems of the hypothalamus are altered in tumour bearing rats (Tisdale (2001))

Hypothalmic monoaminergic neurotransmission importance in homeostasis and food intake has been demonstrated by Meguid *et al* (2004). Increased serotonin is directly related to the presence of the tumour, decreased dopamine levels are related to reduced food intake. After tumour resection these neuro-mediators reverted to the non tumour bearing levels, with an increase in food intake reversing cancer associated anorexia. Therefore inhibition of NPY is associated with an increase in serotonin and decrease in dopamine in the PVN, inhibiting food intake and promoting the onset of cancer anorexia.

Tumour anorexia might be a consequence of altered serotonin activity in cancer. Concentrations of plasma tryptophan in the Walker 256 tumour bearing rats were reduced and there was a high brain concentration of tryptophan (Krause *et al* (1979)). Tryptophan is a precursor of serotonin, which is important in the pathogenesis of cancer anorexia by increasing ventromedial hypothalamic serotinergic activity (Tisdale (2001)). Oral administration of branched chain amino acids competitive to tryptophan decreased the incidence of anorexia. However the serotonin antagonist cyproheptidine did not improve progressive weight loss in patients with cancer anorexia/ cachexia (Kardinal *et al* (1990)).

There are several reasons for development of cancer anorexia varying from physical, psychological, hormonal and as a response to chemotherapy. However, cancer-anorexia cannot fully explain the extent of tissue wasting and rapid deterioration of the host. There is another phenomenon occurring which is cancer cachexia.

1.3 Cachexia

The term cachexia is derived from the Greek *kakos* meaning bad and *hexis* meaning condition. Cachexia refers to a state of severe malnutrition characterized by anorexia, weight loss and muscle wasting. Cachexia may occur in the context of many chronic disease states including cancer, autoimmune-deficiency syndrome, chronic renal failure, chronic gastrointestinal disease, sepsis and AIDS (Tisdale (2001)).

Cachexia is a common manifestation of advanced malignant disease and may occur in the majority of cancer patients prior to death. Warren (1932) proposed 22% of cancer deaths were due to cachexia. Cachectic cancer patients experience decreased performance status, an inferior response to chemotherapy and a shorter survival time (De Wys (1986)).

Therefore it is important to understand the mechanism of cachexia and how the shift in metabolism is affecting the host survival and ultimately how to reverse this process.

1.4 Cachexia and starvation: A comparison

The weight loss in cancer patients differs significantly from that observed in starvation. In the starved state three quarters of weight is lost from body fat and only a small percentage derived from lean body mass. However, with cancer patients, there is equal loss from both adipose tissue and lean body mass (Burke *et al* (1980)).

Body composition analysis of patients with anorexia nervosa has shown that the majority of weight loss arises from fat and only a small proportion from muscle. Lung cancer patients who had lost 30% of their pre-illness weight showed an 85% decrease in body fat and 75% decrease in skeletal muscle protein and conservation of non muscle protein (Fearon (1992)).

Starvation is defined as the state of deprivation of food. The length of survival depends on the nature of reserves in the body and metabolic adaptations. The brain is incapable of deriving energy from the oxidation of fatty acids, the primary fuel is glucose and survival depends on gluconeogenesis. Hormonal responses to starvation include a drop in plasma insulin levels, and an increase in plasma glucagon, which are triggered by a drop in blood glucose levels (Stryer (1998)).

During starvation, metabolism becomes catabolic, causing liver glycogenesis, gluconeogenesis and adipose tissue lipolysis to meet the glucose demands of the brain. Derivation of glucose from muscle proteolysis is minimised, and brain metabolism switches away from glucose to prevent excessive depletion of lean body mass (White *et al* (1993))

Starvation is accompanied by the provision of fatty acids and ketone bodies (acetoacetate and 3-hydroxy-butyrate) as alternative fuels. Ketone bodies have been found to inhibit oxidation of branched chain amino acids in muscle, resulting in inhibition of protein catabolism. Ketone body accumulation could be said to mediate the observed inhibition of protein breakdown (White *et al* (1993)).

1.5 Energy expenditure within the cancer patient :

The normal response to weight loss as seen in starvation does not occur in cachexia. There is a dramatic shift in metabolism and a huge depletion of lean body mass. Cachexia can be explained as a negative balance between calorie intake and calorie expenditure (Theologides (1979)).

Initially it was postulated that weight loss resulted from impaired absorption and enteric nutrient loss (Burke *et al* (1980)). However, it is now well documented that a major contributing factor towards weight loss is the shift in host metabolism and rise in resting energy expenditure (REE) of the host (Tisdale (2001))

Knox *et al* (1982) studied REE in 200 malnourished cancer patients and concluded the majority of cancer patients exhibited aberrations in energy metabolism, with 26% patients experiencing a hypermetabolic REE.

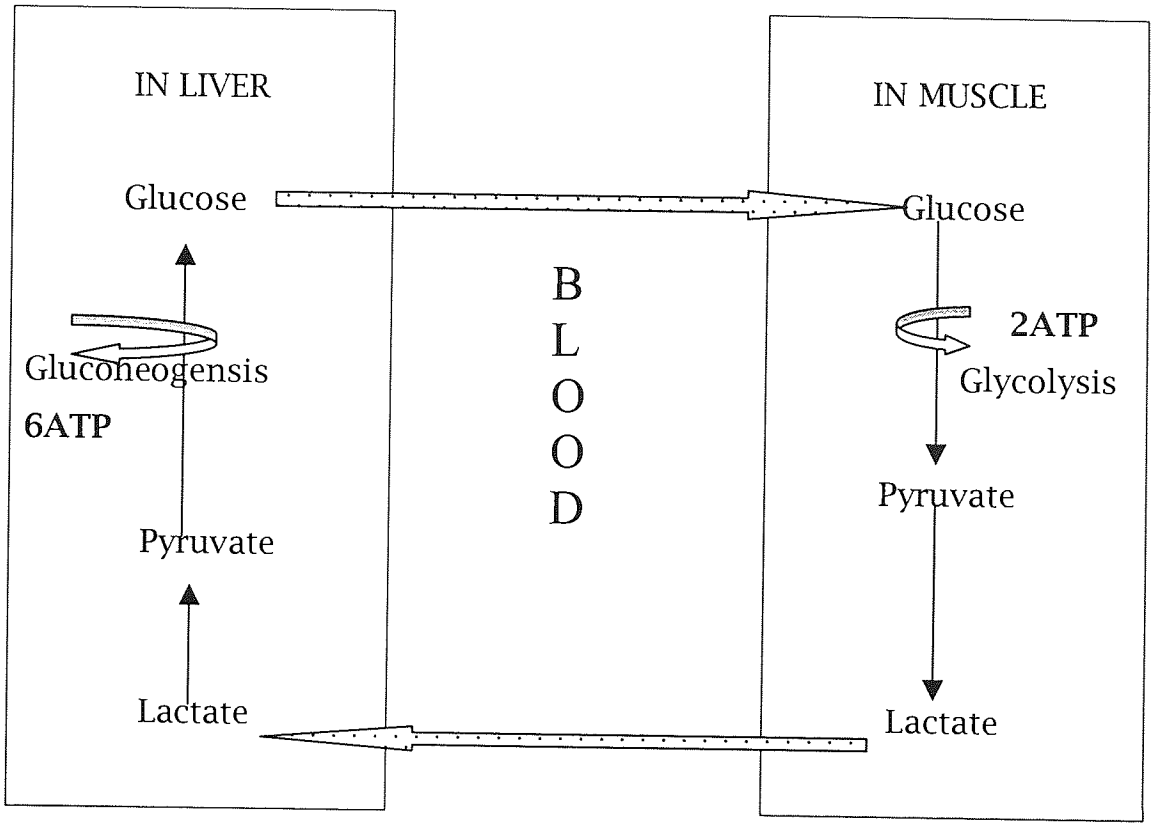
Elevated REE is directly related with weight loss in cancer patients. Resection of the tumour results in a decrease in energy expenditure (Hyltander *et al* (2000)). Increased resting metabolism may be the result of increased adrenergic activity and sensitivity in cancer patients. Administration of β blockers reduced REE corresponding to 0.5-1kg fat or 2-3kg muscle tissue over 4-6 months (Hyltander *et al* (2000)). The REE was significantly greater in patients with an acute phase response (Burke *et al* (1980))

Glucose metabolism is derived from glycolysis, the tricarboxylic acid cycle (TCA) and oxidative phosphorylation where 36 molecules of ATP are derived from 1 molecule of glucose (Stryer (1998)). However, tumours possess pockets of hypoxic tissue where aerobic glycolysis cannot occur. Respiration shifts from aerobic, to the anaerobic Cori cycle. The raw materials for this gluconeogenesis are primarily lactate and alanine normally produced by active skeletal muscle and erythrocytes. The Cori cycle usually occurs as the production of pyruvate exceeds the oxidation rate in the citric acid cycle. Cancer patients experience this shift in metabolism due to hypoxic regions of the tumour and demand for glucose as an energy source. (Tisdale (2001))

Lactate is a metabolic end point and must be converted back into pyruvate for further metabolism. The purpose of reducing pyruvate to lactate is to regenerate NAD+ for normal aerobic glycolysis to continue. (Stryer (1998))

The MAC16 tumour, described in section 1.7, is accompanied by hypoglycaemia of the host, a result of a growing tumour producing excessive glucose demands (Bibby *et al* (1987))). Host glucose metabolism is therefore derived from the energy inefficient cori cycle see figure 1.5a.

Figure 1.5. The Cori cycle



This futile cycle results in a net loss of 4 ATP molecules per molecule of glucose and may account for 300kcal/day loss of energy in cancer patients (Eden *et al* (1984))

An increase in REE is associated with infection, sepsis, trauma, major surgery, and burns and poorly nourished patients have a reduced survival potential (White *et al* (1993))

1.6 The Acute Phase Protein Response (APPR):

The acute phase protein response occurs in response to trauma, inflammation or infection. The purpose of APPR is to assist in tissue repair, blood clotting and aid in the destruction of pathogens.

Sepsis, injury and cancer share a metabolic trend involving the release of amino acids from skeletal muscle, especially alanine and glutamine. Glutamine is taken up by the tumour to sustain energy and nitrogen demands and alanine is channelled to the liver for gluconeogenesis and protein synthesis. The APPR provides tissues with increased amino

acids. If allowed to continue uncontrolled the APPR will lead to muscle wasting and asthenia (Argilés *et al* (2001)).

The APPR is characterised by a series of hepatocyte-derived plasma proteins known as acute phase reactants. These include C-reactive protein (CRP), serum amyloid A (SAA), complement factors B and C3, and a reduced synthesis of transferrin and albumin. In starvation there is no change in albumin synthesis rate whereas cancer patients experience much lower albumin concentrations (Argilés *et al* (2001)).

McMillan *et al* (2001) found low albumin concentrations in male cancer patients reflected loss of body cell mass, and presence of an inflammatory response. The same response in acute injury, gastrointestinal and lung cancer results in an inverse correlation of circulating levels of albumin and C reactive protein. The demand for specific amino acids to fuel the APPR promotes the degradation of available protein, primarily skeletal muscle and over a prolonged period results in death of the cancer patient.

The APPR response is seen in a significant proportion of cancer patients (pancreas, lung, kidney and oesophagus). Pancreatic cancer patients experience an increase in APPR as the disease progresses. The APPR is associated with shorter survival time. The role of the APPR is unclear in cancer and it may be a reaction of the host to inflammation. However the APPR accelerates muscle wasting due to increased demand for amino acids to support hepatic export protein synthesis (Fearon and Moses (2002)), and wastes energy increasing REE and creating a negative energy balance.

1.7 MAC16 Model:

Further investigations into cachexia required the introduction of an animal model. Models that reflect the critical ratio of tumour burden to emaciation are very rare. In the Walker 256 model, tumour burden can reach 50% of body weight. The MCG 101 model initiates cachexia at a small burden of 2-3% however it grows exponentially and can reach 15% body weight in 5 days (Emery (1999)).

Human tumours rarely reach more than 5% body weight, so a suitable animal model was employed to match that seen in cancer patients.

The MAC16 is a slow growing 1,2-Dimethylhydrazine induced transplantable adenocarcinoma of the colon in NMRI mice. The tumour model provides a stable, well-differentiated model for colorectal cancer (Double *et al* (1975)).

The MAC16 model results in significant weight loss without excessive tumour burden (<1% of host weight). Weight loss is directly related to tumour size. A reduction in food or water intake is observed as the animals adjust their food intake to their body weight (Bibby *et al* (1987)).

In male mice weight loss begins when the tumour exceeds 0.3% of total body weight reaching 30% when the tumour mass reaches 3% host body weight. Females suffer more extensive weight loss of 40%, with a tumour burden of 2.5%, which suggests the tumour is not competing with the host for nutrients. The weight loss is accompanied by depletion of skeletal muscle and fat stores directly proportional to tumour size, suggesting the production of tumour catabolic factors (Beck *et al* (1987)).

The MAC13 is a closely related adenocarcinoma (Double *et al* (1975)) which does not induce cachexia *in vivo* (Beck *et al* (1987)).

1.8 Circulatory Factors in Cachexia:

Cancer anorexia, increased resting energy expenditure and acute phase protein response alone do not account for the massive emaciation and tissue wasting observed with certain cancers. This suggests there are circulatory factors involved in the induction of cachexia.

Norton *et al* (1985) demonstrated a control rodent parabiotically joined to a tumour-bearer, exhibited homologous emaciation in the absence of metastasis. These findings prompted the successful isolation and characterisation of numerous humoural factors from various models.

1.8.1 Lipolytic factors and cancer cachexia

In the past 40 years, many lipolytic factors have been isolated from various tumour models and patients. Characterisation of these factors offers a potential target for therapeutic intervention.

The major calorific reservoir in the body is adipose tissue, consisting of triglycerides. Mobilisation of these triglyceride stores is achieved by lipase hydrolysis, yielding free fatty acids (FFA) and glycerol. FFA can then be released into the bloodstream or re-esterified within the adipocyte. There is a relative absence of the enzyme glycerol kinase within adipose tissue, and glycerol is released into the bloodstream. Glycerol present in plasma is indicative of breakdown of lipid stores (Jeevanadam *et al* (1981)).

It was suggested by Kralovic *et al* (1977) that the Walker 256 carcinoma might produce a lipolytic factor. As the tumour reached 10% body weight, total lipid content fell by 50% and ascites serum from tumour bearing rats stimulated lipoysis *in vitro* (Kralovic *et al* (1977))

C57BL mice bearing the preputial gland tumour ESR-586 displayed significant mobilisation and depletion of carcass lipid stores, suggesting a tumour secreted humoural factor with a direct effect on adipose tissue (Thompson *et al* (1981)).

Costa and Holland (1962) demonstrated male swiss mice bearing the Krebs-2-carcinoma to experience profound weight loss during tumour growth whilst food intake remained normal.

It was reported by Kitada *el al* (1980) that a lipid mobilising factor was detected in AKR mice bearing a thymic lymphoma. This lipid mobilising factor was present in tumour bearing mice, but absent from controls (Kitada *et al* (1980)). Characterisation of this factor and determined a low molecular weight protein of 5 kDa which acts directly on adipocytes causing glycerol and fatty acid release from triglycerides. Later studies demonstrated this low molecular weight form to be inactive until aggregation at low temperature (Kitada *et al* (1982)).

This factor was present in both tumour extract and culture medium therefore secreted directly by the tumour (Kitada *et al* (1980)). This factor, *in vivo,* stimulated the release of unsaturated fatty acids, required by the tumour for growth and formation of membrane phospholipids (Kitada *et al* (1980)).

1.8.1.1 Lipolysis Promoting Factor

Taylor *et al* (1992) identified a novel Lipolysis Promoting Factor isolated from the A375 melanoma human cell line. LPF was characterised as heat stable, rich in glutamic acid with molecular weight of 6 kDa. LPF was immunologically and biochemically distinct from TNFα and produced a loss of carcass lipid *in vivo* and *in vitro* via an increase in cellular lipase activity.

1.8.1.2 Anaemia inducing substance (AIS)

Ishiko *et al* (1999) characterised an Anaemia Inducing Substance (AIS) isolated from plasma of VX2 carcinoma bearing rabbits using a phenyl-Sepharose column. AIS was

characterised as a 50 kDa glycoprotein produced from tumours, alkaline resistant and heat unstable. After injection into non-tumour bearing rabbits total body fat was reduced by 24% and lipolytic activity was increased by 51.5% over the experimental period. However there is no mention of biochemical mechanisms and there is limited evidence in support of this AIS.

1.8.1.3 Azaftig :

A 24kDa chondroitin-SO_4 containing proteoglycan was isolated from the urine of pancreatic cancer patients experiencing pronounced weight loss. This factor was termed Azaftig (Figueroa *et al* (1999)). This 24kDa protein produced dose dependant stimulation of lipolysis *in vitro* using isolated human and rat adipocytes. It was proposed Azaftig mediated a lipolytic effect, to be cAMP and Hormone Sensitive Lipase dependant, and implicated this purified 24kDa proteoglycan as a potential anti obesity treatment (Figueroa *et al* (2002)).

However, it appears that this group have co purified LMF and PIF from cachectic patient urine. McDevitt *et al* (1995) isolated a similar lipolytic material, purified from pancreatic cancer patient urine and MAC16 tumour homogenate, with a molecular weight of 24kDa.

1.8.1.4 ToxoHormone

Toxohormone was isolated from malignant tissue by Nakahara *et al* (1949) and was found to be heat stable, water soluble, and interfered deleteriously with catalase production in the liver.

1.8.1.5 Toxohormone L :

Masuno *et al* (1981) reported body triglyceride content to decrease during sarcoma 180 growth in mice. Successful isolation of a lipolytic substance was possible from ascites fluid of mice bearing the 180 sarcoma, but not ascites fluid of peritonitis.

Characterisation revealed an acidic protein with an isoelectric point of 4.7 and molecular weight of 75kDa. Toxohormone L was heat labile and non dialyzable. Trypsin digestion yielded a heat stable, dialyzable protein of 10kDa, suggesting that the entire protein was not required for lipolytic activity (Masuno *et al* (1981))

Masuno *et al* (1984) reported an anorexigenic effect of Toxohormone L. Administration *in vivo* resulted in lipid mobilisation, immunosuppression and a significantly repressed food and water intake, suggesting Toxohormone L to be a contributing factor towards weight loss in cancer.

1.8.2 The 43kDa Tumour Derived Lipid Mobilising Factor (LMF)

Groundwater *et al* (1990) speculated cancer weight loss could be linked to tumour-produced factors. Urine and serum of weight losing cancer patients were examined. Control urine and serum was derived from Alzheimer's patients and healthy individuals. Urinary and serum lipolytic activity was assessed according to the method of Weiland (1974). Control and Alzheimer's serum and urine lipolytic activity were significantly lower than weight losing cancer patients. This indicated a circulatory factor lacking species specificity, which was able to stimulate lipolysis in murine adipocytes.

Groundwater *et al* (1990) identified a lipid mobilising factor with a similar charge and molecular weight as detected in the MAC16 tumour from the serum and urine of cachectic cancer patients. This factor lacked species specificity as human derived material was able to stimulate lipolysis in isolated murine adipocytes.

Lipid mobilising factors present in plasma of tumour bearing mice suggests lipolytic activity is not local, but a circulatory effect to mobilise host lipids required for tumour growth. The lipolytic factor associated with the MAC16 model is non dialyzable and destroyed by heat and acid. Taylor *et al* (1992) found insulin and 3-hydroxybutyrate to suppress lipolysis; however, propanalol and indomethacin had no effect on lipolytic activity suggesting this factor to be distinct from adrenaline and prostaglandins. A lipid mobilising factor was purified from excised MAC16 tumours (McDevitt *et al* (1995)) and urine of cachectic cancer patients, and resolved on a 12% SDS-PAGE gel to yield an approximate molecular weight of 43kDa (McDevitt *et al* (1995)).

Characterisation of this 43kDa lipolytic protein found an increase in cAMP levels of white adipocytes (Tisdale and Beck (1991)) and an increase in adenylate cyclase. The adenylate cyclase inhibitor MDL_{12330A} attenuated lipolysis, suggesting that cAMP was the intracellular mediator inducing lipolysis (Khan & Tisdale 1999)).

Khan and Tisdale (1999) found GDP-associated G proteins to be involved in the signal transduction of the lipid mobilising factor (LMF). G proteins regulate the adenylate cyclase pathway via stimulatory G_s and inhibitory G_i (Levitzi 1987). Stimulation of adenylate

cyclase by LMF is GTP dependant, with a maximum response at 0.1μM (Khan and Tisdale (1999).

Islam-Ali *et al* (2001) reported enhanced G_{α_s} expression and reduced G_{α_i} expression in cachectic cancer patients was observed compared to non cachectic controls. Cachexia inducing tumours and the production of tumour derived LMF were demonstrated to up-regulate expression of G_{α_s}, G_{α_i} and HSL (hormone sensitive lipase) (Islam-Ali *et al* (2001)).

Adipocyte lipolysis is initiated by Hormone Sensitive Lipase (HSL). HSL is regulated by reversible phosphorylation at a single serine residue, catalysed by cAMP dependent protein kinase A (Stralfors *et al* (1987)). HSL catalyses the hydrolysis of triacylglycerols into free fatty acids (Stryer (1998)). This lipolysis was attenuated with the PKA inhibitor H8 *in vitro*, (Khan and Tisdale (1999)), suggesting LMF induced lipolysis to be PKA dependant.

LMF administration to NMRI mice induces a decrease in blood glucose, confirming stimulation of glucose consumption. Protein synthesis is enhanced in tumour cells suggesting LMF to be a growth factor for the tumour (Islam-Ali and Tisdale (2001)). Russell and Tisdale (2002) determined that treatment of ex-breeder male mice with LMF, isolated from the urine of cachectic cancer patients caused a significant increase in glucose oxidation to CO_2, compared to PBS controls. Altered carbohydrate metabolism, loss of adipose tissue and increased whole body fatty acid oxidation in cachectic patients may be a result of tumour production of LMF.

LMF administered *in vivo* to *ob/ob* mice is similar to the effect of leptin, both produced weight loss and reduced body fat content. However leptin associated weight loss is accompanied by a reduced food and water intake, whereas rodents receiving LMF do not exhibit altered food or water intake (Hirai *et al* (1998)). The method which LMF induces degradation of adipose tissue is analogous to the lipolytic hormones and not cytokines. Animals treated with TNFα experienced hypertriglyceridema through inhibition of lipoprotein lipase (LPL) (Mahoney *et al* (1988)).

Human derived LMF administered to *ob/ ob* mice demonstrated a significant increase in 3-hydroxybutyrate and 3 fold increase in oxygen consumption in BAT tissue, suggesting an increase in thermogenesis, oxygen consumption in BAT is mediated through the β_3 subtype (Mahoney *et al* (1988)).

Russell *et al* (2002) determined that LMF inducted lipolysis in murine white adipocytes and stimulated adenylate cyclase in adipocytes plasma membranes, this effect was attenuated with the specific β_3 adrenoreceptor antagonist SR59230A. This study suggested LMF to induce lipolysis through the β_3 adrenoreceptor.

Bing *et al* (2002) studied the effect of LMF isolated from the urine of cachectic cancer patients injected intravenously into mice over 52h. LMF was found to induce a significant reduction in body weight, fat mass, accompanied by a decrease in plasma leptin levels. Brown adipose tissue was found to have increased mRNA levels of uncoupling proteins -1, -2 and -3. There was increased uncoupling protein-2 mRNA in the liver and skeletal muscle. Up-regulation of the uncoupling proteins -1, -2 and -3 in brown adipose tissue and uncoupling protein -2 in skeletal muscle and the liver suggests they may serve to utilize excess lipids mobilised during fat catabolism during cancer cachexia (Bing *et al* (2002)).

LMF is produced by cachexia-inducing tumours and is involved in the degradation of adipose tissue, increased oxidation and release of fatty acids through induction of uncoupling protein (UCP) expression (Bing *et al* (2002)). UCP-2 is thought to be involved in detoxification of free radicals (Ricquier and Bouillaud (2000)), therefore, LMF induced UCP-2 expression in tumour cells might attenuate free radical toxicity. Sanders and Tisdale (2004) investigated the effect of LMF on MAC13 cells, and found it to induce an increase in UCP-2 expression. This effect was attenuated by the β_3 adrenreceptor antagonist SR59230A. Co-incubation of LMF with MAC13 cells reduced the growth inhibitory effects of bleomycin, paraquat and hydrogen peroxide, which are free radicals generators. This data suggests that LMF antagonises the antiproliferative effect of chemotherapeutic agents working through a free radical mechanism (Sanders and Tisdale (2004)). This may explain the partial unresponsiveness to chemotherapy of cachexia –inducing tumours.

1.8.3 Zinc-α2 glycoprotein (ZAG)

A substantial loss of body fat (up to 85%) is a hallmark of cancer cachexia, one mediator is thought to be Zinc-α2-glycoprotein (ZAG) which is a 43kDa protein originally isolated from human plasma, and has been shown to be identical to LMF (Todorov *et al* (1996)).

LMF purified from the urine of cachectic cancer patients has demonstrated homology to the plasma protein Zn-α_2 glycoprotein in terms of amino acid sequence, electrophoretic mobility and immunoreactivity (Hirai *et al* (1998)). Both LMF and Zn-α_2-glycoprotein stimulate lipolysis in isolated murine epididymal adipocytes and this in turn is associated

with activation of adenylate cyclase in adipocyte plasma membranes in a GTP dependent manner. LMF and Zn-α_2-glycoprotein both achieve maximum stimulation at 0.1μM (Hirai *et al* (1998)).

Russell *et al* (2004) found human derived ZAG to stimulate glycerol release from isolated murine epididymal adipocytes in a dose-dependent manner. This effect was attenuated by the β_3 antagonist SR59230A. *In vivo* ZAG was found to induce a significant decrease in body weight, from the depleation of adipose tissue. UCP-1 expression in brown adipose tissue was increased, suggesting ZAG to increase energy expenditure (Russell *et al* (2004)).

Bing *et al* (2004) determined the MAC16 tumour to be accompanied by a significant increase in ZAG mRNA and protein expression in white adipose tissue and brown fat. ZAG mRNA was also detected in 3T3-L1 cells and it was suggested that ZAG is a new adipose tissue protein factor, which may be responsible for lipolysis in adipocytes.

ZAG induced a concentration dependent increase in expression of UCP-1 in primary cultures of brown, but not white adipocytes, and the effect was attenuated by the β_3 adrenoreceptor antagonist SR59230A (Sanders and Tisdale (2004)). ZAG also increased UCP-2 expression in C_2C_{12} murine myotubes. The increase in UCP-2 expression was attenuated by SR59230A and potentiated by isobutylmethylxanthine which suggested a cyclic AMP-mediated process through interaction with a beta 3 adrenoreceptor. UCP-3 expression in murine myotubes also increased; however, this effect appeared to require mitogen activated protein kinase. This study confirmed the ability of ZAG to directly influence UCP expression, which may be important in lipid utilisation during cancer cachexia (Sanders and Tisdale (2004)).

1.9 A Tumour Derived Proteolysis Inducing Factor (PIF)

Beck and Tisdale (1991) isolated tumour extract and plasma from MAC16-tumour bearers and demonstrated significant proteolytic activity over that from control MAC13 bearers. This suggested production of a circulatory proteolytic factor by the MAC16 tumour.

MAC16 tumour-bearers demonstrated significantly reduced protein synthesis *in vivo* and *in vitro* with increased protein degradation compared to controls (Beck *et al* (1991)). Administration of 50μM of the polyunsaturated omega three fatty acid eicosapaentanoic acid (EPA) significantly attenuated protein degradation but had no effect on protein synthesis.

MAC16-tumour bearers with weight losses between 16-25% demonstrated massive depletion of lean body mass, reduced protein synthesis and increased protein degradation. Serum of these rodents induced protein degradation in isolated gastrocnemius muscle *ex vivo* (Smith *et al* (1993)).

Serum of weight stable mice bearing the MAC16 and MAC13 tumours was assessed for antibodies specific to the induction of cachexia. Immunoblotting detected a protein of molecular weight 24kDa using serum from MAC16 tumour bearing mice, which was absent from sera of MAC13 tumour bearing mice. Fractionation and purification of cachectic pancreatic cancer patient urine yielded the same molecular weight protein, using serum from mice bearing the MAC16 tumour (McDevitt *et al* (1995)). Again this peptide was absent from urine of normal subjects (Todorov *et al* (1996)).

A monoclonal antibody was obtained from fusion of splenocytes from MAC16 tumour bearers with Balb/c myeloma cells. Resulting hybridomas were screened for production of a monoclonal antibody against the 24kDa peptide. Administration of affinity purified 24kDa factor induced lipolysis and enhanced protein degradation in isolated gastrocnemius muscle (Todorov *et al* (1996)). *In vivo* studies demonstrated attenuated weight loss in mice bearing the MAC16 tumour and inhibition of tumour growth when accompanied by administration of the monoclonal antibody (Tisdale *et al* (1996)).

Antisera to the 24kDa protein does not cross-react with the cytokines TNFα and IL-6, deeming this factor distinct. Biosynthetic labelling studies of MAC16 cells to produce this material *in vitro* confirmed this material to arise from the tumour rather than host cells (Todorov *et al* (1997)).

Cachectic cancer patient urine was purified to yield material homologous to the 24kDa material found in MAC16 tumour bearers. Administration of this 24kDa protein to NMRI mice induced significant weight loss, (without a reduction in food or water intake) depression of protein synthesis and increased skeletal muscle proteolysis. The effect was inhibited by pre-treatment with mouse monoclonal antibody to the 24kDa material (Kien *et al* (1983)).

This proteolytic factor isolated from MAC16 tumour bearing mice was found to be heat stable at 60°C for 5 minutes, suggesting this factor is not a cytokine. Proteolytic action was not inhibited by 1mM phenylmethyl-sulfonyl fluoride, suggesting it is not a serine protease. Inhibition was achieved by 0.2mM indomethacin, 0.5mM EPA and 1.7mM BWA4C suggesting prostaglandin E_2 (PGE_2) to be the intracellular signalling pathway (these drugs

interfere with PGE_2 production). Incorporation of EPA into muscle phospholipids causes competition with arachidonate for cyclooxygenase in response to phospholipase A_2. (Smith *et al* (1993)).

Todorov *et al* (1996) affinity purified this cachectic factor from excised MAC16 tumours. 12% SDS-PAGE electrophoresis yielded two immuno-reactive bands with apparent molecular weights of 69kDa and 24kDa. This 24kDa factor binds tightly to mouse albumin, as the sulphate residues in the carbohydrate chains cause electrostatic linkage, thus yielding a molecular weight of 69kDa. Further fractionation by reverse phase high performance liquid chromatography elucidated the proteolytic factor to have a molecular weight of 24kDa (Todorov *et al* (1996)).

Further structural analysis elucidated this 24kDa protein to have a central polypeptide chain of 4kDa. Functional and immunological studies suggest biological activity is mediated through *N*- and *O*-linked oligosaccharide chains in the molecule with molecular weights of 10kDa and 6kDa. Therefore this 24kDa material is a heavily glycosylated proteolytic factor (Todorov *et al* (1997)).

Amino acid sequence analysis confirmed the 24kDa material to be unique, with no homology to cytokines, and to be resistant to proteolytic digestion. The amino acid sequence of the 24kDa glycoprotein is YDPEAASAPGSGNPSHEA(S)(A) (Todorov *et al* (1996)).

This Proteolysis Inducing Factor is associated with skeletal muscle catabolism and depletion of lean body mass, therefore it was termed PIF. PIF has been shown to be expressed in tumours and mesenchymal tissue of patients that have PIF detectable in their urine (Mansano *et al* (2001)).

Unlike the cytokines TNFα, IL-1 and IL-6, PIF increased protein degradation *in vivo* and *in vitro*, suggesting a direct effect on skeletal muscle protein homeostasis (Smith *et al* (1993)).

There are three major proteolytic pathways involved in the intracellular degradation of proteins in skeletal muscle: (i) The lysosomal system is concerned with proteolysis of endocytosed proteins and receptors. Llovera *et al* (1994) found activity of the lysosomal system (cathepsin B and B+L) to be decreased in gastrocnemius muscle of rats bearing Yoshida AH-130 tumour, indicating lysosomal proteolysis not to be involved in cancer cachexia (Llovera *et al* (1994)). (ii) The cytosolic calcium activated cysteine proteases are

mainly concerned with tissue injury, necrosis and autolysis. (iii) The ATP-ubiquitin-dependent pathway plays an important role in muscle protein degradation induced by starvation, sepsis, metabolic acidosis, denervation atrophy and cancer cachexia (Lorite *et al* (1998)).

The cancer related catabolic response assumed in skeletal muscle is primarily caused by stimulation of protein degradation, particularly myofibrillar protein. Intracellular protein breakdown is regulated by multiple proteolytic pathways, including lysosomal, calcium dependent and the ATP-ubiquitin-dependent proteolytic pathway. Recent studies in rodents with experimental tumours suggest muscle proteolysis in cancer is regulated mainly by the ATP-ubiquitin-dependent proteolytic pathway (Williams *et al* (1999)).

Ubiquitin and 20S proteasome subunit mRNA levels have been found to be 2-4 times higher in muscle from patients with cancer compared to control patients (Williams *et al* (1995)).

Cachectic mice bearing the MAC16 tumour demonstrated enhanced expression of the components of the ATP-ubiquitin-dependent proteolytic pathway (Lorite *et al* (1998)). To confirm the direct action of PIF, parallel studies using the surrogate muscle model, C_2C_{12} murine myoblast cells confirmed an increase in protein degradation in response to PIF (Smith *et al* (1999)).

Enhanced expression of components of the ATP-ubiquitin-dependent proteolytic pathway occurred within 24h of administration of PIF *in vivo*. Northern blot analysis indicated an increase in mRNA levels for the ubiquitin conjugating enzyme E2 and the proteasome subunit C9 in gastrocnemius muscle (Lorite *et al* (2001)). Increased proteasome activity was accompanied by increased expression of the 20S proteasome catalytic core and the 19S regulatory subunit in gastrocnemius muscle. There was no increased mRNA transcript for E2 in heart or liver (visceral tissue), where atrophy is not observed, suggesting PIF activity to be restricted to skeletal muscle, while visceral protein is preserved (Lorite *et al* (2001)).

In vitro administration of PIF to C_2C_{12} myoblasts induced increased protein degradation, 'chymotrypsin-like' activity of the 26S proteasome and increased expression of the ATPase MSS1. Pre-treatment with MG115 and lactacystein, (potent proteasome inhibitors) attenuated protein degradation, confirming the relevance of activation of the proteasome in catabolism of muscle proteins (Lorite *et al* (2001)).

Gomes-Marcondes *et al* (2002) determined C_2C_{12} myotubes to exhibit PIF-induced increase in expression of the 19S regulatory subunit, 20S catalytic core and the ubiquitin conjugating enzyme $E2_{14k}$ with a decrease in the myofibrillar protein myosin.

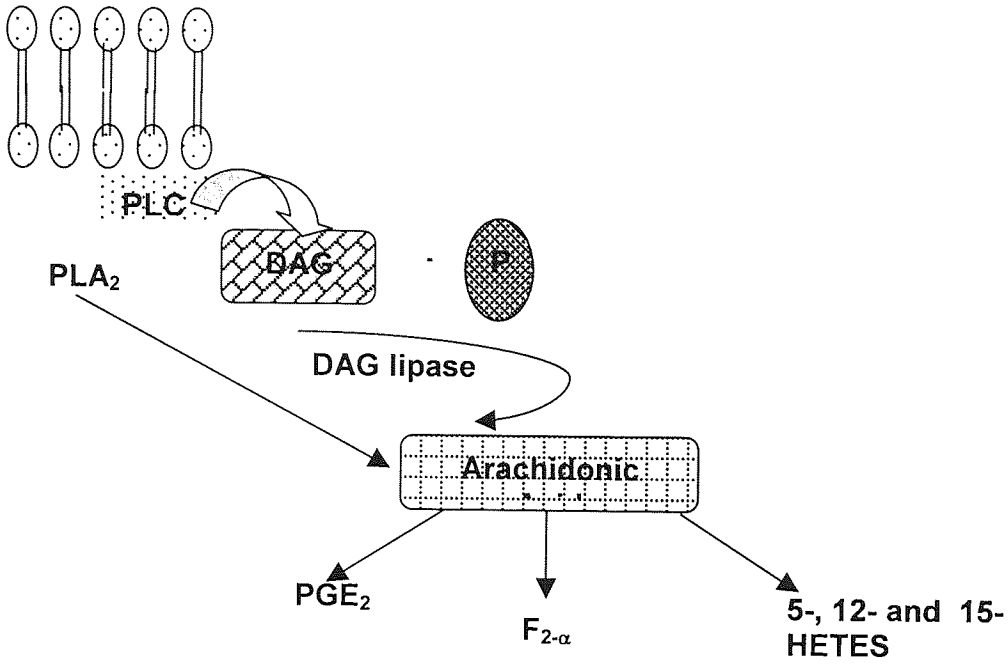
Prostaglandin E_2 (PGE_2) has been implicated as a mediator of muscle protein degradation (Rodemann *et al* (1982)). There is increased PGE_2 production in isolated gastrocnemius muscle when incubated with serum of cachectic mice bearing the MAC16 tumour (Cariuk *et al* (1997)). Protein degradation *in vitro* was associated with elevated PGE_2 production, suggesting it to be an intracellular mediator for protein degradation in muscle; the rise was attenuated by treatment with the monoclonal antibody to PIF (Cariuk *et al* (1997)).

Induction of protein degradation *in vitro* by PIF was reduced by pre-treatment with the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA), which inhibited the rise in PGE_2. This effect could not be produced by the related fatty acid γ linolenic acid (GLA), suggesting EPA acts pharmacologically to attenuate muscle catabolism by blocking metabolism of arachidonic acid (Tisdale (1998)).

EPA failed to affect either the lysosomal or calcium-dependent proteolytic pathways, but ATP-dependent proteolysis was completely attenuated in isolated gastrocnemius muscle from MAC16 tumour bearing mice (Whitehouse *et al* (2001)). *In vivo* studies demonstrated EPA to significantly decrease proteasome activity and expression in gastrocnemius muscle, while myosin expression returned to control levels (Whitehouse *et al* (2001)).

There is evidence to suggest that metabolism of arachidonic acid through the cyclooxygenase or lipoxygenase pathways may be important in skeletal muscle catabolism (Hussey *et al* (1997)). The lipoxygenase inhibitor 2,3,5-trimethyl-6-3(3-pyridylmethyl),1,4-benzoquinone (CV6504) has been shown to inhibit tumour growth and development of cachexia in the MAC16 model (Hussey *et al* (1996)). The effect arose through the inhibition of either 12- or 15-lipoxygenase (Hussey *et al* (1997)).

Figure 1.9 : Mechanism of PIF action on arachidonate metabolism



Arachidonic acid is released from cell membranes by the action of phospholipases. PIF activates phospholipase A_2 (PLA_2), which catalyses the release of fatty acids from the sn-2 position of all membrane phospholipids with the formation of lysophospholipids (Smith and Tisdale (2003)). Phospholipase C hydrolyses the glycerophosphate ester bond of varied phospholipids with the formation of diacylglycerol (DAG) and a phosphate monoester, which can be hydrolysed to arachidonic acid by DAG lipase (Smith and Tisdale (2003)).

PIF-induced activation of PLA_2 causes the metabolism of arachidonate to release arachidonic acid from C_2C_{12} myotube membrane phospholipids. There is increased production of PGE_2 and $F_{2\alpha}$ together with metabolism through the lipoxygenase pathways to yield 5-, 12- and 15-hydroxyeicosatetraenoic acids (HETEs) (Smith *et al* (1999)).

Arachidonic acid release and subsequent metabolism was shown to be directly related to the induction of protein catabolism, and was completely attenuated in the presence of EPA, which also inhibited PIF-induced protein degradation (Smith *et al* (1999)). Of the arachidonate metabolites, only 15(*S*)-HETE produced a significant increase in protein degradation alone, suggesting a role as an intracellular signal for the action of PIF (Smith *et al* (1999)).

PIF-induced protein catabolism is mediated via up-regulation of the ATP-ubiquitin-dependent proteolytic pathway (Gomes-Marcondes *et al* (2002)). EPA attenuated protein degradation and the increased expression of key components of the ubiquitin-proteasome pathway by inhibiting the production of 15(*S*)-HETE. EPA directly attenuated protein degradation and increased proteasome expression induced by 15(*S*)-HETE. This implies that EPA affects a downstream transcription pathway involved in the induction of proteasome expression by 15(*S*)-HETE (Whitehouse *et al* (2003)). There is evidence to suggest that the formation of eicosanoids from arachidonic acid is important in PIF induced protein catabolism, mediated through the ubiquitin proteolytic pathway (Whitehouse *et al* (2003)).

In addition to the role of PIF in protein catabolism, it may also contribute to the pro-inflammatory response observed in cachexia. Incubation of primary cultures of human hepatocytes and Hep G2 cells with PIF produced activation of the transcription factors nuclear factor κB (NF-κB) and the signal transducer and activator of transcription (STAT3). STAT3 activation results in increased production of interleukin-8 (IL-8), interleukin-6 (IL-6) and C- reactive protein (CRP) and decreased production of transferrin (Watchorn *et al* (2002)).

PIF expression appears confined to cachexia-inducing tumours in the adult (Mansumo *et al* (2001)). However, it has been suggested PIF may play a role in embryonic development. PIF expression peaks between embryonic days E8 and E9, a stage that is crucial in development of skeletal muscle and liver. At this point activation of NF-κB and STAT3 was observed (Takeda *et al* (1997)). Mouse embryos were analysed at several gestation times and revealed STAT3 deficient embryos showed rapid degradation between embryonic days 6.5 and 7.5, demonstrating STAT3 as essential for early development of mouse embryos (Takeda *et al* (1997)).

Screening of adult tissue suggests the primary binding sites for PIF are skeletal muscle and the liver. PIF binding to liver endothelial cells activates NF-κB resulting in production of pro inflammatory cytokines and modulates expression of adhesion molecules. PIF induces a local inflammatory response, which attracts monocytes, increases adhesion molecules and contributes to a broader inflammation response. Increased release of IL-8 and IL-6 from endothelial cells and hepatocytes contributes towards the acute phase response (Watchorn *et al* (2002)).

In vitro studies utilising C_2C_{12} myotubes demonstrate NF-κB to provide a positive signal for protein degradation, resulting in increased expression of proteasome subunits and ubiquitin-dependent proteolysis (Whitehouse and Tisdale (2003)). Nuclear accumulation of NF-κB is increased by PIF at the same concentrations that induce protein catabolism and up-regulation of 20Sα proteasome subunit expression. This is accompanied by disappearance of IκBα from the cytosol for the same concentrations of PIF which induce nuclear accumulation of NF-κB (Whitehouse and Tisdale (2003)).

A possible human analogue of PIF, Human Cachexia-Associated Protein (HCAP) was recently identified from a breast cancer library, based on the 20 amino acid sequence of PIF. HCAP appears 90% homologous to PIF and is absent from non-tumour bearing libraries. HCAP is also 100% homologous to the 458 bp dermicidin, an antimicrobial peptide secreted by sweat glands and 100% homologous to a pre-proteolysin gene (AF418981) (Wang *et al* (2003)).

1.10 Mechanisms and factors contributing to cachexia:

1.10.1 The Role of Prostaglandins

Prostaglandins are derived from prostanoids which belong to the eicosanoid family. The eicosanoid family is composed of prostanoids (prostaglandins and thromboxanes) which are synthesised by the cyclooxygenase pathway, the leukotrienes and mono-, di- and tri-hydroxyl acids formed by the lipoxygenase pathways (Smith (1989)).

Eicosanoids are synthesised from C_{20} polyunsaturated fatty acids which contain three to five *cis*, methylene-interrupted double bonds. These include 8,11,14-eicosatrienoic acid and 5,8,11,14-eicosatetraenoic acid (arachidonic acid) which are ω6 polyunsaturates and 5,8,11,14,17-eicosapentaenoic acid (EPA) which is a member of the ω3 family of polyunsaturated fatty acids (Llovera *et al* (1996)).

Prostanoids are formed by the release of arachidonic acid from precursor glycerophospholipids followed by oxygenation of free arachidonic acid by prostaglandin endoperoxide G/H (PGG/H) synthase (cyclooxygenase) and finally metabolism of PGH_2 to biologically active PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 (prostacyclin), or thromboxane A_2 (TxA_2) (Smith (1989)).

Arachidonic acid (AA) is an essential fatty acid, derived from the diet either directly or via its precursor, linoleic acid (LA). Upon entry to the cell it is esterified to phospholipids and can be released by activation of phospholipase A_2 (Camandola *et al* (1996)).

Within mammalian cells conversion of LA to AA is achieved by the $\Delta 5$ and $\Delta 6$ desaturases and the product becomes the precursors of the PG_1 and PG_2 series of prostaglandins (PG) (Dunbar *et al* (1974)).

In vitro experiments have demonstrated muscle is capable of endogenous PG production; PGE_2 stimulates muscle protein degradation and $PGF_{2\alpha}$ activates synthesis of muscle proteins (Reeds *et al* (1985)). PGE_2 is a bone resorbtion stimulating factor, leading to excessive production of PGE_2 in the HSDM tumour model, resulting in hypocalcaemia, which can be improved by administration of the cyclooxygenase inhibitor indomethacin (Tashjian *et al* (1974)).

Ruff *et al* (1984) found that muscle wasting and impaired contractility of skeletal muscle associated with infection is mediated through production of PGs. PGs activate intracellular proteases including cathepsin B, and Indomethacin prevented muscle protein breakdown.

The Yoshida Ascites Hepatoma (YAH) rat tumour model has also implicated the role of PGE_2 in the progression of cancer cachexia. A large proportion of muscle wasting in the YAH model results from excessive PGE_2 production. Administration of naproxen (an inhibitor of PGE_2 production) significantly reduced protein degradation and restored the protein synthesis rates to that of normal controls (Strelkov *et al* (1989)).

Elevation of PGE_2 production in tumour bearing animals has a direct effect on isolated muscle, causing increased protein degradation (Strelkov *et al* (1989)) and hypocalcaemia (Tashjian *et al* (1974)). High levels of Ca^{2+} activate protein degradation in skeletal muscle. It may be that increased Ca^{2+} levels indirectly result in the muscle wasting observed in the YAH-bearing rats (Strelkov *et al* (1989)).

Cyclooxygenase catalyses the oxidation of AA, and is expressed in two isoforms; Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in most tissues and is important in maintaining cellular homeostasis. COX-2 is frequently undetectable at baseline in normal tissues, but becomes readily expressed in gastrointestinal epithelial cells in response to inflammatory cytokines, LPS and ROS (Williams *et al* (1996)).

COX-2 is constitutively expressed in some cancers; COX-2 specific inhibitors suppress cell proliferation and induce apoptosis in pancreatic cancer cells (Ding *et al* (2000))

NF-κB has been found to regulate COX-2 expression and PG synthesis in AGS cells. Inhibition of constitutive NF-κB results in inhibition of COX-2 expression and proliferation of gastric cancer cells (Lim *et al* (2001)). NF-κB translocates to the nucleus and induces the COX-2 gene, which catalyses the synthesis of pro-inflammatory prostaglandins, particularly PGE_2 (Rossi *et al* (2000)).

Growth of the androgen-unresponsive PC-3 human prostate cancer cell line was stimulated *in vitro* by the presence of LA. LA is metabolised by desaturase and elongase to AA, the immediate precursor for PG and leukotriene (LT) eisocanoids. EPA and docosahexaenoic acid (DHA) inhibited PC-3 cell growth in a dose dependant manner. EPA competes with AA for incorporation into phospholipids and for the enzymes involved in PG synthesis (Rose *et al* (1991)).

The effects of ω3 fatty acids on prostaglandin synthesis result from the ability of ω3 fatty acids such as EPA, to replace the more common ω6 polyunsaturated fatty acids at the sn-2 position of glycerophospholipids, which are the precursors of the eicosanoids (Smith (1989)).

AA strongly up-regulates NF-κB nuclear translocation in the human promonocytic cell line U937 (Camandola *et al* (1996)). EPA did not influence the activity of this transcription factor. The effect of AA was inhibited by indomethacin, indicating AA eicosanoid metabolites to be responsible for NF-κB up-regulation as enhanced NF-κB binding activity was seen in response to PGE_2 (Camandola *et al* (1996)).

However, the role of prostaglandins in the regulation of muscle protein breakdown is controversial. McKinely and Turinskey (1986) found rats with hindlimb scald burn injury experienced a 354% increase in PGE_2 in the affected limb. However, net proteolysis could not be attenuated with 90% inhibition of PGE_2 production by aspirin or indomethacin.

Hasselgren *et al* 1990 examined the influence of AA, PGE_2 and indomethacin on total and myofibrillar protein breakdown in rat extensor digitorum longus and soleus muscle. The addition of AA and PGE_2 did not affect total or myofibrillar protein breakdown. Indomethacin inhibited muscle PGE_2 production in incubated muscles from septic rats but

did not lower proteolytic rates. *In vivo* administration of indomethacin did not affect total or myofibrillar muscle protein breakdown either.

Roe *et al* (1997) examined the role of prostaglandins in T-cell induced leukaemia which results in rapid and severe cachexia. The study demonstrated peripherally produced prostaglandins to be involved in the increased energy metabolism and body temperature of leukemic rats. However, prostaglandins are not likely to be involved in the long term changes in food intake and body weight as observed in cachexia.

This suggests protein breakdown in skeletal muscle is not regulated by PGE_2 or other prostaglandins, although they may contribute in some models.

PIF stimulated AA release from skeletal muscle cells, which is metabolised to prostaglandins E_2, $F_{2\alpha}$ and to 5- 12- and 15- hydroxyeicosatetraenoic acids (HETES) (Smith *et al* (1999)).

Total protein breakdown in response to PIF was related to the release of AA and formation of PGE_2. However PGE_2 is not the eicosanoid responsible for the effect. Although AA is converted into a range of prostaglandins and HETEs, only 15(*S*)-HETE was capable of directly inducing protein degradation (Smith *et al* (1999)).

Cyclooxygenase (COX) is a rate-limiting step in the conversion of AA into prostaglandin endoperoxide. Increased expression of cyclooxygenase isoforms COX-1 and COX-2 have been reported in various tumour models, leading to speculation of a role for COX inhibition of apoptosis and increased tumour invasiveness (Hussey and Tisdale (2000)).

The specific COX-2 inhibitor meloxicam was administered to mice bearing the MAC13 and MAC16 adenocarcinomas (Hussey and Tisdale (2000)). Meloxicam significantly suppressed the growth of both tumours and attenuated cachexia in the MAC16 bearers. *In vitro,* meloxicam significantly inhibited PIF-induced protein degradation in C_2C_{12} myotubes, suggesting this agent to directly antagonise muscle catabolism.

Further studies have demonstrated 15(*S*)-HETE to induce an increase in expression of the ubiquitin-proteasome pathway, responsible for the initiation of protein catabolism involving the transcription factor NF-κB (Whitehouse *et al* (2003)).

It was observed that PIF activated PLA_2 which is responsible for catalysing the release of AA from membrane phospholipids (Smith and Tisdale (2003)). PLA_2 is also involved in PIF

induced proteasome expression (Gomez-Marcondes *et al* (2002)). In addition to PLA_2, Phospholipase C (PLC) was shown to be involved in PIF-induced proteasome expression, attenuated by using PLC inhibitors U73122 and D609. Diacylglycerol (DAG) derived from PC-PLC is suggested to provide a positive feedback signal to Protein Kinase C (PKC) (Smith and Tisdale (2003)).

PIF induces translocation of PKCα from the cytosol to the membrane at the same concentrations that induce proteasome expression. PKCα is an upstream activator of the I-κB kinase complex, which phosphorylates IκBα at Ser^{32} and Ser^{36} leading to ubiquitination and subsequent proteolysis (Hasselgren *et al* (1990)). This indicates a mechanism of how PIF induces IκBα degradation and subsequent translocation and nuclear accumulation of NF-κB. This results in increased expression of the ATP-ubiquitin-dependent proteolytic pathway responsible for the massive catabolism of lean body mass as observed in cancer cachexia (McKinely and Turinskey (1986)).

1.10.1.1 Glucocorticoids

Glucocorticoids have been found to activate protein catabolism during fasting and are deleterious to skeletal muscle, by inducing atrophy of fast twitch myofibres. Glucocorticoids impair myofibrillar protein synthesis and increase protein degradation (Llovera *et al* (1996)). Excess glucocorticoid levels have been linked with pathological conditions such as Cushing's syndrome, and are implicated in muscle catabolism associated with trauma, sepsis and cancer cachexia (Llovera *et al* (1996)).

Glucocorticoids regulate the ubiquitin-proteasome system in muscle, and when present in a high physiological range they activate proteolysis. Glucocorticoids administered *in vivo* to rats induce muscle atrophy, causing a transient increase in mRNA encoding for the ubiquitin-proteolytic pathway (Price (2003)).

Muscle protein catabolism present in rats with insulin-dependent diabetes is associated with increased glucocorticoid production and mRNA encoding components of the ubiquitin-proteasome system (Marinovic *et al* (2002)).

Metabolic acidosis, acute diabetes, sepsis and starvation studies in rats have demonstrated that glucocorticoids are required for the rise in mRNA encoding ubiquitin and proteasome subunits as well as the increase in protein degradation (Mitch *et al* (1999)).

Marinovic *et al* (2002) found that glucocorticoids regulate the transcription of ubiquitin, by *UbC* gene induction involving the Sp1 and MEK1 and MAPK pathway in rats with insulin dependant diabetes.

The role of glucocorticoids and their involvement in the induction of cancer cachexia was investigated by Llovera *at al* (1996). Rats bearing the Yoshida AH-130 ascites hepatoma show rapid body weight loss and tissue wasting, especially depletion of skeletal muscle as a result of increased protein degradation. The anti-progestational agent RU38486 (mifepristone) is a glucocorticoid receptor antagonist with no agonist activity. RU38486 was unable to prevent the accelerated protein breakdown and consequent muscle depletion in the Yoshida AH-130 tumour model.

Although glucocorticoids may produce emaciation in sepsis and increased mRNA encoding for the ubiquitin proteasome pathway in insulin dependent diabetes, they do not appear to play an essential role in the activation and hypercatabolism of skeletal muscle during cancer cachexia.

1.10.2 Other factors implicated in cancer cachexia

1.10.2.1 Leptin

Leptin is a peptide member of the cytokine receptor family, an anorexigenic peptide, primarily produced by fat cells. Leptin is a 16kDa hormone product of the *ob* gene which is produced by adipocytes and is tightly associated with body weight homeostasis. Leptin regulates the central processes of appetite control and metabolic rate (Aleman *et al* (2002)).

Rodent models demonstrate *ob* gene expression and circulating leptin levels to be modulated by a variety of host factors including; insulin, glucocorticoids, catecholamines and cytokines. In humans circulating leptin levels correlate closely with total body fat mass. However, glucose and insulin do not appear to modulate *ob* gene expression and leptin production (Hardie *et al* (1996)).

Anorexia and hyper-metabolism play a role in the development of cancer cachexia, it has been hypothesised that increased leptin secretion could be involved in cancer cachexia pathogenesis.

Administration of leptin *in vivo* causes a dramatic reduction in fat deposits in white adipose tissue (WAT) of rodents. Leptin exerts a direct effect on peripheral tissues and adipocytes characterised by the stimulation of acetyl CoA degradation (Ceddia *et al* (2000)).

Simons *et al* (1997) reported low or undetectable leptin concentrations in patients with lung cancer and weight loss. Wallace *et al* (1998) found circulating leptin concentrations in gastrointestinal cancer not to be related to the inflammatory response. Lopez-Soriano *et al* (1999) demonstrated that anorexia was not related to leptin changes in experimental cancer, thus suggesting cancer cachexia is not due to a dysregulation of leptin production.

Aleman *et al* (2002) concluded that serum leptin levels are not raised in patients with advanced non-small cell lung cancer and production is not induced by the inflammatory response. Concentrations of leptin were appropriate for the amount of body fat.

Therefore it appears that cancer anorexia and cachexia are not caused by a dysregulation of leptin production.

1.10.2.2 Lipoprotein Lipase Inhibitors

Tsujimoto *et al* (2000) postulated that a reduction in lipoprotein lipase (LPL) activity to be involved in the induction of cachexia in cancer patients. LPL activity was reduced in C57BL/6 mice transplanted with the cachexigenic EL-4 lymphoma. Elevated blood triglyceride and non-esterified fatty acid levels were observed in EL-4 bearing mice. This increase was associated with impaired plasma LPL activity.

Mori *et al* (1989) analysed conditioned medium of the human melanoma cachexigenic SEKI cell line. A 40kDa protein was isolated, effective in inhibiting LPL activity in 3T3-LI adipocytes and termed melanoma-derived LPL inhibitor (MLPLI). Amino acid sequencing revealed 13 amino acids present in the amino-terminal portion of MLPLI to be identical to those found in leukaemia inhibitory factor (LIF).

Mori *et al* (1991) further characterised MLPLI and LIF. Four melanoma xenografts, SEKI, G361, A375 and MEWO were analysed. Both SEKI and G361- bearing nude mice developed cancer cachexia, and northern blot analysis revealed increased expression of LIF mRNA in these two xenografts. The A375 and MEWO bearing nude mice did not develop cachexia. This implies that LIF may be involved in the development of cachexia.

Kawakami *et al* (1991) also purified a heat liable peptide >25 kDa from the SEKI cell line, capable of inhibiting LPL activity in differentiated 3T3-L1 adipocytes. This factor was not immunoreactive with any cytokines implicated in cachexia. However conditioned medium from four different types of tumour cell including human and murine melanomas failed to suppress LPL activity. This study suggested that the secretion of an LPL suppressing substance is not a specific characteristic of all cultured tumour cells.

Nara-Ashizawa *et al* (2001) analysed conditioned media from LS180, MKN-1, MMG-1, C32 and LX-1 cachexigenic human cell lines. The media from all cell lines significantly suppressed LPL activity in 3T3-L1 cells. Media from LX-1 and C32 promoted lipolytic activity in a dose dependant manner. RT-PCR and ELISA analysis identified the major factor responsible for LPL inhibition as a 65kDa heat vulnerable protein and a heat stable lipolytic factor < 1kDa. These factors were not IL-1β, IL-6, IL-11, TNF-α, TGF-β1 or LIF. In the same study Nara-Ashiwaza could not generate significant lipolysis *in vitro* with ZAG at a dose of 10mg/ml.

Although lipoprotein lipase inhibitors may be important in the development of cachexia, there is no compounding evidence to suggest their sole responsibility for the massive depletion of adipose tissue as associated with cancer cachexia.

1.11 The role of cytokines in cancer cachexia

A lot of work has been undertaken to identify specific factors responsible for the induction of cachexia. Investigations have identified many possible candidates for instigating cachexia. These factors can be defined by two categories: of tumour origin and humoral origin.

The immune system produces a large number of cytokines which target a variety of tissue types such as bone marrow, myocytes, hepatocytes and adipocytes. This culminates in a biological response and leads to the wasting associated with cancer cachexia.

Matthys and Billiau (1997) reviewed cytokines as a diverse group of soluble glycoproteins, and low molecular weight peptides. Cytokines are hormone-like proteins acting as chemical messengers between cells and the body, acting as paracrine or autocrine rather than endocrine messengers

Cytokines interact with cells via high-affinity receptors, usually glycoproteins that are located on cell membranes, linked to intracellular second messenger signalling pathways. Cells are rarely confronted with a single cytokine, but several sets interacting synergistically or antagonistically with each other. Cytokines form local immune networks, essential in the defence against noxious influences or foreign factors such as viruses, bacteria and parasites as well as cancer cells.

The concept that cytokines are involved in cachexia arose from the observation that the metabolic derangements in cancer patients, was similar with those seen in infectious diseased states (Matthys and Billiau (1997)).

Sharma *et al* (2002) found levels of cytokines and catabolic hormones correlated with the reduction of muscle, fat and bone tissue content in cardiac cachexia. X-ray absorptiometry confirmed chronic heart failure patients to have reduced body fat, lean tissue mass and bone mineral density.

Plata-Salaman (2001) reviewed cytokine involvement in cancer cachexia in mice, following intracerebral or peripheral tumour implantation. It was concluded that central nervous activities (CNS) of cytokines are involved in the induction of anorexia and wasting. Hypothalmic feeding-associated sites are targets from cytokine action and regulate cytokine-induced anorectic responses.

The cytokines implicated in cancer cachexia include tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin-6 (IL-6), interferon gamma (IFN-γ) and leukaemia inhibitory factor (LIF).

1.11.1.1 Leukemia Inhibitory Factor (LIF)

Mori *et al* (1989) isolated a 40kDa melanoma derived lipoprotein lipase inhibitor (MLPLI) from the conditioned media from the SEKI (human melanoma) cell line. MLPLI shared amino acid sequence homology with LIF. Mori *et al* (1991) demonstrated that cachexigenic SEKI and G361 xenografts in nude mice induced cancer cachexia, accompanied by increased mRNA expression of LIF.

Mice inoculated with tumour cells of a hemopoietic cell line that over express LIF developed a fatal syndrome characterised by weight loss, pancreatitis, thymus atrophy and abnormalities in the adrenal cortex and calcium metabolism (Matthys and Billiau (1997)).

Metcalf *et al* (1990) reported that exogenously added recombinant LIF induced body weight loss in experimental animals.

However these findings do not mean that MLPLI or LIF are solely responsible for cancer associated cachexia in cancer patients.

1.11.1.1 Ciliary Neurotrophic Factor (CNTF)

Ciliary neurotrophic factor (CNTF) is a naturally occurring protein with a molecular mass of 22kDa. CNTF is structurally and functionally related to a number of cytokines including LIF, IL-6, IL-11 and oncostatin M (OSM).

Sleeman *et al* (2000) found *in vivo* administration of CNTF to suppresses appetite and induces fat loss in *ob/ob* mice. These effects may be mediated by specific activation of hypothalamic areas, which regulate food intake and body weight and express the CNTF receptor α (CNTFRα).

Espat *et al* (1996) reported that administration of 25g/kg of CNTF to healthy C57B1/6 mice induced profound anorexia and lean tissue wasting, with 21% loss of carcass protein due a 218% increase in protein degradation when compared to controls.

Similarly Martin *et al* (1996) demonstrated muscle wasting properties of recombinant human CNTF (rhCNTF) when administered to rats. Recombinant inhibitors of the cachectic cytokines, TNF-α and IL-1 did not significantly alter the wasting effects of rhCNTF. Suggesting that rhCNTF induces atrophy of skeletal muscle involving anorexia and cachexia, independent of the classic cytokines involved in cachexia.

However, Lambert *et al* (2001) reported that CNTF-induced weight loss to be distinct from that experienced by cachectic cancer patients. CNTF mimics the ability of leptin to cause fat loss in *ob/ob* mice but can also activate hypothalamic leptin-like pathways in diet induced obesity models unresponsive to leptin. CNTF acts in a different manner than the prototypical cachectic cytokines.

It is difficult to elucidate a role for CNTF in cancer cachexia. It is possible that it may be involved in some manner, but the mechanisms of action need to be fully elucidated.

1.11.1.2 Tumour Necrosis Factor – alpha (TNF-α)

Tumour necrosis factor-α is a 17kDa protein, a potent proinflammatory cytokine important in immunity and inflammation. TNF-α acts as an endogenous mediator during host immune, inflammatory and metabolic processes. A primary function of TNF-α is its involvement in the host response to invasive pathogens.

TNF-α was originally termed "cachectin" because of the apparent association between this cytokine and cachexia (Espat *et al* (1994)).

Exposure of cells to TNF-α can result in the activation of a caspase cascade leading to apoptosis. More commonly, the binding of TNF-α to its receptors causes activation of two major transcription factors, AP-1 and NF-κB, which in turn induce genes involved in chronic and acute inflammatory responses (Baud and Karin (2001)).

TNF-α binding to surface receptors stimulates a stereotypical cascade of events, resulting in degradation of I-κBα, TNF-α thereby activates NF-κB, with translocation to the nucleus (Baud and Karin (2001)).

Truyens *et al* (1995) investigated the role of TNF-α in cachexia induced in BALB/c male mice infected with Trypamosoma cruzi, the causative agent of Chaga's disease. These rodents experienced extreme cachexia, with a weight loss of 20%. This was associated with massive depletion of fat stores (60 - 80%) and an increase in water gain, with no alterations in food or water intake. Administration of anti TNF-α monoclonal antibody during the first two weeks of infection significantly attenuated the weight loss. Similar administration of anti IL-6 or anti IFN-γ did not improve tissue wasting. Therefore it was concluded that TNF-α is an important factor in this cachexia model (Truyens *et al* (1995)).

Tayek *et al* (1990) studied the effect of TNF-α in the Walker 256 carcinoma model in Sprague-Dawley rats. Administration of TNF-α stimulated tumour protein degradation, with an overall decrease in tumour growth. This may be the result of increased synthesis of proteases, which enhance protein catabolism in the tumour tissue.

Tessitore *et al* (1993) investigated weight loss associated with the YAH-130 ascites hepatoma. They found increased catabolism of skeletal muscle is associated with high plasma concentrations of PGE_2 and TNF-α, suggesting a network of factors including classical hormones and cytokines to concur, resulting in enhanced protein catabolism.

Costelli *et al* (1993) found treatment of YAH-130 tumour bearing rats with a polyclonal goat anti-TNF IgG preparation decreased protein degradation rates in heart, liver and gastrocnemius muscle but did not affect weight loss.

Skeletal muscle was isolated from rats treated with TNF-α (Llovera *et al* (1997)) and from Yoshida AH-130 tumour bearing rats, (Llovera *et al* (1994)), demonstrated an increase in skeletal muscle polyubiquitin gene expression. Llovera *et al* (1996) administered a polyclonal goat anti-TNF IgG preparation to YAH-130 tumour bearing rats, and found it abolished the increase in muscle ubiquitin gene expression as seen in the controls. TNF-α is important in activating the ubiquitin-dependent proteolytic pathway resulting in increased skeletal muscle catabolism (Llovera *et al* (1997)).

Yi-Ping *et al* (2000) determined the importance of NF-κB as an essential mediator of TNF-α induced catabolism in differentiated C_2C_{12} muscle cells. Using C_2C_{12} myoblasts transfected with viral plasmid constructs inducing over-expression of mutant IκBα proteins, insensitive to degradation, therefore selectively inhibiting NF-κB activation in response to TNF-α.

Yi-Ping *et al* (2000) found differentiated myotubes with the control (empty) viral vector experienced a drop in total protein and fast-type myosin heavy chain content, however neither were affected by TNF-α in the transdominant negative cell lines.

Karayiannakis *et al* (2001) evaluated serum levels of TNF-α in 63 pancreatic cancer patients and detected sTNF-α in 36.5% patients. sTNF-α levels were associated with metastasis and advanced disease and correlated with poor nutritional status. Patients with sTNF-α demonstrated significantly lower body weights, lower haemoglobin and total serum protein levels.

Mahony *et al* (1988) compared weight loss produced by TNF-α, with pair fed controls with restricted food and water intake, and mitozolomide (at a toxic dose induces weight loss by anorexia). It was established that TNF-α produced a dose related weight loss proportional to decreased food and water intake. Therefore TNF-α was found capable of inducing anorexia.

Further studies into the relationship of TNF-α and weight loss were reported by Mahony *et al* (1988). It was established that the degree of weight loss following administration of TNF-α is directly proportional to the decreased food and water intake. In contrast, the

MAC16 tumour model produces weight loss, without a reduction in fluid or nutrient intake.

Both the MAC16 tumour and TNF-α produced hypoglycaemia and a reduction in circulatory FFA, but had opposite effects on plasma triglyceride levels. In the MAC16 tumour model a decrease is observed whereas TNF-α produced an increase (Mahoney *et al* 1988). A significant finding of this study was that no TNF was detected in the MAC16 tumour, or in the serum of tumour bearing mice. However both tumour and non-tumour bearers responded with a similar elevation of TNF-α levels 90 min after injection with endotoxin.

Therefore the weight loss and extensive depletion of skeletal muscle and adipose tissue in the MAC16 model is occurring independently of TNF-α. Beck *et al* (1990) investigated the ability of megestrol acetate to reverse weight loss induced by TNF-α and the MAC16 tumour model. In both cases there was a significant increase in food and water intake. However weight gain in TNF-α and MAC16 tumour bearers was the result of increased body water content and not due to any increase in lean body mass.

Mulligan *et al* (1992) reported weight loss in MAC16 tumour bearers to occur without a decrease in food or water intake, hence distinct from weight loss associated with TNF-α. Anti-TNF monoclonal antibodies at a sufficient dose against endotoxinaemia were ineffective in reversing weight loss in MAC16 tumour bearers.

Episodic administration of TNF-α has proved unsuccessful in inducing cachexia *in vivo* (Argilés *et al* (2003)). Initially TNF-α administration produces a cachectic effect, although tolerance to the cytokine develops, whereby food intake and body weight return to normal (Argilés *et al* (2003)).

Porter *et al* (2002) reported that rat epididymal adipose tissue exposed to TNF-α at a dose of 4nmol/L responded significantly with increased glucose metabolism and lactate production and enhancement of lipolysis.

The catabolic effects of TNF-α on adipocytes include a decrease in activity of lipoprotein lipase (Grunfield *et al* (1989)), a decreased expression of the glucose transporter GluT4 (Stephens *et al* (1991)) and increased lipolysis which suggests increased activity of the hormone sensitive lipase (Patton *et al* (1986)).

