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**Investigation into the Mechanisms  
Responsible for Muscle Atrophy in Cancer  
Cachexia**

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Doctor of Philosophy

ASTON UNIVERSITY

September 2007

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Aston University

# Investigation into the Mechanisms Responsible for Muscle Atrophy in Cancer Cachexia

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## Summary

Cachexia is characterised by progressive muscle wasting and is seen in a number of illnesses including AIDS, heart disease and cancer. Cachexia inducing tumours are known to produce a glycoprotein called proteolysis inducing factor (PIF), which induces skeletal muscle atrophy via increased protein degradation and decreased protein synthesis. PIF induced protein degradation is believed to be through up-regulation of the ubiquitin-proteasome proteolytic pathway. However, the mechanism involved in the depression of protein synthesis is unknown. The objective of this study was to investigate the signalling pathway by which PIF reduces protein synthesis in skeletal muscle and to determine the link, if any, to the ability to induce protein degradation.

In murine myotubes PIF induced an increase in expression of the active form of the dsRNA dependent protein kinase (PKR), as well as the phosphorylated form of the translation initiator eIF2 $\alpha$ , possibly through the release of calcium, at the same concentration as that inhibiting protein synthesis. Inhibition of PKR reversed the inhibition of protein synthesis by PIF and also the induction of protein degradation through the ubiquitin-proteasome pathway by a reduction in the nuclear migration of NF- $\kappa$ B. The expression of phosphorylated forms of PKR and eIF2 $\alpha$  was also increased in the muscle of cancer patients experiencing weight loss, and in gastrocnemius muscle of mice bearing the cachexia inducing MAC16 tumour, as well as in the tumour itself. Treatment of mice bearing the MAC16 tumour with a PKR inhibitor attenuated muscle atrophy and inhibited tumour growth, through the inactivation of PKR and the consequent reduction of nuclear accumulation of NF- $\kappa$ B. Nutritional supplementation with leucine, and its metabolite HMB, reversed muscle loss of mice bearing the MAC16 tumour through a reduction in protein degradation, through the ubiquitin-proteasome pathway, together with a stimulation of protein synthesis. This was associated with an inhibition of PKR and eIF2 $\alpha$  phosphorylation, and increased eIF4F assembly, combined with an attenuation of NF- $\kappa$ B mobilization, and proteasome subunit expression.

A decreased translational efficiency of the eIF4F complex of initiation factors, through dephosphorylation of 4E-BP1 and an increased eEF2 phosphorylation, was seen in response to PIF *in vitro*. The same pattern of events also occurred in gastrocnemius muscle of mice bearing the MAC16 tumour demonstrating weight loss, where a depression of mTOR and p70S6K activation was also observed as weight loss increased.

These results suggest that PIF acts through activated PKR resulting in the phosphorylation of translation factor eIF2 $\alpha$  to inhibit protein synthesis. This process was also linked to up regulation of proteasome expression, and activity, and increased protein degradation, possibly through activation of NF- $\kappa$ B. It is likely that PIF can decrease translation initiation via interference with the eIF4F complex which would also lead to a depression of protein synthesis.

**Keywords:** Cachexia, PKR, eIF2 $\alpha$ , ubiquitin-proteasome, eIF4F.

# Dedication

To my parents, brother and granddad whose love and support makes everyday special



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# Abbreviations

20S	Alpha proteasome subunit
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AIS	Anaemia-inducing factor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Ang I	Angiotensin I
Ang II	Angiotensin II
APP	Acute-phase protein
APS	Ammonium persulphate
A-Smase	Acidic sphingomyelinase
AT1	Angiotensin type 1 receptor
AT2	Angiotensin type 2 receptor
ATP	Adenosine triphosphate
AVP	Vasopressin
BAD	Bcl-2-associated death promoter
BAT	Brown adipose tissue
BCAA	Branched chain amino acids

BSA	Bovine serum albumin
C-26	Colon-26 tumour
Ca <sup>2</sup>	Calcium
Ca/Cam	Calcium/calmodulin
camK	Calcium/calmodulin dependent protein kinase
Caspases	CysteinyI aspartate specific proteinases
CGN2	General control nonderepressible 2
CHO	Chinese hamster ovary
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPM	Counts per minute
CRP	C-reactive protein
Ctl	Control
DAG	Diaglycerol
ddH <sub>2</sub> O	Double distilled water
DFF	DNA fragmentation factor
DMEM	Dulbecco's modified eagles media
DMSO	Dimethyl sulfoxide
DNA	Deoxy nucleic acid
DNTs	Deoxy nucleotides
DPM	Disintegrations per minute
dsRNA	Double stranded ribonucleic Acid
DTT	Dithiothreitol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2

EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid
eIF	Eukaryotic initiation factor
EMSA	Electrophoretic mobility shift assay
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FKHR	Forkhead protein
g	Gram
G $\beta$ L	G-protein beta-subunit-like protein
GAP	GTPase activating protein
GCN4	General control nonderepressible 4
GDP	Guanosine diphosphate
GH	Growth hormone
GHS-Rs	GH secretagogue receptors
GLUT	Glucose transporters
GNEF	Guanine nucleotide exchange factor
GSH	Glutathione synthetase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
h	Hour
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HA	Heamagglutinin
HCL	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic Acid
HIV	Human immunodeficiency virus
HMB	$\beta$ -Hydroxy- $\beta$ -methylbutyrate
HPSF	High-purity salt-free
HRI	Heme-regulated inhibitor

HSV	Herpes Simplex Virus
I $\kappa$ B	Inhibitor of kappaB
I $\kappa$ K	I $\kappa$ B kinase complex
IFN $\gamma$	Interferon gamma
IGF-1	Insulin like growth factor 1
IL-1	Interleukin 1
IL-1 $\beta$	Interleukin 1 beta
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
Ins	Insulin
IP3	Inositol 1,4,5-triphosphate
Iso	Isoleucine
kDa	Kilo Dalton
kg	Kilo gram
KOH	Potassium hydroxide
L	Litre
Leu	Leucine
LIF	Leukemia inhibitory factor
LMF	Lipid mobilizing factor
LMP2	Large multifunctional protease 2
LMP7	Large multifunctional protease 7
LPS	Lipopolysaccharide
m	Milli
M	Mole
m <sup>7</sup> GTP	7-methylguanosine triphosphate



MAC	Murine adenocarcinoma
MAFbx	Muscle atrophy F-box protein
MAPK	Mitogen activated kinase
MDA	Malondialdehyde
MEC1	Multicatalytic endopeptidase complex
Met-tRNA	Methionyl tRNA
min	Minute
Mr	Molecular mass
mRNA	Messenger RNA
mTOR	Mammalian target of Rapamycin
MuRF1	Muscle specific ring finger protein 1
n	Nano
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NaOH	Sodium hydroxide
NF- $\kappa$ B	Nuclear factor kappa B
NIK	NF- $\kappa$ B inducing kinase
NK cells	Natural killer cells
NO	Nitric oxide
NPY	Neuropeptide Y
N-Smase	Neutral sphingomyelinase
NTB	Non tumour bearing
OD	Optical density
P42	ATPase 19S proteasome subunit
p70S6K	70 kDa ribosomal protein S6 kinase
PA700	Proteasome activator 700
PACT	PKR activating protein
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEK	Pancreatic eIF2 $\alpha$ kinase
PERK	ER resident PKR-like endoplasmic reticular kinase
PGE2	Prostaglandin E2
PGE2 $\alpha$	Prostaglandin E2 alpha
PI-3K	Phosphatidylinositol-3 kinase
PIF	Proteolysis inducing factor
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PKR	dsRNA dependent protein kinase
PKR I	PKR inhibitor
PLA2	Phospholipase A2
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl fluoride
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
RAAS	Renin-angiotensin-aldosterone system
Rap	Rapamycin
Raptor	Regulatory associated protein of mTOR
REE	Resting energy expenditure
RHEB	Ras homolog enriched in brain
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Repetitions per minute
rpS6	Ribosomal protein S6
s.c.	Subcutaneously

Sal	Salubrial
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Standard error mean
SOD	Superoxide dimutase
TCA	Tricarboxylic Acid
T-cells	Thymus dependent lymphocytes
TEMED	Tetramethylethylenediamine
TNF $\alpha$	Tumour necrosis factor alpha
TOP	Terminal oligopyrimidine
TPPII	Tripeptidyl-peptidase II
tRNA	Transfer RNA
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
UCP	Uncoupling protein
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UCP3	Uncoupling protein 3
UKCCR	UK Co-ordinating Committee on Cancer Research
Val	Valine
WAT	White adipose tissue
ZAG	Zn- $\alpha$ 2-glycoprotein

# Chapter 1

## Introduction

### 1.1 Cachexia

Cachexia is a wasting disease characterized by anorexia, anaemia, asthenia and loss of lean body mass, leading to an increased mortality rate. Cachexia is seen in a number of life threatening diseases such as cancer, AIDS, sepsis, chronic heart failure, severe burns, diabetes and rheumatoid arthritis and is a highly complex metabolic disorder involving lipolysis, activation of the immune response and insulin resistance [3].

In cancer related cachexia the condition leads to a poor prognosis and quality of life. More than half of cancer patients develop cachexia and 22% of these patients die from cachexia rather than from the tumour itself [3].

The negative nitrogen balance underlying cancer cachexia leads to a significant wasting of skeletal muscle. Loss of both adipose tissue and lean muscle mass is seen in cachexia, however it is the chronic loss of muscle that most often disrupts the physiological functions of patients. Muscle loss reduces patient mobility,

jeopardizes respiratory function, relates to reduced immunity, and is associated with reduced response to therapy [2]. Muscle wasting is an important component of the process of cachexia. In a study of lung cancer patients who had lost 30% of their pre-illness stable weight, the overall change reflected a 75% decrease in skeletal muscle mass while visceral protein reserves were preserved, and kidney and heart muscle weights remained unchanged. In fact there was shown to be a 2-fold increase in non-skeletal muscle protein synthesis (most likely arising from an increased hepatic production of acute phase proteins) [111].

It has been suggested that a tumour constitutes a new metabolically active organ requiring its own sustenance and so increasing demand for nutrients and causing weight loss if these are not forthcoming. However, the presence and severity of cachexia often correlates poorly with the size of the tumour [113], where cachexia can arise in a patient with a tumour comprising <0.01% of the host weight [190].

It seems that the weight loss observed in the cachectic patient is as a result of decreased protein biosynthesis (anabolism) and increased protein breakdown (catabolism). Hypoanabolism implies a deficit in the supply of amino acids, or energy, or a failure of normal anabolic stimuli required for muscle protein synthesis to proceed. This is demonstrated in a study of lung and colon cancer patients with cachexia, where muscle protein synthesis only accounted for 8% of total body protein synthesis, compared with 53% for healthy controls. Hypercatabolism involves participation of catabolic mediators and activation of intracellular proteases [113]. Competition for nutrients between the tumour and the host leads to hypermetabolism resulting in an accelerated starvation state.

A diverse picture of energy expenditure has been described in cancer patients with resting energy expenditure varying between less than 60% and more than 150% of predicted values, where patients with tumour types associated with cachexia (such as lung and pancreas) tend to exhibit an elevated resting energy expenditure [64]. Even normal energy expenditure could be classified as being in excess since cachectic cancer patients have reduced calorific intake. An increase in basal metabolic rate by just 12% can account for the loss of up to 1-2kg of body weight per month. Elevated energy expenditure can be attributed to increases in Cori cycle activity (catalytic conversion of lactic acid to glucose), glucose and triglyceride-fatty acid cycling and gluconeogenesis. Most solid tumours rely mainly on anaerobic metabolism for glucose,

due to their poor vascularization, leading to hypoxic conditions where most of the glucose is converted to lactate. Tumours in patients with progressive cancer have also shown an increased production of glucose from alanine and glycerol [186]. Thus it may be the tumour itself that is regulating the increase in energy expenditure. One report showed that glucose uptake in the tumour exceeds that of the peripheral tissues, and that glucose utilization is second only to that of the brain. The increased glucose recycling has been estimated to cause a potential loss of 0.9kg of body fat per month. An increased heart rate due to elevated production of catecholamines has also been suggested to play a role in increasing energy expenditure in cancer patients, while another contributing factor to raised metabolic rate is the presence of brown adipose tissue (BAT). BAT plays a role in energy balance in many mammals, including humans where cachectic cancer patients bear evidence of brown adipose tissue [185].

Cancer-related nutritional deterioration is traditionally attributed to continued loss of lean body mass, altered carbohydrate and lipid metabolism; the latter may ensue from increased metabolic rates, and the production and release of proinflammatory cytokines, hormones and cancer produced factors such as proteolysis inducing factor (PIF) and lipid mobilizing factor (LMF). However the progressive caloric deficit may be exacerbated by anorexia, where patients have a reduction in food intake due to factors such as physical obstruction of the gastrointestinal tract, vomiting and malabsorption associated with cancer itself, constipation, psychological problems including depression and pain and the side effects of treatment with opiates, radiotherapy and chemotherapy [64]. The relative contribution of the above conditions to nutritional depletion is thought to differ according to progression of the cancer, cancer type or site e.g. patients with neck and gastro-oesophageal cancer were, at early stages, already severely depleted and showed significant dietary reductions [148], because cancer of the digestive organs such as the oesophagus, stomach or pancreas means the cancer patient is subjected not only to the localized and systemic effects of carcinoma but to the body's inability to properly nourish itself [74].

During the early days of starvation the brain will utilize liver and muscle glycogen stores and increase glucose production from lactate and gluconeogenic amino acids by the liver. Long term starvation leads to the production of ketone bodies from fatty acid metabolism which the brain will depend for energy upon, reducing the requirement for glucose and thus preserving lean body mass. Therefore in anorexia the



depletion of adipose tissue exceeds that of lean muscle mass [188]. However, there is an equal loss of both adipose tissue and muscle protein in the cachectic state, which nutritional supplementation can not reverse. This suggests that anorexia is not the main cause of weight loss in cachexia since starvation leads to a decrease in fat reserves but preserves body protein reserves [189]. In fact a study of 297 cancer patients showed that the caloric intake of cancer patients demonstrating weight loss was the same if not higher than that of weight stable cancer patients [190]. There are also changes in plasma amino acid concentrations where a decrease of gluconeogenic amino acids is seen. This is different from malnourished states where the concentration of branched chain amino acids in plasma is normal or increased [188]. Therefore the metabolic changes in anorexia and cachexia are likely to be different where other host and tumour produced factors may be involved [189].

The majority of patients with advanced cancer lose weight and exhibit to some extent, the features of cachexia. In terms of weight loss, this is more common in patients with lung and gastrointestinal cancers and less often in breast cancer. In fact 85% of patients with pancreatic cancer are diagnosed with cachexia [188]. However, although certain tumour types are more commonly associated with cachexia, it is remarkable how patients with the same tumour type can vary in the extent to which they become cachectic. Such observations point to variations in tumour phenotype or host genotype contributing to the development of cachexia and emphasise the importance of host tumour interaction in the genesis of the syndrome [64].

## 1.2 MAC16 Tumour

The MAC16 tumour is a cachexia inducing murine transplantable colonic adenocarcinoma which has pathogenic similarities to wasting in human gastrointestinal and pancreatic cancers. The MAC16 induces severe weight loss, which is a combination of both muscle and fat depletion, without stimulation of hunger, which is normally seen during starvation conditions [26]. MAC-16 induced cachexia occurs with very small tumour burdens (<1% of body weight) and in the absence of metastases. The cachexia is reversible if the tumour is removed, suggesting that the wasting is due to tumour produced products such as Lipid

mobilizing Factor (LMF) and Proteolysis Inducing Factor (PIF) which are released into the system and have catabolic actions on secondary tissues [26].

The MAC13 tumour is histologically similar but does not induce cachexia or produce PIF which makes it useful for experimental comparison with MAC16 tumours.

### 1.3 Tumour Derived Factors

#### Proteolysis Inducing Factor

Another potential mediator of cachexia is the 24kDa glycoprotein called proteolysis inducing factor (PIF), which was first isolated from the cachexia inducing murine MAC16 tumour and from the urine of cancer patients with weight loss [187]. The presence of PIF in the urine was strongly associated with weight loss in patients with gastrointestinal tumours and it was determined that the tumour itself is the source of PIF [187]. PIF is not detectable in the urine of patients with benign weight losing disease states, which supports the concept that PIF is a cancer specific cachectic factor [206]. Structural studies of PIF indicate a Mr 24kDa glycoprotein consisting of a Mr 4kDa central polypeptide chain that may be attached to a polypeptide or a short oligosaccharide chain containing glucosamine; one Mr 6kDa O-linked sulfated oligosaccharide chain containing glucosamine; and one Mr 10kDa N-linked sulfated oligosaccharide chain also containing glucosamine. The PIF sequence contains a single Asn residue, which must be the site for N-glycosylation [195], and this glycosylation is necessary for functional activity [206]. Although some cytokines, such as tumour necrosis factor alpha ( $TNF\alpha$ ) have been shown to induce cachexia, there is no evidence for cytokine involvement in the MAC16 cachexia model [167]. Instead PIF appears to be solely responsible for the onset of skeletal muscle atrophy. PIF expression has been found in carcinomas of the prostate, colon, lung, esophagus, liver and pancreas, when weight loss is apparent, but not in normal tissue [167]. It has been shown that in adult tissue PIF only exhibits substantial binding affinity for skeletal muscle and liver [204]. PIF is able to induce both the proinflammatory response and the acute phase response through production of cytokines like IL-8 and IL-6, both of which have also been



linked to muscle proteolysis [167]. Thus PIF is able to induce muscle wasting directly, as well as indirectly through upregulation of cachexia inducing cytokines. PIF may also have a role in tumour development and progression through the induction of syndecan shedding. Syndecans are a family of transmembrane proteoglycans, which are able to interact with adhesion molecules. Loss of syndecan-1 has been linked to the uncontrolled proliferation and differentiation of tumour cells. Therefore the interference of PIF with syndecan expression may contribute to the pathogenesis/metastasis of the tumour [204].

PIF inhibits the translation process of protein synthesis, activates protein degradation specifically, and also stimulates the ATP-proteasome-dependent pathway [8]. Thus, evidence for increased levels of ubiquitin-conjugated proteins, 14kDa ubiquitin carrier protein E2, and C8 and C9 proteasome subunits, in atrophying gastrocnemius muscle, have been reported [9], where proteasome inhibitors MG115 and lactacystin completely attenuated protein degradation induced by PIF, confirming the relevance of activation of the proteasome pathway to catabolism of skeletal muscle proteins [111]. Induction of proteasome expression by PIF is associated with an increased DNA-binding activity of the transcription factor NF- $\kappa$ B [210]. PIF has also been shown to increase activity of the protease tripeptidyl-peptidase II (TPPII), which is believed to be regulated in parallel with the proteasome, where both proteasome proteolytic activity and TPPII activity increased with increasing weight loss in mice bearing the MAC16 tumour [38]. Moreover, PIF appears to have a direct inhibitory effect on the glucose consumption by skeletal muscle, since uptake of 2-deoxyglucose by C2C12 myoblasts in vitro is inhibited by PIF [9].

The compound, when injected into healthy animals, is able to mimic the muscle-wastage associated with experimental cancer cachexia. In vitro studies on C2C12 myoblasts have shown that eicosapentaenoic acid is able to block PIF action on proteolysis, which suggests that PIF acts intracellularly, via the arachidonate metabolite 15-hydroxyeicosatetraenoic acid (15-HETE) [8]. In fact it has been demonstrated that PIF induces activation of PLA2 [165], which produces an increased release of arachidonic acid from membrane phospholipids, which is then metabolised to prostaglandins, PGE2 and PGF2 $\alpha$ , and 15-HETE. However only 15-HETE was shown to produce a significant increase in protein degradation [111]. The signals mediating the response of PIF to the coordinate upregulation of ubiquitin, E2 and proteasome subunits are unknown but are likely to involve the metabolites of arachidonic acid [111] and PKC [167]. However EPA does not

attenuate the inhibitory effect of PIF on protein synthesis, therefore the block of the translation process by PIF must be activated by factors not involved with the arachidonic acid pathway [9].

PIF has also been shown to induce apoptosis in C2C12 myotubes where an increase in free nucleosome formation and increased DNA fragmentation was observed concomitant with an upregulated expression of the apoptotic initiator caspases-8 and 9 and the apoptotic effector caspases-2, 3 and 6. It was also demonstrated that PIF can increase the cytosolic content of cytochrome c (precedes morphological change associated with apoptosis), as well as expression of the pro-apoptotic protein bax. Increased apoptosis may contribute to the loss of muscle mass in cancer cachexia [164].

In conclusion, PIF might have a constitutive function in normal states and become altered or overproduced during cancer cachexia [8]. No correlation between expression of PIF and anatomical stage of cancer has been identified, which may indicate that expression is determined by the phenotype of the tumour rather than by bulk of disease [206]. There is some evidence that the normal role of PIF is involved in developmental regulation in embryonic development where a peak of expression in the mouse embryo at embryonic day 8.5 has been observed [204].

## **Lipolysis Mobilizing Factor**

Cachectic cancer patients demonstrate a drastic loss of fat mass, where as much as 85% of adipose tissue can be lost during the cachectic process, either through increased lipolysis or decreased lipogenesis [188]. Cancer patients have a high turnover of both glycerol and free fatty acids, which may arise from increased  $\beta$ -adrenergic receptor activity, where patients with weight loss show elevated levels of plasma and urinary catecholamines, an elevated heart rate and increased fat oxidation. Many proinflammatory cytokines such as  $\text{TNF}\alpha$ , IL-6 and LIF can stimulate lipolysis through the inhibition of lipoprotein lipase [188]. However the lipid-mobilizing factor (LMF) is able to induce lipolysis directly in adipose tissue, thus contributing to loss of adipose tissue in cancer patients. This factor was originally purified from the cachexia-inducing mouse colon adenocarcinoma, MAC16, but has also been found in the urine of cancer patients, suggesting that it is able to induce lipid mobilization and catabolism in cachectic cancer patients. LMF initiates lipolysis

in adipose tissue in a manner similar to lipolytic hormones [156] through increases in intracellular cyclic AMP in adipose tissue through a GTP-dependent stimulation of adenylate cyclase. In addition, adipocytes from cachectic mice bearing the MAC16 tumour showed a three-fold increase in lipolytic response to both isoprenaline and LMF compared with those from normal mice. This effect seems to be due to the alteration of G-protein expression by LMF, with an up-regulation of  $G_{\alpha s}$  and a downregulation of  $G_{\alpha i}$ . Therefore, LMF both sensitizes and stimulates the adipocytes to increased lipolysis [187]. A similar LMF was also found to be present in the serum of cancer patients with weight loss, and activity appeared to be directly proportional to the extent of weight loss. LMF has been shown to induce lipolysis through binding to  $\beta 3$ -adrenoreceptor [156]. However LMF has an anabolic effect on skeletal muscle by downregulation of proteasome expression, decreasing protein degradation, and increasing protein synthesis related to an increase in intracellular cyclic AMP levels. This anabolic effect is also induced through a  $\beta 3$ -adrenoreceptor. This anabolic effect tends to oppose the catabolic effect of PIF in skeletal muscle, which partly explains why protein catabolism is not seen until weight loss approaches 15-20%, when LMF production ceases [187].

LMF has been found to produce an increase in oxygen uptake by interscapular brown adipose tissue (BAT), providing evidence for increased lipid utilization. The mitochondrial uncoupling proteins (UCP) -1, -2 and -3 have been suggested to play essential roles in energy dissipation and disposal of excess lipid. Further studies have shown that LMF increases UCP-1, -2 and -3 in BAT and UCP-2 in both skeletal muscle and liver. Up-regulation of these UCPs by LMF may serve to utilize excess lipid mobilized during fat catabolism in cancer cachexia [156]. LMF was originally thought to be only produced by the tumour, although it has now also been shown to be produced in both white and brown adipose tissue [189].

LMF is homologous to the plasma protease, Zn- $\alpha$  2-glycoprotein (ZAG), in its amino acid sequence, electrophoretic mobility and immuno-reactivity [8] and it has been observed that both LMF and ZAG have the same biological activity, producing significant decreases in body fat via the induction of lipolysis by a cyclic AMP-mediated system, through interaction with a  $\beta 3$ -adrenoreceptor [156].

ZAG is a 43kDa protein which has been detected in several organs such as the breast, prostate, liver, lung and skin. Its overexpression has been described in several malignant tumours, and can therefore be

used as an early cancer marker. ZAG is present in subcutaneous and visceral WAT, specifically in white adipocyte cytoplasm and the stroma-vascular fraction during normal expression. It is likely that under normal circumstances ZAG is involved in body weight regulation [43].

### **Anaemia Inducing Factor**

Anaemia-inducing factor (AIS) is a protein of  $\approx 50$ kDa that is secreted by malignant tumour tissue, thus depressing erythrocyte and immunocompetent cell functions. This protein is able to reduce food intake, body weight and body fat in rabbits. In addition, it shows an important lipolytic activity [8].

### **Toxohormone-L**

Toxohormone-L, a polypeptide of  $\approx 75$ kDa was isolated both from the ascites fluid of patients with hepatoma and sarcoma-bearing mice, and was found to induce lipid mobilization, immunosuppression and involution of the thymus [8].

### **Cytokines**

Tumour-produced cytokines might even have a more important role in the anorexia-cachexia syndrome than those produced by the host. Cancer cells are capable of producing cytokines constitutively. These cytokines might act on the cancer cells in an autocrine manner, or on the supporting tissues, such as fibroblasts and blood vessels, to produce an environment that is conducive to cancer growth [8].



## 1.4 Host Derived Cachectic Factors

### Angiotensin

Activation of the rennin-angiotensin-aldosterone system (RAAS) results in the formation of Angiotensin II (Ang II) which leads to vascular hypertrophy, vasoconstriction, salt and water retention, hypertrophy, and cardiac and renal tissue damage. Circulating renal derived rennin cleaves hepatic derived angiotensinogen to form the decapeptide angiotensin I (Ang I), which is then converted to the active Ang II by angiotensin converting enzyme (ACE). ACE is present in all major organs, including the heart, brain, blood vessels, adrenals, kidney, liver, and reproductive organs. Chymotrypsin-like serine protease may represent an ACE-independent pathway for formation of Ang I and Ang II in the human heart, kidney, and vasculature. Ang II is a vasoactive octapeptide hormone which has a role in cardiac hypertrophy, myocardial infarction, hypertension, and atherosclerosis. It mediates these results directly through interaction with two receptors: the Ang II type 1 (AT1) and type 2 (AT2) receptors, and indirectly through release of other factors and cross talk with intracellular signalling cascades of vasoactive agents, growth factors, and cytokines. Most of the biological functions of Ang II are thought to be mediated by the AT1 receptor. The AT1 receptor belongs to the seven-membrane spanning G-protein coupled receptor family and are widely distributed throughout the cardiovascular, renal, endocrine, and nervous system in humans. Activation of the AT1 receptor stimulates vasoconstriction, vascular cell hypertrophy and hyperplasia, and sodium retention as well as production of reactive oxygen species (ROS), induction of inflammatory, thrombotic, and fibrotic responses, and in some circumstances apoptosis in some cell types. It does this through the activation of a variety of intracellular pathways including transactivation of receptor tyrosine kinases, PLA2 production which is responsible for arachidonic acid release from cell membrane phospholipids, 15-HETE and calcium release, activation of PKC and NF- $\kappa$ B. The most characterized of the signalling pathways linked to AT1 receptor activation is that of the protein kinase C (PKC) pathway, involving stimulation of phospholipase C (PLC), with subsequent inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) generation, leading to the activation of PKC [73]. The AT2 receptor is also a member of the seven transmembrane type G-protein coupled receptors although it has low sequence homology ( $\approx 34\%$ ) with the AT1 receptors. The AT2 receptor is detectable in the pancreas, heart, kidney, adrenals, brain, and vasculature. Its vasodilator,

antigrowth, and apoptotic actions appear to counterbalance the effects of the AT1 receptor demonstrating a physiological role in regulating blood pressure and renal function [106, 21]. Congestive heart failure is increasing in prevalence and is the leading cause of cardiovascular mortality and morbidity. The syndrome of advanced heart failure often includes muscle wasting, commonly termed cardiac cachexia, which is a predictor of poor outcome [51]. There is recent evidence that increased angiotensin II plays an important role in cardiac cachexia, since the rennin activity was higher in cachectic subjects than in non-cachectic subjects [154]. Ang II has been shown to induce protein degradation in skeletal muscle via increased mRNA levels of the ubiquitin ligases atrogin-1 and Muscle RING finger-1 in skeletal muscle, and can increase the production of TNF $\alpha$  and IL-6 which are known to be pro-cachectic cytokines, although the effect was considered indirect and mediated by intermediate factors such as glucocorticoids. The induction of protein degradation and the ubiquitin proteasome pathways by Ang II has been shown to be similar to PIF in that they both stimulate release of PLA2, PLC and arachidonic acid as well as 15 HETE and PKC production leading to an increased nuclear localization of NF- $\kappa$ B resulting in an increase in transcriptional activation and increased proteasome expression [155]. The stimulation of protein degradation by Ang II in vitro has been determined a direct effect [159]. The ACE inhibitor imidapril attenuated the development of weight loss in mice bearing the MAC16 tumour, suggesting that Ang II may play a role in the development of cachexia in this model [159]. Ang II has also been shown to modify muscle mass through a depression in the rate of protein synthesis. It has been suggested that it does this through an effect on translation initiation, similar to cytokines and possibly PIF [154].

## **Uncoupling Proteins**

Uncoupling proteins (UCPs) translocate protons across the inner mitochondrial membrane, in a process that is not coupled to ADP phosphorylation, stimulating heat production. There are three types of UCP; UCP1 which is present only in brown adipose tissue (BAT); UCP2 which is expressed ubiquitously; and UCP3 which is found only in skeletal muscle of humans and BAT of rodents. BAT is normally only found in human neonates and is not usually present in adult humans. However, BAT was found in perirenal tissue in 80% of cachectic patients compared to 13% of age-matched control subjects. UCP1 mRNA has been shown to be elevated in BAT of mice bearing the cachexia inducing MAC16 adenocarcinoma

while both UCP2 and 3 mRNAs have been found elevated in skeletal muscle of cachectic cancer patients during tumour growth. Implantation of the cachexia inducing Lewis lung carcinoma resulted in profound muscle wasting and a significant increase in both UCP2 and UCP3 gene expression in skeletal muscle and heart [33]. The presence of UCPs would increase the REE of cancer patients and therefore exacerbate weight loss [9, 190].

## **Cytokines**

Elevation of circulating pro-inflammatory cytokines is a common feature of cachexia [201]. In animals, administration of many pro-inflammatory cytokines mimics the symptoms seen in cachexia such as anorexia, weight loss, acute phase protein response, protein and fat breakdown, an increase in levels of glucocorticoids and glucagon and a decrease in insulin levels, insulin resistance, anaemia, fever and elevated energy expenditure. However, individual cytokines do not work alone in the *in vivo* situation, instead a complex network of cytokines in combination with other factors are involved [64].

The major sources of these pro-inflammatory cytokines are monocytes and macrophages, although other cell types, such as T-lymphocytes or even non-immunological cells like endothelial cells are also capable of their production [201].

## **Tumour Necrosis Factor- $\alpha$**

Episodic tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) administration has proved unsuccessful at inducing cachexia in experimental animals. Repetitive TNF $\alpha$  administrations initially induce a cachectic effect, however, tolerance to the cytokine soon develops and food intake and body weight return to normal [9]. It has been demonstrated that escalating doses of TNF $\alpha$  are necessary to maintain the cachectic effects [9].

However other studies have shown that administration of TNF $\alpha$  to rats results in an increased skeletal muscle proteolysis that is associated with an increase in proteasome subunit gene expression and higher levels of free and conjugated ubiquitin, both in experimental animals and humans [8]. Levels of TNF $\alpha$

were found to be elevated in the circulation of cancer patients, and is thought to contribute to multiple aspects of cachexia including the potent stimulation of fat degeneration [3].

Low doses of  $\text{TNF}\alpha$ , injected peripherally or into the brain of laboratory animals, elicits rapid increases in metabolic rate that are not associated with increased metabolic activity but, rather, with an increase in blood flow and thermogenic activity associated with uncoupling protein (UCP1) in brown adipose tissue.  $\text{TNF}\alpha$  is able to mimic the increase in gene expression of UCPs seen in cachectic states. Thus TNF is able to induce uncoupling of mitochondrial respiration and induce weight loss [8].

López-Soriano et al [9] demonstrated that the proteolytic mechanism, which is involved in skeletal muscle wasting during cancer cachexia, can be activated by  $\text{TNF}\alpha$ . In addition to muscle protein degradation, during cancer cachexia muscle DNA is also decreased, resulting from DNA fragmentation and apoptosis.  $\text{TNF}\alpha$  can mimic the apoptotic response in muscle of healthy animals, which is evidence that it is involved in the induction of muscle cell apoptosis seen in cancer cachexia [9].

$\text{TNF}\alpha$  has been shown to induce transcriptional activation of the genes encoding IL-8 and synthesis of IL-8 protein. IL-8 is another proinflammatory cytokine which can recruit neutrophils and lymphocytes to the site of tissue damage. However IL-8 also has angiogenic properties and may have a role in tumour proliferation and implantation [205].