There is contradictory evidence to support the role of TNF-α and the extent of its importance in the development of cancer cachexia. TNF-α may be involved in some models, but its sole importance is questionable.

1.11.1.3 Interleukin- 1 (IL-1)

IL-1 was originally termed 'leukocyte pyrogen' and it was found that in the hypothalamus it stimulated the release of arachidonic acid and synthesis of PGE_2. IL-1 also induces fever and is associated with the acute phase protein response (Baracos *et al* (1983)).

In muscles incubated *ex vivo* at 37°C, IL-1 stimulated net protein degradation by 62 - 118 per cent, with no observed change in protein synthesis. IL-1 also stimulated muscle synthesis of PGE_2, which promotes skeletal muscle protein breakdown (Baracos *et al* 1983)). A tumour induced IL-1 response could therefore potentially account for the catabolism of lean body mass, fever, malaise and increased REE observed in cancer cachexia.

Nakatani *et al* (1998) used C3H/He mice bearing MH-134 tumour cells to investigate circulating IL-1β mRNA levels. They found IL-1β production significantly increased in the spleen of tumour bearing mice compared to control animals. There was no significant increase in other cytokine mRNA, suggesting that over expression of IL-1β mRNA occurs in response to tumour burden

Barber *et al* (2000) studied genomic DNA from 64 pancreatic cancer patients compared to 101 healthy controls. A polymorphism of the IL-1β gene was detected and had a significant effect on patient survival time. The possession of the genotype resulting in increased IL-1β production was associated with a shorter survival time and increased serum C- reactive protein levels. This suggested a role of IL-1β in inducing an acute phase protein response in cancer cachexia.

However Costelli *et al* (1995) found no correlation between the induction of cancer cachexia in the Yoshida AH-130 model and IL-1 production. The IL-1 receptor antagonist (IL-ra) failed to modify the acceleration of muscle protein turnover in the untreated YAH-130 bearers.

Observations' concerning the circulating levels of IL-1 present in the serum of cancer patients appears inconsistent. Moldawer et al (1988) failed to detect any IL-1 bioactivity in

the plasma of weight losing cancer patients. Similarly, Costelli *et al* (1995) didn't detect any circulating levels of IL-1 in the YAH-130 tumour bearers.

Moldawer *et al* (1987) investigated the ability of human and murine recombinant IL-1α, IL-1β and TNF-α to affect skeletal muscle protein synthesis and degradation *in vitro* and *in vivo*. It was found that neither recombinant IL-1 or TNF-α had any impact on skeletal protein balance.

It would therefore appear that IL-1 is not a direct mediator of weight loss and depletion of lean body mass as observed in cancer cachexia, although, it may be a contributing factor in some cancer models.

1.11.1.4 Interleukin- 6 (IL-6)

IL-6 is attributed multiple biological activities such as regulation of immune responses, haematopoiesis and inflammation. IL-6 mediates rheumatoid arthritis, Castleman disease, psoriasis, cardiac myxoma and cachexia. The IL-6 receptor interacts with the signal transducer gp130, and activates the *Ras* dependant MAPK cascade. Several other cytokines use gp130 as a common signal transducer, including, oncostatin M, IL-11, LIF and CNTF (Ogata *et al* (1999)).

The development of cancer cachexia has been linked to IL-6 related cytokines. Barton *et al* (2001) investigated the role of IL-6, IL-11 and Oncostatin M (OSM) involvement in cancer cachexia. They used a lung carcinoma line grown in C57BL/6J mice and found cytokine expression detectable within 3 days for IL-6, IL-11 and 1 day for OSM. Cytokine expression preceded the onset of cachexia which developed at 7-14 days.

Strassman *et al* (1992) investigated the C-26.IVX cell line derived from the murine colon 26 adenocarcinoma cachexia model, which retains transplantability of the original tumour and induces cachexia in synergistic hosts. Increased levels of IL-6 were observed in C-26.IVX tumour bearing mice that correlated with development of cachexia. Weight loss began at a tumour burden of <3% (0.7g) and constituted wasting of muscle and adipose tissue. A monoclonal antibody to murine IL-6 successfully suppressed the development of cachexia in the tumour bearing mice.

Scott *et al* (1996) looked at IL-6 levels in non-small-cell lung cancer patients (NSCLC), n=21, with and without weight loss. Circulating levels of IL-6 were consistently higher in the

group with at least 5% weight loss. There was a significant correlation between circulating levels of IL-6 and C-reactive protein, consistent with IL-6 as a modulator of the acute phase protein response. This suggested a role for IL-6 in the development of cancer cachexia in NSCLC.

Ebisui *et al* (1995) concluded IL-6 to be an important mediator capable of inducing intracellular proteolysis in C_2C_{12} myotubes. Human recombinant IL-6 increased activity and transcription of cathepsin B and L, and the 26S and 20S proteasomes. IL-6 directly increased protein degradation of intracellular proteins (Ebisui *et al* (1995)).

However García-Martínez *et al* (1994) failed to correlate IL-6 with detectable protein breakdown in rat skeletal muscle. Recombinant human IL-6 was added to either soleus or extensor digitorum longus (EDL) muscle, and failed to exert an effect on protein degradation or protein synthesis.

Soda *et al* (1994) transplanted the cachexigenic subclone 20 adenocarcinoma and a similar, non cachexigenic subclone 5, of the colon26 adenocarcinoma into CDF_1 mice. Induction of cachexia in mice transplanted with clone-20 was accompanied by a rise in blood IL-6 levels. However, this was also observed in mice transplanted with clone-5, which were not experiencing cachexia, thus some other circulatory factor may be contributing to cachexia in the subclone 20 group.

Fujimoto *et al* (1995) investigated the mechanisms of cachexia in the murine colon 26 carcinoma subclone 20 (cachexia inducing) and subclone 5 (non cachectic) tumour bearing mice. Neither TNF-α, IL-1 or IFN-γ was detected in the serum of mice bearing each clone, whereas IL-6 serum levels were increased with both clones. Administration of anti IL-6 monoclonal antibody suppressed cachexia in the clone 20 tumour bearing mice. However, raised IL-6 serum levels were detected in both clones, therefore cachexia induction in clone -20 tumour bearing mice cannot be fully attributed to the role of IL-6.

Soda *et al* (1994) found that mice bearing the clone-20 tumour experienced massive depletion of adipose tissue. However, this was not linked with the increased circulatory levels of IL-6. IL-6 had no intrinsic lipolytic activity *in vitro* when incubated with 3T3 L-1 adipocytes. Therefore, the massive weight loss and depletion of adipose tissue in the clone-20 mice could not be attributed to IL-6.

Yasumoto *et al* (1995) administered an anti IL-6 antibody to subclone 20 mice and found it to partially inhibit weight loss. The findings from this study suggested IL-6 contributes to the establishment, but is not sufficient for the development of cachexia.

This was further demonstrated by Soda *et al* (1995). Human recombinant IL-6 was administered to CDF_1 mice bearing clone 20 and clone 5 adenocarcinomas, derived from the colon 26 model at 1-10μg/ day. Administration of IL-6 did not induce weight loss or decreased food intake in clone-20 tumours.

Circulating levels of IL-6 were not elevated in MAC16 tumour bearers, suggesting that these cytokines are not involved in cancer cachexia produced by the MAC16 adenocarcinoma (Mulligan *et al* (1992)).

Hussey *et al* (2000) found the fluorinated pyrimidine nucleoside, 5'-deoxy-5-fluorouridine (5-dFU Urd) to attenuate the progress of cachexia in the MAC16 and colon 26 murine adenocarcinoma models. Mice bearing the colon 26, clone 20 variant demonstrated evidence of PIF in the tumour, serum and urine. There was no evidence for the presence of PIF in animals bearing the clone 5 variant of the colon 26 adenocarcinoma. Treatment of mice bearing the clone 20 variant with 5'-dF Urd led to the disappearance of PIF from the tumour, serum and urine concomitant with the attenuation of the development of cachexia. These results suggest that cachexia appears to be correlated with the production of PIF, and not the result of IL-6 in the colon 26 adenocarcinoma model.

It seems that IL-6 is a permissive factor in the development of cachexia. IL-6 is capable of inducing some of the typical symptoms associated with cachexia, but it cannot induce the full cachectic syndrome.

1.11.1.5 Interferon gamma (IFN-γ)

IFN-γ is produced by T lymphocytes and has anti viral activity, inducing expression of MHC class I and class II in macrophages and other cells. IFN-γ induces differentiation of cytotoxic T lymphocytes and promotes synthesis of IgG2a by activated B cells. IFN-γ is also able to antagonise several IL-4 actions (Roitt (1997)).

Cahlin *et al* (2000) transplanted MCG101 tumours on wild type C57B1 and gene knockout mice to assess the role of host produced cytokines in tumour growth, anorexia and carcass weight loss. The absence of host derived IL-12 and IFN-γ did not attenuate tumour

growth or improve cachexia. The results from this study support the conclusion that host production of IFN-γ and IL-12 is not involved in the development of cachexia in the MCG101 model.

Maltoni *et al* (1997) investigated the correlation between serum levels of circulating cytokines in cancer anorexia-cachexia syndrome (CACS), which is characterised by weight loss and anorexia. There was no significant correlation between circulating levels of TNF-α, IL-1, IL-6 and IFN-γ and weight loss.

These results imply that IFN-γ is not directly related to the onset of cachexia.

All the data on cytokine involvement in the development of cancer cachexia implies that cachexia can rarely be attributed to a single cytokine, but rather a set of cytokines working together (Matthys and Billiau (1997)). Cancer cachexia is a consequence of many metabolic disorders and is not the result of one cytokine or factor. It is important to understand the factors that are involved *in vitro* and *in vivo* to develop a greater understanding and improve strategies of treatment.

1.12 Therapeutic Intervention

The most effective method to alleviate cancer cachexia is to remove the cancer. In many advanced cases, as with unresectable pancreatic cancer this is not possible. Other means of intervention are required to ameliorate cancer cachexia.

1.12.1 Anticytokine Therapy.

Specific antibodies against IFN-γ, IL-6 and TNF-α combined with the cyclooxygenase inhibitor indomethacin have been used to reverse cancer cachexia (Yoshikawa *et al* (1996)).

Haslett *et al* (1998) reported a trial using TNF-α inhibitors pentoxyfylline and thalidomide, in reversing weight loss in cachexia and chronic disease. Pentoxyfylline did not show any efficacy in reversing weight loss. Fearon and Moses (2002) reported pentoxyfylline was able to inhibit TNF-α production and suppress protein degradation in a tumour-bearing rat model. However in a controlled trial involving cancer cachexia patients, no benefits were reported when compared to the placebo.

The administration of thalidomide to patients with HIV or tuberculosis, experiencing weight loss consistently resulted in weight gain. However, the metabolic benefits of thalidomide to its complex effect on the immune system are not well understood (Haslett *et al* (1998)).

1.12.1.1 Total Parenteral Nutrition (TPN)

TPN is an alternative to nutrition via the gastrointestinal tract and can provide sufficient nutrients to achieve the positive nitrogen balance and weight gain in non cancer cachexia patients.

Nixon *et al* (1981) studied a group of metastatic colon cancer patients in receipt of TPN. These patients gained an average 2.9kg/ 3-4 weeks. Copeland (1984) reported similar results with a larger group of 175 patients. However the weight gain associated with TPN treatment is the result of increased water and fat, rather than an increase in lean body mass (Nixon *et al* (1981)).

Weinger *et al* (1985) reported a study involving 119 small cell lung cancer patients where serious complications occurred in patients receiving TPN. Patients experienced sepsis, fluid overload, hyponatraemia and hyperglycaemia and there was no clinical benefit observed.

Although TPN is undoubtedly important in overall patient survival, in cancer cachexia there is no reported benefit to patients. Any weight gain is the result of increased water and fat, with no increase in lean body mass.

1.12.1.2 Megestrol Acetate

Megestrol acetate is a synthetic, orally active progesterone, widely used as therapy for advanced breast cancer and clinically produces a weight gain in more than 80% of all treated patients (Aisner *et al* 1988)

López *et al* (2004) reviewed megestrol acetate in the treatment of anorexia-cachexia syndrome (ACS) and found it to have beneficial effects when compared to placebo effects in patients with ACS, and no adverse side effects were observed.

Beck and Tisdale (1990) investigated the effect of megestrol acetate on TNF-α and MAC16 tumour bearing mice weight loss. Megestrol acetate reversed weight loss induced by TNF-α

by increasing food and water intake. MAC16 tumour bearers also experienced weight gain, however body composition analysis determined this to be result of increased water intake and tumour weight. Weight reversal in TNF-α treated animals by megestrol acetate appears to be solely due to rehydration (Beck and Tisdale (1990)).

Loprinzi *et al* (1994) conducted a randomised trial with 342 patients suffering with cancer anorexia-cachexia. Patients received varied doses of megestrol acetate of 160, 480, 800 and 1280 mg/day. The trial concluded that patients experienced appetite increase and weight gain, however the weight gain was from increased fat mass

Fearon and Moses (2002) reviewed the effects of megestrol acetate on weight loss in cancer patients. Doses varying between 240 - 1600 mg per day increase appetite and food intake, with weight loss stabilising at 500mg twice a day. However there are a number of side effects associated with the administration of megestrol acetate, including, venous thrombosis and peripheral oedema. Weight gain in patients receiving megestrol acetate consists of fat and water retention.

More recently it has been implicated that megestrol acetate led to poorer survival in patient response to chemotherapy (Fearon and Moses (2002))

The frequency of oedema and the fact that patient weight increase is due to increased fat mass and water retention, with no attenuation in atrophy of lean body mass questions the use of megestrol acetate in the treatment of cancer cachexia.

1.12.1.3 Ketogenic Diets

Cancer cachexia involves extensive mobilisation of body fat reserves but ketosis does not occur. Ketone bodies are important in the regulation of lean body mass during starvation. It was suggested that a ketogenic diet of high fat/ low carbohydrate should preserve lean body mass during cancer cachexia (Brennan and Tisdale (1988)).

Medium chain triglycerides (MCT) and long chain triglycerides (LCT) were administered to MAC16 tumour bearing mice. MCT fed mice experienced reduced weight loss and tumour size, with no effect observed with the LCT diet (Brennan and Tisdale (1988)). MAC16 tumour bearers, in receipt of MCT, experienced increased plasma acetoacetate levels and 3-hydroxybuturate, a factor previously shown to inhibit the lipolytic and proteolytic factors associated with cachexia (Beck and Tisdale (1987)).

Fearon *et al* (1988) reported that a ketogenic diet containing 70% MCT, supplemented with D-3-hydroxybuturate caused weight gain in cachectic cancer patients.

1.12.2 Eicosapentaenoic acid (EPA)

Tisdale and Dhesi (1990) found cancer cachexia as observed in the MAC16 model is effectively reversed by a diet rich in the polyunsaturated fatty acids EPA and DHA. There was a reduction in tumour growth rate and an increase in total body fat and muscle mass. The results from this study suggested that fish oil is a non toxic, highly effective anti cachectic agent with anti tumour activity.

Tisdale and Beck (1991) demonstrated EPA to be effective inhibiting lipolytic activity of the MAC16 tumour extract. Elevation of cAMP in response to tumour derived LMF and EPA inhibited lipolytic hormones. *In vivo* administration of EPA to MAC16 tumour bearers prevented weight loss and tumour growth rate. Other ω3 fatty acids, including DHA, and LA found in marine fish oil were ineffective in inhibiting weight loss or MAC16 tumour growth (Tisdale and Beck (1991)).

MAC16 tumour bearers demonstrated increased protein degradation and decreased protein synthesis in skeletal muscle. EPA significantly attenuates protein degradation, with no effect on protein synthesis. Protein degradation is accompanied by an increase in PGE_2 content, which is inhibited by EPA. EPA becomes incorporated into membrane phospholipids and competes with arachidonic acid in response to PLA_2 (Tisdale (1996)).

Using isolated gastrocnemius muscle from MAC16 tumour bearers in the presence of inhibitors, there was no effect of EPA on lysosomal or calcium-dependent proteolysis pathways, but ATP-dependent proteolysis was completely inhibited. EPA antagonises the loss of skeletal muscle protein in cancer cachexia by down-regulation of proteasome expression (Whitehouse *et al* (2001)).

EPA inhibits 15(*S*)-HETE production, which has shown to induce protein degradation in murine myoblasts (Smith *et al* (1999)). Whitehouse *et a*l (2003) demonstrated 15(*S*)-HETE to induces degradation of myofibrillar proteins in differentiated C_2C_{12} myotubes via increased expression of the ubiquitin-proteasome proteolytic pathway, mediated by NF-κB. EPA inhibits degradation of IκBα and subsequent translocation of NF-κB to the nucleus. Therefore, down-regulating expression of the ATP-ubiquitin dependent proteolytic

pathway, responsible for the catabolism of lean muscle mass observed in cancer cachexia (Whitehouse *et al* (2003)).

Ross *et al* (2003) investigated the effect of EPA on NF-κB in the pancreatic cell line MIA PaCa2 and EPA was again found to perturb the NF-κB signalling pathway.

Jho *et al* (2002) reported rats implanted with the methylcholanthrene induced fibrosarcoma (MCA) responded well to treatment with EPA. EPA supplementation of 5g/kg per day resulted in weight stabilisation, reduced tumour volume and increased total liver and serum protein levels.

Wigmore *et al* (1996) investigated dietary supplementation with MaxEPA, a complex fish oil containing EPA. Eighteen patients were in receipt of 1g capsules/ day (containing 18% EPA and 12% DHA). Patients with a median weight loss of 2.9kg/ month prior to supplementation at 3 months experienced a median weight gain of 0.3kg/ month. In addition to weight change there was a significant reduction in the acute phase protein response and REE.

Several small-scale clinical studies have ensued and Wigmore *et al* (2000) found that EPA stabilised body weight and is well tolerated, with no toxic side effects.

Fearon *et al* (2002) conducted a randomised double blind trial with 200 advanced pancreatic cancer patients, investigating the benefit of a protein and energy dense N-3 fatty acid enriched supplement. This supplement resulted in a net gain of weight, especially lean body mass and an improved quality of life for the cancer patient.

It is apparent that cancer cachexia is a multifactoral problem and is difficult to treat. The complex nature of the disease means that TPN and megestrol acetate are unable to reverse weight loss. However ω3 fatty acid administration shows great promise in attenuating cachexia.

1.13 The role of the ubiquitin-dependent proteolytic pathway in cancer cachexia

Baracos *et al* (1995) investigated the role of the ATP-dependent proteolytic pathway in cancer cachexia as observed in rats implanted with YAH-130, experienced accelerated muscle proteolysis and atrophy in tumour bearing rats, was the result of activation of ATP-dependent proteolysis. Proteolysis was not affected by blocking the Ca^{2+} dependent

system or the lysosomal pathway. Inhibition of ATP production reduced muscle proteolysis to that of control levels.

Temparis *et al* (1994) implanted the Yoshida sarcoma in young rats to determine whether the tumour activates the Ca^{2+} dependent, lysosomal or the ATP-ubiquitin-dependent proteolytic pathway. Inhibitors of lysosomal and Ca^{2+}- dependent proteases failed to attenuate the increased proteolysis in skeletal muscle associated with cachexia. However, ATP depletion almost totally suppressed the increased protein breakdown. To confirm the role of the ATP-dependent proteolytic pathway, mRNA levels for ubiquitin conjugating enzyme E2, C8 and C9 proteasome subunits were found to be increased in the atrophying muscle (Temparis *et al* (1994)).

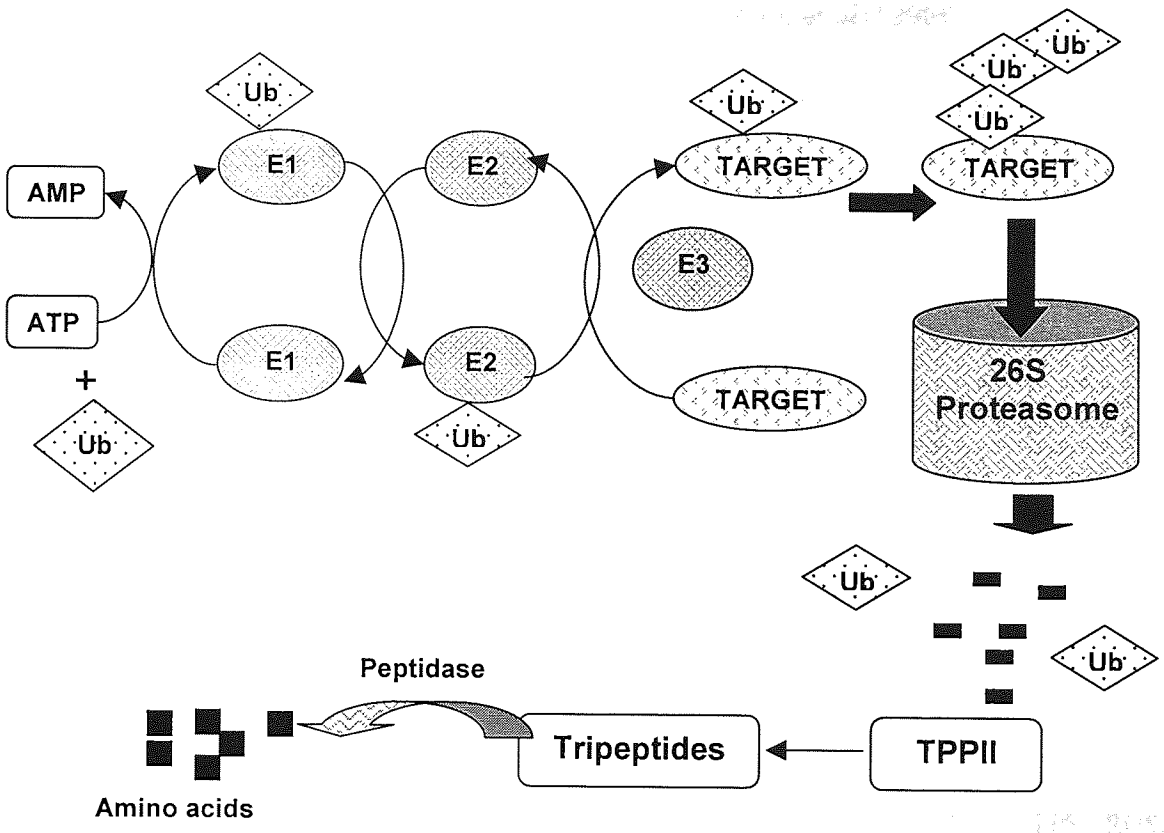
Bossola *et al* (2001) studied muscle ubiquitin mRNA levels in gastric cancer patients and confirmed an over-expression of muscle ubiquitin mRNA in cancer cachexia.

MAC16 tumour bearing mice also demonstrated increased expression of the components of the ATP-ubiquitin-dependent proteolytic pathway in skeletal muscle. The ATP-dependent proteolytic pathway is important in cancer cachexia and is responsible for the massive depletion of skeletal muscle and wasting of the cachectic host (Lorite *et al* (1998)).

Ubiquitin-dependent proteolysis of key regulatory proteins affects various cellular processes; cell cycle progression, transcription, antigen presentation, receptor endocytosis and signal transduction. Ubiquitin is a small 76 amino acid, highly conserved protein that is conjugated to other proteins through an energy dependent enzyme pathway (Voges *et al* (1999)).

Conjugation is initiated by activation of ubiquitin by the ubiquitin-activating enzyme, E1 which forms a high-energy ubiquitin-thiol ester bond in the presence of ATP. Activated ubiquitin is transferred to a ubiquitin conjugating enzyme, E2, to form an E2-thiol ester bond. Finally ubiquitin is transferred to a target substrate protein through an isopeptide linkage between the conserved C-terminal glycine residue of ubiquitin and the ε-amino group of the lysine residue of the substrate which is achieved via the ubiquitin ligase, E3. Sequential conjugation of the internal lysine residue of ubiquitin to the C- terminal residue of a new ubiquitin molecule results in a poly-ubiquitin chain, which targets proteins for degradation by the 26S proteasome (Sakamoto (2002)).

Figure 1.13.a: The ATP-ubiquitin dependent proteolytic pathway



The 20S proteasome is an intracellular protease responsible for the majority of cytosolic and nuclear protein degradation. The 20S proteasome forms the core of the larger 26S proteasome, essential for maintaining cell integrity; degrading abnormal proteins, short lived proteins responsible for regulation of the cell cycle, gene transcription and metabolism (Harris *et al* (2001)).

The 20S proteasome consists of 4 heptameric rings stacked upon each other to form a cylindrical particle. Two inner β-rings contain catalytic subunits with active sites facing the central proteolytic chamber. Two flanking α-rings separate the proteolytic chamber from the external solvent and association with the 19S cap complex (PA700) yields the 26S proteasome, responsible for the ATP-ubiquitin-mediated degradation of proteins (Harris *et al* (2001)).

Nuclear factor -κB was first identified by Sen and Baltimore (1986) as a B-cell specific nuclear binding protein. Virtually all vertebral cells express at least one of the five Rel/ NF-κB members; p50/p105 (NF-κB1), p52/100 (NF-κB2), c-Rel, p65 (RelA) and RelB which

are assembled into dimers. The most common complex is the p65/p50 dimer (Ghosh *et al* (1998). The composition of these dimers determines the affinity with which they bind to a loosely related set of 10 bp DNA sites (the κB sites) (Gilmore *et al* (1996)).

All Rel proteins contain a highly conserved 300 amino acid amino-terminal domain, the Rel Homology (RH) domain, containing the necessary sequences for the formation of dimmers, nuclear localisation, DNA and IκB binding (Gilmore *et al* (1996)). There are two classes of Rel proteins, distinguished by sequences to the carboxy-terminal to the RH domain. One class of Rel proteins include p50/p105 and p52/p100 which are precursor proteins (p105 and p100), regulated by proteolytic processing of the carboxy-terminal sequence to yield mature DNA binding proteins (p50 and p52) containing the RH sequence. Rel proteins contain carboxy-terminal ankyerin repeats which interact intramolecularly with amino terminal RH sequences (Gilmore *et al* (1996)).

The second class of Rel proteins including RelA, RelB and c-Rel are not processed and contain carboxy-terminal sequences to function as transcription activation domains. Heterodimers form between these two classes of Rel proteins; the best-characterised heterodimer is NF-κB, composed of p50 and RelA subunits (Gilmore *et al* (1996)).

NF-κB is a latent transcription factor, requiring cell stimulation (TNF-α, LPS, ROS, γ radiation and antineoplastic agents) for activation (Ghosh *et al* (1998)). Following stimulation a dedicated kinase complex, IKK, is induced. This results in phosphorylation-dependent degradation of IκB. Activation of NF-κB is the hallmark of the innate immune response, with the induction of genes encoding cytokines, chemokines, enzymes and anti microbial peptides. NF-κB helps mount the first line of defence against bacteria and fungi (Baldwin and Baltimore (1996)).

Almost all kinase pathways participate in NF-κB activation, including PKC, AKT/ PKB, JNK, MAP3Ks and MEKKs. All signals converge to activate IKK, leading to IκB phosphorylation, ubiquitination and degradation, enabling nuclear translocation and DNA-binding of NF-κB dimers (Amit *et al* (2003)).

The role of NF-κB is to provide a rapid response against environmental challenges. NF-κB already exists in the cytosol; therefore the immune and inflammatory responses regulated by NF-κB are very efficient (Lee and Buckart (1998)).

NF-κB is present in the cytoplasm as an inactive complex associated with an inhibitor called IκB. External or internal stimuli results in the dissociation of the NF-κB/ IκB complex following degradation of IκB. NF-κB subsequently translocates to the nucleus, where NF-κB binds to a specific DNA motif and regulates transcription of target genes (Amit *et al* (2003)).

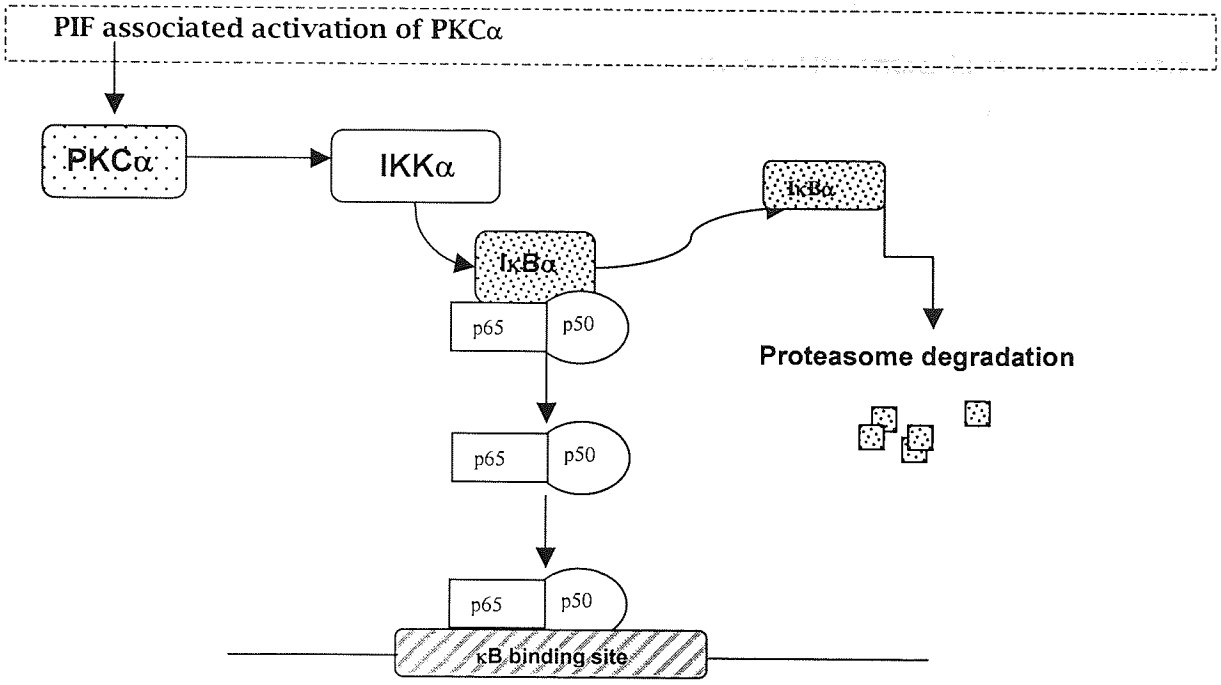
The transcriptional activity of NF-κB is regulated from the cytoplasmic compartment by IκBα, which is rapidly degraded during cellular activation (Beg *et al* (1992) and Beg *et al* (1993)). It is the N-terminal regulatory sequence in IκBα that integrates proximal biochemical signals leading to the induced nuclear translocation of NF-κB (Brockman *et al* (1995)).

Brockman *et al* (1995) studied the effect of a deletion mutant of IκBα lacking amino acids 1-36 (IκBαΔN) which failed to support NF-κB directed transcription when stimulated with potent NF-κB inducers, including HTLV-1, Tax, and TNF-α. However the wild type IκBα cDNA expressed in this system recapitulated the inhibitory and signal-transducing functions of endogenous IκBα.

The IKK complex phosphorylates IκBα at specific serine residues Ser^{32} and Ser^{36}. Phosphorylation alone is insufficient to activate NF-κB, but targets IκBα for ubiquitination at specific lysine residues by E3, the ubiquitin protein ligase. Replacement of $serine^{32}$ and $serine^{36}$ with alanine prevents hyperphosphorylation of IkBα, repressing NF-κB directed transcription (Brockman *et al* (1995)). It is phosphorylation of serine residues Ser^{32} and Ser^{36} of IκBα which target the protein to the ubiquitin-proteasome pathway (Chen *et al* (1995)). The conjugation reaction catalysed by E2 is specific, the enzyme does not recognise the mutated form of IκBα with alanine replacing serine residues at 32 and 36. The mutant species of IκBα cannot be phosphorylated and conjugated following extracellular signal stimulation (Gonen *et al* (1999)).

IκBα is targeted to the 26S proteasome for degradation. The degradation of IκBα unmasks the nuclear localisation sequence, allowing NF-κB to translocate to the nucleus (Schwartz *et al* (1999)). The gene encoding for IκBα contains upstream binding sites for NF-κB. There is rapid synthesis of IκBα; the newly synthesised IκBα can remove NF-κB from DNA, re-sequestering it back to the cytoplasm, and therefore attenuating the response (Gilmore *et al* (1996)).

Figure 1.13.b: Induction of IκB kinase pathway by PIF



Aberrant NF-κB activity has been described in a number of solid tumours. Breast cancer, melanoma, colon and pancreatic cancer all demonstrate high levels of NF-κB activity; however, the mechanism for this constitutive activation has not been fully delineated. (Schwartz *et al* (1999)).

NF-κB up regulates cytokines IL-1, IL-6, IL-8 and TNF-α, which in turn have been reported to contribute to the development of cachexia. Kawamura *et al* (1999) utilised the colon26 model of induced cachexia to investigate the effectiveness of a synthetic, double stranded oligonucleotide (ODN) as a 'decoy' to block the binding of the transcription factor NF-κB. They found this 'decoy' was successful in inhibiting cachexia in the colon26 model. However, this could not be fully explained as a net result of cytokine interference alone.

Aberrant activation of NF-κB has been observed in muscle disuse atrophy, this muscle loss is similar to that seen in cachexia, whereby protein degradation is mediated by the ubiquitin-proteasome pathway (Lecker *et al* (1999)). The activation of NF-κB during disuse atrophy involves a pathway distinct from that mediated by cytokines (Hunter *et al* (2002)). Skeletal muscle fibres are post mitotic and multinucleated. There is a loss of myonuclei during unloading atrophy, yet whole muscle cells are not lost. Up regulation of survival

genes such as bcl-2 or HO-1 may temper the stimuli, leading to myonuclear loss from destroying the entire cell (Hunter *et al* (2002)).

Several mechanisms can cause constitutive NF-κB activity. Alterations in rel of IκB family genes may occur in approximately 1-10% of human T- and B- cell cancers (Fracchiolla *et al* (1993)). Viral oncoproteins such as TAX of human T-cell leukaemia virus (HTLV)-1 can directly interact with the IKK complex. The Epstein Barr virus nuclear antigen (EBNA)-2 and latent membrane protein-1 (LMP-1) can also increase NF-κB DNA binding (Greten *et al* (2004)).

Chromosomal translocations, amplifications, deletions and mutations that affect genes coding for NF-κB and IκB proteins can also result in constitutive NF-κB activity. Amplifications of c-Rel in non-Hodgkin's B-cell lymphoma and chromosomal rearrangements affect the NF-κB2 locus, detected in B- and T- cell lymphomas. These include chronic lymphocytic leukaemia (CLL), multiple myeloma, T- cell lymphoma and cutaneous B- and T- cell lymphomas (Greten *et al* (2004).

Tumour cells that have undergone DNA damage induce NF-κB activity to confer resistance to apoptosis. NF-κB target gene products block apoptosis, including the Bcl-2 family and interfering with p53 mediated apoptosis (Greten *et al* (2004)).

Tumours with constitutive NF-κB activity demonstrate increased resistance to chemotherapy. Many chemotherapy agents induce NF-κB activity and increase drug resistance in tumour cells. Inhibition of NF-κB leads to enhanced apoptosis, increased sensitivity to radiation and chemotherapy (Whal *et al* (1998)).

1.14 Aims of Study

Previous studies have characterised the tumour-derived Proteolysis-Inducing Factor (PIF), see **section 1.9**. However, at present the signalling pathway for this glycoprotein in cancer cachexia remains unclear.

The aim of this study is to further characterise this glycoprotein. As our understanding of this glycoprotein increases, the role for this molecule in cancer cachexia becomes more evident. The work conducted in this study looks at many aspects of the molecule and its role in cancer cachexia, which may in turn generate more successful therapeutics against cancer cachexia.

The aim of chapter 3 is to determine the role of 15(*S*)-HETE and its involvement in increased expression of proteasome subunits, activation of PKCα and subsequent activation of the IKK complex. The eicosanoid 15(*S*)-HETE is the only metabolite of AA to directly induce protein degradation (Smith and Tisdale (1999)), which occurs as a result of PIF-induced release of AA from membrane phospholipids and metabolism via the cyclooxygenase pathway to 15(*S*)-HETE. Therefore the aim of chapter 3 is to focus on the role of PKCα and NF-κB as up-regulatory signals of the ATP-ubiquitin dependent proteolytic pathway in response to 15(*S*)-HETE.

Chapter 4 and 5, focus on the importance of NF-κB as a transcription factor involved in induction of the PIF-induced increase in expression of the ATP-ubiqutin dependent proteolytic pathway. Chapter 4 determines the importance of NF-κB signalling in the upregulation of this proteolytic pathway by using two 'mutant' dominant negative IκBα plasmid DNA inserts (IκBα S32/A36 and IκBα ΔN) and a control vector (pCMV4). Chapter 5 examines NF-κB inhibitors and assesses their functional ability *in vitro* and *in vivo* to attenuate protein degradation and their potential as therapeutic agents for the treatment of cancer cachexia.

The role of chapters 6 and 7 is to determine the importance of PKC as an intracellular signal to activate the IKK complex, which results in the phosphoryltation and subsequent degradation of IκBα and increased DNA binding of NF-κB. PIF-induced activation of PKCα is investigated in chapter 6 and relays the importance of PKCα in upregulation of ubiquitin dependent proteolysis via NF-κB. Chapter 7 looks at the role of PKCα in protein degradation of skeletal muscle in response to the phorbol ester TPA. Both chapters

support the theory that PKC is an important intracellular signal involved in the induction of skeletal muscle proteolysis by the ATP-ubiquitin dependent proteolytic pathway.

The aim of chapter 8 is to determine the cellular signalling pathways further upstream of PKC in response to PIF. Chapter 8 determines a role of Akt involvement in activation of NF-κB and upregulation of ATP-ubiquitin dependent proteolysis in response to PIF

Finally chapter 9 aims to characterise the PIF receptor by using a polyclonal rabbit antibody commercially produced and the potential use as therapeutic intervention for the treatment of cancer cachexia.

Chapter 2

Materials and Methods

2.1 Animals

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

2.2 Chemicals

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

Affiniti Research Products, Exeter UK

Anti 20Sα proteasome subunit antibody, mouse (Rabbit)
Anti 19S proteasome subunit, P42, antibody, mouse (Rabbit)
Anti ATPase proteasome subunit, MSSI, antibody, mouse (Rabbit)

Amersham Biosciences (Buckinghamshire, UK)

ECL Western blotting detection reagents
HyBond nitrocellulose membrane
Hyperfilm ECL
Hypercassette
L-[2,6-^{3}H] Phenylalanine (sp act 2.07 TBq/ mmol)
Rainbow molecular weight markers

Baylor College of Medicine, Houston, Texas, USA

Provided (pCMV4-IκBαS32/ A36 and pCMV4-IκBαΔN) mutant and (pCMV4) wild type IκBα plasmid DNA inserts for work in chapter 4.

BDH (Poole UK)

pH indicator paper

Biomol Reseach Laboratories, USA

Anti IκBα antibody, rabbit (goat)

Anti IκBα to phosphorylated form IκBα antibody, mouse (rabbit)
15 (*S*) - Hydroxyeicosatetraenoic acid
Resveratrol, SN50

Bio-Rad Laboratories (Richmond, CA, USA)
Ammonium Persulphate (APS)
Electrophoresis and transferring apparatus
Inner and outer glass electrophoresis plates
Protein Assay reagents
Silver protein stain kit

British Oxygen Company Ltd (London, UK)
5% CO_2 in air

Calbiochem (San Diego, CA, USA)
Anti IκBα Antibody rabbit (goat)
Anti IκBα phosphorylated form at serine 32 and 36 Antibody, mouse (rabbit)
Anti PKC-α Antibody rabbit (goat)
SN50 and SN50M
Calphostin C
D609
PD98059
LY294002

Dako Cytomation (Glostrop, Denmark)
Rabbit ANTI Mouse HRP conjugated secondary antibody
Goat ANTI Rabbit HRP conjugated secondary antibody

Fischer Scientific Apparatus (England, UK)
Acetic Acid (Glacial)
Sodium Dihydrogen Orthophosphate

Fison Scientific Equipment (Loughborough, UK)
Sodium dihydrogen orthophosphate
Magnesium sulphate

Gelman Science (Northampton, UK)

YM membranes, 10kDa MWCO

Microcon concentrators, 10kDa MWCO

GIBCO BRL Life Technologies (Paisley, Scotland, UK)

DMEM (X1)

Foetal Calf Serum (FCS)

Glutamine (100X)

Horse Serum

Penicillin Streptomycin (100X)

Trypsin (100X)

Oxoid (Basingstoke, UK)

Phosphate Buffered Saline (PBS) tablets

Panomics (CA, USA)

NF-κB EMSA 'Gel-Shift' kit

NF-κB Probe

Cold probe

Pierce (UK)

EMSA light shift chemiluminescence kit

Standard Laboratory Reagents

Acetone

Deionised water

Ethanol

Hydrochloric Acid

Methanol

Methylated spirits

Perchloric acid

2.3.1 Purification of a Tumour-Derived PIF

2.3.1a Phsophate buffered Saline

1 X PBS Tablet	1 tablet/ 500ml dH_20

2.3.1b 100mM PMSF

M.W 174.2	0.218g to 12.5ml iso-propanol

2.3.1c 0.2M Glycine HCl pH 2.5

M.W 75.07	1.5g to 90mls dH_20, pH to 2.5 with conc. HCL, make upto 100ml

2.3.1d 0.2M Sodium Barcarbonate

M.W 84.01	1.68g to 100ml dH_20

2.3.1e QS1 Buffer

Tris HCl pH 8.0 (3.03g)	10mM
PMSF 12.5ml (100mM solution)	0.5mM
DTT (0.386g)	1.0mM
EGTA (0.475g)	0.5mM

- Use ratio of 1g MAC16 tumour to 5mls QS1 buffer (typically use 20g)
- Homogenise tumour on ice
- Centrifuge at 4,500 rpm for 20 min at 4°C
- Discard pellet and ammonium sulphate precipitate the supernatant using 38% ammonium sulphate. Add over 8h.
- Leave in cold room to equilibrate overnight.
- Centrifuge again at 4,500 for 20 min at 4°C
- Circulate on washed affinity column, pH 7.0 overnight, on pump setting 1.5.
- Wash column for 1h with PBS, at pump setting 3.0.
- Elute PIF with 0.2M Glycine-HCl pH 2.5, into 5ml 0.2M sodium bicarbonate, over 1.5h at pump setting at 1.5.
- Equilibrate with PBS, bring volume down to 1-2ml, yield should be between 150-200μg
- Store on ice in the cold room until use.

2.3.2 Bradford Protein Assay

Bradford Biorad Reagent	200µl
dH_20	790µl
Protein sample	10µl

Add all components to a cuvette and read in a spectrophotometer at an absorbance of 595nM. For a blank, add biorad reagent and 800µl dH20 and zero the spectrophotometer. To determine protein concentration µg/ µl follow the equation below :

$$\frac{A^{595nM}}{0.053} \times 0.1 = \text{protein concentration } \mu g/\ \mu l$$

(0.053 is the gradient obtained from a BSA calibration curve, while 0.1 is the dilution factor)

2.3.3 SDS PAGE Gel Electrophoresis

2.3.3a 12% Resolving Gel

Assemble gel apparatus and ensure there are no leaks within the casting equipment. Add the following components into a universal and mix. Gently pour into the glass plates, leave space at the top of the gel for the stacking gel. .

30% bis acrylamine	5ml
Distilled water	6.8ml
Buffer B	3ml
10% SDS	450µl
TEMED	24µl
10% APS	200µl

Add a layer of ethanol to remove air bubbles. Once the resolving gel has set add the following components into a universal, mix and add to the casting apparatus. Insert a sample comb (10-15 lanes).

2.3.3b 5% Stacking Gel

30% bis acrylamine	0.8ml
Distilled water	3.6ml
Buffer C	0.5ml
10% SDS	50µl
TEMED	15µl
10% APS	200µl

2.3.3c Electrophoresis Buffer (X10)

Glycine	144g
Trizma base	30.3g
SDS	10g

Dissolve in 1l of distilled water. Dilute 1:10 with deionised water for use.

2.3.3d Transfer Buffer (X10)

Glycine	72.08g
Trizma base	15.52g
SDS	5g

Dissolve in 1l of distilled water. For use add 100ml stock : 200ml methanol : 700ml distilled water.

2.3.3e 0.1% PBS-Tween

PBS	5 tablets
Tween 20	0.5g
Deionised water	500ml

2.3.3f 0.5% PBS-Tween

PBS	5 tablets
Tween 20	2.5g
Deionised water	500ml

2.3.3g Membrane Blocking Solution

Marvel non-fat milk	5g
Deionised water	95ml

Myotubes were incubated with various concentrations of PIF, after which the medium was removed. Cells were washed with PBS and scraped from the culture flask. They were then

sonicated at 4°C in 500-1500µl of 20mM Tris-HCl, pH 7.5, 2mM ATP, 5mM $MgCl_2$ and 1mM dithiothreitol (DTT). Samples of cytosolic protein (5µg) formed by centrifugation at 18,000 x g for 10 min at 4°C and whole cell lysate (30µg) were resolved on 12% sodium dodecylsulfate, polyacrylamide gels (SDS/ PAGE) and transferred to 0.45µm nitrocellulose membranes, which had been blocked with 5% Marvel in Tris-buffered saline, pH 7.5, at 4°C overnight. The primary antibodies were used at a dilution of 1:1000 except for actin (1:250) and myosin (1:100), and the secondary antibodies were also used at a concentration of 1:1000. Incubation was for 1h at room temp and development was enhanced by chemiluminescence (ECL) (Amersham, UK). Blots are scanned by a densitometer to quantitate differences.

2.4.1 Gel Staining

2.4.1a Coomassie Brilliant Blue Stain

Coomassie Brilliant Blue R259 Stain	0.1%
Glacial Acetic Acid	10%
Methanol	25%

Adjust volume to 100ml with deionised water

2.4.1b Coomassie Blue Gel Destain solution

Glacial Acetic Acid	10%
Methanol	25%

Adjust volume to 100ml with deionised water.

2.4.1c Silver Stain Fixing Solution

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

2.4.1d Silver Stain Oxidising Solution

Dilute 1:10 with deionised water for use

2.4.1e Silver Stain Silver Nitrate Solution

Dilute 1:10 with deionised water for use

2.4.1f Silver Stain Developing Solution

Developer	6.4g

Deionised water	200ml

2.4.1g Silver Stain Stopping Solution

Glacial Acetic Acid	5%
Deionised water	95%

2.5.1 Tissue Culture

2.5.1a C_2C_{12} Growth Media

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

2.5.1b C_2C_{12} Differentiation Media

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

2.5.1c Transfected C_2C_{12} Ampicillin Resistant Myotubes Selection Growth Media

Dulbecco's modified eagles media (DMEM)	500ml
Foetal Calf Serum	10%
Ampicillin	5%
Glutamine	1%

C_2C_{12} myoblasts were propagated in DMEM supplemented with 10% FCS, 1% glutamine and 1% penicillin-streptomycin under an atmosphere of 10% CO_2 in air at 37°C. Myotubes were formed by allowing confluent cultures of myoblasts to differentiate in DMEM containing 2% HS, with media changes every 2 days. Differentiation was complete in 5-7 days.

2.5.1d 3[H] Phenylalanine Working Stock Solution

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

This method was described by Whitehouse and Tisdale (2003). C_2C_{12} myotubes are pre-labelled for 24h with L-[2,6^{3}H] phenylalanine (0.67mCi / mmole) followed by extensive washing in PBS. Further incubation of 2h in DMEM without phenol red, until no more radioactivity appeared in the supernatant. Protein degradation was determined by the release of [2,6^{3}H] phenylalanine into the medium after 24h in the presence of various concentrations of PIF or TPA, together with 2mM cold phenylalanine to prevent reincorporation of radioactivity in the cells.

2.5.1e 'Chymotrypsin-like' assay

N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin	0.1mM
Lactacystin	10μm

'Chymotrypsin-like' enzyme activity was determined fluorimetrically by the method of Orino *et al* (1991), by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC (Whitehouse and Tisdale (2003)). Myotubes were washed with ice cold PBS and sonicated in 20mM Tris-HCl, pH 7.5, 2mM ATP, 5mM $MgCl_2$ and 1mM DTT at 4°C. The supernatant formed by centrifugation at 18,000 x g for 10 min was used to measure the 'chymotrypsin-like' enzyme activity by the release of aminomethylcoumarin (AMC) from the floureogenic peptide succinyl LLVY-AMC (0.1mM). Activity was measured in the absence and presence of the specific proteasome inhibitor lactacystin (10μM). Only lactacystin suppressible activity was considered to be proteasome specific.

2.6.1 Molecular Biology

2.6.1a TBE (X10) Buffer (1L)

107.80 g	Tris base
55 g	Boric acid
7.44 g	Disodium EDTA, dehydrate

Add components in the above order to 800ml distilled water. Add slightly less than the total amount of boric acid. Mix until completely dissolved, check the pH and adjust to 8.3 with boric acid. Bring the final volume to 1L with distilled water.

2.6.1b Antibiotic Stock Solution

Ampicillin 100mg/ ml dissolved in deionised water (sterile filtered)
Store at -20°C

2.6.1c LB Medium

10g Bacto ®- tryptone

5g Bacto ® - yeast extract

5g NaCl

Add deionised water to approximately 1L. Adjust pH to 7.5 with 10N Na OH and autoclave.

2.6.1d LB/ antibiotic plates (1L)

Add 15g of agar to 1L of LB medium and autoclave. Allow the medium to cool to 55°C before adding antibiotic to the specified final concentration (ampcillin 100μg/ ml). Pour 30-35ml of medium into 85mm Petri dishes. Flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden overnight. Store at 4°C for ≤1 month.

2.6.1e Appropriate Risk Assessment/ Assignment of contamination level

See appendix for appropriate risk assessment.

2.6.2a Transformation of competent DH5α cells.

Plasmid DNA of pCMV4, IκBαS32/36A and IκBαΔN (0.015μg) was added to 70μl of commercially available competent DH5α E. coli cells in a chilled microcentrifuge tube, mixed and left on ice for 30 min. Cells were then heat shocked for 30 sec at 37°C and immediately put back on ice for 2 min. 500μl of LB medium was added to the cells and incubated at 37°C for 40 min. 200μl aliquots of the transformed cells were spread onto LB agar plates containing ampicillin (100μg/ ml). The plates were incubated overnight at 37°C. Control plates were conducted alongside all transformations, including the control plasmid PUC19 transformation, which confers ampicillin resistance and also untransformed DH5α cells.

2.6.2b PCR analysis of successful transformations.

To determine if colonies present on the amipicillin selection plates have the correct plasmid insert, PCR analysis is employed. Primer sequences were derived from the IκBα and pCMV4 gene sequence. The primer to detect IκBα was produced by MWG Biotech and contained the following sequence FORWARDS : 5' GCT GTG ATC ACC AAC CAG C 3' and the REVERSE : 5' C TCT GGC AGC ATC TGA AGG 3' with an annealing temp of 60°C. The size of the target from the IκBα gene is 462 bases long. The primer designed to detect the pCMV4 vector was synthesised by MWG biotech according to the following sequence

FORWARDS : 5' GGT CTA TTC GGG AAC CAA G 3' and REVERSE : 5' CAC ATT CCA CAG AAG CTG C 3'. The size of the target from the pCMV4 gene is 444 bases long.

To conduct the PCR

1X	PCR buffer
20 pmol	Forwards primer
20 pmol	Reverse primer
dNTPs (10mM)	5µl
1 unit	Taq DNA Polymerase (0.25µl)
dH_2O	Make final volume up to 25µl

2.6.2c PCR protocol

Use sterile 200µl PCR tubes. Pick 1 colony from the successfully transformed plates, using a sterile pipette tip, scrape the tip around the base of the PCR tube and place on ice. With a new amipicillin selection plate, spread the tip over ¼ of the plate and label appropriately. Repeat this process for 10 colonies.

Add the components listed above into each PCR tube, ensure that Taq is the final component added. Ensure that PCR tubes are kept on ice throughout additions. Finally whirlymx and quickly centrifuge the PCR tubes and put into PCR machine. Select the 2 ½ hour PCR cycle.

2.6.2d 2% Agarose Gel electrophoresis

150 ml	TBE buffer (x1)
3g	Agarose

Microwave the above components in a sterile glass bottle to ensure complete dissolution. Allow to cool slightly and add 15µl ethidium bromide. Ensure the gel casting stand is adequately sealed with autoclave tape and pour the 2% agarose gel into gel caster.

Ensure that 100bp DNA markers are loaded onto the gel in the first and last lanes. Take 15µl of PCR product and mix with 5µl of loading buffer. Fill the gel tank with 1X TBE buffer and switch on the power pack. Run the gel at 100mV for 1 hour. To visualise PCR product DNA, use a UV transilluminator.

2.6.2e Glycerol Stocks of transformed colonies

After successful PCR analysis, glycerol stocks can be established and stored at -70°C until further required.

Prepare ampicillin at 100μg/ml and sterile filter. Add this stock ampicillin to 10.5ml LB medium and select a colony of transfected E. coli that gave the best PCR result and mix with the LB medium. Leave ½ volume of container to allow in air, as the E. coli are aerobic bacteria. Seal the container and place in hot shaking vessel overnight. The following day add 4.5mls of 50% sterile glycerol. Whirly mix to ensure even distribution. Aliquot 1ml of this combination to screw capped mini universals. Whirly mix and store at -70°C until required for further use.

2.6.2f Preparation of E. coli for plasmid DNA extraction

Select a colony which yielded the most successful PCR product. Innoculate this colony into 5-125ml of LB medium containing ampicillin at 100μg/ml. The inoculated medium should be left 12-16 hours at 37°C in a hot shaking vessel. Incubations over 16h result in excessive cell death and cell lysis may occur, resulting in plasmid loss.

2.6.2g Wizard ®Purefection Plasmid DNA Purification System (Promega)

Midipreps (5-25ml Bacterial Cultures) Protocol in brief, for full version see appendix II.

For a full protocol see appendix II

2.6.2h Spectrophotometric quantitation of extracted plasmid DNA.

Using a spectrophotometer with the wavelength of 260nM. According to the spectrophotometer 50μg/ml will give an absorbance of 1.0. Therefore blank the spectrophotometer using 1ml endotoxin free water in a quartz cuvette. Add 5μl DNA to 1ml endotoxin free water in a quartz cuvette. The value should be subjected to the following calculation : A^{260nM} x 50 = (value ÷ 1000) x 200 = DNA μg/μl

2.6.3 Transfection Procedure in C_2C_{12} myoblasts

Cells should be at 50-80% confluence prior to transfection. Each 25cm² culture flask will require 200μl serum free media and 6μl GeneJuice™ . Mix by vortexing and incubate at room temp for 5 min. Each 25cm² flask requires 1μg DNA to be added to the GeneJuice™/ serum free media and gently mixed. Incubate this composition for 5-15 min. Add the entire volume in drops to the cells in complete growth medium (10% FCS supplemented DMEM) in a volume of 5ml. Distribute the drops gently and evenly over the surface of the culture flask.

Remove transfection mixture 2-8 h later. Incubate cells with complete growth medium and ampicillin (5g/l). A lethal death curve was conducted in non transfected C_2C_{12} myoblasts

and found that 5g/l was effective against normal C_2C_{12} myoblasts. Incubate cells 24-72 h in selective media and non transfected cells expire.

When cells have reached 90% confluence replace selective media with 2% HS supplemented DMEM to allow myoblasts to differentiate. Once myotubes are clearly visible the transfected C_2C_{12} myotubes are ready for experimental analysis.

2.7 Indirect ELISA Protocol (adapted from Perlmann and Perlmann (1994)

The antigen (PIF ® peptide antagonist) was used in immunisations. This antigen has to be diluted and used in the ELISA to determine specific antibody activity.

2.7.1a Antigen information

Antigen peptide was synthesised by Severn Biotech, Kidderminster, Worcs, UK.
The sequence is 19 amino acids long and derived from the N terminus : D I N G G G A T L P Q K L Y L I P N V

Amino acid composition : Aspartic acid, Isoleucine, Asparagine, Glycine, Glycine, Glycine, Alanine, Threonine, Leucine, Proline, Glutamine, Lysine, Tyrosine, Leucine, Isoleucine, Proline, Asparagine and Valine.

2.7.1b Coating solution

50mM sodium carbonate pH 9.6
20mM Tris-HXl pH 8.5
10mM PBS pH 7.2
Protein concentration of antigen (10μg/ml).

2.7.1c Blocking solution

1% BSA in PBS

2.7.1d Primary/ secondary antibody solution

Dilute primary and secondary antibodies in 1X blocking solution to prevent non-specific binding.

2.7.1e Wash solution

0.1M phosphate-buffered saline (PBS) pH 7.4 with the detergent Tween 20 (0.02% w/v)

2.7.1f Substrate solution

1% 3,3',5'5'-tatramethylbenzidine (TMB) and 0.0025% hydrogen peroxide in 100mM sodium acetate at pH 5.5. Stop reagent : 50µl 0.6M sulphuric acid.

- Add 100µl antigen diluted in coating solution to a 96 well vinyl microtitre plate, (Costar MA, USA).
- Incubate 1h at room temperature.
- Empty plate and tap out residual liquid.
- Add 300µl blocking solution to each well.
- Incubate 5 min, empty plate and tap out residual liquid.
- Add 100µl diluted primary antibody to each well
- Incubate 1h at room temperature
- Empty plate and tap out residual liquid.
- Fill each well with wash solution, invert plate to empty and tap out residual liquid.
- Repeat 3-5 times.
- Add 100µl diluted secondary antibody to each well
- Incubate for 1h room temperature.
- Empty plate, tap out residual liquid and wash as before. Give a final 5 min soak with wash solution, tap residual liquid from plate.
- Dispense 100µl substrate into each well
- After development of desired colour, add 100µl of appropriate stop solution to each well
- Read the plate with Anthos Labtech 2001 Microplate reader at Absorbance 450_{nM}. End values are calculated by subtracting sample absorbances from the absorbance achieved in a 'no primary antibody' well control.

To determine the specific activity (titre mg/ml) adhere to the following calcultation : Titre of antibody samle

Total protein concentration (mg/ml)

2.7.2 Protein A antibody purification kit

Materials required but not supplied:

Sterile filters	0.45μm
12x75mm test tubes	
Beakers	50ml
PBS	
Additional 5ml and 10ml syringes	
Timer	
Clamp and ring stand	

- Serum must be clarified by centrifugation or filtration wth a 0.45μm filter prior to use with the protein A cartridge.
- The protein A cartridge will bind approximately the IgG content contained in 2ml of serum. Add 4ml of binding buffer per 2ml of serum.
- The cartridge is designed to use in the vertical position during loading, washing and elution steps. Avoid the intoducton of air bubbles into the cartridge.
- Add 10ml HEPES buffer to a 10ml syringe and attach to the desalting cartridge. Regenerate the cartridge by passing the buffer through at 1ml/ min.
- Add 5ml regeneration buffer to the 5ml syringe and connect to the top of the protein A cartridge. Regenerate the cartridge by passing buffer through at 1ml/min.
- Fill the 10ml syringe with 4ml binding buffer. Equilibrate the protein A cartridge by passing buffer through at 1ml/min.
- Fill the 10ml syringe with the sample serum/ binding buffer mixture. Load the protein A cartridge by passing the mixture through at a flow rate of 0.5ml/min.
- Rinse the cartridge with 10ml of water. Refil syringe with 6ml of binding buffer and wash the protein A cartridge by passing buffer through at 1ml/min.
- Attach the female end of the protein A cartridge to the male Luer lock fitting of the desalting cartridge.
- Fill the 5ml syringe with 5ml elution buffer. Elute the cartridges by passing the buffer through at an approximate rate of 0.5ml/min. The eluate contains the purified immunoglobulin at a physiological pH.
- Detach the two cartridges. Regenerate the protein A cartridge by passing 5ml of regeneration buffer through the cartridge.
- Regenerate the desalting cartridge by passing 10ml HEPES buffer through the cartridge.

- For long term storage wash the cartridge with 10ml PBS containing 0.02% sodium azide. Cap the cartridges and store at 2-8°C.

2.7.3 Isolating PIF receptor from C_2C_{12} membranes

2.7.3a Receptor Buffer :

HEPES	20mM pH 7.4
EDTA	1mM
PMSF	0.5mM
DTT	1mM

2.7.3b WGA Affinity column buffer

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

2.7.3c WGA Affinity Column Elution Buffer

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

- Sonicate at 4°C harvested C_2C_{12} myotubes in receptor buffer to isolate the cell membranes
- Cetrifuge at 15,000 rpm and keep the pellet
- Wash the pellet with receptor buffer
- Solubilise 200µl membranes in 1% Triton X for 30 min.
- Dialyse against PBS overnight at 4°C.
- Incubate dialysed membranes with PIF for 48h in the presence of protease inhibitors.
- Purifiy the PIF receptor using a WGA column (glass pipette, glass wool and WGA immobilised on sepharose beads)
- Load the column with sample, wash with 20ml WGA column buffer.
- Elute with 0.1M N-acetyl-glucosamine. Collect the 1ml fractions.
- Store the purified receptor with protease inhibitors as the fractions are susceptible to protease degradation.

2.8.1 Extraction of DNA binding proteins (Andrews and Faller (1991)

2.8.1a Buffer A

HEPES-KOH pH 7.9	10mM
$MgCl_2$	1.5mM
KCl	10mM
Dithiothreitol	0.5mM
PMSF	0.2mM

2.8.1b Buffer C

HEPES-KOH pH 7.9	20mM
Glycerol	25%
NaCl	420mM
$MgCl_2$	0.5mM
EDTA	0.2mM
Dithiothreitol	0.5mM
PMSF	0.2mM

- Add 400µl buffer A to prepared cell pellet
- Allow cells to swell on ice for 10 min
- Vortex for 10 sec
- Centrifuge for 10 sec and discard the supernatant
- Re-suspend pellet in 100µl of cold buffer C
- Incubate on ice for 20 min (high salt extraction)
- Cellular debris is removed by 2 min centrifugation at 4°C.
- Discard the pellet, the supernatant contains the DNA binding proteins.
- Determine the protein concentration by the Bradford Biorad assay.
- Store DNA binding proteins at -70°C.

2.8.2 EMSA 'Gel-Shift' kit protocol (PANOMICS)

The majority of components are provided with the kit, however you also need :

30% Bis-acrylamide	Dry oven
Glycerl	Hyperfilm ECL
Ammonium persulfate	
TEMED	
10 X TBE stock solution	
Whatmann 3MM paper	
Electrophoresis unit	

- For each nuclear extract add the following components into a sterile 0.5ml centrifuge tube. It is recommended to incubate with poly d(I-C) at room temperature for 5 min prior to adding the probe.
- To run a sample with the 'cold' (unlabelled) probe, prepare another sample as described below, but instead of adding 5µl of H_20, add 2µl of cold TF probe and 3µl H_20 to the sample.

Nuclear extract (5µg)	e.g. 1µl if 5µg = 1µl
5 X Binding buffer	2.0µl
Poly d(I-C) (1µg/µl)	1.0µl
TF probe (10ng/µl)	1.0µl
Distilled water	5.0µl
Total volume	10µl

- Combine these components and incubate at 15-20°C for 30 min.
- Prepare a 6.0% polyacrylamide native running gel. Mix the components into a sterile 50ml centrifuge tube, add in the order listed below :

10 X TBE	1ml
30% bis-acrylamide	4ml
80% glycerol	625µl
dH20	14.375ml
10% APS	300µl
TEMED	20µl
TOTAL VOLUME	20ml

- Allow to set and then add a 5% native stacking gel

dH_2O	6.93ml
1.5M Tris-HCl pH 8.8	1.3ml
10% APS	100µl
TEMED	10µl
30% bis-acrylamide	1.67ml

- Pre-chill 0.5 X TBE to 4°C prior to running the gel.
- Run the gel in chilled 0.5 X TBE for 10 min at 120V before loading samples into gel. Flush the wells using a transfer pipette before loading samples.
- Mix samples with 1µl of loading dye and add samples to gel.
- Run the gel at 4°C in an ice bath at 120V, until the dye reaches 1 inch from the bottom of the gel (approx 50-55 min).
- Follow standard electroblotting transfer procedure using usual gel electrophoresis transfer apparatus (SDS free). Transfer for 30 min at 300mA.
- After transfer, remove membrane from the apparatus and bake the membrane for 30 min at 85°C in a dry oven to immoilise the bound oligonucleotides.
- Tranfers the membrane to a new container, containing 20ml of 1 X blocking buffer.
- Block the membrane by incubating at room temperature with gently shaking for 15 min.
- Dilute 20µl of Streptavidin-HRP conjugate 1:1000 with 1X blocking buffer. Vortex the diluted Streptavidin and transfer onto the membrane. Continue incubation for a further 15 min with gentle shaking.
- Decant the Streptavidin-HRP solution. Wash each membrane three times at room temperature with 20ml of 1X wash buffer, 8 min each wash.
- Add 20ml of 1X detection buffer to each membrane and incubate at room temperature for 5 min.
- Overlay each blot with 2ml working substrate solution; mix 200µl solution I with 200µl solution II, briefly vortex and add 1.6ml solution III. Throughly mix. Using clingfilm, place each membrane on a sheet, then pipette 2ml of the mixed substrate solution onto each membrane and overlay with clingwrap. Ensure the substrate is evely distributed over the membrane with no air bubbles. Incubate at room temperature for 5 min.
- Remove excess substrate by gently applying pressure over the top sheet.
- Expose the membranes using hyperfilm™ ECL

2.9.1 Tyrosine Release Assay

2.9.1a Krebs-Ringer Bicarbonate Buffer

$NaHC0_3$	25mM
NaCl	118mM
KCl	4.7mM
$MgSO_4$	1.2mM
NaH_2PO_4	1.2mM
$CaCl_3$	1.2mM

2.9.1b Krebs-Heinsleit buffer (make on the day by adding components to Krebs buffer)

Glucose (α-D[+]-Glucose)	6mM
Bovine Serum Albumin	0.12%
Cyclohexamide (3-[2-(3,5 dimethyl-2-oxocyclohexyl] glutarimide	0.5mM

- Excise intact soleus muscle and place in ice cold PBS
- Incubate muscle for 2h at 37°C in 3mls Kresb-Heinsleit buffer. Gas samples with 5% CO_2 and 95% O_2, seal and put in water bath.
- Blot and weight muscles accurately. Remove 2ml of incubation medium into a glass centrifuge tube. Set water bath to 55°C.
- Add 20µl ice cold 30% TCA (trichloracetic acid)
- Mix by centrifuging at 2800 x g for 10 min. Keep the supernatant.
- Add 1ml 0.1% 1-nitroso-2-naphthol (0.1g/ 100mls in 95% ethanol) and 1ml nitric acid to the supernatant. Mix and incubate at 55°C for 30 min.
- Allow to cool and add 5ml dichlorethane (to extract the unchanged 1-nitroso-2-napthol).
- Mix by inversion and centrifuge at 2800 x g for 10 min.
- Read the aqueous phase, measuring the fluorescence at 460 excitation and 570 emission.
- To calculate : $\frac{\text{Fluoresence}}{\text{Muscle weight}}$ = Fluorescence/ g/ 2h

Chapter 3

Signalling pathways in the induction of proteasome expression by proteolysis-inducing factor (PIF) in murine myotubes

Introduction

Weight loss and depletion of lean body mass in cancer patients are important prognostic factors in determining overall patient survival (Tisdale (1993)). Muscle wasting results in; fatigue, immobility, asthenia and reduced quality of life for the cancer patient. It is important to determine the underlying mechanisms that drive the massive protein catabolism observed in cancer patients (Tisdale (2002)).

Studies utilising *in vivo* cachexia models (Baracos *et al* (1995), Temparis *et al* (1994), Lorite *et al* (1998)) suggest that skeletal muscle catabolism occurs through an increased expression of the key components of the ATP-dependent ubiquitin-proteasome proteolytic pathway.

Todorov *et al* (1996) successfully isolated a 24kDa sulphated glycoprotein from murine and human cachexia-inducing tumours. This 24kDa glycoprotein was found to initiate protein catabolism both *in vitro* (Todorov et al (1996)) and *in vivo* (Lorite et al (1997)) and therefore termed Proteolysis-Inducing factor (PIF).

Studies *in vitro* and *in vivo* have characterised PIF-induced protein catabolism as the result of increased expression of proteasome subunits and catalytic activity (Lorite *et al* (2001). PIF-induced protein catabolism is attenuated by the polyunsaturated fatty acid EPA (Smith *et al* (1999)). Whitehouse *et al* (2001) demonstrated EPA to attenuate skeletal muscle atrophy in MAC16 tumour bearing mice, by suppressing proteasome subunit expression and catalytic activity.

In vitro studies demonstrate PIF-induced protein degradation linked with release of arachidonic acid from membrane phospholipids (Smith *et al* (1999)). Arachidonic acid is metabolised to prostaglandins and hydroxyeicosatetraenoic acids (HETEs). Of the metabolites formed, only 15(*S*)-HETE initiated a significant increase in protein degradation (Smith *et al* (1999)), suggesting the eicosanoid acts as an intracellular signal for PIF.

Whitehouse *et al* (2003) recently demonstrated 15(*S*)-HETE to induce protein degradation in differentiated C_2C_{12} myotubes through increased expression of the ubiquitin-proteasome proteolytic pathway. It was suggested that this was possible due to the intervention of the transcription factor nuclear factor -κB (NF-κB).