### **Ciliary Neurotrophic Factor**

Ciliary Neurotrophic Factor (CNTF) is a member of the family of cytokines that includes IL-6 and LIF, and is produced predominantly by glial cells of the peripheral nervous system; however, this cytokine also seems to be expressed in skeletal muscle. Henderson et al [78] have demonstrated that CNTF induced potent cachectic effects and acute-phase protein (APP) synthesis in mice implanted with C6 glioma cells, genetically modified to secrete this cytokine. The cytokine however, exerted divergent direct effects on in vitro muscle preparations, which were dependent on the dose and the time of exposure [9].



## Interleukin-6

Interleukin-6 (IL-6) is the main inducer of liver synthesis of acute phase proteins. IL-6 is also involved in rapid weight loss, which frequently accompanies short-term disease such as sepsis, trauma, or burns [129] and is also elevated in chronic diseases such as rheumatoid arthritis [61]. Some human studies have shown an increased IL-6 response in nutritionally depleted cancer patients [129]. IL-6 shares a structural and functional similarity to the anorexogenic hormone called leptin. Both are released in response to injury at a similar time and can activate the same receptor in the hypothalamus. This may explain why IL-6 can still cause a reduction in fat mass in experimental rodents without causing an acute phase response [205] (discussed in section 1.5).

Strassmann and co-workers [172] have shown that treatment with an anti-mouse IL-6 antibody was successful in reversing the key parameters of cachexia in colon adenocarcinoma tumour-bearing mice [8]. The atrophy of skeletal muscle observed in IL-6 transgenic mice and rats treated with IL-6 was associated with increased mRNA levels of cathepsin (B and L) and ubiquitins and myofibrillar protein degradation. In vitro studies of C2C12 murine myotubes administered IL-6 showed an increase in the activity of the 26S proteasome and lysosomal proteolytic pathways, which are often associated with the cachexia syndrome [185]. CHO cells transfected with the IL-6 gene have been shown to produce a syndrome of cachexia in nude mice [27]. Moreover, Serum levels of IL-6 have been found to increase to similar levels in one clone of colon 26 adenocarcinoma, which produced the syndrome of cachexia when transplanted into syngenic mice [169]. Conversely, other studies, using a very similar mouse tumour model to that of Strassmann, have revealed that IL-6 is not involved in cachexia. These results indicate that, at least in certain types of tumours, IL-6 could have a direct involvement in the cachectic state [8].

## Interferon- $\gamma$

Another candidate for cachexia is interferon- $\gamma$  (IFN- $\gamma$ ), which is produced by activated T and NK cells. Using a monoclonal antibody against IFN- $\gamma$ , Matthys et al [118] were able to reverse the wasting syndrome associated with the growth of the Lewis lung carcinoma in mice, thus indicating that endogenous production

of IFN- $\gamma$  occurs in the tumour-bearing mice, and is instrumental in bringing about some of the metabolic changes that are characteristic of cancer cachexia [8]. Inoculation of CHO cells transfected with the IFN- $\gamma$  gene into mice resulted in a dose dependent development of anorexia and cachexia. However, other studies have shown that IFN- $\gamma$  on its own can not induce cachexia, suggesting that it is not the sole mediator in the development of cachexia [185].

Other cytokines such as leukaemia inhibitory factor (LIF), transforming growth factor- $\beta$  (TGF- $\beta$ ), interleukin 1 (IL-1), interleukin 2 (IL-2) and interleukin 8 (IL-8) have also been suggested as mediators of cachexia. However, although IL-1 has anorectic and pyrogenic effects it does not seem to have a role in the cachectic process. The final wasting status is determined by the balance between the already mentioned pro-cachectic cytokines and the anti-cachectic cytokines, such as interleukins-4, 10 and 13, and also by soluble receptors for TNF $\alpha$  and interleukin-6 [9].

## Hormones

Increase in the plasma levels of hormones such as vasopressin (AVP), prostaglandin E2 (PGE2), glucagon, cortisol, catecholamines and reduction of insulin levels have been reported to contribute to the development of cachexia [142]. Infusion of hydrocortisone or cortisol, glucagon and adrenaline in humans produce features of cachexia such as protein loss, an acute phase protein response, increased energy expenditure and glucose intolerance.

In humans with cancer, elevated levels of cortisol and glucagon have been observed, and these may amplify the acute phase protein response. A blunted insulin secretory response to a glucose load has been described in patients with colorectal cancer. In addition, insulin resistance in terms of glucose metabolism has been noted, particularly in pancreatic cancer, due to the production of islet amyloid polypeptide (amylin) by the surrounding normal pancreatic tissue. The production of amylin appears to be stimulated by the presence of pancreatic cancer cells. Amylin will induce anorexia and weight loss when administered to rats. Changes in hormone levels and target-organ sensitivity have been described in both animals and humans with cachexia, where profound changes are seen in hormone levels in experimental tumour-bearing animals [64].

## Ghrelin

Ghrelin is a 28-amino-acid peptide that is the natural endogenous ligand for growth hormone (GH) secretagogue receptors (GHS-Rs), which were originally isolated from rat and human stomach and then subsequently identified in various tissues, including small bowel, pancreas, kidney, pituitary, and hypothalamus. Ghrelin exerts various endocrine and non-endocrine effects, including the control of energy homeostasis and food intake [138].

It has been demonstrated that weight loss and restriction of caloric intake increase ghrelin expression and secretion, which suggest it may have a role to play in the weight loss experienced in cancer patients with cachexia. Indeed, in human's ghrelin circulating levels are elevated in anorexia and in cachexia [138].

## Leptin

Leptin is a 164kDa adipocyte derived hormone. Leptin is secreted from adipocytes in white adipose tissue when fat stores are full and suppresses appetite by acting on the hypothalamus. Circulating leptin concentrations regulate energy intake and expenditure by a control feedback loop involving neuropeptide-Y (NPY). In humans leptin's major role is believed to be in the adaptation to reduced energy intake and reduced body fat stores [205]. Leptin production is linked to the induction of anorexia and weight loss in patients with chronic illness and inflammation. However, in many diseases associated with cachexia, such as chronic inflammatory bowel disease, there is an inflammatory status where leptin levels are decreased related to body fat mass. In cancer cachexia leptin has also been demonstrated to be lower reflecting the depleted energy stores and alterations of energy metabolism seen in these patients. It has been suggested that leptin levels are modulated by invading macrophages secreting TNF- $\alpha$  or prostaglandin E2 in the early stages of cancer cachexia [112, 139]. In rats implanted with the cachexia inducing Walker 256 tumour a reduced concentration of leptin was observed in WAT depots and in the plasma, while in mice bearing the MAC16 tumour a decrease in leptin mRNA in the gonadal WAT was discovered [113].

## 1.5 The Acute Phase response

An acute phase protein response may be seen in a significant proportion of patients with cancer of the pancreas, lung, kidney and oesophagus and the proportion of pancreatic cancer patients exhibiting an acute phase response tends to increase with disease progression [64]. In fact at the time of diagnosis approximately 50% of patients with pancreatic cancer will have an elevated acute phase response. The acute phase response has been associated with an increased energy expenditure, reduced nutritional intake and weight loss [205]. The presence of an acute phase response has been related to accelerated weight-loss in both lung and pancreatic cancer and melanoma. Moreover, the presence of such a response in cancer patients is strongly associated with a shortened survival in renal, pancreatic and colorectal cancer [64]. Activation of the acute phase response can be detected from an elevated serum C-reactive protein (CRP). CRP can bind to denatured proteins, LPS and nucleic acids which lead to complement formation and phagocytosis by macrophages through their complement receptors. Activation of complement causes a release of IL-6 by macrophages which stimulates synthesis of more C-reactive protein, causing a positive feedback loop for C-reactive protein secretion. This process is energy intensive requiring essential amino acids which are obtained from skeletal muscle, since skeletal muscle accounts for almost half of the body protein mass. In chronic conditions such as cachexia this process depletes skeletal muscle tissue faster than it can be replaced, since there is no recovery respite, which leads to severe muscle wasting [115].

The acute phase protein response is known to aid tissue repair, blood clotting, the prevention of ongoing tissue damage and the destruction of infective organisms. In this process liver protein synthesis shifts from synthesis of albumin to production of acute phase proteins such as CRP, serum amyloid A protein,  $\beta_2$  macroglobulin and  $\alpha_1$  antitrypsin. The value of the acute phase protein response in patients with cancer is not clear, and it may be that it occurs simply as part of a response to inflammation. It is stimulated at least in part by proinflammatory cytokines, particularly interleukin IL-6. Tumour factors associated with cachexia, such as PIF can upregulate the acute phase response through the activation of NF- $\kappa$ B, which upregulates target genes such as proinflammatory cytokines and CRP. Furthermore, some tumour cell lines produce proinflammatory cytokines, and it is possible that the tumour may benefit from changes associated

with the acute phase response like altered energy substrate metabolism, such as release of amino acids from skeletal muscle, which may aid nutrition of the tumour [188, 64].

## 1.6 Apoptosis and Caspases

Cells die in two different ways. When they die through accidental injury, they swell and burst, and flood their surroundings with their internal components, in a process called necrosis. Cells may also undergo a controlled or programmed cell death in a process called apoptosis. This process removes obsolete, damaged or infected cells. Caspases are the executioners of apoptosis. Caspases are a structurally related group of cysteine proteases that cleave peptide bonds following aspartate residues. They play a central role in activating the apoptotic cell death machinery. During apoptosis DNA fragmentation factor (DFF) is activated, leading to fragmentation and condensation of the cell's genetic material. The ATP-dependent ubiquitin proteolytic pathway rapidly removes products of caspase actions. Adhesion proteins are cleaved, releasing the cell from the surrounding tissue, and the lipids in the cell membrane are subtly changed to signal removal by immune cells such as macrophages [72]. Enhanced DNA fragmentation has been observed in the skeletal muscle of rats bearing the cachexia inducing Yoshida AH-130 ascites hepatoma, and in the early stage of tumour development in rabbits bearing the VX2 carcinoma cells, together with an increased expression of the apoptosis promoting protein, bax. A significant increase in the activity of caspase-1, 3, 8 and 9, together with cleavage of PARP, one of the caspase protein substrates cleaved during apoptosis by caspase-3 and 7, in skeletal muscle from MAC16 mice shows that protease activation and apoptosis may be involved in the atrophy of skeletal muscle in cancer cachexia. After all apoptosis has also been shown to play a role in atrophy of slow skeletal muscle in chronic heart failure, muscle damage after mild exercise [17, 164], diabetes, chronic uremia and muscular dystrophy [55].



## 1.7 Cancer Anorexia

Tumour growth is frequently associated with the development of anorexia where it is seen in 15-40% of cancer patients at presentation [185]. Anorexia and reduced food intake are physiological responses prompted by the growing tumour. Initially, these changes contribute to fight tumour growth; the beneficial effects of the initial anorexia during disease being supported by a classic study in which force-feeding of mice experimentally infected with a pathogen increased mortality. However, long-lasting anorexia compromises host defence and delays recovery [108].

Anorexia, together with pain and fatigue, is consistently among the 10 most prevalent symptoms associated with cancer. Anorexia contributes to the development of malnutrition and cachexia, since it reduces the oral intake of calories, thus promoting skeletal muscle wasting [108].

Accumulating evidence indicates that cancer anorexia is multifactorial in its pathogenesis, and most of the hypothalamic neuronal signalling pathways modulating energy intake are likely to be involved. Several factors are considered to be putative mediators of cancer anorexia, including hormones (eg, leptin), neuropeptides (eg, NPY), cytokines (eg, interleukin 1 and 6, and  $TNF\alpha$ ), and neurotransmitters (eg, serotonin and dopamine). These pathways are not isolated and distinct mechanisms but are closely inter-related and inter-linked [108].

However, evidence suggests that cytokines have a vital role, triggering the complex neurochemical cascade, which leads to the onset of cancer anorexia. Increased expression of cytokines during tumour growth prevents the hypothalamus from responding appropriately to peripheral signals [108]. For example, cytokines can increase the levels of corticotrophin-releasing hormone, a CNS neurotransmitter that suppresses food intake and the function of glucose sensitive neurons, which also decreases food intake [8].

Cytokines could have a role in cancer-induced anorexia because they modulate gastric motility and emptying, either in the gastrointestinal system itself, or via the brain, by altering efferent signals that regulate satiety.

IL-1, in particular, has been clearly associated with the induction of anorexia, by blocking NPY induced feeding. The levels of this molecule (a feeding-stimulating peptide) are reduced in anorectic tumour-bearing rats where a correlation between food intake and brain-IL-1 has been found in anorectic rats with cancer. The mechanism involved in the attenuation of NPY activity by cytokines might be related to an inhibition of cell firing rates, an inhibition of NPY synthesis or an attenuation of its postsynaptic effects [8].

## 1.8 Oxidative Stress

Oxidative stress is a state where oxidants, also known as reactive oxygen species (ROS) such as hydroxyl radicals and superoxide radicals, have exceeded detoxification capacity of the antioxidants in the body. ROS are products of normal aerobic metabolism and can be useful in the immune response. However they are toxic to the hosts cells at high levels and thus there are systems to regulate their actions. One such counteracting mechanism is that supplied by the diet, such as glutathione synthetase (GSH). Glucose has an important role in the production of GSH and since the glucose metabolism and nutritional input is altered in cachectic patients this leads to an inadequate detoxification of ROS, which is produced at excess levels due to an increase in energy metabolism in these patients [112]. Oxidative stress has been attributed to the chronic inflammation seen in patients with advanced cancer. Furthermore, some of the components released during inflammation such as TNF- $\alpha$  can also induce oxidative stress and nitric oxide synthase. The use of antineoplastic drugs such as alkylating agents and cisplatin, may exacerbate the excessive immune response, since they are able to produce an excess of ROS, while several studies have shown that chemotherapy and radiation therapy are also associated with increased formation of ROS and depletion of critical plasma and tissue antioxidants [116]. Production of ROS has been proposed as a mechanism of muscle wasting in cachexia, since its actions can induce muscle proteolysis, probably through the induction of proteasome expression, via activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B) [191]. Treatment with antioxidants has also been shown to prevent muscle wasting in a murine model of cachexia [50].



## 1.9 NF- $\kappa$ B and the Proteasome

Nuclear Factor- $\kappa$ B is a key transcription factor which regulates genes involved in the immune and inflammatory response and in the regulation of growth (18). NF- $\kappa$ B can be activated by a host of factors including TNF- $\alpha$ , IL-1 $\beta$ , bacterial cell wall and viral products, dsRNA, mitogens and reactive oxygen species (ROS). In response to these activators NF- $\kappa$ B can increase production of many components such as cytokines and chemokines, immune and antigen presenting receptors, stress response and acute phase proteins, and other regulators of apoptosis, growth and host defence [35].

NF- $\kappa$ B is a dimer composed of various DNA-binding subunits forming a homo-dimer or hetero-dimer. The most frequent NF- $\kappa$ B being a hetero-dimer of the p50 and p65 (RelA) subunits. The DNA-binding NF- $\kappa$ B dimer is held in an inactive state in the cytoplasm by inhibitory I $\kappa$ B proteins, which masks the nuclear localization sequence of NF- $\kappa$ B. Activation of NF- $\kappa$ B (see Figure 1.1 for diagram) comprises of the phosphorylation and ubiquitination of I $\kappa$ B $\alpha$  and  $\beta$  which targets them for proteolytic degradation via the ubiquitin-proteasome pathway. This process unmasks the nuclear localization sites of the DNA binding subunits allowing the NF- $\kappa$ B to enter the nucleus, bind to its consensus DNA sequence and up regulate transcription of target genes [150]. NF- $\kappa$ B activation has been shown to be important in the induction of proteasome expression and protein degradation in cancer cachexia [210], as well as a number of other muscle wasting conditions such as ageing, diabetes, denervation and immobilization [35].

There are three major proteolytic pathways involved in intracellular degradation of proteins; the lysosomal pathway, calcium/calpain pathway and the ubiquitin-proteasome system. Lysosomal proteolysis plays a minor role in skeletal muscle protein breakdown [111]. In muscle cachexia induced by severe injury, sepsis and cancer there is an increased gene expression and activity of the calcium/calpain and ubiquitin-proteasome proteolytic pathways. The calcium/calpain pathway releases myofilaments from the sarcomere, which is an early component of this catabolic response in muscle [187]. The released myofilaments degradation is mediated through the ubiquitin-proteasome pathway [82]. The ubiquitin-proteasome system is present in both the nucleus and the cytosol. Alterations in the activity of this pathway are probably due to changes

in the rate of ubiquitin conjugations, and several hormonal and immunological factors seem to be involved in a regulatory fashion [201].

Muscle atrophy results when there is accelerated degradation of myofibrillar proteins, which make up approximately 80% of the volume in muscle. The ubiquitin-proteasome pathway is the major proteolytic system for myofibrillar protein breakdown under both normal and disease states, where upregulation of the ubiquitin-proteasome pathway has been identified as a key factor behind the accelerated muscle wasting of cancer cachexia [82], starvation, sepsis, metabolic acidosis, weightlessness, severe trauma and denervation atrophy [38].

The proteasome exists as different oligomeric assemblies i.e., the 20S, 26S, and immunoproteasome, that

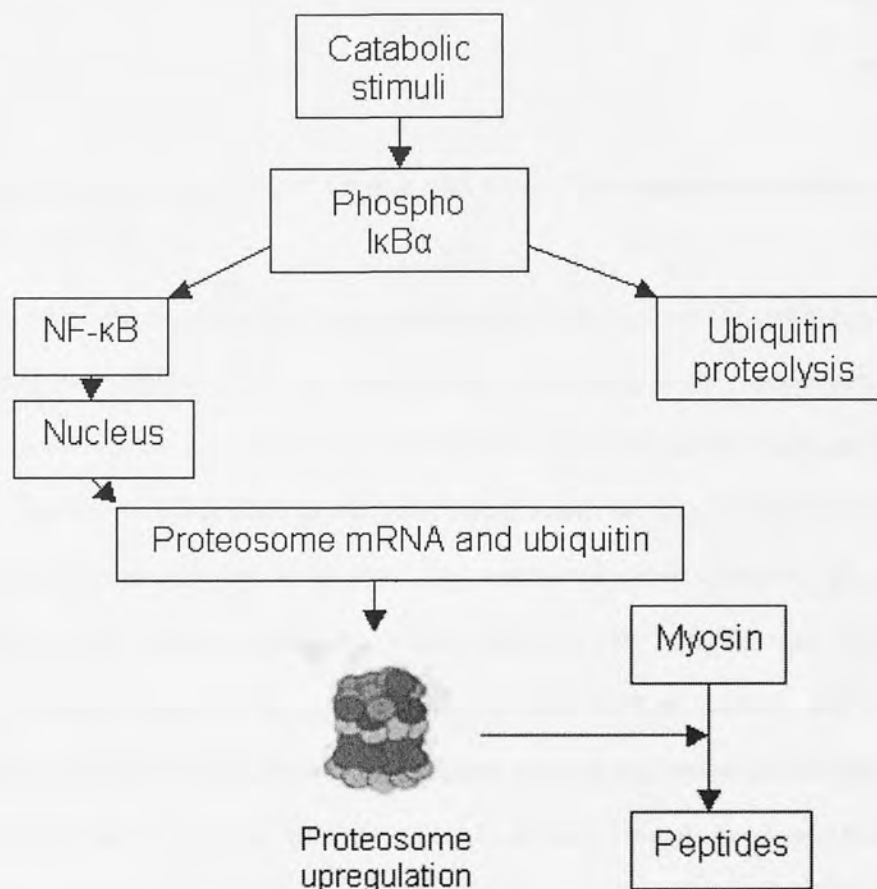


Figure 1.1: **Outline of the NF-κB activation pathway by catabolic agents and ensuing upregulation of the proteasome.** Phosphorylation and proteolytic degradation of IκB releases NF-κB, allowing it to translocate to the nucleus and up regulate transcription of target genes

are defined by both the type of regulatory complex associated with the catalytic core and the composition of the catalytic subunits (see Figure 1.2 for diagram of the proteasome and some of its regulatory complexes). The 20S proteasome, also known as the catalytic core, is composed of four stacked rings of seven subunits each. The outer rings are made up of the constitutively expressed alpha subunits that interact with the regulatory complexes, PA28 and PA700 [82].

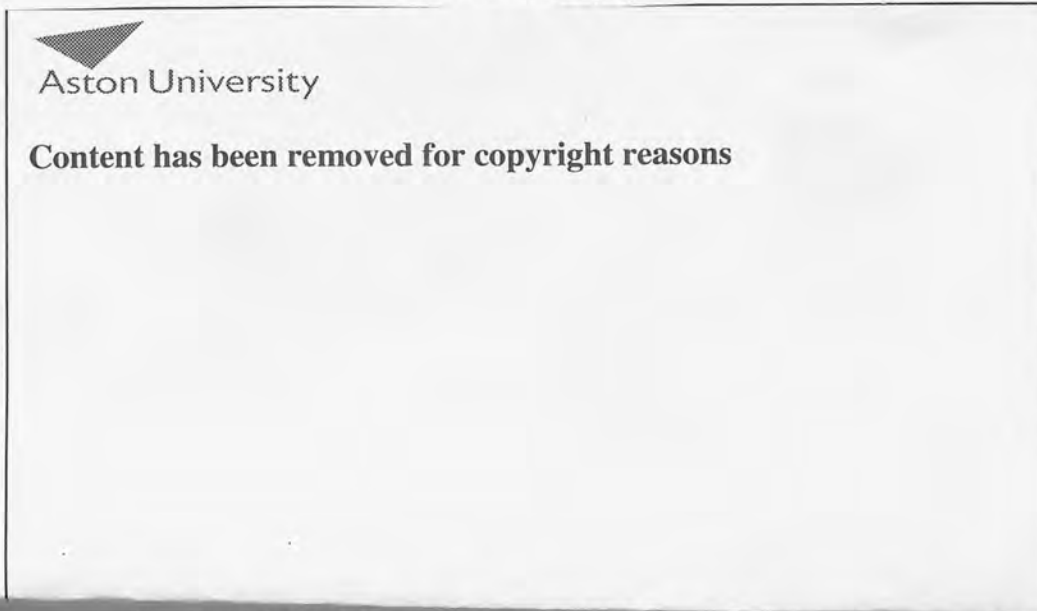


Figure 1.2: Interaction of the 20S proteasome with alternative regulatory complexes. Diagram cited from [80]

The two inner rings are composed of the beta subunits and contain three pairs of active sites ( $\beta 1$ ,  $\beta 2$  and  $\beta 5$ ) that perform distinct proteolytic activities. Based on the proteolysis of model peptide substrates, the active sites have been classified as peptidylglutamyl hydrolyzing, trypsin-like, and chymotrypsin-like for cleavage after acidic, basic, and hydrophobic amino acids, respectively. As a consequence of an inflammatory stimulus, such as exposure to  $\text{TNF-}\alpha$  or  $\text{IFN}\gamma$ , the constitutive catalytic subunits  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  can be replaced in nascent proteasomes by the inducible subunits LMP2, MECL, and LMP7, respectively. Association of PA28, a heptameric complex composed of alpha and beta subunits, with the catalytic core containing one or more of the cytokine-induced subunits produce the immunoproteasome. Association of PA700, a complex made up of at least 18 distinct subunits, with the catalytic core forms the 26S proteasome. The PA700 complex binds and unfolds ubiquitin-modified proteins so that they can be degraded by the catalytic core [82]. In the ubiquitination process (see Figure 1.3 for diagram outlining this process), ubiquitin is first activated by a ubiquitin activating enzyme (E1), which transfers the ubiquitin to a carrier protein (E2), which either ligates the ubiquitin directly to the target protein, or does so in the presence of

a ubiquitin protein ligase (E3) [111].

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Figure 1.3: **The ubiquitin proteasome pathway.** ubiquitin (Ub) is activated by a ubiquitin activating enzyme (E1), which transfers the ubiquitin to a ubiquitin conjugating enzyme (E2), which ligates the ubiquitin to the target protein in the presence of a ubiquitin ligase (E3). Diagram cited from [190]

Each proteasome species performs distinct functions. In vitro evidence has shown that the 20S proteasome selectively degrades oxidized proteins in an ATP-independent manner, and has been suggested as the primary mechanism for degrading damaged proteins following an oxidative insult. The 26S proteasome requires ATP for activation and is responsible for most cellular degradation of both ubiquitinated and some non-ubiquitinated proteins. The immunoproteasome is especially effective in generating immunogenic peptides for major histocompatibility complex class 1 presentation, and is hence intimately linked to the cellular immune response. Thus, the cellular concentration of each proteasome species could influence the ability to respond to a variety of stressors [82].

The 26S proteasome is not only involved in the degradation of myofibrillar proteins, but also in that of

transcription factors, oncoproteins and cell cycle regulators and thus plays a role in the cell cycle progression, oncogenesis, transcriptional control, development and differentiation [187].

## 1.10 Translation Initiation: Regulation and Control

Cachectic factors are thought to regulate muscle wasting by several mechanisms, one of which is a depression in protein synthesis. This may occur through inhibition of amino acid uptake or the reduction of RNA. Indeed, in some tumour models total RNA can decrease as much as 40% compared to control animals [3]. Inhibition of protein synthesis by PIF is believed to result from an ability to block translation, because no significant changes in protein synthesis were observed after treating C2C12 myoblasts with PIF beyond six hours, which would reflect a change at the transcriptional level. The increase in protein synthesis after short term incubation with insulin has been attributed to an increase in translational efficiency, and since pretreatment of cells with insulin prior to addition of PIF abolished the inhibitory effect on protein synthesis in C2C12 myoblasts, this supports the suggestion that PIF affects translation. A similar short term inhibition of protein synthesis has been observed in hepatocytes after incubation with vasopressin, and this effect has been attributed to a reduction in eIF2B activity arising from an increase in phosphorylation of eIF2 $\alpha$  [166]. Ang I and II has also been demonstrated to inhibit protein synthesis in C2C12 myotubes, which can be attenuated by pretreatment with insulin like growth factor-1 (IGF-1). IGF-1 is known to enhance human skeletal muscle protein anabolism, which suggests that angiotensin can also modify protein synthesis through an effect on translation initiation, similar to cytokines [154].

Regulation of gene expression occurs at multiple levels, including transcription, mRNA processing and stability, translation, and protein modification and turnover. mRNA translation represents an important control point in gene expression [147]. The apparent advantages of the regulation of gene expression at the translational level are the speed and the readily reversible nature of the response to altering physiological conditions [124]. Translational regulation in eukaryotic cells plays a critical role in cell growth, proliferation, and development. mRNA translation is divided into three stages; initiation, elongation, and termination,



where initiation appears to be the primary regulation point [147]. Translation initiation of protein synthesis in eukaryotes is a highly complex and conserved process involving at least 13 initiation factors, many of which are themselves assembled from numerous subunits [140]. Translation initiation has at least two stages that are subjected to regulation in vivo. The first stage is the binding of the initiator methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit (see Figure 1.4 for diagram) and the second is the binding of mRNA to the 43S preinitiation complex (see Figure 1.5 for diagram) [147].



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Figure 1.4: **Translational control by eIF2 $\alpha$** . Regulation of the 43S preinitiation complex by the eIF2 $\alpha$  signaling pathway. Modified from Cell Signaling technology Inc 2002, [www.cellsignal.com](http://www.cellsignal.com)

The binding of the met-tRNA to the ribosome is mediated by the eukaryotic initiation factor eIF2 during initiation of translation of all cytoplasmic mRNAs in eukaryotic cells. eIF2 is bound to GTP and it is this ternary complex eIF2.GTP.met-tRNA that binds the 40S ribosomal subunit, along with eIF3 forming the 43S preinitiation complex. The newly assembled 43S ribosome associates with an mRNA transcript in a 3' direction until an AUG start codon is located within the context of an appropriate Kozak sequence. Following start codon recognition, the GTP is hydrolysed to GDP in a process that involves eIF5, a GTPase activator and the met-tRNA is released from the complex to initiate nascent peptide chain synthesis [13]. To undergo further rounds of initiation the GDP must be exchanged for GTP. The guanine nucleotide exchange factor eIF2B is responsible for this process. Modulation of the rate of GDP/GTP exchange is a key regulatory step for translation. The eIF2 can be regulated by phosphorylation on its alpha subunit, which converts it from a substrate in to a competitive inhibitor of eIF2B. Since in most cell types the level of eIF2 is higher than eIF2B, even low levels of eIF2 $\alpha$  phosphorylation can block global translation initiation.

Considerable evidence exists to indicate that in mammalian cells hormonal, mitogenic and stress response signalling cascades can regulate protein synthesis through post-translational modification of eukaryotic initiation factors (eIFs) [140]. Mammalian cells possess four different eIF2 $\alpha$  kinases which can regulate the activity of eIF2. Heme-regulated inhibitor (HRI), double stranded RNA-dependent protein kinase (PKR), ER resident PKR-like endoplasmic reticular kinase (PERK) or pancreatic eIF2 $\alpha$  kinase (PEK) and GCN2. HRI is responsible for inhibiting translation when cells are deprived of heme, thus controlling normal globin production. However HRI has also been shown to phosphorylate eIF2 $\alpha$  in response to heat shock and arsenite by mechanisms independent of its heme control [147]. PKR is activated following infection with different viruses and phosphorylates eIF2 $\alpha$  to suppress viral replication. eIF2 $\alpha$  is the best characterized substrate for PKR, but it can also affect other components including NF- $\kappa$ B, stat1 and 3, P53, and P38 mitogen activated kinase (MAPK) [161]. As well as double stranded RNA, PKR can also be activated by lipopolysaccharide, cytokines such as IL-1 and TNF- $\alpha$  [97], polyanions, PACT (PKR activating protein), cellular stresses such as serum depletion and calcium depletion from the ER [174], and ceramide [157] to control a host of cellular responses including growth regulation, transcription, the cell cycle, and cell death [212]. PERK and PEK can undergo dimerisation and activation in response to



ER, nitrosative and oxidative stress, shutting down translation of ER-destined proteins and allowing the organelle a reprieve to deal with accumulation of misfolded proteins. GCN2 has a C-terminal domain that resembles histidyl-tRNA synthetases. It is thought that during amino acid starvation the uncharged tRNAs accumulate and bind to the histidyl-tRNA synthetase related domain activating its kinase activity causing eIF2 $\alpha$  phosphorylation. However it is possible that, like the yeast homologue, GCN2 can actually increase translation of GCN4 mRNA, encoding a transcriptional activator of amino acid biosynthetic genes [147]. However, at present GCN4 has not been found in mammalian systems [7]. Mammalian GCN2 has also been found to phosphorylate eIF2 $\alpha$  in response to ultraviolet radiation and possibly glucose and serum starvation [147].

eIF2B can also be regulated by phosphorylation on its epsilon subunit by glycogen synthase kinase 3 (GSK3) which down regulates the activity of eIF2B. GSK3 can be inactivated by insulin and growth factors, providing a mechanism by which they can affect a key step in translation initiation [147].

The second important control point of translation initiation is mediated by the eIF4F triad of translation initiation factors. This complex consists of the scaffold protein eIF4G, which binds eIF4E the m<sup>7</sup> GTP-cap binding protein, eIF4A an RNA helicase, and the RNA-binding protein eIF4B. eIF4E binds the 5'-mRNA cap; eIF4A and 4B then unwind the secondary structure in the 5'-untranslated end, enabling the 40S-met-tRNA complex, aided by eIF3, to scan the mRNA 5'-untranslated end to the AUG start site. The availability of eIF4E for incorporation into the eIF4F complex is regulated by the 4E-BP polypeptides. The 4E-BP competes for the same binding site on eIF4E as eIF4G. The eIF4F triad is regulated by phosphorylation. Thus eIF4E can be phosphorylated by mitogens, or stress causing an increased affinity for the mRNA, and phosphorylation of 4E-BP by insulin and mitogens causes a decreased affinity for eIF4E thus allowing eIF4G to bind and upregulate translation [76].

Another mechanism for controlling selection of mRNA for translation involves the 70kDa ribosomal protein kinase (p70S6K). p70S6K phosphorylates the ribosomal protein S6 (rpS6) which increases the affinity of ribosomes for terminal oligopyrimidine (TOP) mRNAs which contain an uninterrupted stretch of pyrimidine residues adjacent to the 5'-cap structure. Examples of protein encoded by TOP mRNAs are ribosomal

proteins, elongation factors and poly(A) binding protein. Thus p70S6K increases the protein synthetic potential of the cell. The eIF4F complex and p70S6K can be regulated by upstream mediators, such as mammalian target of rapamycin (mTOR). mTOR is an evolutionary conserved kinase that integrates signals from nutrients to regulate cell growth and cell cycle progression co-ordinately [65]. Signalling through mTOR is increased by nutrients and anabolic hormones such as insulin and IGF, and depressed by catabolic stimuli such as AMP-activated protein kinase (AMPK), which is activated under conditions of hypoxia, exercise, ischemia, heat shock and low glucose [196]. Recent studies have suggested that



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Figure 1.5: **Translational control by Akt/mTOR.** Regulation of eIF4F and p70S6 kinase through the Akt/mTOR signalling pathway. Cited from Cell Signalling technology Inc 2002, [www.cellsignal.com](http://www.cellsignal.com)

nutrient and hormone signalling is through a common mediator rather than a direct effect on mTOR. It is likely that anabolic stimuli from insulin and IGF would signal via the phosphatidylinositol-3 kinase (PI-3K)/protein kinase B (PKB) pathway [98], which in turn would phosphorylate TSC2 repressing the inhibitory action of the tuberous sclerosis complex TSC1/TSC2 on mTOR, while AMPK would upregulate the inhibition of mTOR by TSC1/TSC2. Leucine is believed to act on mTOR through a TSC1/TSC2 dependent and independent mechanism, possibly by altering the association of mTOR with its regulatory proteins, such as the regulatory associated protein of mTOR (raptor), and G-protein  $\beta$ -subunit-like protein ( $G\beta L$ ) [97]. mTOR can also regulate the phosphorylation of elongation factor eEF2. eEF2 mediates the translocation step of elongation. When phosphorylated at Thr56, eEF2 can no longer bind to the ribosome, thus blocking elongation. However, via mTOR anabolic stimuli like insulin can dephosphorylate eEF2 and activate elongation [146].

Both the eIF4F triad and eEF2 can also be regulated by an mTOR independent mechanism, through an indirect action of the eIF2 $\alpha$  kinase PKR. It does this via interaction and phosphorylation of the B56 $\alpha$  subunit of the protein phosphatase PP2A [212]. It is possible that PKR can even affect mTOR activity via the P38/MAPK pathway and MK2 which is an upstream kinase of the TSC1/TSC2 complex [97]. In yeast mTOR may be able to enhance eIF2 $\alpha$  kinase activity through phosphorylation of GCN2 [7], thus demonstrating that control of translation is a multicomponent system, involving multiple regulation points, and crosstalk between signalling pathways to produce a complex system to allow the cell to respond to ever changing physiological conditions.

## 1.11 Treatments for Cachexia

### Insulin Like Growth Factor

It has been reported that there are disturbances in the GH-IGF1 axis in patients with cancer cachexia. Abnormalities in the GH-IGF1 axis have also been reported in other catabolic states such as sepsis, AIDS, congestive heart failure, diabetes and severe burns. Therefore, treatment to restore IGF levels by admin-

istration of IGF-1 or GH have a large therapeutic potential [25]. IGF-1 has been used to reverse muscle protein degradation in cachectic burned rats, and has also shown anticatabolic effects in patients with thermal injury. It is believed to reduce muscle protein breakdown by multiple mechanisms, including the PI3K/PKB mediated inactivation of GSK3- $\beta$  and FOXO transcription factors [63]. Decreased circulating levels of insulin and impaired signalling through PI3K have been linked with apoptosis via increased caspase-3 activation in many catabolic conditions, such as diabetes and chronic uremia. Since PI3K activity is a major influence controlling apoptosis, supplementation with insulin may reverse the proteolysis of muscle through inhibition of caspase activation [55].

### **$\beta$ -Hydroxy- $\beta$ -Methylbutyrate**

$\beta$ -Hydroxy- $\beta$ -methylbutyrate (HMB) is a metabolite of the amino acid leucine. Leucine is a member of the branched chain amino acids (BCAA) [192]. In patients with cancer cachexia a decrease in plasma concentrations of BCAA has been observed. Some human colon tumours require the BCAA valine, leucine and isoleucine, thus depleting the plasma amino acid pool [185]. Therefore supplementation of cachectic cancer patients with BCAA, or their derivatives, may have a beneficial effect on the hosts nutritional status. HMB has been shown to attenuate loss of body weight and preserve lean body mass in tumour bearing mice. HMB can also attenuate the increase in protein degradation found during cachexia by interfering with the activation of NF- $\kappa$ B and downregulating the ubiquitin-proteasome proteolytic pathway. HMB has been combined with other nutritional components such as leucine, L-arginine and L-glutamine, which have been shown to increase weight gain and lean body mass in cachectic AIDS and cancer patients [192].

Nutritional strategies for counteracting cachexia are not sufficient alone, emphasizing the role of the metabolic changes in cancer induced cachexia. Therefore any therapeutic approach using nutritional supplementation is generally combined with a pharmacological agent to deal with metabolic abnormalities [9].

## **Megestrol Acetate**

Megestrol acetate is a synthetic progestogen which is used as a treatment for cancer cachexia due to its positive effect on weight gain, appetite, nausea and mood. However the weight that is gained is due to an increase in fat mass and water retention and does not stimulate lean body mass improvement. Corticosteroids such as dexamethasone have been used to increase appetite and sensation of well being to improve the quality of life in terminally ill patients with a limited survival time since the beneficial effects are only seen for a few weeks.

## **Thalidomide**

Inhibitors of the nuclear factor  $\kappa$ B have proven most effective in combating cancer cachexia. Thalidomide is believed to reduce activation of the ubiquitin-proteasome pathway by down regulating the I $\kappa$ B kinase and thus prevent activation of NF- $\kappa$ B. It has been used as a anticachectic agent in AIDS and tuberculosis patients, and more recently cancer patients, where it was shown to increase gain of weight and lean body mass, as well as improve the Karnofsky index of cachectic patients with esophageal cancer [192].

## **Eicosapentaenoic Acid**

Another agent that has been shown to inhibit the nuclear binding activity of NF- $\kappa$ B is eicosapentaenoic acid (EPA). EPA is an omega-3 polyunsaturated fatty acid which is a component of fish oil. It has been shown to attenuate the actions of PIF by inhibiting the release of arachidonic acid from membrane phospholipids and depressing the upregulation of the ubiquitin-proteasome pathway, thus inhibiting protein degradation. However, EPA has no effect on protein synthesis [166]. In clinical trials EPA administered for a sustained period improves appetite and can stabilize body weight through a preservation of lean body mass [192].

## Hydrazine Sulfate

Hydrazine sulfate, an agent that inhibits the enzyme phosphoenolpyruvate carboxykinase, has a positive effect on normalizing glucose and protein metabolism in cachectic cancer patients and maintain, or even increase body weight. Cyclooxygenase inhibitors like ibuprofen have been seen to reduce the REE and CRP levels in patients with pancreatic cancer which may have a beneficial effect on weight maintenance [185].

To conclude, cancer-related weight loss is a multi-dimensional manifestation where multiple mediator pathways including pro-inflammatory cytokines, neuroendocrine hormones and tumour-specific factors are involved in combination with a reduction of food intake, vomiting and other such factors related to the cancer itself or the side effects of therapy [143, 74]. Many therapeutic approaches have been applied over the years which have attenuated the cachectic state, improved quality of life and extended survival time. However none, have completely abolished the effects of cachexia and are unlikely to do so, due to the complex multifactorial nature of the disease, which we are still far from completely understanding.



# Aim

Previous studies have shown that the proteolysis inducing factor (PIF) (37), Angiotensin (Ang) (35) and cytokines (61) are able to depress protein synthesis to induce a cachectic state. However the mechanism involved in this process is largely unknown. The aim of this project was to investigate the mechanisms involved in cancer cachexia related depression of protein synthesis by PIF and Ang II, and to ascertain if this process is linked with, or distinct from, the pathway by which PIF/Ang II induces protein degradation. Emery et al (60) suggested that muscle mass in cancer cachexia is regulated primarily by alterations in the protein synthetic rate, while changes in protein degradation are largely secondary. Clearly this demonstrates the importance of elucidating the pathway by which PIF inhibits protein synthesis. A further goal was to determine if nutritional supplementation could reverse the depression in protein synthesis as a possible therapeutic potential to alleviate the symptoms of cachexia and to improve quality of life

## Chapter 2

# Materials and Methods

### 2.1 Materials

#### **Animals**

Pure strain NMRI mice were obtained from our inbred colony. 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 (Lilico, Wonham Mill, Bletchworth, Surrey) with fresh tap water ad libitium. Animals were humanely sacrificed prior to 25% loss of body weight.

#### **Antibodies**

All antibodies were obtained from Cell Signaling Technologies (Danvers, MA, USA) unless specified otherwise.

## Chemicals

All chemicals were obtained from Sigma-Aldrich (Dorset, UK) at the highest available purity unless specified otherwise below.

### Abbott (Illinois, USA)

$\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB)

### Abcam (Cambridge, UK)

eIF2 $\alpha$  (phospho S51) rabbit polyclonal primary antibody

### Alomone Laboratories (Jerusalem, Israel)

Rapamycin

### Beckman Coulter (Buckinghamshire, UK)

Coulter Particle Counter

### Bioline (London, UK)

PCR Buffer (10x)

Taq polymerase

## **Biomol (Exeter, UK)**

Lactacystin

19S regulator, ATPase subunit Rpt 4 (P42) mouse monoclonal antibody

20S proteasome subunits alpha 1,2,3,5,6 and 7 mouse monoclonal antibody

Anti-I $\kappa$ B $\alpha$ , C-terminal, rabbit polyclonal antibody

Phospho Anti-I $\kappa$ B $\alpha$  (pS232, pS36), rabbit polyclonal

## **Chemical Abstracts (London, UK)**

## **Bio-Rad laboratories (Richmond, CA, USA)**

Ammonium persulphate (APS)

Electrophoresis and transferring apparatus

Electrophoresis glass plates

Protein assay reagents

## **Chemical Abstracts (London, UK)**

## **British Oxygen Company Ltd (London, UK)**

CO<sub>2</sub>

O<sub>2</sub>

Liquid nitrogen

## **Chemical Abstracts (London, UK)**

## **Calbiochem (San Diego, CA, USA)**

Phosphosafe Extraction Reagent

Glutathione, reduced, free acid ( $\gamma$ -Glu-Cys-Gly) (GSH)

Calphostin C, Cladosporium cladosporioides (UCN-1028c)

RNA-Dependent Protein Kinase (PKR) inhibitor

eIF-2 $\alpha$  inhibitor, Salubrinal

Protease inhibitor cocktail set III

1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid Tetra(acetoxymethyl) Ester (BAPTA/AM)

### **Cambridge Biosciences (Cambridge, UK)**

HA.11 mouse monoclonal antibody

### **Canberra UK Ltd (Oxfordshire, UK)**

1600 TR Liquid Scintillation Counter

### **Dako Cytomation (Glostrup, Denmark)**

Rabbit anti mouse HRP conjugated secondary antibody

Goat anti rabbit HRP conjugated secondary antibody

### **MWB Biotech (Ebersberg, Germany)**

Oligonucleotides (primers), HPSF purified

### **Fermentas Life Sciences (York, UK)**

MassRuler DNA ladder high range (ready to use)

### **Fisher Scientific (Loughborough, Leicestershire, UK)**

Hydrochloric acid (HCl), specific gravity 1.16

Methanol

Ethanol, absolute

Perchloric acid 60%

Nitric acid, specific gravity 1.42

OptiPhase 'HighSafe' 3

3MM Whatman Filter paper

Propan-2-ol (iso-propyl alcohol)

Sodium hydrogen carbonate ( $\text{NaHCO}_3$ )

Magnesium sulphate ( $\text{MgSO}_4$ )

Isoton

### **GE Healthcare (Amersham, Bucks, UK)**

ECL detection reagents

Hybond Nitrocellulose membrane

Hyperfilm ECL

Hypercassette

Rainbow coloured protein molecular weight markers

L-(2,6- $^3\text{H}$ ) Phenylalanine, specific activity 2.07 TBq/mmol

L-(4- $^3\text{H}$ ) Phenylalanine, specific activity 46.3mCi/mmol

7-methyl GTP sepharose 4B

Streptavidin horseradish peroxidase conjugate

Novaspec II visible spectrophotometer

### **Gibco BRL Life Technologies (Paisley, Scotland)**

Dulbecco's Modified Eagle Medium (DMEM) (1x)

DMEM (1x) without phenol red

Foetal calf serum

Glutamine

Horse serum



Penicillin Streptomycin

RPMI 1640 (1x)

Trypsin (100x)

Agarose (molecular biology grade)

E.Coli DH5 $\alpha$  cells (SupE44  $\Delta$ LacU169 ( $\phi$ 80 Lac Z  $\Delta$ M15) hsdR17 recA1 end A1 gyrA96 thi-1 rel A1)

### **Insight Biotechnology (Wembly, UK)**

Phospho eIF2 $\alpha$  (pS51) rabbit monoclonal antibody

Phospho PKR (T446) rabbit polyclonal antibody

### **Invitrogen Life Technologies (Carlsbad, CA, USA)**

Lipofectamine 2000 reagent

### **Marvel (Ireland, UK)**

Dried Skimmed milk

### **Millipore (Billerica, MA, USA)**

Amicon Ultra centrifugal filter devices

Ultrafiltration membrane 10,000 $\mu$ M pore size

### **Molecular Devices Corporation (Sunnyvale, CA, USA)**

Spectra Max Gemini XS (fluorometer)

## **Nalgene (Rochester, NY, USA)**

0.2 $\mu$ m syringe filters

## **New England Biolabs (Hertfordshire, UK)**

Buffer 2 (10x)

Hind III

## **Novacastra (Newcastle Upon Tyne, UK)**

Myosin heavy chain mouse monoclonal antibody

## **Omnigene (UK)**

PTC-100 thermal cycler (PCR machine)

## **Oxoid (Basingstoke)**

Phosphate buffered saline (PBS) tablets

Bacto tryptone

Yeast extract

## **Panomix Inc (Fremontica, CA, USA)**

NF- $\kappa$ B Probe

## **Pierce (Rockford, IL, USA)**

Lightshift EMSA kit

Chemiluminescent nucleic acid detection module

## **Promega (Madison, Wis, USA)**

PCR Master Mix

Wizard plus maxipreps DNA purification system

100-1000bp DNA ladder

## **Santa Cruz (Santa Cruz, CA, USA)**

eIF2 $\alpha$  (FL-315) rabbit polyclonal antibody

PP1 (E-9) mouse monoclonal antibody

## **SynGene (Cambridge, UK)**

GeneTools image analysis programme

GeneSnap transilluminator photographic systems

## **Gifts**

Expression vectors (pcDNA) encoding mouse Akt proteins fused in-frame to the hemagglutinin (HA) epitope, were kindly supplied by Dr. Kenneth Walsh (Tufts University, School of Medicine, Boston, USA). The plasmid vector pcDNA3(-) containing wild-type PKR and the catalytically inactive variant PKR $\Delta$ 6, were a gift from Dr G.N. Barber (University of Miami School of Medicine, Miami, FL). PKR $\Delta$ 6 lacks six amino acids (361-366) between catalytic domains IV and V, which render the PKR variant unable to autophosphorylate or activate substrate proteins.

Samples of rectus abdominus muscle (obtained from the edge of the patients abdominal wound after induction of general anaesthesia) were supplied by Dr Richard JE Skipworth (University of Edinburgh, Tissue Injury and Repair Group).

## 2.2 Buffers and Solutions

### Animal Studies

PBS Tables	1 tablet per 500mL ddH <sub>2</sub> O
BCAA (leucine, valine and methionine)	1g/kg
Vitamin E	1mg/kg
PKR inhibitor	1mg/kg and 5mg/kg

### Cell Counting using the Coulter Counter

Isoton	Dilute 200 $\mu$ L of cells in 10mL of Isoton for use
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### Cell Culture

10% Trypsin:

Trypsin	10mL
Sterile PBS	90mL

10% Fetal Calf Serum:

DMEM	440mL
10% Foetal Calf Serum	50mL
1% Glutamine	5mL
1% Penicillin Streptomycin	5mL

2% Horse Serum:

DMEM	480mL
2% Horse Serum	10mL
1% Glutamine	5mL
1% Penicillin Streptomycin	5mL

RPMI:

RPMI 1640	440mL
10% Foetal Calf Serum	50mL
1% Glutamine	5mL
1% Penicillin Streptomycin	5mL

10% Fetal Calf Serum for Mutants:

DMEM	445mL
10% Foetal Calf Serum	50mL
1% Neomycin	5mL

2% Horse Serum for mutants:

DMEM	485mL
2% Horse Serum	10mL
1% Neomycin	5mL

### Chymotrypsin Assay

Homogenisation Buffer:

Trizma Base-HCl (pH7.5)	20mM
ATP	2mM
MgCl <sub>2</sub>	5mM
Dithiothreitol (DTT)	1mM

Lactacystin 10 $\mu$ M

Chymotrypsin Substrate: 0.1mM

Dilute in 600 $\mu$ L of DMSO

Dilute 1:10 in 100mM Trizma Base (pH8)

Trizma Base (pH 8) 100mM

### Electrophoretic Mobility Shift (EMSA)

Buffer A:

HEPES-KOH (pH 7.9) 10mM

MgCl<sub>2</sub> 1.5mM

KCl 10mM

DTT 0.5mM

PMSF (dissolved in isopropyl alcohol) 0.2mM

Buffer C:

HEPES-KOH (pH 7.9) 20mM

Glycerol 25%

NaCl 420mM

EDTA 0.2mM

DTT 0.5mM

PMSF (dissolved in isopropyl alcohol) 0.2mM

10x TBE Buffer:

Trizma Base 107.8g

EDTA 7.44g



Boric acid 55g

Dissolve in 800mL double distilled water (ddH<sub>2</sub>O)

Dilute to 0.5x for use

80% Glycerol:

Glycerol 80mL

ddH<sub>2</sub>O 20mL

6% Polyacrylamide Gel:

10x TBE 1mL

30% Acrylamide/bis-Acrylamide 4mL

80% Glycerol 0.625mL

10% APS 14.375mL

TEMED 0.3mL

ddH<sub>2</sub>O 0.02mL

5% Stacking Gel:

1.5M Trizma Base (pH8.8) 1.3mL

30% Acrylamide/bis-Acrylamide 1.67mL

10% APS 0.01mL

TEMED 0.001mL

ddH<sub>2</sub>O 6.93mL

1x Wash Buffer:

4x Wash Buffer 40mL

ddH<sub>2</sub>O 120mL

Conjugate/Blocking buffer:

Blocking Buffer	20mL
Stabilized Streptavidin-Horseradish Peroxidase Conjugate	0.0667mL

Substrate Working Solution:

Luminol/Enhancer Solution	6mL
Stable Peroxide Solution	6mL

### **Immunoprecipitation of HA.11 Tagged Akt**

1x Catch and Release Wash Buffer:

4x Wash Buffer	2.5mL
ddH <sub>2</sub> O	7.5mL

1x Denaturing Elution Buffer:

Add Beta-Mercaptoethanol	
immediately before use	5% (v/v)

### **PIF Purification**

PMSF (100mM):

PMSF	0.218g
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Dissolve in 12.5mL iso-propranol

QS1 Buffer:

Trizma Base-HCl (pH8.0)	10mM
PMSF 12.5mL (100mM solution)	0.5mM
DTT	1mM
EGTA	0.5mM

Glycine-HCl (pH 2.5)	0.2M
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Sodium Bicarbonate	0.2M
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### **m<sup>7</sup> GTP Sepharose Purification**

Lysis Buffer:

NaCl	150mM
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Tergitol (NP-40)	1%
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Tris (pH 7.4)	50mM
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Deoxycholic acid	0.25%
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EGTA	2mM
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EDTA	1mM
------	-----

Sodium orthovanadate	0.2mM
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NaF	20mM
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Protease inhibitor	1%
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Dissolved in ddH<sub>2</sub>O

### **Protein Degradation Assay (In Vitro)**

Phenylalanine [ <sup>3</sup> H] Working Stock:	0.5mL
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L-[2,6- <sup>3</sup> H] Phenylalanine	60mg
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L-Phenylalanine	4.5mL
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Sterile PBS

Chase Media:

DMEM without Phenol Red	480mL
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2% Horse Serum	10mL
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1% Glutamine	5mL
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1% Penicillin Streptomycin 5mL

### Protein Synthesis (Ex Vivo)

L-[4-<sup>3</sup>H] Phenylalanine 20 $\mu$ Curries

2% Perchloric Acid 83.33mL/500mL ddH<sub>2</sub>O

### Protein Synthesis (In Vitro)

Unsupplemented Media:

DMEM without Phenol Red 490mL

1% Glutamine 5mL

1% Penicillin Streptomycin 5mL

Phenylalanine [<sup>3</sup>H] Working Stock:

L-[2,6-<sup>3</sup>H] Phenylalanine 0.002mL

Sterile PBS 0.008mL

NaOH 0.3M

Perchloric Acid 0.2M

### Transformation of E.Coli DH5 $\alpha$ Cells

LB Broth:

Bacto tryptone 1% (w/v)

Yeast extract 0.5% (w/v)

NaCl 0.5% (w/v)

LB Agar:

Bacto tryptone	1% (w/v)
Yeast extract	0.5% (w/v)
NaCl	0.5% (w/v)
Bactoagar	1.5% (w/v)

SOB Broth:

Bacto tryptone	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	1% (w/v)
KCl	0.25% (w/v)
MgCl <sub>2</sub>	1% (w/v)
MgSO <sub>4</sub>	1% (w/v)

TAE 50x (pH8):

Tris acetate	2M
EDTA	0.05M

Diluted 1:50 in ddH<sub>2</sub>O for use

Loading Buffer (6x)

Glycerol	30% (v/v)
Xylene Cyanol and/or Bromophenol blue	0.025% (w/v)

Make up to 100% with ddH<sub>2</sub>O

RFB 1 Buffer:

RbCl	100mM
MnCl <sub>2</sub> 4H <sub>2</sub> O	50mM
Potassium acetate	30mM

CaCl <sub>2</sub> ·2H <sub>2</sub> O	10mM
Glycerol	15% (w/v)

RFB 2 Buffer:

MOPS	10mM
RbCl	50mM
CaCl <sub>2</sub> ·2H <sub>2</sub> O	10mM
Glycerol	15% (w/v)

Ampicillin sodium salt solution	50µg/mL
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Agarose Gel:

Agarose	1 or 2% (w/v)
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Dissolve in TAE buffer 1x

### Transfection of C2C12 Myoblasts with Plasmid DNA

Selection Media:

DMEM	425mL
10% Foetal Calf Serum	50mL
5% Neomycin	25mL

### Tyrosine Release Assay (Ex Vivo)

Krebs-Ringer Bicarbonate Buffer:

NaHCO <sub>3</sub>	25mM
NaCl	118mM
KCl	4.7mM
MgSO <sub>4</sub>	1.2mM
NaH <sub>2</sub> PO <sub>4</sub>	1.2mM



CaCl <sub>3</sub>	1.2mM
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Krebs-Heinsleit Buffer:

Components added to Krebs-Ringer bicarbonate buffer

Glucose ( <i>alpha</i> -D[+]-Glucose)	6mM
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Bovine Serum Albumin	0.12%
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Cyclohexamide	0.5mM
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30% Trichloroacetic Acid (TCA):

Trichloroacetic Acid	30mL
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ddH <sub>2</sub> O	70mL
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95% Ethanol:

Ethanol	95mL
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ddH <sub>2</sub> O	5mL
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0.1% 1-Nitroso-2-Naphthol:

1-Nitroso-2-Naphthol	0.1g
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95% Ethanol	100mL
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## Western Blotting

Buffer B:

Trizma Base-HCl (pH 8.8)	1.875M
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Buffer C:

Trizma Base-HCl (pH 6.8)	1.25M
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10% SDS:

SDS	10%
dH <sub>2</sub> O	90%

10% Ammonium Persulphate:

APS	10%
dH <sub>2</sub> O	90%

6% Tris-Glycine SDS-Polyacrylamide Gels:

dH <sub>2</sub> O	10.6mL
30% Acrylamide/bis-Acrylamide	4mL
Trizma Base (pH 8.8)	1.5M
10% SDS	0.2mL
10% APS	0.2mL
Temed	0.016mL

10% Tris-Glycine SDS-Polyacrylamide Gels:

dH <sub>2</sub> O	6.8mL
30% Acrylamide/bis-Acrylamide	5mL
Buffer B	3mL
10% SDS	0.45mL
10% APS	0.2mL
Temed	0.024mL

15% Tris-Glycine SDS-Polyacrylamide Gels:

dH <sub>2</sub> O	4.6mL
30% Acrylamide/bis-Acrylamide	10mL
Trizma Base (pH 8.8)	1.5M

10% SDS	0.2mL
10% APS	0.2mL
Temed	0.008mL

Stacking Gel:

dH <sub>2</sub> O	3.6mL
30% Acrylamide/bis-Acrylamide	0.8mL
Buffer C	0.5mL
10% SDS	0.05mL
10% APS	0.2mL
Temed	0.015mL

Sample Buffer:

Trizma Base-HCl	62.5mM
2-Mercapto-ethanol	5mL
10% Glycerol	10mL
SDS	2g
0.1% Bromophenol Blue	0.1g

10x Electrode Buffer:

Tizma Base	30.3g
Glycine	72.08g
SDS	5g

Dissolve in 1L of dH<sub>2</sub>O

Dilute 100mL in 900mL dH<sub>2</sub>O for use

10x Transfer Buffer:

Tizma Base	30.3g
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Glycine 72.08g

SDS 5g

Dissolve in 1L of dH<sub>2</sub>O

Dilute 100mL in 200mL ethanol and 700mL dH<sub>2</sub>O for use

0.1% PBS Tween:

PBS 500mL

Tween-20 0.5mL

0.5% PBS Tween:

PBS 500mL

Tween-20 2.5mL

Membrane Blocking Buffer:

Marvel 5%

0.1% PBS Tween 500mL

ECL:

Detection Solution 1 2mL

Detection Solution 2 2mL

Fixer:

Fixer 103mL

Make up to 470mL with ddH<sub>2</sub>O

Developer:

Developer 103mL

Make up to 470mL with ddH<sub>2</sub>O

## Catabolic Factors

Angiotensin II	0.1, 0.5, 1 and 2.5 $\mu$ M
Proteolysis Inducing Factor	2.1, 4.2, 10.5 and 16.8nM

## Anabolic Factors

L-Leucine	2mM
L-Isoleucine	2mM
L-Valine	2mM
L-Arginine	2mM
L-Methionine	2mM
HMB	25 and 50 $\mu$ M
Insulin Like Growth Factor-1 (Mouse recombinant)	75 and 100ng/mL
Insulin (From Bovine Pancreas)	0.1 and 1nM

## Protein Kinase Inhibitors

Rapamycin	25ng/mL
Genestein	30 $\mu$ M
Calphostin C	2 $\mu$ g/mL
LY294002	100 $\mu$ M
PKR inhibitor	50, 100, 200, 300, 500 and 1000nM

## eIF2 $\alpha$ Inhibitors

Salubrinal	3, 15 and 30 $\mu$ M
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## Antioxidants

Vitamin E  $10^{-5}$ M

GSH 3mM

## Calcium Chelators

BAPTA/AM  $25\mu$ M

## 2.3 Methods

### 2.3.1 Animal Studies

Animals were transplanted with fragments of the MAC16 tumour subcutaneously into the flank by means of a trochar, as described [24], selecting from donor animals with established weight loss. Weight loss was evident 12 to 15 days after tumour transplantation and animals were entered into experimental studies when they had lost approximately 5% of their starting body weight. Animals were randomised into groups of 6 to receive control solvent or experimental drug treatment. Both tumour volume and body weight were monitored daily. Animals were terminated by cervical dislocation when the body weight loss reached 25%, and all animal experiments followed a strict protocol approved by the British Home Office, and the ethical guidelines that were followed meet the standards required by the UKCCR guidelines [208].

Male NMRI mice bearing the MAC16 tumour were treated daily with the compounds detailed in Table 2.1. Upon treatment completion animals were sacrificed humanely and tissue/tumour was excised and either snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ , or used immediately for experimentation.



Table 2.1: Animal experiments

Drug Name	Drug Concentration	Drug Volume	Route Administered	Drug Vehicle	Treatment Span (days)
PKR inhibitor	1 and 5mg/kg	100 $\mu$ L	Subcutaneously	10% DMSO in PBS	5
BCAA (Leucine, Valine and Methionine)	1 g/kg	100 $\mu$ L	Gavage	PBS	5
Vitamin E	1 mg/kg	100 $\mu$ L	Subcutaneously	Olive oil	4
HMB	0.25g/kg	100 $\mu$ L	Gavage	PBS	4

### 2.3.2 Bio-Rad Protein Analysis

Protein concentration of samples was analysed by diluting 10 $\mu$ L of each sample in 790 $\mu$ L of double distilled water (ddH<sub>2</sub>O) with a blank of 800 $\mu$ L ddH<sub>2</sub>O. 200 $\mu$ L of the Bio-Rad reagent was added and the components mixed together by inversion. After 5min incubation at room temperature the samples were measured on a spectrophotometer at 595nm and the protein concentration measured using the following calculation:

$$\text{Protein } (\mu\text{g}/\mu\text{L}) = \text{Absorbance (595nm)} \times 0.1^* / 0.053^{**}$$

\* Dilution factor

\*\* Gradient of the BSA standard curve

### 2.3.3 Body Composition Analysis

After animals were humanely sacrificed their bodies were heated to 80-90°C for 48h, or until a constant weight was achieved. The water content was determined from the difference between wet weight and dry weight. Lipid were extracted from the dried carcass with chloroform/methanol (1:1). ethanol/acetone (1:1) and diethyl ether, which was then allowed to evaporate. The fat content was determined from the weighed residue. The non-fat carcass mass was calculated as the difference between the initial weight of the carcass and the weight of water and fat.

### 2.3.4 Cell Counting

MAC16 and MAC13 cells were grown (using the method described in Section 2.3.5) until reaching 90% confluence and then treated with the PKR inhibitor (0, 100, 200, 300, 500 and 1000nM) and incubated for a further 48h. 200 $\mu$ L of cells were counted 3 times to get an average value. Cells were counted on the basis that MAC16 cells have a diameter of 9.7 $\mu$ m and MAC13 cells have a diameter of 8.6 $\mu$ m.

### 2.3.5 Cell Culture

The C2C12 cell line, a clone of the C2 mouse satellite cell line derived from adult mice was used. C2C12 myoblasts were grown in 75cm<sup>2</sup> stock flasks in 25mL of DMEM medium containing 10% Fetal calf serum (FCS) until they neared (90-95%) confluency. The media was then poured off and the cells washed twice with sterile PBS. 3mL of 10% trypsin was added and incubated with the cells for 3-5min. 7mL of DMEM containing 10% FCS was added to neutralize the trypsin and this cell suspension was used to seed 6 well plates or 25cm<sup>2</sup> flasks for experimentation. 2mL of DMEM containing 10% FCS was added to the 6 well plates and 10mL of DMEM containing 10% FCS was added to 25cm<sup>2</sup> flasks.

When C2C12 myoblasts were 90-95% confluent the cells were induced to differentiate into multinucleated myotubes by exchanging the growth media with DMEM medium containing 2% horse serum. Myotubes were used no later than 4 days post differentiation.

MAC16/MAC13 cells were grown in 25mL of RPMI 1640 medium containing 10% FCS in 75cm<sup>2</sup> stock flasks. The MAC13 cells were trypsinised using the same method as with the C2C12 myoblasts and this cell suspension was used to seed 6 well plates or 25cm<sup>2</sup> flasks. However the MAC16 cells are non-adherent and therefore grow in a suspension. Thus 6 well plates and 25cm<sup>2</sup> flasks were seeded directly from the stock flask. MAC16/MAC13 cells were grown in RPMI 1640 containing 10% FCS until reaching 90-95% confluency upon which time they were used for experimentation.

Akt and PKR mutant C2C12 cells were grown in the same way as normal C2C12's except that the DMEM containing 10% FCS and 2% horse serum was supplemented with 1% Neomycin instead of Glutamine and Penstrep.

The media was changed every 3-4 days and all cell types were kept at 37°C and 5% CO<sub>2</sub> during growth and experimentation procedure unless specified otherwise.

### **2.3.6 Chymotrypsin Assay**

C2C12 myotubes were treated with the drug of choice and incubated for 2h prior to the addition of PIF or Ang II. Myotubes were then incubated for a further 24h. Cells were washed three times with sterile ice cold PBS before they were scraped and centrifuged down into a pellet. The cell pellet was either snap frozen and stored at -70°C, or used immediately for experimentation. All subsequent stages were performed on ice.

The cell pellet was resuspended in 500µL of homogenisation buffer and sonicated three times for 10sec. The cells were then centrifuged at 15,000rpm for 15min at 4°C. The supernatant was kept and the protein

content analysed using the Bio-Rad method (Section 2.3.2).

10 $\mu$ L of the supernatant was added in triplicate to a chymotrypsin plate followed by 10 $\mu$ L of lactacystein added to half the plate. The plate was read on a fluorometer set for reading 360nm excitation and 460nm emission. 100 $\mu$ L of the chymotrypsin substrate (N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methyl-Coumarin) was then added to every well and the plate incubated at room temperature for 1h with gentle shaking. After an hour the plate was read again at an excitation wavelength of 360nm and an emission wavelength of 460nm. The chymotrypsin activity was measured as activity per  $\mu$ g of protein.

### **2.3.7 Electrophoretic Mobility Shift Assay (EMSA)**

All the following steps were performed on ice or at 4°C. C2C12 myotubes were treated with the drug of choice and incubated for 2h. PIF or Ang II was then added and the myotubes incubated for a further 30min. Cells were washed in ice cold PBS, then scraped and centrifuged down. The cell pellet was snap frozen and stored at -70°C until use.