This chapter examines the role of 15(*S*)-HETE and its involvement in increased expression of proteasome subunits, activation of PKCα and subsequent activation of the IKK complex.

O'Flaherty *et al* (2001) concluded AA activates protein kinase C (PKC) directly, and could be a regulatory signal during cell stimulation. However, Alpert *et al* (1999) suggested that 15(*S*)-HETE may participate in intracellular signalling through the formation of modified diacylglycerols (DAGs). DAGs containing monohydroxy-substituted fatty acids have been found to activate and/or inhibit PKC isotypes.

Therefore, a potential role of the lipoxygenase metabolite 15(*S*)-HETE is the activation of PKC. PKC can phosphorylate many cellular substrates. A possible target for PIF action is the IκBα kinase (IKK) complex. This IKK complex is involved in the subsequent activation of NF-κB (Amit *et al* (2003)).

This mechanism could provide a signalling pathway for PIF, from the release of eicosanoids from membrane phospholipids to increased proteasome expression through increased nuclear binding of NF-κB (Whitehouse and Tisdale (2003)).

Methodology

Purification of PIF

See section 2.3.1

Tissue Culture

See section 2. 5. 1

Western Blot Analysis

For a full protocol see section 2.3.3

The primary antibodies were used at 1:1000, except for actin (1:250) and PKCα (1:100), while secondary antibodies were used at a dilution of 1:1500. Incubations were between 1 and 3 hours at room temperature and development was enhanced by chemiluminescence (ECL). Blots were scanned using a densitometer to quantitate differences, and a parallel gel was silver stained to ensure equal loading.

Protein Degradation Assay

See section 2.5.1.d

Measurement of proteasome activity

'Chymotrypsin-like' activity was determined fluorimetrically by the method of Orino *et al* (1991), by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC. For a full protocol see section 2.5.1.e

Statistical Analysis

Results are expressed as a mean ± S.E.M Differences were determined by one-way ANOVA, followed by the Tukey-Kramer multiple comparison test.

Results

Western blot analysis of 20S proteasome α subunit expression in C_2C_{12} myotubes in response to PIF (24h)

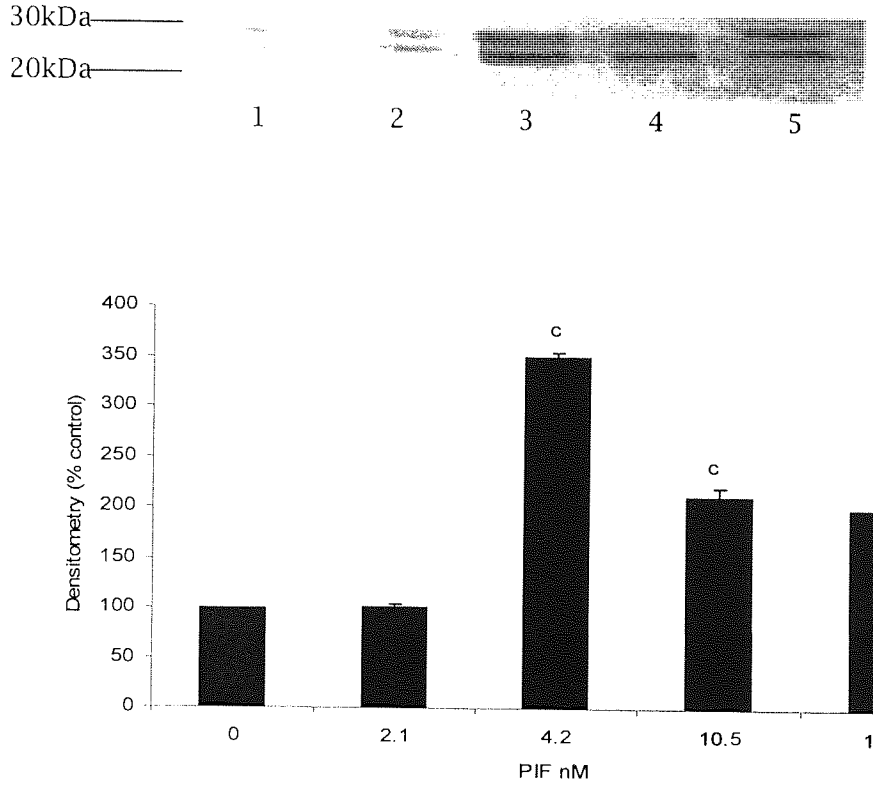


Figure 3. 1a

Cells were incubated with 0 (lane 1), 2.1 (lane 2), 4.2 (lane 3), 10.5 (lane 4), 16.8nM PIF (lane 5) and expression was determined after 24h. A representative blot is shown and densitometric analysis is based on three replicate blots. Results are expressed as mean ± S.E.M, representing the two major bands. Differences from control were determined by one-way ANOVA followed by Tukey's test and indicated as b, $p < 0.01$ and c, $p < 0.001$.

Western blot analysis of $E2_{14K}$ expression in C_2C_{12} myoutbes in response to PIF (24h)

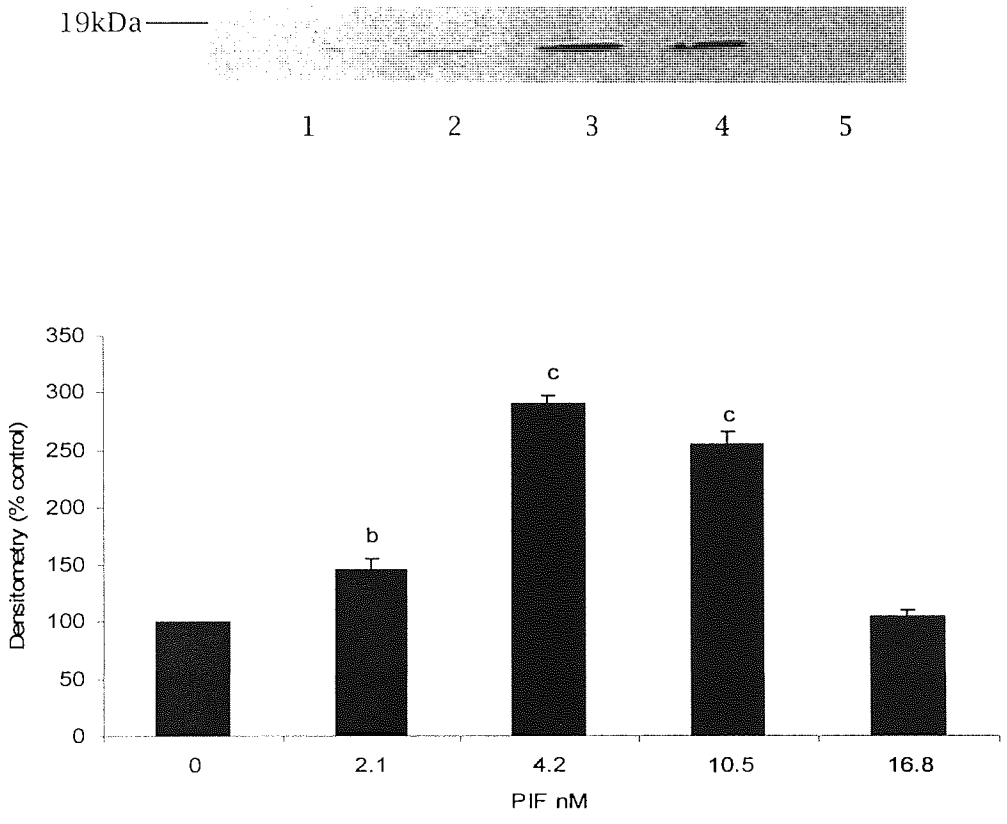


Figure 3. 1b

Cells were incubated with 0 (lane 1), 2.1 (lane 2), 4.2 (lane 3), 10.5 (lane 4) or 16.8 nM PIF (lane 5), and expression was determined after 24h. A representative blot is shown and densitometric analysis was based on three replicate blots. Results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$.

'Chymotrypsin-like' activity of the proteasome of C_2C_{12} myotubes in response to PIF (24h)

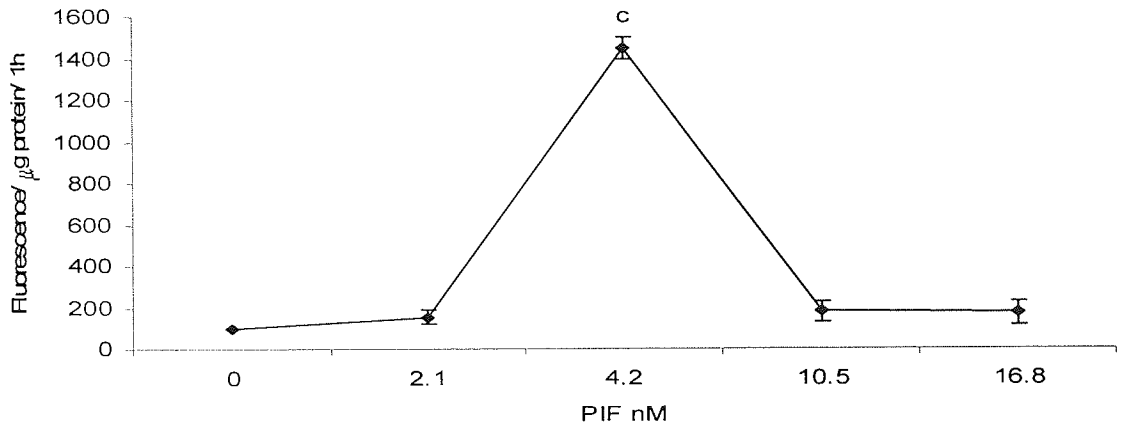


Figure 3. 1c

The 'chymotrypsin-like' enzyme activity was determined fluorometrically as described in section 2.5.1.e. Results are based on three replicate experiments and are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukeys test. Differences from 0nM PIF are indicated as c, $p < 0.001$.

Total protein degradation in C_2C_{12} myotubes in response to PIF (24h)

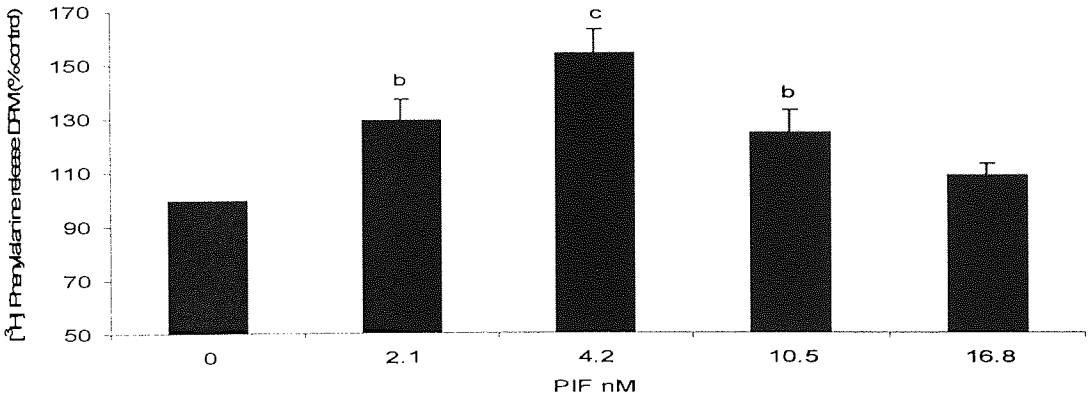


Figure 3. 1d

Results are expressed as mean ± S. E. M. of one experiment, where n=6. The experiment was repeated three times on different days with similar results. Statisical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$.

Figures 3. 1a- 3.1 d

PIF was found to increase 20S proteasome α subunit expression, a maximum increase of 350% at a dose of 4.2nM PIF (figure 3. 1a). This was accompanied by an increase in $E2_{14K}$ expression, the ubiquitin-conjugating enzyme, by 300% at the same concentration of PIF.

Chymotrypsin-like enzyme activity increased in response to PIF, with a maximum effect at 4.2nM PIF. Total protein degradation increased by 50% at 4.2nM PIF. This data correlates with that previously reported by Lorite *et al* (2001) and Gomes-Marcondes *et al* (2002) that PIF directly affects murine myotubes to create an increase in proteasome activity, total protein degradation and protein expression of the components within the ATP-ubiquitin dependent proteolytic pathway.

PIF concentrations in figure 3. 1a - 3. 1d represent the range whereby PIF exerts an effect *in vitro*; increased activity of the proteasome, increased protein degradation and increased expression of components within the ATP-ubiquitin dependent proteolytic pathway. Therefore this range was employed with all subsequent experiments.

Western blot analysis of the effect of 15(*S*)-HETE on cytoplasmic PKCα in C_2C_{12} myotubes in the absence or presence of calphostin C (300nM)

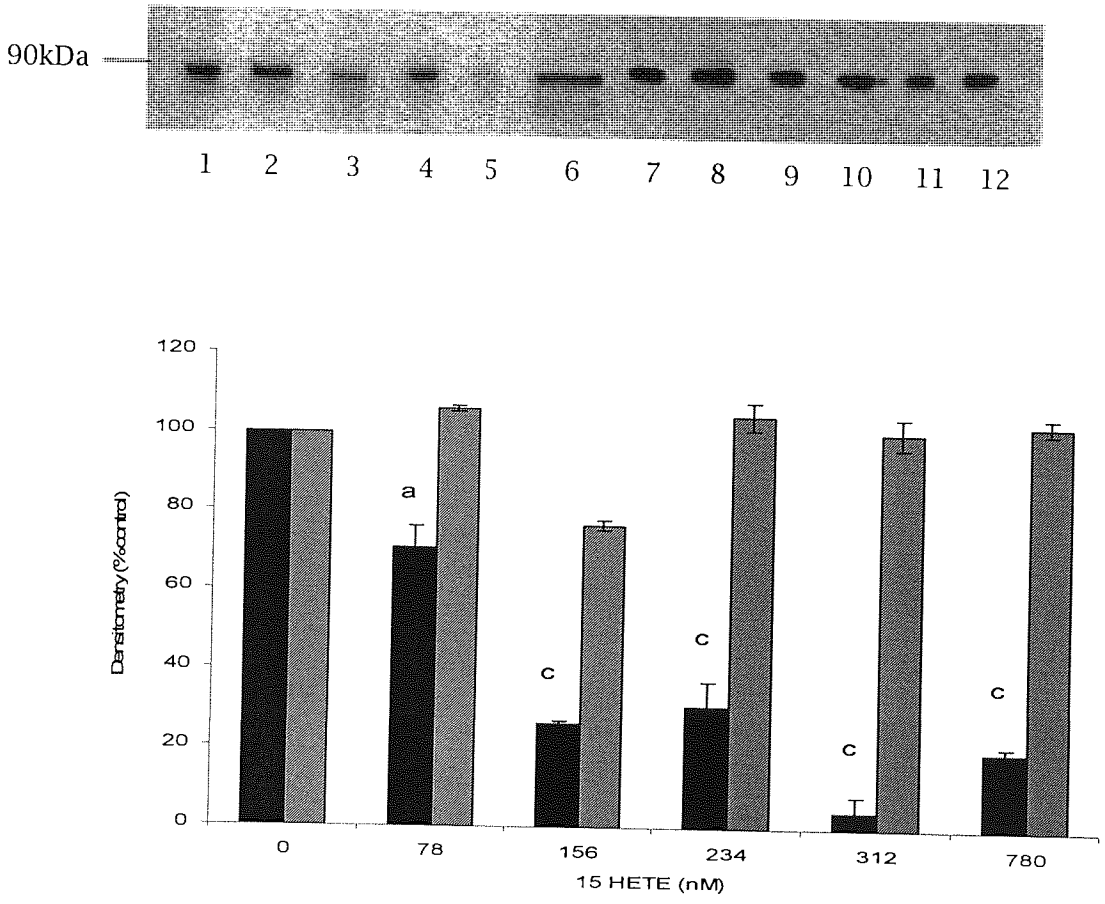


Figure 3. 2a

Myotubes were treated with 0 (lanes 1 and 7), 78 (lanes 2 and 8), 156 (lanes 3 and 9), 234 (lanes 4 and 10), 312 (lanes 5 and 11) or 780nM 15(S)-HETE (lanes 6 and 12) in the absence (lanes 1-5, ■) or presence (lanes 6-10, ▨) of calphostin C. Densitometric analysis is of 3 replicate blots, results are expressed as mean ± S.E.M and differences were determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as a, $p<0.05$, b, $p<0.01$ and c, $p<0.001$.

Western blot analysis of the effect of 15(*S*)-HETE on membrane bound PKCα in C_2C_{12} myotubes in the absence or presence of calphostin C

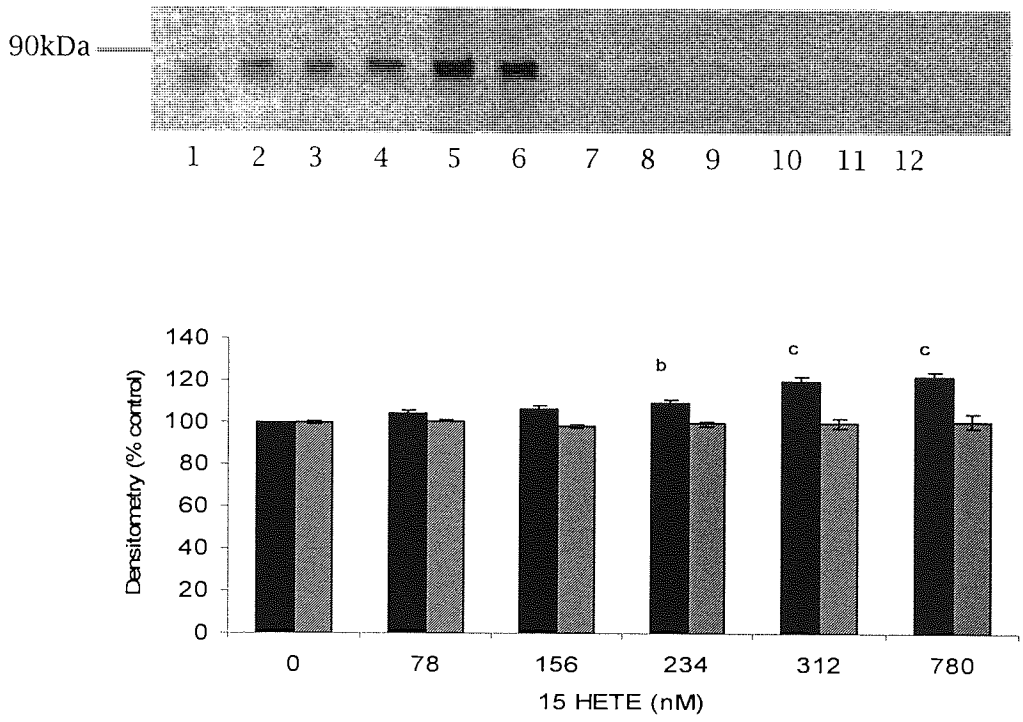


Figure 3. 2b

Myotubes were treated with 0 (lanes 1 and 7), 78 (lanes 2 and 8), 156 (lanes 3 and 9), 234 (lanes 4 and 10), 312 (lanes 5 and 11) or 780nM 15(S)-HETE (lanes 6 and 12) in the absence (lanes 1-5, ■) or presence of calphostin C (lanes 6-10, ▨). Densitometric analysis is of 3 replicate blots and results are expressed as mean ± S.E.M. Differences were determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as a, $p<0.05$, b, $p<0.01$ and c, $p<0.001$.

Actin loading control

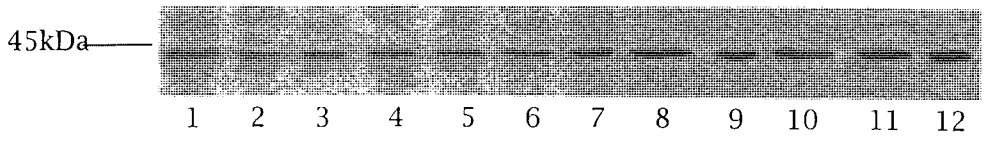


Figure 3. 2c

Lane discipline as figures 3. 2a and 3. 2b

Figures 3. 2a - 3. 2b

One possible action of 15(*S*)-HETE is the activation of PKCα. Figure 3. 2b demonstrates the effect of 15(*S*)-HETE on membrane bound PKCα. The activated form of the enzyme is induced in response to 15(*S*)-HETE. However, in the presence of the highly specific PKCα inhibitor calphostin C, activation of PKCα was inhibited. 15(*S*)-HETE activates PKCα and stimulates translocation of cytoplasmic PKCα (figure 3. 2a), accompanied by an increase in membrane bound PKCα (figure 3. 2b), demonstrating activation of PKCα. The maximal effect was observed at concentrations of 15(*S*)-HETE between 156 and 780nM. When myotubes were incubated with 15(*S*)-HETE in the presence of calphostin C (300nM) no activation of PKCα was observed.

To determine whether PKCα played a role in increased proteasome expression by 15(S)-HETE, C_2C_{12} myotubes were incubated with 15(*S*)-HETE in the absence and presence of calphosin C.

Western blot analysis of 20S proteasome α subunit expression in response to 15(S)-HETE in C_2C_{12} myotubes in the absence or presence of calphostin C (300nM)

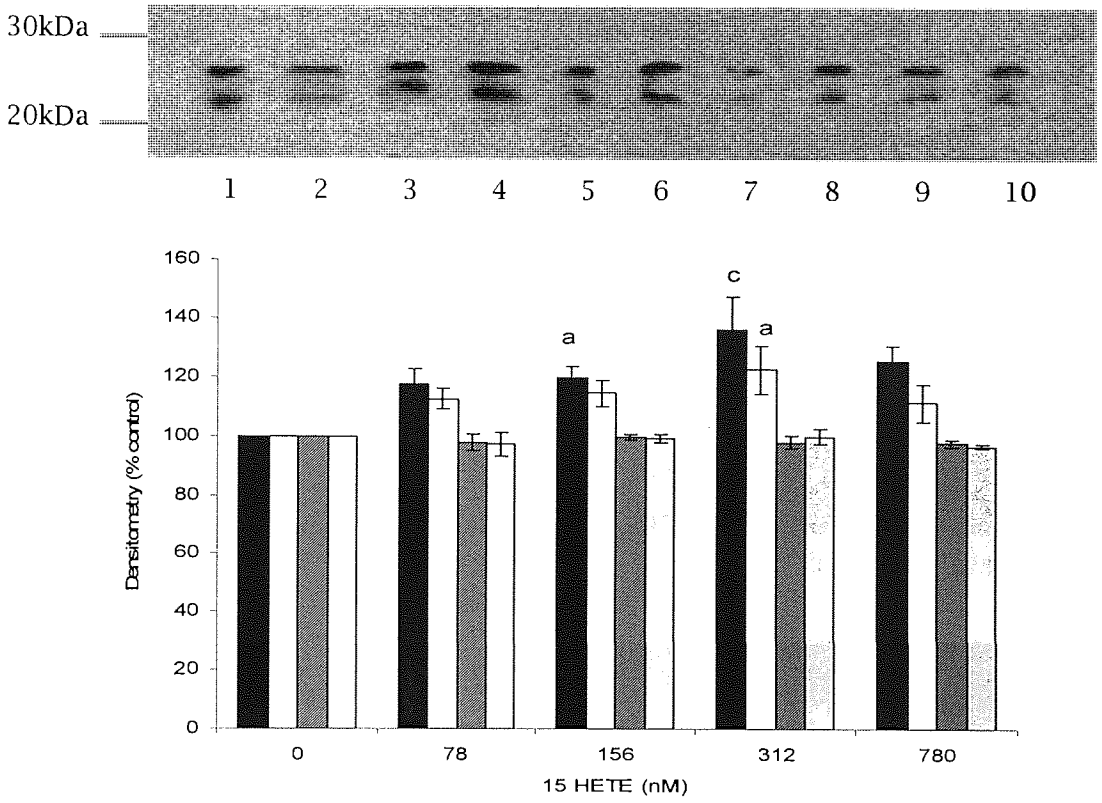


Figure 3. 3a

Myotubes were treated with 0 (lanes 1 and 6), 78 (lanes 2 and 7), 156 (lanes 3 and 8), 312 (lanes 4 and 9) or 780nM 15(*S*)-HETE (lanes 5 and 10) in the absence (lanes 1-5, ■ and □) or presence of calphostin C (lanes 6-10, ▨ and ⊡) Densitometric analysis is of three separate blots, results are expressed as mean ± S.E.M. Differences were determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as a, $p<0.05$ and c, $p<0.001$.

Actin loading control

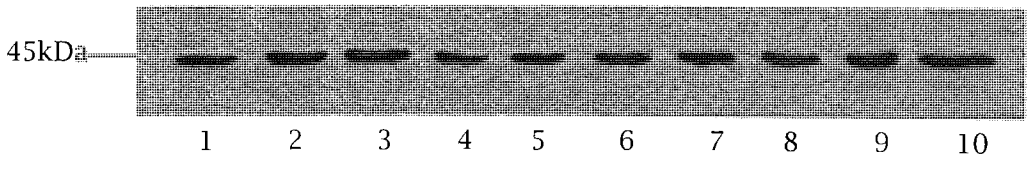


Figure 3. 3b

Lane discipline as figure 3. 3a

'Chymotrypsin-like' activity of the proteasome in C_2C_{12} myotubes in response to 15(*S*)-HETE in the absence and presence of calphostin C (300nM)

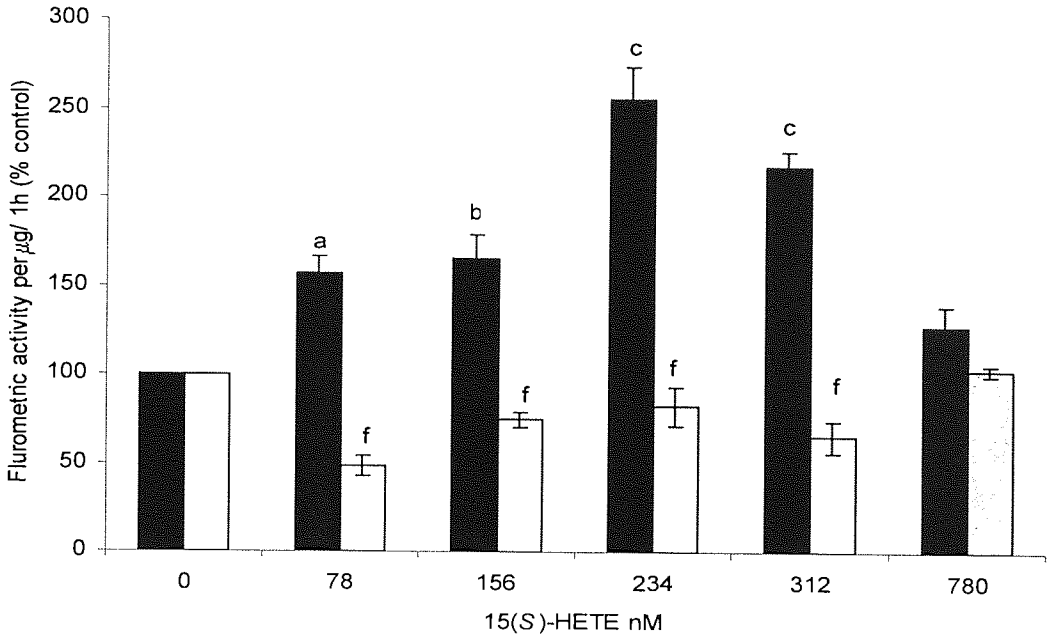


Figure 3. 3c

The results expressed as mean ± S.E.M of three repeats (n=3) in the absence (■) or presence of calphostin C (⊡). Differences were determined by one-way ANOVA, followed by Tukey's test. Differences from 0mM and 15(*S*)-HETE are indicated as a, $p<0.05$, b, $p<0.01$ and c, $p<0.001$. Differences between calphostin C treated and 15(*S*)-HETE at the same concentration are indicated as f, $p< 0.001$

Figures 3. 3a – 3. 3c

Whitehouse and Tisdale (2003) previously reported 15(*S*)-HETE to induce proteasome expression and activity. This effect was replicated here, but the PKCα inhibitor calphostin C completely attenuated the increase in 20S proteasome α subunit expression (figure 3. 3a) and proteasome activity (figure 3. 3c).

The increased expression of 20S proteasome α subunits in response to 15(S)-HETE has previously been shown to involve NF-κB (Whitehouse and Tisdale (2003)). NF-κB is retained in the cytosol by an inhibitory kinase complex (IκBα), which shields the nuclear localisation sequence of NF-κB. PKC has been suggested to be an upstream activator of the IKK complex (Vertegaal *et al* (2000) and Huang *et al* (2003)). This is turn phosphorylates IκBα at serine residues 32 and 36, rendering IκBα sensitive to degradation by the addition of a ubiquitin tag, targeting IκBα for degradation by the 26S proteasome complex (Brockman *et al* (1995)).

The role of PKCα in the phosphorylation and subsequent degradation of IκBα in response to 15(*S*)-HETE was assessed with various concentrations of the eicosanoid for 30 min in the presence or absence of calphostin C (administered 2h prior to adding 15(*S*)-HETE.

Western blot analysis of phospho-IκBα in C_2C_{12} myotubes in response to 15(*S*)-HETE in the absence and presence of calphostin C (300nM) determined 30 min after addition of 15(S)-HETE

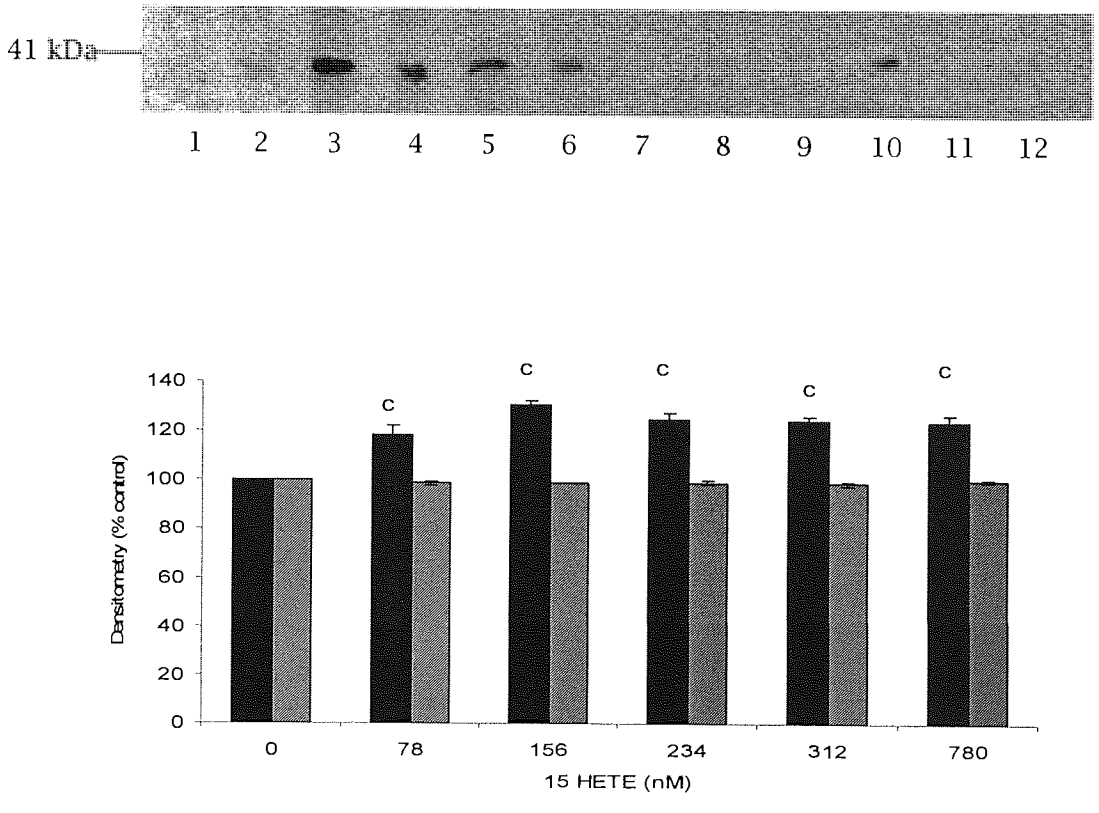


Figure 3. 4a

Myotubes were treated with 0 (lanes 1 and 7), 78 (lanes 2 and 8), 156 (lanes 3 and 9), 234 (lanes 4 and 10), 312 (lanes 5 and 11) or 780nM 15(*S*)-HETE (lanes 6 and 12) in the absence (lanes 1-6, ■) or prescence of calphostin C (lanes 7-12, ▨). Densitometric analysis is of three separate blots. Results are expressed as mean ± S.E.M and differences were determined by one-way ANOVA followed by Tukey's test. Differences from control are indicated as c, $p < 0.001$.

Western blot analysis of IκBα in C_2C_{12} myotubes in response to 15(*S*)-HETE in the absence and presence of calphostin C (300nM) determined 30 min after addition of 15(S)-HETE

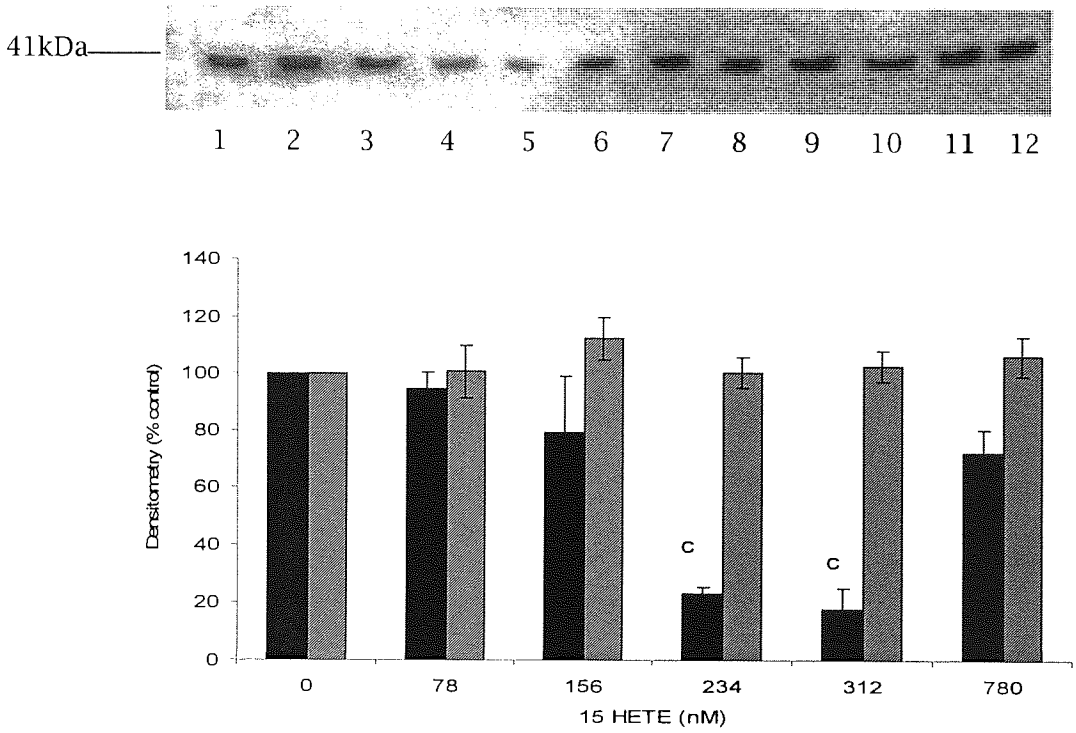


Figure 3. 4b

Myotubes were treated with 0 (lanes 1 and 7), 78 (lanes 2 and 8), 156 (lanes 3 and 9), 234 (lanes 4 and 10), 312 (lanes 5 and 11) or 780nM 15(*S*)-HETE (lanes 6 and 12) in the absence (lanes 1-6 ■) or presence of calphostin C (lanes 7-12 ▨). Densitometric analysis is of three replicate blots. Results are expressed as mean ± S.E.M., with differences determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as c, $p<0.001$.

Actin loading control

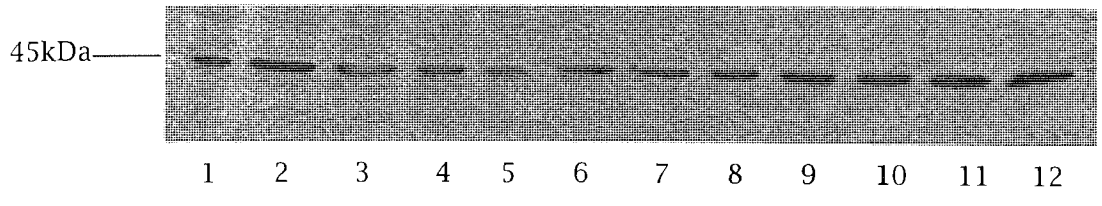


Figure 3. 4c

Lane discipline as figures 3. 4a and 3. 4b

Figure 3. 4a - 3. 4b

The role of PKC in the phosphorylation and subsequent degradation of IκBα in response to 15(*S*)-HETE was assessed with various concentrations of the eicosanoid for 30 min in the absence or presence of calphostin C. It was found that 15(*S*)-HETE increased IκBα phosphorylation (figure 3. 4a) at the same concentrations that induced 20S proteasome α subunit expression (figure 3. 3a). This action was attenuated by calphostin C and suggests mediation by PKC. 15(*S*)-HETE induced a decrease in IκBα (figure 3. 4b) that correlated inversely with levels of phosphorylated IκBα (figure 3. 4a). There was no decrease in IκBα in myotubes treated with 15(*S*)-HETE together with the PKC inhibitor calphostin C, suggesting that activation of PKC is responsible for IκBα degradation in response to 15(*S*)-HETE.

Discussion

Increased activity of the ubiquitin-proteasome proteolytic pathway is responsible for the enhanced proteolysis of myofibrillar proteins in skeletal muscle in a range of catabolic states including fasting, diabetes, sepsis, burn injury, neuromuscular disease, uremia and hyperthyroidism as well as in cancer cachexia (Lecker *et al* (1999)). In all of these conditions there is an increase in the levels of mRNA encoding ubiquitin and proteasome subunits attributed in part, to increased gene transcription. However, the mechanism by which this occurs has not been fully defined. Du *et al* (2000) demonstrated glucocorticoids to stimulate C3 subunit expression by antagonizing interaction between NF-κB and its response element in the C3 proteasome subunit promoter region. TNF-α has also been reported to rapidly activate NF-κB in differentiated skeletal muscle myotubes and mediate the loss of protein (Li and Reid (2000)). *In vitro* studies with PIF indicate increased nuclear binding of NF-κB correlating with increased proteasome expression, whilst the NF-κB inhibitor peptide, SN50, attenuated the increase in proteasome activity and expression induced by PIF (Whitehouse and Tisdale (2003)).

Previous studies have suggested increased nuclear binding of NF-κB to be involved in increased transcription of proteasome subunits induced by PIF and 15(*S*)-HETE (Whitehouse and Tisdale (2003)). Both PIF and 15(*S*)-HETE induce rapid breakdown of IκBα, although the mechanism has not yet been fully elucidated. This chapter provides evidence to suggest a role for PKCα in this process. As 15(*S*)-HETE has been shown to activate PKCα in murine myotubes at the same concentrations as those inducing protein degradation and increasing proteasome expression. The PIF-induced increase in proteasome expression was attenuated by the PKC inhibitor, calphostin C, suggesting a role for PKC in this process. The most likely route of involvement is in the phosphorylation and degradation of IκBα, as calphostin C attenuated the effect of 15(*S*)-HETE in this process. The mechanism by which this occurs has not yet been established, but most likely involves the phosphorylation and activation of IKK.

The results presented in this chapter confirm the concentration range of PIF to exert a catabolic effect *in vitro* and suggest potential control points in proteasome activation that could be useful for therapeutic intervention.

Chapter 4

NF-κB mediates proteolysis inducing factor (PIF) induced protein degradation and expression of the ubiquitin proteasome system in skeletal muscle.

Introduction

Skeletal muscle loss results in weakness, immobility and finally death of the cancer patient. Depletion of myofibrillar proteins in muscle is a result of decreased protein synthesis (Funabiki *et al* (1976)) and increased protein degradation (Lundholm *et al* (1982)). The increase in protein breakdown is an important component of muscle cachexia, since anabolic stimuli, such as nutritional supplementation fails to attenuate or reverse muscle wasting (Nixon *et al* (1981), Loprinzi *et al* (1994), Fearon *et al* (1988)).

The major catabolic pathway involved in the degradation of myofibrillar proteins in cancer cachexia is ATP-ubiquitin dependent proteolysis, confirmed by Bossola *et al* (2001). This process requires protein substrates to become conjugated with a polyubiquitin-chain, enabling proteasome recognition and degradation. There are several mediators known to influence the expression of polyubiquitin genes and proteasome subunits, including; glucocorticoids (Price (2003)), TNF-α (Llovera *et al* (1994)) and PIF (Lorite *et al* (2001)). PIF is a sulphated glycoprotein produced by cachexia-inducing murine and human tumours (Todorov *et al* (1996)), which specifically induces degradation of skeletal muscle (Lorite *et al* (1998)).

Several studies have been conducted into the role of the nuclear transcription factor, nuclear factor-κB (NF-κB) and its involvement in the induction of proteasome gene expression. The human proteasome C3 subunit promoter contains elements homologous to the consensus NF-κB binding site (Du *et al* (2000)), suggesting NF-κB may be involved in gene transcription. However, the mechanism by which this occurs appears to be opposite for glucocorticoids (Du *et al* (2000)) and TNF-α (Li and Reid (2000)). Glucocorticoids stimulate C3 subunit proteasome expression by antagonising the interaction of NF-κB with its response element in the proteasome promoter region. Glucocorticoids also induce gene transcription and protein synthesis of the NF-κB inhibitor, IκBα and inhibit the expression of cytokines (Almawi and Melemedjian (2002)). Induction of protein degradation by TNF-α, is mediated through ATP-ubiquitin dependent proteolysis (Li *et al* (1998)). Protein

degradation is mediated through proteasomal degradation of IκBα and translocation of NF-κB to the nucleus (Li and Reid (2000)).

Whitehouse and Tisdale (2003) established both PIF and 15(S)-hydroeicosatetraenoic acid (15(S)-HETE), an intracellular signal for the increased protein degradation induced by PIF (Whitehouse *et al* (2003)), induce degradation of IκBα and nuclear accumulation of NF-κB, associated with an increased proteasome expression. This was inhibited either by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), or the NF-κB inhibitor peptide SN50, with attenuation of the PIF-induced increase in proteasome expression, suggesting NF-κB may act as a transcription factor in the PIF-induced increase in proteasome expression.

To determine whether NF-κB mediates PIF-induced protein degradation and up-regulation of the ubiquitin-proteasome pathway in skeletal muscle, C_2C_{12} murine myoblasts are to be transfected with either two dominant negative mutants of IκBα. In IκBαΔN the phosphorylation sites required for degradation of IκBα (Ser ^{32} and Ser ^{36}) are absent (truncation of amino acids 1-36), while in IκBα S32/A36 there are point mutations of Ser ^{32} and Ser ^{36} to alanine (Li and Reid (2000)). This prevents ubiquitin conjugation and proteolysis of IκBα and subsequent nuclear accumulation of NF-κB (Brockman *et al* (1995)). The control cells are to be transfected with the empty pCMV4 vector, which has no effect on IκBα phosphorylation.

Methodology

Purification of PIF

See section 2.3.1

Production of transformed colonies

See section 2.6

Genetic Modification Risk Assessment

See appendix I

Myogenic cell culture and transfection

See section 2.6

Measurement of protein degradation

See section 2.5.1.d

Measurement of proteasome activity

See section 2.5.1.e

Western Blot Analysis

For a full protocol see section 2.3.3

Samples of total cellular lysate (30μg) were used for detection of IκBα 30 min after the addition of PIF. Cells incubated with PIF for 24h was used to assess proteasome activity and increased proteasome expression.

The primary antibodies were used at a dilution of 1:1000 except for actin (1:250) and myosin (1:100) and secondary antibodies were used at a dilution of 1:1500. Incubation was for 1h at room temperature and development was enhanced by chemiluminescence (ECL) (Amersham, UK). Blots were scanned by a densitometer to quantitate differences.

Electectrophoresis mobility shift assay (EMSA)

DNA binding proteins were extracted from myotubes according to the method of Andrews and Faller (1991) (see section 2.8.1) which utilises hypotonic lysis followed by a high salt

extraction of nuclei. The EMSA binding assay was carried out using a Panomics EMSA 'gel shift' kit according to instructions (see section 2.8.2).

Statstical Analysis

Differences in means between groups was determined by one-way ANOVA, followed by the Tukey-Kramer multilple comparisons post test.

Results

Western blot analysis of IκBα degradation in response to PIF after 30min in 'wild-type' and 'mutant' transfected murine myotubes.

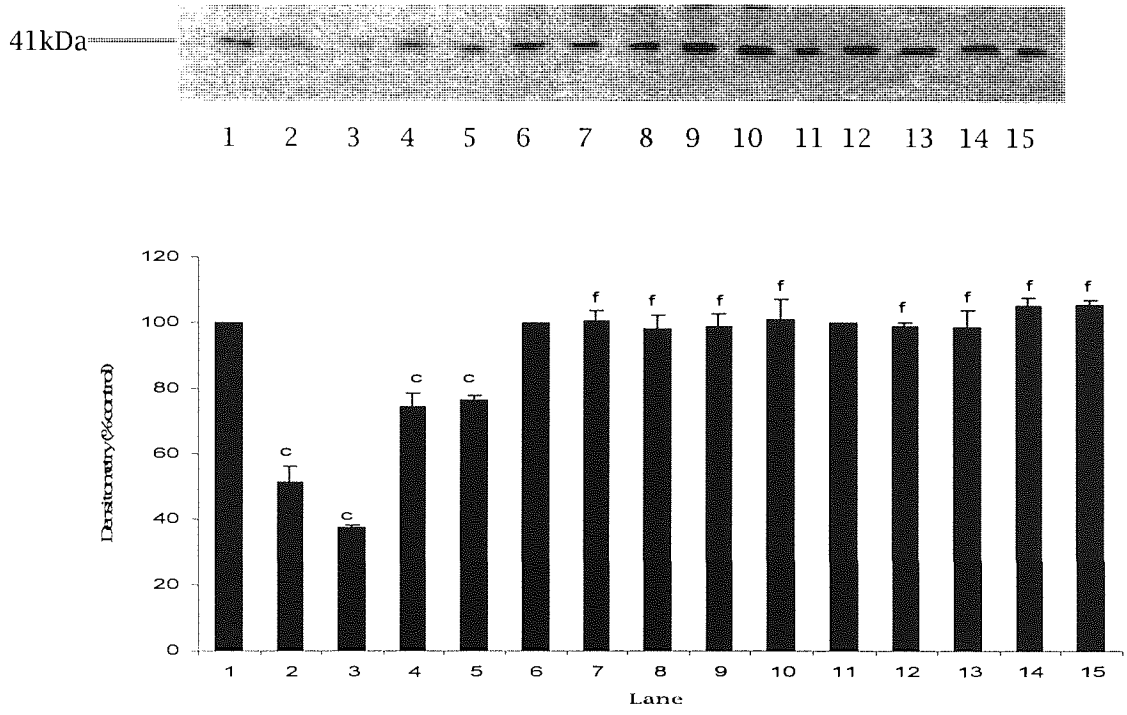


Figure 4. 1a

Myotubes were treated with 0 (lanes 1, 6 and 11), 2.1 (lanes 2, 7 and 12), 4.2 (lanes 3, 8 and 13), 10.5 (lanes 4, 9 and 14) and 16.8 nM PIF (lanes 5, 10 and 15) in wild type pCMV4 (lanes 1-5), IκBα S32/A36 (lanes 6-10) and IκBαΔN (lanes 11-15). Densitometric analysis is of three replicate blots. Results are expressed as mean ± S.E.M., with differences determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and pCMV4 are indicated as c, $p<0.001$ while differences between groups are indicated by f, $p < 0.001$.

Actin loading control

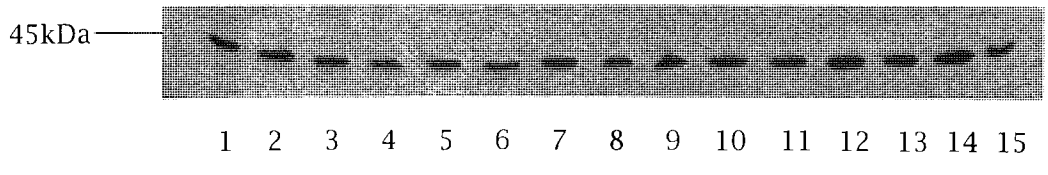


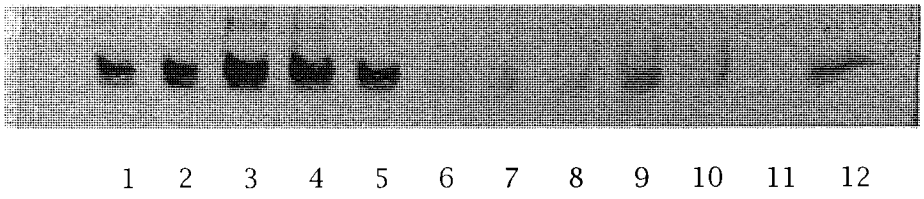
Figure 4. 1b

Lane discipline as figure 4. 1a

Figure 4. 1a

PIF induced a decrease in cytosolic IκBα in C_2C_{12} myotubes transfected with the control plasmid DNA (pCMV4 vector). Myotubes transfected with the dominant negative forms of IκBα, IκBαS32/A36 and IκBαΔN did not demonstrate a response to PIF, with no characteristic degradation of IκBα.

DNA binding of NF-κB in pCMV4 'wild type' and 'mutant' IκBα S32/36A transfected myotubes in response to PIF after 30 min



1 2 3 4 5 6 7 8 9 10 11 12

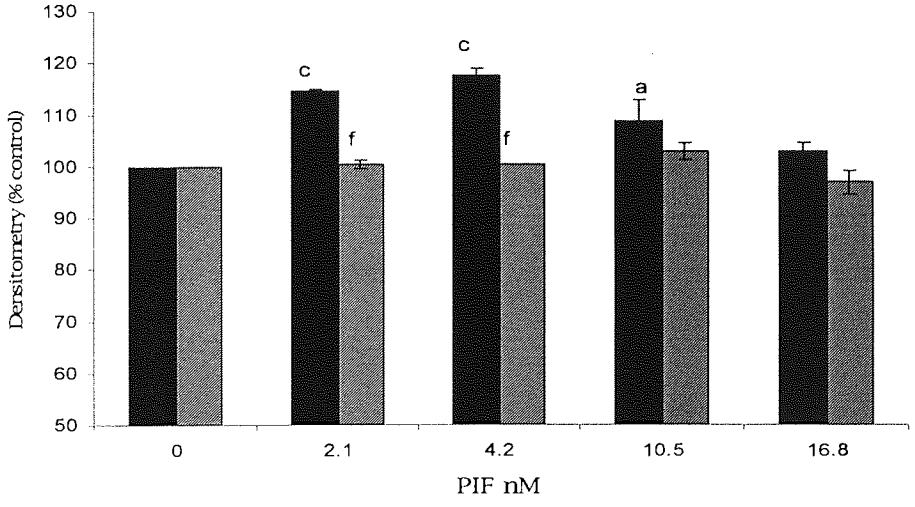


Figure 4. 1c

C_2C_{12} myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10). DNA binding of NF-κB was determined in pCMV4 (lanes 1-5, ■) and IκBα S32/A36 (lanes 6-10, ▨). Lane 11 is a control with 100 fold excess of cold NF-κB probe, and lane 12 is a positive control for NF-κB supplied with the kit. Densitometric analysis is based on 3 replicate EMSAs, results are expressed as mean ± S.E.M. Significance was determined by one-way ANOVA followed by Tukey's test. Differences from control are indicated as a, $p < 0.05$ and c, $p < 0.001$ whereas differences between groups are indicated as f, $p < 0.001$.

DNA binding of NF-κB in pCMV4 'wild type' and 'mutant' IκBαΔN transfected myotubes in response to PIF after 30 min

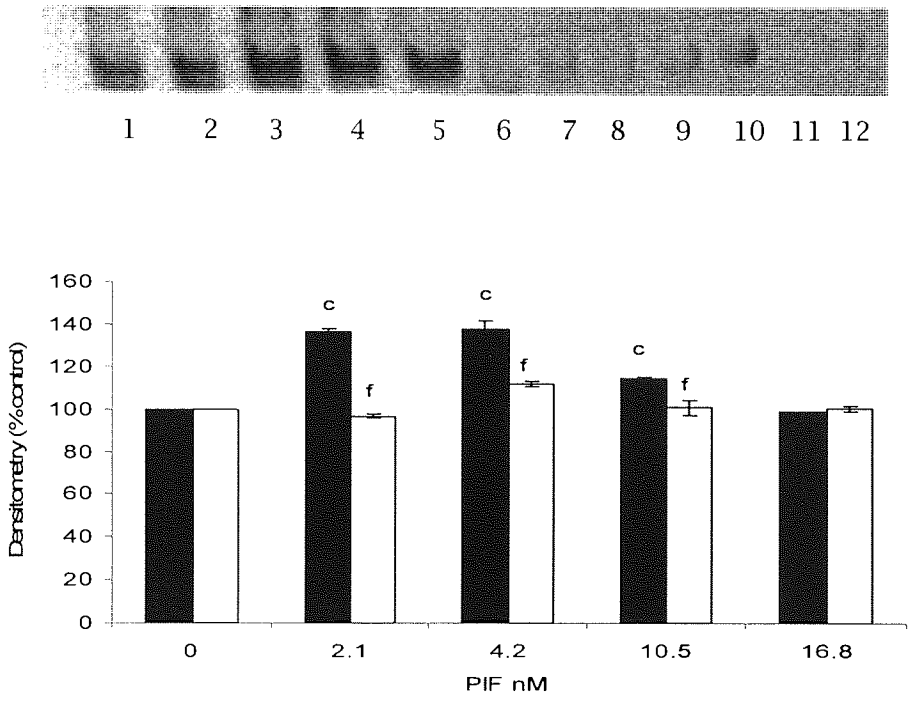


Figure 4. 1d

C_2C_{12} myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10). DNA binding of NF-κB was determined in pCMV4 (lanes 1-5, ■) and IκBαΔN (lanes 6-10, □). Lane 11 is a control with 100 fold excess of cold NF-κB probe, and lane 12 is a positive control for NF-κB supplied with the kit. Densitometric analysis is based on 3 replicate EMSAs, results are expressed as mean ± S.E.M. Significance was determined by one-way ANOVA followed by Tukey's test. Differences from control are indicated as a, $p < 0.05$ and c, $p < 0.001$ whereas differences between groups are indicated as f, $p < 0.001$.

Figure 4. 1c and 4. 1d

PIF-induced nuclear accumulation of NF-κB in control myotubes transfected with the pCMV4 vector, was as previously reported by Whitehouse and Tisdale (2003). However, myotubes transfected with the dominant negative forms of IκBα, IκBαS32/A36 and IκBαΔN did not demonstrate increased DNA binding of NF-κB in response to PIF

Total protein degradation in response to PIF (24h) in 'wild type' and 'mutant' IκBα transfected myotubes

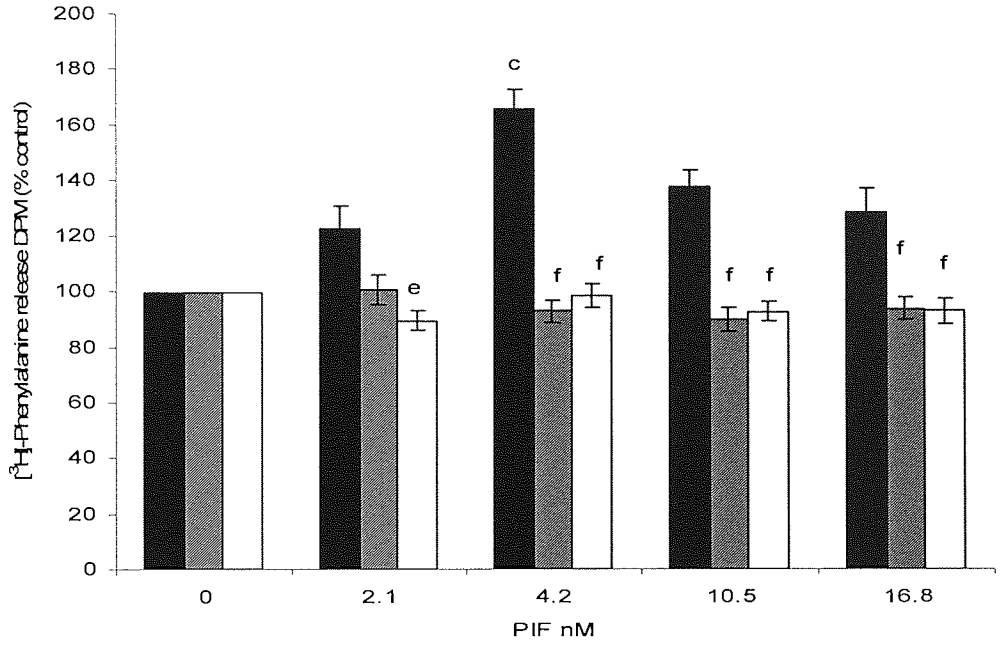


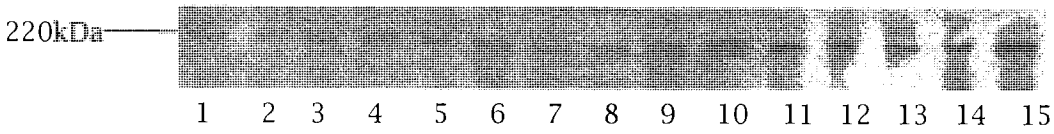
Figure 4. 2

Results expressed as mean ± S.E.M of one experiement, where n=6. The experiment was repeated twice on different days with similar results with pCMV4 (■), IκBα S32/A36 (▨) and IκBαΔN (□) over 24h. Statistical significance was detemined by one-way ANOVA followed by Tukey's test and the difference between 0nM PIF and pCMV4 is shown as c, $p < 0.001$, while differences between pCMV4 and 'mutant' IκBα inserts are shown as e, $p < 0.01$ and f, $p < 0.001$.

Figure 4. 2

The effect of PIF on protein degradation in 'wild-type' and 'mutant' cells is represented in figure 3.2. PIF produced a significant increase in total protein degradation, as measured by [^{3}H] phenylalanine release, over the concentration range of 2 to 16.8nM PIF, in 'wild-type' but not 'mutant' cells. The effect was seen over the same concentration that induced IκBα degradation (figure 4. 1a) and nuclear accumulation of NF-κB (figure 4. 1c and 4. 1d). The effect in wild-type cells was similar to that previously reported for non-transfected myotubes (Gomes-Marcondes *et al* (2002)).

Western blot analysis of myosin in 'wild type' and 'mutant' IκBα transfected myotubes in response to PIF (24h)



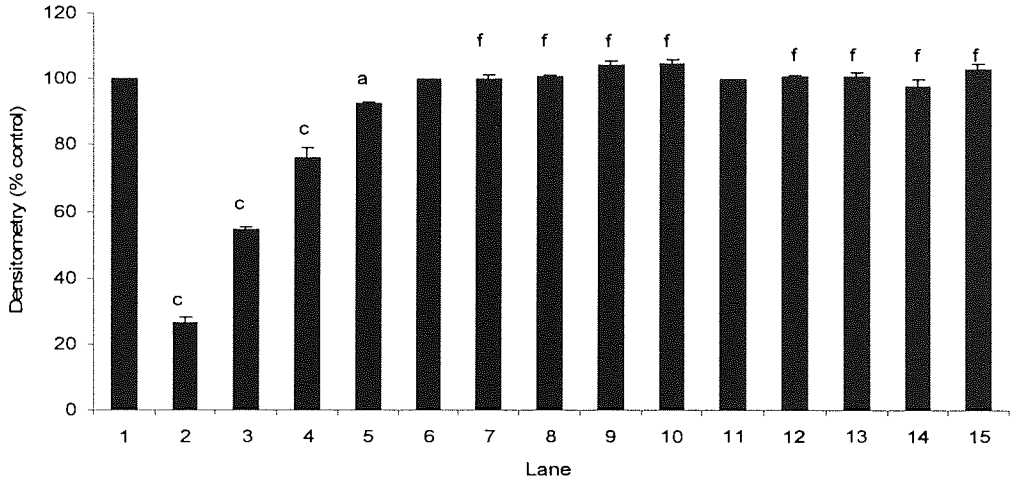


Figure 4. 3a

Wild type pCMV4 (lanes 1-5), IκBα S32/A36 (lanes 6-10) and IκBαΔN (lanes 11-15). Myotubes were incubated with 0 (lanes 1, 6 and 11), 2.1 (lanes 2, 7 and 12), 4.2 (lanes 3, 8 and 13), 10.5 (lanes 4, 9 and 14) or 16.8 nM PIF (lanes 5, 10 and 15) for 24h. Densitometric analysis is based on three replicate blots, results are expressed as mean ± S.E.M, and statistical significance between groups was determined by one-way ANOVA followed by Tukey's test. Differences from wild type pCMV4 are indicated as a, $p < 0.05$ and c, $p < 0.001$, while differences between 'mutant' IκBα and 'wild-type' inserts are indicated as f, $p < 0.001$.

Actin loading control

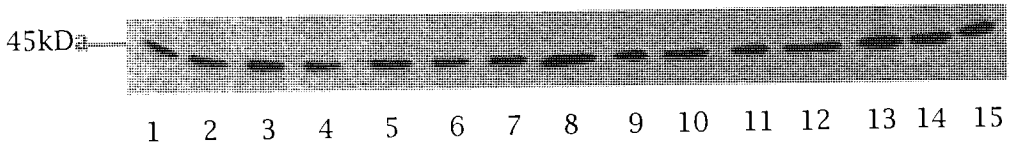


Figure 4. 3b
Lane discipline as figure 4.3a

Figure 4. 3a
PIF induced a significant decrease in the myofibrillar protein myosin in myotubes transfected with the wild-type pCMV4 vector. This was significant at concentrations of PIF between 2 and 16.8nM. There was no decrease in myosin in myotubes expressing the dominant negative forms of IκBα (IκBαS32/A36 and IκBαΔN), therefore the ability of mutant IκBα to inhibit PIF-induced protein loss indicates that this response depends on NF-κB signalling.

Lorite *et al* (2001) and Gomes-Marcondes *et al* (2002) have previously reported that PIF-induced protein degradation was strongly correlated with an increase in expression of key regulatory components of the ubiquitin-proteasome proteolytic pathway.

'Chymotrypsin-like' proteasome activity of 'wild type' and 'mutant' IκBα transfected myotubes in response to PIF (24h)

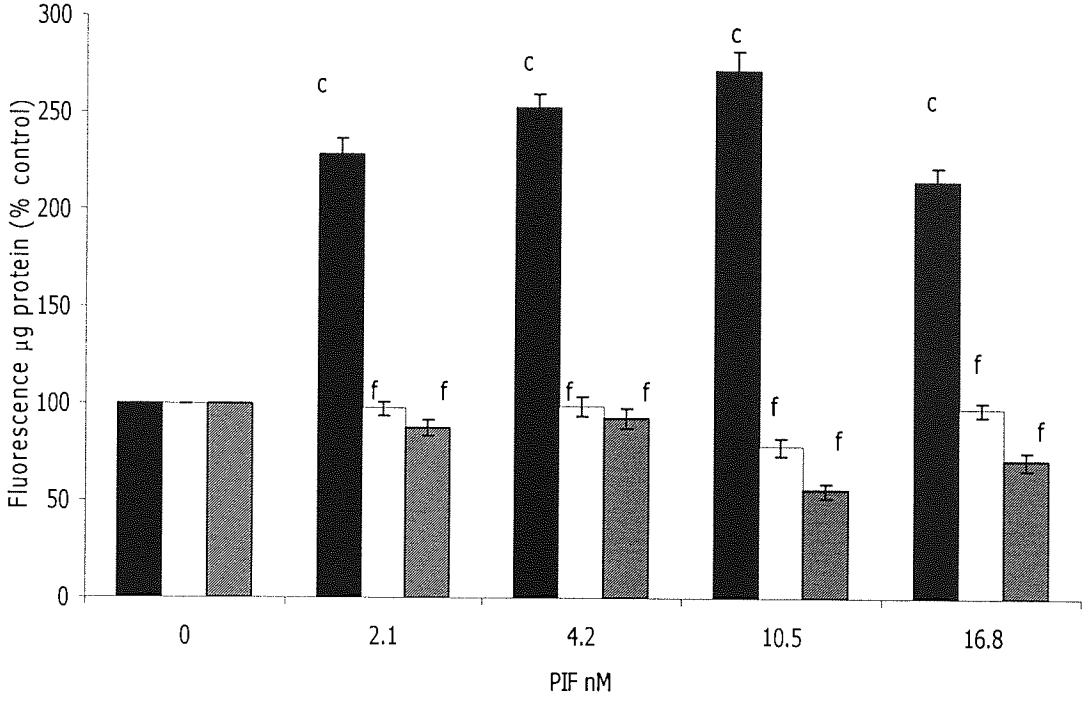


Figure 4. 4

pCMV4 myotubes (■); IκBα S32/A36 (▨) and IκBαΔN (□) were treated with concentrations of PIF ranging from 0nM to 16.8nM. Results are expressed as mean ± S.E.M and are representative of 3 separate experiments. Significance between groups was determined by one-way ANOVA followed by Tukey's test. Difference from 0nM PIF and pCMV4 is indicated as c, $p < 0.001$, whereas differences between 'mutant' IκBα and 'pCMV4' myotubes are indicated as f, $p < 0.001$.

Figure 4. 4

Elucidating the importance of NF-κB in PIF-induced increase in proteasome activity, was achieved by measuring the 'chymotrypsin-like' enzyme activity, the major proteolytic activity of the β-subunits of the proteasome. The specific proteasome inhibtior lactacystin is an antibiotic derived from *Streptomyces,* and spontaneously hydrolyses into *clasto*-lactacystin β-lactone, which becomes the active inhibitor that reacts with the N-terminal threonine of the 20S catalytic core of the proteasome (Craiu *et al* (1997)). An increase in activity was seen at concentrations of PIF between 2 and 16.8nM in myotubes transfected with the control vector pCMV4, which correlated with previously reported observations in

non-transfected myotubes (Gomes-Marcondes *et al* (2002)). Myotubes transfected with both mutant constructs of IκBα showed no increase in 'chymotrypsin-like' enzyme activity in the presence of PIF.

Western blot analysis of 20S proteasome α subunit expression in 'wild type' and 'mutant' IκBα transfected myotubes in response to PIF (24h)

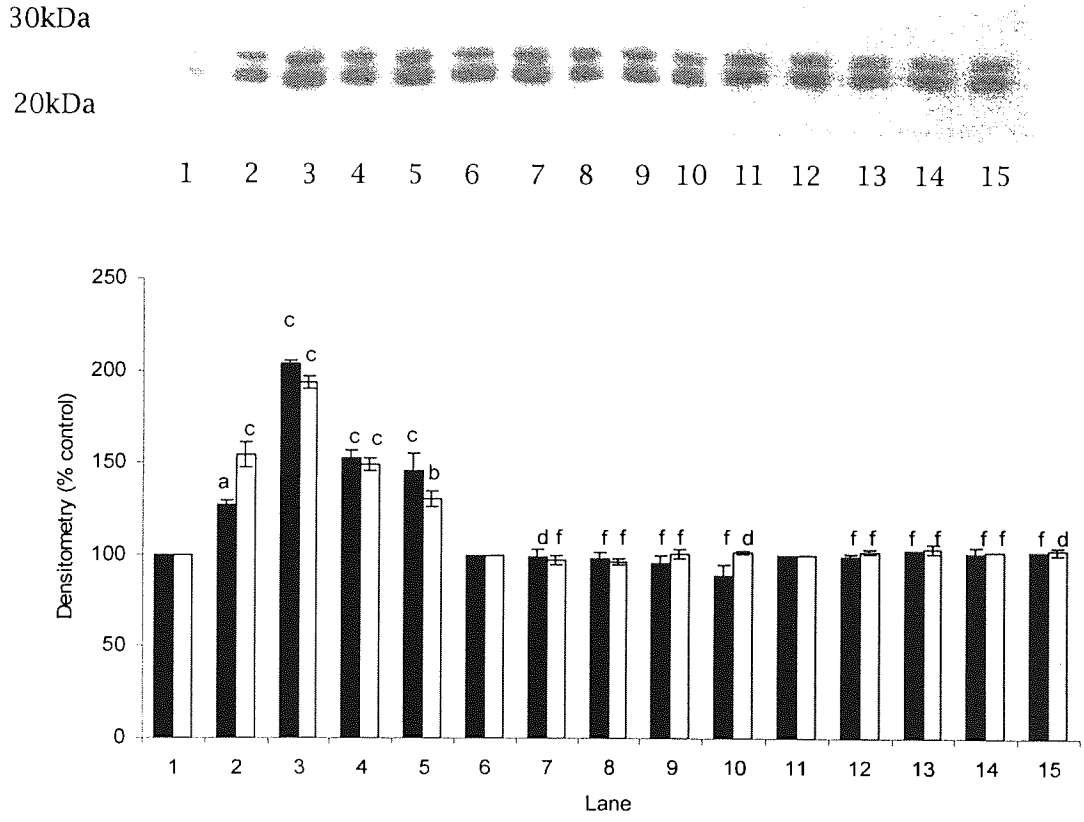


Figure 4. 5a

pCMV4 (lanes 1-5), IκBαS32/A36 (lanes 6-10) and IκBαΔN (lanes 11-15) were incubated for 24h with 0 (lanes 1, 6 and 11), 2.1 (lanes 2, 7 and 12), 4.2 (lanes 3, 8 and 13), 10.5 (lanes 4, 9 and 14) or 16.8nM PIF (lanes 5, 10 and 15). Densitometric analysis of three replicate blots is shown above, (■) represents band 1 and (□) represents band 2. Results are expressed as mean ± S.E.M and significance between groups was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF and pCMV4 are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$ whereas differences between pCMV4 and mutant IκBα constructs are indicated as d, $p < 0.05$, e, $p < 0.01$ and f, $p < 0.001$.