The cell pellet was defrosted then resuspended in 400 $\mu$ L of Buffer A. Gastrocnemius muscle or a tumour sliver from an animal experiment (see Section 2.3.1 for method) was homogenized in 400 $\mu$ L of buffer A. Cells were allowed to swell on ice for 10min. The cells were vortexed for 10sec then centrifuged for 10sec and the supernatant discarded.

The pellet was resuspended in 100 $\mu$ L of ice cold Buffer C and incubated on ice for 20min for high salt extraction. The cell samples were then centrifuged for 2min to remove cellular debris. The supernatant was kept and its protein concentration determined using the Bio-Rad method.

The following reagents were combined:

Sample 5 $\mu$ g

Poly DI 1 $\mu$ L

NF- $\kappa$ B Probe 1 $\mu$ L

MgCl<sub>2</sub> 1 $\mu$ L

NP40 1 $\mu$ L

KCl 1 $\mu$ L

EDTA 1 $\mu$ L

Made up to 20 $\mu$ L with ddH<sub>2</sub>O

Samples were incubated on ice for 30min. Wells in the stacking gel were flushed out and the gel pre-run at 90 volts using 0.5x TBE for running buffer. 1 $\mu$ L of the loading dye was added to the samples, which were then loaded into the gel and run on ice for 2h at 90 volts.

The Biorad B nylon membrane, sponges and Whatman 3mm paper were pre-soaked in 0.5x TBE buffer before use. Proteins were transferred from the gel onto the nylon membrane for 45min at 80 volts. The membrane was placed into a clean weighing boat and heated for 30min at 85°C in an oven.

The membrane was blocked by adding 20mL of blocking buffer (warmed to 37°C before use) and incubated for 15min at room temp with gentle shaking.

The blocking buffer was replaced with conjugate/blocking solution (1:300 dilution) and incubated with the membrane for 15min with gentle shaking.

The membrane was washed four times for 5min with 1x wash buffer (warmed to 37°C before use).

The membrane was then transferred to a new container and 30mL substrate equilibration buffer added and incubated for 5min with gentle shaking.

The membrane was placed into a clean container and the substrate working solution was poured on and incubated for 5min without shaking.

The membrane was covered in plastic wrap and exposed to X-ray film for 2-5min before developing.

### **2.3.8 Human Studies**

#### **Cancer Patients and Controls**

Patients provided written, informed consent, and the study was approved by the Lothian Research Ethics Committee. Twenty-nine patients with newly-diagnosed oesophago-gastric adenocarcinoma who were undergoing elective resection of their primary cancer were recruited for the study. Oesophago-gastric cancer patients have a high incidence of weight loss [53] and were therefore chosen as a representative group of patients who develop cancer cachexia. Muscle biopsies were also collected from ten healthy, weight-stable volunteers who were undergoing elective hernia surgery and who served as controls.

#### **Muscle Biopsy**

A sample of rectus abdominis muscle was obtained from the edge of the patients abdominal wound within 10min of induction of general anaesthesia. The sample was obtained without the use of diathermy and was frozen immediately in liquid nitrogen. Samples were frozen at -70°C until analysis.



### 2.3.9 Immunoprecipitation of HA.11 Tagged Akt

Spin columns from the Catch and Release v2.0 Reversible Immunoprecipitation System were used in this method.

The myoblasts had undergone three passages in culture before being differentiated into myotubes. Wildtype, MyrAkt and DNAkt C2C12 myotubes were washed in ice cold PBS 3 times then scraped and centrifuged down into a pellet. The cell pellets were resuspended in 500 $\mu$ L of Phosphosafe extraction buffer. Cells were incubated for 5min at room temperature allowing them to lyse. The lysed cell suspension was then centrifuged at 15,000rpm for 15min at 4°C. The supernatant was kept and the protein concentration measured using the Bio-Rad method (Section 2.3.2).

Spin columns were centrifuged at 5000rpm for 15-30sec to remove the resin slurry buffer. The resin was then washed twice with 400 $\mu$ L of 1x wash buffer.

Reagents were added to the spin columns in the following order:

1x wash buffer

Cell lysate (500 $\mu$ g)

HA.11 antibody (4 $\mu$ L)

Antibody capture affinity ligand (10 $\mu$ L)

Made up to 500 $\mu$ L with the 1x wash buffer

The reagents were allowed to mix in the spin column overnight at 4°C shaking gently. The spin column was centrifuged at 5000rpm for 15-30sec, then washed 3 times with 400 $\mu$ L of 1x wash buffer, spinning at 5000rpm for 15-30sec for each wash.

The protein was eluted from the column using denaturing buffer. The protein sample was then loaded on to a 10% SDS polyacrylamide gel (see Section 2.3.21 for method). After transfer the nitrocellulose membrane was probed with the Akt antibody. The film was exposed for 10min.

### **2.3.10 Intracellular Amino Acid Pool Measurement**

The method for the protein synthesis assay *in vitro* (see Section 2.3.15) was followed up to the point where the 0.2M perchloric acid was added. Instead of discarding the perchloric acid it was added to a centrifuge tube and mixed with 1mL of potassium chloride, then centrifuged at 4500rpm for 10min. 0.5mL of the supernatant was added to 8mL of OptiPhase HighSafe 3 scintillation fluid and sample radioactivity was analysed over 4min as disintegrations per minute on a scintillation counter.

#### **2.3.11 PIF Purification**

PIF was purified from solid MAC16 tumors excised from mice with a weight loss between 20 and 25%.

All the following steps were performed on ice or at 4°C. MAC16 tumours were homogenised in ice cold QS1 buffer (1g tumour/5mL QS1 buffer) then centrifuged at 4500rpm for 20min. The pellet was removed and the supernatant centrifuged again at 4500rpm for 20min. Ammonium sulphate (38% w/v) was added gradually over 6-8h, whilst stirring. Upon completion of ammonium sulphate addition the homogenate was covered and left stirring overnight.

The homogenate was centrifuged twice at 4500rpm for 20min and the supernatant removed and equilibrated in sterile PBS in an Amicon with a molecular weight 10,000 cut off membrane. Once the total volume reached 10-15mL the homogenate was centrifuged twice at 4500rpm for 20min and the supernatant circulated on an affinity column overnight at 0.1mL/min. The affinity column was prepared by coupling the monoclonal antibody isolated from MAC16 hybridomas to Protein A on an Affi-Gel Hz matrix using

the method performed by Todorov et al [194].

The column was washed with sterile PBS for 1h at 0.3mL/min. The PIF was eluted into an equal volume of 0.2M sodium bicarbonate with 0.2M Glycine (pH2.5) at 0.1mL/min for 1.5h. The eluate was then equilibrated in PBS and concentrated in an Amicon Ultra centrifuged at 4000rpm for 1h. PIF concentration was then measured using the Bio-Rad method (Section 2.3.2).

### 2.3.12 m<sup>7</sup> GTP Sepharose Purification

This method was used to purify eIF4E and eIF4E associated proteins from cell/muscle extracts since eIF4E can bind to 7-methylguanosine triphosphate (m<sup>7</sup> GTP).

The growth medium of C2C12 myotubes was changed from DMEM containing 2% horse to unsupplemented DMEM media 18h prior to experimentation. C2C12 myotubes were incubated with the drug of choice for 2h then PIF was added and incubated with the cells for a further 4h. Cells were scraped and centrifuged down and the pellet was snap frozen and stored at -70°C until used.

All subsequent steps were performed on ice or at 4°C. The cell pellet was defrosted then resuspended in 500μL of lysis buffer and incubated for 5min at room temp with occasional vortexing. The samples were then centrifuged at 15,000rpm for 15min and the supernatant reserved for protein analysis by the Bio-Rad method (see Section 2.3.2).

100μL of m<sup>7</sup> GTP sepharose slurry was washed 3 times with 1mL of lysis buffer, then resuspended in a further 100μL of lysis buffer. 400μg of protein from the supernatant was added to 100μL of m<sup>7</sup> GTP sepharose and left to mix overnight.

The supernatant/m<sup>7</sup> GTP sepharose mixture was centrifuged at 13,000rpm for 15sec and then washed 3 times with 1mL of lysis buffer, centrifuging at 13,000rpm for 15sec for each wash. The m<sup>7</sup> GTP sepharose

beads were resuspended in SDS sample buffer and boiled for 5min. The beads were centrifuged down and the supernatant removed and loaded onto an SDS polyacrylamide gel (see Section 2.3.21 for further method).

### 2.3.13 Preparation of Samples for Western Blotting

For cell samples that were to be detecting components involved in protein degradation, the drugs were added to C2C12 myotubes growing in DMEM containing 2% horse serum. The myotubes were dosed with the required drug and incubated for 2h. PIF or Ang II was then added, and the cells incubated for a further 24h, except when western blotting for phosphorylated and total I $\kappa$ B, where the cells were incubated for 30min and 45min respectively.

For cell samples that were to be detecting components involved in protein synthesis, the cells were washed 3 times with sterile PBS, then unsupplemented media was added 18h prior to experimentation, with the exception of MAC16/MAC13 cells, where the drug was added directly to the RPMI 1640 medium containing 10% FCS, and C2C12 myotubes treated with the calcium chelator BAPTA/AM, where the drug was added directly to the DMEM medium containing 2% horse serum, since it was lethal to the cells in unsupplemented media. The drug was added to C2C12 myotubes and incubated for 2h prior to the addition of PIF or Ang II, which was then incubated with the cells for a further 4h. The MAC16/MAC13 cells were treated with the PKR inhibitor and incubated for 48h.

C2C12 myotubes or MAC13 cells were washed 3 times with 10mL ice cold sterile PBS, then centrifuged in 1mL of PBS at 4500rpm. The PBS was removed and the cell pellet snap frozen in liquid nitrogen, then stored at -70°C until use.

MAC16 cells were centrifuged at 4500rpm for 2min at 4°C, since they were in suspension, and the media removed. The cell pellet was washed 3 times with 10mL ice cold sterile PBS, and centrifuged at 4500rpm for 2min at 4°C for each wash. The PBS was removed and the cell pellet snap frozen, then stored at -70°C

until use.

The cell pellet was resuspended in 500 $\mu$ L of Phosphosafe extraction buffer, and incubated at room temperature for 5min with occasional vortexing.

Mouse tumours and muscle was excised from experimental animals (see Section 2.3.1 for method). The tissue samples were defrosted on ice, then homogenized in 500 $\mu$ L of Phosphosafe extraction buffer. The homogenate was then incubated for 10min at room temperature with occasional vortexing.

The lysed cell /homogenate samples were centrifuged at 15,000rpm for 15min at 4°C. The protein concentration of the supernatant was measured using the Bio-Rad method (Section 2.3.2).

5-15 $\mu$ g of protein was mixed with an equal volume of SDS sample buffer and loaded on to an SDS polyacrylamide gel (see Section 2.3.21 for further method).

### **2.3.14 Protein Degradation Assay (In Vitro)**

Fresh DMEM containing 2% horse serum was added to C2C12 myotubes in 6 well plates, and 10 $\mu$ L of the [<sup>3</sup>H] Phenylalanine working stock was added per well, and myotubes were incubated overnight to allow cellular incorporation.

Cells were washed three times with sterile PBS, and residual PBS removed using a Pasteur pipette. 2mL of chase media was added and cells incubated for a further 2 hours. The chase media was discarded and residual media removed with a Pasteur pipette, then 2mL of fresh chase media was added. The cells were treated with the appropriate drug and incubated for 2h upon which time PIF or Ang II were added and the myotubes incubated for a further 24h.

1mL of media was removed and added to 8mL of OptiPhase HighSafe 3 scintillation fluid and sample

radioactivity was analysed over 4min as disintegrations per minute on a scintillation counter.

### **2.3.15 Protein Synthesis Assay (In Vitro)**

C2C12 myotubes were washed 3 times with sterile PBS, and 2mL of unsupplemented media was added to the 6 well plates 18h before beginning the assay.

Drugs were added as required and incubated with the myotubes for 2h. 10 $\mu$ L per well of the [<sup>3</sup>H] Phenylalanine working stock was added with the PIF or Ang II, and incubated with the myotubes for 4h unless stated otherwise.

Cells were washed 3 times with sterile ice cold PBS, which was then replaced by 1mL of ice cold 0.2M perchloric acid. The myotubes were incubated at 4°C for 20min before replacing the perchloric acid with 1mL of 0.3M NaOH and incubating for a further 30min at 4°C, followed by a 20min incubation at 37°C.

The NaOH, which now contained the cellular proteins, was added to a centrifuge tube. Each well was washed with a further 1mL of 0.3M NaOH which was also added to the centrifuge tube. 0.5mL of 0.2M perchloric acid was added to the tubes, and incubated on ice for 20min, to precipitate cellular proteins. The tubes were centrifuged at 700rpm for 5min at 4°C and the supernatant poured off. The pellet was dissolved in 1mL of 0.3M NaOH and 0.5mL of this was added to 8mL of OptiPhase HighSafe 3 scintillation fluid. The radioactivity of each sample was calculated over 4min on a scintillation counter as disintegrations or counts per minute.

### **2.3.16 Protein Synthesis Assay (Ex Vivo)**

Gastrocnemius muscles were removed from MAC16 mice (see Section 2.3.1 for details) and incubated for 30min at 37°C in RPMI 1640 without phenol red, and saturated with O<sub>2</sub>/CO<sub>2</sub> (19:1). The muscle was



then washed in PBS and placed in 5mL of fresh media containing L-[4-<sup>3</sup>H] phenylalanine and incubated for 2h. The muscles were rinsed in non-radioactive medium then weighed.

Each gastrocnemius muscle was homogenized in 4mL 2% perchloric acid, then centrifuged at 2800rpm for 15min. The supernatant and the pellet were reserved. The supernatant was transferred to a new centrifuge tube, and the pH was adjusted to 6 by adding saturated tripotassium citrate. Insoluble potassium perchlorate was removed by centrifuging at 2800rpm for 15min and 1mL of the supernatant was added to 8mL of OptiPhase HighSafe 3 scintillation fluid, and sample radioactivity was assessed over 4min to measure protein in the intracellular free pool.

The pellet, which was reserved from the earlier step, was washed 3 times with 5mL 2% perchloric acid and centrifuged at 2800rpm for 15min for each wash. The pellet was then hydrolysed in 6N HCl at 110°C for 24h. All residual liquid was evaporated off, and the pellet dissolved in 10mL ddH<sub>2</sub>O. 1mL of this was added to 8mL of OptiPhase HighSafe 3 scintillation fluid and the radioactivity was quantified on a scintillation counter to determine the bound protein. Rate of protein synthesis was calculated by the following calculation:

$$\text{Rate of protein synthesis} = \text{Bound protein} / \text{Unbound protein pool}$$

### 2.3.17 RNA Measurement

The method for the protein synthesis assay in vitro (see Section 2.3.15) was followed up to the point after centrifuge tubes containing dissolved cellular proteins and perchloric acid was centrifuged at 700g for 5min at 4°C. Instead of discarding the supernatant this was kept and 1mL was added to a quartz cuvette. The RNA was measured using the absorbance at 260nm and 232nm. The following equation uses the modified formula of Ashford and Pain [10] which corrects for any absorbance due to the presence of aromatic amino acids:

RNA cellular content( $\mu\text{g}/\text{mL}$ ) = absorbance(260nm) $\times$ 32.9 - absorbance(232nm) $\times$ 6.11

### 2.3.18 Transformation of E.coli DH5 $\alpha$ Cells using PKR and Akt Plasmids

#### Preparation of competent E.coli DH5 $\alpha$ cells

A single colony of DH5 $\alpha$  cells were used to prepare overnight cultures, which were grown in 10mL of SOB broth. 0.25% (v/v) of the overnight culture was added to sterile SOB broth and incubated at 37°C until cells reached the logarithmic stage of growth (approximately 0.4 OD at 550nm). The cells were incubated on ice for 30min, then centrifuged at 2500rpm for 15min at 4°C. The supernatant was discarded. The cell pellet was resuspended in ice cold RFP 1 buffer at 33% volume of the original media and kept on ice for 60min. The cells were centrifuged at 2500rpm for 15min at 4°C and the supernatant discarded. The cell pellet was resuspended in RFB 2 buffer at 8% volume of the original volume of media, and put on ice for 15min. The cells were now competent and ready to use or snap frozen and stored at -70°C until use.

#### Transformation of Competent E.coli DH5 $\alpha$ Cells

100 $\mu\text{L}$  of the competent cells were mixed gently with approximately 5ng of transforming DNA and kept on ice for 30min. The cell/DNA mixture was heat shocked in a water bath for 1min at 37°C, then put on ice for 2min. 0.9mL of sterile LB broth was added to the mixture and incubated for 45min at 37°C to allow expression of phenotypic characterization. 200 $\mu\text{L}$  of transformed cells were spread onto LB agar supplemented with ampicillin for selection purposes. The agar plates were incubated overnight at 37°C. Single colonies were picked the following morning for use in PCR reactions.

#### Colony PCR Reactions

PCR reactions were performed using the PCR master mix (Promega) for the Akt plasmids and 1x PCR buffer (Bioline) for the PKR plasmids. PCR reactions using cells transformed using the Akt plasmids were set up as follows:

PCR master mix (1x) 12.5 $\mu$ L

Forwards primer 0.55 $\mu$ L (5'd GAT TAG GGT GAT GGT TCA 3')

Reverse primer 0.86 $\mu$ L (5'd GTT CAA TCA TGC GAA ACG 3')

DNA template 1 colony

Make up to 25 $\mu$ L with nuclease free ddH<sub>2</sub>O

PCR reactions using cells transformed with PKR plasmids were set up as follows:

PCR buffer (1x)

DNTs (deoxynucleotides) 100 $\mu$ M

MgCl<sub>2</sub> 1.5 $\mu$ M

DNA template 1 colony

T7 forward primer 400pM (5'd TTA ATA CGA CTC ACT ATA GGG 3')

BGH reverse primer 400pM (5'd TAG AAG GCA CAG TCG AGG 3')

Taq polymerase 2 units/50 $\mu$ L

Make up to 25 $\mu$ L with nuclease free ddH<sub>2</sub>O

PCR reactions were performed using the standard 30 PCR cycles using the following temperatures:

95°C for 3min prior to cycle 1

95°C for 30sec

52°C for 30sec (Akt transformations)

55°C for 30sec (PKR transformations)

72°C for 60sec

### Plasmid DNA Purification and Quantification

4 $\mu$ L of loading buffer (6x) was added to 24 $\mu$ L of each PCR product, which was then loaded onto a 1% agarose gel. TAE buffer (1x) was used as the electrophoresis buffer and gels were run at 4volts/cm potential difference. The DNA was visualized using the GeneSnap transilluminator photographic system.

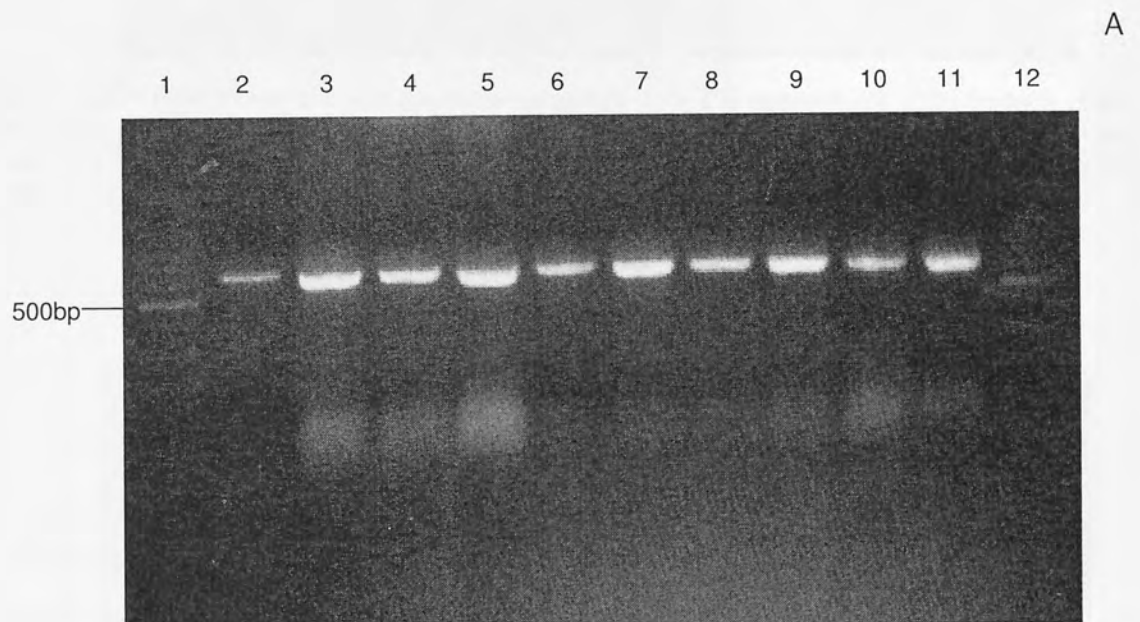


Figure 2.1

B

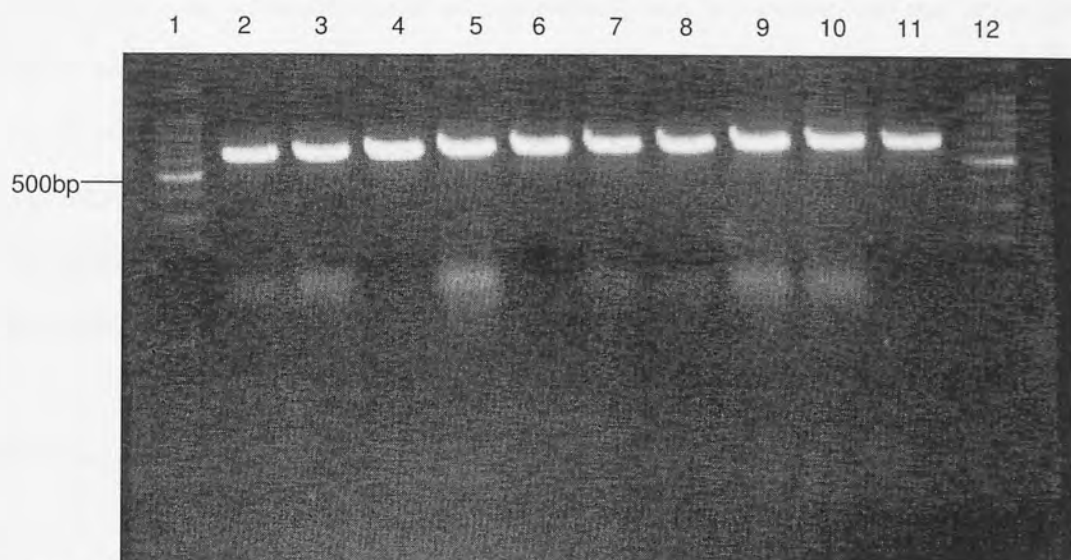


Figure 2.1: **PCR products of the Akt plasmids visualised on a 1% agarose gel.** PCR products of the Akt plasmids were run on a 1% agarose gel to determine the best colonies to use for further experimentation. In lanes 2-11 the PCR product generated was approximately 590bp long for the MyrAkt (A) and DNAkt (B) allele. A 100bp ladder was used in lanes 1 and 12. All colonies were appropriate for further use

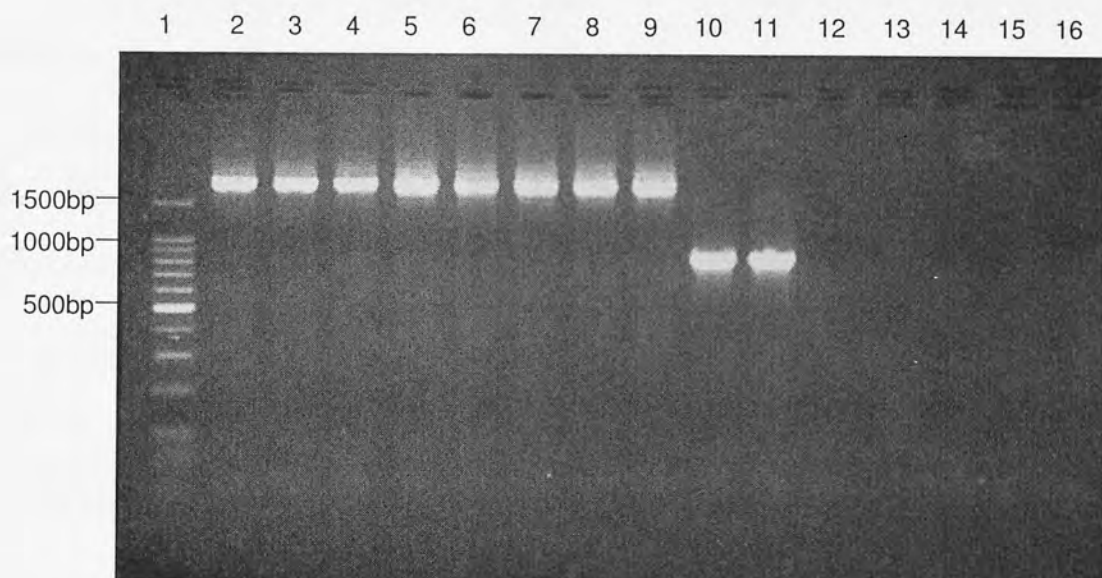


Figure 2.2: **PCR products of the PKR plasmids visualised on a 1% agarose gel.** PCR products of the PKR plasmids were run on a 1% agarose gel to determine the best colonies to use for further experimentation. In lanes 2-9 the PCR reaction yielded a wildtype PKR product of approximately 1656bp (lanes 2-5) and a mutant  $\Delta 6$  PKR product (lanes 6-9) of approximately the same size. The negative control (lanes 13-16) contain PCR mastermix with no cells added while the pcDNA3 control (lanes 10 and 11) comprised of pcDNA3 containing an 800bp insert. A 100bp ladder was used in lane 1. All colonies were appropriate for further use

Products of the relevant size were visualised on the agarose gels (Figure 2.1 and 2.2) confirming that the DH5 $\alpha$  E.coli cells were successfully transformed with the Akt and PKR mutants and that all colonies were suitable for further experimentation.

The best colonies shown by PCR were grown up in 250mL LB media supplemented with ampicillin overnight at 37°C with shaking. Plasmid DNA was recovered from the 250mL liquid culture using the Wizard plus maxiprep DNA purification system following the manufacturers instructions.

DNA was quantified with a spectrophotometer at 260nm absorbance using the following calculation:

$$\text{DNA } (\mu\text{g}/\mu\text{L}) = \text{Absorbance at 260nm} \times 50^* \times \text{dilution factor} / 1000$$

\* From the assumption that an OD of 1 at 260nm equates to 50 $\mu\text{g}/\mu\text{L}$  of double stranded DNA

### **Restriction Digest of Plasmid DNA**

A restriction digest was performed to linearise the purified plasmid DNA containing the PKR wild type and  $\Delta 6$  gene and to ensure that the DNA had been successfully extracted. Restriction digests were carried out using the following components:

DNA 1 $\mu\text{g}$

Buffer 2 (37°C) 4 $\mu\text{L}$

Hind III 1 $\mu\text{L}$

BSA 0.4 $\mu\text{L}$

Make up to 15 $\mu\text{L}$  with nuclease free ddH<sub>2</sub>O



4 $\mu$ L of loading buffer (6x) was added to the products of the restriction digest, which was then loaded onto a 2% agarose gel. TAE buffer (1x) was used as the electrophoresis buffer and gels were run at 5volts/cm potential difference. The DNA was visualized using the GeneSnap transilluminator photographic system. Figure 2.3 shows restriction digest products of the appropriate size and purity confirming that the PKR DNA was successfully extracted and available for use in the transfection of murine myotubes. Restriction digests were performed to determine the validity of the pcDNA3 plasmid since there is only a single Hind III restriction enzyme site in the pcDNA3 plasmid. The correct banding pattern for pcDNA3 was observed as a single band of approximately 2102bp when containing the PKR insert.



Figure 2.3: **Restriction digest of the maxiprep PKR plasmids visualised on a 2% agarose gel.** The PKR plasmid DNA was purified using the maxiprep system then subjected to restriction digest. Products of the restriction digest were run on a 2% agarose gel to determine if the DNA had been successfully extracted from the DH5 $\alpha$  cells. An unlinearised pcDNA3 plasmid was visualised (lanes 2 and 7) of approximately 5446bp. The restriction digest generated linearised PKR wild type (lanes 3-5) and PKR  $\Delta$ 6 plasmids (lanes 8-10) of approximately 7102bp. A 10,000bp ladder was visualised in lanes 1, 6 and 11

### Sequencing of the Maxiprep Products

Sequencing was performed to ensure that the PKR $\Delta$ 6 mutant gene contained the deletion and that the wild type PKR gene did not. Sequencing reactions were performed by the Birmingham University Functional Genomics Laboratory. Sequencing was carried out using the Big Dye 3 chain termination protocol. The

PKR wildtype and  $\Delta 6$  sequence generated was aligned with the human PKR coding sequence extracted from the NCBI website, genbank accession number M35663. DNA sequence alignments were carried out using multiple sequence alignment (MultAlin) by Florence Corpet [47]. Sequencing reactions yielded a PKR $\Delta 6$  sequence that lacked six amino acids (361-366) between catalytic domains IV and V, which render the PKR variant catalytically inactive, and an intact PKR wildtype gene (Figure 2.4), which is compatible with the coding sequence of the human PKR gene.

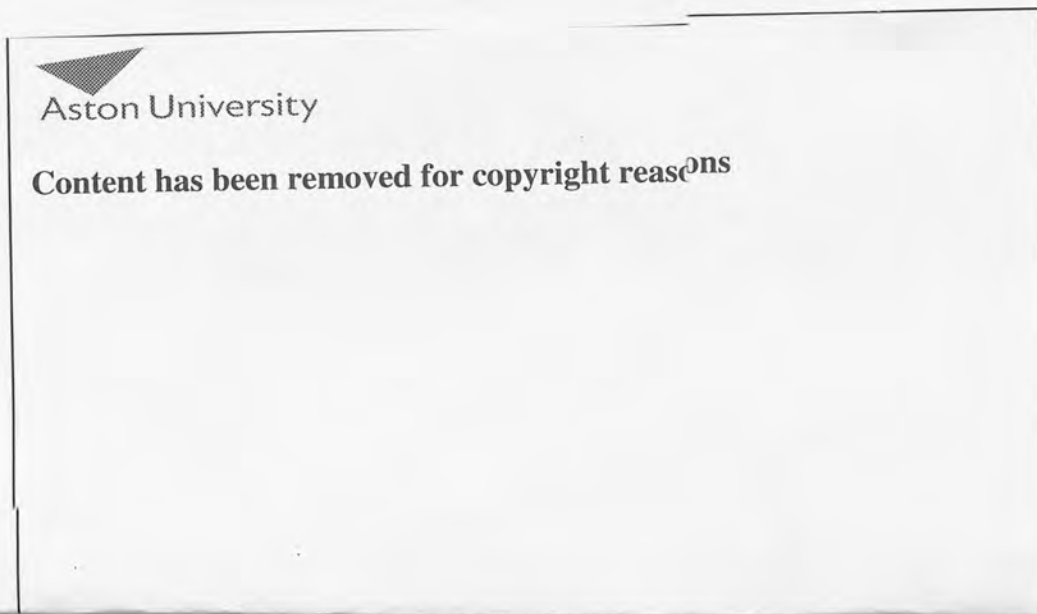


Figure 2.4: **Coding sequence of PKR wildtype and PKR $\Delta 6$ .** Sequencing of the PKR wildtype (A) and PKR $\Delta 6$  genes (B) aligned with the coding sequence of human PKR, obtained from the NCBI website, using multiple sequence alignment

### 2.3.19 Transfection of C2C12 Myoblasts with Plasmid DNA

One day prior to transfection C2C12 myoblasts were plated out into 20cm<sup>2</sup> flasks and grown in 5mL DMEM growth medium without antibiotics so that the cells were 50-60% confluent the following day. 8 $\mu$ g of DNA was diluted in 500 $\mu$ L of DMEM media without serum supplementation and mixed gently. 20 $\mu$ L of lipofectamine was diluted in 500 $\mu$ L of serum free media. The DNA and lipofectamine were incubated for 5min at room temperature. The DNA and lipofectamine were combined and mixed gently, then incubated for 20min at room temperature. 1mL of the coupled complexes were added to the C2C12 myoblasts and

incubated at 37°C, 5% CO<sub>2</sub> for 24h. Cells were passaged and incubated for a further 24h in DMEM media without antibiotics. Selection media was added and cells grown for at least 48-72h prior to use.

To determine whether the expression of the Akt plasmid was stable, cell lysates were immunoprecipitated with anti-HA antibody, followed by Western blotting for Akt (see Section 2.3.9 and 2.3.21 for methods used). Also, to confirm that the plasmids were influencing cellular pathways as expected, myotubes were treated with IGF-I (13.2nM) for 1h and the levels of phosphorylation of the 70kDa ribosomal S6 kinase (p70S6K) and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) was determined.