Actin loading control

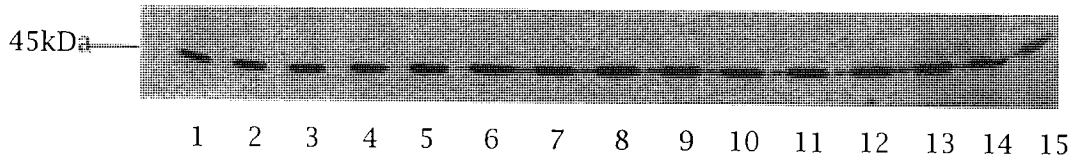


Figure 4. 5b
Lane discipline as figure 4. 5a

Western blot analysis of MSS1 proteasome subunit expression in response to PIF (24h) 'wild type' and 'mutant' IκBα myotubes

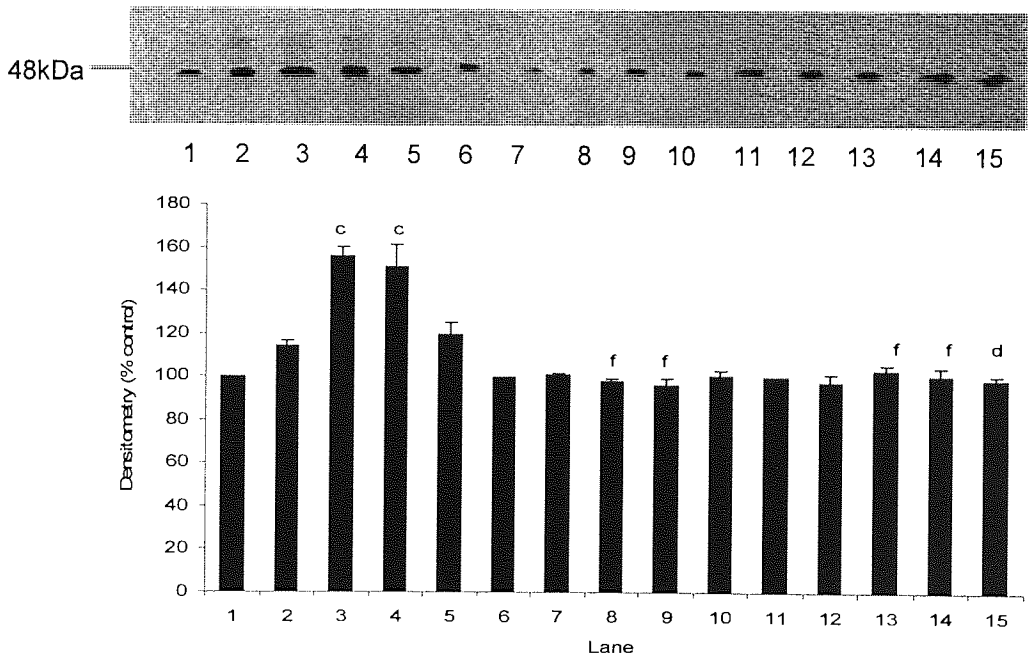


Figure 4. 5c
Myotubes pCMV4 (lanes 1-5), mutant IκBαS32/A36 (lanes 6-10) and mutant IκBαΔN (lanes 11-15) were incubated with PIF for 24h at 0nM (lanes 1, 6 and 11), 2.1nM (lanes 2, 7, 12), 4.2nM (lanes 3, 8, 13), 10.5nM (lanes 4, 9, 14) or 16.8nM (lanes 5, 10 and 15). Densitometric analysis is of three replicate blots amnd results are expressed as mean ± S.E.M. Significance between groups was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF and pCMV4 are indicated as c, $p < 0.001$ while differences between 'wild type' and 'mutant' IκBα are indicated as d, $p < 0.05$ and f, $p < 0.001$.

Actin loading control

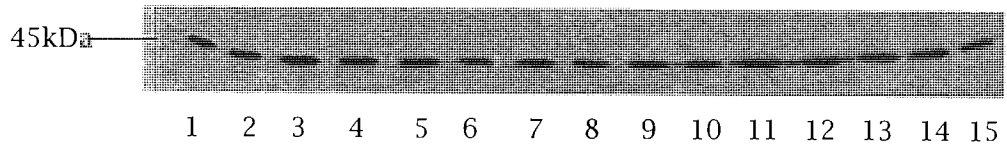


Figure 4. 5d

Lane discipline as figure 4. 5c

Western blot analysis of p42 proteasome subunit expression in 'wild type' and 'mutant' IκBα transfected myotubes in response to PIF (24h)

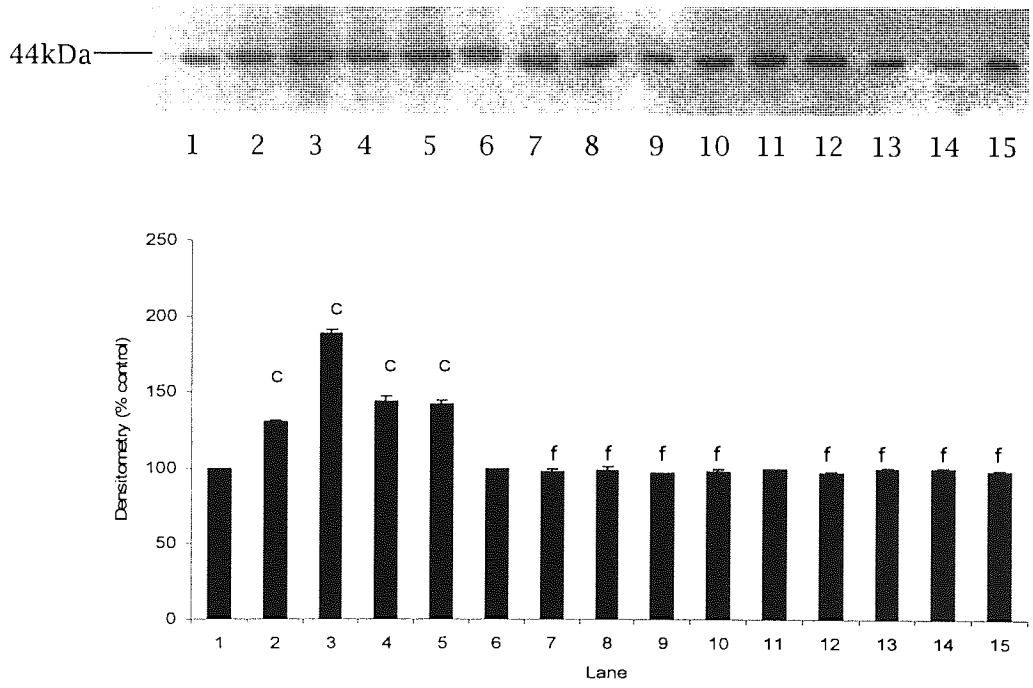


Figure 4. 5e

Myotubes pCMV4 (lanes 1-5), IκBα S32/A36 (lanes 6-10) and IκBαΔN (lanes 11-15). were incubated with 0 (lanes 1, 6 and 11), 2.1 (lanes 2, 7 and 12), 4.2 (lanes 3, 8 and 13), 10.5 (lanes 4, 9 and 14) or 16.8nM PIF (lanes 5, 10 and 15). Densitometric analysis is of three replicate blots and results are expressed as mean ± S.E.M. Significance between groups was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF and pCMV4 are shown as c, $p < 0.001$ whereas differences between pCMV4 and 'mutant' IκBα are shown as f, $p < 0.001$.

Actin loading control

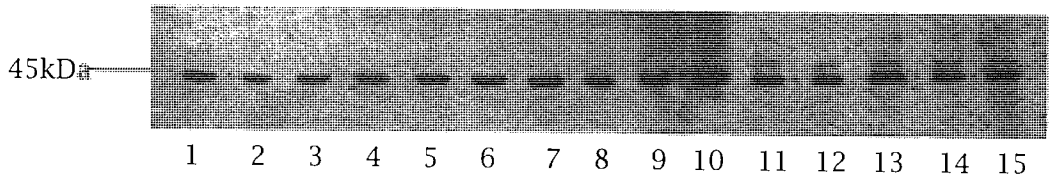
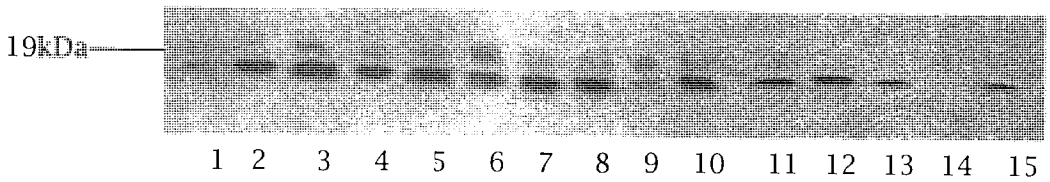


Figure 4. 5f

Lane discipline as figure 4. 5e.

Western blot analysis of $E2_{14K}$ expression in 'wild type' and 'mutant' IκBα transfected myotubes in response to PIF (24h)



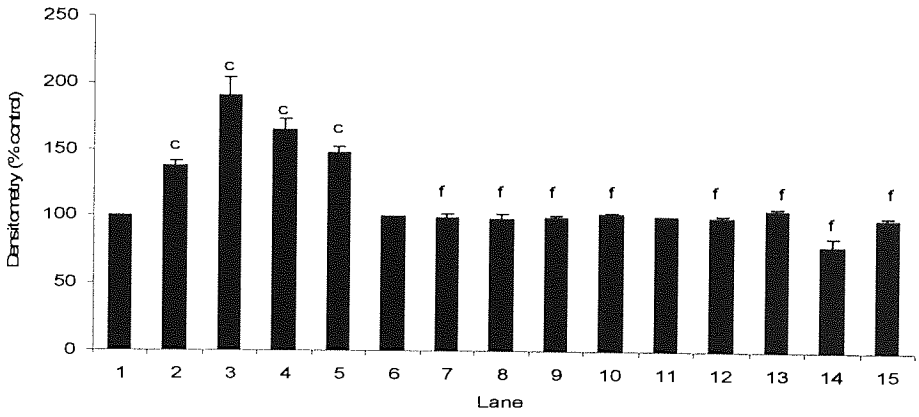


Figure 4. 5g

Myotubes pCMV4 (lanes 1-5), IκBα S32/A36 (lanes 6-10) and IκBαΔN (lanes 11-15) were incubated for 24h with 0nM (lanes 1, 6 and 11), 2.1 (lanes 2, 7 and 12), 4.2 (lanes 3, 8 and 13), 10.5 (lanes 4, 9 and 14) or 16.8 nM PIF (lanes 5, 10 and 15). Densitometric analysis was conducted on 3 replicate blots and results are expressed, as mean ±S.E.M. Significance between groups was determined using one-way ANOVA followed by Tukey's test. Differences from 0nM PIF and pCMV4 are indicated as c, $p < 0.001$, while differences between pCMV4 and 'mutant' IκBα are indicated as f, $p < 0.001$.

Actin loading control

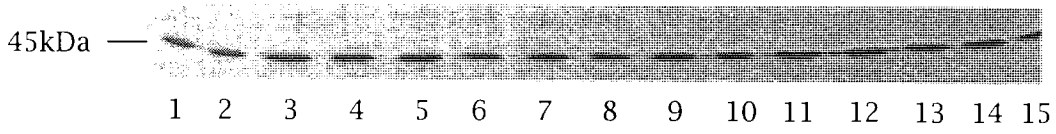


Figure 4. 5h
Lane discipline as figure 4. 5g

Figures 4. 5a - 4. 5h
Expression of proteasome subunits was determined by western blotting of cellular supernantents. The effect of PIF on 20S proteasome α subunit expression in wild type and mutant IκBα transfected C_2C_{12} myotubes (figure 4. 5a) demonstrates how PIF-induced a significant increase in 20S proteasome α subunit expression between 2 and 10.5nM in wild type, but not mutant cells.

PIF also induced an increased expression of MSS1, an ATPase subunit of the 19S regulatory complex of the proteasome in myotubes transfected with the control vector, pCMV4, but not in those transfected with either type of mutant (figure 4. 5c). MSS1 expression increased in pCMV4 myotubes in the same concentration range as previously reported in non-transfected C_2C_{12} myotubes (Gomes-Marcondes *et al* (2002)).

PIF-induced an increase in expression of p42, an ATPase subunit of the 19S regulator that promotes ATP-dependent association of the 20S proteasome with the 19S regulator to form the 26S proteasome (Tanahashi *et al* (1999)), p42 expression was increased in wild-type but not mutant myotubes (figure 4. 5e). The concentrations of PIF that induced an increase in p42 expression were the same as that increased 20S proteasome α subunit expression (figure 4. 5a).

PIF produced an increase in expression of the ubiquitin-conjugating enzyme ($E2_{14K}$) in 'wild-type' but not in myotubes expressing the 'mutant' forms of IκBα (figure 4. 5g). Gomes-Marcondes *et al* (2002) previously demonstrated $E2_{14K}$ expression to parallel that of proteasome subunits. The ability of 'mutant' IκBα to inhibit PIF-induced proteasome expression confirms that this response also depends on inititation of transcription by NF-κB.

Discussion

NF-κB is important in cellular function, providing a rapid response and is an efficient regulator of immune and inflammatory responses (Lee & Buckart (1998)). This chapter looks at the role of mutated IκBα at Ser ^{32} and Ser ^{36} to investigate a role for NF-κB in protein degradation and induction of the ubiquitin-proteasome proteolytic pathway by PIF. The IKK complex phosphorylates IκBα at Ser^{32} and Ser^{36} and targets IκBα for ubiquitination and subsequent degradation by the proteasome (Brockman *et al* (1995)). The conjugation reaction catalysed by E2 is specific, the enzyme does not recognise the mutated form of IκBα with alanine replacing serine at residues ^{32} and $^{36.}$ Therefore the mutant species of IκBα cannot be phosphorylated and conjugated with ubiquitin to target the protein for degradation (Gonen *et al* (1999)).

The observations within this chapter indicate that NF-κB activation is involved in PIF-induced degradation of myofibrillar protein. PIF in this respect appears to be similar to TNF-α (Li and Reid (2000)). An alternative pathway has recently been reported by Fan *et al* (2003), whereby phosphorylation of the tyrosine 42 residue of IκBα mediates NF-κB activation. This pathway is distinct from the canonical proinflammatory pathway, and that taken by PIF, whereby NF-κB activation is mediated through serine phosphorylation of IκBα. It was found that this alternative IKK independent pathway regulated NF-κB through c-Src activation, which for transcriptional activation of NF-κB requires tyrosine and not serine phosphorylation of IκBα and usually occurs in the redox activation of NF-κB. This alternative pathway is not in operation in C_2C_{12} myotubes in response to PIF, due to the lack of nuclear accumulation of NF-κB in cells transfected with IκBα mutant plamids (figure 4. 1b and 4. 1c). The lack of response of PIF treated myotubes with mutant IκBα suggests PIF to mediate an effect through NF-κB.

The observations from this chapter indicate NF-κB activation is involved in PIF-induced skeletal muscle atrophy. Hunter *et al* (2002) found nuclear extracts of soleus muscle from rats undergoing disuse atrophy, to exhibit increased binding of NF-κB consensus oligonucleotide complexes. This complex bound antibodies to p50, c-Rel and Bcl3, but no other NF-κB family members. There was no evidence for the canonical NF-κB pathway involving activation of p65 or IκB, suggesting another alternative NF-κB pathway in skeletal muscle disuse, distinct from that observed induced by PIF in cancer cachexia (Hunter *et al* (2002)).

An important similarity between the muscle loss seen in cachexia and that in disuse atrophy, is that protein degradation is mediated primarily by the ubiquitin-proteasome pathway (Lecker *et al* (1999)). This suggests that activation of NF-κB may be a common mechanism for increased gene expression of key regulatory components of this pathway by agents such as PIF and TNF-α (Li and Reid (2000)). However, this mechanism is opposite to that induced by glucocorticoids in L6 muscle cell nuclei (Du *et al* (2000). In this model, glucocorticoids stimulate C3 subunit expression by antagonizing the interaction of this NF-κB protein with an NF-κB response element in the C3 promoter region.

Tiao *et al* (1994) determined that increase in total and myofibrillar protein breakdown during sepsis was a result of a several fold increase in ubiquitin mRNA. This study suggested that increased muscle proteolysis during sepsis is due to increased non-lysosomal energy dependent protein breakdown, involving ubiquitin-dependent proteolysis. Penner *et al* (2001) showed that during skeletal muscle sepsis NF-κB binding activity increased at 4h, but decreased at 16h below control levels. Treatment with the glucocorticoid receptor antagonist RU38486 increased NF-κB binding activity, suggesting that decreased activity of NF-κB is a result of glucocorticoids. In contrast, AP-1 was activated throughout the entire course of sepsis, which suggests multiple transcription factors may be involved in the development of muscle cachexia during sepsis.

PIF has also been shown to activate NF-κB in primary hepatocytes and the human cancer cell line HepG2, resulting in increased production of interleukins-6 and -8 (IL-6 and (IL-8), C reactive protein, and decreased production of transferrin (Watchorn *et al* (2001)), suggesting the primary effects of PIF on gene expression are mediated through NF-κB. The actual mechanism has not been fully elucidated yet, but recent evidence (Smith and Tisdale (2003)) suggests that PIF induces the activation of both phospholipases A and C (PLA_2 and PC-PLC). PLA_2 causes the release of arachidonic acid from membrane phospholipids, while diacylglycerol derived from PC-PLC has been suggested to be a positive feedback signal to protein kinase C (Smith and Tisdale (2003)). Smith *et al* (2004) demonstrated PKC to be involved in PIF-induced proteasome expression, and PKC may be responsible for NF-κB activation through phosphorylation and activation of IκBα kinase (IKK) (Smith *et al* (2004)).

Induction of protein degradation and expression of the ATP-dependent ubiquitin proteolysis through activation of NF-κB provides a possible mechanism to explain the effect of EPA, in attenuating the process in MAC16 tumour bearing mice (Beck *et al* (1991)). EPA is clinically effective in attenuating loss of lean body mass in patients with

pancreatic carcinoma (Wigmore *et al* (1996)). Whitehouse and Tisdale (2003) showed EPA to prevent degradation of IκBα and nuclear accumulation of NF-κB in murine myotubes in the presence of PIF, through attenuation of upstream signalling pathways. Therefore agents capable of inhibiting activation of NF-κB should potentially be capable of inhibiting muscle protein degradation in cancer cachexia.

Chapter 5

Induction of proteasome expression in skeletal muscle is attenuated by inhibitors of NF-κB activation

Introduction

Loss of skeletal muscle mass in cancer patients has a negative influence on prognosis, resulting in asthenia, immobility and early death. There are three major proteolytic pathways involved in the intracellular degradation of proteins in skeletal muscle. The ubiquitin-proteasome proteolytic pathway is considered to be the predominant system in both experimental models of cancer cachexia (Attaix *et al* (1999)) and in cancer patients (Bossola *et al* (2003)). Expression of the ubiquitin associated enzymes and proteasome subunits are upregulated in cancer cachexia (Lorite *et al* (1998)). Knowledge regarding the mechanism for regulation of proteasome expression in cancer cachexia should facilitate the design of effective inhibitors.

Muscle mass in cancer cachexia has been found to be regulated by a tumour produced sulphated glycoprotein, proteolysis-inducing factor (PIF). Smith *et al* (1993) determined that MAC16 tumour bearers with weight losses between 16-25% demonstrated a massive depletion of lean body mass, a reduction in protein synthesis and increased protein degradation.

Lorite *et al* (2001) determined that protein degradation associated with MAC16 tumour bearers was a result of increased expression of the key components of the ubiquitin-proteasome proteolytic pathway.

In murine myoblasts, the PIF-induced increase in proteasome expression is associated with increased DNA binding of the transcription factor NF-κB. This is accompanied by a transient decrease in cytosolic levels of the NF-κB inhibitor protein IκBα (Whitehouse and Tisdale (2003)).

A high molecular weight complex (IκB kinase or IKK), in the case of PIF, is probably activated by protein kinase C (PKC) (Smith *et al* (2004)). This IKK complex phosphorylates the N-terminal region of IκBα, at serine residures, Ser^{32} and Ser^{36}, targeting IκBα for ubiquitination and degradation by the 26S proteasome complex (Chen *et al* (1995)). The

degradation of IκBα unmasks the nuclear localisation sequence of NF-κB, allowing NF-κB to translocate to the nucleaus and bind to target DNA (Schwartz *et al* (1999)).

The polyunsaturated fatty acid, EPA attenuates skeletal muscle loss in cancer cachexia. Whitehouse *et al* (2001) found EPA to significantly decrease proteasome activity and expression in gastrocnemius muscle, while myosin expression was restored to control levels. Whitehouse and Tisdale (2003) found EPA to prevent PIF induced nuclear accumulation of NF-κB, degradation of IκBα and increased proteasome expression, suggesting agents which interfere with activation of NF-κB may be useful in preventing muscle protein degradation in cancer cachexia.

Two potential agents have been studied to verify this hypothesis. Curcumin, a natural product from turmeric, prevents activation of NF-κB by blocking phosphorylation and subsequent degradation of IκBα. Han *et al* (1999) demonstrated curcumin downregulated the expression of survival genes *erg*-1, *c-myc*, *bcl*-X_L and p53 in B cells. NF-κB binding activity was also downregulated, leading to the suggestion that curcumin caused growth arrest and apoptosis of BKS-2 immature B cell lymphoma by downregulation of growth and survival promoting genes. Chuang *et al* (2002) investigated NF-κB and drug resistance in cancer cells. Basal levels of NF-κB activity were roughly correlated with drug resistance, however, when pre-treated with curcumin, tamoxifen and dexamethasone, doxorubicin-induced NF-κB activation was significantly attenuated. Therefore inhibition of NF-κB may be important in sensitizing cancer cells to chemotherapeutic drugs. Thaloor *et al* (1999) concluded NF-κB to exert a role in regulating myogenesis. Modulation of NF-κB activity within muscle tissue is beneficial for muscle repair, and systematic administration of curcumin, *in vivo* greatly enhanced muscle regeneration.

Resveratrol (3-4'-5-trihydroxystilbene) is a natural phytoalexin present in grape juice and wine. It has been shown to have anticancer, anti-inflammatory and antiaggregating properties (Fulgenzi *et al* (2001)). Carbo *et al* (1999) found resveratrol administered to YAH-130 bearing rats significantly decreased tumour cell content (25%), suggesting resveratrol induces apoptosis in the tumour cell.

Resveratrol mediates its effect through the inhibition of IKK, the key regulatory complex required for NF-κB activation of gene transcription. Holmes-McNary and Baldwin (2000) proposed resveratrol to inhibit upstream signalling components, leading to the activation of IKK as MEKK1, MEKK1 can activate both AP-1 and NF-κB and would explain the dual inhibition demonstrated by resveratrol.

Both agents have been evaluated for their ability to inhibit PIF-induced proteasome expression *in vitro* and as anticachectic agents in mice bearing the MAC16 tumour, which induces profound cachexia involving wasting of skeletal muscle (Beck and Tisdale (1987)).

Methodology

Animals

Pure strain NMRI mice (average weight 25g) were obtained from our won inbred colony and were fed a rat and mouse breeding diet (Special Diet Services, Witham, UK) and water *ad libitium*. Animals were implanted with the MAC16 tumour s.c. in the flank by means of trochar, selected from donor animals with established weight loss (Beck and Tisdale (1987)). Animals with an average weight loss of 5% (10-12 days after transplantation) were randomised to receive resveratrol [1mg/kg^{-1} body weight dissolved in DMSO : PBS (1:20)] i.p. daily, while controls received solvent alone. Body weight and tumour volume were measured daily. Tumour volumes were calculated according to the following formula : **(length x (width)^2) ÷2** . All animal experiments followed a strict protocol, approved by the British Home Office, and the ethical guidelines that were followed met the standards required by the UKCCR guidelines (Workman *et al* (1998)). Animals were terminated by cervical dislocation and the soleus muscles were quickly dissected out, together with intact tendons and maintained in isotonic ice-cold saline before determination of protein degradation. For protein degradation, muscles were fixed by their tendons at approximately resting length in 3ml of oxygenated (95% oxygen : 5% carbon dioxide) Krebs-Henesleit Buffer (pH7.4) containing 5mM glucose and 0.5mM cycloheximide. The protein degradation rate was determined by the release of tyrosine (Waalkes and Udenfriend (1957)) over 2h.

Purification of PIF

See section 2.3.1

Cell Culture

See section 2.5.1

Measurement of protein degradation

See section 2.5.1.d

Measurement of proteasome activity

See section 2.5.1.e

Western blot analysis

See section 2.3.3

Tyrosine Release Assay

See section 2.9

Electrophoresis mobility shift assay (EMSA)

DNA binding proteins were extracted from myotubes according to the method of Andrews and Faller (1991), which utilises hyptonic lysis, see section 2.8.1. The EMSA binding assay was carried out using a Panomics EMSA 'gel shift' kit according to manufacturers instructions. For full protocol see section 2.8.2

Statistical analysis

Differences in mean ± S.E.M. between groups was determined by one-way ANOVA, followed by Tukey-Kramer multiple comparison post test.

Results

To investigate the importance of the activation of NF-κB in PIF-induced proteasome expression and degradation of myofibrillar proteins in murine myotubes, the cell permeable NF-κB inhibitor SN50 (18μM), was used to block nuclear translocation of NF-κB (Lin *et al* (1995). SN50 contains the nuclear localisation sequence of the transcription factor NF-κB p50 linked to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor (K-FGF). The N-terminal K-FGF region confers cell permeability, while the nuclear localisation sequence 360-369 inhibits translocation of the NF-κB active complex into the nucleus, with maximum inhibition at 18μM (Lin *et al* (1995)).

SN50M is an inactive control for the SN50 peptide. SN50M corresponds to the SN50 peptide sequence with substitutions as Asn for Lys^{363} and Gly for Arg^{364} in the nuclear localisation sequence. This peptide had no measureable effect on NF-κB translocation at 18μM (Lin *et al* (1995)).

Effect of NF-κB inhibitor SN50 (18μM) and SN50M (18μM) on PIF-induced proteasome 20S α subunit expression (24h)

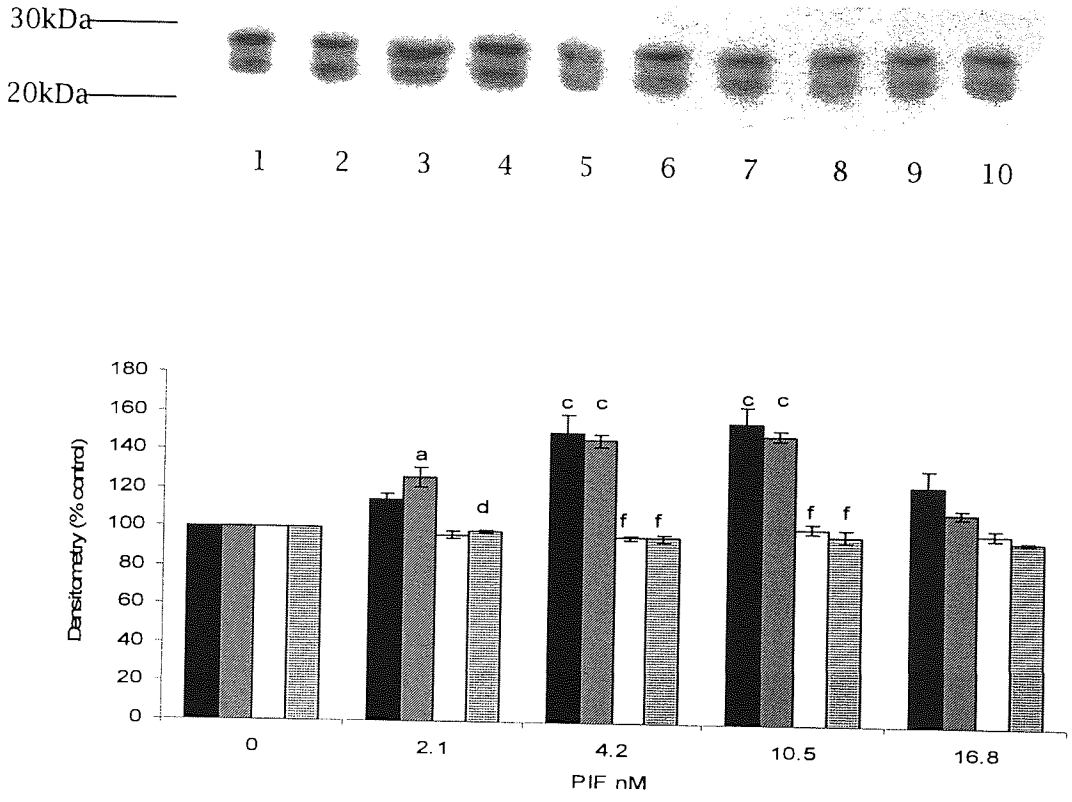


Figure 5. 1a

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M for the two major bands. Values for SN50M are represented by ■ and ▨, and SN50 as □ and ▨ Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and SN50M are indicated as a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$, while differences between SN50M and SN50 are d, $p < 0.05$, e, $p < 0.01$ and f, $p < 0.001$.

Effect of NF-κB inhibitor SN50 (18μM) and SN50M (18μM) on PIF-induced MSS1 proteasome subunit expression (24h)

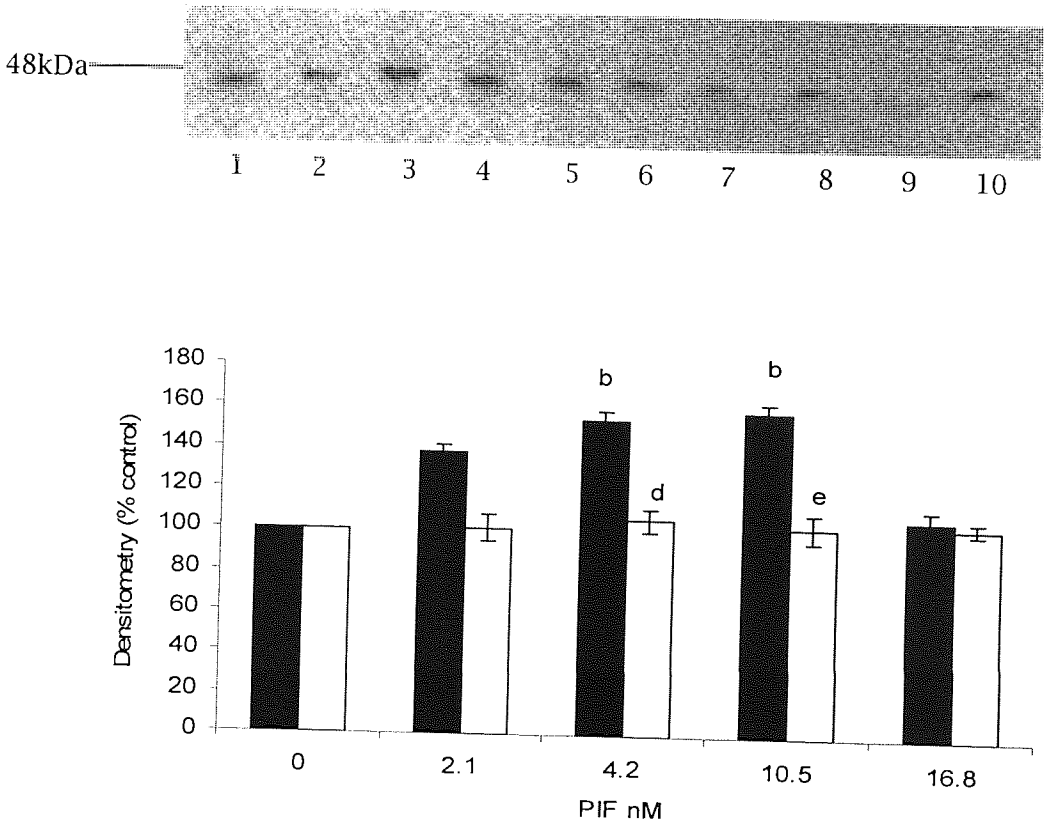


Figure 5. 1b

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. SN50M represented by ■ and SN50 as□ . Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and SN50M are indicated as b, $p < 0.01$, while differences between SN50M and SN50 are d, $p < 0.05$ and e, $p < 0.01$

Effect of NF-κB inhibitor SN50 (18μM) and SN50M (18μM) on PIF-induced p42 proteasome subunit expression (24h)

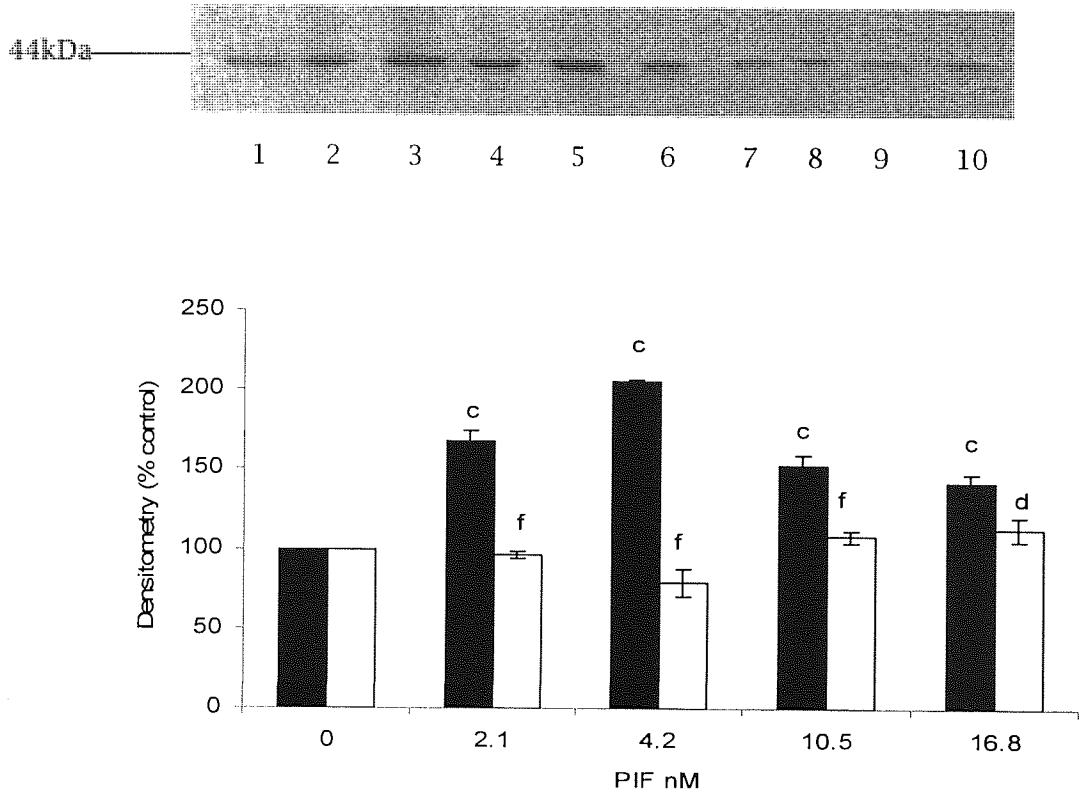


Figure 5. 1c

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). Densitometric analysis was based on three replicate blots, results are expressed as mean ± S.E.M. SN50M represented by ■ and SN50 as □. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and SN50M are indicated as c, $p < 0.001$, while differences between SN50M and SN50 are d, $p < 0.05$ and f, $p < 0.001$

Effect of NF-κB inhibitor SN50 (18μM) and SN50M (18μM) on PIF-induced $E2_{14K}$ expression (24h)

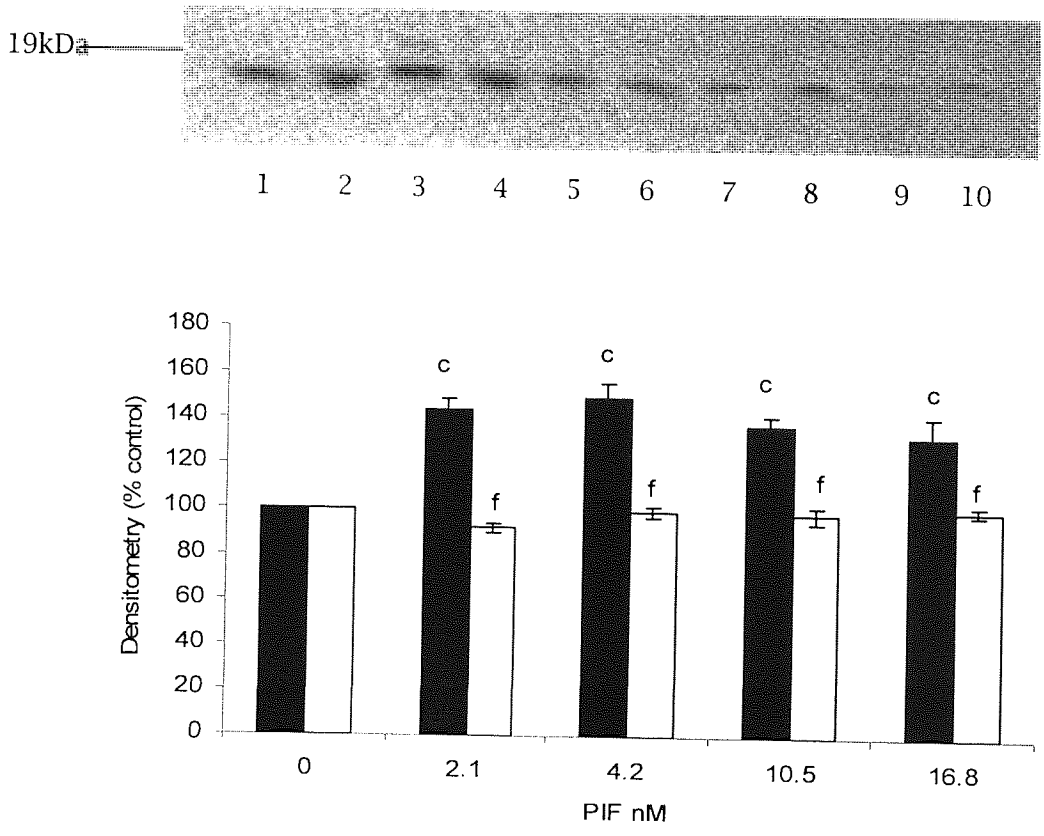


Figure 5. 1d

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. SN50M represented by ■ and SN50 as□ .Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and SN50M are indicated as c, $p < 0.001$, while differences between SN50M and SN50 are f, $p < 0.001$

Effect of NF-κB inhibitor SN50 (18μM) and SN50M (18μM) on PIF-induced depletion of myosin expression (24h).

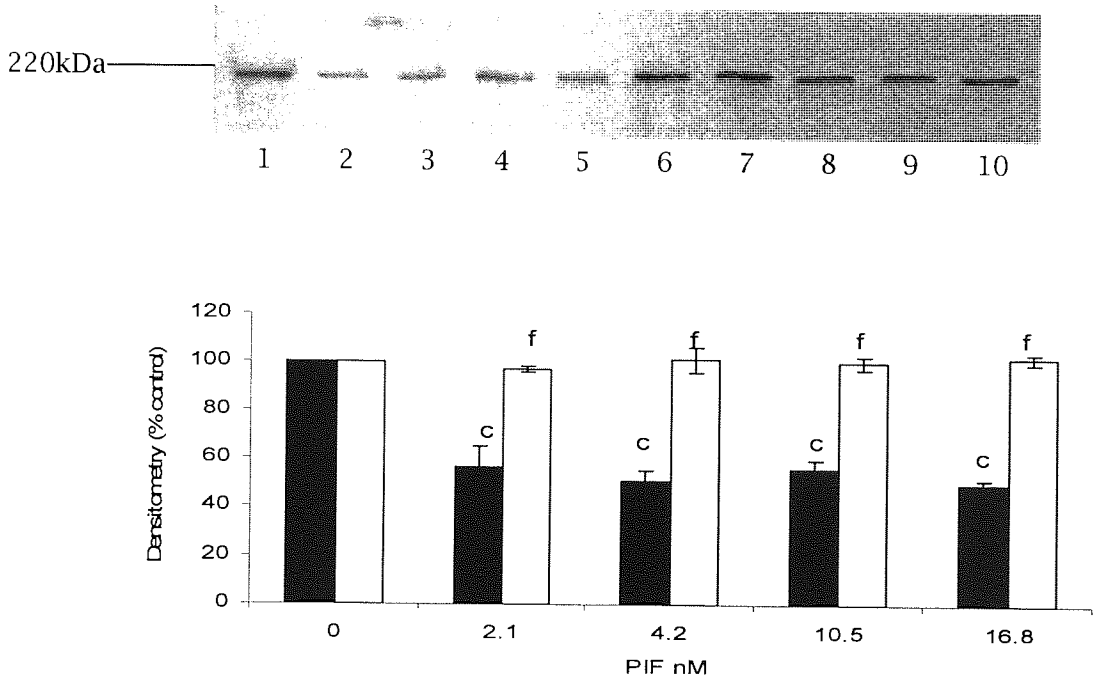


Figure 5. 1e

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. SN50M represented by ■ and SN50 as□ . Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF and SN50M are indicated as c, $p < 0.001$, while differences between SN50M and SN50 are f, $p < 0.001$

Actin loading control

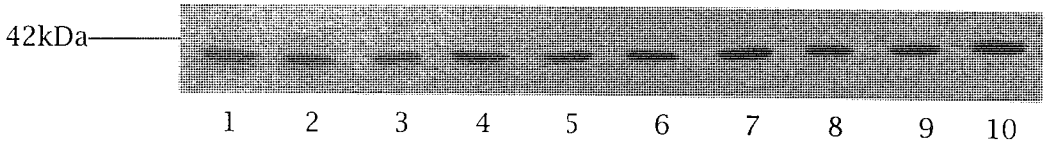


Figure 5. 1f

Lane discilpline as figures 5. 1a - 5. 1e

Figures 5. 1a - 5. 1f

Lin *et al* (1995) found SN50 to inhibit nuclear translocation of NF-κB in a concentration dependant manner in cultured endothelial and monocytic cells stimulated with LPS or TNF-α.

The PIF-induced increase in 20S proteasome α subunit expression in the presence of SN50M was not seen in the presence of SN50 (figure 5. 1a). The same pattern was observed in PIF-induced expression of MSS1 (figure 5. 1b) and p42 (figure 5. 1c).

$E2_{14K}$ is one of the major mammalian E2s that support E3-α dependent ubiquitin conjugate formation (Attaix *et al* (1999). Figure 5. 1d clearly demonstrates an increase in $E2_{14K}$ in response to PIF in C_2C_{12} myotubes treated with SN50M; however, there is no associated increase with PIF and the SN50 peptide.

PIF induced a decrease in myosin expression in C_2C_{12} myotubes treated with SN50M. This response was not observed in C_2C_{12} myotubes treated with SN50 (Figure 5. 1e). In each case PIF produced an effect between 2.1 and 10.5nM, which is the range as demonstrated later in figure 5. 2a, that PIF initiates protein degradation.

These results suggest that nuclear translocation of NF-κB is essential for PIF-induced proteasome expression, ubiquitin conjugation and loss of the myofibrillar protein myosin.

To evaluate the effect of pharmacological inhibition of NF-κB on PIF-induced proteasome expression and protein degradation, the IKK inhibitor curcumin (Thaloor *et al* (1999)) was employed.

Total protein degradation of C_2C_{12} myotubes in response to PIF in the absence or prescence of 50μM curcumin (24h)

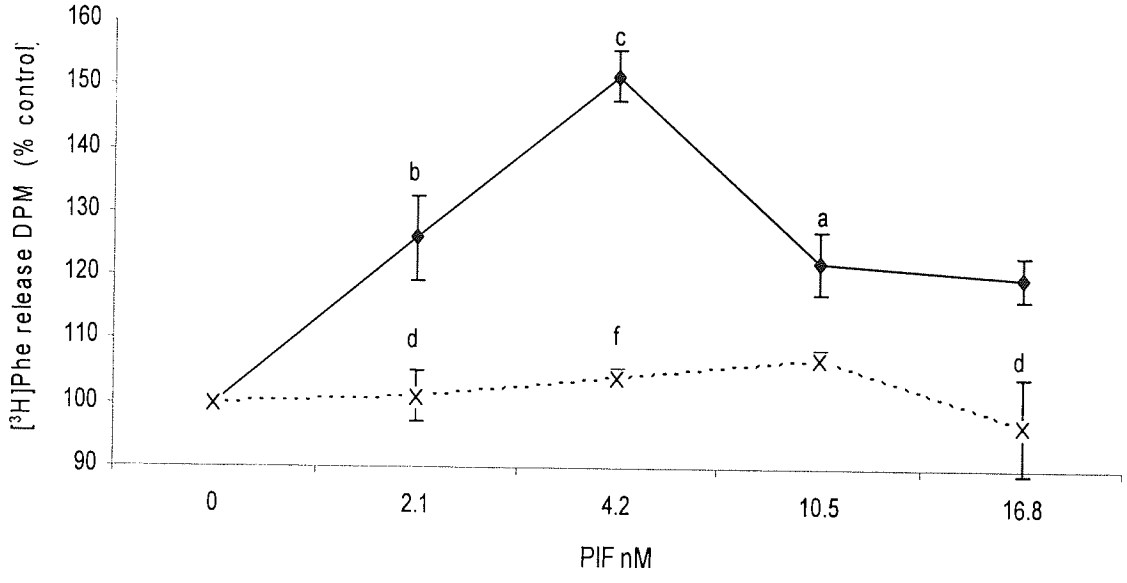


Figure 5. 2a

The effect of curcumin (50μM) on total protein degradation in the presence of PIF for 24h, as measured by the release of [^{3}H] phenylalanine. Myotubes were incubated with PIF alone (◆) or pre-treated with curcumin (X) 2h prior to the addition of PIF. Statistical analysis between group means was determined by one-way ANOVA, followed by Tukey's test. Results are expressed as mean ± S.E.M and differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of curcumin are indicated as d, $p < 0.05$ and f, $p < 0.001$. n=9 and the experiment was repeated 3 times.

'Chymotrypsin-like' activity of the proteasome in C_2C_{12} myotubes treated with PIF in the absence or presence of 50μM curcumin (24h)

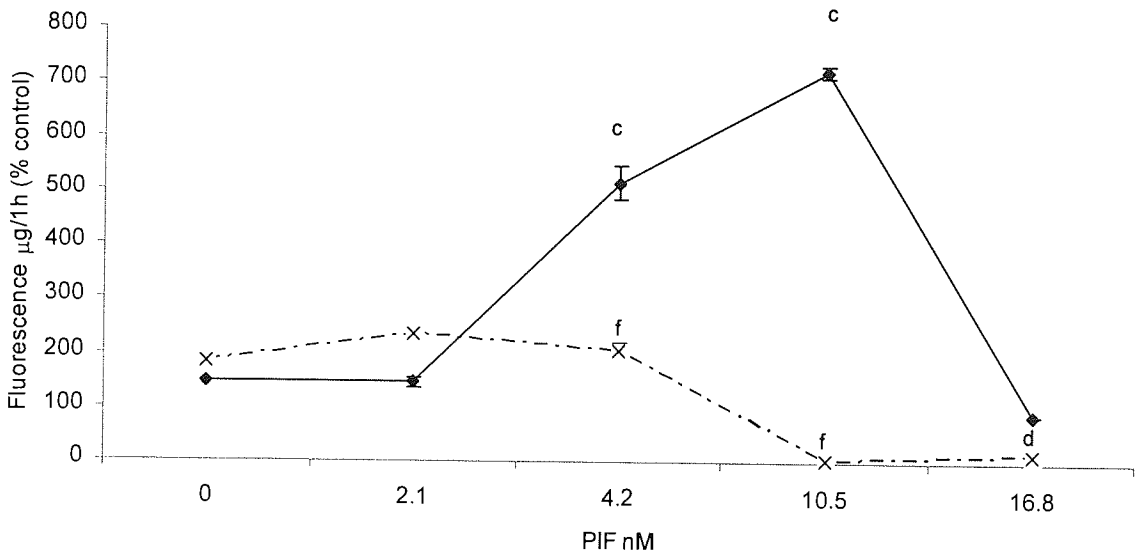


Figure 5. 2b

'Chymotrypsin-like' enzyme activity of murine myotubes in response to PIF 24h in the absence (◆), or presence of 50μM curcumin 2h prior to addition of PIF (X). The experiment was repeated three times (n=9). Statistical analysis between group means was detemined by one-way ANOVA, followed by Tukey's test. Results are expressed as mean ± S.E.M and differences from 0nM PIF are indicated as c, $p < 0.001$, while differences in the presence of curcumin are indicated as f, $p < 0.001$.

Figure 5. 2a and 5. 2b

At a concentration of 50μM curcumin attenuated the PIF-induced increase in protein degradation and 'chymotrypsin-like' enzyme activity, which is the predominant proteolytic activity of the proteasome.

Western blot analysis of PIF-induced expression of 20S proteasome α subunit in C_2C_{12} myotubes in the presence or absence of 50μM curcumin (24h)

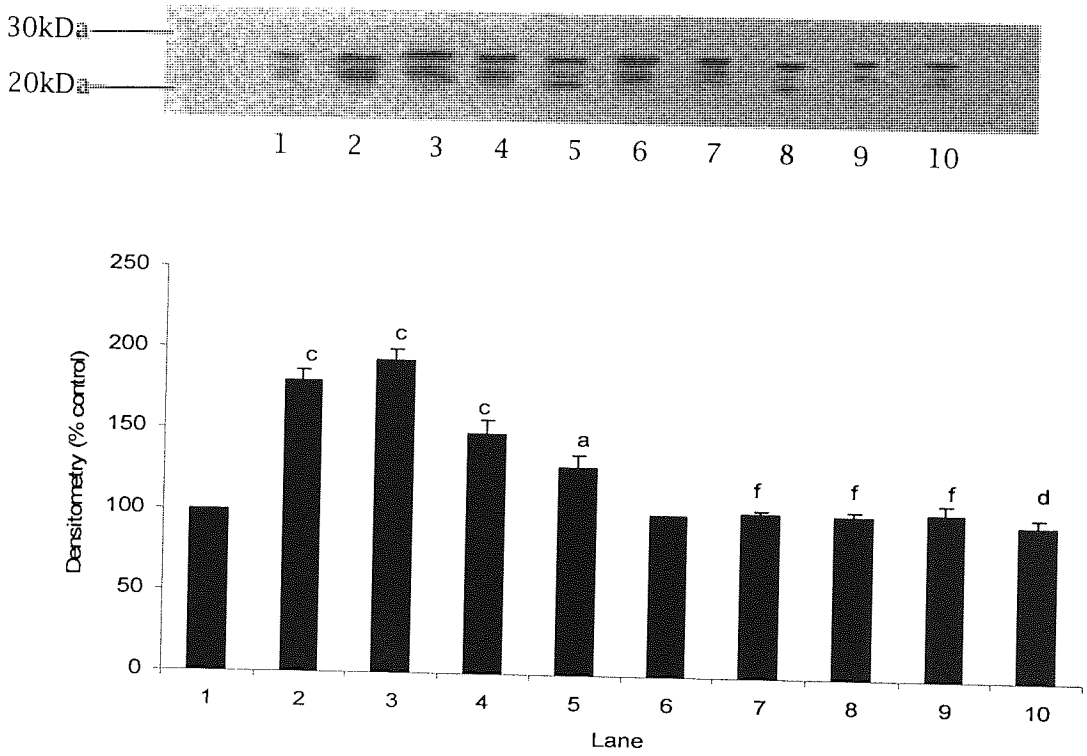


Figure 5.2c

Cells were incubated for 24h with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5) and presence (lanes 6-10) of curcumin (50μM) added 2h prior to the addition of PIF. The densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's post test. Differences from 0nM PIF are indicated as a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of curcumin are shown as d, $p < 0.05$, e, $p < 0.01$ and f, $p < 0.001$.

Western blot analysis of PIF-induced expression of $E2_{14K}$ in C_2C_{12} myotubes in the presence or absence of 50μM curcumin

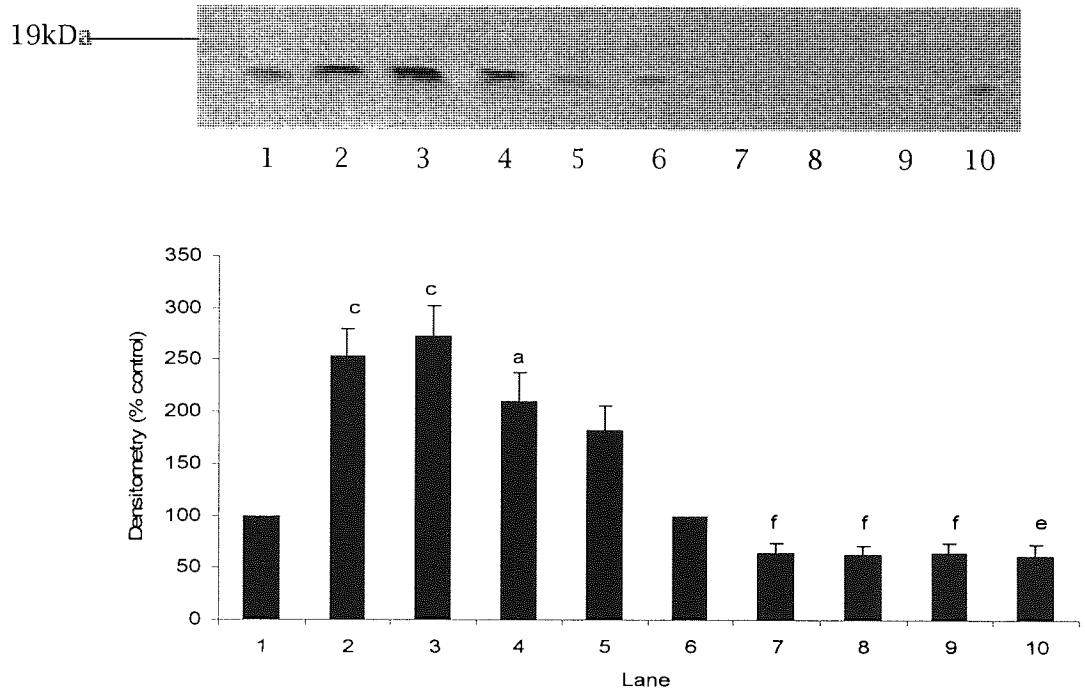


Figure 5.2d

Cells were incubated for 24h with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5) and presence (lanes 6-10) of curcumin (50μM) added 2h prior to PIF. The densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are indicated as a, $p < 0.05$, b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of curcumin are d, $p < 0.05$, e, $p < 0.01$ and f, $p < 0.001$.

Actin loading control for figures 5. 2c and 5. 2d

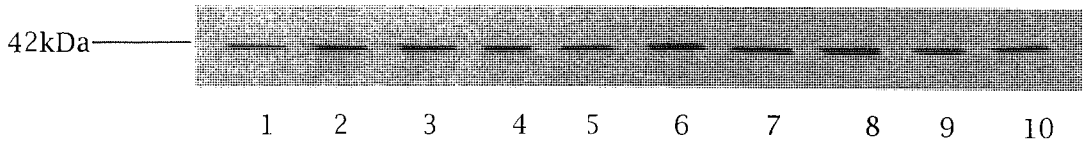


Figure 5. 2e

Lane discipline as figures 5. 2c and 5. 2d

Figures 5. 2c and 5. 2d

Curcumin at 50μM was successful in attenuating the PIF-induced increase in 20S proteasome α subunit expression (figure 5. 2c) and $E2_{14k}$ expression (figure 5. 2d).

These results suggest that curcumin may be effective in the treatment of muscle atrophy in cancer cachexia.

Daily administration of curcumin to MAC16 tumour bearers

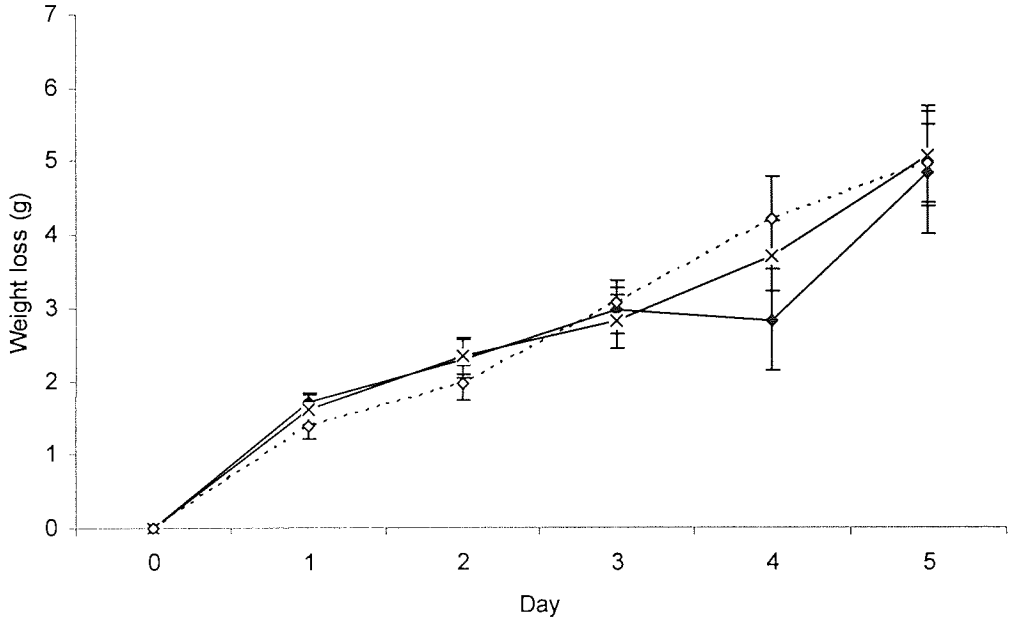


Figure 5. 2f

Effect of curcumin administered p.o. daily at 150 (X) or 300 (◇) mg/kg in PBS compared with PBS alone (◆) on weight loss in mice bearing the MAC16 tumour over 5 days. Statistical analysis between group means was determined by one-way ANOVA, followed by Tukey's test. Results are mean ± S.E.M. There was significant weight loss for all groups at day 5 ($p < 0.001$), but there was no difference in weight loss between groups.

Figure 5. 2f

In vitro results looked very promising, whereby 50μM curcumin was successful in attenuating the PIF-induced increase in protein degradation (figure 5. 2a), 'chymotrypsin-like' activity (figure 5. 2b) and increased 20S proteasome α subunit (figure 5. 2c) and $E2_{14K}$ expression (figure 5. 2d). However, when curcumin was evaluated *in vivo* in MAC16

tumour bearing mice, it was shown to be ineffective in preventing loss of body weight at a dose of 150 and 300mg/kg^{-1}.

The results prompted the search for an alternative inhibitor of NF-κB which may be effective *in vivo*. This led to the use of the IKK inhibitor, resveratrol (Holmes-McNary and Baldwin (2000)), to investigate the effect of resveratrol on PIF-induced protein degradation and subsequent proteasome expression.

Total protein degradation in C_2C_{12} myotubes in response to PIF (24h) in the presence or absence of resveratrol (30μM)

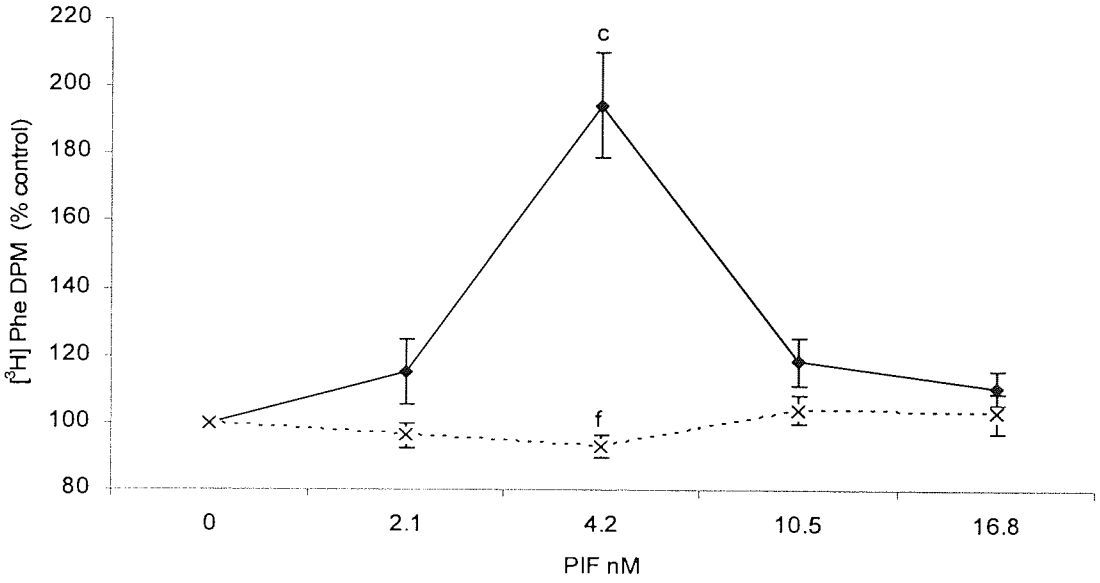


Figure 5. 3a

Myotubes were incubated with PIF alone (◆) or pre-incubated with resveratrol 2h prior to the addition of PIF and maintained in the culture medium over the 24h period (X). Statistical analysis between groups was determined by one-way ANOVA followed by Tukey's test. Differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of resveratrol are f, $p < 0.001$. The experiment was repeated three times (n=9).

'Chymotrypsin-like' activity of the proteasome in C_2C_{12} myotubes in response to PIF (24h) in the absence or prescence of resveratrol (30μM)

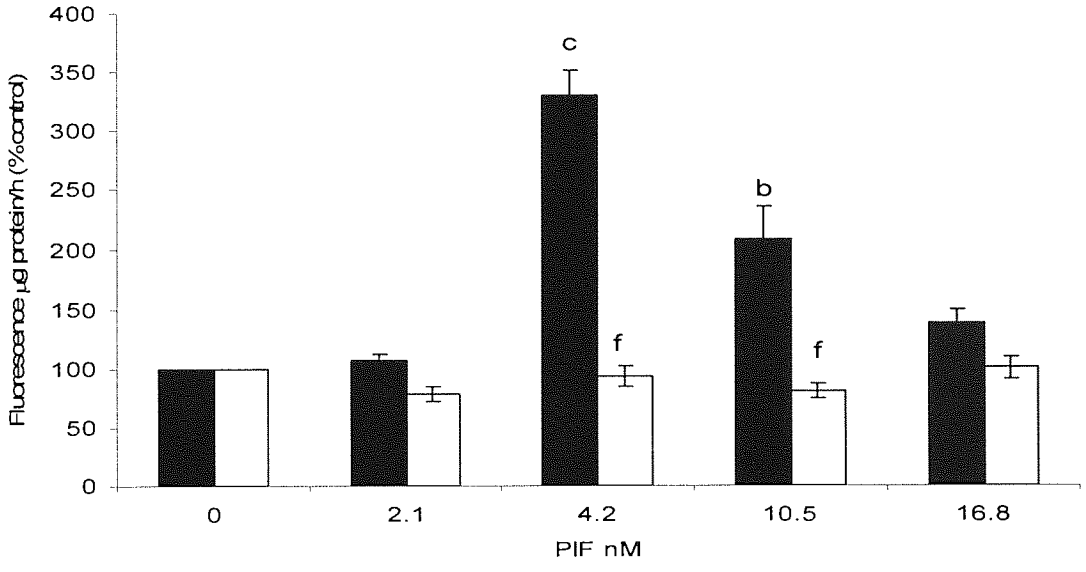


Figure 5. 3b

Myotubes were incubated with PIF in the absence (■) or prescence of resveratrol 2h prior to addition of PIF (□). Results are expressed as mean ± S.E.M and statistical analysis between groups was determined by one-way ANOVA followed by Tukey's test. Differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of resveratrol are indicated as f, $p < 0.001$. The experiment was repeated three times (n=9).

Figures 5. 3a and 5. 3b

At a concentration of 30μM, resveratrol effectively attenuated both PIF-induced protein degradation (figure 5. 3a) and the PIF-induced increase in 'chymotrypsin-like' proteolytic activity of the proteasome (figure 5. 3b).

Western blot analysis of PIF-induced 20S proteasome α subunit expression in C_2C_{12} myotubes in the absence or presence of resveratrol (30μM) (24h)

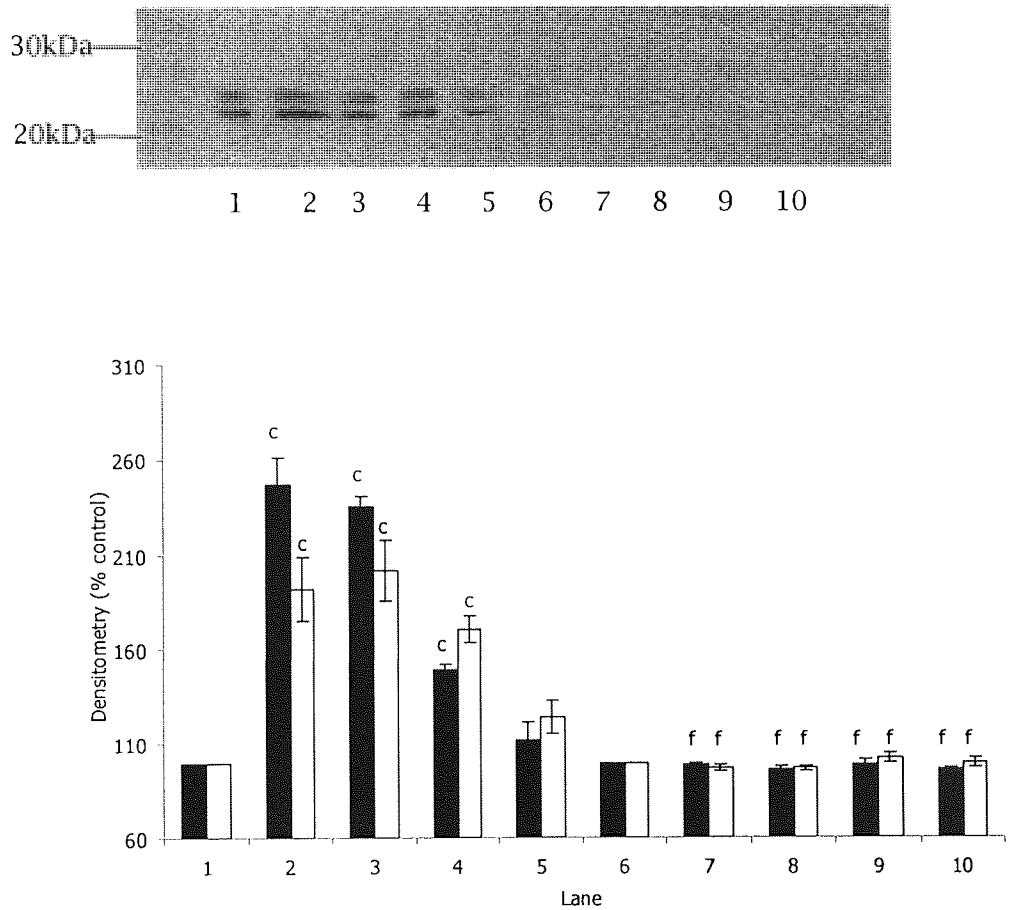
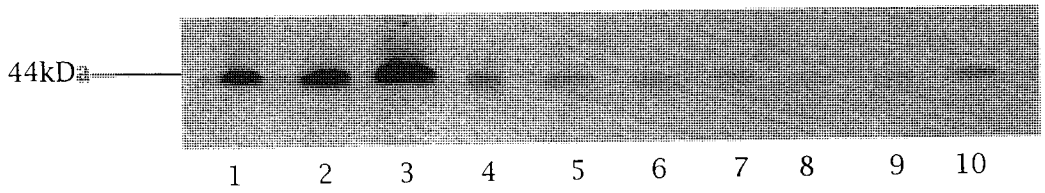


Figure 5. 4a

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) in the absence (lanes 1-5) or prescence (lanes 6-10) of resveratrol (30μM) for 24h. Densitometric analysis was based on three replicate blots and results are expressed, as mean ± S.E.M. Statistical analysis was detemined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are shown as c, $p < 0.001$ while differences between groups are indicated as f, $p < 0.001$.