Figure 2.5: **Western blot of immunoprecipitated HA-tagged Akt in myotubes transfected with Akt mutant plasmid DNA.** HA-tagged Akt was immunoprecipitated from myotubes transfected with empty pcDNA3 plasmid (lane 1), MyrAkt (lane 2) and DNAkt (lane 3) using rabbit polyclonal antisera to Akt. The lower band is the Ig heavy chain.

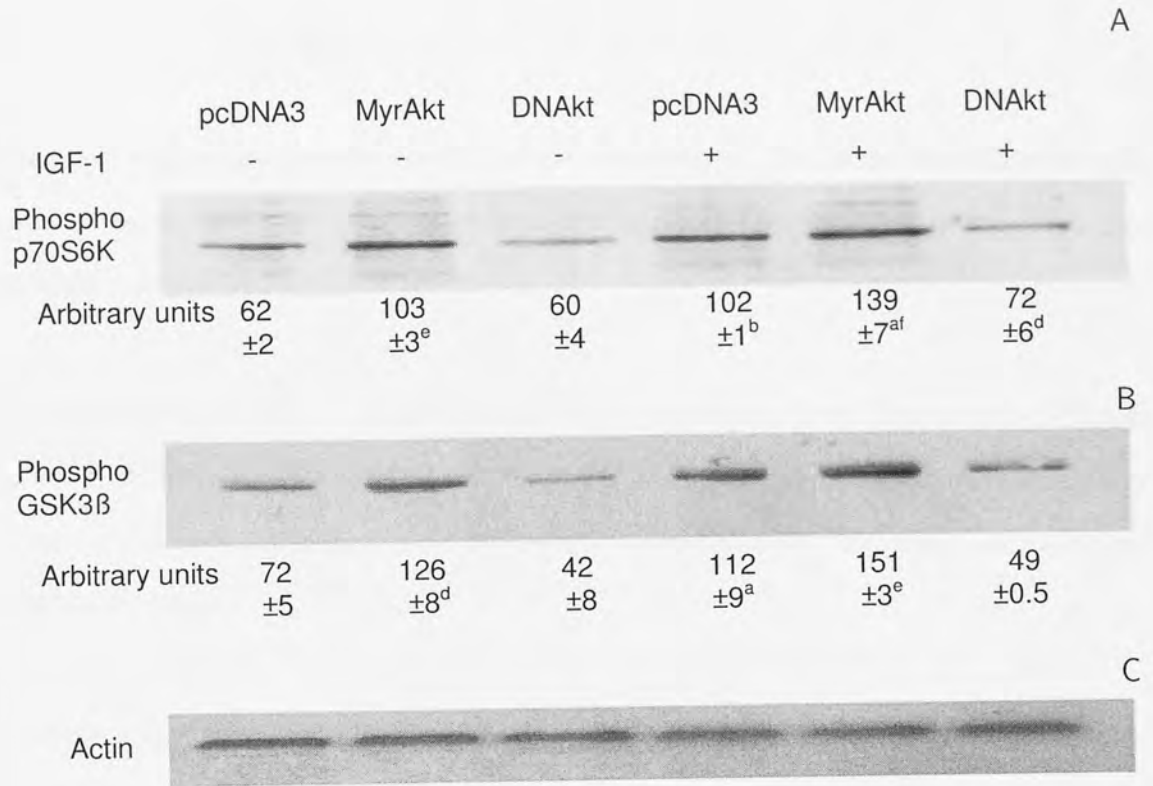


Figure 2.6: **Effect of Akt mutation on the phosphorylation of p70S6K and GSK-3β in myotubes incubated with IGF-1.** Western blot for the effect of IGF-I (13.2nM) on phospho p70S6K (A) and GSK-3β (B) with a corresponding actin loading control (C) in myotubes transfected with pcDNA3(-), MyrAkt and DNAkt. Densitometric analysis is shown below where ± represents SEM of three replicate blots. Differences from control are shown as a, p<0.05 and b, p<0.01 while differences from pcDNA3(-) are shown as d, p<0.05, e, p<0.01 or f, p<0.001

Figure 2.5 confirmed that this was a stable transfection and expression of both dominant negative and constitutively active Akt was observed in myotubes. As expected IGF-I increased the level of phosphorylation of p70S6K (Figure 2.6A) and GSK-3β (Figure 2.6B) in myotubes containing the empty plasmid (pcDNA3(-)), but not in those containing DNAkt. Myotubes containing MyrAkt showed an increased phosphorylation of p70S6K and GSK-3β, even in the absence of IGF-I.

### 2.3.20 Tyrosine Release Assay (Ex Vivo)

Soleus muscle was excised from MAC16 tumour bearing mice (see Section 2.3.1 for details) and placed in ice cold sterile PBS. The PBS was replaced by Krebs-Heinsleit buffer and incubated at 37°C, 5% CO<sub>2</sub> and

95% O<sub>2</sub> for 2h.

Excess liquid was removed from the muscles and they were weighed. 2mL of the Krebs-Heinsleit buffer incubation medium was removed and put into a glass centrifuge tube with 20 $\mu$ L of 30% TCA, then centrifuged at 2800rpm for 10min.

The supernatant was removed and mixed with 1mL 0.1% 1-nitroso-2-naphthol and 1mL nitric acid in a fresh glass centrifuge tube before incubating at 55°C for 30min. The mixture was allowed to cool before mixing with 5mL of dichloroethane (to extract the uncharged 1-nitroso-2-naphthol). The mixture was centrifuged at 2800rpm for 10min.

10 $\mu$ L of the aqueous phase was added to a 96 well plate and the fluorescence measured at 460nm excitation and 570nm emission. To calculate tyrosine release the following calculation was used:

$$\text{Fluorescence/g/2h} = \text{Fluorescence} / \text{Muscle weight(g)}$$

### 2.3.21 Western Blotting

5-15 $\mu$ g of protein was loaded on to an SDS polyacrylamide gel (see table 2.2 for gel types used) and run for 45-50min at 180 volts. The 3MM Whatmann filter paper, sponges and nitrocellulose membrane was pre soaked in 1x transfer buffer. The gel was placed on one sponge and 3 pieces of filter paper and covered by the nitrocellulose membrane. A further 3 pieces of filter paper and one sponge were placed on top of the nitrocellulose membrane and sealed tightly. The cellular proteins were electrotransferred onto the membrane over 2h at 80 volts on ice.

After transfer the membrane was blocked in 50mL of blocking buffer for 1-2h. The membrane was rinsed with 50mL 0.5% PBS-tween then incubated with the primary antibody diluted in 0.1% PBS-Tween. The primary antibody was washed off with 0.1% PBS-Tween, and secondary antibody added (1:1000 dilution

in 0.1% PBS-Tween) and incubated for 1-2h. The secondary antibody was washed off with 0.1% PBS-Tween for 45min, changing the wash every 15min. (see table 2.2 for incubation, wash time, antibody type and antibody dilution). ECL was added to the membrane and incubated without shaking for 2min. Nitrocellulose membrane was wrapped in cling film and exposed to photographic film for 30sec - 15min. Bands were visualized on the X-ray film using developer and fixer.

Western blot band density was quantified using the GeneTools image analysis software.

Table 2.2: Antibody details

<b>Antibody</b>	<b>Dilution</b>	<b>Incubation (h)</b>	<b>Wash Time (min)</b>	<b>Gel Type (%)</b>	<b>Secondary Antibody</b>
Actin	1:200	1-2	45	10	Rabbit
Akt (phospho/total)	1:1000	24	15	10	Rabbit
4E-BP1 (phospho)	1:500	24	15	15	Rabbit
4E-BP1 (total)	1:1000	24	15	15	Rabbit
eEF2 (phospho/total)	1:1000	24	15	10	Rabbit
eIF2 $\alpha$ (phospho)	1:500	24	15	10	Rabbit
eIF2 $\alpha$ (total)	1:1000	1-2	15	10	Rabbit
eIF4E (phospho)	1:500	24	15	10	Rabbit
eIF4E (total)	1:1000	24	15	10	Rabbit
eIF4G (phospho/total)	1:1000	24	15	10	Rabbit
GSK3 $\beta$ (phospho)	1:1000	24	15	10	Rabbit
I $\kappa$ B (phospho/total)	1:1000	24	15	10	Rabbit
mTOR (phospho/total)	1:1000	24	15	6	Rabbit
Myosin	1:100	24	15	6	Mouse
P42	1:1000	1-2	30	10	Mouse
p70S6K (phospho/total)	1:1000	24	15	10	Rabbit
Anti PIF antibody	10 $\mu$ g	24	15	10	SHPC
PKR (phospho/total)	1:1000	24	15	10	Rabbit
20S	1:1000	1-2	30	10	Mouse

Note: SHPC = Streptavidin horseradish peroxidase conjugate







## Chapter 3

# Effect of PIF/Angiotensin II on Protein Synthesis and Degradation

### 3.1 Introduction

Loss of skeletal muscle mass in cancer patients has a negative effect on prognosis, which leads to a poor quality of life and early death. The wasting of skeletal muscle is seen as a combination between an increase in protein degradation and a decrease in protein synthesis. The ubiquitin proteasome proteolytic pathway is considered the predominant system involved in the breakdown of myofibrillar protein in both experimental models of cancer cachexia and cachectic cancer patients (see Figure 1.1 in Chapter 1 for an outline of this pathway). The upregulation of proteolytic activity is believed to be via an increase in DNA binding of the transcription factor NF- $\kappa$ B, which increases the gene and protein expression of key components of the proteasome such as, proteasome subunits and enzymes of ubiquitin conjugation [210].

The relative importance of synthesis and degradation vary between individual studies, however Emery et

al [60] suggested that muscle mass in cachectic cancer patients is regulated mainly through changes in the rate of protein synthesis, rather than protein degradation. The importance of protein synthesis alterations in cancer cachexia was shown, where one study demonstrated a decreased rate of muscle protein synthesis observed in rectus abdominus muscle in weight-losing cancer patients, when compared to age matched controls. However other studies in tumour bearing animals have shown that loss of muscle occurs by both a decrease in protein synthesis and increase in protein degradation, without a decrease in calorie intake [168].

Muscle mass in cancer cachexia is strongly regulated by a tumour produced sulphated glycoprotein called PIF, which inhibits protein synthesis and stimulates protein degradation [210]. PIF has been found to be correlated to muscle wasting in both cancer patients, where PIF has been found in the urine of patients with weight loss greater than 10%, and experimental models, where PIF has been found in the serum of mice bearing the cachexia inducing MAC16 tumour [168]. One study showed that the PIF produced by human melanoma cells was structurally and functionally identical with the mouse factor [193].

In vitro studies using murine C2C12 myotubes treated with PIF over 24 hours, showed an increase in protein degradation with a typical bell shaped dose response curve with a maximal effect at a concentration of 4.2nM. PIF has been demonstrated to induce protein degradation in C2C12 murine myotubes through the activation of the proteasome. The functional activity of the proteasome was assessed by measuring the chymotrypsin like enzyme activity, which is the predominant proteolytic activity of the  $\beta$ -subunits of the proteasome. The maximum increase in chymotrypsin like activity was seen at 4.2nM [167]. PIF has been shown to reduce the rate of protein synthesis in a dose dependent manner, where the maximum effect was also at 4.2nM, as with protein degradation. However, the increase in protein degradation by PIF was attenuated by the addition of the polyunsaturated fatty acid, EPA, which had no effect on protein synthesis inhibition suggesting that protein synthesis and degradation are regulated by PIF through different mediators [166].

Treatment of C2C12 myoblasts with PIF showed a similar effect to that of gastrocnemius muscles of mice after administration of PIF, where protein degradation was increased and protein synthesis decreased. This

demonstrates that the C2C12 cell line is an appropriate model for experimental studies with PIF since it responds similarly to skeletal muscle [166].

Angiotensin II is a vasoactive peptide, which is linked to the modulation of cardiovascular disease and believed to be involved in the development of cardiac cachexia. Ang II is also capable of inducing muscle wasting, where infusion of rats with Ang II produced a significant reduction in body weight caused by the loss of skeletal muscle through an increase in protein degradation and decrease in protein synthesis. It has been demonstrated that like PIF, Ang II can upregulate protein degradation through an increased expression of the proteasome subunits, via the release of arachidonic acid leading to 15-HETE production, resulting in the mobilization of PKC to the membrane. PKC is an upstream activator of the I $\kappa$ B kinase complex (I $\kappa$ K), which leads to a degradation of I $\kappa$ B $\alpha$  allowing NF- $\kappa$ B to translocate to the nucleus and upregulate the target genes, such as those involved in the proteolytic proteasome pathway. When Ang II was added to C2C12 myotubes an increase in protein degradation and chymotrypsin like activity was observed in a parabolic dose response curve with the maximal effect seen at a concentration of 0.5 $\mu$ M [155]. Protein synthesis is also reduced in C2C12 myotubes treated with Ang II with a similar dose response curve to that seen in protein degradation, where again the maximal effect is seen at 0.5 $\mu$ M of Ang II [154]. It is likely that both PIF and Ang II act through similar mediators to increase protein degradation and decrease protein synthesis.

The Aim of this study was to confirm that PIF and Ang II increase protein degradation and decrease protein synthesis, and to investigate the most appropriate time point to measure the decrease in protein synthesis by PIF. A further aim was find out if PIF could alter the levels of RNA or affect the intracellular amino acid pool of C2C12 myotubes to evaluate possible mechanisms for the decrease in protein synthesis.

## 3.2 Results

PIF was purified from MAC16 tumours by affinity chromatography (as described in Section 2.3.11). The protein concentration of the purified PIF sample was measured using the Biorad method (as described in Section 2.3.2), and then mixed with an equal volume of SDS sample buffer (as described in Section 2.3.13), and analysed via western blotting (described in Section 2.3.21) to ensure that the sample was positive for PIF.

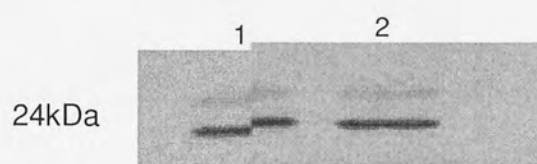


Figure 3.1: **Western blotting for PIF.** PIF was extracted from MAC16 tumours and 5 $\mu$ g was loaded onto a 10% polyacrylamide gel. PIF was detected at 24kDa in lane 1 and 2 proving that the purified sample contained PIF

PIF was demonstrated to be present in the end product purified from the MAC16 tumours of NMRI mice (Figure 3.1). To determine the biological activity of PIF, a chymotrypsin assay (as described in Section 2.3.6) was performed on the affinity chromatography product since previous studies have reported that PIF upregulates the proteasome. Since studies have also shown Ang II to increase proteasome activity, it was also tested for the ability to upregulate chymotrypsin like activity.

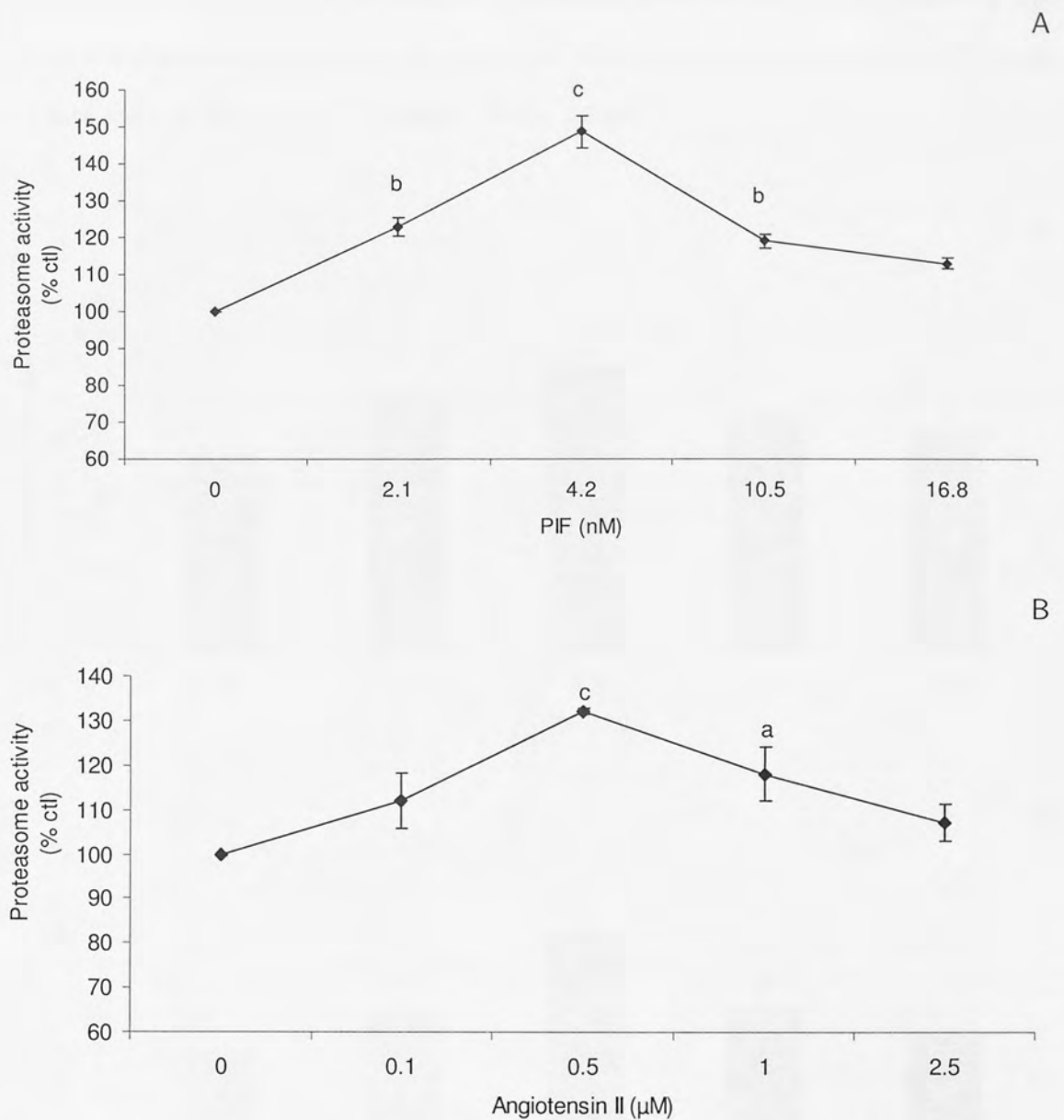


Figure 3.2: **Chymotrypsin like activity in murine myotubes in response to PIF and Ang II.** Murine myotubes were incubated for 24h in the presence of PIF (A) and Ang II (B) and assayed for activity of the beta subunits of the proteasome. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$

Both PIF and Ang II showed a significant increase in proteolytic activity when compared to the PBS control, which was maximal at 4.2nM of PIF (Figure 3.2A) and 0.5 $\mu$ M of Ang II (Figure 3.2B) as previously reported.

The PIF was thus confirmed biologically active and suitable to use in the following assays.

Both PIF and Ang II have been shown to increase total protein degradation in C2C12 myotubes as measured by the release of [<sup>3</sup>H] phenylalanine at the same concentrations which increase proteasome activity. This was confirmed (Figure 3.3) where the protein degradation was initiated significantly at 4.2nM PIF by 45% and 0.5 $\mu$ M Ang II by 53% (method described in Section 2.3.14).

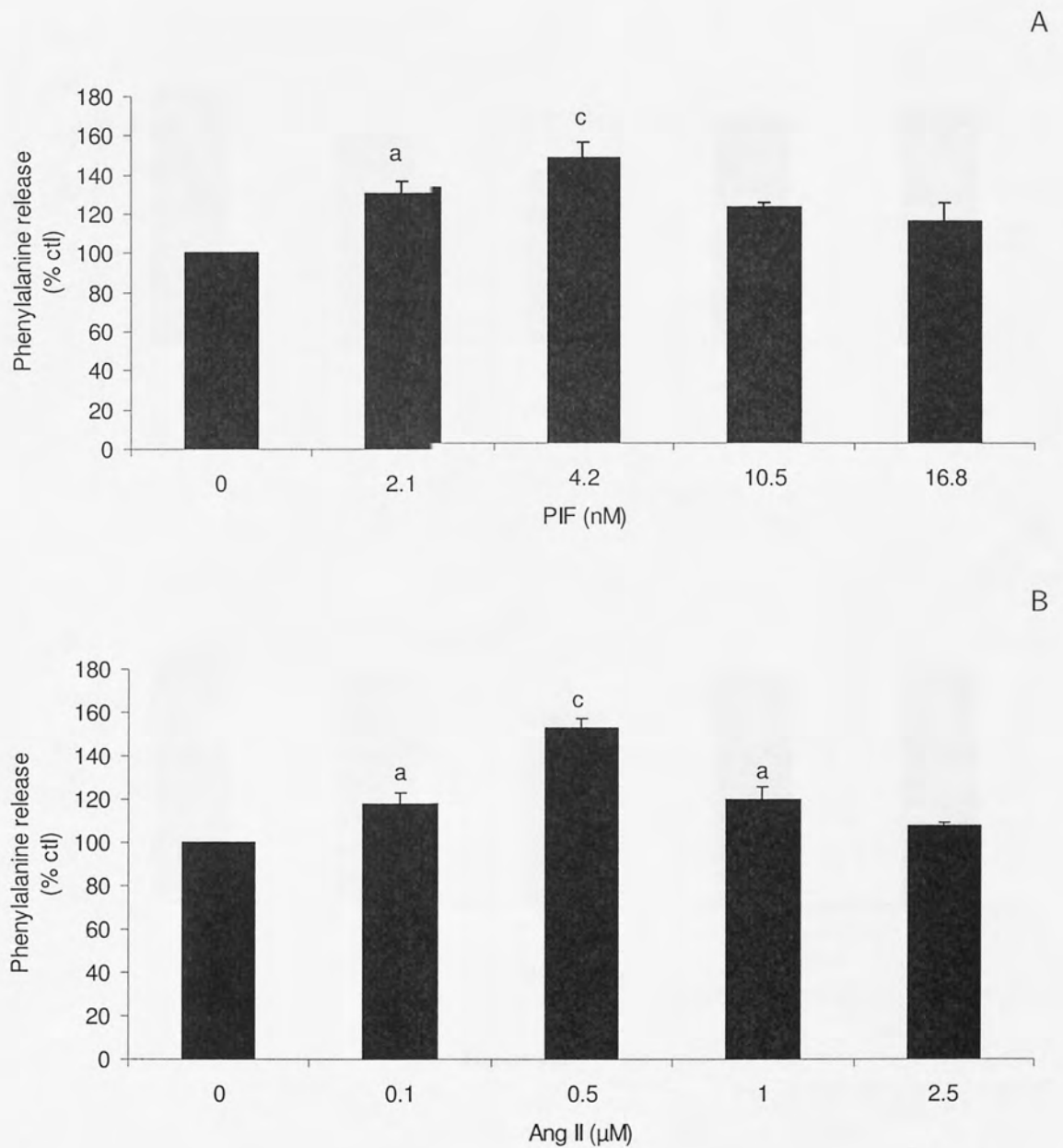


Figure 3.3: **Protein degradation in murine myotubes upon treatment with PIF and Ang II.** PIF (A) or Ang II (B) was incubated with the myotubes for 24h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as a:  $p < 0.05$ , c:  $p < 0.001$

To assess the role of PIF in protein synthesis C2C12 myotubes were incubated in the presence of PIF over 24h and protein synthesis was measured by the incorporation of [<sup>3</sup>H] phenylalanine into the cells after perchloric acid precipitation (described in Section 2.3.15) at variable points throughout this time course.

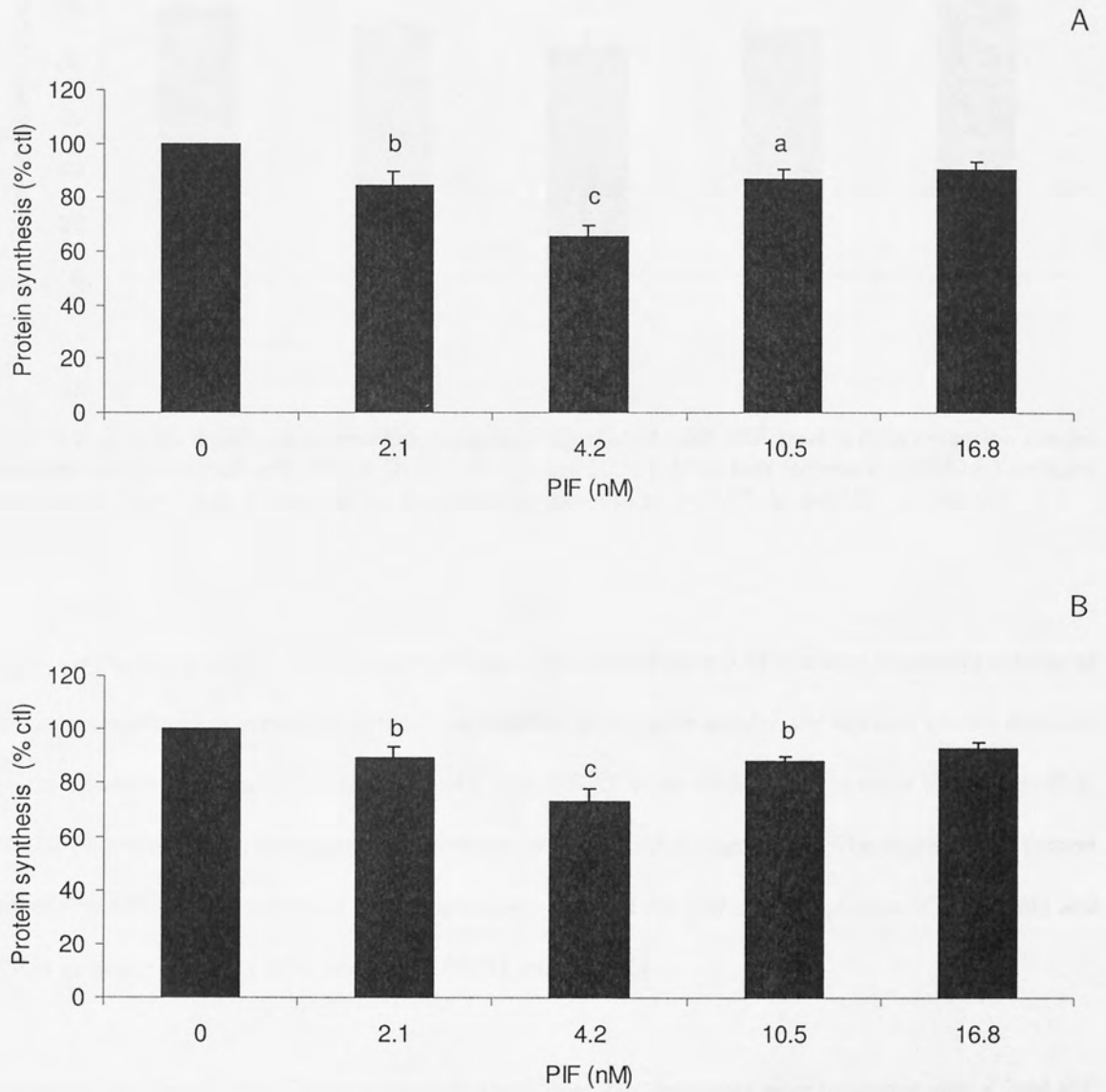


Figure 3.4



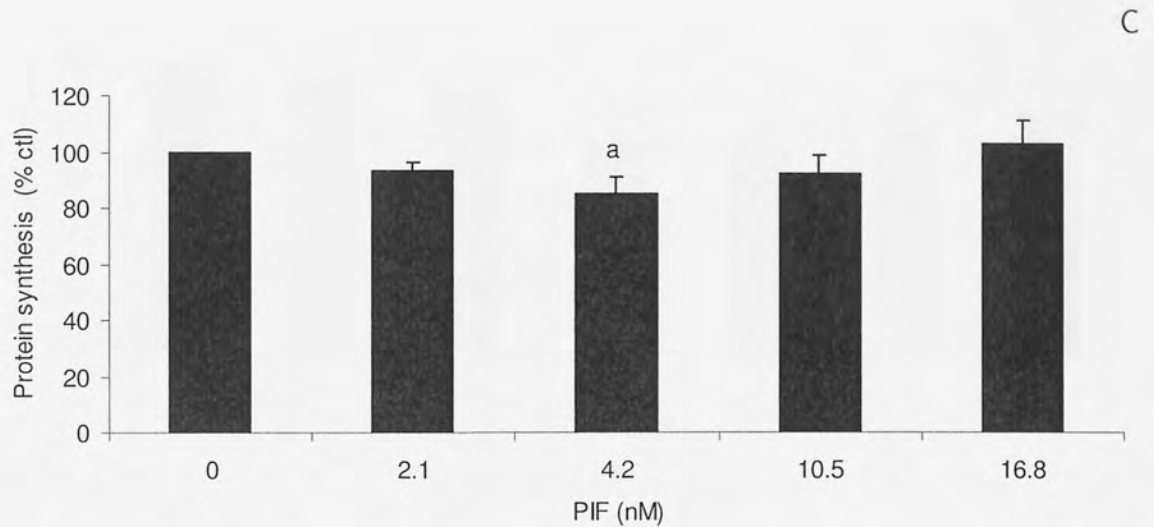


Figure 3.4: **Protein synthesis in murine myotubes incubated with PIF over a concentration range.** Myotubes were incubated with PIF for 4h (A), 6h (B) and 8h (C). Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$

Protein synthesis was decreased significantly between 4 and 8h (Figure 3.4) in a dose dependent manner at the same concentrations previously shown to upregulate proteasome activity and increase protein degradation, which was maximal at a concentration of 4.2nM PIF. Dose response curves at other time points (0.5, 1, 2 and 24h), which were not significant are shown in appendix A.1, Figure A.1. The depression of protein synthesis by PIF (30%) was similar to that previously reported for  $TNF\alpha$  in soleus muscle (22%) [68] and for IL-1 in gastrocnemius (30%) and soleus (25%) muscles [46].

To demonstrate the effect of PIF on protein synthesis over 24h, myotubes were incubated with 4.2nM PIF and protein synthesis measured at various times throughout (method described in Section 2.3.15).

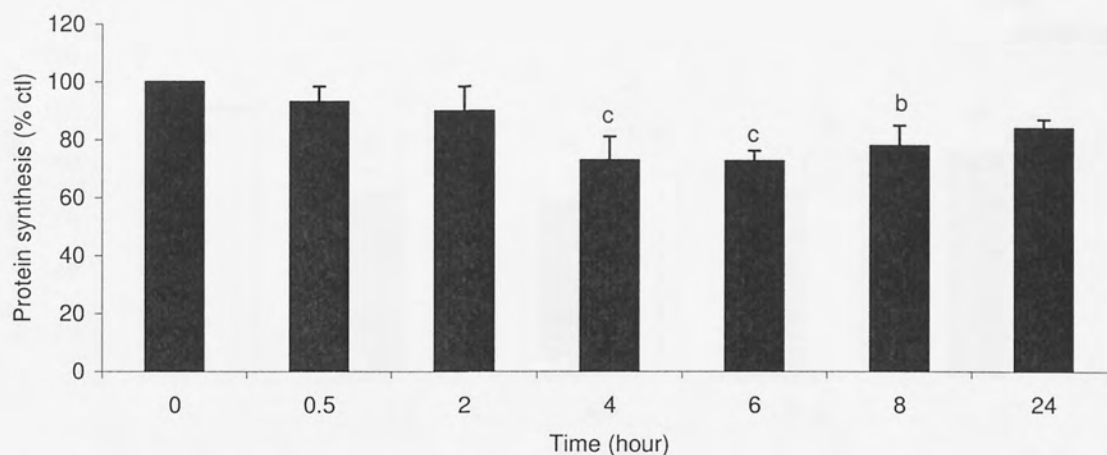


Figure 3.5: **Protein synthesis in murine myotubes in response to 4.2nM PIF incubated over 24h.** Synthesis was measured at various times over the incubation period. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as b:  $p < 0.01$ , c:  $p < 0.001$

As observed previously, PIF had a significant effect on protein synthesis at 4, 6 and 8h time points (Figure 3.5), and the effect was specific since it was attenuated by co-incubation with anti-PIF monoclonal antibody (Figure 3.6). One possible explanation for this is that PIF is affecting the translational apparatus since the effect on synthesis is relatively short term, while long term effects might reflect changes in transcription.