Western blot analysis of PIF-induced expression of p42 proteasome subunit expression in C_2C_{12} myotubes in the presence or absence of resveratrol (30μM)



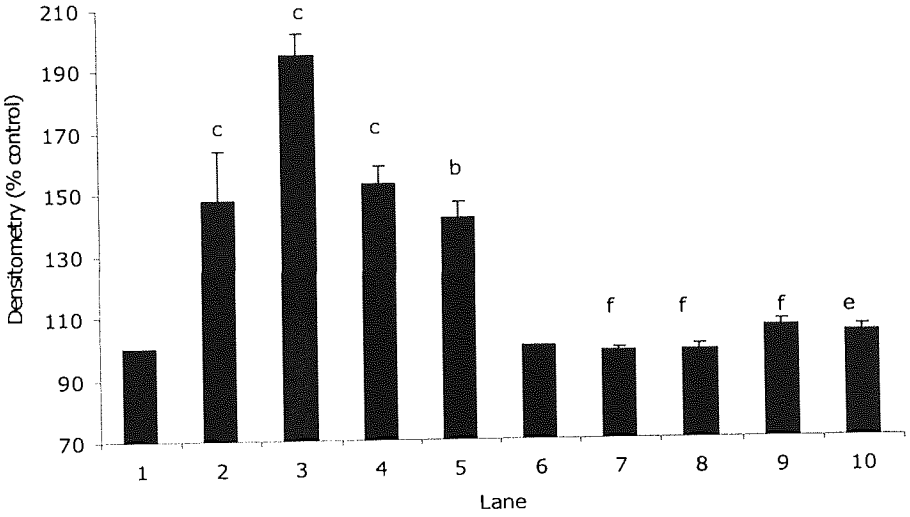


Figure 5. 4b

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) in the absence (lanes 1-5) or presence (lanes 6-10) of resveratrol (30μM) for 24h. Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are shown as b, $p < 0.01$ and c, $p < 0.001$ while differences between groups are indicated as e, $p < 0.01$ and f, $p < 0.001$.

Western blot analysis of PIF-induced expression of MSS1 proteasome subunit in C_2C_{12} myotubes in the absence or presence of resveratrol (30μM) (24h)

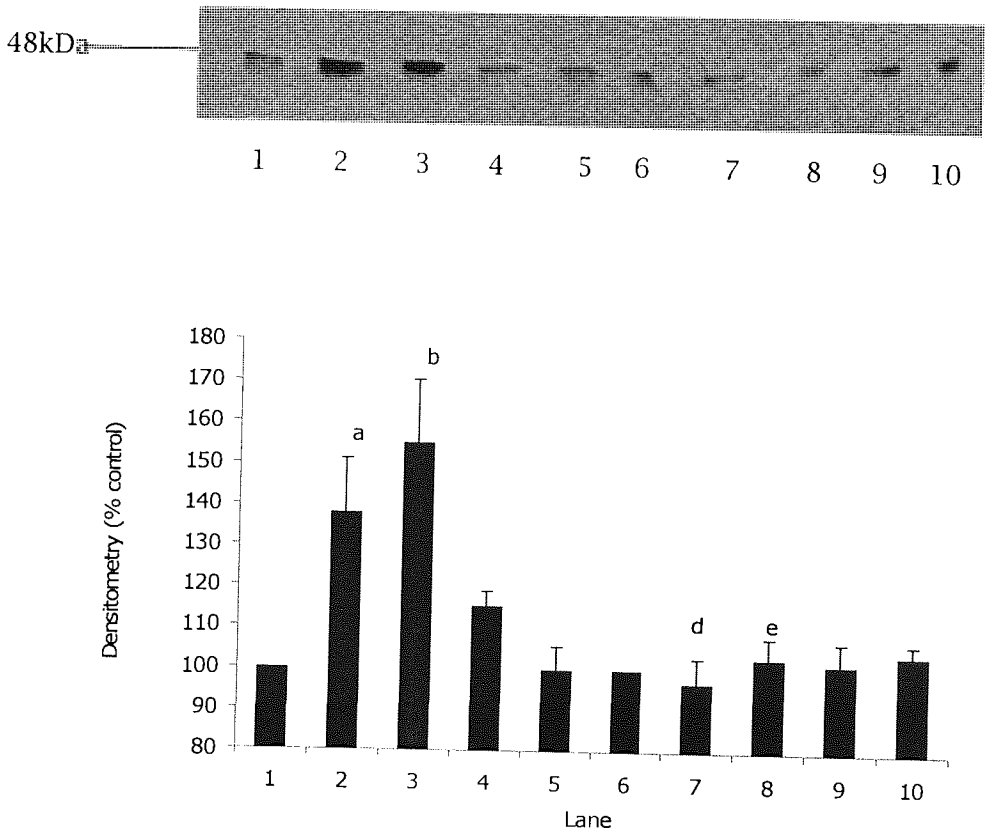


Figure 5. 4c

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) in the absence (lanes 1-5) or presence (lanes 6-10) of resveratrol (30μM) for 24h. Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are shown as a, $p < 0.05$ and b, $p < 0.01$ while differences between groups are indicated as d, $p < 0.05$ and e, $p < 0.01$.

Western blot analysis of PIF-induced depletion of the myofibrillar protein myosin in C_2C_{12} myotubes in the absence or presence of resveratrol (30μM)

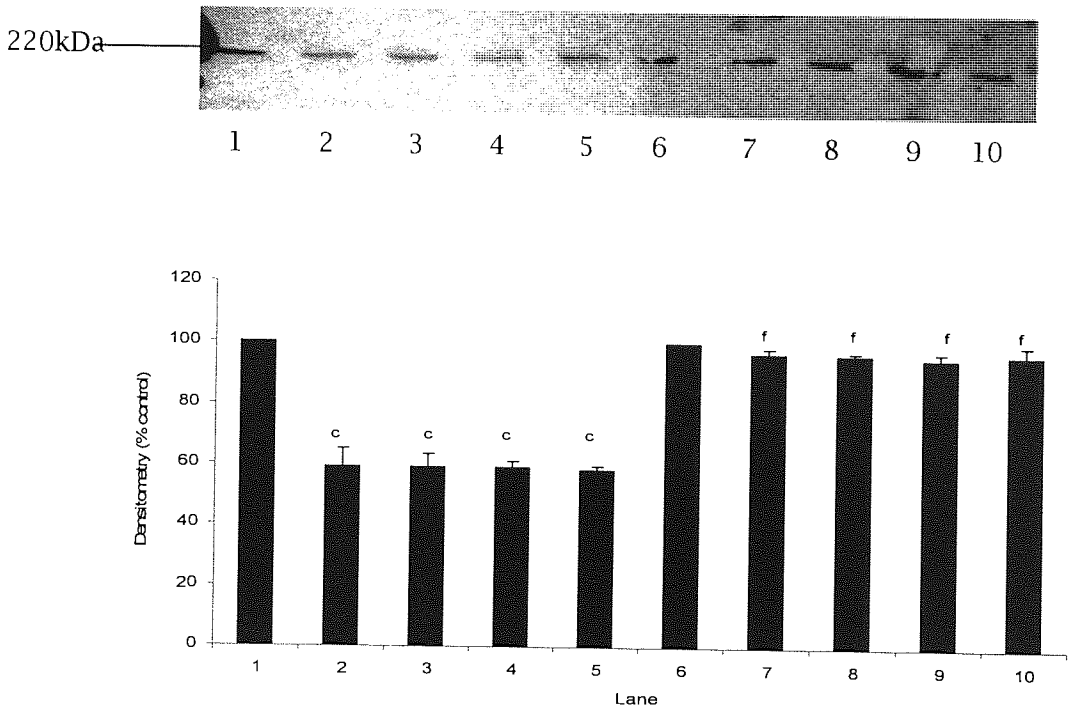


Figure 5. 4d

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) in the absence (lanes 1-5) or prescence (lanes 6-10) of resveratrol (30μM) for 24h. The densitometric analysis was based on three replicate blots. Statistical analysis was detemined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are shown as c, $p < 0.001$ while differences between groups are indicated as f, $p < 0.001$.

Actin loading control

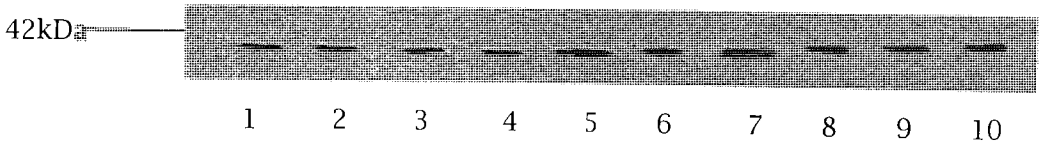


Figure 5. 4e

Lane discipline as figures 5. 4a - 5. 4d.

Figures 5. 4a – 5. 4d

Resveratrol was successful in attenuating the PIF-induced increase in protein degradation (figure 5. 3a) and 'chymotrypsin-like' proteolytic activity of the proteasome (figure 5. 3b). This effect was confirmed by western blot analysis of proteasome subunit expression. Resveratrol attenuated the PIF-induced increase in 20S proteasome α subunit expression (figure 5. 4a), p42 (figure 5. 4b) and MSS1 (figure 5. 4c). Resveratrol was also successful in attenuating PIF-induced degradation of myosin (figure 5. 4d)

To confirm that resveratrol was affecting NF-κB at this concentration, murine myotubes were treated with resveratrol (30μM) 2h prior to the addition of PIF.

The effect of PIF-induced degradation of IκBα in C_2C_{12} myotubes in the absence or presence of resveratrol (30μM)

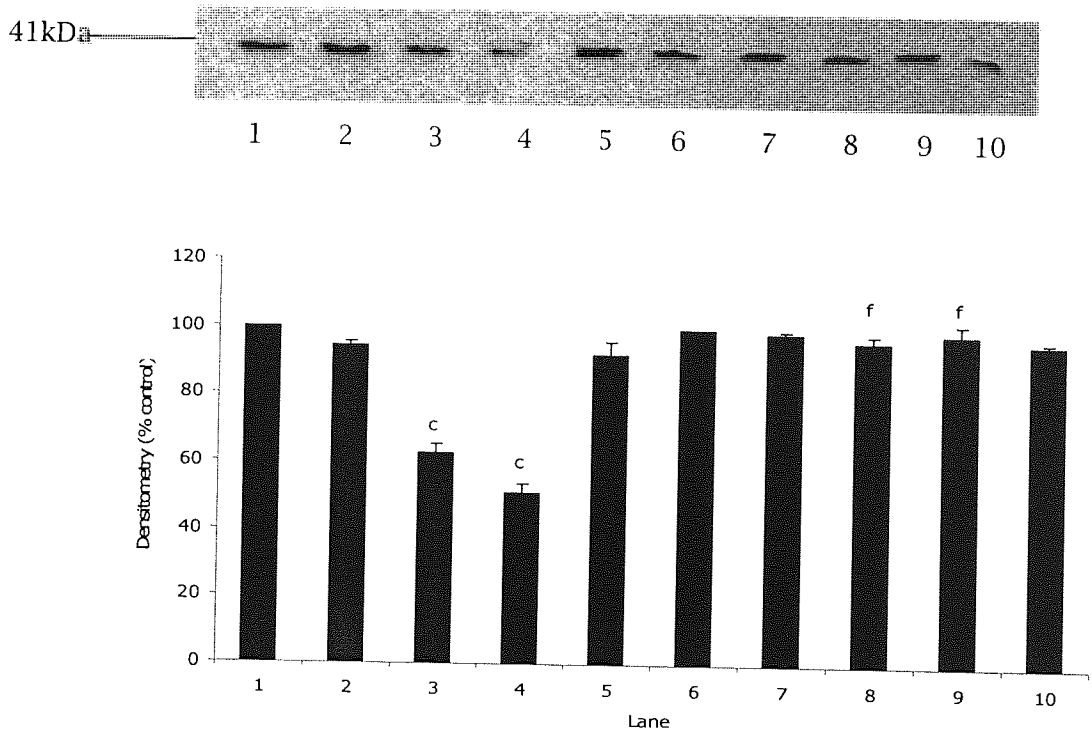


Figure 5. 5a

Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5) or prescence (lanes 6-10) of resveratrol (30μM). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as c, $p < 0.001$ while differences in the presence of resveratrol are shown as f, $p < 0.001$.

Actin loading control

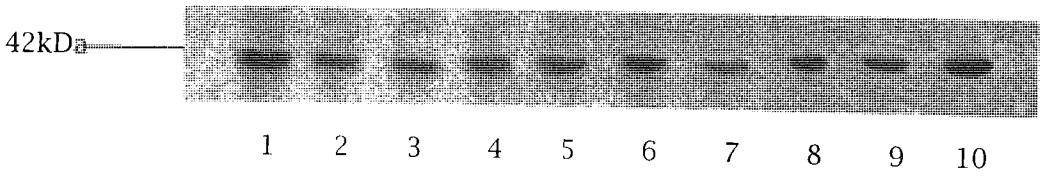


Figure 5. 5b

Lane discipline as figure 5. 5a

The effect of PIF-induced increase in DNA binding of NF-κB in C2C12 myotubes in the absence or presence of resveratrol (30μM)

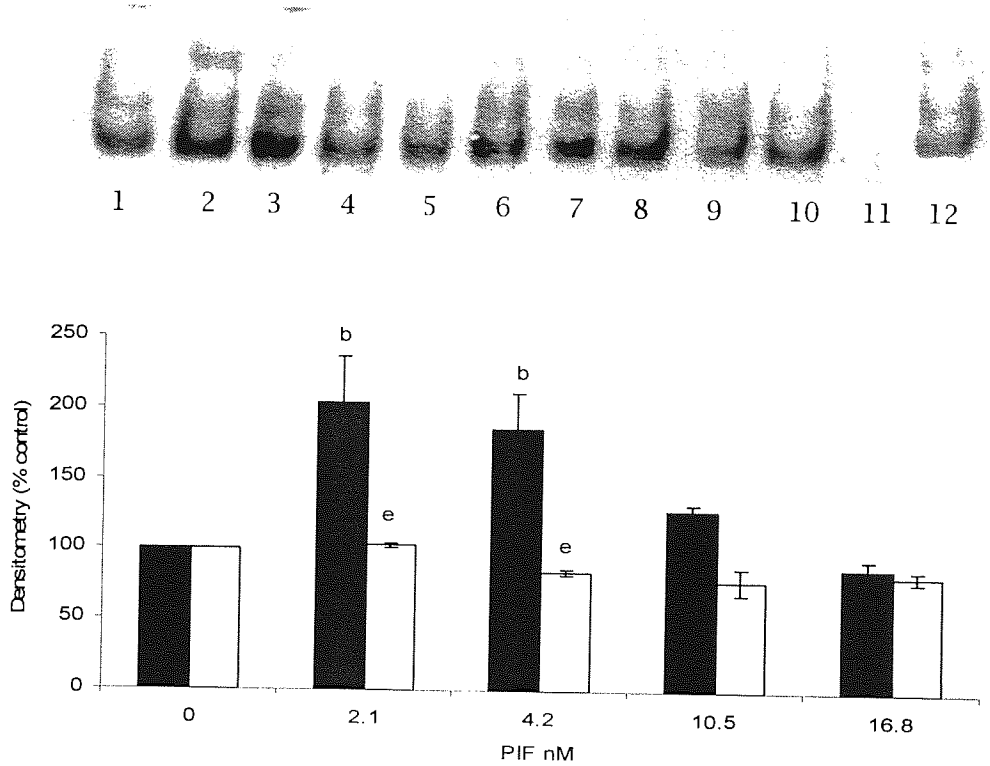


Figure 5. 5c

Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) of resveratrol (30μM). Lane 11 contains a positive control for NF-κB together with 100 fold excess of unlabelled NF-κB. Lane 12 is a positive control for NF-κB. Densitometric analysis was based on three separate experiments and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA, followed by Tukey's test. Differences from control are indicated as b, $p < 0.01$ while differences in the presence of resveratrol are shown as e, $p < 0.01$.

Figures 5. 5a- 5. 5c

These results confirm that resveratrol is producing an effect on NF-κB at the concentration of 30μM. PIF-induced degradation of IκBα was inhibited (figure 5. 5a) as was nuclear accumulation of NF-κB in response to PIF (Figure 5. 5c). These results confirm that the mechanism of action was as expected in murine myotubes.

To evaluate the anti-achectic effect of resveratrol mice bearing the MAC16 tumour, with an established weight loss of 5% were treated with resveratrol (1mg/kg^{-1}) daily.

Effect of resveratrol (1mg/kg) on body weight of MAC16 tumour bearing mice

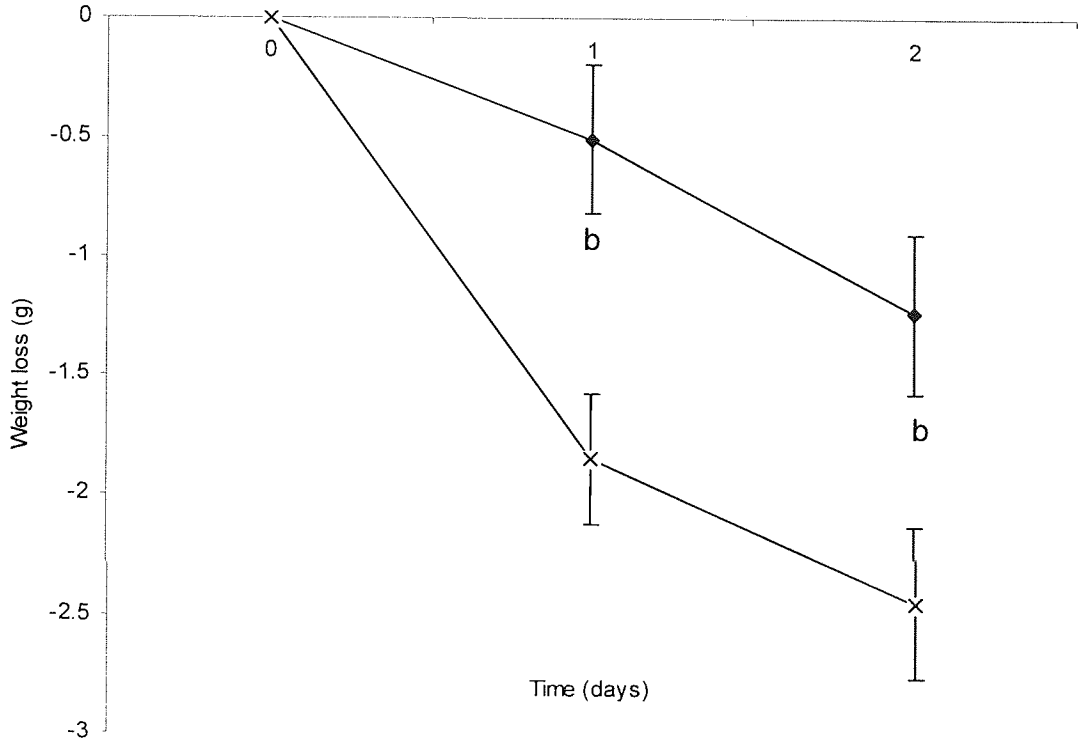


Figure 5. 6a

The effect of resveratrol (1mg/ kg ◆) on body weight in mice bearing the MAC16 colon adenocarcinoma compared with solvent (DMSO : PBS; 1:20) controls (X) where n=6 for both groups. Results are expressed as mean ± S.E.M. Statistical analysis was determined by Student's t test and differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$.

Effect of resveratrol (1mg/ kg) on tumour volume of MAC16 tumour bearing mice

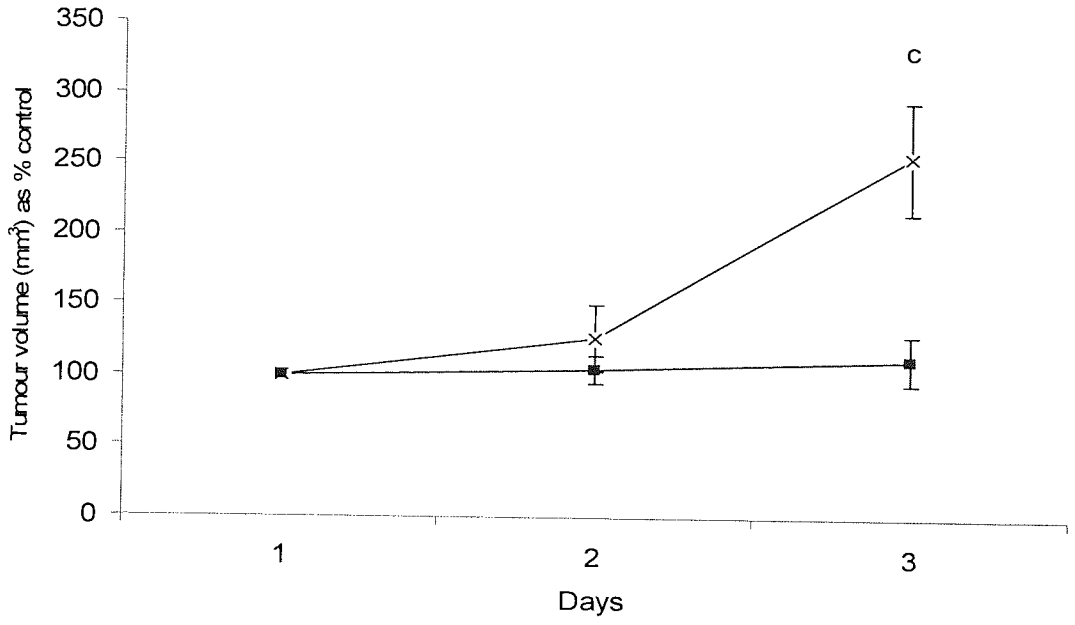


Figure 5. 6b

The effect of resveratrol (1mg/ kg◆) on body weight in mice bearing the MAC16 colon adenocarcinoma compared with solvent (DMSO : PBS; 1:20) controls (X) where n=6. Results are expressed as mean ± S.E.M. Statistical analysis was determined by Student's t test and differences from control are indicated as b, $p < 0.01$ and c, $p < 0.001$.

Effect of resveratrol (1mg/kg) on protein degradation in soleus muscle *ex vivo*

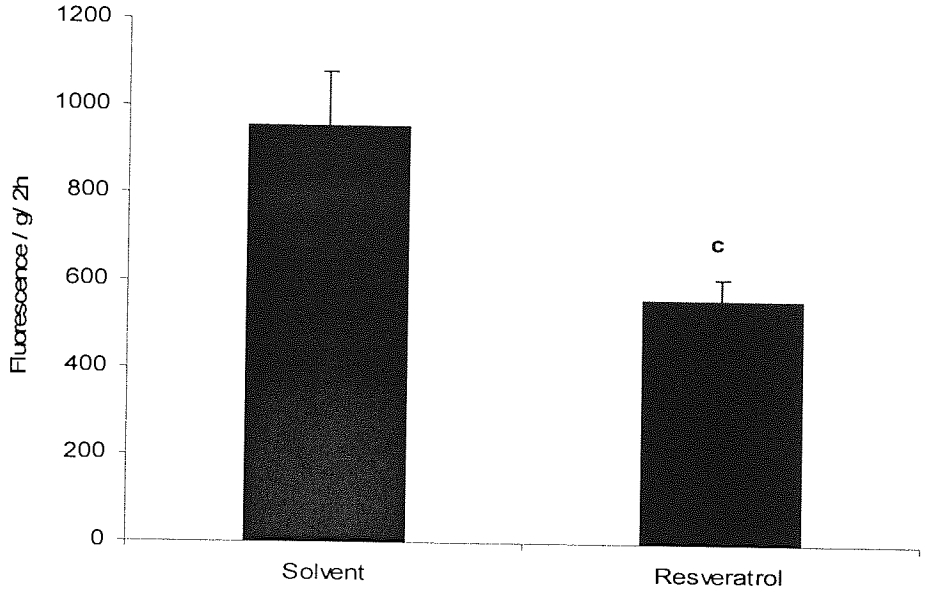


Figure 5. 6c

Effect of resveratrol (1mg/kg) on protein degradation in soleus muscle, as determined by the release of tyrosine, after 48h treatment. Results are expressed as mean ± S.E.M and statistical analysis was determined by Student's t-test. Differences from solvent control are indicated as c, $p < 0.001$ where n=12.

Effect of resveratrol on increased nuclear binding of NF-κB in gastrocnemius muscle of MAC16 tumour bearing mice.

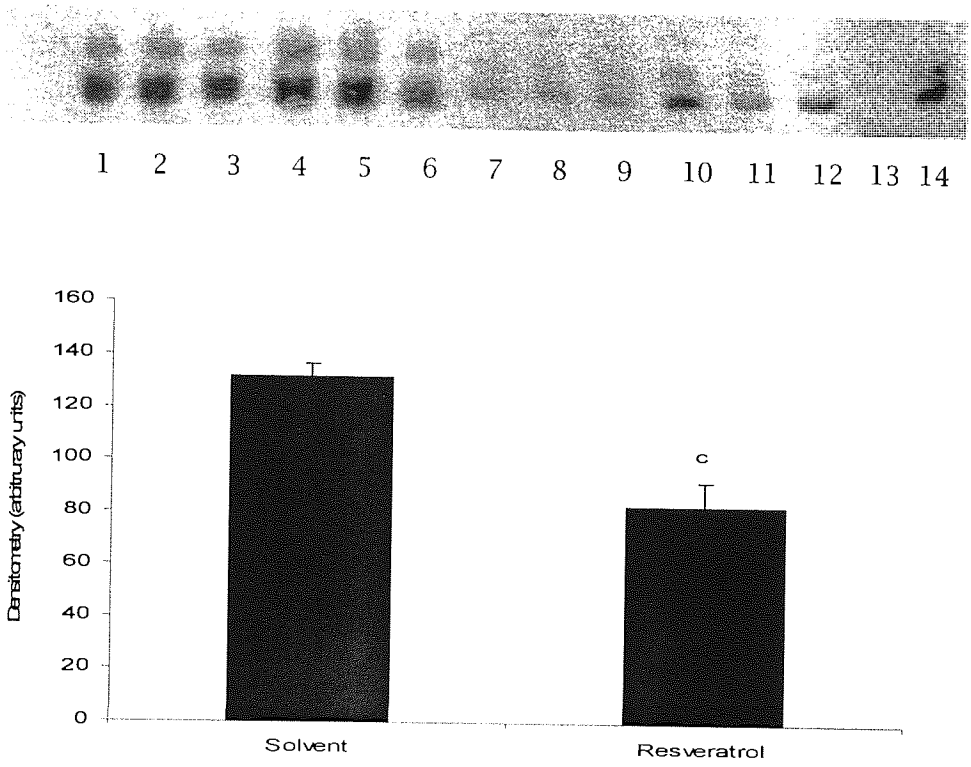


Figure 5. 6d

EMSA to determine nuclear binding of NF-κB in gastrocnemius muscle of mice receiving solvent control (lanes 1-6) or resveratrol (1mg/kg) (lanes 7-12) after 48h treatment. Lane 14 is the positive control for NF-κB and lane 13 is the positive control in the presence of 100 fold excess of unlabelled NF-κB probe. Results are expressed as mean ± S.E.M of densitometry analysis. Statistical analysis was determined by Student's t test. Differences from control are indicated as c, $p < 0.001$.

Figures 5. 6a- 5.6d

Resveratrol was found to significantly attenuate weight loss within 24h of administration to MAC16 tumour bearing mice (figure 5. 6a). This was accompanied by an inhibition of tumour growth within 48h (figure 5. 6b). In order to determine whether the increase in body weight of mice treated with resveratrol arose from a reduction in protein degradation, the rate of protein degradation in soleus muscle was determined by the release of tyrosine (Figure 5. 6c). Previous studies (Beck *et al* (1991) have shown that

muscles from MAC16 tumour bearing mice demonstrate a significant increase in protein degradation compared with non-tumour bearing controls. Soleus muscle of MAC16 tumour bearing mice and those treated with resveratrol showed a significant reduction in protein degradation compared to muscles of mice treated with the solvent alone (Figure 5. 6c).

To verify that this effect arose through an effect on nuclear migration of NF-κB, the amount of DNA bound NF-κB was determined by EMSA. The results depicted in figure 5.6d show a significant reduction in NF-κB DNA binding in the gastrocnemius muscles of mice treated with resveratrol.

The results presented here indicate that agents, which inhibit nuclear translocation of NF-κB may prove useful in the treatment of muscle wasting in cancer cachexia.

Discussion

NF-κB is involved in controlling a number of cellular processes, providing a rapid response against environmental and immune challenges (Lee and Buckart (1998)). NF-κB up-regulates cytokines IL-1, IL-6, IL-8 and TNF-α, all have been reported to contribute towards cachexia (Kawamura *et al* (1999)). NF-κB mediates protein loss induced by TNF-α in differentiated skeletal muscle myotubes (Li and Reid (2000)) and is an important transcription factor in PIF-induced proteasome expression in murine myotubes (Whitehouse and Tisdale (2003)). Aberrant NF-κB activation has been observed in muscle disuse atrophy, whereby protein degradation is mediated by the ubiquitin-proteasome pathway (Lecker *et al* (1999)). Hunter *et al* (2002) found a model of disuse atrophy using hind-limb unloading to result in a ten-fold increase in the activity of NF-κB involving p50, c-Rel and Bcl-3 and not activation of p65 or IκBα, and is thus distinct from cachexia.

Inhibitors of the release and subsequent nuclear translocation of NF-κB, should be evaluated as potential agents to prevent muscle atrophy in cancer cachexia. Agonist-induced nuclear translocation of NF-κB in intact cells can be inhibited by a cell-permeable synthetic peptide representing the nuclear localisation sequence of NF-κB (Lin *et al* (1995)). When the peptide SN50 was incubated together with PIF in C_2C_{12} myotubes it attenuated PIF-induced proteasome subunit expression, $E2_{14K}$ and the loss of the myofibrillar protein myosin. These results suggest that inhibition of NF-κB activity should inhibit muscle protein degradation induced by PIF in cancer cachexia.

Curcumin and Resveratrol are known inhibitors of IKK and both attenuated PIF-induced protein degradation in murine myotubes as well as increased expression of the ubiquitin-proteasome proteolytic pathway. Busquets *et al* (2001) found systemic administration of curcumin (20μgkg) did not influence muscle wasting in YAH-130 ascites hepatoma bearing tumour bearing rats. However, there was inhibition of tumour cell growth (31%) and reduced tumour cell content (24%). This lack of effect was mirrored with *in vivo* administration of curcumin to MAC16 tumour bearing mice at much higher doses of 150 and 300mg/kg^{-1}. This may be a result of poor bioavailability (0.06% absorbtion), which is insufficient to exert a therapeutic effect. Pan *et al* (1999) found that a dose of 1g/kg body weight in the mouse yielded a plasma level of only 0.5μM, which is below the concentration needed to attenuate PIF action *in vitro*. These results prompted the search for alternative NF-κB inhibitors that may be more effective *in vivo*.

Thus the effect of another inhibitor of IκB kinase (IKK), resveratrol (Holmes-McNary and Baldwin (2000) on PIF-induced protein degradation and proteasome expression was studied in murine myotubes. At 30μM resveratrol successfully attenuated PIF-induced protein degradation, 'chymotrypsin-like' enzyme activity and increase in proteasome subunit expression and loss of myosin. Resveratrol (30μM) was found to prevent PIF-induced IκBα degradation and nuclear binding of NF-κB in response to PIF.

Resveratrol was found to significantly attenuate weight loss within 24h of administration to MAC16 tumour bearing mice, accompanied by inhibition of tumour growth within 48h. Resveratrol treated MAC16 tumour bearing mice were found to have a significantly reduced protein degradation compared to MAC16 tumour bearers treated with the solvent alone.

However, Carbo *et al* (1999) found resveratrol at $1mg/kg^{-1}$ unable to prevent body weight loss in rats bearing the YAH-130 ascites hepatoma, although reducing tumour growth and tumour cell content by 25%. However, with this investigation the same dose of resveratrol ($1mg/kg^{-1}$) was effective in attenuating weight loss and tumour growth in MAC16 tumour bearers. The different response between the two cachexia models is unclear. It may be because TNF-α has been implicated in the YAH-130 rat tumour model to be responsible for enhanced protein degradation in skeletal muscle (Costelli *et al* (1993)). However, with mice bearing the MAC16 carcinoma, PIF appears to be responsible for the enhanced protein degradation (Lorite *et al* (1998)).

These results suggest agents, which inhibit NF-κB nuclear translocation of NF-κB may be useful in the treatment of muscle atrophy and wasting in cancer cachexia. Whitehouse and Tisdale (2003) demonstrated EPA to prevent nuclear migration of NF-κB in C_2C_{12} myotubes in response to PIF by stabilising the cytosolic IκBα/ NF-κB complex. EPA probably achieves this by interfering with IκBα-phosphorylation. Novak *et al* (2003) found that pre-treatment of RAW 264.7 cells with an emulsion of ω-3 fatty acids prior to stimulation by LPS resulted in the inhibition of IκBα phosphorylation and significantly decreased NF-κB activity.

EPA has been shown to attenuate the development of further weight loss in weight-losing patients with pancreatic cancer (Wigmore *et al* (2000)) and produce a increase in lean body mass when combined with a high protein energy dense nutritional supplement (Barber *et al* (1999)). Palayoor *et al* (1999) found the anti-inflammatory agent ibuprofen to inhibit the constitutive activation of NF-κB and IKKα in PC-3 and DU-145 prostate tumour cell lines. McMillan *et al* (1999) combined ibuprofen with megestrol acetate in a randomised trial in

gastrointestinal cancer patients. There was a median decrease of 2.8kg in 12 weeks from the group receiving megestrol acetate alone, whereas patients receiving the combination of ibuprofen and megestrol acetate experienced a median weight gain of 2.3kg in 12 weeks. Another anti-inflammatory agent, thalidomide has been shown by Keifer et al to block NF-κB activation through inhibition of IKK.

These results suggest although NF-κB is involved in the control of over 150 target genes (Pahl (1999)), inhibitors of NF-κB activation do not produce overt toxicity and should be evaluated for the treatment of muscle atrophy in cancer cachexia.

Chapter 6

The role of protein kinase C and NF-κB in proteolysis-inducing factor induced proteasome expression in C_2C_{12} myotubes.

Introduction

Loss of skeletal muscle in cancer cachexia results in asthenia, immobility and eventually death through impairment of respiratory function. Nutritional supplementation fails to reverse this wasting process (Nixon *et al* (1981), Loprinzi *et al* (1994) and Fearon *et al* (1988)), suggesting that the balance between protein synthesis and degradation is impaired. Depletion of myofibrillar proteins is a result of decreased protein synthesis (Funabiki *et al* (1976), and increased protein degradation (Lundholm *et al* (1982)). Increased activity of the ubiquitin-proteasome proteolytic pathway is considered to be the major factor contributing towards this increase in protein degradation (Williams *et al* (1999)).

Todorov *et al* (1996) demonstrated tumour production of a sulphated glycoprotein, termed proteolysis-inducing factor (PIF) which may be responsible for the progressive loss of skeletal muscle in cancer cachexia. PIF is only produced by cachexia-inducing tumours, and when purified and administered to non tumour bearing mice, induces specific loss of skeletal muscle, while visceral protein is preserved (Lorite *et al* (1998)). The C_2C_{12} surrogate skeletal muscle model was employed by Smith *et al* (1999) who found PIF to inhibit protein synthesis and increase protein degradation; the latter being due to increased expression of the ATP-ubiquitin dependent proteolytic pathway (Lorite *et al* (2001)).

PIF-induced protein degradation was accompanied by an increased release of arachidonic acid from membrane phospholipids. Subsequent metabolism to 15-hydroxyeicosatetraenoic acid 15(*S*)-HETE, has been related to protein catabolism (Smith *et al* (1999)). Arachidonic acid (AA), has been demonstrated to activate PKC (Shearman *et al* (1989), therefore AA may serve as a regulatory signal, activating PKC during cell stimulation (O'Flaherty *et al* (2001)). AA and lipoxygenase metabolites may play a role in PIF-induced proteasome expression.

Phosphorylation of proteasome subunits may be important in the regulation of proteasome activity, since some proteasome subunits have potential tyrosine (Tanaka *et al* (1990)) and serine/ threonine phosphorylation sites (Heinimeyer *et al* (1994)). Mason *et al*

(1996) found phosphoserine to be present in both C8 and C9 subunits, dephosphorylation by acid phosphotase significantly reduced proteasome activity. The findings by Mason *et al* (1996) implied phosphorylation to be an important mechanism in regulating proteasome function. Alternatively PKC may be involved in the activation of the transcription factor NF-κB, which has been shown to be involved in the production of IL-6, IL-8, C-reactive protein and ICAM-1 in liver cells (Watchorn *et al* (2001)) and may be involved in PIF-induced proteasome expression (Whitehouse and Tisdale (2003)).

PKC has been suggested to be an upstream activator of IκB kinase complex (IKK) (Huang *et al* (2003); Catley *et al* (2004) and Amit *et al* (2003)), leading to IκBα phosphorylation, ubiquitination and subsequent degradation by the 26S proteasome, followed by the translocation of NF-κB into the nucleus. The previous chapters 4 and 5 have demonstrated NF-κB to be essential for the increased expression of components in the ATP-ubiquitin-dependent proteolytic pathway.

This chapter investigates the role of PKC in PIF-induced proteasome expression and its relationship to the activation of NF-κB in C_2C_{12} murine myotubes.

Methododology

Purification of PIF

See section 2.3.1

Protein Degradation Assay

See section 2.5.1.d

'Chymotrypsin-like' activity assay

See section 2.5.1.e

Myogenic cell culture and transfection

The C_2C_{12} myoblast cell line was grown in DMEM supplemented with 10% FCS plus 1% penicillin and streptomycin under 10% CO_2 in air. Cells were transfected c/o Dr. H. Smith with 2 forms of PKCα. The insert A25E PKCα is constitutively active due to deletion of amino acids 22 and 28 in the N-terminal region and is expressed via the pCO_2 vector (Pears *et al* (1990)). PKCα(T/A)3 is a dominant-negative mutant expressed in PKS1 (Bornacin and Parker (1996)).

Transfection was carried out on cells at 50% confluency using GeneJuice™ transfection reagent, according to the manufacturers protocol (for full protocol see section 2.5.2.3) and selected by resistance to ampicillin ($5gl^{-1}$) (c/o Dr. H. Smith).

Transfected myoblasts were stimulated to differentiate by replacing growth medium with DMEM supplemented with 2% HS, when cells reached confluence. Differentiation was allowed to continue for 5-9 days until myotubes were clearly visible.

Western Blot Analysis

For a full protocol see section 2.3.3

Cytoplasmic proteins from the supernatant extract of the sonicated lysate, and the pellet were both used for western blotting of PKCα, 20Sα and $E2_{14K}$. The pellet was dissolved in sonicating buffer supplemented with 0.1% Nonident P40 and was the source of membrane proteins. Analysis of IκBα required the use of the sonicated lysate, without separation by centrifugation at 18,000g for 10min. Extracts were loaded at 5μg protein of cytoplasmic and membrane fractions, and 20μg of whole lysate. The primary antibody for PKCα was used at a dilution of 1:100, while antibodies for IκBα and $E2_{14K}$ were at 1:1000 and 20S

proteasome α-subunits at 1:1500. The secondary antibodies were used at a dilution of 1:2000. Incubation was carried out for 2h at room temperature, and development was by ECL.

Electrophoresis mobility shift assay (EMSA)

DNA binding proteins were extracted from myotubes by the method of Andrews and Faller (1991), which utilises hypotonic lysis, followed by high salt extraction of nuclei (see section 2.8.1). The EMSA binding assay was carried out using a Panomics EMSA 'gel shift' kit according to the manufacturers instructions. For full protocol, see section 2.8.2

Statistical Analysis

Differences of means between groups was determined by one-way ANOVA followed by Tuker-Kramer Multiple comparison Test.

Results

To evaluate the role of PKC in PIF-induced proteasome expression, calphostin C, a highly specific inhibitor of PKC was employed (Jarvis *et al* (1994)), which selectively inhibits PKCα.

Western blot analysis of 20S proteasome α expression in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of calphostin C (300nM).

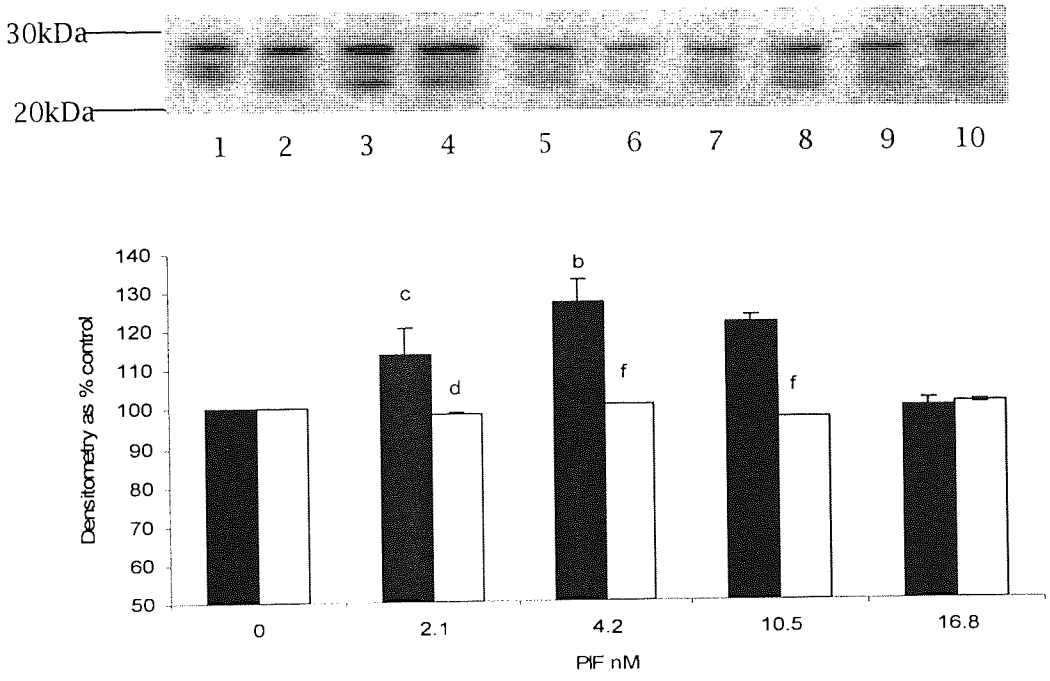


Figure 6. 1a

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence of calphostin C 300nM (lanes 6-10, □). Expression was determined after 24h. Densitometric analysis is based on three replicate blots and results are expressed as mean ± S.E.M, representing the two major bands. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM and PIF are indicated as a, $p<0.05$; b, $p<0.01$ and c, $p<0.001$, while differences between calphostin C and PIF are shown as d, $p<0.05$; e, $p<0.01$ and f, $p<0.001$.

Western blot analysis of $E2_{14K}$ expression in response to PIF (24h) in the absence or presence of calphostin C (300nM) in C_2C_{12} myotubes

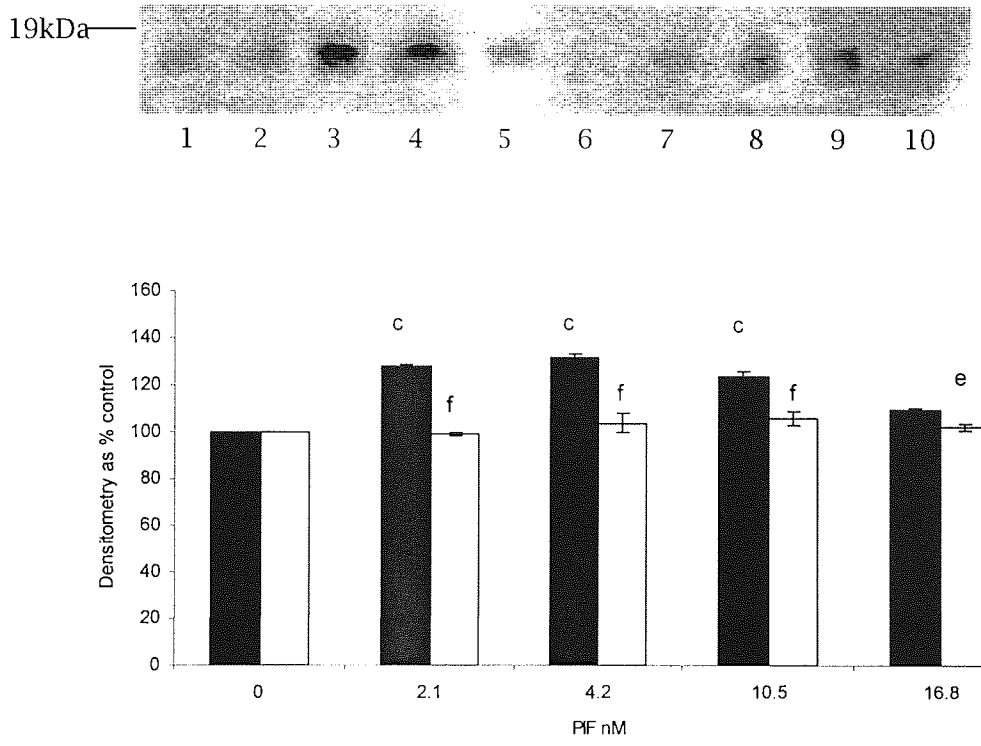


Figure 6. 1b

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5,■) or presence of calphostin C 300nM (lanes 6-10,□), expression was determined after 24h. Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM and PIF are indicated as a, $p<0.05$; b, $p<0.01$ and c, $p<0.001$, with differences between calphostin C and PIF as d, $p<0.05$; e, $p<0.01$ and f, $p<0.001$.

Figure 6. 1a and 6. 1b

Calphostin C completely attenuated the PIF-induced increase in 20S proteasome α subunit expression (figure 6. 2a) and $E2_{14K}$ (Figure 6. 2b), suggesting a role for PKC in the induction of the ubiquitin-proteasome pathway.

To corroborate that this effect was mediated by PKC, cytosolic and membrane fractions of PKC were subjected to western blot analysis.

Western blot analysis of cytosolic PCKα in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of calphostin C (300nM)

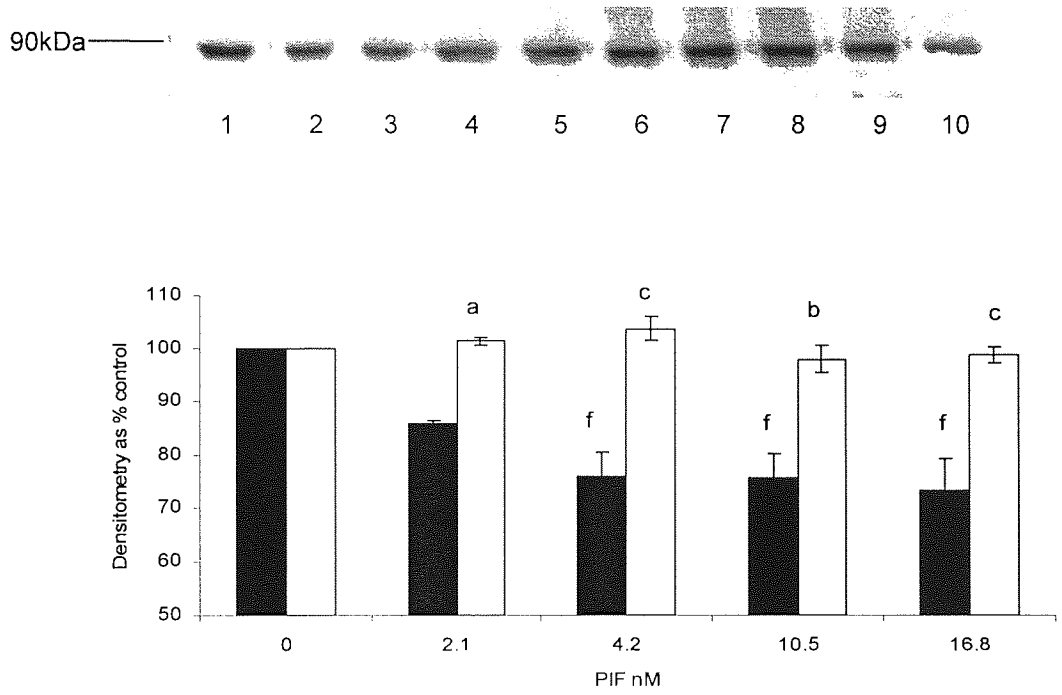


Figure 6. 2a

Cytoplasmic PKCα expression after incubation with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) for 24h in the absence (lanes 1-5,■) or presence (lanes 6-10,□) of calphostin C (300nM). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statististical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from control at 0nM and PIF are shown as c, $p<0.001$, with differences between calphostin C and PIF as d, $p<0.05$; e, $p< 0.01$ and f, $p<0.001$.

Western blot analysis of membrane bound PCKα in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of calphostin C (300nM)

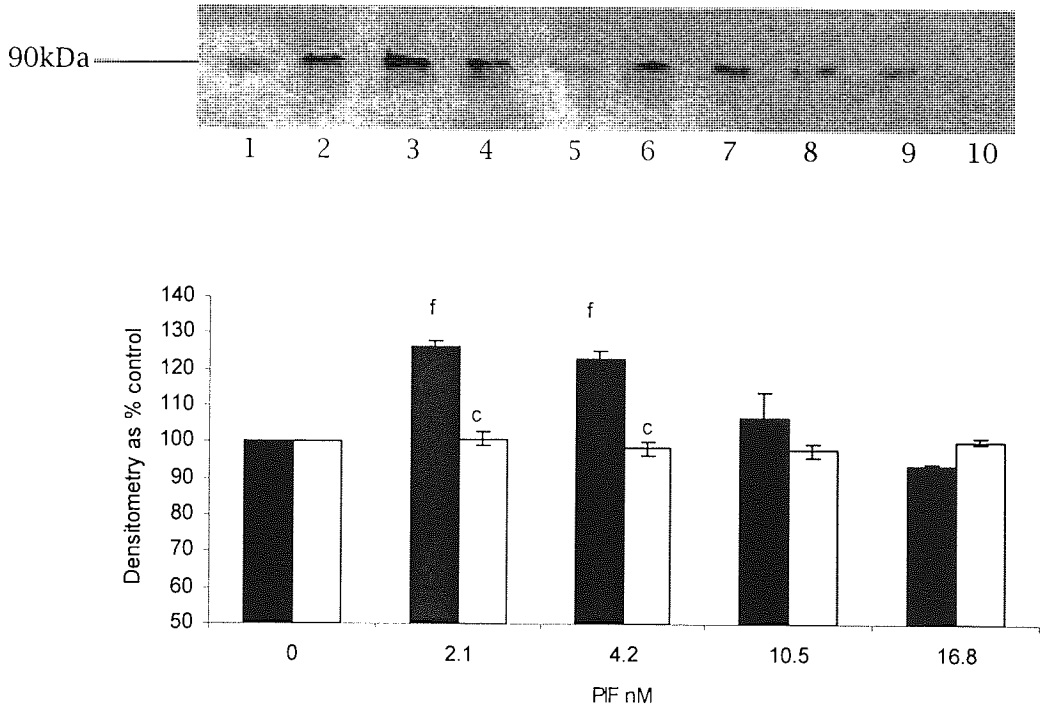


Figure 6. 2b

Membrane bound PKCα expression after incubation with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8 nM PIF (lanes 5 and 10) for 24h in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) calphostin C (300nM). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statististical analysis was determined by one-way ANOVA followed by Tukey's test. Differences between 0nM and PIF are shown as f, $p<0.001$, with differences between calphostin C and PIF as c, $p<0.001$.

Actin loading control

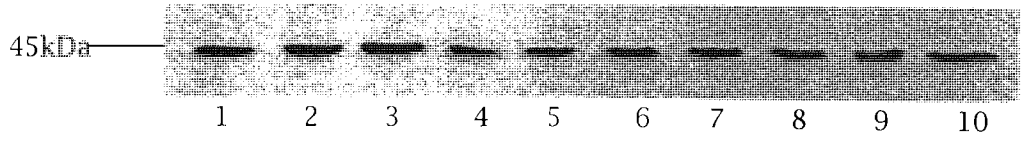


Figure 6. 2c

Lane discipline as figures 6. 2a and 6. 2b.

Figures 6. 2a and 6. 2b

Calphostin C completely attenuated the PIF-induced increase in 20S proteasome α subunit expression (figure 6. 1a) and $E2_{14K}$ (figure 6. 1b). Western blot analysis of cytosolic and membrane bound PKCα determined PIF to induce a decrease in cytosolic PKCα (figure 6. 2a), accompanied by a transient increase in membrane bound PKCα (figure 6. 2b) at the same concentrations that increased proteasome (figure 6. 1a) and $E2_{14K}$ expression (figure 6. 1b). This effect was attenuated by the PKCα inhibitor calphostin C and confirms a role for PKC in PIF-induced proteasome expression.

To further substantiate PKC involvement in the activation of IKK, degradation of IκBα and DNA binding of NF-κB, murine myotubes were subjected to western blot analysis of cytosolic IκBα in response to calphostin C added 2h prior to the addition of PIF for 30min.

Western blot analysis of cytoplasmic IκBα degradation in C_2C_{12} myotubes in response to PIF (30 min) in the absence or presence of calphostin C (300nM)

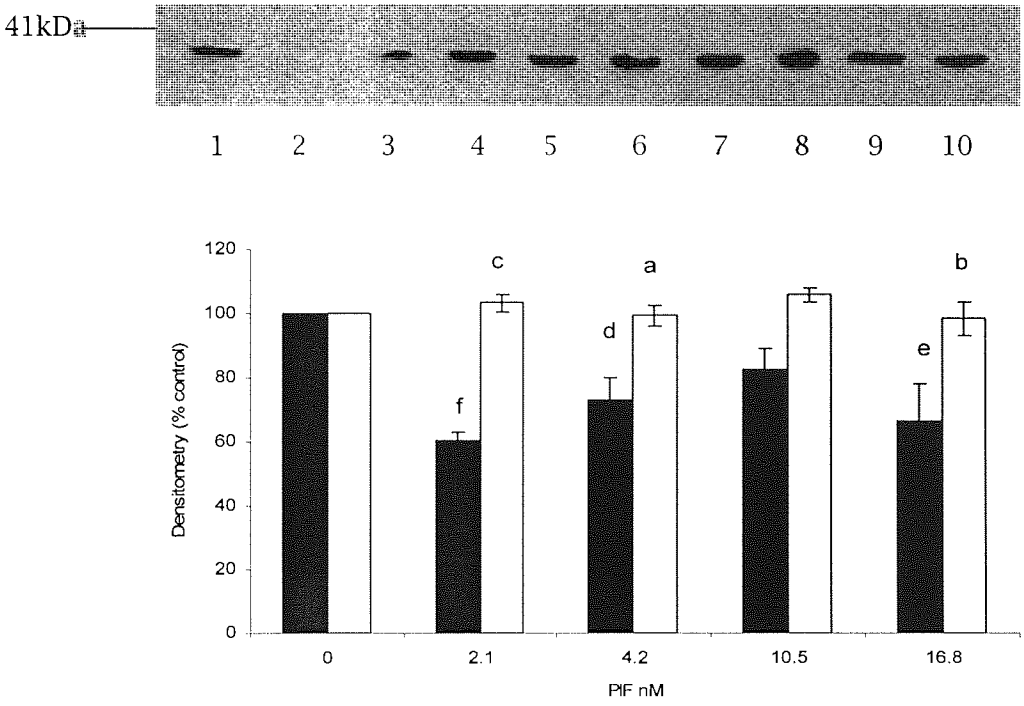


Figure 6. 3a

Effect of PIF and calphostin C on cytoplasmic IκBα in C_2C_{12} myotubes determined 30min after addition of PIF to murine myotubes. Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in absence (lanes 1-5, ■) or presence (lane 6-10, □) of calphostin C (300nM) added 2h prior to PIF. Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statististical analysis was determined by one-way ANOVA followed by Tukey's test. Differences between 0nM and PIF are a, $p<0.05$; b, $p<0.01$ and c, $p<0.001$, with differences between calphostin C and PIF as d, $p<0.05$; e, $p< 0.01$ and f, $p<0.001$.

Actin loading control.

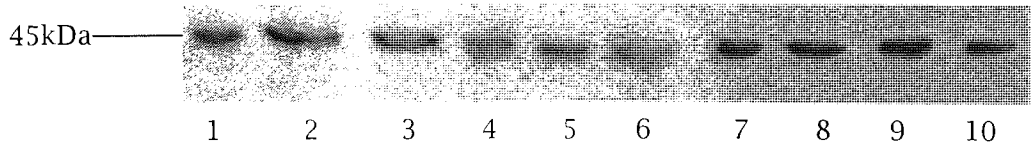


Figure 6. 3b

Lane discipline as figure 6. 3a

Nuclear accumulation of NF-κB in C_2C_{12} myotubes in response to PIF (30min) in the absence or presence of calphostin C (300nM)

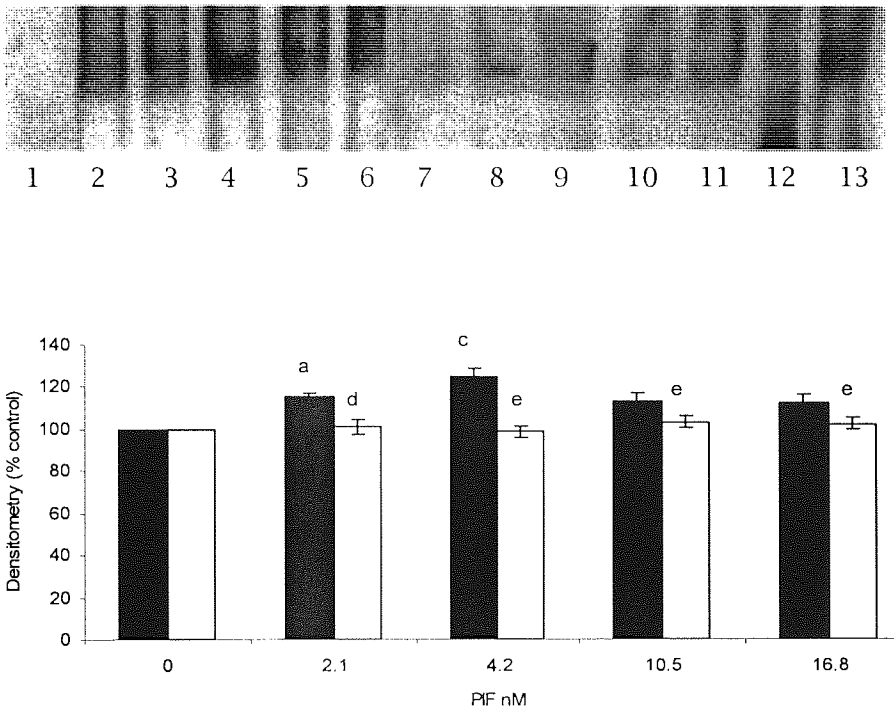


Figure 6. 3c

Nuclear bound levels of NF-κB determined 30min after the addition of PIF. Myotubes were treated with 0 (lanes 2 and 7), 2.1 (lane 3 and 8), 4.2 (lanes 4 and 9), 10.5 (lanes 5 and 10), or 16.8nM PIF (lanes 6 and 11) in the absence (lanes 2-6, ■) or presence of calphostin C (300nM) (lanes 7-11 □). Lane 1 is a negative control and lane 13 a positive control, lane 12 contains excess unlabelled NF-κB. Densitometry is based on three replicate EMSAs with results expressed as mean ± S.E.M. Statisitcal analysis was determined using one-way ANOVA followed by Tukey's test. Differences from control are indicated as a, $p<0.05$ and c, $p<0.001$, while differences in the presence of calphostin C are indicated as d, $p<0.01$ and e, $p< 0.001$.

Figures 6. 3a - 6. 3c

Whitehouse and Tisdale (2003) have previously shown PIF-induced proteasome expression appears to require the activation of NF-κB. There is evidence to support this theory in chapter 4 and 5. One mechanism by which PKC may function in the PIF signalling pathway

is the activation of IKK, with subsequent phosphorylation and degradation of IκBα and translocation of NF-κB from the cytosol to the nucleus (Vertegaal *et al* (2000)).

PIF was found to induce a decrease in cytosolic IκBα (figure 6. 3a) and a subsequent increase in nuclear accumulation of NF-κB (figure 6. 3c). This effect was however attenuated with the specific PKCα inhibitor calphosin C.

To further substantiate a role for PKC in the activation of IKK and subsequent degradation of IκBα and activation of NF-κB, C_2C_{12} myoblasts were transfected with plasmids encoding constitutively active PKCα (pCO_2) and a dominant negative PKCα (pKS1).

Western blot analysis of cytoplasmic IκBα in response to PIF (30 min) in constitutively active (pCO_2) and mutant (pKS1) PKCα transfected cells.

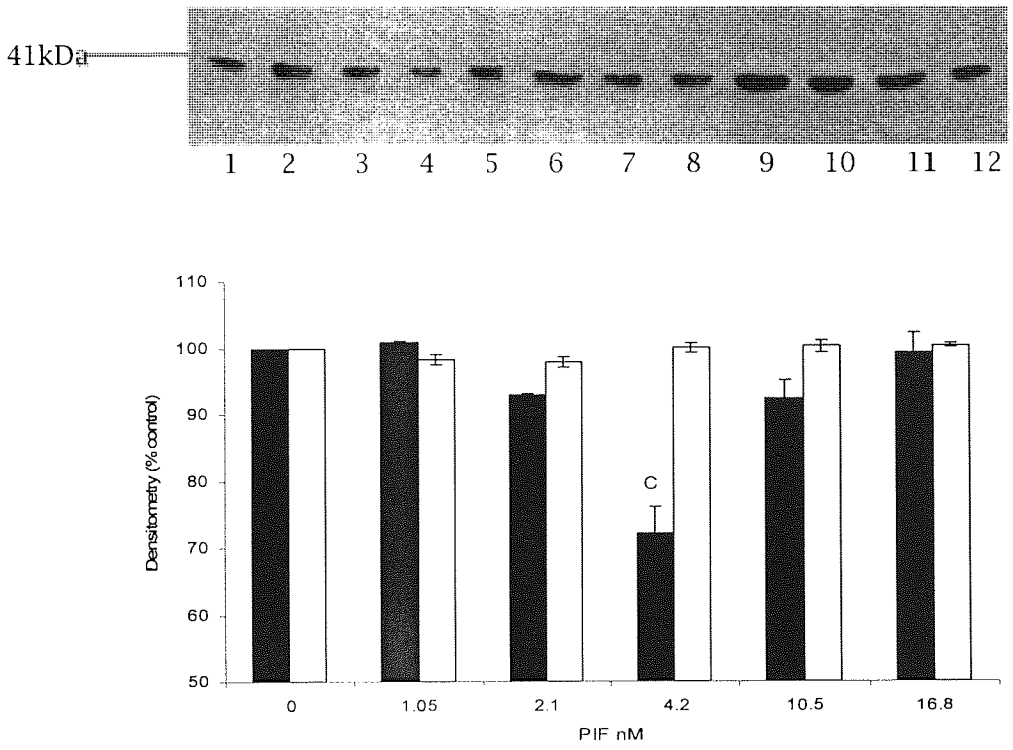


Figure 6. 4a

Effect of PIF on cytoplasmic IκBα determined 30min after PIF addition to murine myotubes transfected with constitutively active (pCO_2 □) and mutant (pKS1 ■) PKCα. Myotubes were transfected with either pCO_2 (lanes 1-6) or pKS1 (lanes 7-12) and treated with 0 (lanes 1 and 7), 1.05 (lanes 2 and 8), 2.1 (lanes 3 and 9), 4.2 (lanes 4 and 10), 10.5 (lanes 5 and 11) or 16.8nM PIF (lanes 6 and 12). Densitometric analysis is an average of 3 replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA and Tukeys post test. Differences from 0nM PIF are shown as c, $p< 0.001$.

Actin loading control

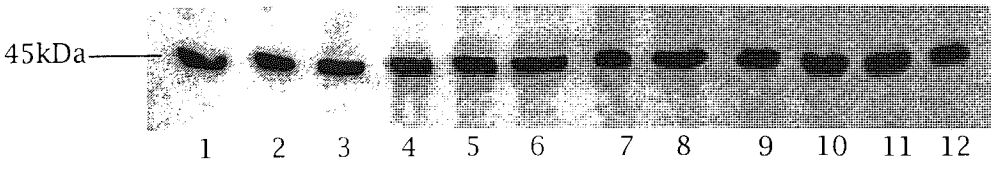
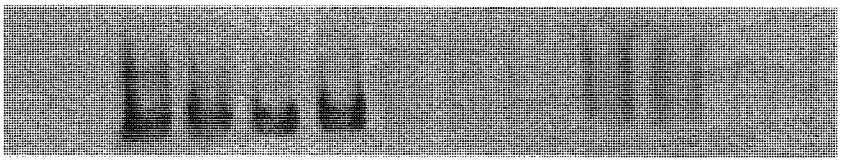


Figure 6. 4b

Lane discipline as figure 6. 4a

DNA binding of NF-κB in response to PIF (30 min) in constitutively active (pCO_2) and mutant (pKS1) PKCα transfected cells.



1 2 3 4 5 6 7 8 9 10 11 12 13

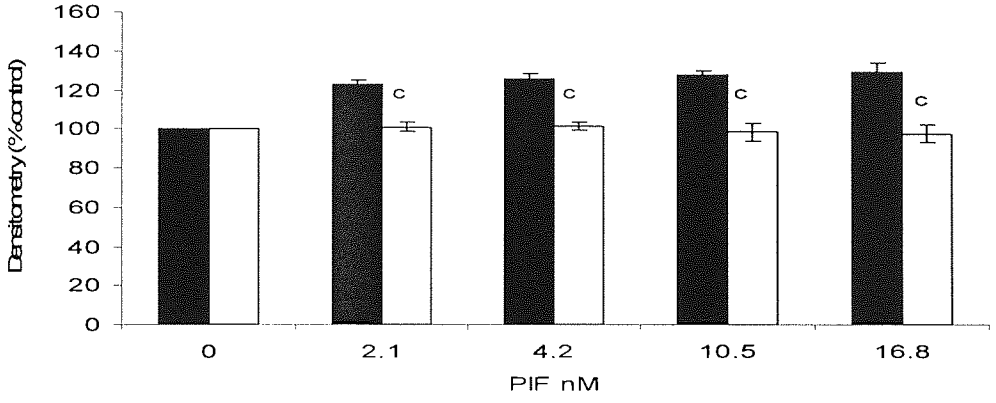


Figure 6. 4c

Effect of PIF on nuclear accumulation of NF-κB determined 30min after PIF addition to murine myotubes transfected with constitutively active (pCO_2 □) and mutant (pKS1 ■) PKCα. Myotubes were transfected with either pCO_2 (lanes 2-6) or pKS1 (lanes 7-11) and were treated with 0 (lanes 2 and 6), 2.1 (lanes 2 and 8), 4.2 (lanes 3 and 9), 10.5 (lanes 4 and 10) or 16nM PIF (lanes 6 and 11). Densitometric analysis is an average of 3 replicate EMSAs and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are shown as c, $p< 0.001$.

Figures 6. 4a-6. 4c

The specific PKCα inhibitor calphostin C successfully attenuated the degradation of cytosolic IκBα (figure 6. 3a) and subsequent increased DNA binding of NF-κB (figure 6. 4c) in response to PIF. In addition PIF induced a decrease in IκBα (figure 6. 4a) in myotubes transfected with constitutively active PKCα (pCO_2), but not in those containing the

dominant-negative insert of PKCα (pKS1). This effect was accompanied by an increase in DNA binding of NF-κB (figure 6. 4c). These results suggest that activation of PKCα by PIF in muscle cells leads to IκBα degradation, nuclear accumulation of NF-κB and increased proteasome expression, which results in increased intracellular protein degradation.

The importance of PKC as an intracellular signal for the translocation of NF-κB to the nucleus was further corroborated by using the potassium salt D609, a selective inhibtor of phosphatidylcholine (PC) specific phospholipase C (PLC) (Monick *et al* (1999)).

Western blot analysis of cytosolic IκBα in C_2C_{12} myotubes in response to PIF (30 min) in the absence or prescence of D609 (10μM)

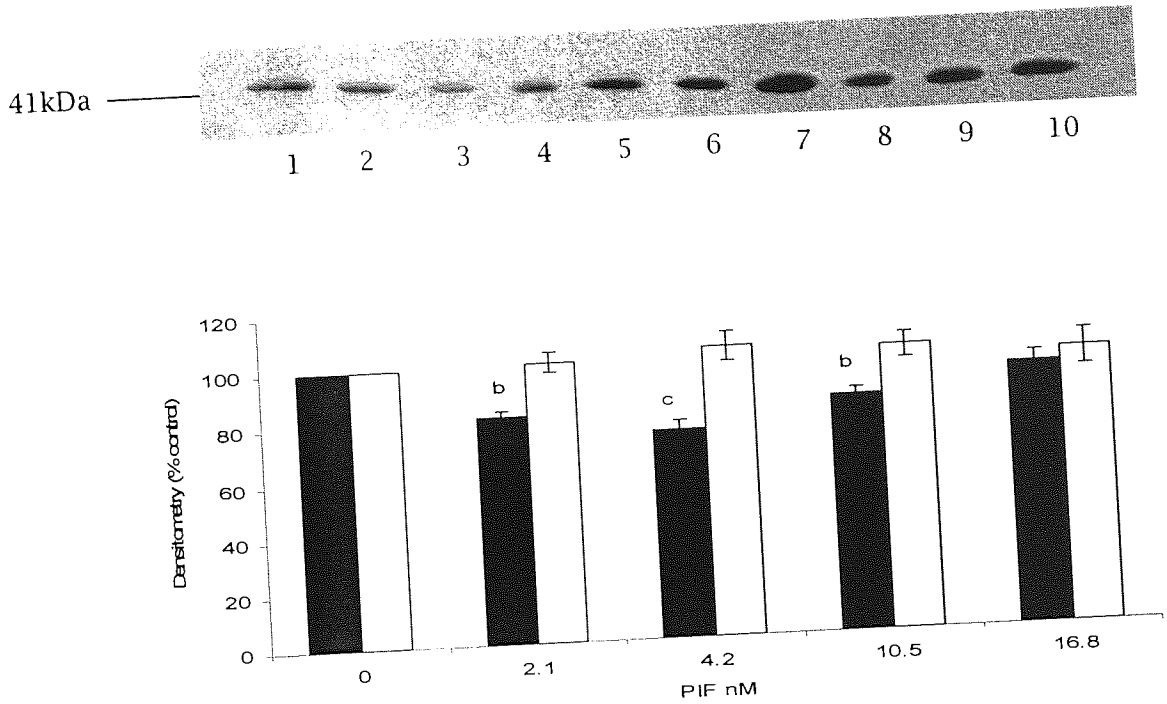


Figure 6. 5a

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) of 10μM D609. Densitometry is based on three separate blots and results are expressed as mean ± S.E.M. Statisitcal analysis was determined by one-way ANOVA followed by Tukeys test. Differences between 0nM and PIF are indicated by , $p < 0.01$ and c, $p < 0.001$.