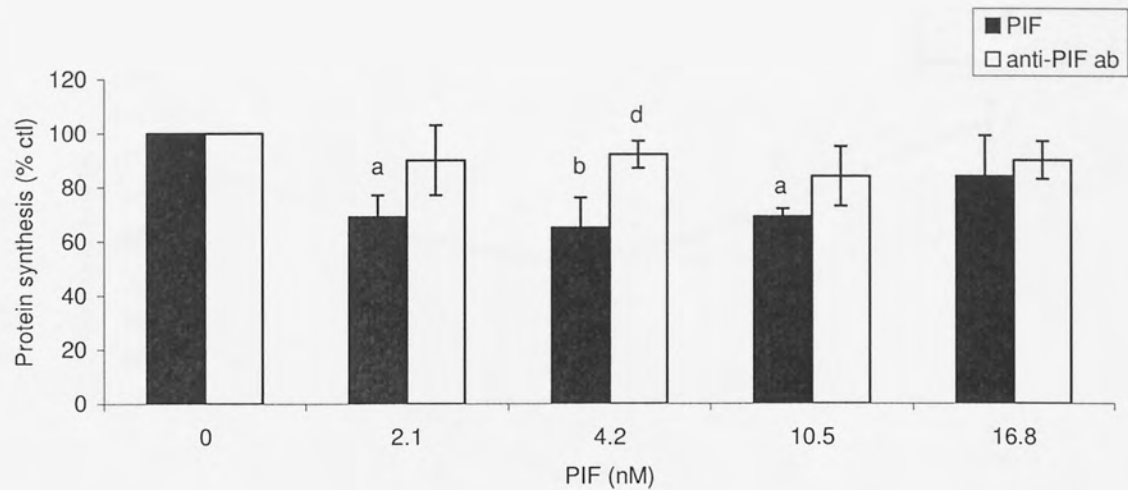


Figure 3.6: **Protein synthesis in murine myotubes incubated with PIF and co-treated with the anti-PIF antibody.** Myotubes were incubated with the anti-PIF antibody ( $10\mu\text{g}/\text{mL}$ ) for 2h prior to the addition of PIF with a further incubation of 4h. Error bars represent  $\pm\text{SEM}$  of 3 replicate experiments. Significant differences from control are shown as a:  $p<0.05$ , b:  $p<0.01$ , while differences in the presence of the anti-PIF antibody are shown as d:  $p<0.05$

To investigate why the effect of PIF on protein synthesis diminishes after prolonged incubation (above 8h), myotubes were treated with 4.2nM PIF for 4h, then either dosed again with another 4.2nM PIF, or PBS, and incubated for a further 4h. Protein synthesis was measured at 4 and 8h time points (see Method Section 2.3.15).

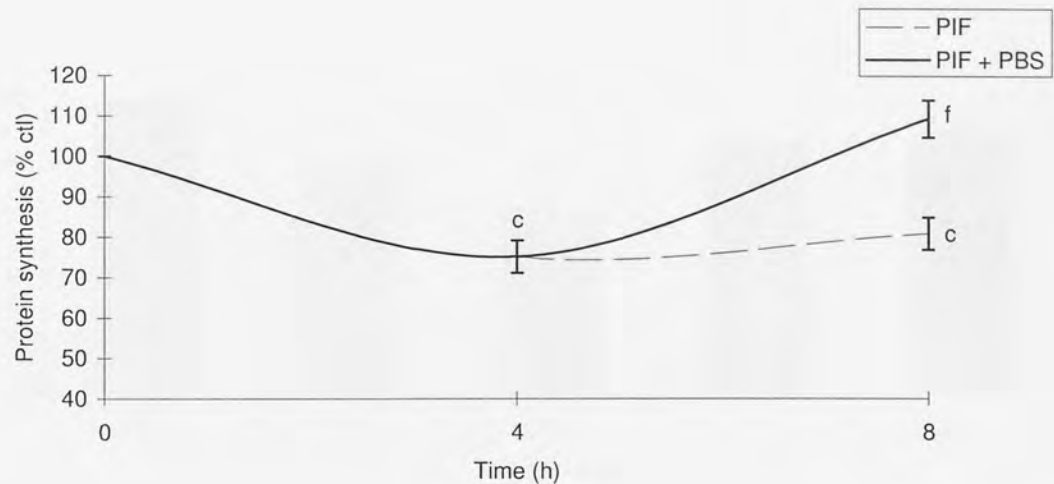


Figure 3.7: **Effect on protein synthesis of continual or transient incubation with PIF.** Protein synthesis in murine myotubes in response to incubation with PIF (4.2nM) over 4h followed by a further 4h incubation with either PIF or PBS. Error bars represent  $\pm$ SEM of 6 replicate experiments. Significant differences from control are shown as c:  $p < 0.001$ , while differences from PIF treatment is shown as f:  $p < 0.001$

When PIF was added at the two consecutive time points (0 and 4h) the level of protein synthesis remained attenuated and significant from the untreated control, however after PBS was added at the second time point (4h) synthesis levels began to return to control levels, which was significantly different from the double dose of PIF over 8h (Figure 3.7) indicating that, *in vivo*, it is likely that PIF is produced continuously to maintain repression of protein synthesis, and thus sustain weight loss, in the cachectic condition.

Since PIF and Ang II have very similar actions it was proposed that Ang II could also decrease protein synthesis. Indeed Ang II has been attributed to the cause of cardiac cachexia and *in vitro* Ang II has been demonstrated to depress protein synthesis [154]. To support this data myotubes were incubated with Ang II over a concentration range, the same as that used for the induction of protein degradation (Figure 3.3), and protein synthesis measured after 4h, since this has been demonstrated as the optimal time for a maximum effect on protein synthesis by PIF (see Section 2.3.15 for method).

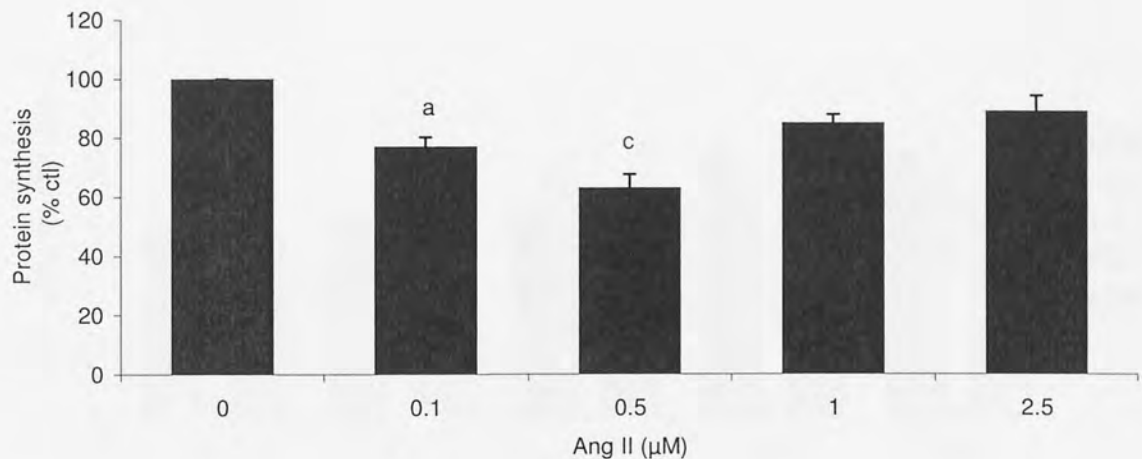


Figure 3.8: **Protein synthesis in murine myotubes in response to Ang II.** Myotubes were incubated with Ang II for 4h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as a:  $p < 0.05$ , c:  $p < 0.001$

As expected, treatment of myotubes with Ang II resulted in a dose dependent reduction of protein synthesis, where  $0.5\mu\text{M}$  had the most potent effect. It is possible, since both PIF and Ang II affect proteasome activity and protein degradation in a similar manner [59], that PIF and Ang II also decreases protein synthesis by comparative mechanisms.

There are several potential mechanisms that are involved in the regulation of protein synthesis. Both the number of ribosomes per cell and translational efficiency per ribosome determine the rate of tissue protein synthesis [4]. RNA has been observed to decrease in some tumour models, affecting the rate of protein synthesis [3]. To investigate whether PIF had any effect on RNA levels, the total cellular RNA concentration was measured in murine myotubes after incubation with PIF over 4h (described in Section 2.3.17). There was no significant change in total cellular RNA observed (Figure 3.9) and, since 85% of RNA is ribosomal, this suggests PIF does not decrease protein synthesis by affecting the number of ribosome's for translation elongation [4].

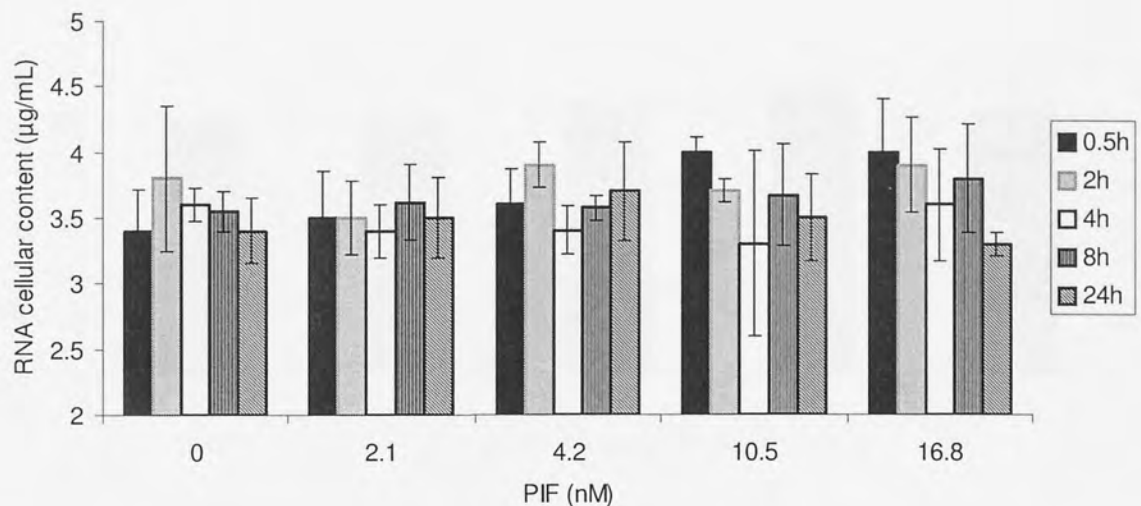


Figure 3.9: **Effect of PIF on total cellular RNA.** Murine myotubes were incubated with PIF over a period of 24h and cellular RNA content was measured. Error bars represent  $\pm$ SEM of 3 replicate experiments. There was no significant difference in total cellular RNA in response to PIF at any concentration or time point

The intracellular amino acid pool was measured by the uptake of labelled phenylalanine into myotubes (described in Section 2.3.10) to discover whether PIF was decreasing protein synthesis by blocking the uptake of amino acids into the cells. However, there was no significant alterations in amino acid concentration intracellularly upon treatment with PIF (Figure 3.10), suggesting that depletion of amino acids was not a contributing factor to the depression of protein synthesis. However this evidence does not completely rule out an effect of PIF on amino acid uptake since the effect could be obscured by the high levels of intracellular amino acids which are released by protein degradation in response to PIF.

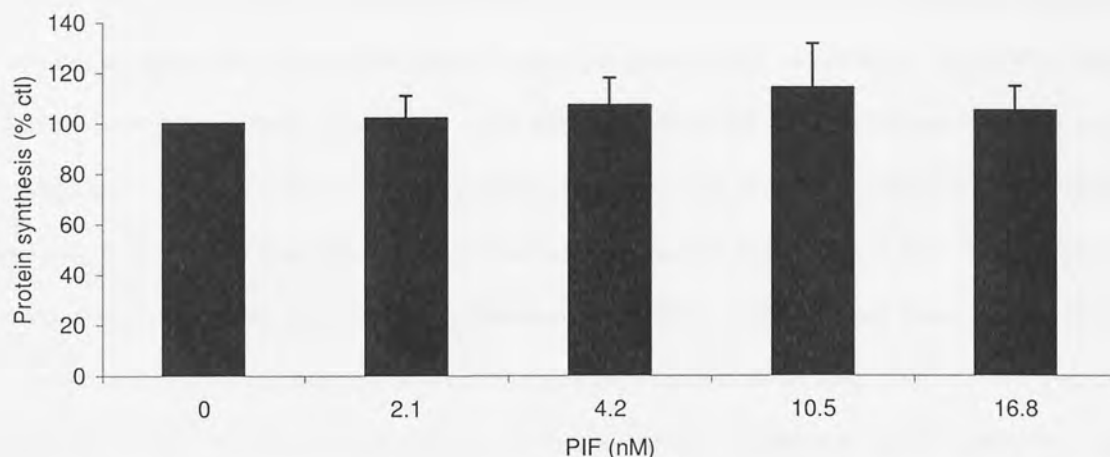


Figure 3.10: **Effect of PIF on the intracellular levels of amino acids.** PIF was incubated with murine myotubes over 4h and the amino acid pool was precipitated then measured). Error bars represent  $\pm$ SEM of 3 replicate experiments. There was no significant change in intracellular amino acid content in response to PIF

To conclude from these results, PIF and Ang II increase protein degradation through a proteasome dependent mechanism, which reflects previous studies performed on PIF and Ang II in vitro. The effect that the catabolic agents have on protein synthesis seem to be through a manner independent of amino acid uptake into the cell or alteration in cellular RNA content. Taking the time course into consideration, it is likely that protein synthesis is inhibited at the translational level.

### 3.3 Discussion

Muscle mass is controlled by the rate of protein synthesis and the rate of protein degradation, which in the adult are normally in balance so that muscle mass remains constant [162]. A decrease in protein synthesis has been attributed to the depletion of skeletal muscle mass in a number of weight losing conditions including congestive heart failure [51], weightlessness and cancer [59]. Cancer cachexia is associated with an increase in morbidity and mortality. Unfortunately even small and persistent changes in protein synthesis



as well as protein degradation lead to large protein deficits because the rate of protein turnover is so high (240-310 g/day). To improve prognosis, mechanisms that cause loss of muscle must be understood [125]. A number of agents have been nominated as causative factors, such as cytokines, like  $\text{TNF}\alpha$ , for the reduction in protein synthesis observed in many catabolic conditions [125]. Changes in muscle protein synthesis and degradation rate in cancer cachexia probably arise from the presence of tumour-derived factors, such as PIF, or increased production of endogenous factors such as Ang II [59]. It is possible that tumour produced catabolic products and cytokines work together, with the former inducing total changes in cytokine production that then contribute to overall development of cachexia [188]. In contrast, Jatoi et al failed to find a correlation between the presence of PIF and weight loss tied to patient survival in the clinical setting of metastatic gastric/oesophageal cancer [85]. It should be noted that this study had prominent differences from the early studies aimed at characterizing PIF, which casts doubt on the identity of the product purified in this more recent study. For example, Choudhary et al [41] identified PIF as a minor peak when analysing urine from weight losing cancer patients by MALDI-TOF mass spectrometry, while Jatoi showed a major peak which they identified as PIF. Moreover PIF is a 24kDa glycoprotein which is inconsistent with the western blot demonstrated by Jatoi, where a band was observed at a molecular weight closer to 30kDa. Therefore this band may represent immunoglobulin rather than PIF.

Both PIF and Ang II decreased protein synthesis by approximately 40%, and the concentrations of both agents that are maximally effective in the depression of protein synthesis are the same as those that are maximally effective in the induction of protein degradation. The effect of PIF on protein synthesis is suggested to result from an effect on translation, as total RNA levels were not affected by incubation with PIF at all concentrations. Since about 85% of the total RNA is ribosomal, this indicates that short term exposure to PIF does not affect protein synthesis by decreasing the availability of ribosomes for initiation factors and mRNA assembly, and subsequent elongation [4]. However, since changes in RNA content are generally considered long term changes (hours to days) rather than acute (minutes to hours) it is possible that PIF will affect RNA content over an extended time period, such as, in the MAC16 in vivo model where exposure to PIF is continuous. In fact this has been demonstrated to occur in animals bearing the MAC16 tumour where a significant reduction in the RNA content was noted in all tissues studied [23]. The PI3K-mTOR signalling pathway (discussed in Chapter 6) can induce long term changes (occurring

after several hours), which are typically a result of an increase in the number of ribosomes available to translate mRNA. A significant downregulation of the mTOR pathway in MAC16 mice was demonstrated (Chapter 6), and since PIF is the supposed catabolic factor in this cachexia model, this suggests that PIF may indeed cause a reduction in RNA and thus ribosomal levels in the long term, which would result in a decreased capacity of the cells to synthesize protein leading to accelerated weight loss [30].

The mechanism for increased protein degradation both in vitro and in vivo has been attributed to an increased expression and activity of the ubiquitin proteasome proteolytic system [211]. Both PIF and Ang II have been noted to increase protein degradation by this mechanism [155]. The PIF-induced increase in protein degradation can be attenuated by pre-treatment with eicosapentaenoic acid (EPA), which has no effect on PIF induced protein synthesis depression, suggesting that the two effects are mediated differently [166]. The ubiquitin proteasome pathway plays a major role in protein loss in gastrocnemius muscle of MAC16 tumour bearing mice, up to 20% weight loss, but a reduction in protein synthesis may be the determining factor contributing to loss of protein at weight losses greater than 20% [93]. Although a number of studies have shown that protein synthesis is inhibited in the skeletal muscle of cachectic subjects, there is little information on the processes involved [154]. Tissue catabolism can be stimulated by many mechanisms in response to a diverse range of stresses. One study suggested that the decrease in protein synthesis by vasopressin administration was as a result of an increased phosphorylation of the  $\alpha$ -subunit of the translation initiation factor eIF2 [96]. Furthermore a significant increase in protein synthesis upon leucine administration was demonstrated in gastrocnemius muscle of cachectic mice. Leucine stimulates protein synthesis through the activation of the mRNA binding step in translation initiation [162]. Hydrogen peroxide [135] production and glucocorticoid administration [109], which accompanies Ang II induced wasting [51], exert a permissive role in muscle protein loss through the destabilization of the eIF4F active complex and deregulation of the Akt/mTOR signalling pathway. Release of calcium [48] and reactive oxygen species (ROS) [128] are linked with muscle atrophy in many diseases which manifest cachexia, such as inflammatory and autoimmune disorders, as well as sepsis and cancer. Taking into account the above observations, further studies were concentrated on the effect of PIF and Ang II on the various translational components and regulators and the involvement of ROS and calcium in these processes. The results from these studies may suggest potential control points in protein synthesis deregulation by catabolic mediators

that could be useful for therapeutic intervention [211].

### 3.4 Conclusion

PIF induced a time and concentration-dependent decrease in protein synthesis in murine myotubes, and the maximum effect was observed after 4h incubation and at a concentration of 4.2nM. Ang II also initiated a depression of protein synthesis which was decreased maximally at 0.5 $\mu$ M. The concentrations of PIF and Ang II causing significant inhibition of protein synthesis were the same as those shown to stimulate protein degradation and upregulate the ubiquitin proteasome pathway and the dose-response curve showed a similar parabolic relationship. There was no effect of PIF on the uptake of labelled phenylalanine into myotubes, confirming that the effect was on protein synthesis, rather than depletion of the amino acid pool in the cell. Incubation of murine myotubes with PIF was ineffective at altering the total cellular RNA, suggesting that the depression in protein synthesis resulted from an effect on translational efficiency rather than ribosome availability and elongation.

## Chapter 4

# Role of PKR/eIF2 $\alpha$ Pathway in Catabolic Processes In Vitro

### 4.1 Introduction

As demonstrated previously (Section 3.1) both PIF and Angiotensin II can induce an increase in protein degradation through the upregulation of the ubiquitin proteasome proteolytic pathway via increased nuclear migration of NF- $\kappa$ B. However, much less is known about how PIF and Ang II affect protein synthesis, although with PIF at least this has been suggested to arise from an effect on translation efficiency as discussed in Section 1.10.

There are two important points of control for translation initiation. The first is the binding of methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit and the second is the binding of mRNA to the 43S preinitiation complex. This chapter will concentrate on the first point of control [59]. eIF2 is a trimeric complex involved in the first step of translation initiation. The complex is made up of three subunits

designated  $\alpha$ ,  $\beta$  and  $\gamma$ , and behaves in a manner similar to the trimeric G-coupled proteins [181]. The eIF2 coupled with GTP and initiator met-tRNA participates in the ribosomal recognition of the AUG start codon. Once the AUG codon has been recognized the GTP is converted to GDP and eIF2 dissociates from the ribosome. The guanine nucleotide exchange factor (GNEF), eIF2B, replaces the GTP allowing translation initiation to continue. In response to diverse stress conditions eIF2 is phosphorylated at serine 51 on its alpha subunit resulting in a 100-fold increase in its affinity for eIF2B, which is a rate limiting component of translation. Since eIF2 is found in excess over eIF2B in cells, a small percentage of phosphorylated eIF2 can sequester all of the available eIF2B in the cell leading to a general reduction in translation initiation [88, 37].

One study reported an inhibition of protein synthesis in rat livers perfused with vasopressin which was attributed to an increase in eIF2 $\alpha$  phosphorylation [96], while another report showed a rise in eIF2 $\alpha$  levels due to an increased proportion of eIF2 $\alpha$  mRNA associated with the polysomes in sepsis [198].

The double stranded RNA dependent protein kinase, or PKR, is an eIF2 $\alpha$  kinase which is involved in the anti-viral immune response following infection. It is activated by the extensive secondary structure of viral RNA and upon dsRNA binding undergoes autophosphorylation [133] on multiple serine and threonine residues, which may induce a conformational change that leads to the disclosure of the ATP-binding site and/or catalytic domain. Human PKR contains at least 15 autophosphorylation sites, but only threonine 446 and 451 in the activation loop are found to be critical for kinase activity in yeast [218]. This is followed by PKR dimerization, which is thought to promote the intermolecular autophosphorylation of PKR molecules, resulting in maximal activation of the enzyme [180]. Human PKR consists of 551 amino acids, is located on chromosome 2p21 [13] and has a bipartite structure that forms two functional domains which consist of an N-terminal dsRNA binding regulatory domain that contains two dsRNA binding motifs responsible for dsRNA binding, and a C-terminal catalytic domain [212, 174]. Approximately 80% of PKR is present in the cytoplasm, mostly associated with ribosomes, although a significant fraction also resides in the nucleolus [45]. One of the best known substrates for PKR is eIF2 $\alpha$  which, once phosphorylated by PKR, blocks synthesis of viral proteins [133].

Although the majority of research on PKR has focused on its inhibitory participation in viral infection, there



is substantial evidence for PKR's role in uninfected cells also [145]. For example PKR is also considered a stress kinase and its levels increase during ageing [104].

PKR is present at low constitutive levels in cells and its expression can be induced by IFN- $\gamma$  [136]. Evidence has been found that suggests the cytokine TNF $\alpha$  also acts through PKR [147]. Interestingly cytokines such as IFN- $\gamma$  and TNF $\alpha$  have been implicated as causative factors in cachectic states [185] such as AIDS [107] or sepsis [125]. Although the most well known activator of PKR is dsRNA it can also be activated by a number of other components in response to cellular stress including polyanionic agents [136]. PIF is also a polyanionic molecule and therefore may be able to activate PKR. It is unlikely that Ang II could affect PKR directly, however Ang II can induce the mobilization of calcium and generation of ceramide [21] both of which lead to the phosphorylation of PKR, providing possible alternative mechanisms for PKR activation.

In addition to translational regulation, another important part of the cellular functions of PKR is its role in regulation of cell proliferation, survival, and apoptosis. PKR mediates apoptosis by controlling the activation of several transcriptional factors, regulation of selective pro-apoptotic molecules such as Bax or Fas and selective activation of caspases [174]. Bax and Fas are preferentially translated despite translational impairment due to an increased eIF2 $\alpha$  phosphorylation [11]. Because of its pro-apoptotic actions, forced expression of PKR in mouse, insect, and yeast cells causes translational inhibition and cell death. Indeed, inappropriate activation of PKR has been associated with certain disease states characterized by high levels of apoptosis. For example, PKR and the PKR-like kinase PERK have been implicated as important pathogenic factors in the Alzheimer's, Parkinson's, Huntington's [219] and arthritic disease [70] all of which have symptoms of weight loss and muscle atrophy [143, 202]. The possibility that protein depletion is a consequence of apoptotic events is supported by the observation that muscle atrophy occurring in heart failure or Duchenne muscular dystrophy is associated with a reduction in the number of myonuclei [48].

The receptor associated adaptor protein, FADD, is required for the apoptotic response to PKR activation. PKR mediated apoptosis also involves caspase 3 and caspase 8, the initiator caspase down stream of FAS [86, 69]. It is of interest to note that PIF can also cause apoptosis through an upregulation of caspase 3 and 8 [164].

PKR activation can also alter transcriptional activity through the activation of NF- $\kappa$ B. Recombinant PKR can directly phosphorylate I $\kappa$ B $\alpha$  resulting in its polyubiquitination and degradation by the proteasome allowing NF- $\kappa$ B translocation to the nucleus [217]. PKR has also been shown to physically associate with the I $\kappa$ K complex stimulating NF- $\kappa$ B transcriptional function through the action of I $\kappa$ K and NF- $\kappa$ B inducing kinase (NIK). Other studies have shown that eIF2 $\alpha$  phosphorylation is central to the activation of NF- $\kappa$ B [88]. It is possible that any of the eIF2 $\alpha$  kinases (HRI, GCN2, PERK and PKR) may be activated by individual apoptotic stimuli, but the best evidence so far concerns the role of PKR in cell death regulation [4].

Both eIF2 $\alpha$  and NF- $\kappa$ B activation is believed to be necessary for PKR-induced apoptosis [86], where PKR regulates cell survival and cell death in a chronological manner. Cell survival is induced via the activation of NF- $\kappa$ B to delay apoptosis prior to cell death, which is subsequently stimulated through the phosphorylation of eIF2 $\alpha$  [54]. Thus eIF2 $\alpha$  phosphorylation might provide a link between the depression of protein synthesis and the increase in protein degradation observed in skeletal muscle of cachectic subjects [59]. Since treatment of murine myotubes with PIF can lead to the activation of NF- $\kappa$ B and apoptosis it seems reasonable to assume that PIF may activate PKR as an early signalling event that not only leads to a decrease in protein synthesis through activation of eIF2 $\alpha$  but can also increase protein degradation via the NF- $\kappa$ B transcriptional pathway. AT2 receptor activation by Ang II results in growth inhibition and apoptosis, and since activators of PKR are a part of the intracellular signalling cascade of Ang II it is possible that PKR may also be a downstream effector of Ang II [21].

The aim of this study was to examine the mechanism for the depression of protein synthesis in skeletal muscle by both PIF and Ang II using C2C12 murine myotubes as a surrogate model. This was done by measuring the phosphorylation levels of PKR and eIF2 $\alpha$  upon exposure of murine myotubes to PIF and Ang II, and determine if these components of the translational pathway were involved in the depression of protein synthesis and increased degradation in response to both agents.

A further goal was to investigate the effectiveness of a PKR inhibitor on growth of the MAC16 tumour cells, compared to the effect on MAC13 tumour cells, which are histologically similar to MAC16 cells,



but do not induce cachexia once implanted into NMRI mice, and to investigate the possible mechanisms involved in tumour cell growth.

## 4.2 Results

Phosphorylation of the  $\alpha$ -subunit of eIF2 is an essential control element in the regulation of translation initiation. Since the results from the previous chapter (Chapter 3) suggested that the depression of protein synthesis by both PIF and Ang II is likely to occur at the translational level the phosphorylation of eIF2 $\alpha$  was determined as an important component to study. Mammalian cells possess at least four different eIF2 $\alpha$  kinases [147] and since a PKR inhibitor was commercially available this was chosen as the eIF2 $\alpha$  kinase to study further. Suppression of protein synthesis through phosphorylation of PKR resulting in eIF2 $\alpha$  phosphorylation has been shown to occur in response to many types of cellular stress [136]. Since both PIF and Ang II can be considered as stress factors in cancer cachexia and cardiac cachexia respectively, the effect on PKR and eIF2 $\alpha$  phosphorylation was determined. To determine the optimum dose of the PKR inhibitor used in the following assays, the dose response of the PKR inhibitor in the presence of PIF (4.2nM) was measured in murine myotubes. The results shown in Figure 4.1, show the inhibitor to attenuate the reduction of protein synthesis induced by PIF at concentrations between 100 and 210nM but not at 300nM. Thus 210nM was chosen as the concentration of PKR inhibitor to use in further experiments. C2C12 myotubes were incubated with PIF or Ang II in the absence and presence of the PKR inhibitor (210nM) and protein synthesis was measured (method described in Section 2.3.15).

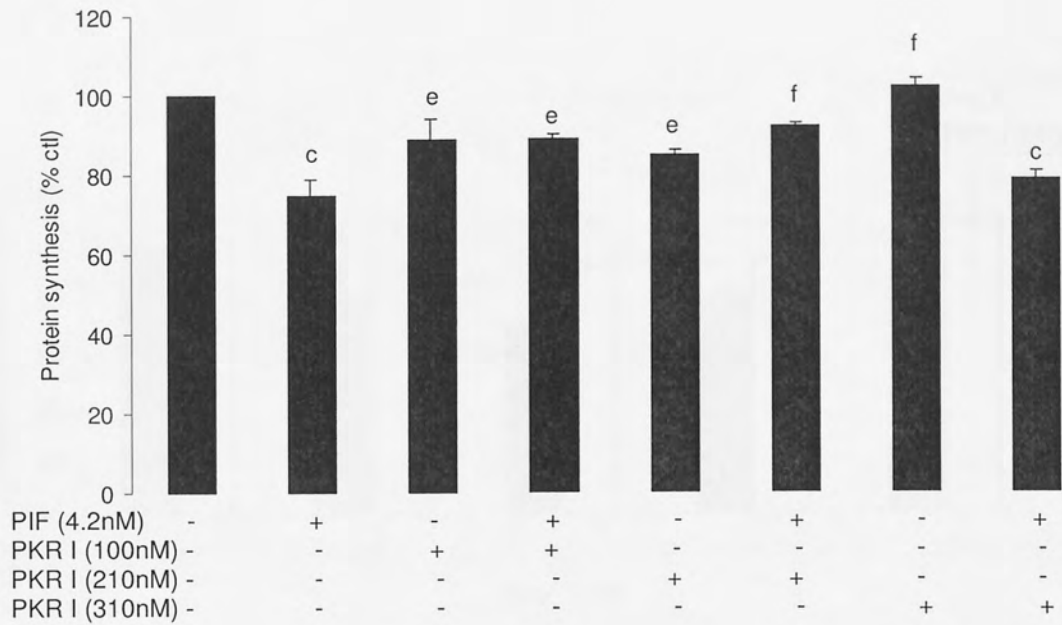


Figure 4.1: **Effect of different concentrations of the PKR inhibitor on protein synthesis in murine myotubes in the presence of PIF.** Myotubes were pretreated with the PKR inhibitor (PKR I) for 2h followed by addition of PIF (4.2nM), which was incubated with the myotubes for a further 4h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as e:  $p < 0.01$  or f:  $p < 0.001$ .

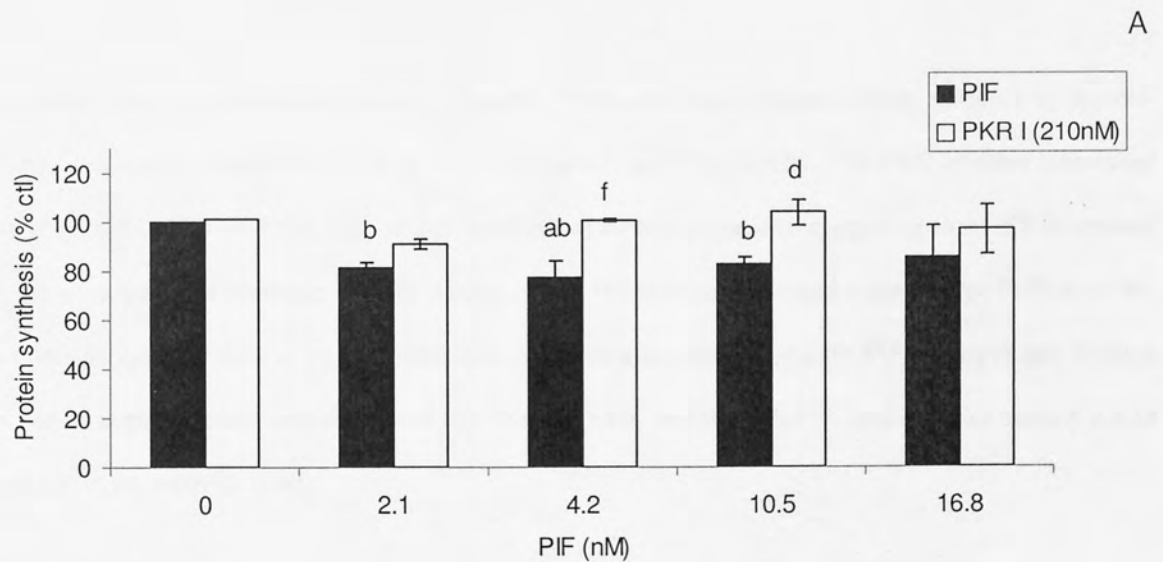


Figure 4.2

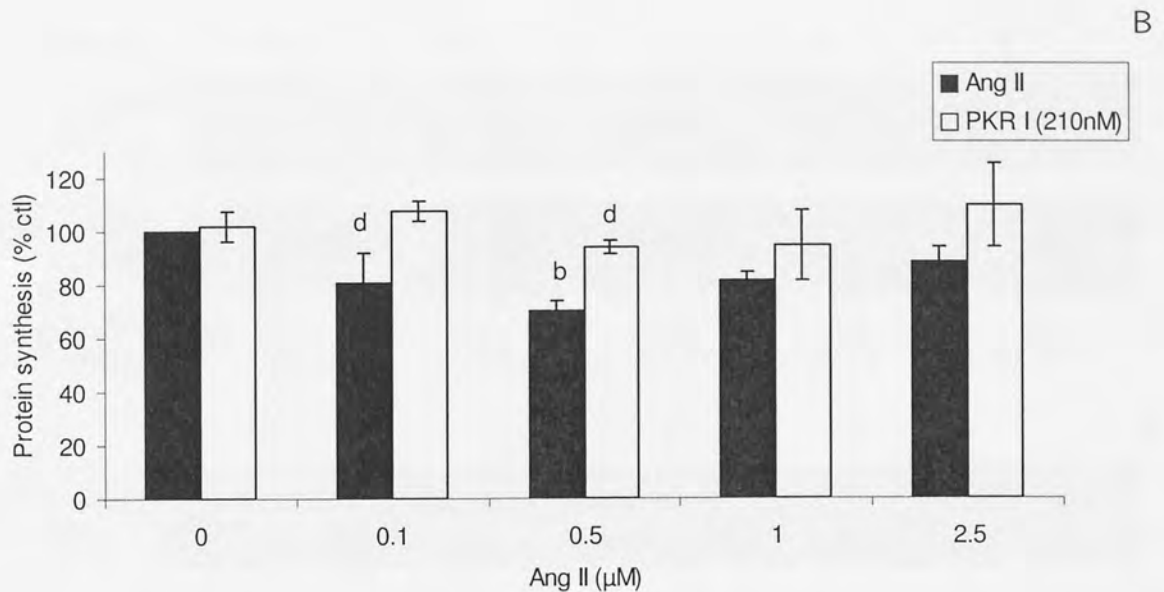


Figure 4.2: **Effect of a PKR inhibitor on protein synthesis in murine myotubes upon treatment with PIF and Ang II.** Myotubes were pretreated with the PKR inhibitor (PKR I) (210nM) for 2h followed by addition of PIF (A) or Ang II (B), which were incubated with the myotubes for a further 4h. Incubation with the PKR inhibitor significantly attenuated the depression in protein synthesis by both agents. Error bars represent  $\pm$ SEM of 3 replicate experiments. Significant differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , f:  $p < 0.001$

As demonstrated in the previous chapter (Chapter 3) PIF and Ang II depress protein synthesis by approximately 40% which is maximal at 4.2nM PIF and 0.5 $\mu$ M Ang II (Figure 4.2). The PKR inhibitor attenuated the effect of both PIF and Ang II on protein synthesis at all concentrations, suggesting that PKR is involved in the suppression of synthesis by both agents. Since the best characterised substrate for PKR is eIF2 $\alpha$ , the phosphorylation state of its  $\alpha$ -subunit was measured after incubation with PIF or Ang II over 4 hours as well as in the presence and absence of the PKR inhibitor, and the anti-PIF antibody (see Section 2.3.13 and 2.3.21 for methods used).

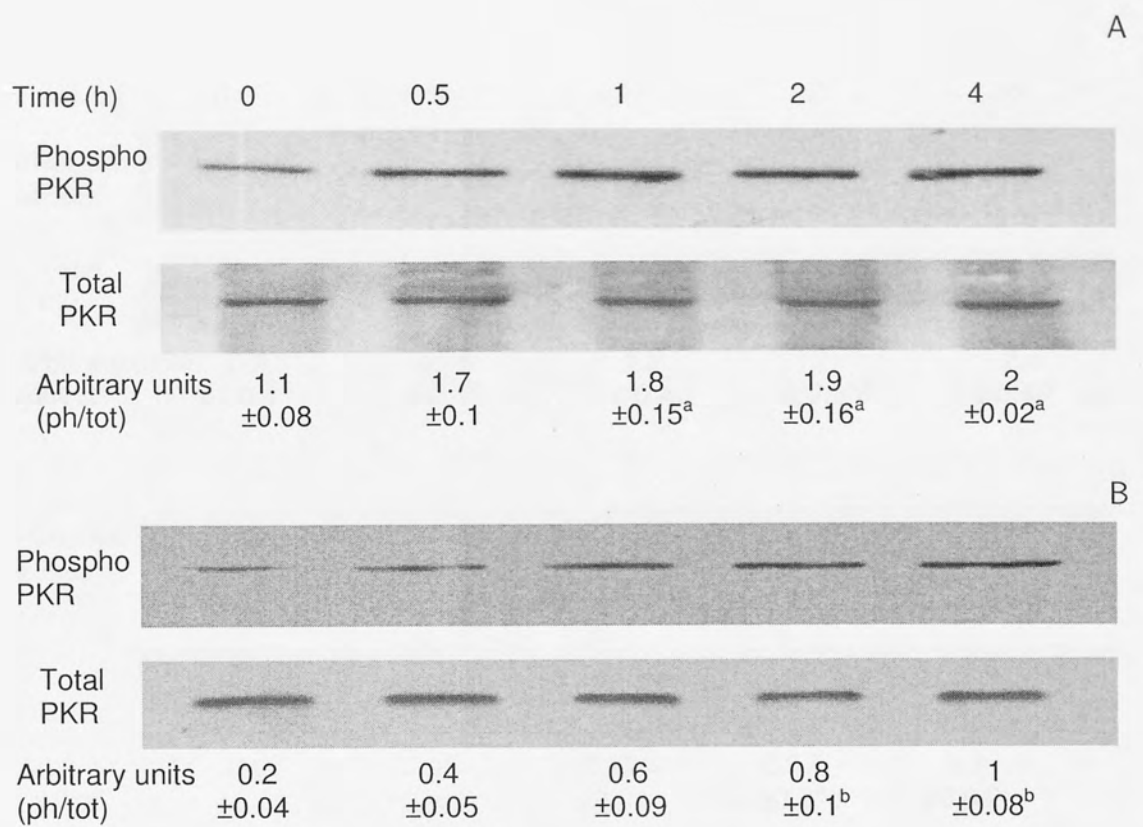


Figure 4.3: **Western blot of a time course for the effect of PIF and Ang II on phosphorylation of PKR.** PIF (4.2nM) (A) or Ang II (0.5 $\mu$ M) (B) were incubated with the myotubes over 4h. A blot of total PKR was used to normalize the phosphorylated protein. A densitometric analysis of phospho PKR is shown underneath where  $\pm$  represent the SEM of three replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$ .

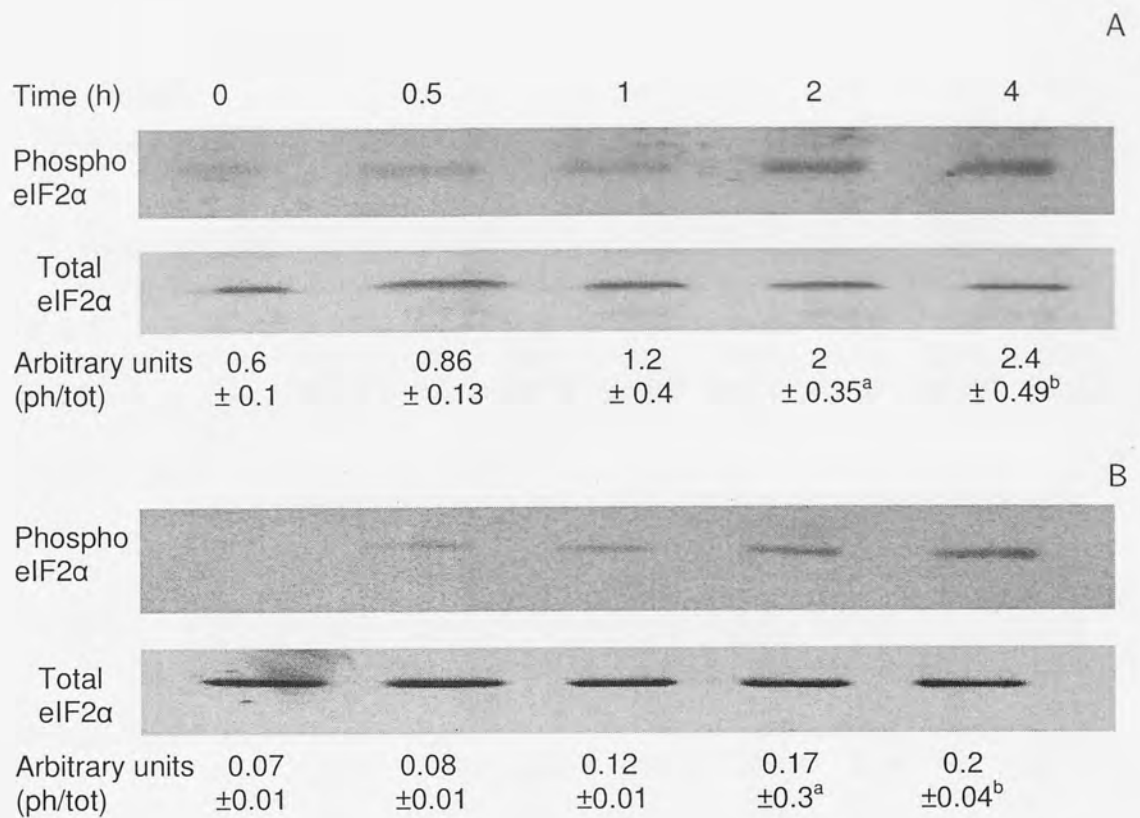


Figure 4.4: **Western blot of a time course for the effect of PIF and Ang II on phosphorylation of eIF2 $\alpha$ .** PIF (4.2nM) (A) or Ang II (0.5 $\mu$ M) (B) were incubated with the myotubes over 4h. A blot of total eIF2 $\alpha$  was used to normalize the phosphorylated protein. A densitometric analysis of phospho eIF2 $\alpha$  is shown underneath where  $\pm$  represent the SEM of 3 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$

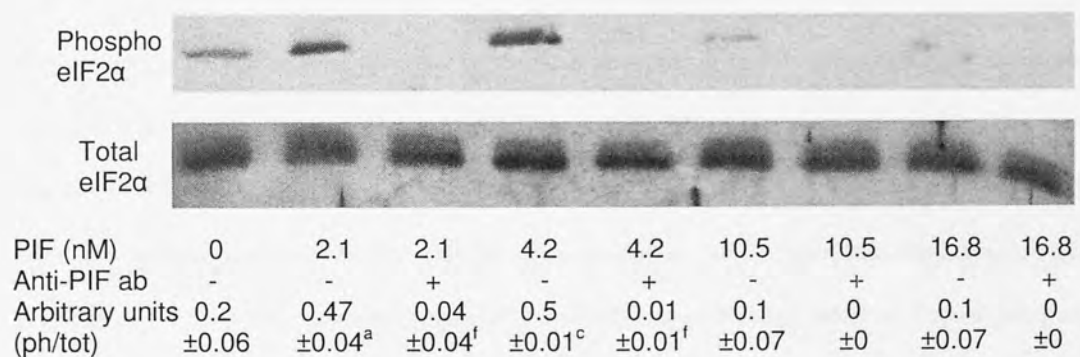


Figure 4.5: **Western blot of eIF2 $\alpha$  phosphorylation after 4h incubation with PIF co-treated with the anti-PIF antibody.** The anti-PIF antibody (10 $\mu$ g/mL) was added to the myotubes 2h prior to the addition of PIF (4.2nM). A blot of total eIF2 $\alpha$  was used to normalize the phosphorylated protein. A densitometric analysis of phospho eIF2 $\alpha$  is shown underneath where  $\pm$  represent the SEM of 3 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , c:  $p < 0.001$ , while differences in the presence of the anti-PIF antibody are shown as f:  $p < 0.001$

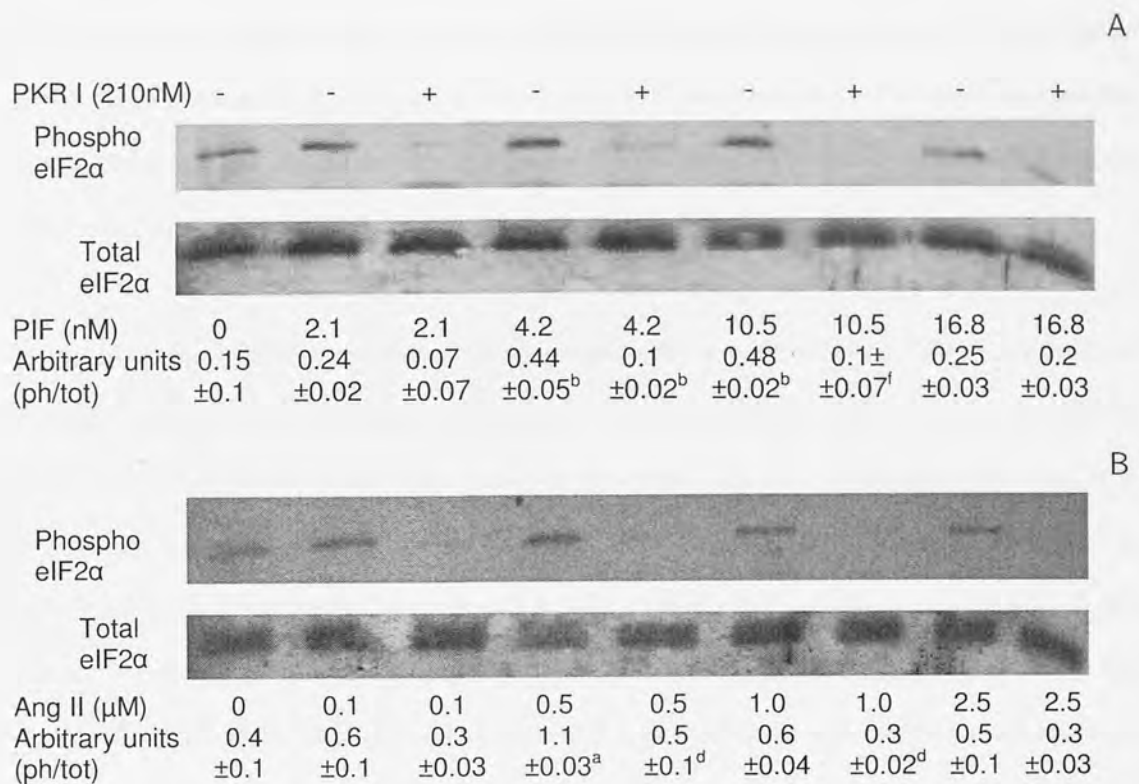


Figure 4.6: **Western blot of eIF2 $\alpha$  phosphorylation after 4h incubation with PIF and Ang II co-treated with the PKR inhibitor.** The PKR inhibitor (PKR I) (210nM) was added to the myotubes 2h prior to the addition of PIF (A) or Ang II (B). Blots of total PKR and eIF2 $\alpha$  were used to normalize the phosphorylated proteins. A densitometric analysis of phospho eIF2 $\alpha$  is shown underneath where  $\pm$  represents the SEM of three replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , f:  $p < 0.001$

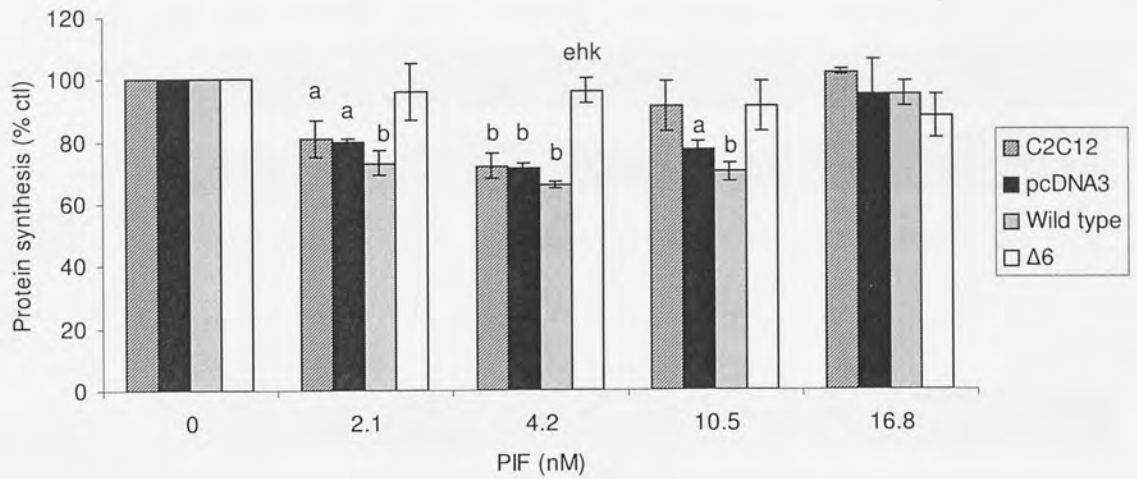
Phosphorylation of PKR increased over the time course of exposure to PIF and Ang II and was significant between 1 and 4h for PIF or 2 and 4h for Ang II (Figure 4.3). This pattern was mirrored by the phosphorylation of the  $\alpha$ -subunit of the translation initiation factor eIF2 (Figure 4.4), where PKR phosphorylation precedes, but overlaps the time course for eIF2 $\alpha$  phosphorylation. eIF2 $\alpha$  phosphorylation was increased in the presence of PIF in a dose dependent manner, where the concentrations which increased this phosphorylation maximally are the same as those affecting protein synthesis (Figure 4.5 and 4.6). This elevation of the active form of eIF2 $\alpha$  was attenuated by a murine monoclonal antibody raised against PIF (Figure 4.5). This anti-PIF antibody also attenuated the depression in protein synthesis, demonstrated in Chapter 3, which may prove a causal relationship between the depression of protein synthesis by PIF and the increase in eIF2 $\alpha$  phosphorylation, where these actions are specific to PIF.

The PKR inhibitor was effective at reversing the phosphorylation of eIF2 $\alpha$  by both PIF and Ang II (Figure 4.6), which may be associated with the reversal of protein synthesis when co-treated with these agents. In controls (Figures 4.5 and 4.6) there was a mild expression of phosphorylated eIF2 $\alpha$ , which was possibly due to the effects of serum deprivation during experimental procedure, as previously suggested for cortical neurons treated with A $\beta$  peptide [174].

These results suggest that PIF and Ang II are responsible for the activation of PKR, which leads to the phosphorylation of eIF2 $\alpha$ , resulting in a depression in protein synthesis. This conclusion is based on experiments in which an inhibitor was used. Although there have been few publications on the specificity of the PKR inhibitor, the original publication [84] claims the inhibitor to be highly specific for PKR. The potential non-specific effects however need to be considered, therefore to investigate further the involvement of PKR in the cellular signalling of PIF and Ang II, and determine its role in eIF2 $\alpha$  phosphorylation, and ensuing depression of protein synthesis, murine myoblasts were transfected with plasmids expressing wild-type or catalytically inactive PKR variant dominant negative PKR $\Delta$ 6. As an additional control murine myoblasts were also transfected with the non-recombinant empty vector pcDNA3(-) [59]. These mutants were then allowed to differentiate into myotubes before use (see Section 2.3.18 for methods used). The control and transfected myotubes were incubated in the presence of PIF over 4h. Protein synthesis, (method described in Section 2.3.15) and expression of activated PKR and eIF2 $\alpha$  were established by western blotting, (2.3.13 and 2.3.21).



A



B

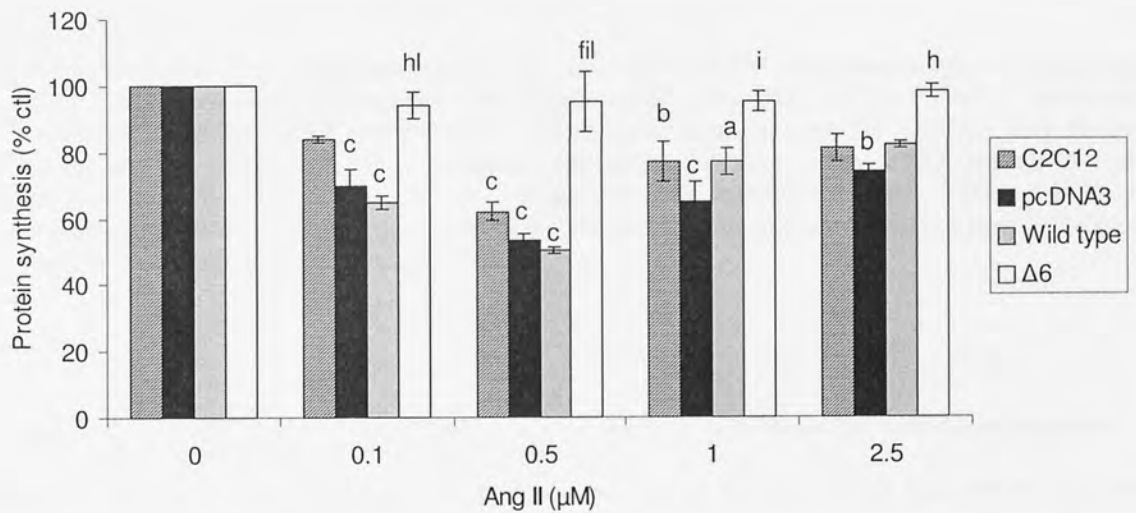


Figure 4.7: **Protein synthesis in murine myotubes transfected with PKR mutant plasmids upon treatment with PIF and Ang II.** PIF (A) or Ang II (B) were incubated with murine myotubes transfected with pcDNA3(-), wildtype PKR, PKRΔ6 or non-transfected myotubes for 4h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from non transfected cells are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$ , differences from pcDNA3(-) g:  $p < 0.05$ , h:  $p < 0.01$  or i:  $p < 0.001$  and differences from wildtype j:  $p < 0.05$ , k:  $p < 0.01$  or l:  $p < 0.001$

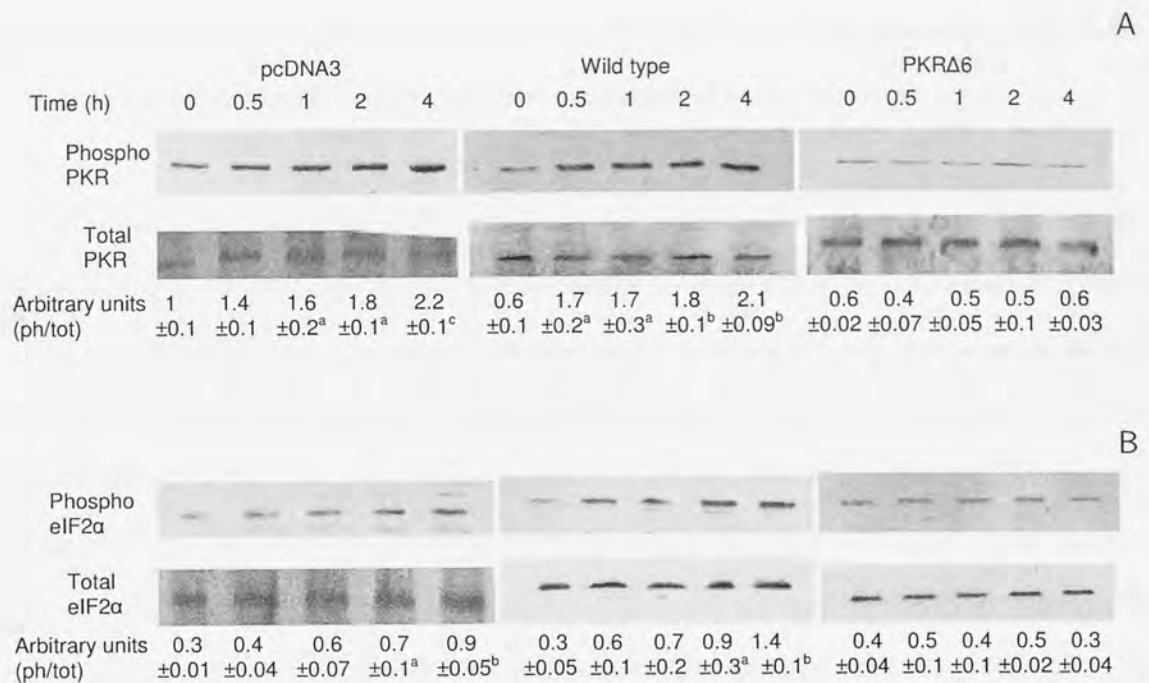


Figure 4.8: **Western blot of a time course for the effect of PIF on phosphorylated PKR and eIF2 $\alpha$  in C2C12 myotubes transfected with PKR mutant plasmids.** C2C12 myotubes transfected with pcDNA3(-), wildtype PKR and PKR $\Delta$ 6 plasmids were incubated with PIF (4.2nM) over 4h and blotted for phosphorylated PKR (A) or phosphorylated eIF2 $\alpha$  (B). Blots of total PKR and eIF2 $\alpha$  were used to normalize the phosphorylated proteins. A densitometric analysis of phospho PKR and eIF2 $\alpha$  is shown underneath where  $\pm$  represent the SEM of 3 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$

In response to both PIF and Ang II, pcDNA3 and PKR wildtype cells showed an analogous depression in protein synthesis to non-transfected cells. In contrast, the PKR $\Delta$ 6 cells showed no response to treatment with either agent (Figure 4.7). The mechanism by which the mutant PKR inhibits cellular PKR activity is likely either through formation of inactive heterodimers with endogenous PKR, or through competition for endogenous PKR activators [11]. Cells expressing PKR $\Delta$ 6 proteins have been observed to have diminished levels of phosphorylated eIF2 $\alpha$  even after stimulation with dsRNA, which significantly increases eIF2 $\alpha$  phosphorylation in both control cells and those over-expressing PKR (wildtype PKR) [11]. When incubated with PIF, both the pcDNA3 and wildtype myotubes demonstrated a significant increase in the phosphorylation of PKR and the  $\alpha$ -subunit of eIF2, which reflected the time course seen in non-transfected cells. Cells expressing PKR $\Delta$ 6 displayed a lack of both PKR and eIF2 $\alpha$  phosphorylation in response to PIF

at all time points (Figure 4.8). This observation not only demonstrates that the myotubes were expressing the PKR $\Delta$ 6 mutants but also confirms that activation of PKR by PIF and most likely Ang II is responsible for the phosphorylation of eIF2 $\alpha$  and the consequent depression of protein synthesis.

Previous studies have reported that PKR autophosphorylation is an important mediator of both pIC and TNF $\alpha$  signalling to NF- $\kappa$ B [217]. Therefore it is plausible to suggest that the involvement of PKR in PIF/Ang II signalling may not be limited to reducing protein synthesis, but may also be responsible for increasing protein degradation through activation of the ubiquitin proteasome proteolytic pathway. Activation of NF- $\kappa$ B by PKR has been well documented, yet still remains controversial [13]. For example, PKR has been reported to regulate NF- $\kappa$ B by directly phosphorylating I $\kappa$ B [101], other groups however, reported that a non phosphorylated PKR can activate the NF- $\kappa$ B pathway via directly interacting with I $\kappa$ K [42]. In contrast, alternative studies have shown that dsRNA mediated activation of NF- $\kappa$ B activity does not require PKR [110]. Finally, recent data demonstrates that NF- $\kappa$ B can be regulated through eIF2 $\alpha$  activation [52, 88].

To further clarify the role of PKR in the NF- $\kappa$ B signalling pathway stimulated by PIF and Ang II, the effect of the PKR inhibitor on chymotrypsin like activity (see Section 2.3.6 for method used) and proteasome subunit expression (see Section 2.3.13 and 2.3.21 for methods used) was determined in murine myotubes, to measure proteasome involvement in the PKR signalling pathway in response to PIF or Ang II stimulation, at the same concentrations shown to decrease protein synthesis. The signal stimulating NF- $\kappa$ B translocation to the nucleus, induced by TNF $\alpha$ , is transduced through degradation of I $\kappa$ Bs [217]. Moreover, the induction of the ubiquitin proteasome pathway in response to both PIF and Ang II also requires the release of NF- $\kappa$ B from the inactive complex with I $\kappa$ B in the cytosol via I $\kappa$ B phosphorylation and degradation, and subsequent nuclear localisation of NF- $\kappa$ B. Thus levels of I $\kappa$ B $\alpha$  (see Section 2.3.13 and 2.3.21 for methods used) and NF- $\kappa$ B DNA binding (see Section 2.3.7 for method used) were also determined after treatment with PIF or Ang II in the presence and absence of the PKR inhibitor. Myosin levels were measured using the western blotting technique (2.3.13 and 2.3.21), to determine the effect of alterations in proteasome activity on muscle protein breakdown, since loss of myosin has been demonstrated to contribute significantly to the reduction in lean mass associated with a cachectic state [3].