Actin loading control

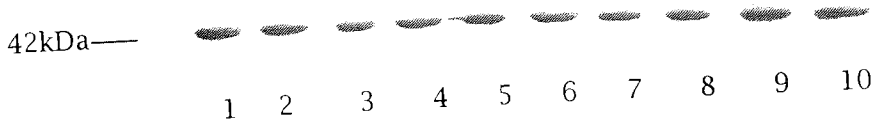


Figure 6. 5b

Lane discipline as figure 6. 5a

DNA binding of NF-κB in C_2C_{12} myotubes in response to PIF (30 min) in the absence or presence of D609 (10μM)

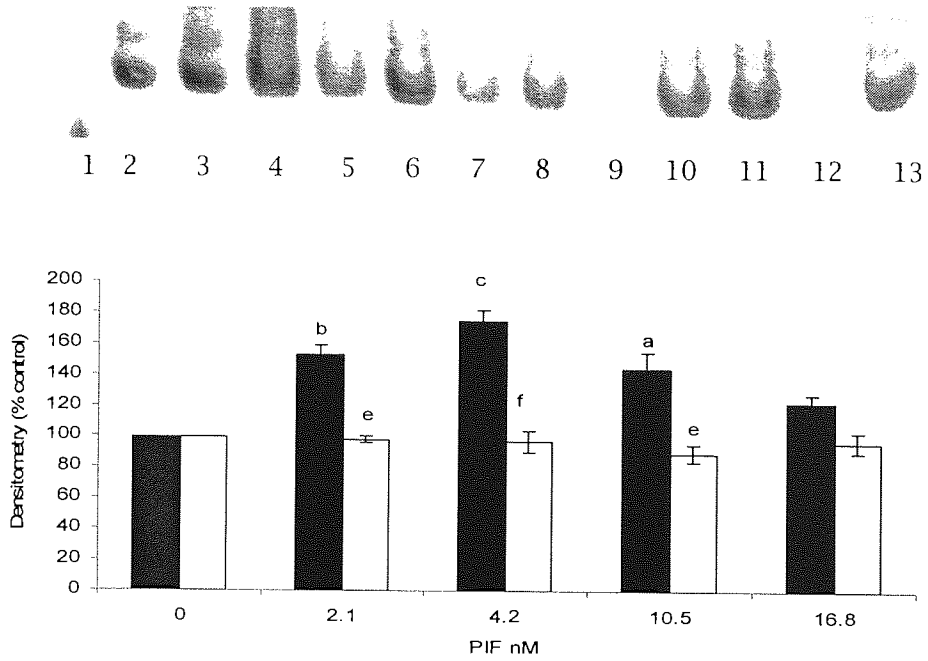


Figure 6. 5c

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) D609 (10μM). Lane 11 is a positive control for NF-κB, while lane 12 contains excess unlabelled NF-κB. Statistical analysis was detemined using one-way ANOVA followed by Tukey's test. Differences from control are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$, while differences in the presence of D609 as e, $p < 0.01$ and f, $p < 0.001$.

Figures 6. 5a-6. 5c

Smith and Tisdale (2003) previously demonstrated PIF to induce activation of phospholipase C (PLC) which is an important signalling event involved in the induction of proteasome expression. Activation of PLC results in the generation of diacylglycerol (DAG) which induces the translocation of PKC from the cytosol to the membrane and thus activating the kinase. This data suggests that activation of PKC by PC-PLC is an important step in the activation and upregulation of ATP-ubiquitin dependent proteolysis. The PC-PLC specific inhibitor D609 attenuated PIF-induced degradation of IκBα (figure 6. 5a) and subsequent nuclear accumulation of NF-κB (figure 6. 5c).

Discussion

Increased intracellular protein catabolism is a common feature of many disease states; however, there is little knowledge of the cellular signalling pathways involved. Initial studies suggested that prostaglandin E_2 (PGE_2) was involved in total protein degradation in skeletal muscle. Elevated PGE_2 production in tumour bearing animals has been found to cause increased protein degradation (Strelkov *et al* (1989)) and hypocalcaemia (Tashjian *et al* (1974)). However, other studies (Hasselgren *et al* (1990), McKinely and Turinskey (1986)) found no evidence that total or myofibrillar protein breakdown in normal or septic muscle is regulated by PGE_2.

Studies with PIF demonstrated protein breakdown was related to release of AA and formation of PGE_2. However, PGE_2 was not found to be the eicosanoid responsible for protein breakdown (Smith *et al* (1999)). Arachidonic acid is converted into a number of metabolites including PGE_2 and HETEs, however, only 15(*S*)-HETE was capable of inducing protein degradation (Smith *et al* (1999)). Whitehouse *et al* (2003) found 15(S)-HETE to induce an increase in expression of the ubiquitin-proteasome pathway, responsible for the initiation of protein catabolism, involving the transcription factor NF-κB.

The results presented in this chapter support the suggestion that PKC plays a central role in the activation of NF-κB by PIF. Previous studies (Smith and Tisdale (2003)) have shown PIF to induce activation of phospholipase C (PLC) as an important signalling event in inducing proteasome expression. Activation of PLC results in the generation of diacylglycerol (DAG), which acutely regulates PKC activity (Bornacin and Barber (1996)). DAG induces the translocation of PKC from the cytosol to the membrane, resulting in the complex activation of the kinase. PIF has been shown to induce translocation of PKCα from the cytosol to the membrane at the same concentrations inducing proteasome expression.

PKCα is an upstream activator of IκBα kinase complex (IKK) (Vertegaal et al (2000)), phosphorylating IκBα at Ser^{32} and Ser^{36} leading to ubiquitination and subsequent degradation, suggesting a mechanism by which PIF may induce degradation of IκBα and stimulate nuclear binding of NF-κB (Whitehouse and Tisdale (2003)). NF-κB regulates the transcription of many genes and is an essential mediator of TNF-α induced protein catabolism in differentiated muscle cells (Li and Reid (2000)).

This chapter has demonstrated how the degradation of IκBα and subsequent translocation of NF-κB to the nucleus in response to PIF is attenuated by calphostin C and is not observed in myotubes expressing the mutant PKCα, suggesting PKC to act as an important mediator in the activation of NF-κB in response to PIF.

Chapter 7

Activation of Protein Kinase C by a phorbol ester initiates protein degradation in skeletal muscle through the ubiquitin-proteasome pathway.

Introduction

Skeletal muscle atrophy is common in a number of unrelated conditions including sepsis, metabolic acidosis, weightlessness, severe trauma, diabetes, AIDS and cancer cachexia. Despite the different aetiology of these disease states, muscle protein catabolism has been attributed to an increased expression of the ubiquitin-proteasome proteolytic pathway (Lecker *et al* (1999). Ubiquitin is a 76 amino acid heat-stable polypeptide and is an essential co-factor in ATP-dependent proteolysis. Proteins marked for degradation by ubiquitin are digested to small peptides within the 20S core of the 26S proteasome. This ubiquitinated protein substrate, once bound, is cut multiple times to small peptides ranging from 3 to 25 residues in length, via enzymatic hydrolysis. Peptides released by the proteasome are either rapidly hydrolyzed to amino acids in the cytosol or used in MHC class I antigen presentation (Rock *et al* (1994)). Bossola *et al* (2001) confirmed the over-expression of muscle ubiquitin mRNA in cancer cachexia, therefore it is important to understand the mechanisms which lead to an up-regulation in the expression of the proteasome system.

A tumour derived proteolysis-inducing factor (PIF) has been isolated from cachexia inducing human and murine tumours (Todorov *et al* (1996)). This tumour derived PIF has been found to have an important role in the degradation of skeletal muscle mass in cancer cachexia (Todorov *et al* (1996)). PIF induces protein degradation directly in isolated skeletal muscle and myotubes *in vitro*, through increased expression and activity of the ubiquitin-proteasome proteolytic pathway (Gomes-Marcondes *et al* (2002)).

PIF-induced proteasome activity and increased protein degradation involves phospholipase A_2 (PLA_2) mediated release of AA from membrane phospholipids (Smith and Tisdale (2003)). AA is metabolised to 15-hydroxyeicosatetraenoic acid (15-HETE) via the lipoxygenase pathway (Smith *et al* (1999)). Phospholipase C (PLC) is also involved in the release of arachidonic acid in response to PIF (Smith and Tisdale (2003)). As a result of PLA_2 or PLC activity, a product derived from this reaction is involved in PIF-induced

activation of protein kinase C (PKC), which is crucial to the induction of proteasome expression (Smith *et al* (2004)).

PKC activation results in phosphorylation of IκBα, possibly through the phosphorylation and activation of the IKK complex (Vertegaal *et al* (2000)) and nuclear accumulation of NF-κB occurs in response to PIF (Whitehouse and Tisdale (2003)). This action of PIF is thought to be responsible for increased gene transcription of proteasome subunits and the ubiquitin-conjugating enzyme (Whitehouse and Tisdale (2003)).

Previous studies suggest activation of PKC as central to the action of PIF-induced protein degradation in skeletal muscle, and non-specific stimulation of PKC should lead to protein breakdown through increased expression of the ubiquitin-proteasome proteolytic pathway. The PKC family is involved in signalling by phorbol esters (Dekker and Parker (1994)). Hong *et al* (1995) found pre-incubation of L6 and L8 muscle cells with the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) to cause rapid depletion of PKCα and PKCδ from the cytoplasm and subsequent translocation to the membrane, however, long term exposure of myotubes to TPA eventually caused down-regulation of PKCs in the membrane, resulting with a loss of α, δ and ε isoforms. This evidence suggests that these isoforms may be involved in PIF action, since this is blocked by high concentrations of TPA (Smith *et al* (2004)). High concentrations of TPA have been shown to inhibit myofibrillar protein degradation in C_2C_{12} myotubes, as measured by the release of 3-methylhistidine (Thompson *et al* (1996)) and to attenuate the PIF-induced increase in proteasome activity and expression (Smith *et al* (2004)).

Methodology

Tissue Culture

See section 2.5.1

Measurement of proteasome activity

See section 2.5.1.e

Measurement of protein degradation

See section 2.5.1.d

Western Blot Analysis

Myotubes were incubated with various concentrations of TPA as depicted in the figure legends, (from 0 to 100nM). Samples of total cellular lysate (30μg) were used for detection of IκBα 30 min after the addition of TPA. Cells incubated with TPA for 24h was used to assess proteasome activity and increased proteasome expression. Cytosolic protein formed by centrifugation at 18,000g for 10 min was used for the proteasome activity assay and at 5μg for western blotting.

For a full protocol see section 2.3.3

The primary antibodies were used at a dilution of 1:1000 except for actin (1:250) and myosin (1:100) and secondary antibodies were used at a dilution of 1:1000. Incubation was for 1h at room temperature and development was enhanced by chemiluminescence (ECL) (Amersham, UK). Blots were scanned by a densitometer to quantitate differences.

Electectrophoresis mobility shift assay (EMSA)

DNA binding proteins were extracted from myotubes according to the method of Andrews and Faller (1991), which utilises hypotonic lysis followed by a high salt extraction of nuclei (see section 2.8.1). The EMSA binding assay was carried out using a Panomics EMSA 'gel shift' kit according to instructions (see section 2.8.2).

Statstical Analysis

Differences beween means between groups was determined by one-way ANOVA, followed by the Tukey-Kramer multilple comparisons post test.

Results

Total protein degradation in C_2C_{12} myotubes in response to TPA

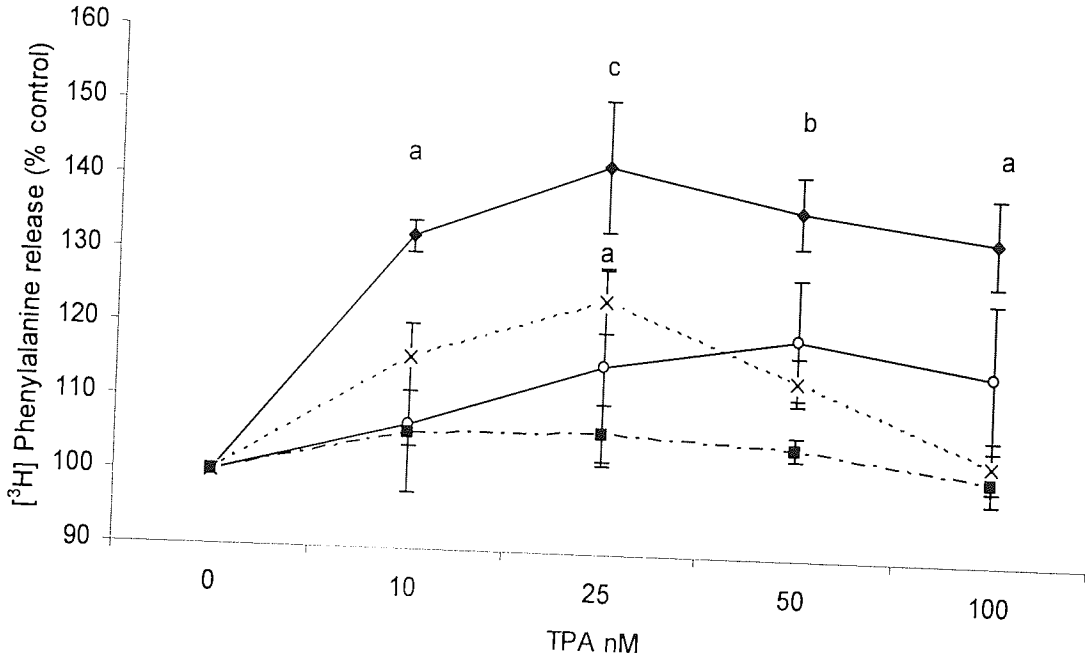


Figure 7. 1a

The effect of incubating C_2C_{12} murine myotubes with various concentrations of TPA for 1h (0), 2h (X), 3h (◆) and 4h (■) on total protein degradation, measured by release of [^{3}H] phenylalanine. Results are expressed as mean ±.S. E. M of 3 repeats, where n = 6. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$.

Figure 7. 1a

The effect of various concentrations of TPA and various incubation times on total protein degradation in murine myotubes, as measured by the release of [^{3}H] phenylalanine from pre-labelled cells is demonstrated in figure 3. 1a. The dose response curve was similar at all incubation times, with TPA enhancing protein degradation at a maximum concentration of 25nM. The effect increased at low concentrations and decreased at higher concentrations of TPA. This effect was dependent on time, increasing up-to 3h,

with the overall effect decreasing after this incubation time. This overall dose response curve is similar to that induced by PIF, as seen in chapters 4, 5 and 6.

To investigate whether the action of TPA was mediated through the ubiqutin-proteasome proteolytic pathway, proteasome activity was determined by the 'chymotrypsin-like' enzyme activity, which represents the predominant proteolytic activity of the β subunits of the proteasome.

The effect of TPA on 'chymotrypsin-like' activity of the proteasome in C_2C_{12} myotubes

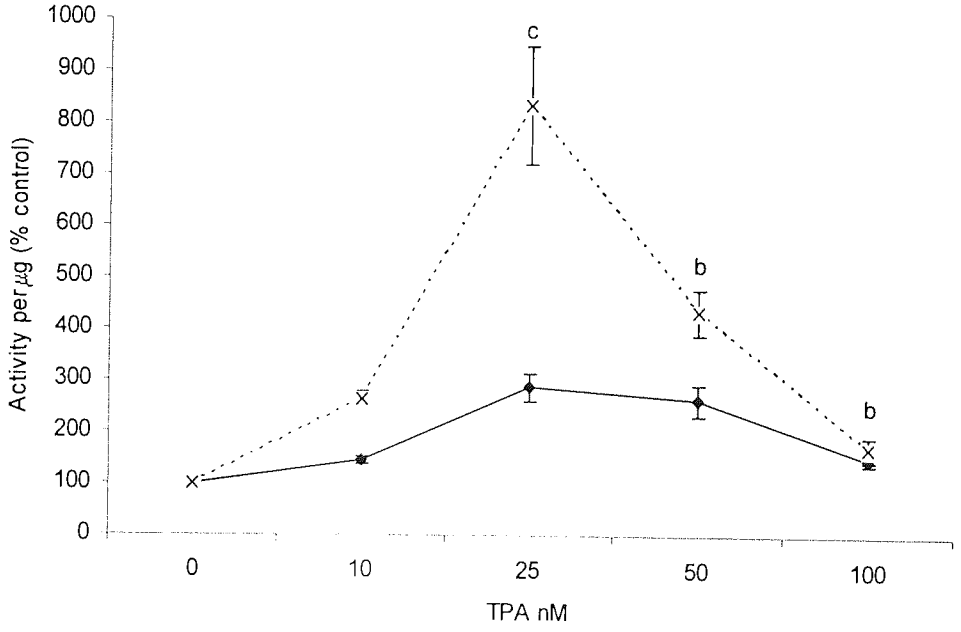


Figure 7. 1b

The effect of TPA at 2h (◆) and 3h (X) on 'chymotrypsin-like' enzyme activity of the proteasome. Results are expressed as mean ± S. E. M of 3 repeats, where n = 6. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$.

Figure 7. 1b

The concentration and time profile for the effect of TPA on 'chymotrypsin-like' activity in murine myotubes is similar to that observed for protein degradation (see figure 7. 1a). Thus, TPA produced a significant increase in 'chymotrypsin-like' activity, with a maximum

effect at 3h and incubation with 25nM TPA. The dose-response curve follows the parabolic pattern as seen with the protein degradation results. This data suggests that TPA stimulates protein degradation as a result of increased activity and expression of the ubiquitin-proteasome proteolytic pathway, through the association and interaction of TPA with PKC. Smith *et al* (2004) have demonstrated that PIF mediates its proteolytic effect through PKC; therefore, it would appear PKC is an essential signalling transducer in the initiation of intracellular protein degradation and up-regulation of the ATP-ubiquitin dependent proteolytic pathway.

To evaluate this suggestion, C_2C_{12} myotubes were treated with various concentrations of TPA for 2h and 3h, and expression of the components within the ATP-ubiquitin dependent proteolytic pathway was assessed by western blotting.

Western blot analysis of 20S proteasome α subunits in C_2C_{12} myotubes in response to TPA

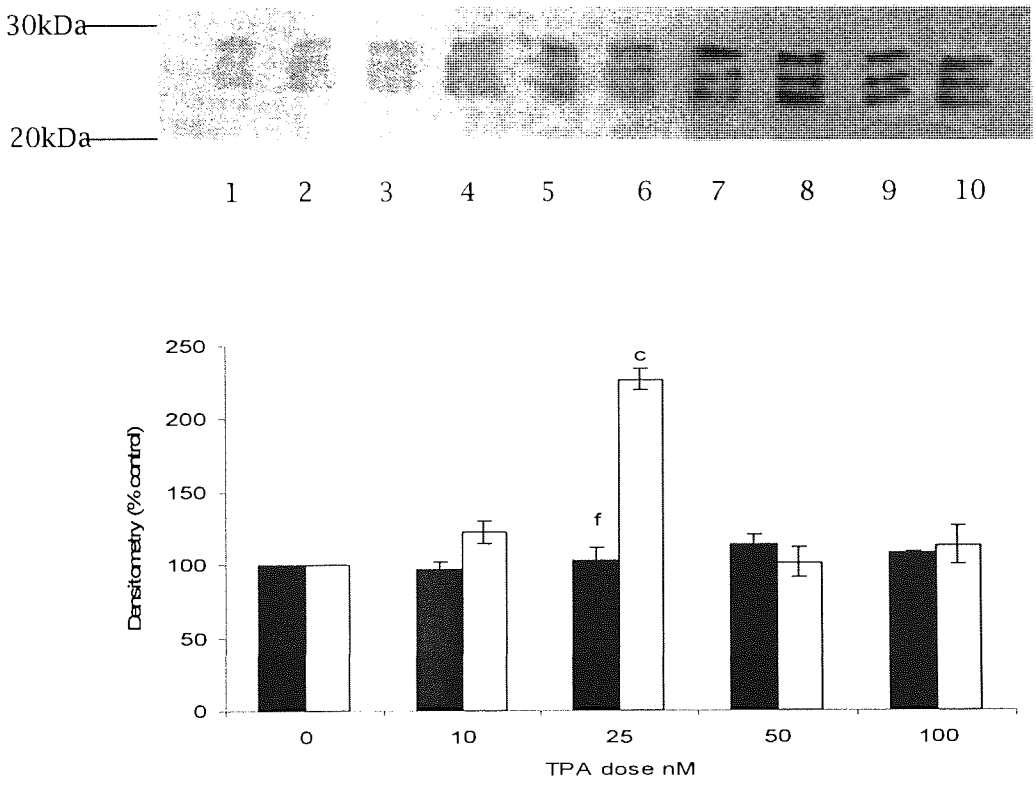


Figure 7. 2a

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometry analysis was based on three replicate blots and results are expressed as mean ± S. E. M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$, while differences between 2h and 3h TPA are indicated as d, $p < 0.05$ and f, $p < 0.001$.

Western blot analysis of MSS1 proteasome subunit expression in C_2C_{12} myotubes in response to TPA in C_2C_{12} myotubes

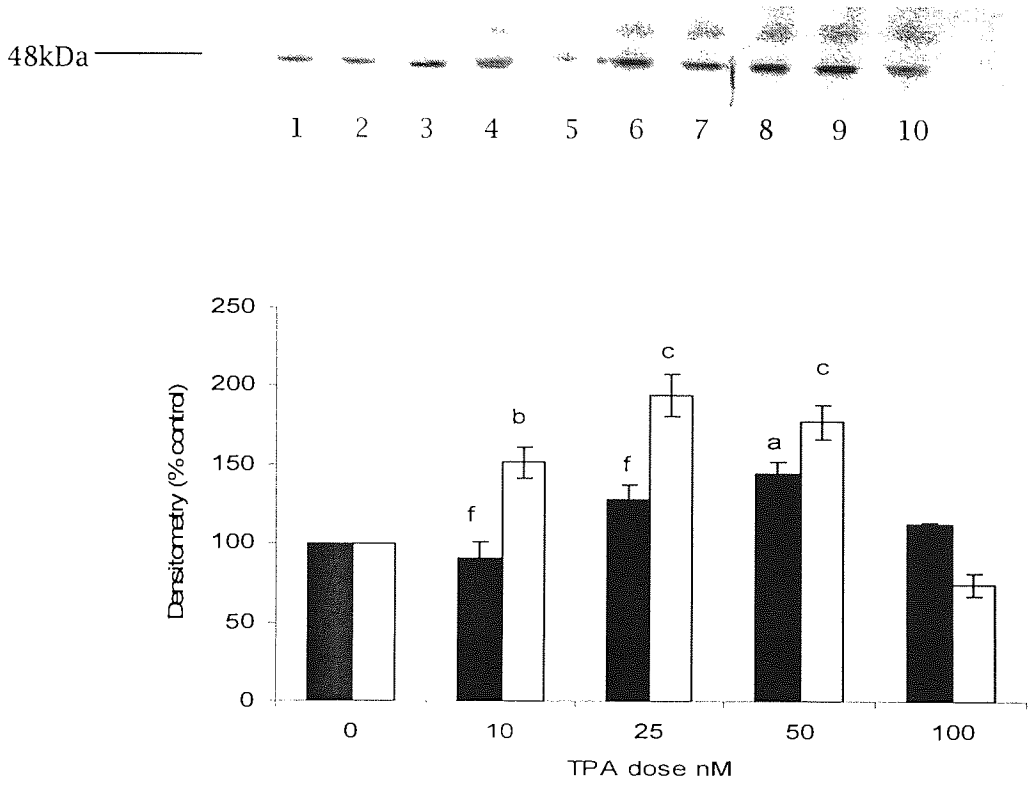


Figure 7. 2b

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$; b, $p < 0.01$ and c, $p < 0.001$, while differences between 2h and 3h TPA are indicated as d, $p < 0.05$ and f, $p < 0.001$.

Western blot analysis of p42 proteasome subunit expression in C_2C_{12} myotubes in response to TPA

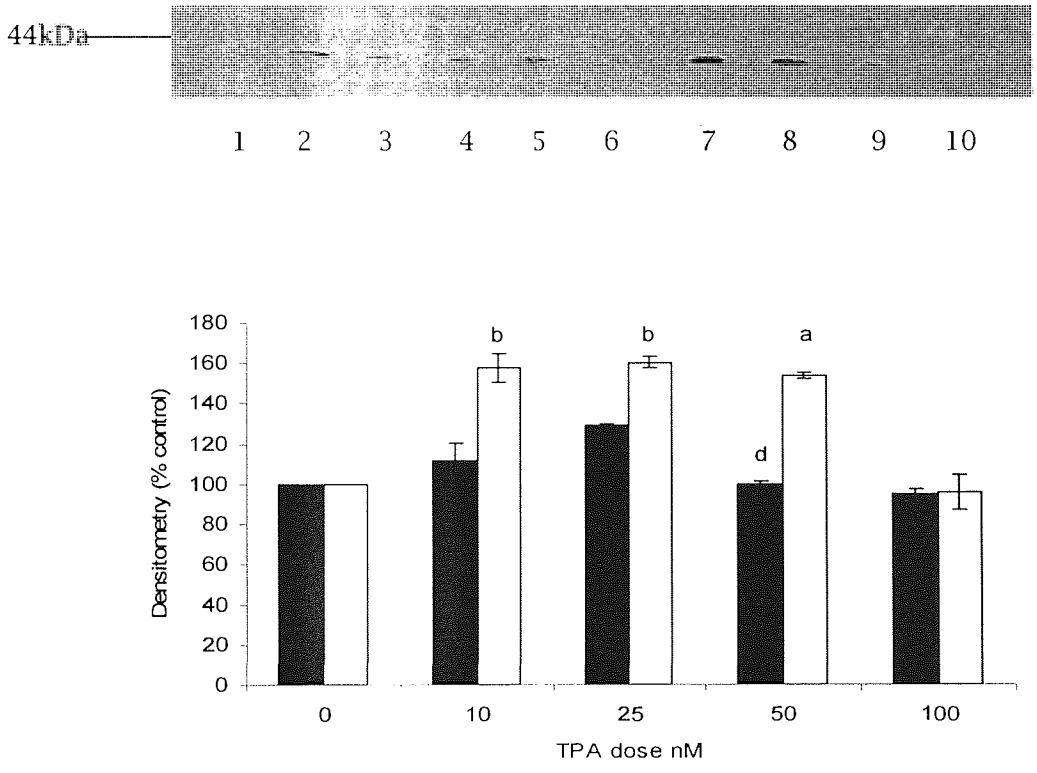


Figure 7. 2c

C_2C_{12} myotubes were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometric analysis was based on three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$ and b, $p < 0.01$, while differences between 2h and 3h TPA are indicated as d, $p < 0.05$.

Western blot analysis of $E2_{14K}$ expression in C_2C_{12} myotubes in response to TPA

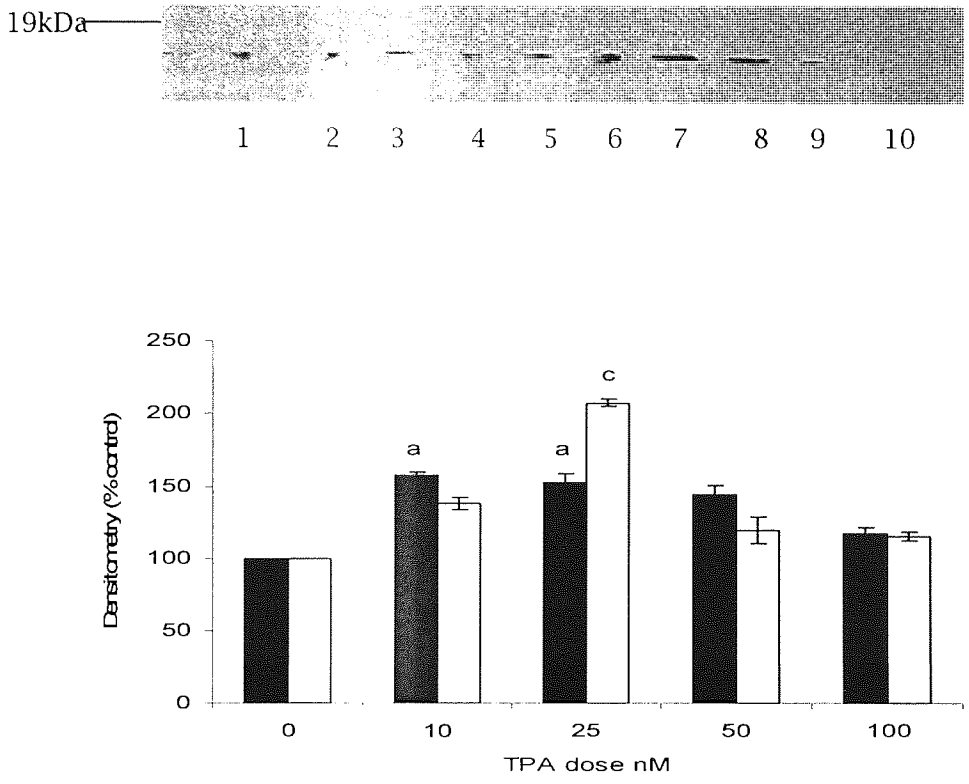


Figure 7. 2d

C_2C_{12} myotubes were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometric analysis was the average of 3 separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$ and c, $p < 0.001$.

Western blot analysis of myosin expression in response to TPA in C_2C_{12} myotubes

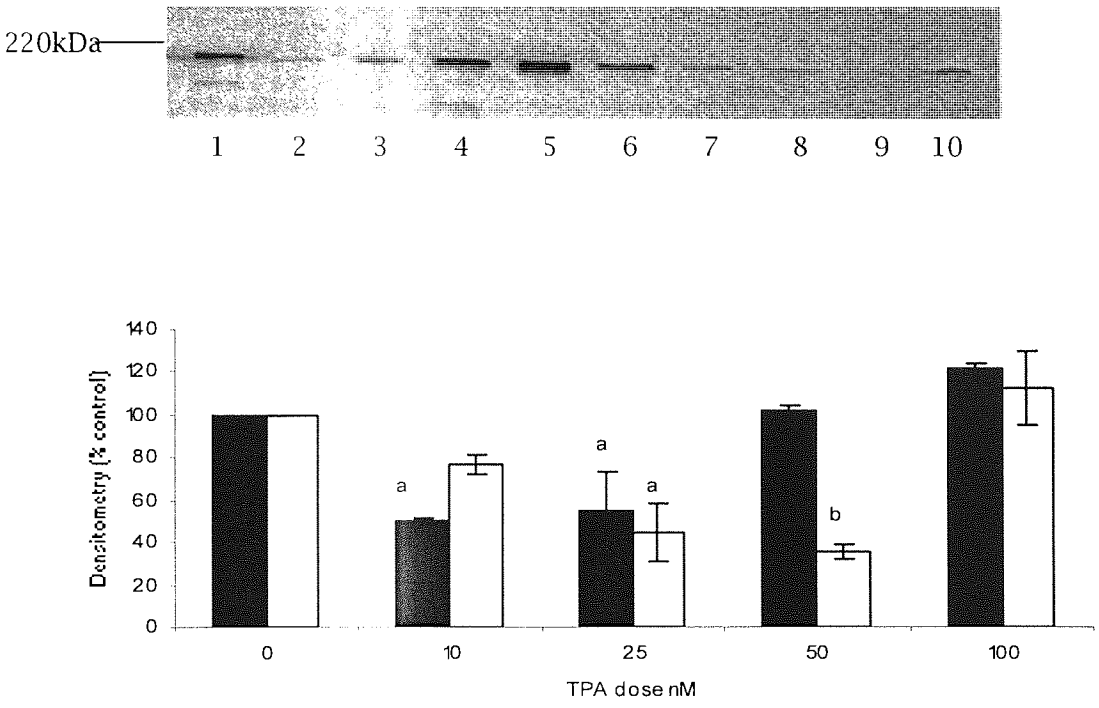


Figure 7. 2e

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometric analysis was the average of 3 separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as a, $p < 0.05$ and b, $p < 0.01$.

Figures 7. 2a- 7. 2e

The results generated from western blot analysis of the components within the ATP-ubiquitin dependent proteolytic pathway paralleled the effect of TPA on the 'chymotrypsin-like' enzyme activity. Thus, 20S proteasome α subunit expression (figure 7. 2a), MSS1 (figure 7. 2b), p42 (figure 7. 2c) expression is increased in response to increasing concentrations of TPA, up-to 25nM at 3h and then decreased.

$E2_{14K}$ (figure 7. 2d) is one of the major mammalian E2s that support E3α-dependent ubiquitin conjugate formation (Lecker *et al* (1999)) and expression followed a similar pattern at concentrations between 10 and 50nM TPA, with a maximal effect again seen at 25nM at 3h. These dose and time response relationships are the same as observed for TPA effect on protein degradation (figure 7. 1a).

TPA produced a reciprocal decrease in the level of the myofibrillar protein myosin (figure 7. 2e), confirming intracellular protein degradation and confirms that TPA induces protein degradation in murine myotubes through an increased expression and activity of the ubiquitin-proteasome proteolytic pathway.

To investigate whether this occurred through the activation of the transcription factor NF-κB, the cytosolic levels of IκB were subjected to western blot analysis and an EMSA was conducted to investigate the effect of TPA on nuclear binding of NF-κB after 2h and 3h in C_2C_{12} myotubes.

Western blot analysis of cytosolic IκBα in response to TPA in C_2C_{12} myotubes

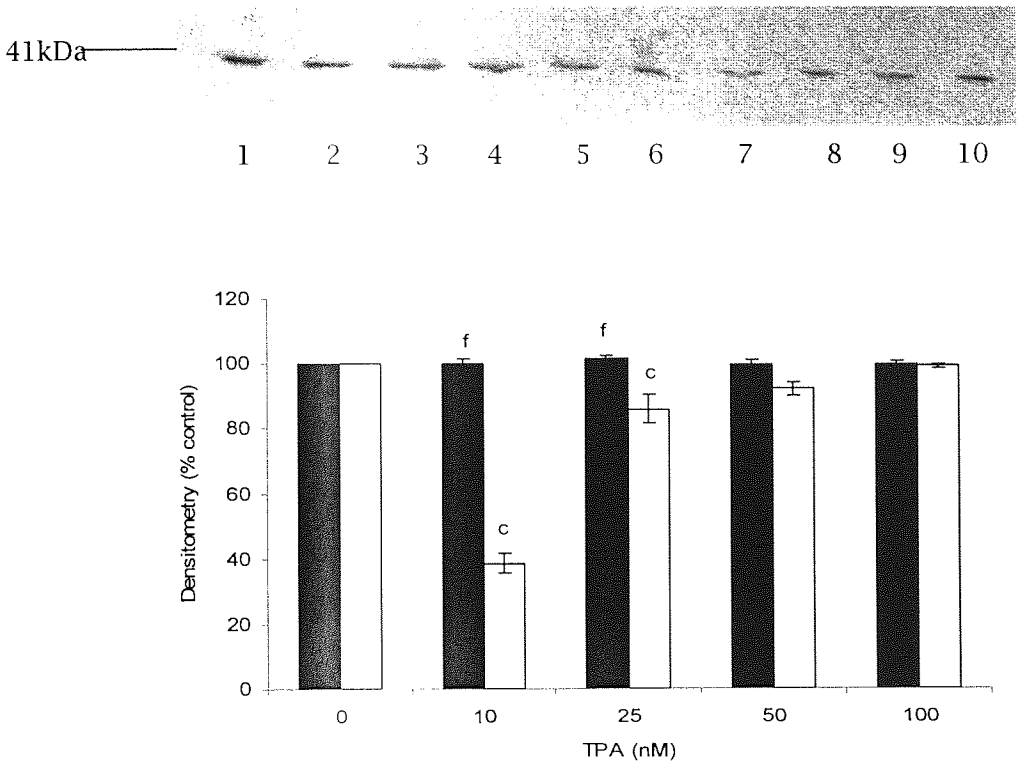


Figure 7. 3a

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, ⊡). Densitometric analysis was the average of 3 separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test Differences from 0nM TPA are indicated as c, $p < 0.001$, while differences between groups are indicated as f, $p < 0.001$.

Actin control loading blot

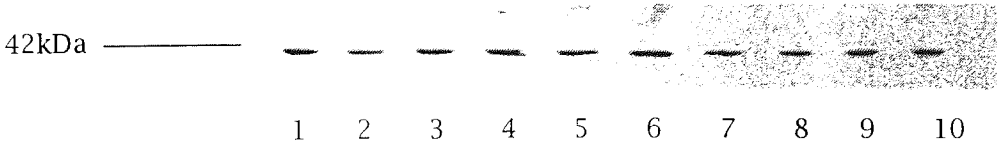


Figure 7. 3b

Lane discipline as figure 7. 3a.

DNA binding activity of NF-κB in response to TPA in C_2C_{12} myotubes

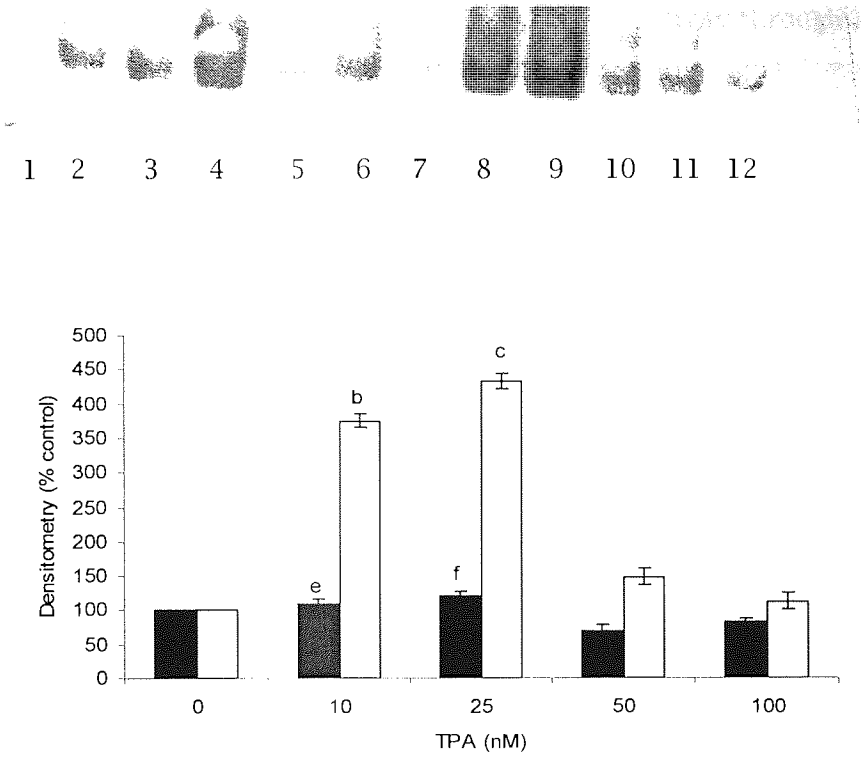


Figure 7. 3c

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Lane 11 is a positive control for NF-κB (supplied by the manufacturer of the kit), while lane 12 contains a positive control for NF-κB together with 100-fold excess of unlabelled NF-κB probe. Densitometric analysis was based on 3 separate EMSAs and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences between groups are indicated as e, $p < 0.01$ and f, $p < 0.001$.

Figure 7. 3a - 7. 3c

There was no effect of TPA on IκBα degradation after 2h incubation, but a concentration dependant decrease occurred after 3h incubation with TPA (see figure 7. 3a). The maximal effect was observed at 10 and 25nM. Degradation of cytosolic IκBα should lead to an increase in nuclear accumulation of NF-κB, and this was confirmed by EMSA (figure 7. 3c).

There is an inverse relationship evident between cytosolic IκBα and nuclear accumulation of IκBα, with an increase in DNA binding occurring at 3h incubation with TPA. A maximal effect was again observed at a concentration of 10 and 25nM. The results presented here suggest that TPA induces proteasome expression and activity through activation of NF-κB in a similar manner to PIF, that they both utilise PKC as the signal transducer in initiating activation of protein degradation.

To investigate whether activation of PKC by TPA leads to activation of ERK and subsequently MAPK as part of a signalling cascade, the phosphorylated form of MAPK was assessed at the same time intervals after addition of TPA.

Western blot analysis of active (phosphorylated) ERK 1/2 in response to TPA in C_2C_{12} myotubes

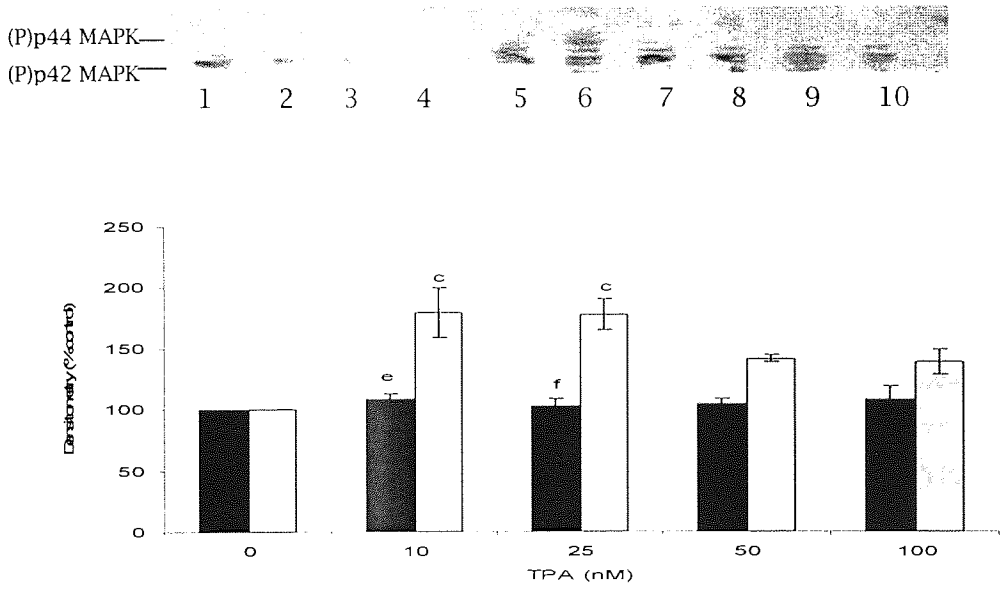


Figure 7. 4a

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, ⊡). Densitometric analysis was based on 3 separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM TPA are indicated as c, $p < 0.001$, while differences between groups are indicated as e, $p < 0.01$ and f, $p < 0.001$.

Western blot analysis of total ERK 1/2 in response to TPA in C_2C_{12} myotubes

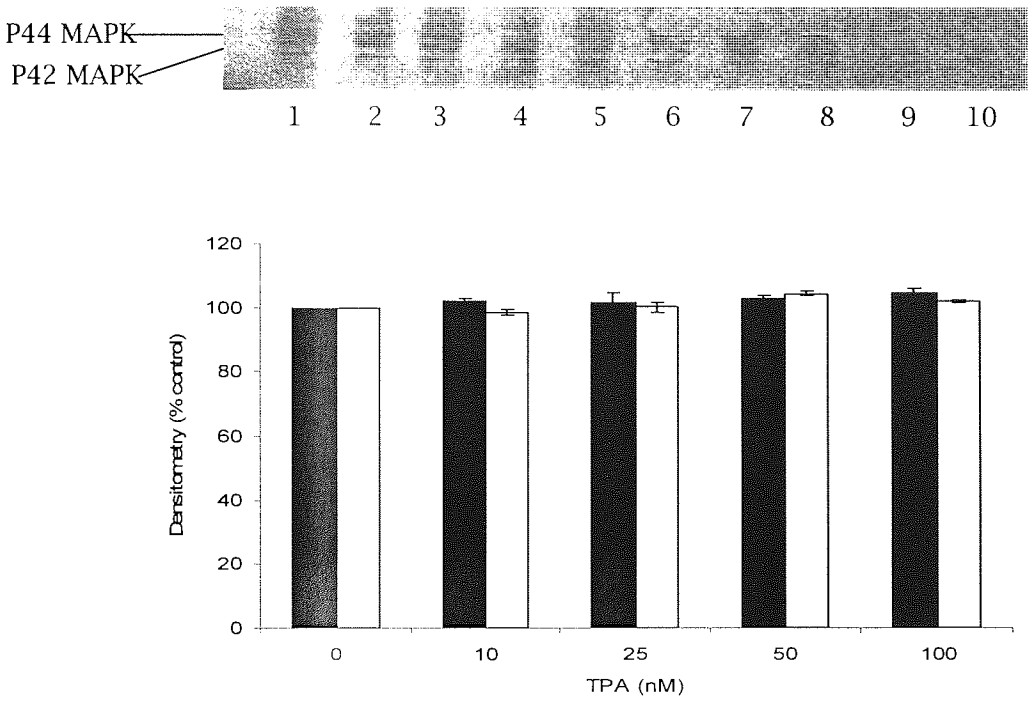


Figure 7. 4b

Cells were treated with 0 (lanes 1 and 6), 10 (lanes 2 and 7), 25 (lanes 3 and 8), 50 (lanes 4 and 9) or 100nM TPA (lanes 5 and 10) for 2h (lanes 1-5, ■) or 3h (lanes 6-10, □). Densitometric analysis was based on 3 separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. There was no significant difference between groups, compared to 0nM TPA.

Figures 7. 4a and 7. 4b

TPA induced phosphorylation of the p44/ p42 MAPK at the same concentrations as those inducing proteasome expression and activation of NF-κB, with a maximal effect at 10 and 25nM (figure 7. 4a). There was no corresponding effect on total levels of ERK 1/ 2. These results suggest a role for MAPK activation via TPA-induced proteasome expression.

The phorbol ester TPA has successfully demonstrated the importance of the PKC signalling cascade in murine myotubes, involved in the upregulation of expression of the ATP-ubiquitin dependent proteolytic pathway (figure 7. 2a - 7. 2e), protein degradation (figure 7. 1a) and activity of the proteasome (figure 7. 1b).

Discussion

Muscle atrophy is a common feature of many disease states however; there is little information on the molecular mechanisms leading to degradation of myofibrillar proteins. PIF has been demonstrated to directly stimulate protein degradation in skeletal muscle myotubes, through an increased expression of the ubiquitin-proteasome proteolytic pathway (Gomes-Marcondes *et al* (2002)). It is likely this pathway is involved with other common initiators of protein degradation, since EPA and lipoxygenase inhibitors, key steps in PIF signalling (Smith *et al* (1999)), also attenuate muscle protein degradation and induction of the ubiquitin-proteolytic pathway in acute starvation (Whitehouse and Tisdale (2001).

Smith *et al* (2004) demonstrated myotubes transfected with constitutively active PKCα to exhibit increased proteasome expression and activity in response to PIF, compared with their 'wild type' counterparts, while myotubes transfected with a dominant-negative PKCα, did not demonstrate any activation of PKCα in response to PIF and no associated increase in proteasome expression or activity.

PIF-induced proteasome expression was also attenuated by the PKC inhibitors Ro31-8220, staurosporine, calphostin C and Gö6976, and high concentrations of TPA (Smith *et al* (2004)), suggesting activation of PKC as a key step in the induction of proteasome expression and protein degradation by PIF in skeletal muscle. Phorbol esters are commonly used to evaluate the effect of PKC in cells, since they can activate PKC and in some cell types, produce diacylglycerols, via the activation of phosphatidyl inositol-phospholipase C and phospatidyl choline-phospholipase D (Madani *et al* (2004)). Therefore if activation of PKC is a general process involved in muscle protein degradation through activation of the ubiquitin-proteasome pathway, then TPA should also activate this process in muscle cells.

This chapter has demonstrated TPA to induce protein degradation in murine myotubes in a concentration and time-dependant manner, with a maximal effect at a concentration of 25nM at a 3h incubation period. The dose-response shows a typical 'bell shaped' curve, with the optimum concentration of TPA, while higher concentrations and incubations greater than 3h lead to over-stimulation and down-regulation of PKC. The dose-response curve for stimulation of protein degradation and up-regulation of the ubiquitin-proteasome pathway by PIF follows a similar parabolic relationship (Gomes-Marcondes *et*

al (2002) and Smith *et al* (2004)), and is probably a result of the PKC signalling step involved in the signal transduction pathway (Smith *et al* (2004)).

TPA-induced protein degradation was mediated through increased expression and activity of the ubiquitin-proteasome proteolytic pathway, as determined by measuring the 'chymotrypsin-like' activity of the β subunits of the proteasome, as well as expression of the proteasome subunits and $E2_{14K}$, which all follow the same pattern as protein degradation. Induction of the ubiquitin-proteasome pathway by TPA was matched by a concomitant decrease in the myofibrillar protein myosin.

Activation of NF-κB by PIF is important in the induction of proteasome expression (Whitehouse and Tisdale (2003)). TPA also caused an increase in nuclear binding of NF-κB, concomitant with a decrease in cytosolic IκBα at the same concentrations, which induced protein degradation and increased proteasome expression. This is most likely due to the activation of the IKK complex by TPA (Vertegaal *et al* (2000)).

PIF also induced phosphorylation of the p44 / p42 MAPK at the same concentrations as those that induced proteasome expression. This was thought related since the MAPK inhibitor PD98059 also inhibited PIF-induced proteasome expression (Smith and Tisdale (2003)). TPA induced an increase in phosphorylation of ERK1 and ERK2 at the same concentrations as those inducing protein degradation, confirming the effect to arise through activation of PKC (Madani *et al* (2004)).

The results from this chapter confirm the importance of PKC in muscle protein degradation through the ubiquitin-proteasome pathway. The particular isozyme of PKC to be involved is probably PKCα, since both PIF (Smith *et al* (2004)) and TPA (Hong *et al* (1995)) have both been shown to activate PKCα and this isozyme is involved in the activation of ERK 1 / 2 and the stimulation of IKK and NF-κB. (Amit *et al* (2003). The results from this chapter suggest that PIF-induced activation of PKCα is central to the activation of other signalling pathways involved in the induction of the ubiquitin-proteasome-proteolytic pathway and protein degradation.

Chapter 8

The role of phosphatidylinositol 3- kinase and Akt in the induction of proteasome expression by proteolysis- inducing factor in murine myotubes

Introduction

The ubiquitin-proteasome proteolytic pathway is the major catabolic pathway involved in the degradation of myofibrillar proteins in cancer cachexia (Bossola *et al* (2001). This pathway is also involved in muscle wasting observed in sepsis and severe infection (Luo *et al* (2000)), disuse atrophy (Hunter *et al* (2002)), and diabetes (Merforth *et al* (1999)). Despite the importance of this catabolic pathway in muscle atrophy, there is little information regarding the signalling pathways in skeletal muscle, which result in increased gene transcription of proteasome subunits and enzymes involved in ubiquitin conjugation. In cancer cachexia-inducing tumours, a 24kDa proteolysis-inducing factor (PIF) has been shown to intiate muscle protein breakdown through increased expression and activity of the ubiquitin-proteasome proteolytic pathway (Gomes-Marcondes *et al* (2002)). Therefore cellular signalling in response to PIF can be used as a model to study pathways involved in skeletal muscle atrophy and gene induction in cancer cachexia.

Smith and Tisdale (2003) established the first step in this PIF-induced pathway as the release of AA from membrane phospholipids through the action of phospholipase A_2 (PLA_2) and C (PLC). Free AA released from membrane phospholipids is rapidly metabolised to prostaglandins E_2 and $F_{2\alpha}$ as well as the lipoxygenase metabolites 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETE) (Smith *et al* (1999)). Smith *et al* (1999) found only 15-HETE able to produce a significant increase in protein degradation in murine myotubes. Whitehouse *et al* (2003) determined that this effect was mediated through an increased expression and activity of the ubiquitin-proteasome proteolytic pathway. PIF (Whitehouse and Tisdale (2003)) and 15-HETE (Whitehouse *et al* (2003)) were both found to cause a transient decrease in IκBα and increased nuclear migration of the transcription factor NF-κB. The NF-κB inhibitor peptide SN50 attenuated both the PIF and 15-HETE induced increase in proteasome activity, suggesting that NF-κB may be involved in the increase in proteasome expression.

Activation of NF-κB is initiated by phosphorylation and degradation of IκBα, leading to the release of NF-κB. IκB kinase (IKK) is involved in activation of NF-κB and is itself activated by phosphorylation and is a substrate for a protein kinase C (Vertegaal *et al* (2000)). Smith *et al* (2004) looked at upstream signalling involved in the action of PIF and found PKC to be a potential mediator of the process. Inhibition of PKC activity by low molecular weight inhibitors, or transfection of myotubes with a dominant negative mutant of PKCα, attenuated the PIF-induced increase in proteasome expression (Smith *et al* (2004)).

An alternative pathway involving PI3K and Akt can also phosphorylate IKK. Inappropriate activation of the PI3K/ Akt pathway has been associated with the development of diabetes, auto-immunity and cancer (West *et al* (2002)). Induction of NF-κB activation by TNF-α requires Akt (Shah *et al* (2001)). Akt (also called protein kinase B) is a serine/ threonine protein kinase that mediates a potent survival signal when activated. Akt is normally transiently activated in response to various growth factors or cytokines and generally occurs via the phosphatidylinositol 3- kinase (PI3K) pathway (Fahy *et al* (2004)). PI3K exerts many biological effects via its downstream target Akt. D3-phosphoinositides (the biologically active products of PI3K) bind to the N-terminal plextrin homology domain (PH) of Akt to induce translocation of the kinase to the plasma membrane. Subsequently two kinases, PDK1 and PDK2 phosphorylate Akt in the activation loop at Thr^{308} and Ser^{473} to fully activate Akt (Choudhury and Abboud (2004)). Once phosphorylated Akt subsequently phosphorylates and activates a number of substrate targets within the cell.

This chapter investigates the possibility that the PI3K/ Akt pathway is involved in the induction of proteasome expression and activity of PIF in murine myotubes, through stimulation and increased activity and DNA binding of NF-κB.

Methodology

Tissue Culture

See section 2.6

Purification of PIF

See section 2.3.1

Measurement of proteasome 'chymotrypsin like' activity

See section 2.5.1.e

Western Blot Analysis

For a full protocol see section 2.3.3

The primary antibodies were used at a dilution of 1:1000 and the secondary antibodies were used at a dilution of 1:2000. Incubation was carried out for 2h at room temperature, and development was enhanced by ECL (Amersham, UK). Total cellular actin was used as a loading control. Blots were scanned by a densitometer to quantitate differences.

Electrophoresis mobility shift assay (EMSA)

DNA binding proteins extracted from myotubes according to the method of Andrews and Faller (1991), which utilises hypotonic lysis followed by high salt extraction of nuclei. The EMSA binding assay was carried out using a panomics EMSA 'gel-shift' kit according to the manufacturers instructions (see section 2.8).

Statistical analysis

Differences in means between groups was determined by one-way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Results

The effect of LY294002 (100μM) on the induction of 'chymotrypsin-like' enzyme activity by 15(*S*)-HETE (24h)

15(S)-HETE production has been suggested to be an important event in the induction of proteasome-expression by PIF (Whitehouse *et al* (2003)). Therefore the effect of 2-(4-morpholinyl)-8-phenylchromone (LY294002), a PI3K inhibitor (Baumann and West (1998) on the 15(S)-HETE induced increase in 'chymotrypsin-like' enzyme activity was assessed.

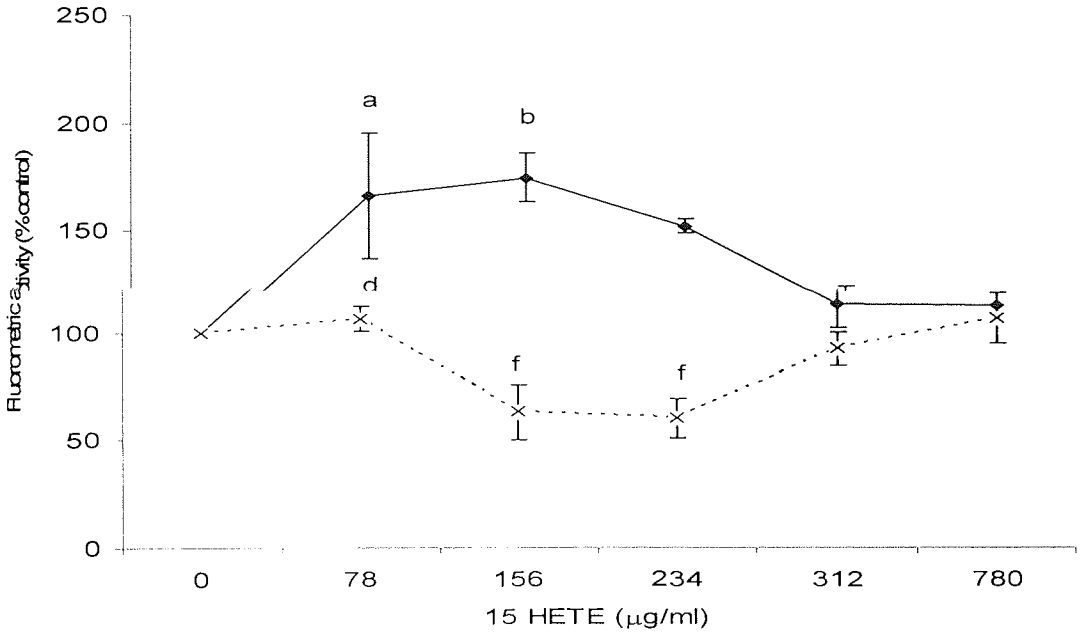


Figure 8. 1a

Murine myoblasts were incubated in the absence (X) or presence (◆) of LY294002 2h prior to the addition of 15(*S*)-HETE (24h). Results are expressed as mean ± S.E.M and statistical analysis was determined by one-way ANOVA followed by Tukey's test. This data is representative of three separate experiments. Differences from 0nM 15(S)-HETE are indicated a, $p < 0.05$ and b, $p < 0.01$, whilst differences between groups in the presence of LY294002 (100μM) are d, $p < 0.05$ and f, $p < 0.001$.

Figure 8. 1a

15(*S*)-HETE induced a significant increase in 'chymotrypsin-like' enzyme activity in murine myotubes, as previously reported by Whitehouse *et al* (2003). This effect was completely attenuated by pre-treatement with the PI3K inhibitor LY294002 (100μM), suggesting a role for PI3-K in proteasome activation.

Western blot analysis of 20S proteasome α subunit expression in response to 15(*S*)-HETE (24h) in the presence or absence of LY294002 (100μM)

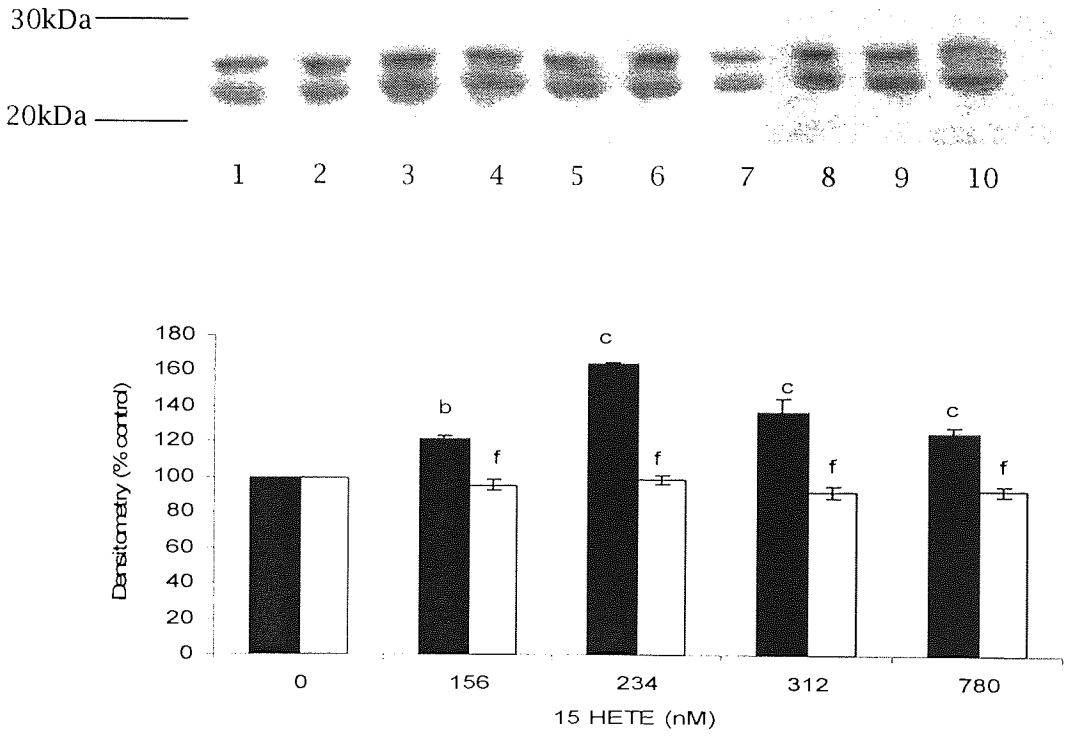


Figure 8. 1b

Murine myotubes were treated with 0 (lanes 1 and 6), 156 (lanes 2 and 7), 234 (lanes 3 and 8), 312 (lanes 4 and 9) or 780nM 15(*S*)-HETE (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) of 100μM LY294002. Densitometric analysis is an average of three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukeys test. Differences from 0nM 15(S)-HETE are indicated as b, $p < 0.01$ and c, $p < 0.001$ while differences between groups, in the presence of LY294002 are indicated as f, $p < 0.001$.

Western Blot analysis of $E2_{14K}$ expression in response to 15(S)-HETE in the presence or absence of LY294002 (100μM)

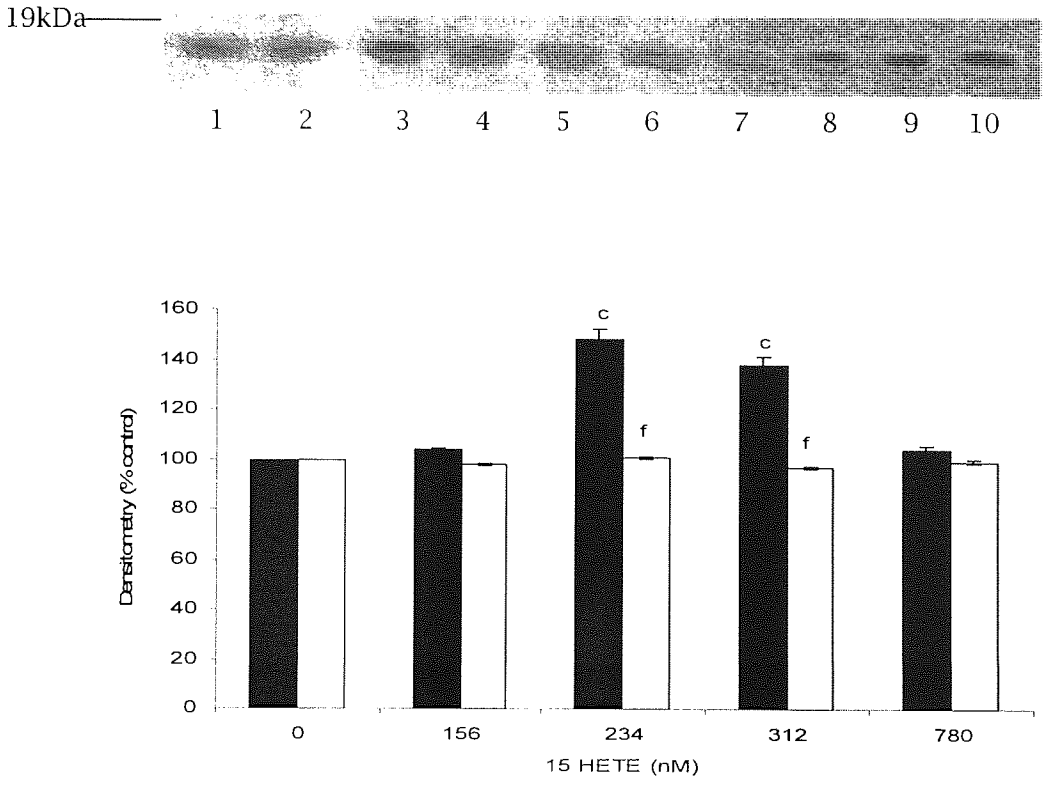


Figure 8. 1c

Murine myotubes were treated with 0 (lanes 1 and 6), 156 (lanes 2 and 7), 234 (lanes 3 and 8), 312 (lanes 4 and 9) or 780nM 15(*S*)-HETE (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) of 100μM LY294002. Densitometric analysis representative of three replicate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM 15(S)-HETE are indicated as b, $p < 0.01$ and c, $p < 0.001$ while differences between groups, in the presence of LY294002 are indicated as f, $p < 0.001$.

Actin loading control

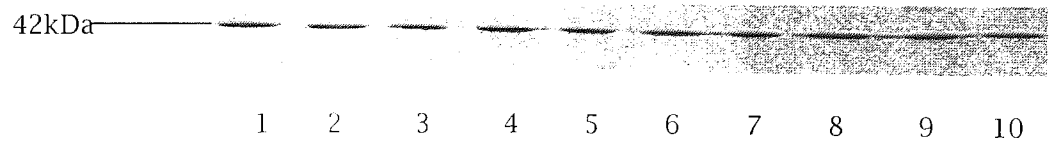


Figure 8. 1d

Lane discipline as figures 8. 1b and 8. 1c.

Figure 8. 1b – 8. 1d

Western blot analysis of the 20S proteasome α subunit (figure 8. 1b) and $E2_{14K}$ expression (figure 8. 1c) corresponds with the 15(*S*)-HETE induced increase in 'chymotrypsin-like' activity of the proteasome in murine myotubes as seen in figure 8. 1a. This effect was completely attenuated by pre-treatment with the PI3K inhibitor LY294002 (100μM) 2h prior to the addition of 15(*S*)-HETE, as was 20S proteasome α subunit and $E2_{14K}$ expression.

These results suggest activation of PI3K occurs after the formation of 15(*S*)-HETE. PIF induces the formation of 15(*S*)-HETE, causing membrane phospholipids to release arachidonic acid, of which the metabolite 15(*S*)-HETE, stimulates muscle protein degradation *in vitro* (Smith et al (1999)) and up-regulate expression of components within the ATP-ubiquitin dependent proteolytic pathway (Whitehouse and Tisdale (2003)).

Western blot analysis of the effect of PIF (30 min) on phosphorylation of Akt in the presence or absence of LY294002 (100μM) in C_2C_{12} myotubes

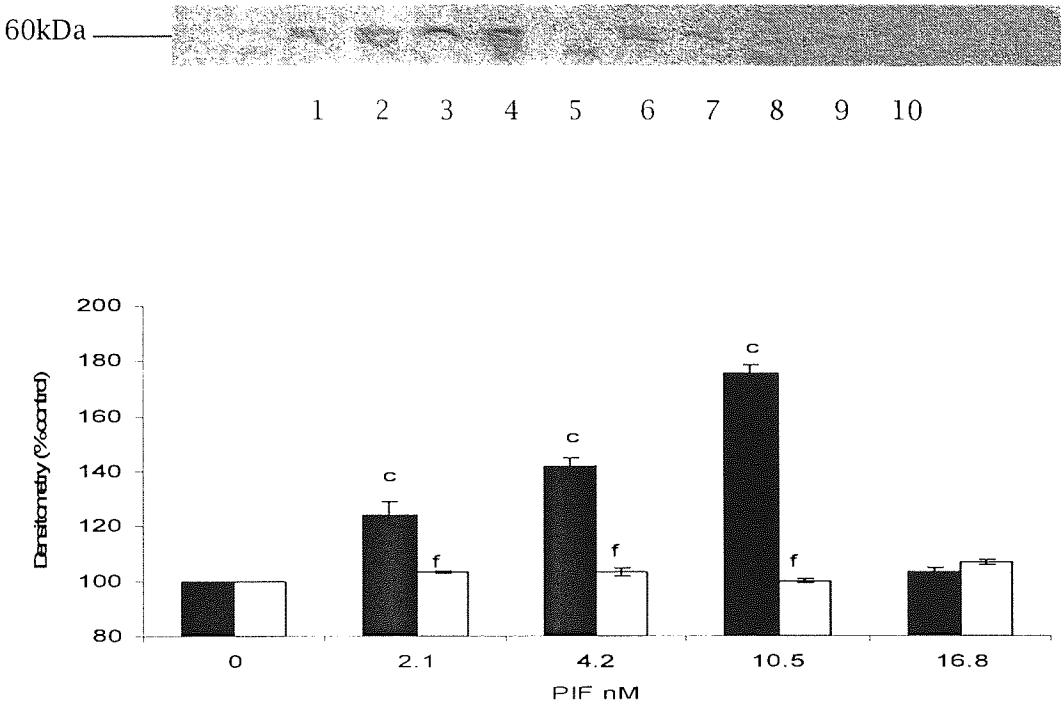


Figure 8. 2a

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) for 30 min in the absence (lanes 1-5, ■) or presence of 100μM LY294002 (lanes 6-10, □) added 2h prior to the addition of PIF. Densitometri analysis is based on three replicate blots and the results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as c, $p < 0.001$, while differences between groups in the presence of LY294002 are represented by f, $p < 0.001$.

Western blot analysis of total Akt in response to PIF (30 min) in the presence or absence of LY294002 (100μM) in C_2C_{12} myotubes

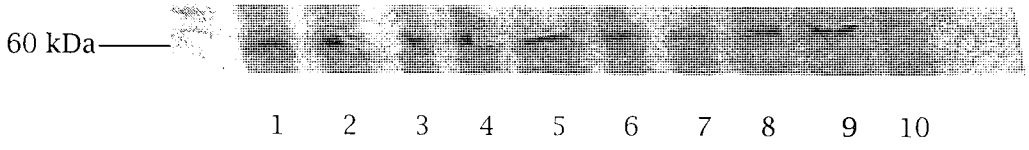


Figure 8. 2b
Myotubes were treated in the same manner as figure 8. 2a and lane discipline remains the same. There was no significant difference in the level of total cellular Akt in response to PIF or the PI3K inhibitor LY294002 (100μM).

Figures 8. 2a and 8.2b
Activation of PI3K results in the phosphorylation and activation of Akt. The results presented in figures 8. 2a and 8. 2b demonstrate PIF increased Akt phosphorylation at Ser^{473} within 30min of addition. This was accompanied by no alteration or effect on total Akt within the cell. The effect of PIF on Akt phosphorylation was completely attenuated by LY294002, confirming this increase occurred through the PI3K pathway.

To investigate the possibility that activated Akt was responsible for the phosphorylation and consequent degradation of IκBα, the effect of LY294002 on this process was determined.

Western blot analysis of PIF-induced increase in IκBα phosphorylation in murine myotubes in the presence or absence of LY294002 (100μM) (30 min)

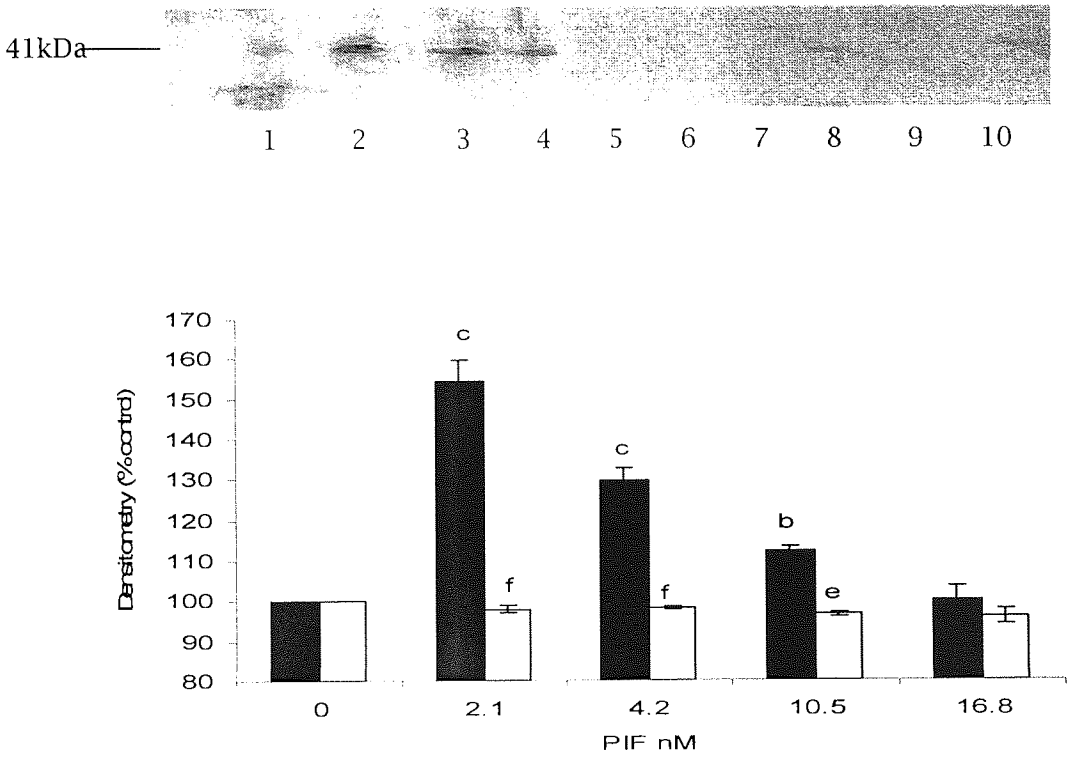


Figure 8. 3a

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the presence (lanes 6-10,□) of LY294002 or absence (lanes 1-5, ■). Densitometric analysis is based on three separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences between groups in the presence of LY294002 are represented by e, $p < 0.01$ and f, $p < 0.001$.

Western blot analysis of PIF-induced decrease in IκBα in murine myotubes in the presence or absence of LY294002 (100μM) (30 min)

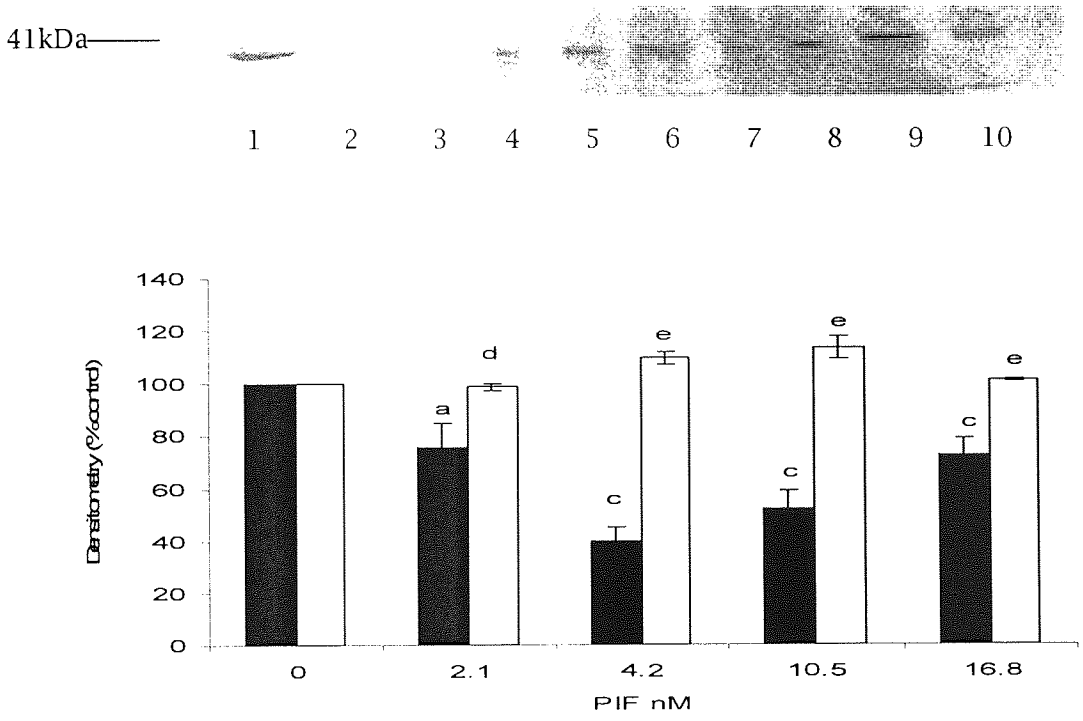


Figure 8. 3b

Myotubes were treated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the presence (lanes 6-10,□) of LY294002 or in the absence (lanes 1-5, ■). Densitometric analysis is based on three separate blots and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as a, $p < 0.05$ and c, $p < 0.001$, while differences between groups in the presence of LY294002 are d, $p < 0.05$ and e, $p < 0.01$.

Actin loading control

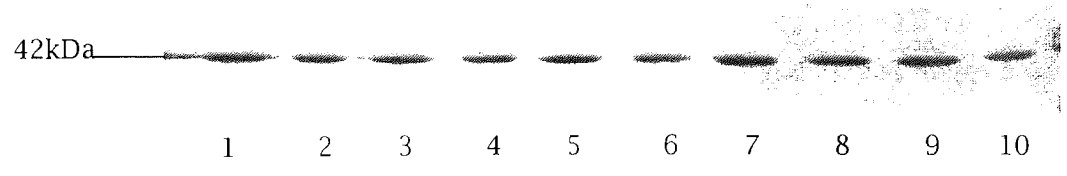


Figure 8. 3c

Lane discipline as figures 8. 3a and 8. 3b.

Figures 8. 3a - 8. 3c

PIF induced an increase in phosphorylation of IκBα (figure 8. 3a) and a reciprocal decrease was seen in cytosolic levels of IκBα in murine myotubes (figure 8. 3b). This effect was completely attenuated by the PI3K inhibitor LY294002 (100μM). These results suggest that activation of Akt by PIF results in the phosphorylation and subsequent degradation of IκBα.

To investigate if 15(*S*)-HETE exerts the same effect, murine myotubes were treated with 15(*S*)-HETE for 30min in the presence or absence of LY2944002 (100μM) added 2h prior to the addition of 15(*S*)-HETE.

Western blot analysis of cytosolic IκBα in murine myotubes in response to 15(S)-HETE in the presence or absence of LY294002 (100μM)

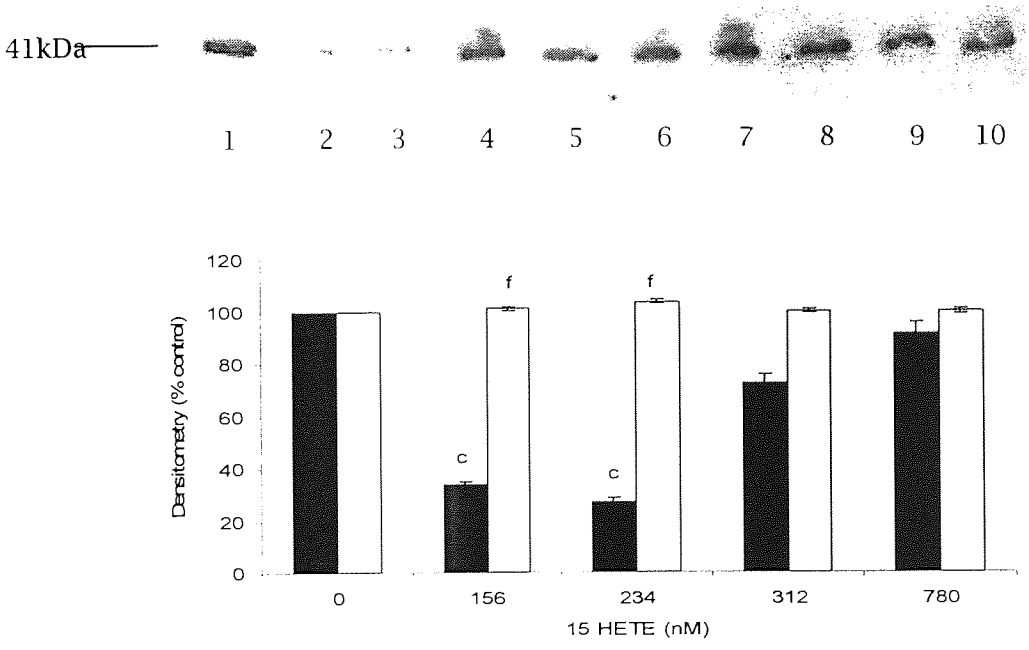


Figure 8. 4a

Myotubes were treated with 0 (lanes 1 and 6), 156 (lanes 2 and 7), 234 (lanes 3 and 8), 312 (lanes 4 and 9) or 780nM 15(*S*)-HETE (lanes 5 and 10) in the presence (lanes 6-10, □) of LY294002 or absence (lanes 1-5, ■). Densitometry is based on three separate experiments and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukeys test. Differences from 0nM 15(*S*)-HETE are indicated as c, $p <$

0.001, while differences between groups in the presence of LY294002 are represented by f, $p < 0.001$.

Actin loading control

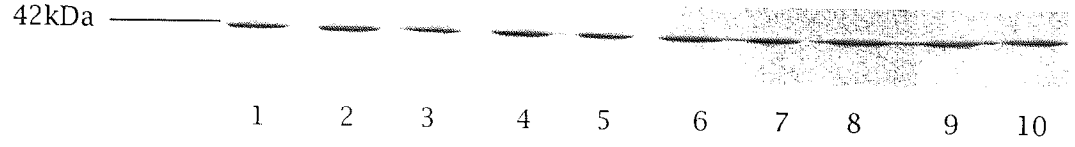


Figure 8. 4b
Lanes discipline as figure 8. 4a

Figure 8. 4a

15(*S*)-HETE successfully induced a decrease in cytosolic IκBα in murine myotubes, as previously reported by Whitehouse and Tisdale (2003). This was attenuated by pre-treatment for 2h prior to the addition of 15(*S*)-HETE with the PI3K inhibitor LY294002 (100μM). These results mirrored what was observed in figures 8. 3a-8. 3c, whereby PIF induced a decrease in cytosolic IκBα and increased phosphorylation of IκBα. This suggests that PIF exerts an effect through the PI3K signalling pathway and subsequently activates the transcription factor NF-κB.

To substantiate this theory, nuclear accumulation of NF-κB in response to PIF and the inhibitor LY294002 was assessed in murine myotubes.

DNA binding of NF-κB in response to PIF (30 min) in the presence or absence of LY294002 (100μM).

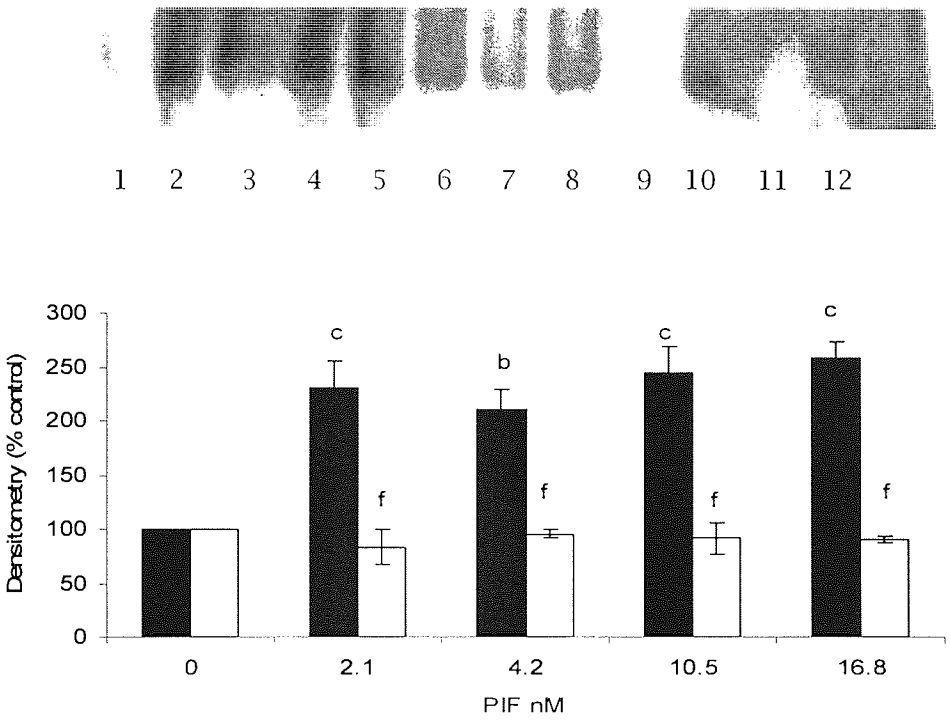


Figure 8. 5

Effect of PIF on nuclear translocation of NF-κB in murine myotubes as determined by EMSA, in the absence (lanes 1-5, ■) or presence (lanes 6-10, □) of 100μM LY294002. Myotubes were treated for 30min with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7) 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) and 16.8nM PIF (lanes 5 and 10). Lane 11 is a positive control for NF-κB in the presence of a 100 fold excess of unlabelled NF-κB probe while lane 12 is a positive control for NF-κB. Densitometric analysis is based on three replicate EMSAs and results are expressed as mean ± S.E.M. Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$, while differences between groups in the presence of LY294002 are represented by f, $p < 0.001$.

Figure 8. 5

PIF was found to induce degradation of IκBα (figure 8. 4b) and increase phosphorylation of IκBα (figure 8. 3a). These results were corroborated by EMSA analysis of nuclear accumulation of NF-κB in response to PIF. However, this effect was attenuated with the PI3K inhibitor LY294002 at 100μM. This data suggests that activation of PI3K by PIF results in activation of NF-κB.

Discussion

This chapter suggests the involvement of Akt in PIF-induced proteasome expression and activity in murine myotubes, through phosphorylation and subsequent degradation of IκBα. This leads to increased nuclear accumulation of NF-κB, which has been suggested to be a transcription factor involved in proteasome expression (Whitehouse and Tisdale (2003)). Activation of Akt appears to occur through the PI3K signalling pathway; West *et al* (2002) reported that both wortmannin and LY294002 inhibit PI3K activity by targeting the ATP binding domain of the p110 subunit. Dardevet *et al* (1996) demonstrated wortmannin and LY294002 to regulate muscle protein metabolism in epitrochloearis muscle *in vitro* in response to insulin or IGF-1, suggesting a role for PI3K in the regulation of muscle protein metabolism.

Class-1 PI3Ks are heterodimers composed of a catalytic subunit (p110) and an adaptor/ regulator subunit (p85). This class-1 is further divided into subclass 1A, which is activated by receptors with protein tyrosine kinase activity and the subclass 1B which is activated by receptors coupled with G-proteins. The substrate for class 1 PI3ks is phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to generate the second messenger phosphadtylinositol-3,4,5-triphosphate (PI-3,4,5-P_3) (Vara *et al* (2004)). The composition of this signalling pathway may provide a mechanism that PIF can activate PI3K, as Smith and Tisdale (2003) found the tyrosine kinase inhibitors genistein and tyrophostin A23 to attenuate PIF-induced proteasome expression.

The data presented in this chapter suggests that Akt is involved in a catabolic pathway in skeletal muscle; other data suggests that Akt is important in tumourigenesis and resistance to chemotherapeutic drugs (Neri *et al* (2002)). Akt-1 activity is required for insulin-like growth factor (IGF)-1 mediated hypertrophy by phosphorylation and activation of the mammalian target of rampamycin (mTOR) (Dardevet *et al* (1996)). Vara *et al* (2004) described how transforming effects of PI3K/ Akt promoting cell cycle and growth are mediated by mTOR, stimulating the 70kDa ribosomal protein S6 kinase (mTOR/$p70^{S6K}$) that is involved in protein synthesis (Vara *et al* (2004)).

Therefore it appears the action of Akt depends on environmental conditions and other signalling molecules within the muscle cell, which may influence the targets for phosphorylation within the cell. 15(*S*)-HETE appears to be involved in Akt activation in response to PIF, this lipoxygenase metabolite has been shown to activate PKCα over the same concentrations that induce proteasome expression (see chapter 4).

Activation of Akt may provide an alternative pathway from PKC for the phosphorylation and degradation of IκBα and the nuclear accumulation of NF-κB. Alternatively there may be some cross talk between the pathways, Hassan *et al* (2004) determined neurotensin (NT) to stimulate mitogenesis in PC3 cells by a PKC-dependent ligand mediated tranactivity of EGFR, leading to stimulation of the Raf-MEK-ERK pathway in a PI3K dependent manner and Burchfield *et al* (2004) demonstrated cross talk between PKC and Akt in response to insulin *in vivo* and *in vitro.*

Phosphorylation of substrates within muscle cells by PKC or Akt may influence the overall outcome, as it is evident that stimulation by the same pathway may lead to different outcomes. Canico *et al* (2001) reported a myogenic signalling cascade initiated by IGF-II that leads to biochemical and morphological skeletal muscle cell differentiation involving PI3K and NF-κB activation. PI3K was shown to activate IKKα, IKKβ and NF-κB inducing kinase (NIK) by IGF-II in the IGF-II-dependent myoblast differentiation process (Canico *et al* (2001)). Hu *et al* (2003) compared ANBL6 cells stably transfected with mutant K or N-ras genes, with wild type ras-expressing control cells. Both ras-containing myeloma cell lines demonstrated constitutive activation of ERK, PI3K/ Akt and NF-κB. LY294002 significantly curtailed the growth of mutant ras-containing cells. Park *et al* (2002) investigated the effect of LY294002 pre-treatment of RAW264.7 in response to IFN-γ and LPS and found the PI3K inhibitor to attenuate degradation of IκBα and production of TNF-α and IL-6.

Meng *et al* (2002) suggested NF-κB is required for TNF-α mediated Akt activation, which lies upstream from the activation of Akt. Treatment of NIH3T3 cells with TNF-α and LPS resulted in the stimulation of Akt phosphorylation, however, the activation of Akt was only detected after IκBα degradation was induced, suggesting DNA binding of NF-κB precedes Akt phosphorylation.

The results presented in this chapter confirm the importance of NF-κB activation in the induction of proteasome gene expression induced by PIF in murine myotubes. Genes encoding proteasome subunits contain sequences for the transcription factors NF-κB and activator protein-1 (AP-1). AP-1 consists of subunits of the jun and fos family that can activate and repress gene transcription. It is possible that gene expression is normally repressed by AP-1 (Thompson and Palmer (1998)) Therefore the balance between activation of NF-κB and repression of AP-1 may govern the overall expression of the ubiquitin-proteasome proteolytic pathway.

Chapter 9

Characterisation of the PIF Receptor

Introduction

Cancer patients often experience weight loss and severe depletion of lean body mass and adipose tissue. Cancer associated weight loss and its associated metabolic abnormalities account for 25% of cancer deaths (Warren (1932)). This deleterious syndrome accompanying cancer is known as cancer cachexia, derived from the Greek *kakos* meaning bad and *hexis* meaning condition. Cancer cachexia is distinct from starvation, as there is significant emaciation of skeletal muscle while visceral protein and organs are unaffected (Burke *et al* (1980)). Cachectic cancer patients also experience a dramatic shift in metabolism and a rise in resting energy expenditure (Knoxx *et al* (1982)).

A number of factors, including cytokines have been implicated to have a role in the development of cancer cachexia including : prostaglandins (Ruff *et al* (1984) and Strelkov *et al* (1989)); glucocorticoids (Llovera *et al* (1996) and Marinovic (2002)); CNTF (Espat *et al* (1996) and Martin *et al* (1996)); TNF-α (Truyens *et al* (1995) and Tessitore *et al* (1993)), IL-1 (Nakatani et al (1998) and Barber *et al* (2000)); IL-6 Strassman *et al* (1992) and Ebisui *et al* (1995)). However none of the above mentioned factors are directly responsible for the development of cachexia. Current data suggests that cachexia can rarely be attributed to a single cytokine or factor, but rather a combination of components working together.

Todorov *et al* (1996) successfully isolated a circulatory catabolic factor from the MAC16 murine adenocarcinoma. This material was found to be a sulphated glycoprotein with relative molecular mass of 24kDa and was also isolated from the urine of cachectic cancer patients, but absent from normal subjects. This suggested that cachexia in mice and humans may be induced by the same material (Todorov *et al* (1996)). Administration of this material, purified from cachectic cancer patient urine, to mice caused a significant reduction in body weight when compared to controls. Weight loss occurred without a reduction in food or water intake, characterised by decreased protein synthesis and increased protein degradation (Cariuk *et al* (1997)).

Lorite *et al* (1998) determined this Proteolysis-Inducing Factor (PIF) to increase protein degradation through activation of the ATP-ubiquitin dependent proteolytic pathway.

Administration of PIF *in vivo* to non tumour bearing mice caused significant decrease in weight of the spleen, soleus and gastrocnemius muscle, with no effect on the heart or kidney and an increased weight of the liver, which is similar to the effects seen in cachectic cancer patients (Lorite *et al* (1998).

Lorite *et al* (2001) determined that intravenous administration of PIF to normal mice produced a rapid depletion in body weight, accompanied by increased mRNA levels for ubiquitin and the C9 proteasome subunit. This was accompanied by increased protein expression of the 20S catalytic core and 19S regulatory subunit of the proteasome. Gomes-Marcondes *et al* (2001) demonstrated that the C_2C_{12} *in vitro* model muscle system experienced an increased protein degradation in response to PIF, accompanied by increased expression of the α-type subunit of the proteasome and increased 'chymotrypsin-like' enzyme activity of the proteasome (Gomes-Marcondes *et al* (2001)).

Cabal-Manzano *et al* (2001) provided some evidence to suggest that tumours are the source of PIF in gastrointestinal cancer patients. PIF was found to be expressed in tumours and mesenchymal tissue of patients who tested positive for PIF in their urine.

Todorov *et al* (1997) conducted functional and immunological studies and found biological activity of PIF to be mediated through N- and O- linked oligosaccharide chains. Both human and mouse PIF appear to contain identical carbohydrate components, due to their reactivity with a murine monoclonal antibody directed towards the oligosaccharide residues (Todorov *et al* (1996)). Amino acid sequence analysis of the N- terminal residues demonstrated homology between species (YDPEAASAPGSGNPSHEA(S) (Todorov *et al* (1996) and Cariuk *et al* (1997)) which suggests protein degradation in cancer cachexia may be identical in mouse and man.

In order for PIF to exert a catabolic effect on skeletal muscle, there must be a specific cell surface receptor, capable of relaying the biological response to instigate the activation of PLA_2 (Smith and Tisdale (2003)). Activation of PLA_2 results in the mobilisation of arachidonic acid from membrane phospholipids and subsequent metabolism to 15(*S*)-HETE, which has been shown to be directly related to the induction of protein catabolism (Smith *et al* (1999)). Whitehouse *et al* (2003) demonstrated 15(*S*)-HETE to increase expression of ATP-ubiquitin dependent proteolytic pathway components. 15(*S*)-HETE was found to mediate this action through the activation of NF-κB (Whitehouse *et al* (2003)).

This chapter aims to characterise the PIF receptor on muscle cells. Previous studies (Todorov and Tisdale, unpublished data) had identified specific PIF binding to membranes

and muscle cells, and a partial N- terminal sequence of the putative receptor had been obtained. This N- terminal amino acid sequence was n- DINGGGATLPQPLYQTAAVLTAGFA. This sequence has homology to a peptide fragment from synovial fluid protein p205 (Hain *et al* (1996)). Further analysis has yielded several internal amino acid peptide fragments that do not share sequence homology with other proteins in public databases. Therefore the PIF receptor represents a novel protein, most likely a combination of these novel internal peptides.

Todorov and Tisdale (unpublished data) provided evidence for specific, high affinity binding sites for PIF in muscle cells. High concentrations of the monoclonal antibody used in the purification of PIF were able to displace PIF from the membrane receptor and explain why high concentrations of the antibody were required to neutralise the biological effect of PIF (Todorov *et al* (1997)). As with binding of PIF to the antibody, binding to the receptor is probably mediated through the sulphated oligosacharide chains, since binding was specifically inhibited by chondroitin sulfate, but not the related proteoglycans dermatan and heparan sulfate (Cariuk *et al* (1997) and Todorov *et al* (1996)).

An antibody has been produced commercially (Severn Biotech) raised in rabbits immunised with a peptide containing the partial N-terminal sequence of PIF : ASPARTIC ACID, ISOLEUCINE, ASPARAGINE, GLYCINE, GLYCINE, GLYCINE, ALANINE, THREONINE, LEUCINE, PROLINE, GLUTAMINE, LYSINE, LEUCINE, TYROSINE, LEUCINE, ISOLEUCINE, PROLINE, ASPARAGINE and VALINE.

This antibody is to be tested *in vitro* and if successful *in vivo,* to assess any functional ability in inhibiting the PIF-induced increase in skeletal muscle degradation and upregulation of ATP-ubiquitin dependent proteolysis. This chapter aims to determine if the PIF receptor antibody interferes with PIF binding enough to disrupt signal transduction and skeletal muscle catabolism in response to PIF.

Methodology

PIF Receptor Polyclonal Antibody

A polyclonal antibody was raised commercially (Severn Biotech, Kidderminster, UK) by immunising rabbits with a peptide containing the partial N- terminal sequence of PIF : ASPARTIC ACID, ISOLEUCINE, ASPARAGINE, GLYCINE, GLYCINE, GLYCINE, ALANINE, THREONINE, LEUCINE, PROLINE, GLUTAMINE, LYSINE, LEUCINE, TYROSINE, LEUCINE, ISOLEUCINE, PROLINE, ASPARAGINE and VALINE.

Indirect ELISA

See secton 2.7

Isolation of PIF receptor from C_2C_{12} myotube membranes

See section 2.7.3

Protein A Antibody purification

See section 2.7.2

Tissue Culture

See section 2. 5. 1

'Chymotryspin-like activity of the proteasome

See section 2. 5. 1 e

Total protein degradation

See section 2. 5. 1d

Tyrosine Release Assay

See section 2. 7

Western Blotting

See section 2. 3. 3

Results

An ELISA is an enzyme-linked immunosorbent assay for the detection and quantitation of an antibody or antigen using a ligand (e.g. IgG) conjugated to an enzyme which changes the colour of a substrate (Roitt (1997).

Specific activity of the rabbit polyclonal PIF receptor antibody

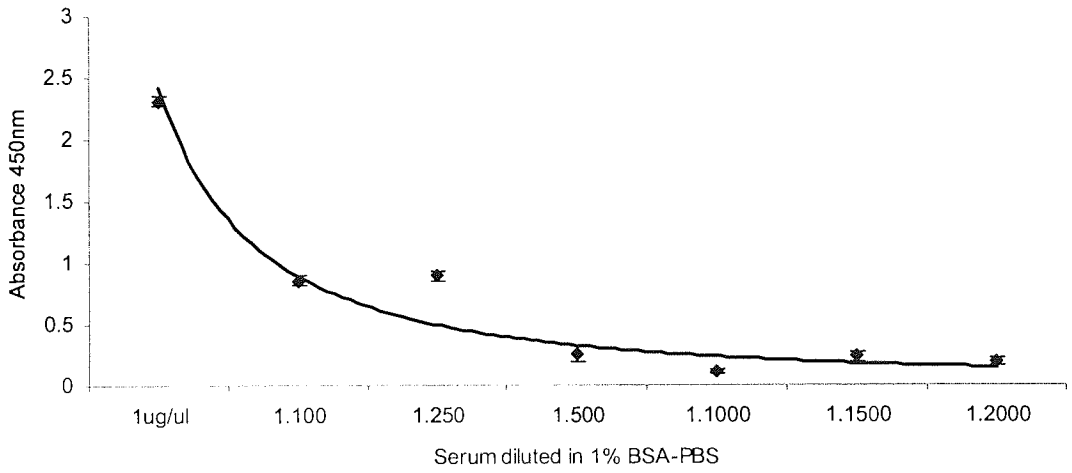


Figure 9. 1a

The ELISA of rabbit serum is represented by a line of best fit (——). The PIF receptor peptide antagonist was immobilised on the ELISA plate at a final concentration of 0.1µg/well.

Antibody Titre of Rabbit Serum

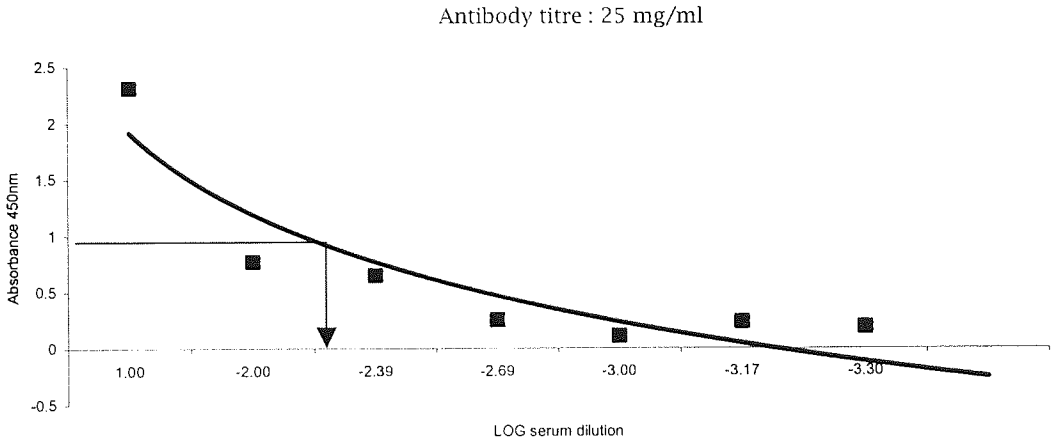


Figure 9. 1b

Titration of rabbit serum IgG specific to the synthetic PIF receptor antagonist was achieved by constructing a log graph, the line of best fit is shown as (——). Rabbit serum was found to have an antibody titre of 25mg/ml specific to the synthetic PIF receptor antagonist peptide.

To characterise the rabbit polyclonal sera, SDS-PAGE gel electrophoresis was conducted.

SDS PAGE gel electrophoresis of rabbit serum

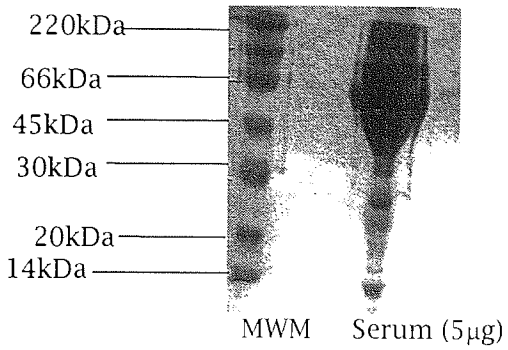


Figure 9. 1c

SDS gel electrophoresis revealed the main contaminant as albumin with the heavy and light chain of IgG barely visible. To further purify the IgG fraction containing the PIF receptor antibody a Protein A antibody purification system was employed. (Sigma, UK).

SDS-PAGE gel electrophoresis of protein A purified PIF receptor antibody

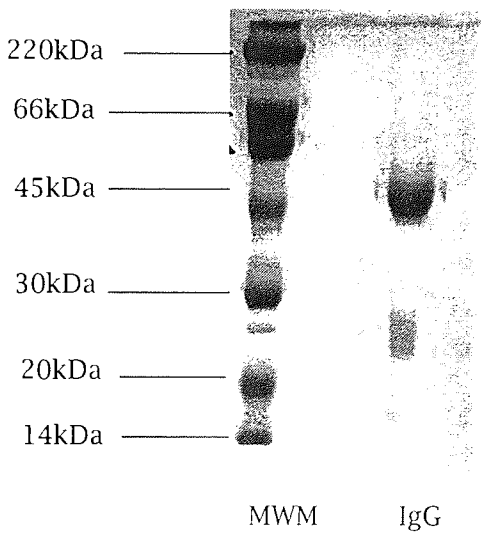


Figure 9. 2a

SDS-PAGE gel electrophoresis of the protein A purified rabbit serum indicates the major contaminating proteins including albumin have been removed, leaving IgG, visible with a heavy chain of 45kDa and a light chain of 22kDa.

Specific activity of protein A purified rabbit polyclonal PIF receptor antibody

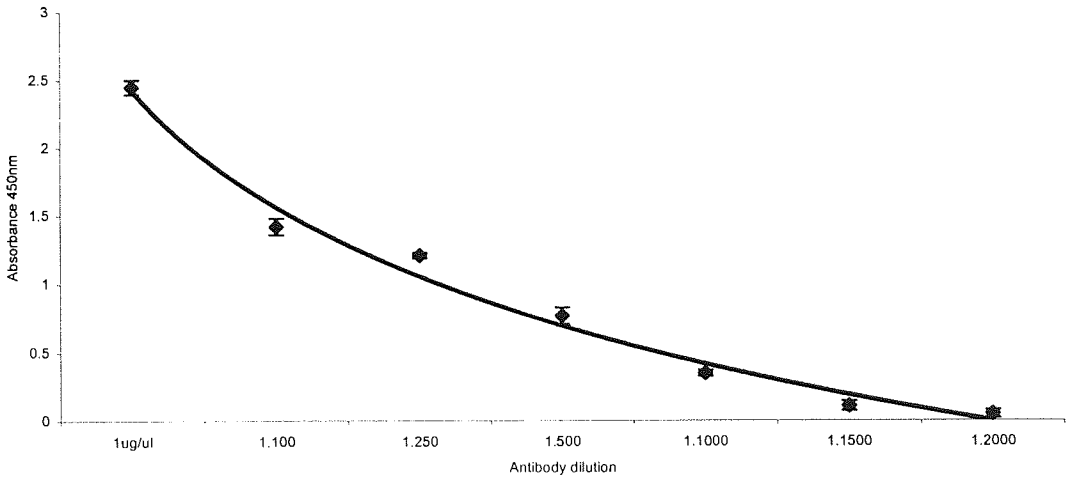


Figure 9. 2b

The ELISA of protein A purified polyclonal IgG is represented by a line of best fit demonstrated as (——). The PIF receptor peptide antagonist was immobilised on the ELISA plate at a final concentration of 0.1μg/well.

Antibody Titration of Protein A Purified rabbit IgG

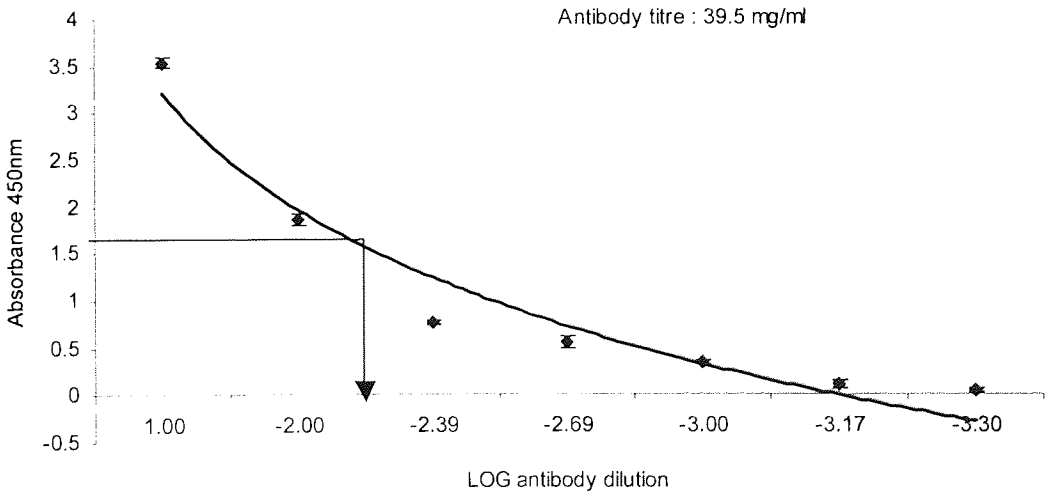


Figure 9. 2c

Titration of protein A purified polyclonal IgG specific to the synthetic PIF receptor antagonist was achieved by constructing a log graph, the line of best fit is shown as (——). Protein A purified rabbit IgG had an increased antibody titre of 39.5mg/ml specific to the synthetic PIF receptor antagonist peptide.

Figures 9. 1a - 9. 2c

The commercially produced rabbit polyclonal antibody raised against the partial N-terminal sequence was successful by indirect ELISA in recognising the antigen from rabbit serum (figure 9. 1a) with an antibody titre of 25mg/ml (figure 9. 1b). However, the major contaminant of serum was albumin (figure 9. 1c) and this was successfully removed with a protein A antibody purification system (Sigma, UK) (figure 9. 2a). This improved the specific activity of the antibody (figure 9. 2a) and antibody titre was increased by 58% (figure 9. 2c).

Isolation of the PIF receptor from C_2C_{12} myotube membranes

Membranes were prepared according to section 2. 7. 3

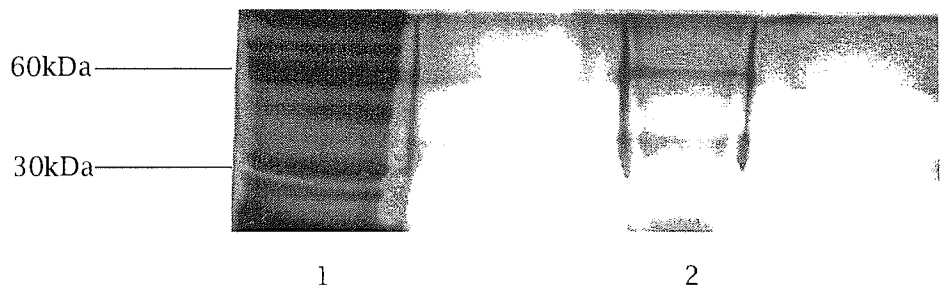


Figure 9. 3a

Silver stain of C_2C_{12} myotube membranes isolated using a WGA affinity column. Lane 1 is composed of molecular weight markers, whereas lane 2 consists of the prepared C_2C_{12} extract. Albumin is clearly visible at 60kDa and there is a double band visible between 30 and 40kDa

Western blot analysis of the C_2C_{12} extract using protein A purified rabbit serum at 1:1000

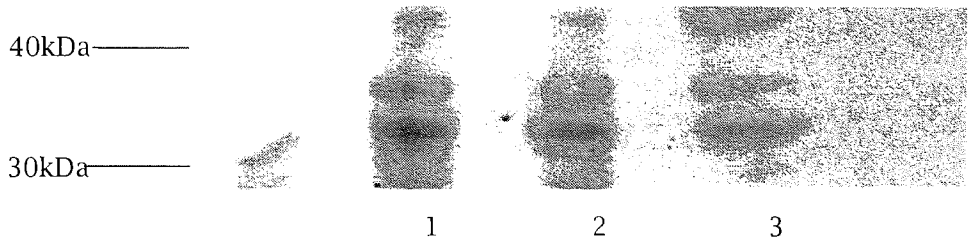


Figure 9. 3b

Western blot analysis of C_2C_{12} membrane expression of the PIF receptor using protein A purified rabbit polyclonal antibody serum at a dilution of 1:1000 at 37°C for 3h.

Figures 9. 3a and 9. 3b

Silver stain analysis yielded a double band visible between 30 and 40kDa (figure 9. 3a). Subsequent western blot analysis was successful in isolating a double band at the same molecular weight and suggests the PIF receptor is a dimer. The N terminal sequence peptide used as an antigen to raise the PIF receptor antibody was tested *in vitro* to establish its potential in antagonising the interaction of PIF with the PIF receptor.

'Chymotrypsin-like' activity of the proteasome in response to PIF (24h) and the PIF receptor peptide in C_2C_{12} myotubes

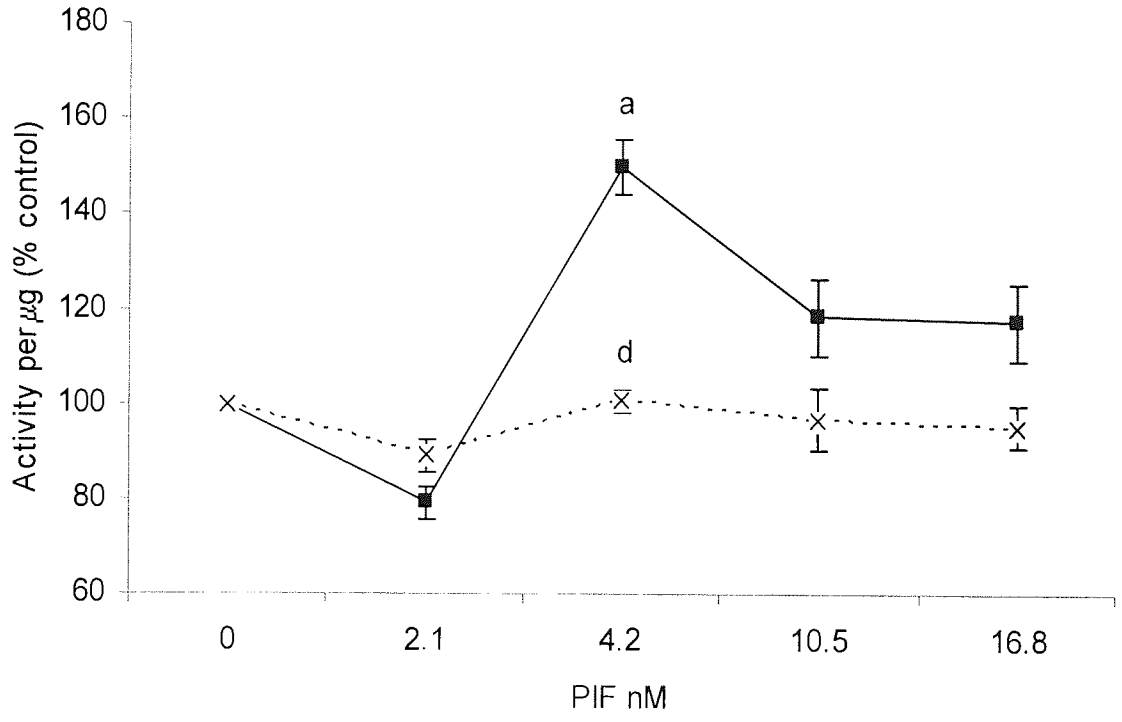


Figure 9. 4a

C_2C_{12} myotubes were pre-treated with 10μM receptor peptide 2h prior to the addition of PIF for 24h. C_2C_{12} myotubes and PIF alone are represented by ■ and in the presence of 10μM receptor peptide as X. The experiment was repeated 3 times where n=3. Results are expressed as mean ± S.E.M and statistical analysis between groups was determined by one-way ANOVA followed by Tukey's test. The difference from 0nM PIF is indicated as a, $p < 0.05$ while the difference between groups is indicated as d, $p < 0.05$.

Total protein degradation of C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of receptor peptide (10μM)

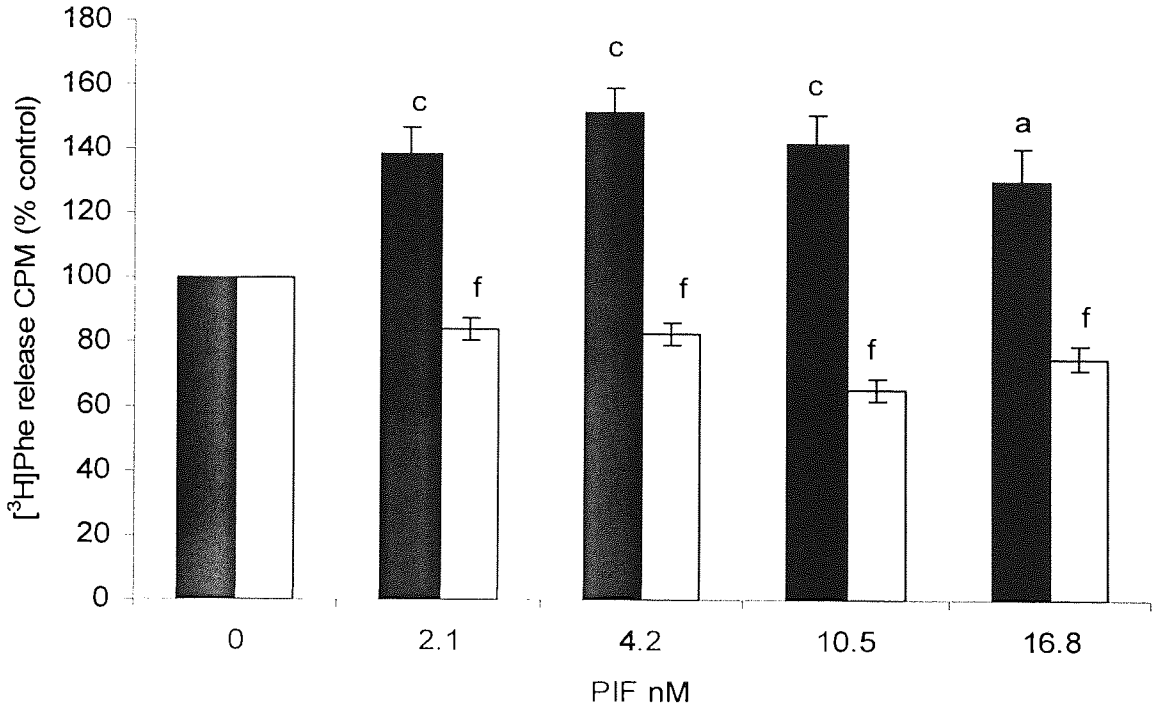


Figure 9. 4b

Myotubes were incubated with PIF alone (■) or in the presence of receptor peptide (10μM) (□) added 2h prior to the addition of PIF. Results are expressed as mean ± S.E.M and statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF in the absence of rececptor peptide are indicated as c, $p < 0.001$ while differences between groups are indicated as f, $p < 0.001$.

Figures 9. 4a and 9. 4b

Pre-treatment (2h) prior to the addition of PIF with the N-terminal peptide sequence was successful in attenuating PIF-induced increase in 'chymotryspin-like' activity of the proteasome (figure 9. 4a) and total protein degradation (figure 9. 4b)

Western blot analysis of 20S proteasome α subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence and presence of 10μM receptor peptide

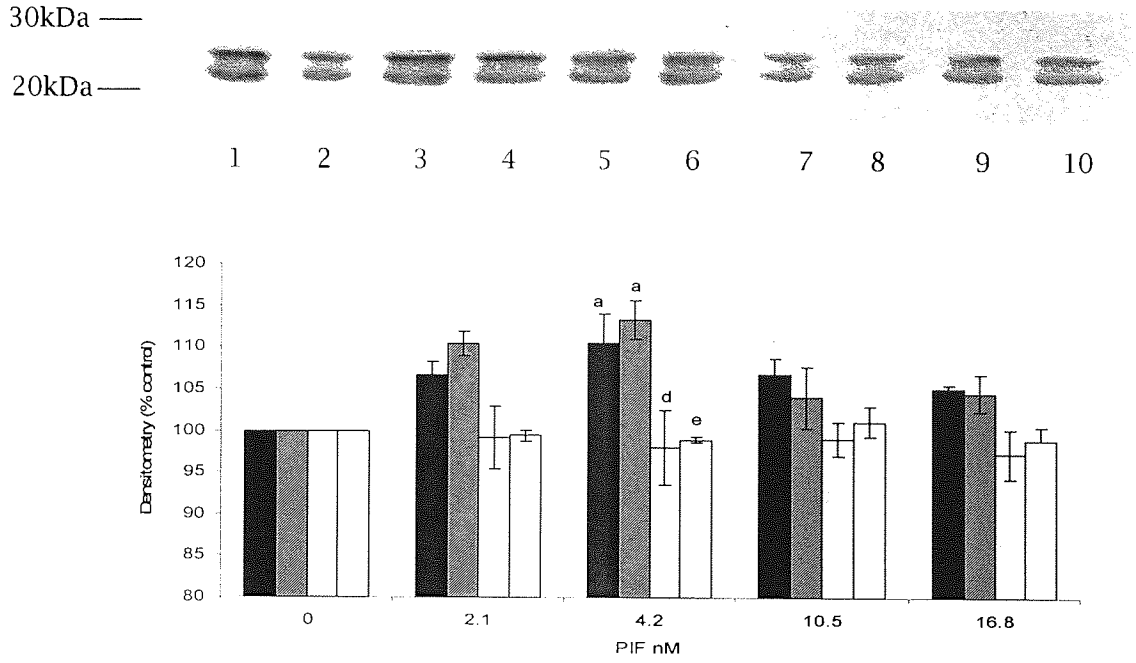


Figure 9. 5a

Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with 10μM receptor peptide, 2h prior to addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). A representative blot is shown, densitometric analysis is based on three separate blots. Values in the presence of PIF are indicated as (■) and (▨) whilst in the presence of PIF and 10μM receptor peptide as (⊡) and (□) Statistical analysis between groups was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as a, $p < 0.05$ whilst differences between groups are d, $p < 0.05$ and e, $p < 0.01$.

Actin loading control

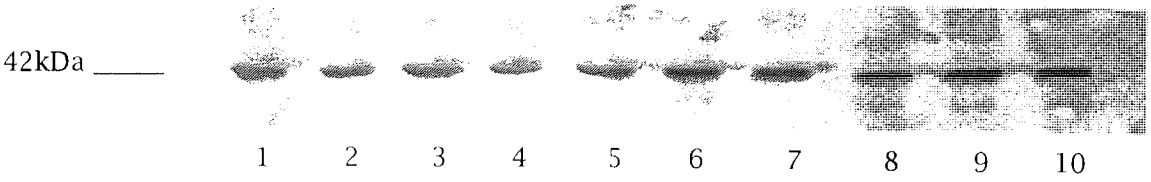


Figure 9. 5b

Lane discipline as figure 9. 5a

Western blot analysis of MSS1 proteasome subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence and presence of 10μM receptor peptide

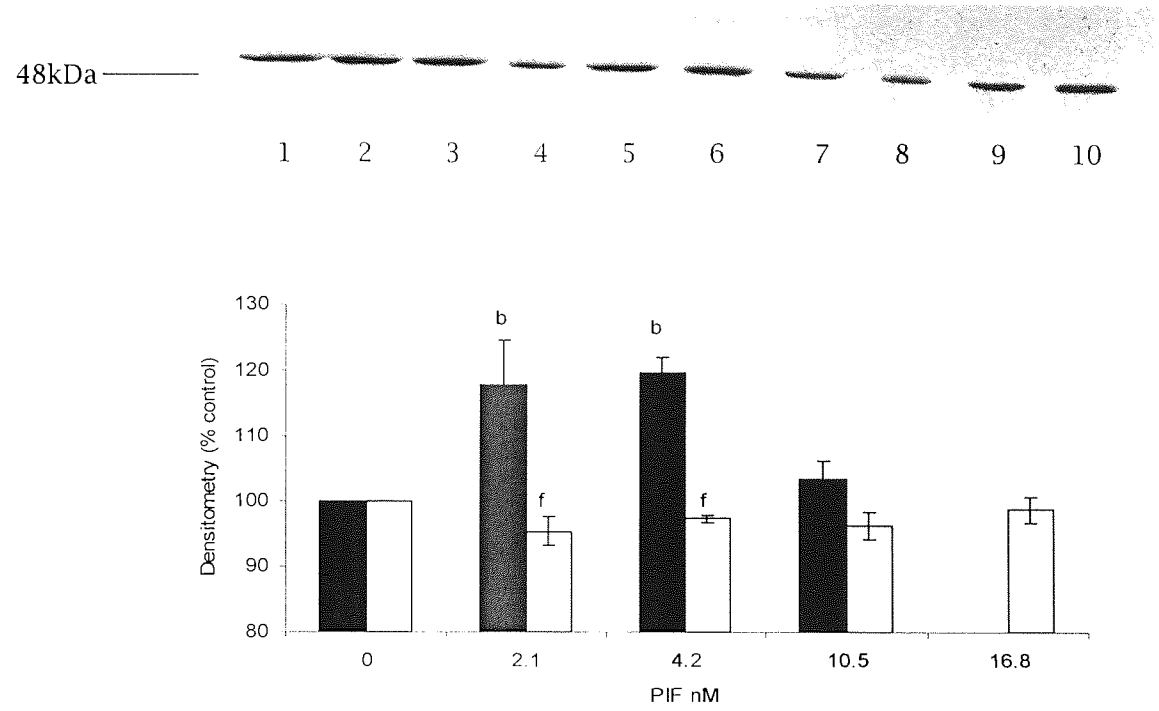


Figure 9. 5c

Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with 10μM receptor peptide, 2h prior to addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). A representative blot is shown, densitometric analysis is based on three separate blots. Values in the presence of PIF are indicated as (■) whilst in the presence of PIF and 10μM receptor peptide as (□). Statistical differences between groups was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ whilst differences between groups are f, $p < 0.001$.

Actin loading control

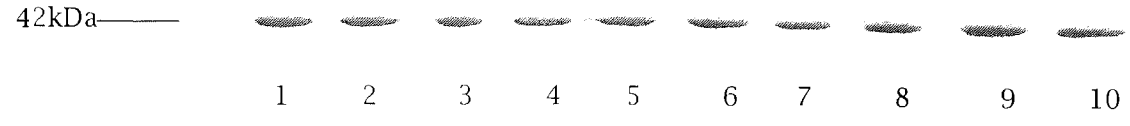


Figure 9. 5d

Lane discipline as figure 9. 5c

Western blot analysis of p42 proteasome subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence and presence of 10μM receptor peptide

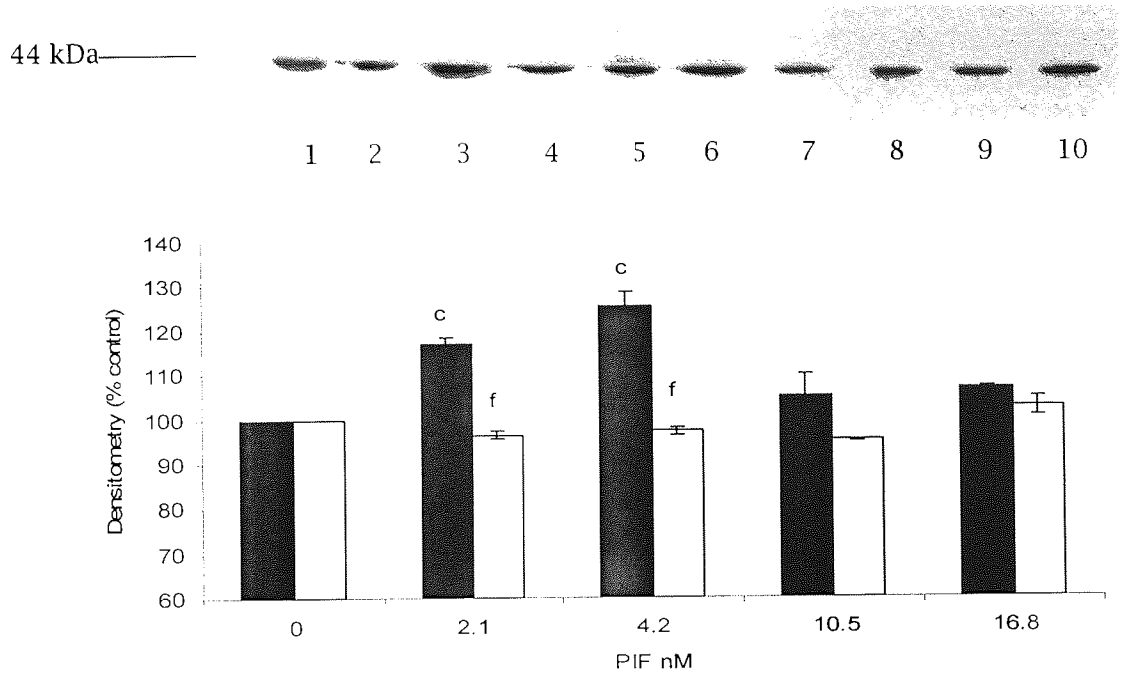


Figure 9. 5e

Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with 10μM receptor peptide, 2h prior to addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). A representative blot is shown, densitometric analysis is based on three separate blots. Values in the presence of PIF are indicated as (■) whilst in the presence of PIF and 10μM receptor peptide as (□). Statistical analysis was determined by one-way ANOVA followed by Tukey's post test. Differences from 0nM PIF are indicated as c, $p < 0.001$ whilst differences between groups are f, $p < 0.001$.

Actin control loading blot

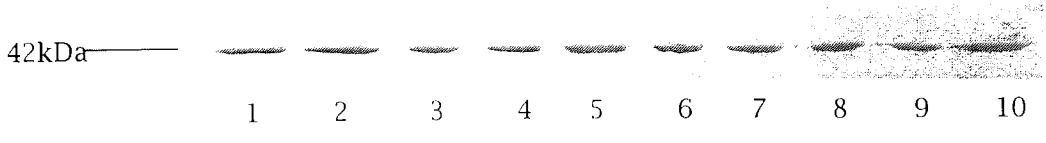


Figure 9. 5f

Lane discipline as figure 9. 5e

Western blot analysis of $E2_{14K}$ expression in C_2C_{12} myotubes in response to PIF (24h) in the absence and presence of 10μM receptor peptide

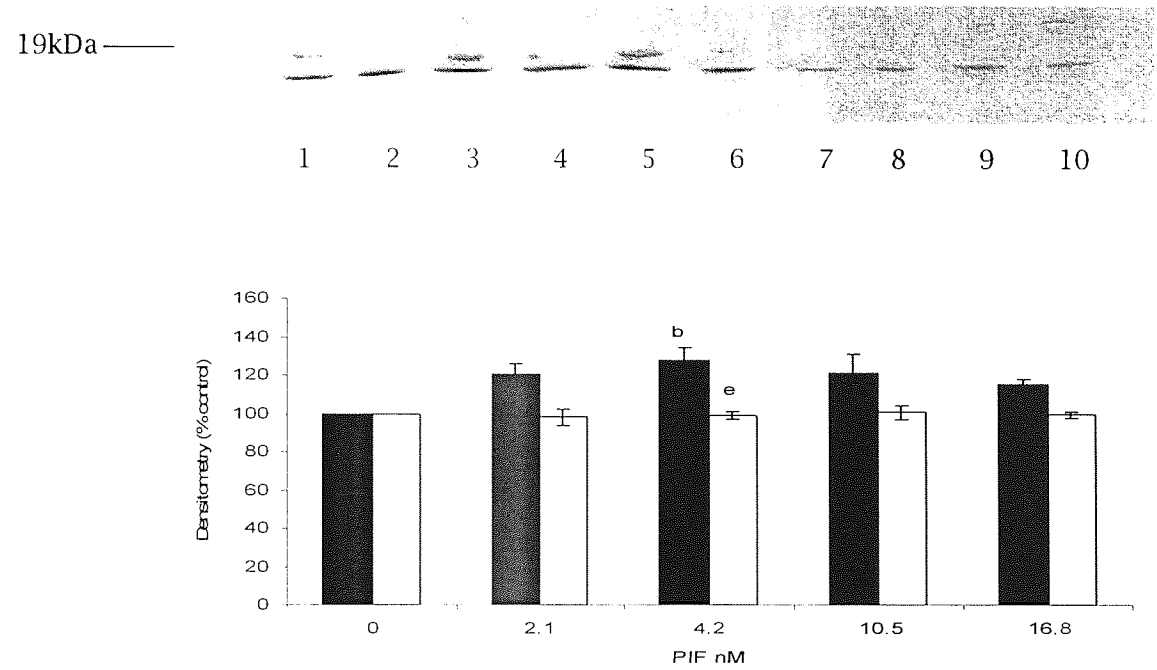


Figure 9. 5g

Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with 10μM receptor peptide, 2h prior to addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). A representative blot is shown, densitometric analysis is based on three separate blots. Values in the presence of PIF are indicated as (■) whilst in the presence of PIF and 10μM receptor peptide as (□). Statistical analysis was determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ whilst differences between groups as e, $p < 0.01$.

Actin loading control

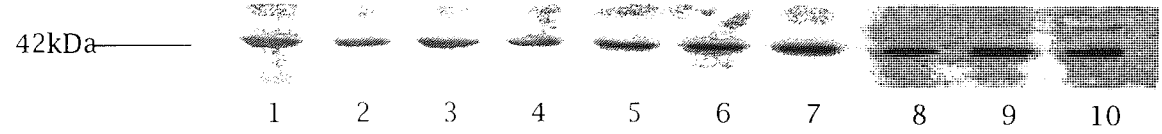


Figure 9. 5h

Lane discipline as figure 9. 5g

Western blot analysis of Myosin expression in C_2C_{12} myotubes in response to PIF (24h) in the absence and presence of 10μM receptor peptide

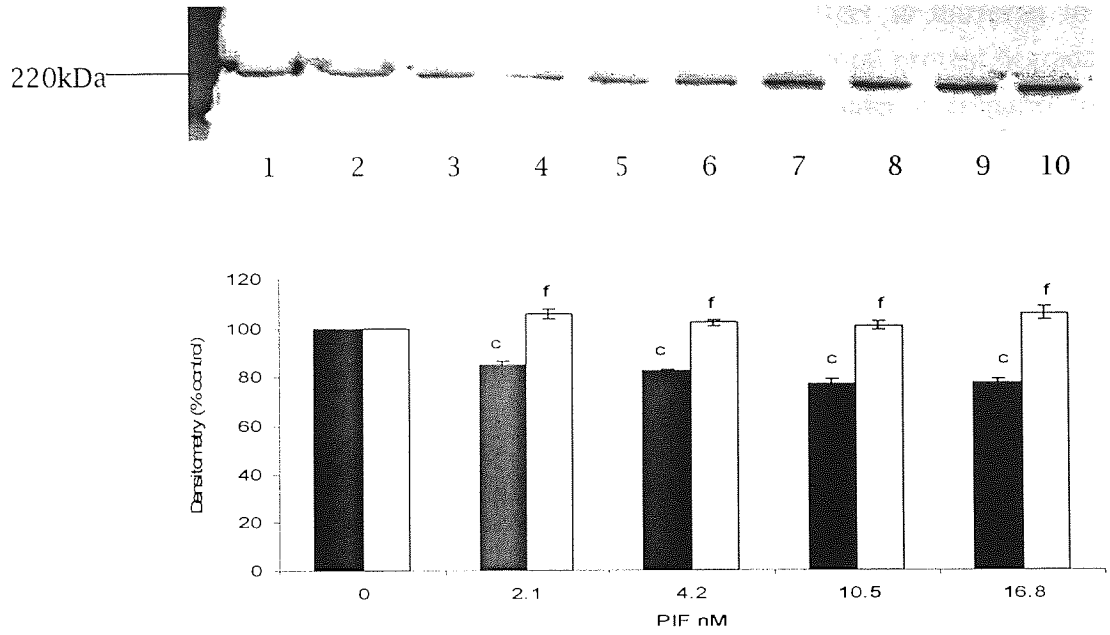


Figure 9. 5i

Myotubes were incubated with PIF alone (lanes 1-5) or pre-treated with 10μM receptor peptide, 2h prior to addition of PIF (lanes 6-10). Myotubes were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10). A representative blot is shown; densitometric analysis is based on three separate blots. Values in the presence of PIF are indicated as (■) whilst in the presence of PIF and 10μM receptor peptide as (□). Statistical differences between groups were determined by one-way ANOVA followed by Tukey's test. Differences from 0nM PIF are indicated as c, $p < 0.001$ whilst differences between groups as f, $p < 0.001$.

Actin loading control

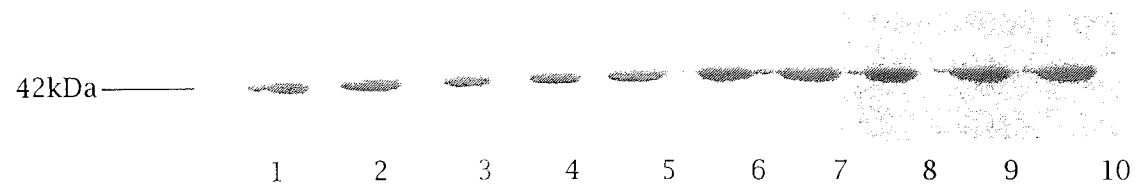


Figure 9. 5j

Lane discipline as figure 9. 5i.

Figures 9. 5a – 9. 5j

Western blot analysis of the proteasome subunits 20S α (figure 9. 5a), MSS1 (figure 9. 5c) and p42 (figure 9. 5e) demonstrate the N-terminal peptide sequence to attenuate the PIF-induced increase in proteasome subunit expression. This effect is mirrored in the 'chymotrypsin-like' activity of the proteasome (figure 9. 4a) and total protein degradation (figure 9. 4b). Ubiquitin conjugating enzyme expression is up-regulated in response to PIF, but this effect is attenuated with pre-treatment of 10μM receptor peptide (figure 9. 5g)

The data presented suggests that the peptide sequence is interfering with PIF-induced up-regulation of the ATP-ubiquitin dependent proteolytic pathway responsible for protein catabolism in cancer cachexia (Lorite *et al* (2001)). This is corroborated in figure 9. 5i whereby myosin expression is depleted in response to PIF but remains as control levels in C_2C_{12} myotubes when pre-treated with 10μM receptor peptide. Therefore, it appears that the N-terminal peptide sequence is acting as a receptor antagonist, with no agonist effects against the PIF receptor on skeletal muscle cells.

The *In vitro* data into the effects of the receptor peptide was very encouraging. Therefore the PIF receptor antibody was assessed as a therapeutic agent against PIF *in vitro*.

Total protein degradation *in vitro* in response to PIF (24h) and the PIF receptor antibody.

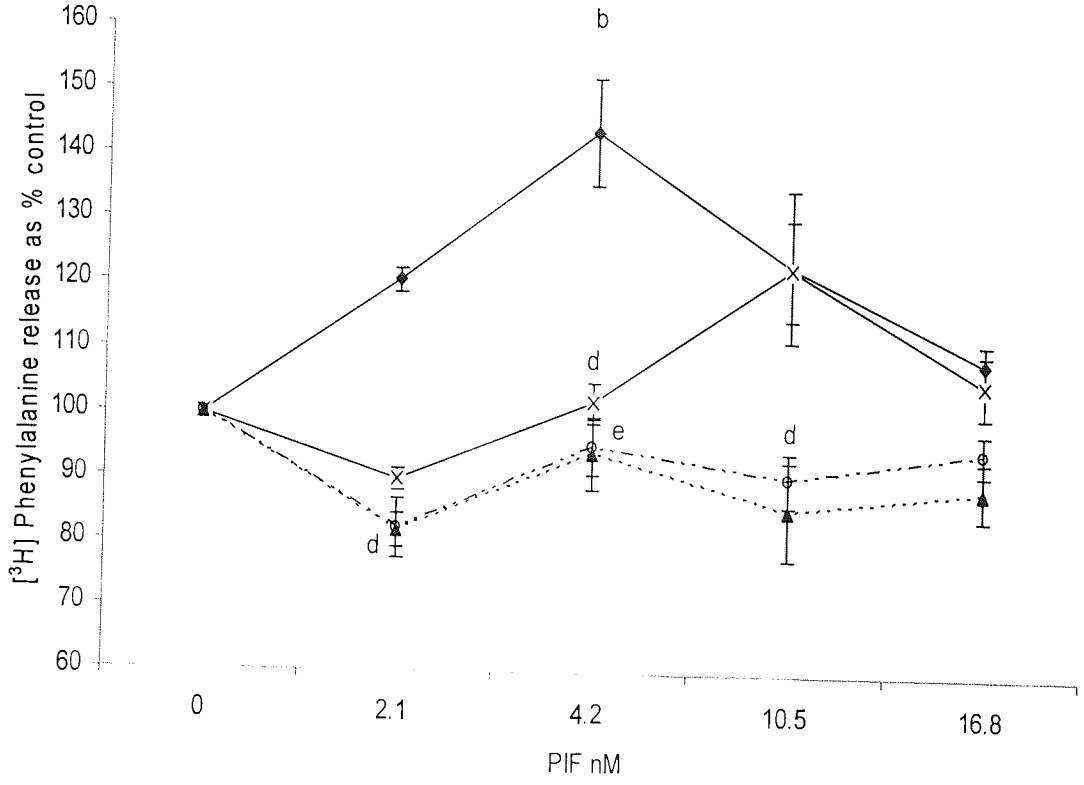


Figure 9. 6a

Myotubes were incubated with PIF alone (■), or pre-treated 2h prior to the addition of PIF with 5μg/ ml antibody (X), 10μg/ ml antibody (▲) or 20μg/ml antibody (○). Results are expressed as mean S.E.M and the experiment was repeated twice where n=6. Statistical analysis between group means was determined by one-way ANOVA followed by Tukey's test. The difference from 0nM PIF is indicated as b, $p < 0.01$ whereas differences between PIF and PIF receptor antibody dilutions are indicated as d, $p < 0.05$ and e, $p < 0.01$.

'Chymotrypsin-like' activity of the proteasome in C_2C_{12} myotubes in response to PIF (24h) in the absence or prescence of the PIF receptor antibody

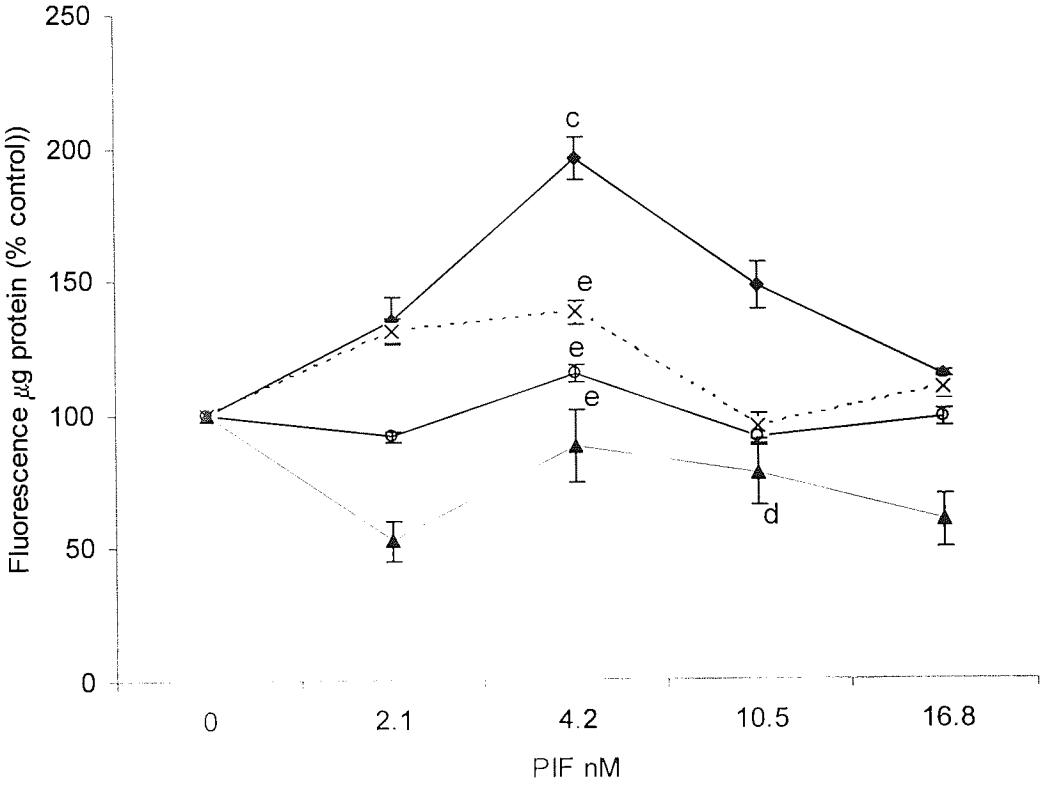


Figure 9. 6b

Myotubes were incubated with PIF alone (■), or pre-treated 2h prior to the addition of PIF with 5μg/ ml antibody (X), 10μg/ ml antibody (▲) or 20μg/ml antibody (○). Results are expressed as mean S.E.M and experiment was repeated twice where n=6. Statistical analysis between group means was determined by one-way ANOVA followed by Tukey's test. The difference from 0nM PIF is indicated as c, $p < 0.001$ whereas differences between PIF and PIF receptor antibody dilutions are indicated as d, $p < 0.05$ and e, $p < 0.01$.

Figure 9. 6a and 9. 6b

The PIF receptor antibody at 10μg/ml was found to be most successful attenuating PIF-induced increase in 'chymotrypsin-like' activity of the proteasome (figure 9. 6a) and total protein degradation (figure 9. 6b). Therefore 10μg/ml of PIF receptor antibody was assessed *in vitro* against PIF-induced increase in the ATP-ubiquitin dependent proteolytic pathway

Western blot analysis of 20S proteasome α subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of 10μg/ml PIF receptor antibody

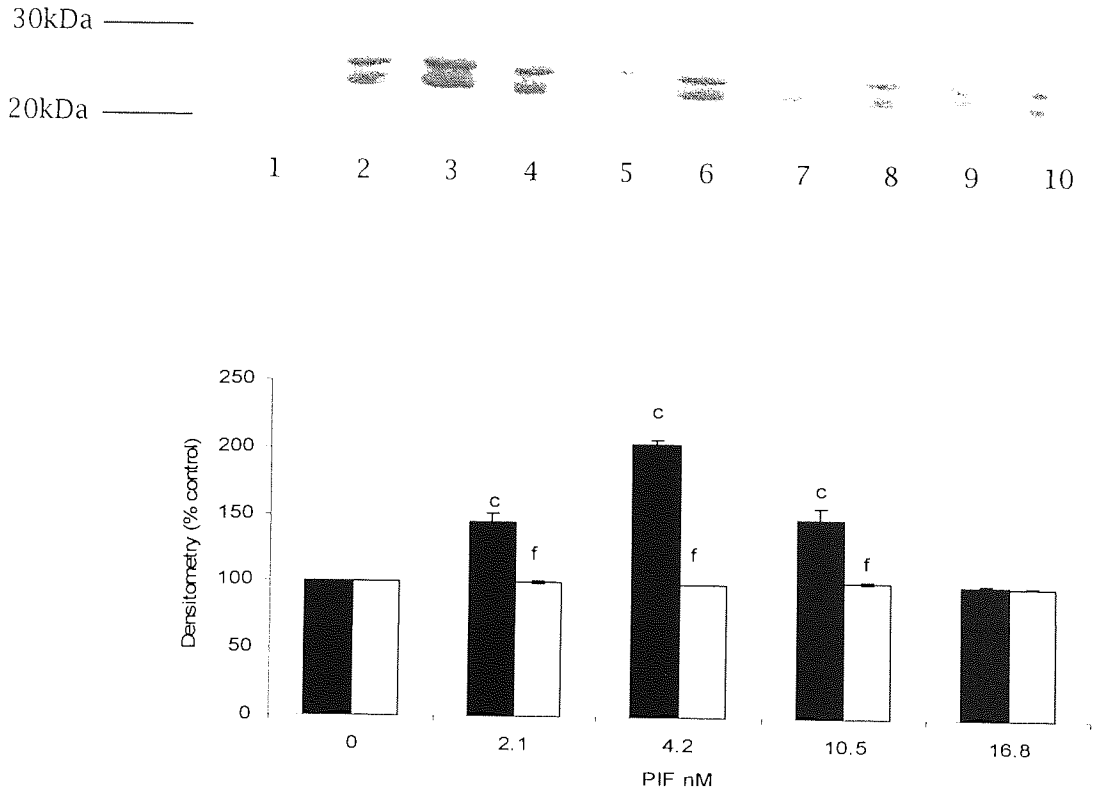


Figure 9. 6c

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence of 10μg/ml PIF receptor antibody (lanes 6-10, □). Densitometric analysis was based on three replicate blots. Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. The difference from 0nM PIF is indicated as c, $p < 0.001$ while differences between groups as f, $p < 0.001$.

Western blot analysis of MSS1 proteasome subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of 10μg/ml PIF receptor antibody

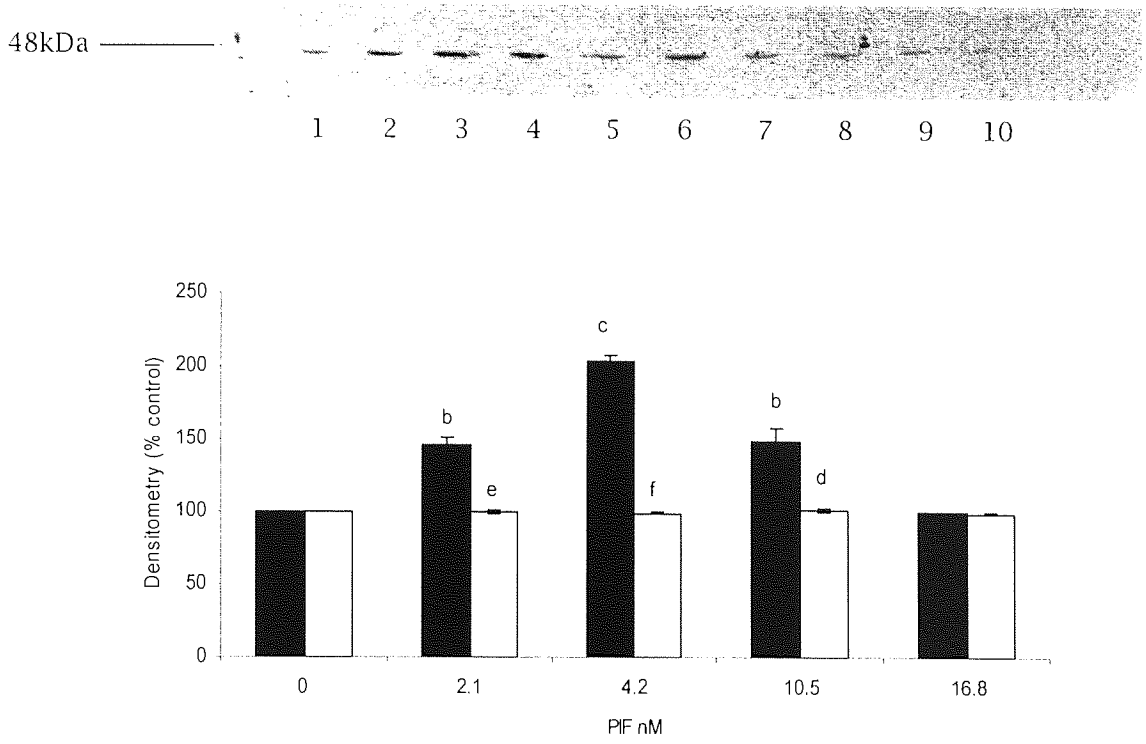


Figure 9. 6d

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence of 10μg/ml PIF receptor antibody (lanes 6-10, □). Densitometric analysis was based on three replicate blots. Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are indicated as b, $p < 0.01$ and c, $p < 0.001$ while differencs between groups as d, $p < 0.05$; e, $p < 0.01$ and f, $p < 0.001$.

Western blot analysis of p42 proteasome subunit expression in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of 10μg/ml PIF receptor antibody

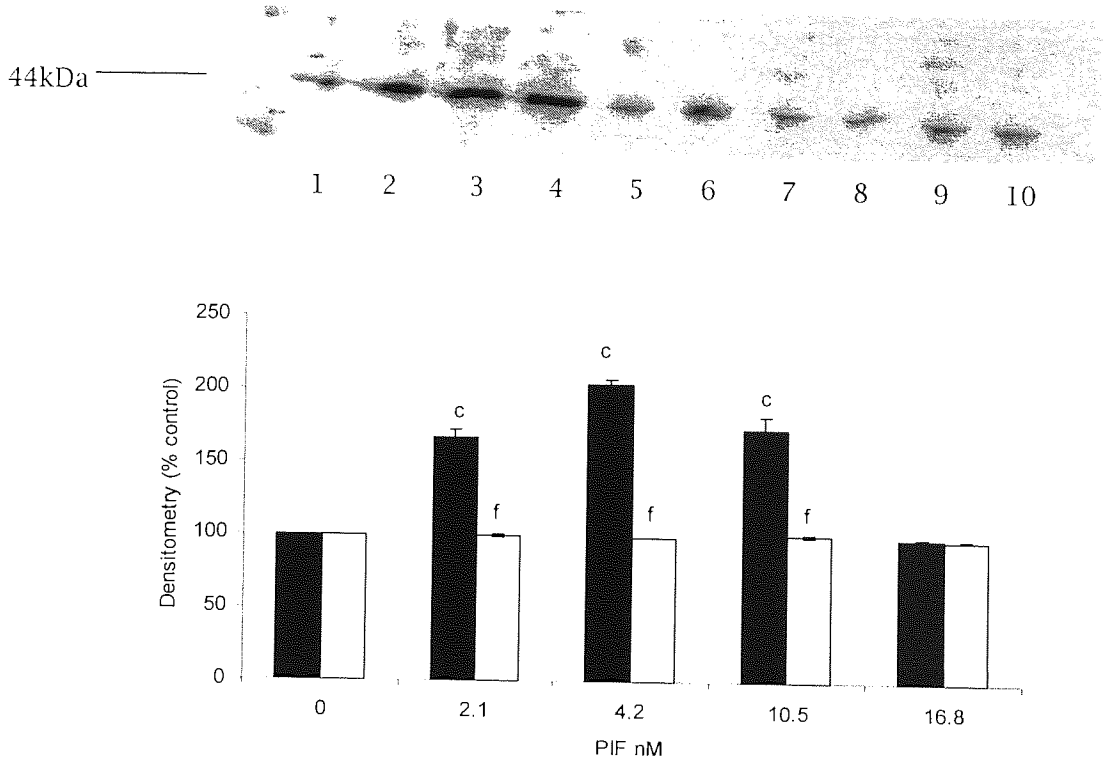


Figure 9. 6e

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence of 10μg/ml PIF receptor antibody (lanes 6-10, □). Densitometric analysis was based on three replicate blots. Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. The difference from 0nM PIF is indicated as c, $p < 0.001$ while differences between groups as f, $p < 0.001$.

Western blot analysis of $E2_{14K}$ expression in C_2C_{12} myotubes in response to PIF (24h) in the absence or presence of 10μg/ml PIF receptor antibody

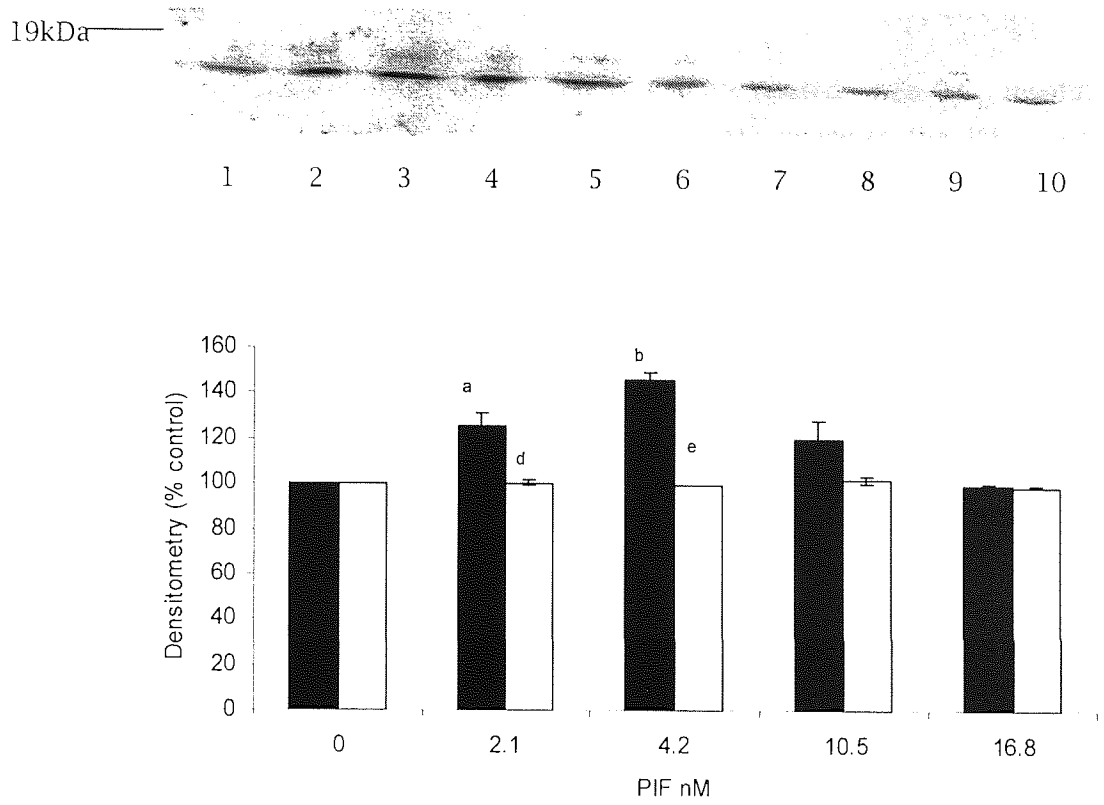


Figure 9. 6f

Cells were incubated with 0 (lanes 1 and 6), 2.1 (lanes 2 and 7), 4.2 (lanes 3 and 8), 10.5 (lanes 4 and 9) or 16.8nM PIF (lanes 5 and 10) in the absence (lanes 1-5, ■) or presence of 10μg/ml PIF receptor antibody (lanes 6-10, □). Densitometric analysis was based on three replicate blots. Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. Differences from 0nM PIF are indicated as a, $p < 0.05$ and b, $p < 0.01$ while between groups as d, $p < 0.01$ and e, $p < 0.01$.

Actin loading control

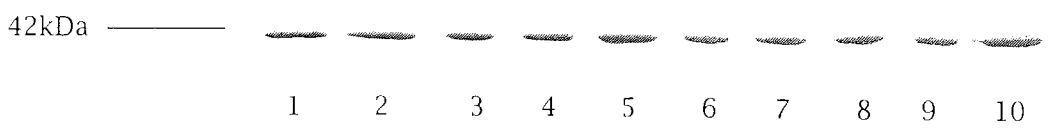


Figure 9. 6g

Lane discipline as figures 9. 6c - 9. 6f

Figures 9. 6c – 9. 6f

Western blot analysis of the proteasome subunits 20S α (figure 9. 6c), MSS1 (figure 9. 6d) and p42 (figure 9. 6e) demonstrate the PIF receptor antibody at 10μg/ml to attenutate the PIF-induced increase in proteasome subunit expression. This effect was also mirrored in 'chymotrypsin-like' activity (figure 9. 6b) and protein degradation (figure 9. 6a).

Up-regulated $E2_{14K}$ expression in response to PIF is also attenuated when C_2C_{12} myotubes are pre-treated with the PIF receptor antibody. This data suggests the PIF receptor antibody is capable of attenuating the response to PIF in vitro, therefore it was analysed for its effectiveness in vivo.

The PIF receptor antibody was tested *in vivo* in MAC16 tumour bearing mice with a weight loss of <5% and n=5.

In vivo administration of PIF receptor antibody (3.47mg/kg) S.C to MAC16 tumour bearing mice and effect on body weight

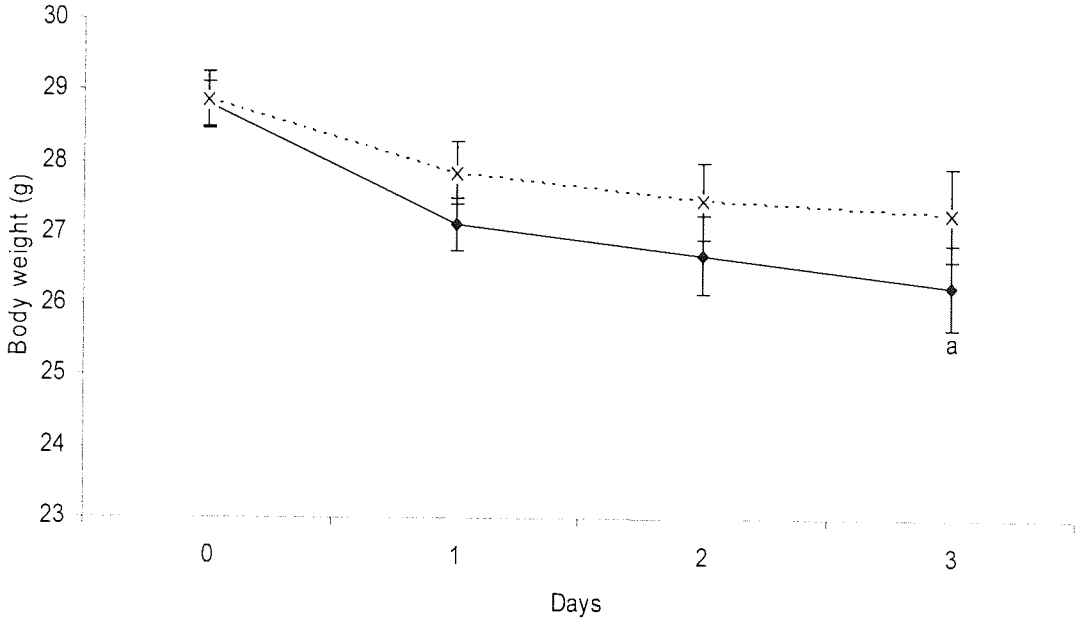


Figure 9. 7a

The PIF receptor antibody and control was administered twice daily by S. C injection ad diluted in PBS. Results are expressed as mean S.E.M, significance between groups was determined by Student's t test. Control mice, represented by (———) experienced a significant weight loss, indicated by a, $p < 0.05$ within 3 days and there was no significant weight loss in the group receiving PIF receptor antibody, represented by (X).

Total protein degradation *ex vivo* determined by tyrosine release in soleus muscle of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

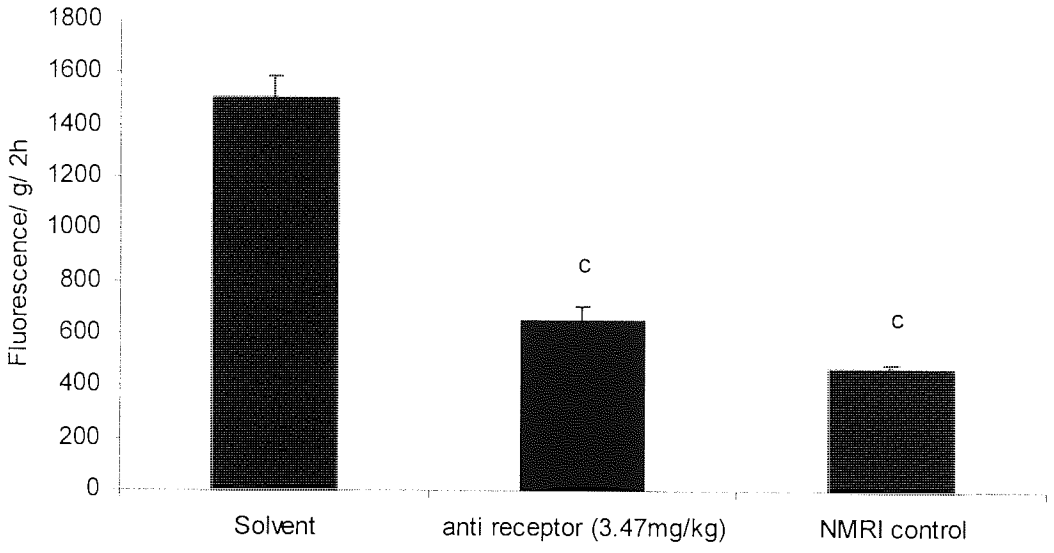


Figure 9. 7b

Results are expressed as mean S.E.M where n=5, significance between groups was determined by one-way ANOVA, followed by Tukey's test. Differences from MAC16 control are represented by c, $p < 0.001$, with no significance between NMRI controls and mice in receipt of PIF receptor antibody. MAC16 tumour bearing mice in receipt of the PIF receptor antibody saw total protein degradation return to control levels as seen in NMRI mice.

Figure 9. 7a and 9. 7b

Weight loss associated with the cachexia-inducing MAC16 tumour appears to be attenuated by administration of the PIF receptor antibody (figure 9. 7a) and follows the encouraging data from the *in vitro* work. The increase in total protein degradation, previously described by Smith and Tisdale (1993) is returned to control levels in MAC16 mice in receipt of the PIF receptor antibody (figure 9. 7b). This suggests that weight loss, catabolism and depletion of skeletal muscle is attenuated when in receipt of the PIF receptor antibody.

'Chymotrypsin-like' activity of the proteasome from gastrocnemius muscle *ex vivo* in MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

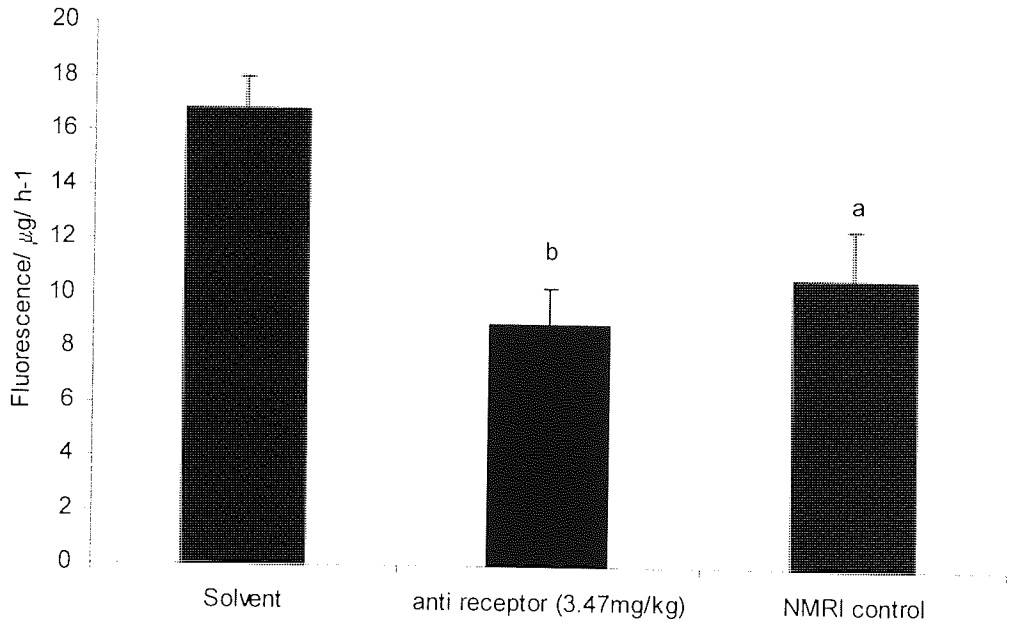


Figure 9. 7c

Results are expressed as mean S.E.M where n=5, significance between groups was determined by one-way ANOVA, followed by Tukey's test. Differences from MAC16 control are represented by a, $p < 0.05$ and b, $p < 0.01$ with no significant difference between NMRI controls and mice in receipt of PIF receptor antibody. MAC16 tumour bearing mice in receipt of the PIF receptor antibody saw 'chymotrypsin-like' activity of the proteasome return to control levels as seen in NMRI mice.

Figure 9. 7c

MAC16 tumour bearers demonstrated an increase in 'chymotrypsin-like' activity as reported by (Whitehouse *et al* (2001)) but this effect was negated by administration of the PIF receptor antibody, whereby activity of the β subunits of the proteasome returned to control levels.

Western blot analysis of gastrocnemius muscle 20S proteasome α subunit expression of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

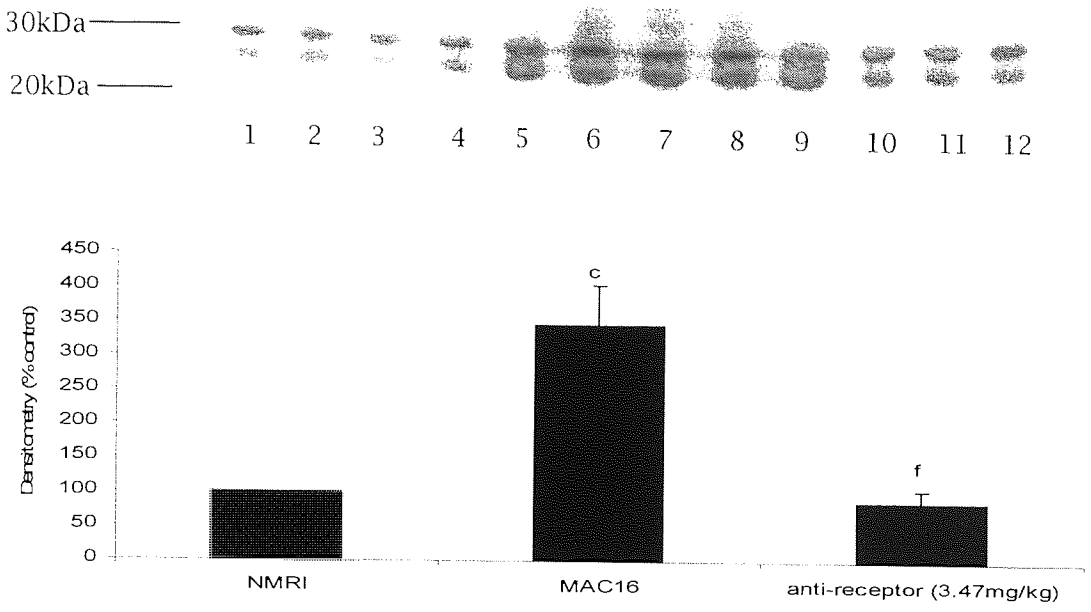


Figure 9. 8a

Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test. Results are expressed as mean S.E.M. The difference between NMRI control and MAC16 tumour bearers is indicated as c, $p < 0.001$ while between MAC16 in receipt of solvent and PIF receptor antibody as f, $p < 0.001$. There is no statistical significance in expression between NMRI controls and mice in receipt of the PIF receptor antibody

Actin loading control

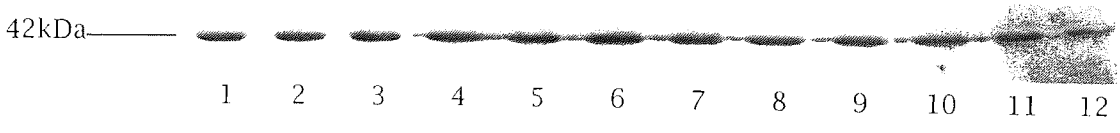


Figure 9. 8b

Lane discipline as figure 9. 8a

Western blot analysis of gastrocnemius muscle MSS1 proteasome subunit expression of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

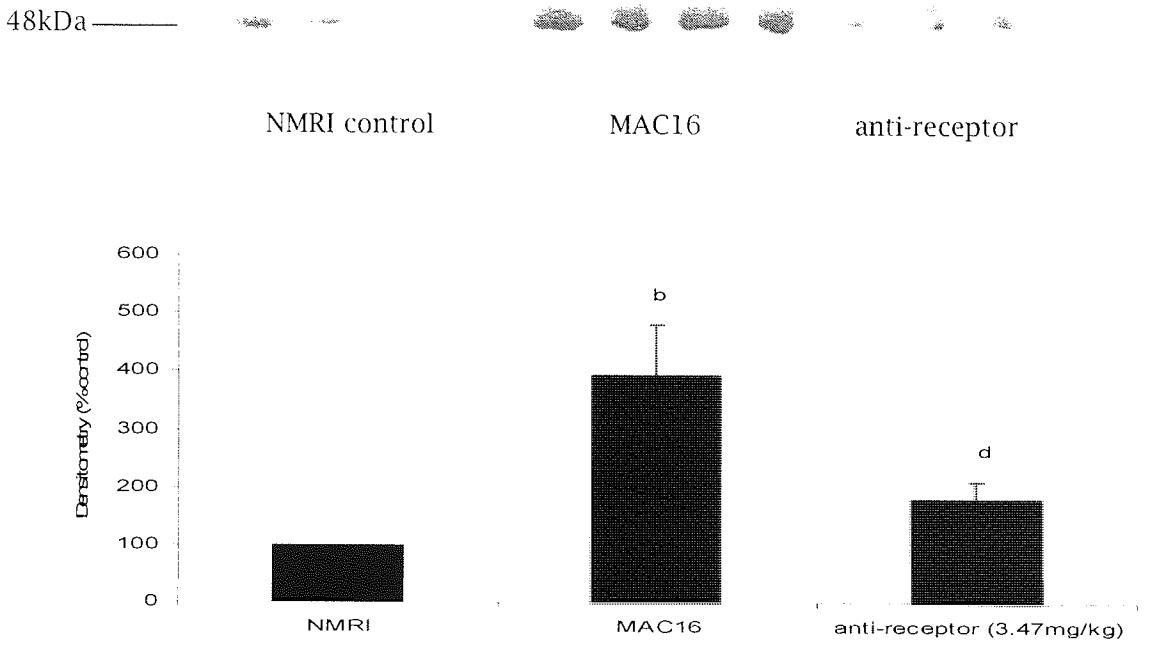


Figure 9. 8c

Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test, results are expressed as mean S.E.M. The difference between NMRI control and MAC16 tumour bearers is indicated as b, $p < 0.01$ while between MAC16 in receipt of solvent and PIF receptor antibody as d, $p < 0.05$. There is no statistical significance between NMRI controls and mice in receipt of PIF receptor antibody expression of MSS1.

Actin loading control

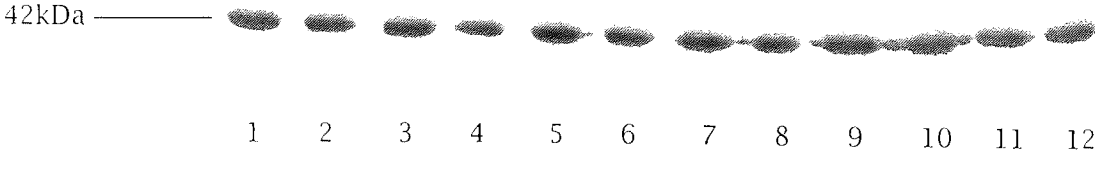


Figure 9. 8d

Lane discipline as figure 9. 8c

Western blot analysis of gastrocnemius muscle p42 proteasome subunit expression of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

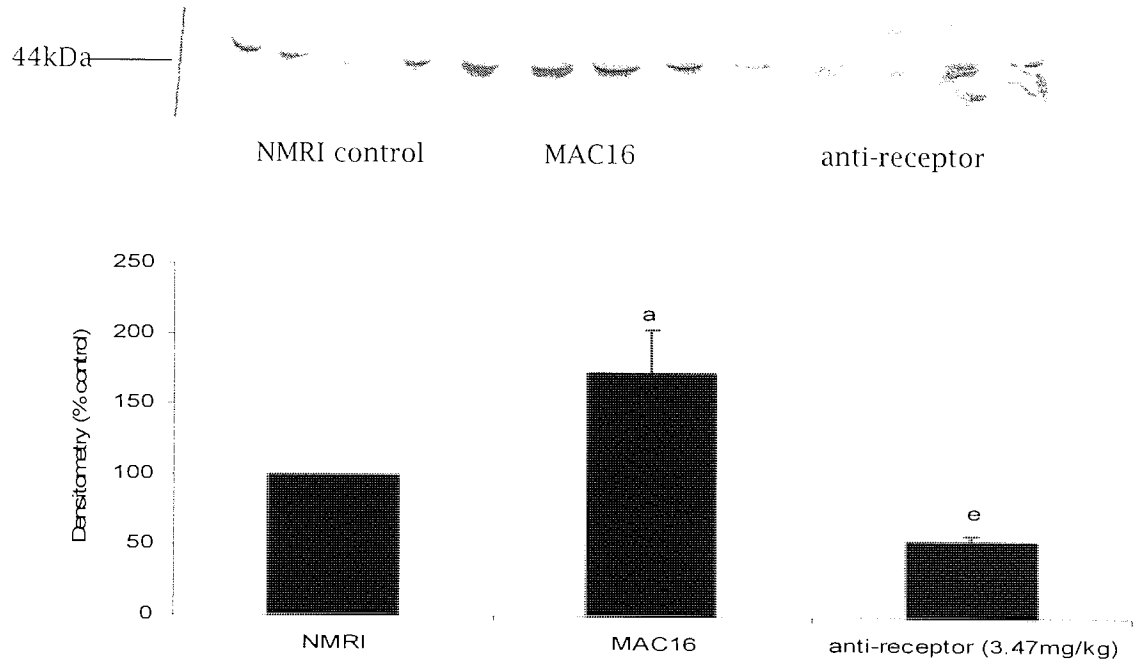


Figure 9. 8e

Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test, results are expressed as mean S.E.M. The difference between NMRI control and MAC16 tumour bearers is indicated as a, $p < 0.05$ while between MAC16 in receipt of solvent and PIF receptor antibody as e, $p < 0.01$. There is no significance between NMRI controls and mice in receipt of PIF receptor antibody expression of p42.

Actin loading control

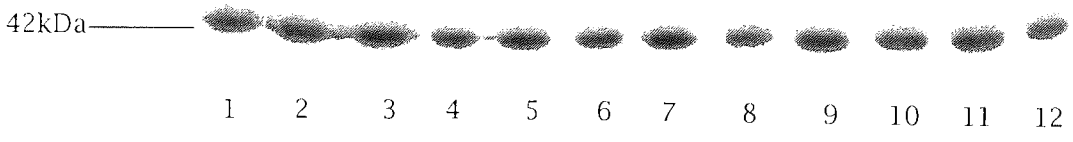


Figure 9. 8f

Lane discipline as figure 9. 8e

Western blot analysis of gastrocnemius muscle $E2_{14K}$ expression of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

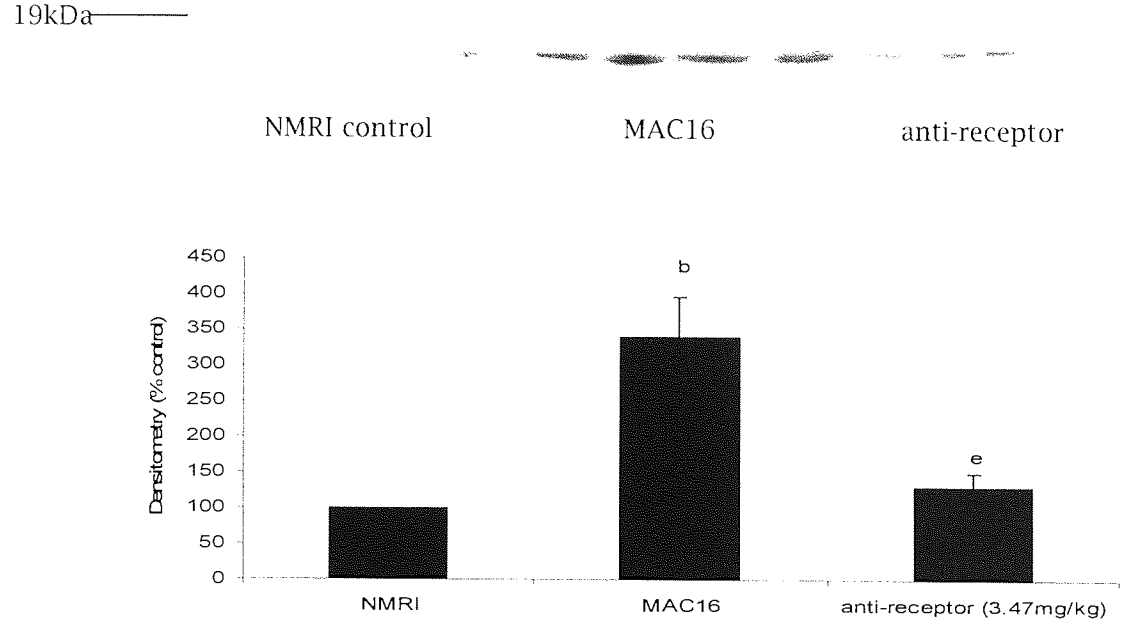


Figure 9. 8g

Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test, results are expressed as mean S.E.M. The difference between NMRI control and MAC16 tumour bearers is indicated as b, $p < 0.01$ while between MAC16 in receipt of solvent and PIF receptor antibody as e, $p < 0.01$. There is no statistical significance in $E2_{14K}$ expression between NMRI control and mice in receipt of PIF receptor antibody.

Actin loading control

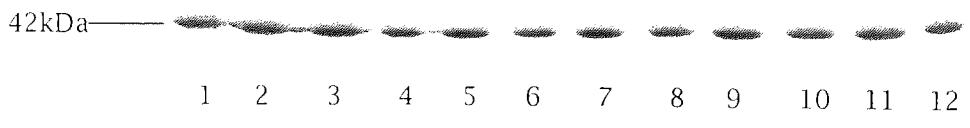


Figure 9. 8h

Lane discipline as figure 9. 8g

Western blot analysis of gastrocnemius muscle myosin expression of MAC16 tumour bearers in receipt of solvent, PIF receptor antibody (3.47mg/kg) or NMRI controls

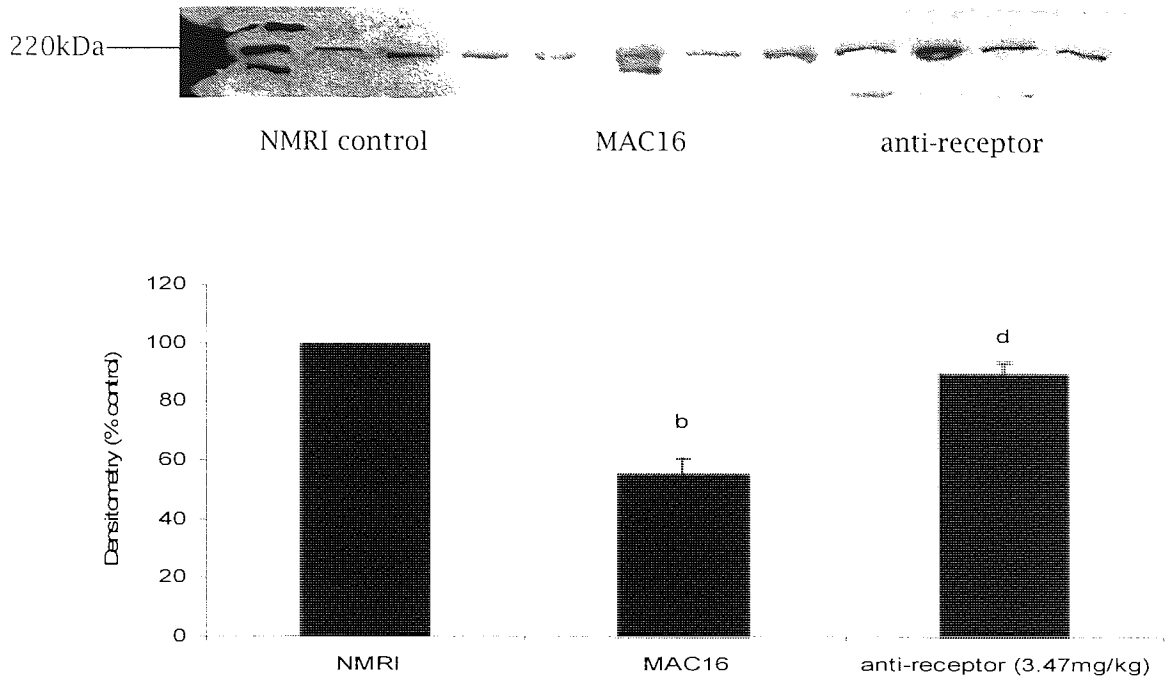


Figure 9. 8i

Statistical analysis between groups was determined by one-way ANOVA, followed by Tukey's test, results are expressed as mean S.E.M. The difference between NMRI control and MAC16 tumour bearers is indicated as b, $p < 0.01$ while between MAC16 in receipt of solvent and PIF receptor antibody as d, $p < 0.05$. There is no significant difference in expression of myosin between NMRI control and mice in receipt of PIF receptor antibody

Actin loading control

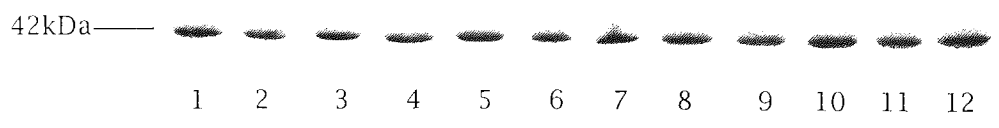


Figure 9. 8j

Lane discipline as figure 9. 8i

Figures 9. 8a- 9. 8e

Proteasome subunit expression in MAC16 tumour bearers was significantly elevated in comparison to NMRI controls including 20S proteasome α subunit expression (figure 9. 8a), MSS1 (figure 9. 8c) and p42 (figure 9. 8e). This data correlated with previous findings (Whitehouse *et al* (2001)). Administration of the PIF receptor antibody at 3.47mg/kg successfully attenuated this increase in proteasome subunit expression, which correlated with the return to control activity of 'chymotrpsin-like' activity of the proteasome (figure 9. 6c).

$E2_{14K}$ expression was attenuated in MAC16 tumour bearing mice in receipt of the PIF receptor antibody (figure 9. 8g). The combination of this data suggests a decrease in activity of the ATP-ubiquitin dependent proteolytic pathway in MAC16 mice administered the PIF receptor antibody.

Total protein degradation was returned to control levels (figure 9. 6a) and this was further corroborated by western blot analysis of myosin. Myosin expression returned to control levels as seen in NMRI mice in MAC16 mice administered the PIF receptor antibody (figure 9. 8i).

Discussion

In order for PIF to induce protein degradation in skeletal muscle, there must be a specific interaction with a muscle protein receptor capable of activating signal transduction and thus the intracellular protein degradative system. PIF is a highly glycosylated and sulphated glycoprotein (Todorov *et al* (1997)) it is likely that PIF remains membrane bound and induces release of arachidonic acid from membrane bound phospholipids. The subsequent metabolism of A.A to 15(*S*)-HETE is then responsible for proteolysis of skeletal muscle (Smith and Tisdale (1999)).

Todorov *et al* (1996) successfully obtained a monoclonal antibody against PIF and subsequently found high concentrations of the monoclonal antibody were capable of displacing PIF from the membrane receptor and successful in neutralising the biological effect of PIF (Cariuk *et al* (1997) and Todorov *et al* (1996)).

Todorov *et al* (1997) found binding of the PIF antibody to be mediated through carbohydrate moieties, as antibody reactivity was destroyed by treatment with periodate. The activity of PIF was also reduced by treatment with PNGase F and O-glycosidase and completely destroyed by treatment with chondriotinase ABC. This confirmed the N- and O-linked sulphated oligosaccharide chains were both antigenic and biological determinants in the action of PIF and enzyme deglycosylation resulted in loss of specific binding of PIF to the PIF receptor. Sulphate residues provide a strong electrostatic linkage to cellular receptors, resulting in high affinity binding of PIF.

Pre-treatment of C_2C_{12} myotubes with the antigenic peptide, used to raise the polyclonal PIF receptor antibody was successful in attenuating the response to PIF *in vitro.* However this peptide was subject to proteolytic digestion and was unsuccessful when tested *in vivo* (data not shown).

The PIF receptor antibody was successful in recognising the antigen (determined by ELISA), and also capable of identifying the PIF receptor by western blotting, isolated from prepared C_2C_{12} myotubes membranes. The PIF receptor is visible as a double band, suggesting it to be a dimer with a relative molecular weight between 40 and 30 kDa.

When tested *in vitro*, the PIF receptor was found to attenuate the action of PIF, inhibiting the PIF-induced increase in 'chymotrypsin-like' activity of the proteasome, protein degradation and up-regulated expression of components of the ATP-ubiquitin dependent

proteolytic pathway. When tested *in vivo* the PIF receptor antibody was found to attenuate cachexia in MAC16 tumour bearing mice.

This chapter presents some very encouraging data and there is great potential for future work into the development of this antibody as a therapy against cancer cachexia.

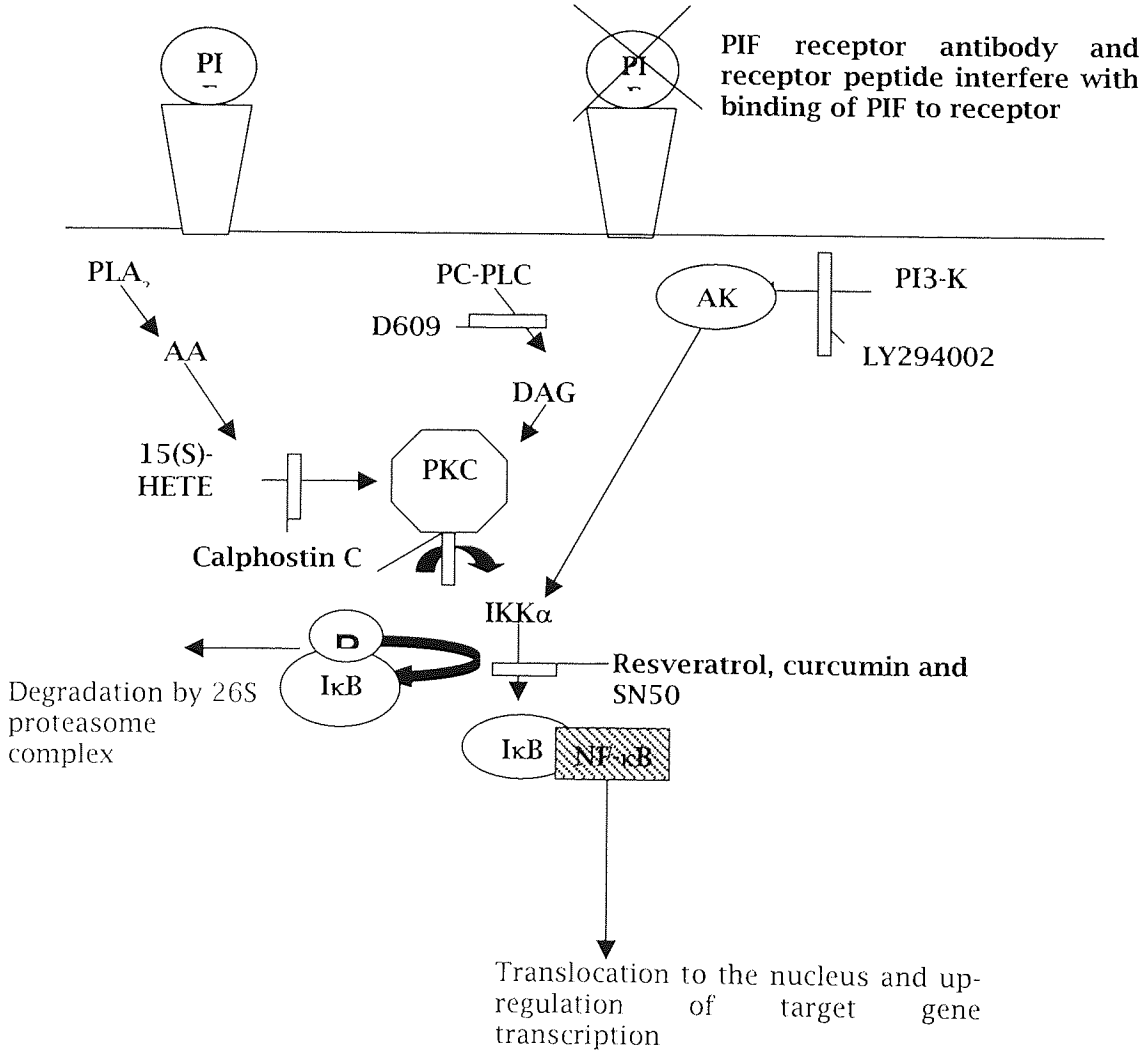
Chapter 10

Conclusions

Cancer cachexia is a wasting syndrome that accounts for a significant proportion of cancer-related deaths. A number of factors have been reported to be involved including prostaglandins, glucocorticoids and cytokines. However, cachexia is predominantly mediated by a 24kDa Proteolysis-Inducing Facor (PIF) and a 43kDa lipid mobilising factor.

An overview is demonstrated in figure 10. 1

Figure 10. 1



PIF stimulates the activation of phospholipase A_2 which results in the release of arachidonic acid from membrane phospholipids, which is subsequently metabolised to a number of end products, of which 15(*S*)-HETE directly stimulates protein degradation and increases nuclear accumulation of NF-κB. This work demonstrates 15(*S*)-HETE to be directly involved in the activation of PKCα, which is responsible for the activation of IKK, phosphorylation of IκBα and upregulation of ATP-ubiquitin dependent proteolysis. The action of 15(*S*)-HETE was successfully attenuated with the PKC inhibitor calphostin C, suggesting PKC to be an intermediate, directly involved in the action of PIF.

The transcription factor NF-κB has a direct role in the induction and upregulation of protein degradation via the ATP-ubiquitin dependent proteolytic pathway. Dominant negative mutant IκBα inserts and control plasmid DNA were employed and transfected into C_2C_{12} myoblasts, when differentiated into C_2C_{12} myotubes the response to PIF was attenutated in those cells transfected with the dominant negative inserts. The PIF-induced increase in total protein degradation, activity of the proteasome and proteasome expression were all attenuated in myotubes containing the mutant IκBα insert, suggesting the transcription factor NF-κB to have a crucial role in translating and mitigating the effect of PIF.

The importance of NF-κB in PIF-induced signal transduction was further corroborated by the use of NF-κB inhibitors *in vitro* and *in vivo*. The SN50 cell permeable NF-κB inhibitor proved NF-κB to be directly involved in the upregulation and expression of the ATP-ubiquitin dependent proteolytic pathway in response to PIF. The IKK inhibtor resveratrol was also effective, attenuating PIF-induced protein degradation and skeletal muscle proteolysis *in vitro* and *in vivo* in MAC16 tumour bearing mice. This data suggests the potential use of NF-κB inhibitors as agents against cancer cachexia and suggests the intereference with signal transduction can combat the effect of PIF.

The data presented within this thesis demonstrates the importance of protein kinase C cellular signalling in response to PIF and 15(*S*)-HETE. Using constitutively active PKCα and dominant negative PKCα mutant plasmid DNA, transfected C_2C_{12} myoblasts, were successful in demonstrating the importance of PKC. C_2C_{12} myotubes transfected with mutant PKCα were unable to illicit a response to PIF, with no associated increased DNA binding of NF-κB, suggesting PKCα as an important intermediate involved in the upregulation of the ATP-ubiquitin dependent proteolytic pathway and skeletal muscle proteolysis.

The phorbol ester TPA, further demonstrated the importance of PKC involvement in upregulation of the ATP-ubiquitin dependent proteolysis achieved through activation of the transcription factor NF-κB.

The Akt signalling pathway has also been suggested to be involved in the action of PIF, which is achieved through the activation of PI3 kinase and may suggest an alternative pathway from PKC for the phosphorylation and degradation of IκBα.

It is apparent that there is no one universal signalling mechanism for PIF to exert its catabolic effect and elicit a response in skeletal muscle. It appears that there are different pathways and involve 'cross-talk' between them. However, they utlimatey result in increased nuclear accumulation and DNA binding of NF-κB. This culminates in up-regulation and increased expression of the components within the ATP-ubiquitin dependent proteolytic pathway.

The work involving the PIF receptor antibody is very encouraging, providing a new avenue to explore therapeutic intervention against cancer cachexia. The antibody appears to be well tolerated and successful in attenuating the *in vitro* response to PIF and cancer cachexia *in MAC16* tumour bearing mice.

Chapter 11

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Appendix I

GENETIC MODIFICATION RISK ASSESSMENT / ASSIGNMENT OF CONTAINMENT LEVEL

Transfection of C_2C_{12} murine myoblasts with IκBα

Laboratory containment : MB 633 / 634

Named Worker : Miss Stacey Wyke

Named Person Responsible : Prof Mike Tisdale

Overview

NF-κB has been shown to be involved in the signalling pathways activated during muscle catabolism in cancer cachexia. The aim of this project is to transfect C_2C_{12} myoblasts with one of three plasmids. The first plasmid pCMV4 is an empty control vector, containing no mutant IκBα insert. The second plasmid is pCMV4-IκBαΔN (truncation of amino acids 1-36). Finally the third plasmid pCMV4-IκBα S32/A36 (point mutations of Ser^{32} and Ser^{36} to alanine).
Each of the latter mutant plasmids lacks Ser^{32} and Ser^{36} phosphorylation sites that are required for IκBα degradation, rendering NF-κB inactive.

Plasmids will first be transformed into E. coli DH5α, the successful transformants will be identified by PCR, and then purified plasmid DNA will be transfected into C_2C_{12} myoblasts.

Hazard Identification In Respect of Human Health and Environmental Safety

The cloning vector pCMV4 is derived from the cytomegalovirus (CMV) and contains a 968bp insert of mutant IκBα. The pCMV4 vector lacks full viral genetic information. There is no danger of genetic recombination with super-infection. The role of IκBα is to hold NF-κB within the nucleus and render it inactive, until appropriate stimuli initiates IκBα degradation, releasing NF-κB from the complex. The IκBα insert is not considered toxic to humans. The mutant IκBα insert when expressed causes truncation of amino acids 1-36 (IκBαΔN) and point mutation of Ser^{32} and Ser^{36} to alanine (IκBαS32/A36). Both mutations prevent phosphorylation of Ser^{32} and Ser^{36} within IκBα, rendering it unable to dissociate from NF-κB, therefore inhibiting the action of NF-κB.

The empty control vector pCMV4, pCMV4-IκBαΔN and pCMV4-IκBαS32/A36 were provided courtesy of Dr Yi Ping Li, from the department of Medicine, Baylor college of Medicine, Houston, Texas.

E. coli strain and genotype : DH5α F'Φ80d/acZΔM15 Δ(lacZYA-argF) U169deoRrecA1 end A1 hsdR17(m_k^- m_k^+)phosA supE44λ-thi-1 gry A96 relA1. This is a K12 derivative that is recognised as non-colonising disabled and considered to be equivalent ACDP hazard group 1. They are not considered to be pathogenic to humans or animals with survival due to their auxotrophic requirements, being unlikely outside laboratory culture. Purchased from Invitrogen.

C_2C_{12} Myoblasts are a subclone from a myoblast cell line established from normal adult C3H mouse leg muscle. They provide a simple model to study *in vitro* myogenesis and cell differentiation. Cells have not been virally transformed and would not survive outside

laboratory culture. The cells are not considered to be hazardous to humans. Cells were purchased from the European Collection of Culture Cells (ECACC).

Since there is no risk to health or the environment from the plasmids, inserts, bacterium or mammalian cells has been identified, containment is designated Class 1.

Anything contaminated (cultures, media etc.) with either E. coli or C_2C_{12} cells, even though non-hazardous, will be autoclaved. Any spillages will be bleached immediately.

Appendix II

Wizard ®Purefection Plasmid DNA Purification System (Promega)

Midipreps (5-25ml Bacterial Cultures) Extended Version (Appendix)

The majority of components are provided in the kit. However, prior to use you need to prepare the following :

4.2M	Guanidine-HCL/ 40% isopropanol solution (4/40 wash solution).
80% Ethanol	Ethanol wash solution 70%, 80%, 95% and 100%
High quality water	e.g., Sigma endotoxin-free water
7.5M	Ammonium acetate
50ml	Sterile centrifuge tubes
15ml	Sterile centrifuge tubes, stable at 15,000g
100%	Isopropanol

Pellet 5-25ml of bacterial solution by centrifugation at 10,000g for 5-10 min at 20-25°C. Discard the supernatent and blot excess liquid from the tube on a paper towel.

Add 1.25ml of Cell Resuspension Solution and completely resuspend the cell pellet by vortexing or pipetting. It is essential to thoroughly resuspend the cells. If not already in a 15ml tube, transfer the cells to a sterile 15ml tube.

Add 1.25ml of Cell Lysis Solution and mix thoroughly by inverting the tube 6-8 times to ensure cell lysis. Incubate for 5 min at 20-25°C.

Add 1.75ml of Neutralisation Solution. Mix immediately and thoroughly by inverting the tube 6-8 times.

Centrifuge the bacterial lysate at 10,000g for 20 min at 20-25°C. Transfer the supernantent into a new 15ml tube and repeat the centrifugation.

Before use, completely and thoroughly resuspend the Endotoxin Removal Resin by shaking and swirling the bottle. Add 0,25ml of resin to the supernatant. Mix vigorously. Incubate for 10 min at 20-25°C, shaking vigorously for 5 seconds several times during the incubation.

Place the tube on the MagneSil ™ Magnetic Separation Unit and allow the solution to clear. Invert the magnetic unit and tube to rinse residual resin from the cap and tube walls. Allow the solution to clear for 30 sec. Keep the tube on the magnet and transfer the supernantent to a new tube, by pipetting or pouring. Discard the resin.

Add 1.0ml of 5M Guanidine Thiocyanate (GTC) to the supernatant-containing tube and mix vigorously. It is important to add sufficient GTC to achieve good DNA binding results.

Before use. Completely and thoroughly re-suspend the MagneSil™ Paramagnetic Particles by shaking and swirling the bottle. Add 0.75ml of the particles to the tube; shake vigorously to mix and incubate at 20-25°C for 2-3 min.

Place the tube onto the magnetic unit and allow the solution to clear. Invert the magnet and tube to rinse residual particles from the cap and tube walls. Allow the solution to

clear for 30 sec, then discard all supernatant. Leave the tube on the magnet for 2-3 min and discard the residual liquid. Save the particles.

Remove the tube from the magnet and wash the particles by adding 1.0ml 4/40 Wash Solution (4.2M guanidine-HCL/ 40% isopropanol). Resuspend the particles by vortexing for at least 10 sec. 4/40 Wash Solution helps to remove extraneous proteins, such as nucleases, from the DNA.

Place the tube on the magnetic unit and allow the solution to clear. Invert the magnet and tube to rinse residual particles from the cap and tube walls. Allow the solution to clear for 30 sec, then discard all supernatant. Leave the tube on the magnet for 2-3 min and discard all residual liquid. Save the particles.

Remove the tube from the magnet and add 2.5ml of 80% ethanol wash solution to the particles. Resuspend completely by vortexing for 10 sec. Place the tube on the magnet and allow the solution to clear. Invert the magnet and tube to rinse residual particles from the cap and tube walls. Allow the solution to clear for 30 sec, then discard all supernatant. Save the particles.

Repeat the previous step for 3 washes.

After the final wash, leave the cap off the tube and leave the tube on the magnet for 10 min. Remove all residual liquid from the bottom of the tube.

Remove the tube from the magnet and add 1.2ml high quality water. Vortex for 10 sec. Incubate for 1 min at 20-25°C place the tube back on the magnet and allow the solution to clear. Invert the magnet and rinse residual particles from the cap and tube walls. Leave the tube on the magnet for 3 min to capture all particles. Carefully transfer the supernatant to a new centrifuge tube.

To ensure all magnetic particles are removed, centrifuge the tube at 14,000g for 1 min. Quantify the supernatant and transfer to a new tube.

To precipitate DNA, add 0.5 volumes of 7.5M ammonium acetate to the supernatent. Add 2.5 volumes of 95% ethanol to the combined volume of supernatant and ammonium acetate. Mix well. Centrifuge at 14,000g for 15 min at 20-25°C. Aspirate the supernatant; avoid disturbing the translucent DNA pellet. Rinse the pellet briefly in 1-2ml of 70% ethanol and centrifuge at 14,000g for 5 min. Carefully aspirate the ethanol and air-dry the pellet for 3-5 min to allow the residual ethanol to evapourate.

Resuspend the DNA pellet in 100µl-300µl of high quality water.

Abstracts and Publications

ACTIVATION OF PKC AND NF-κB BY A PROTEOLYSIS INDUCING FACTOR (PIF) IN CANCER CACHEXIA

Stacey M Wyke, Helen J Smith and Micheal J Tisdale. British Journal of Cancer 88 (Suppliment 1) : S68

P173
ACTIVATION OF PKC AND NFkB BY A PROTEOLYSIS INDUCING FACTOR (PIF) IN CANCER CACHEXIA.
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Proteolysis Inducing Factor (PIF) is a 24kDa glycoprotein that can be isolated from cachexia-inducing murine and human tumours and from the urine of cachectic cancer patients. PIF causes breakdown of skeletal muscle, both *in vivo* and in vitro through the activation of the ubiquitin – dependant proteolytic pathway. The mechanism by which this occurs was studied in C2C12 myotubes as a surrogate model for skeletal muscle. PIF increased 26S proteasome activity (represented by 'chymotrypsin like' activity in C2C12 myotubes at concentrations between 1 – 20nM and maximum stimulation was achieved at 4.2nM ($p=<0.001$). This effect was attenuated by the PKC inhibitors Ro31-8220 and calphostin C.

The PKC inhibitor calphostin C demonstrated inhibition of IkBa degredation in response to PIF, inhibiting translocation of NFkB to the nucleus. Calphostin C also inhibited translocation of PKC in response to PIF. The action of PIF was also attenuated by the NFkB inhibitor curcumin, with regard to chymotrypsin like activity, 20S and E2 expression, suggesting that NFkB may be involved in the regulation of the ubiquitin – dependant proteolytic pathway.

These results suggest that the PKC and NFkB pathways are exploited by PIF in cancer cachexia.

PHYSIOPATHOLOGY OF CACHEXIA IN PANCREATIC CANCER

Stacey M Wyke and Micheal J Tisdale presented at the XXXVI meeting of the E.P.C.

Abstract

Cachexia is a progressive wasting syndrome, resulting in massive loss of both adipose tissue and skeletal muscle mass. Cachexia is very common in patients with pancreatic cancer, with 85% losing weight, and this is evident even at time of diagnosis (average weight loss of 14% of pre-illness stable weight). Weight loss is progressive until death, which usually occurs at weight loss of 30%. Cachexia reduces the response of cancer patients to drug therapy and accounts for the short survival time (average is only 4.1 months). Wasting of skeletal muscle is probably the most important factor regulating morbidity and mortality.

Pancreatic tumours that induce cachexia produce a sulphated glycoprotein, called proteolysis-inducing factor (PIF), that acts directly to stimulate muscle protein breakdown, through inducing an increased expression of the ubiquitin-proteasome proteolytic pathway. This pathway is considered to be the most important in intracellular protein degradation, and expression is elevated in skeletal muscle of pancreatic cancer patients with a weight loss greater than 10%. Administration of PIF to normal mice produces 10% weight loss in 24h arising entirely through the loss of skeletal muscle mass. PIF not only increases protein degradation in skeletal muscle, but also inhibits protein synthesis (by 50%). The combined effects result in muscle atrophy.

The catabolic action of PIF is attenuated by the polyunsaturated fatty acid eicosapentaenoic acid (EPA). EPA prevents muscle breakdown by interfering with key regulatory steps leading to increased gene expression of proteasome subunits and ubiquitin-conjugating enzymes. One of the most important steps inhibited by EPA is the nuclear accumulation of the transcription factor nuclear factor -κB (NF-κB). EPA has no effect on the inhibition of protein synthesis by PIF. Clinical studies in patients with pancreatic cancer show EPA to effectively attenuate further development of weight loss. When combined with a high energy, high protein, nutritional supplementation weight gain was seen, and this was entirely due to increase in lean body mass. The results show that it is possible to intervene therapeutically in the development of cachexia in pancreatic cancer patients, and this results in an improved quality of life and survival.

British Journal of Cancer (2004) 90, 1850 – 1857

www.bjcancer.com

Role of protein kinase C and NF-κB in proteolysis-inducing factor-induced proteasome expression in C_2C_{12} myotubes

British Journal of Cancer (2004) 91, 1742 – 1750

www.bjcancer.com

Induction of proteasome expression in skeletal muscle is attenuated by inhibitors of NF-κB activation

[CANCER RESEARCH 64, 8731–8735, December 1, 2004]

Mechanism of the Attenuation of Proteolysis-Inducing Factor Stimulated Protein Degradation in Muscle by β-Hydroxy-β-Methylbutyrate

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Signalling pathways in the induction of proteasome expression by proteolysis-inducing factor in murine myotubes

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Effect of cancer cachexia on the activity of tripeptidyl-peptidase II in skeletal muscle

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Abstract

[illegible] myofibrillar proteins in skeletal muscle