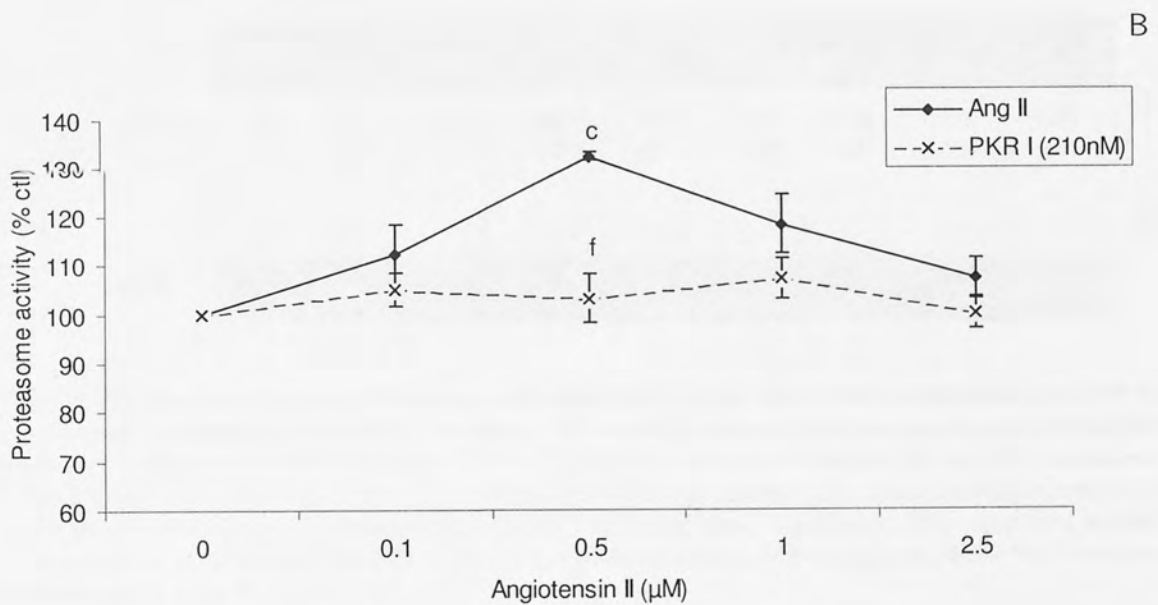
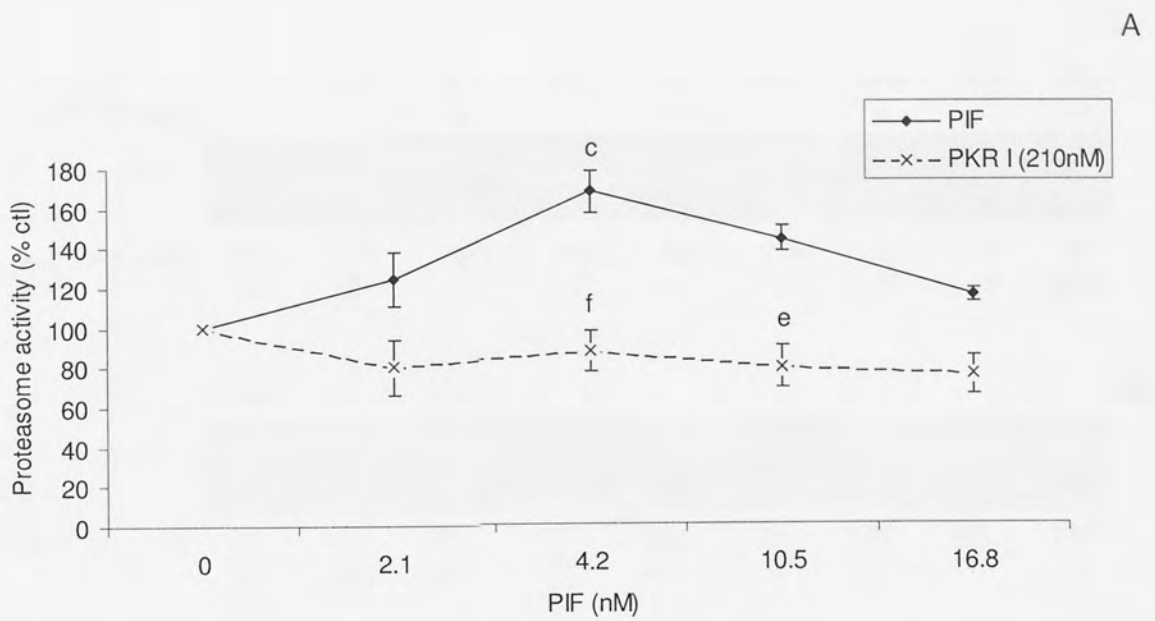


Figure 4.9: **Effect of a PKR inhibitor on chymotrypsin like activity in murine myotubes upon treatment with PIF and Ang II.** The PKR inhibitor (PKR I) (210nM) was incubated with the myotubes for 2h prior to the addition of PIF (A) or Ang II (B), which were incubated with the myotubes for a further 24h. Incubation with the PKR inhibitor significantly attenuated the increased proteasome activity by both agents. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as e:  $p < 0.01$ , f:  $p < 0.001$

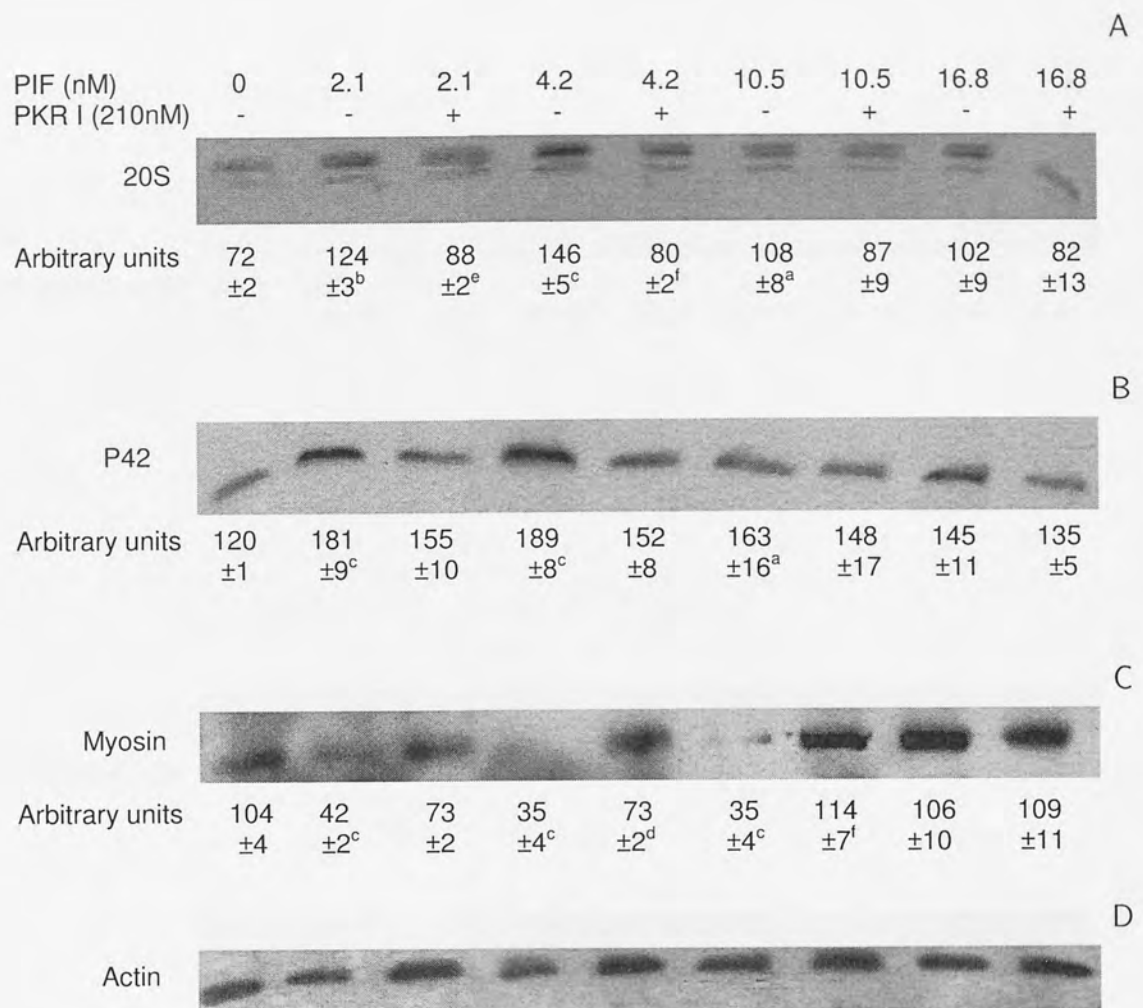


Figure 4.10: **Western blot of proteasome subunits and myosin after 24h incubation with PIF in the absence or presence of the PKR inhibitor.** PIF was incubated with murine myotubes for 24h in the absence or presence of the PKR inhibitor (PKR I) (210nM) and western blotted for the 20S proteasome  $\alpha$ -subunit (A), P42 (B) and myosin (C) with an actin loading control (D). A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , f:  $p < 0.001$

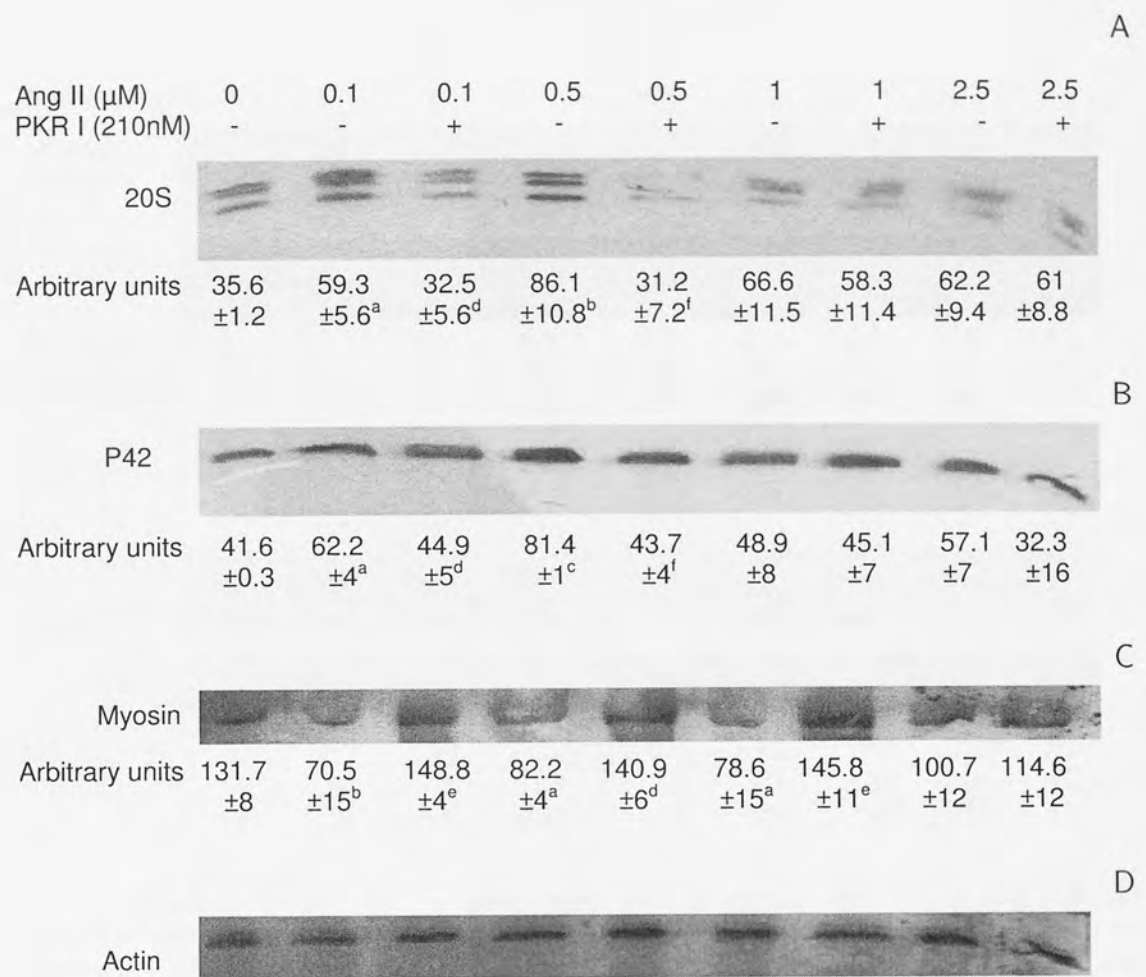
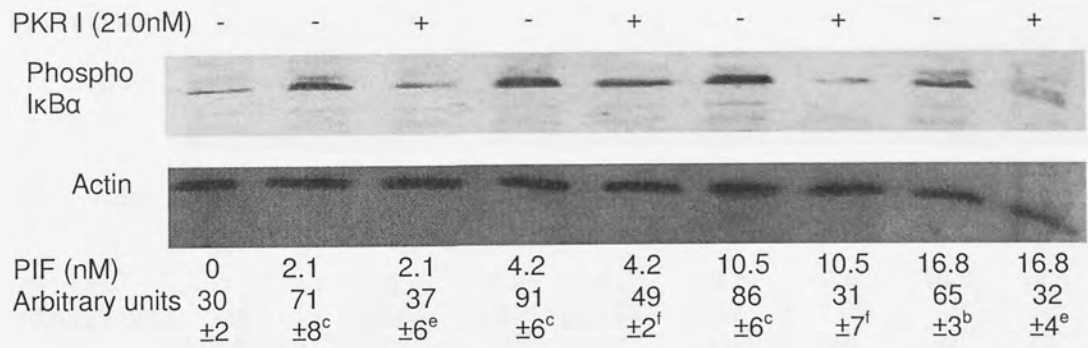


Figure 4.11: **Western blot of proteasome subunits and myosin after 24h incubation with Ang II in the absence or presence of the PKR inhibitor.** Ang II was incubated with murine myotubes for 24h in the absence or presence of the PKR inhibitor (PKR I) (210nM) and western blotted for the 20S proteasome  $\alpha$ -subunit (A), P42 (B) and myosin (C) with an actin loading control (D). A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$



A



B

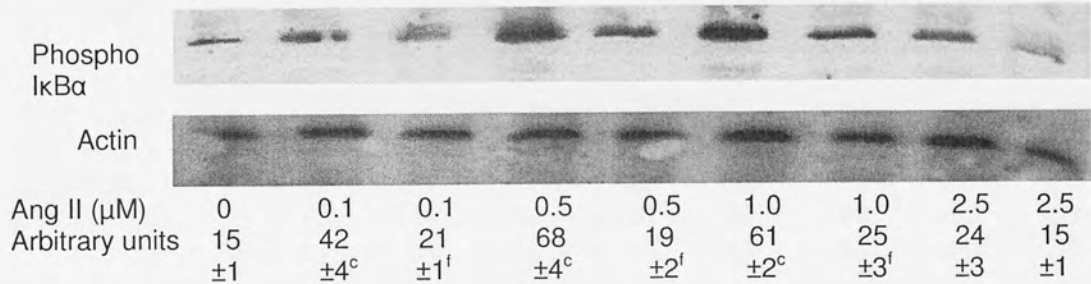
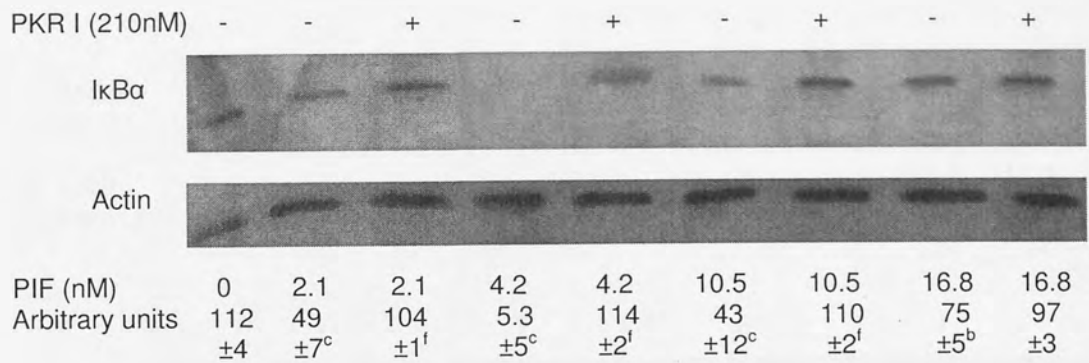


Figure 4.12: Western blot of IκBα phosphorylation after incubation with PIF or Ang II in the absence or presence of a PKR inhibitor. The PKR inhibitor (PKR I) (210nM) was incubated with the myotubes for 2h prior to the addition of PIF (A) or Ang II (B), which was incubated with the myotubes for a further 0.5h. Actin was used as a loading control. A densitometric analysis of phospho IκBα is shown underneath where ± represents the SEM of 3 replicate blots. Significant differences from control are indicated as b:  $p < 0.01$ , c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as e:  $p < 0.01$ , f:  $p < 0.001$



A



B

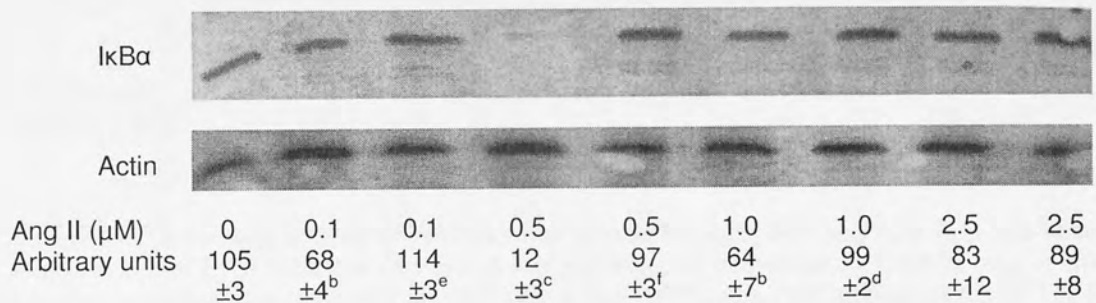


Figure 4.13: Western blot of total I $\kappa$ B $\alpha$  after incubation with PIF or Ang II in the absence or presence of a PKR inhibitor. The PKR inhibitor (PKR I) (210nM) was incubated with the myotubes for 2h prior to the addition of PIF (A) or Ang II (B), which was incubated with the myotubes for a further 0.5h. Actin was used as a loading control. A densitometric analysis of total I $\kappa$ B $\alpha$  is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are indicated as b:  $p < 0.01$ , c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$

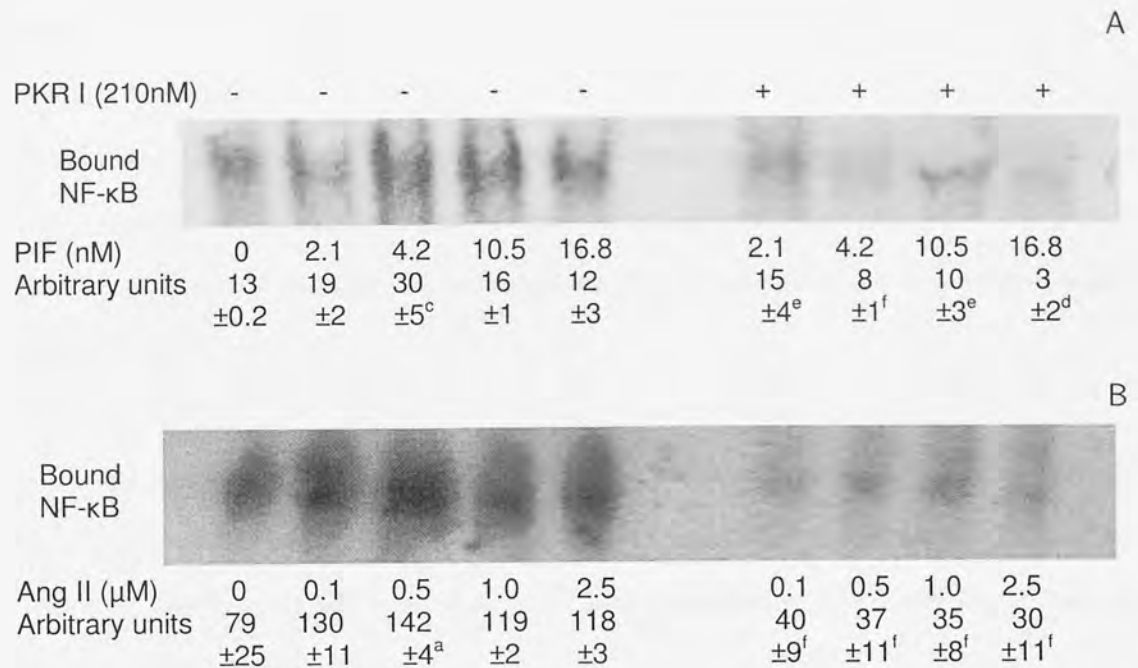


Figure 4.14: **DNA binding activity of NF- $\kappa$ B after incubation with PIF and Ang II in the absence or presence of the PKR inhibitor.** An EMSA was performed to determine the DNA binding of NF- $\kappa$ B after murine myotubes were co treated with PIF (A) or Ang II (B) and a PKR inhibitor (PKR I). The PKR inhibitor (210nM) was incubated with the myotubes for 2h prior to the addition of PIF or Ang II, which was incubated with the myotubes for a further 0.5h. A densitometric analysis of DNA bound NF- $\kappa$ B is shown underneath where  $\pm$  represents the SEM of 2 replicate blots. Significant differences from control are indicated as a:  $p < 0.05$  or c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$

As shown in the previous chapter (Chapter 3) PIF and Ang II are able to increase proteasome activity significantly which is maximum at concentrations of 4.2nM PIF and 0.5 $\mu$ M Ang II (Figure 4.9). Expression of the 20S proteasome  $\alpha$ -subunit and the P42 ATPase 19S proteasome subunit were both upregulated in the presence of PIF and Ang II (Figure 4.10 and 4.11) at the same concentrations as those stimulating proteasome activity. In contrast levels of the myofibrillar protein myosin were decreased upon incubation with PIF and Ang II. For the upregulation of proteasome subunit expression and resulting stimulation of ubiquitin proteasome proteolytic activity to occur the NF- $\kappa$ B transcriptional pathway must first be activated. This is demonstrated in response to PIF and Ang II treatment where I $\kappa$ B phosphorylation is upregulated (Figure 4.12), which is followed by the degradation of total I $\kappa$ B levels (Figure 4.13), and movement of NF- $\kappa$ B to the nucleus (Figure 4.14). Whether this process requires the involvement of eIF2 $\alpha$

phosphorylation is unclear and invites further work to examine the role of eIF2 $\alpha$  in PKR mediated NF- $\kappa$ B activation.

The PKR inhibitor reduced the activation of NF- $\kappa$ B by preventing the phosphorylation and degradation of I $\kappa$ B $\alpha$ . Co-treatment with the PKR inhibitor also completely attenuated the increase in proteasome activity, abrogated the expression of proteasome subunits 20S and P42 and restored myosin levels to those seen in unstimulated cells.

Since increased activity and expression of the ubiquitin proteasome pathway is involved in the induction of protein degradation and cell death, the effect of the PKR inhibitor on protein degradation (see Section 2.3.14 for method used) was analysed using the same concentrations of PIF and Ang II shown to increase proteasome expression and activity.

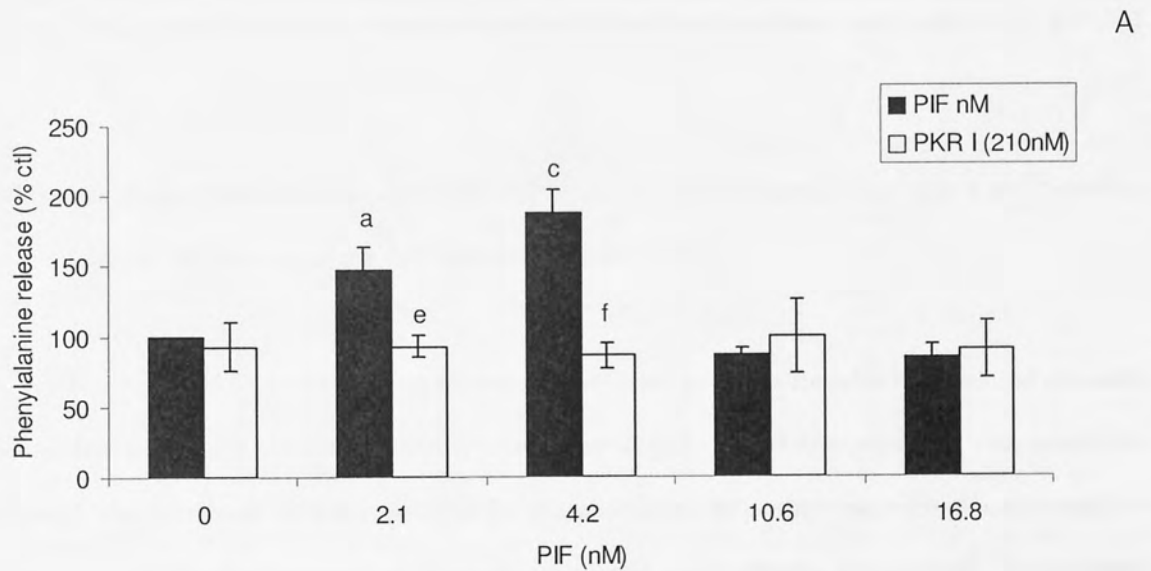


Figure 4.15

B

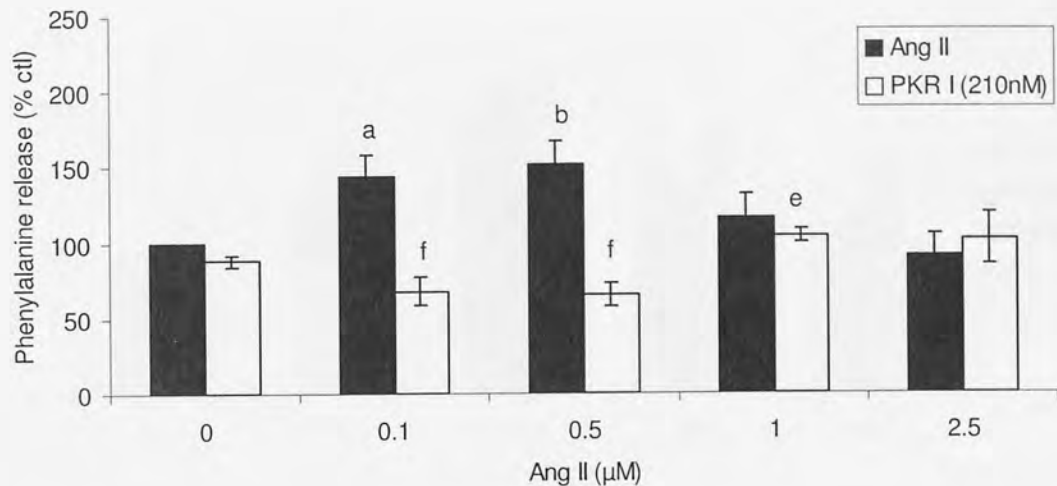
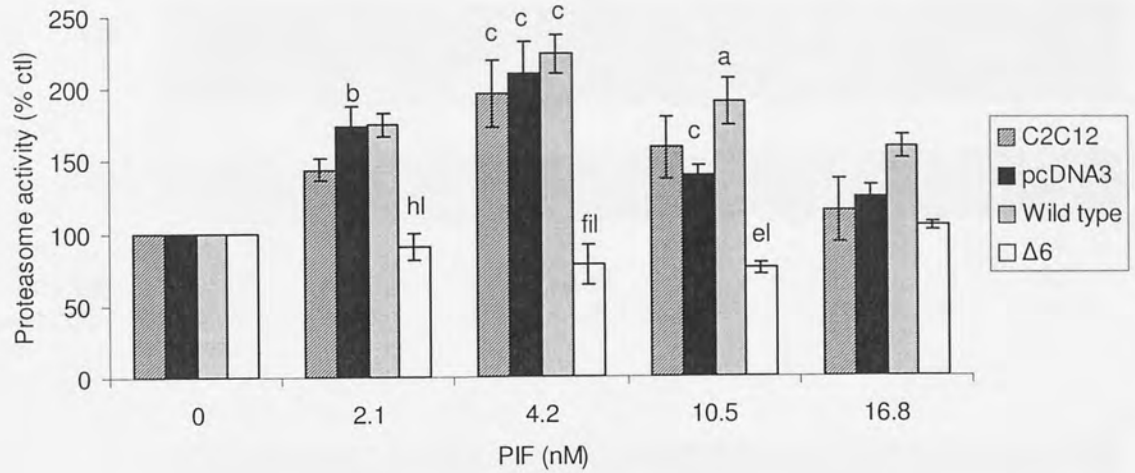


Figure 4.15: **Effect of a PKR inhibitor on protein degradation in murine myotubes upon treatment with PIF or Ang II.** The PKR inhibitor (PKR I) (210nM) was incubated with the myotubes for 2h prior to the addition of PIF (A) or Ang II (B), which was incubated with the myotubes for a further 24h. Incubation with the PKR inhibitor significantly reduced the increased protein degradation by both agents. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences in the presence of the PKR inhibitor are shown as e:  $p < 0.01$  or f:  $p < 0.001$

As expected protein degradation was increased maximally at 4.2nM PIF and 0.5 $\mu$ M Ang II and this effect was abolished in the presence of the PKR inhibitor (Figure 4.15).

Cells expressing PKR $\Delta$ 6 have been demonstrated to be resistant to double stranded RNA induced cell death through the reduction of transcripts involved with apoptosis [11]. NF- $\kappa$ B activation is an early marker for cell death when combined with sequential eIF2 $\alpha$  phosphorylation [54], which has been shown to occur in correlation to PKR phosphorylation in response to PIF and Ang II (Figure 4.3 and 4.4). To determine whether PKR is responsible for NF- $\kappa$ B nuclear mobilization, and subsequent upregulation of proteasome activity, and protein degradation by PIF and Ang II, chymotrypsin like activity was measured in PKR mutant C2C12 myotubes (Methods Section 2.3.6) along with I $\kappa$ B $\alpha$  (Section 2.3.13 and 2.3.21), NF- $\kappa$ B (Section 2.3.6) and proteasome subunit expression (Section 2.3.13 and 2.3.21) and protein degradation (Section 2.3.14) upon exposure to both agents.

C



D

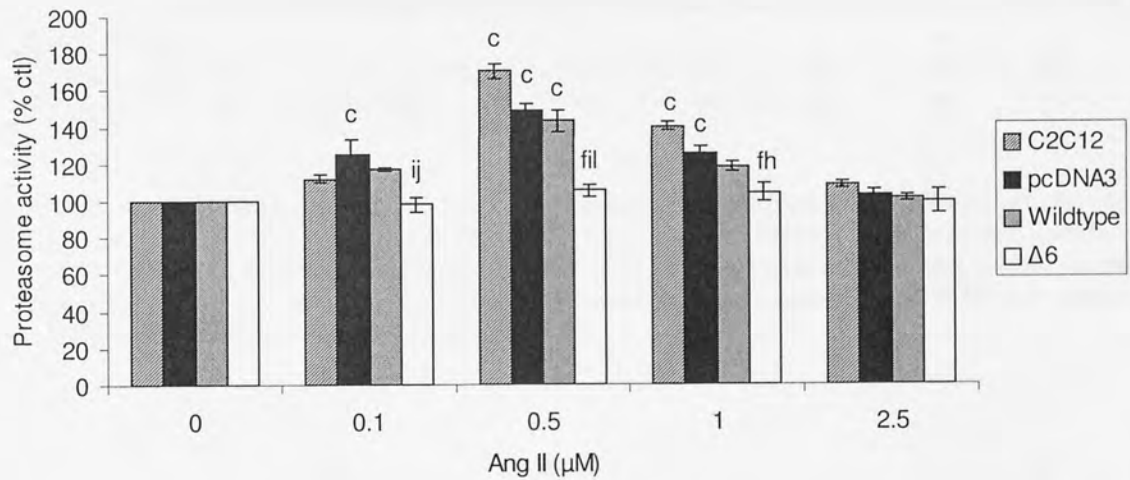


Figure 4.16: **Chymotrypsin like activity in murine myotubes transfected with PKR mutant plasmids upon treatment with PIF or Ang II.** PIF (A) or Ang II (B) were incubated with murine myotubes transfected with pcDNA3(-), wildtype PKR, PKR $\Delta$ 6 or non-transfected myotubes for 24h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from non-transfected cells are shown as e:  $p < 0.01$ , f:  $p < 0.001$ , differences from pcDNA3(-) h:  $p < 0.01$ , i:  $p < 0.001$  and differences from wildtype j:  $p < 0.05$ , l:  $p < 0.001$

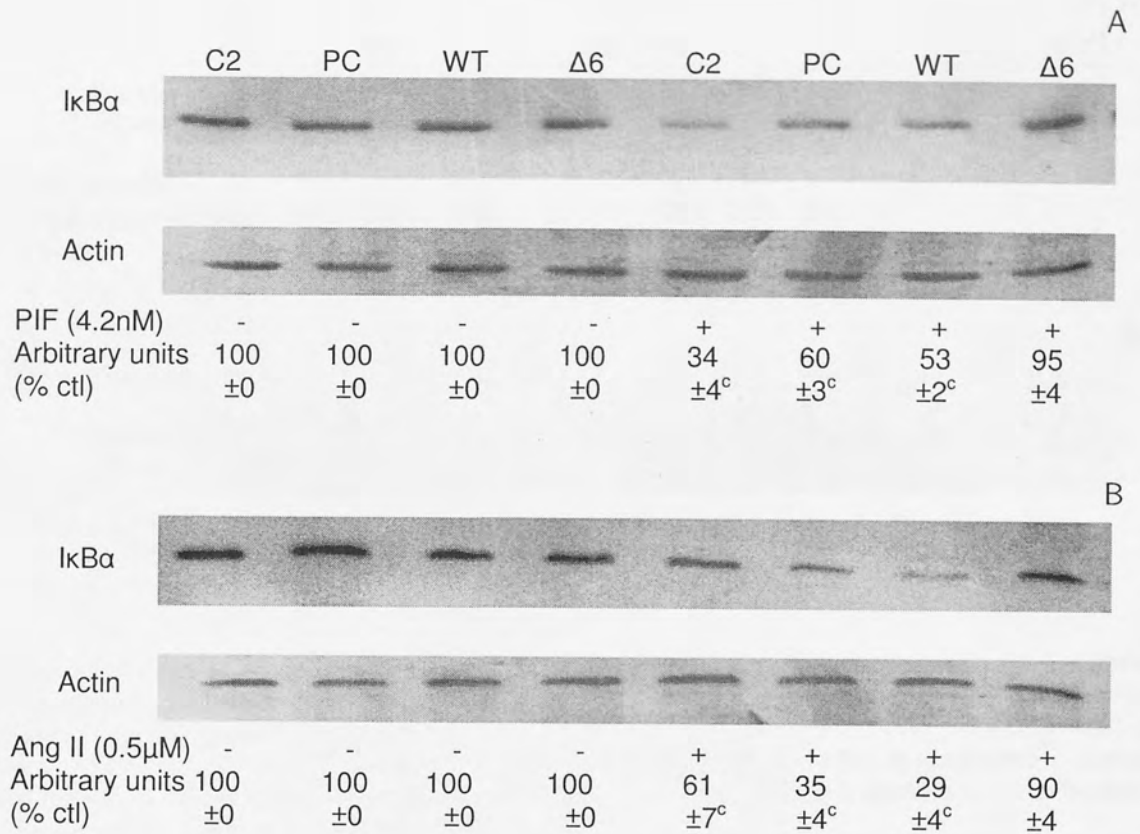


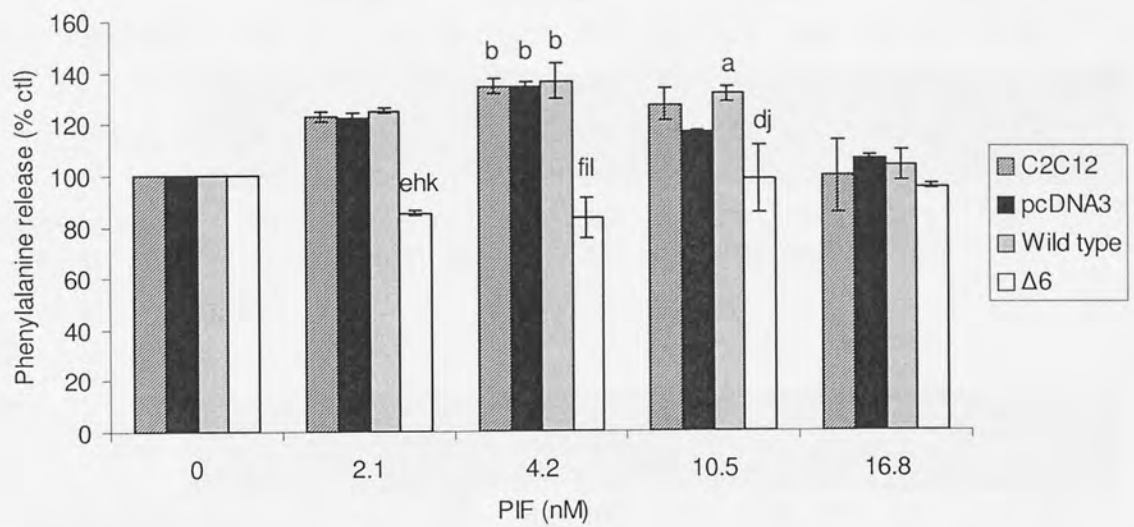
Figure 4.17: **Western blot of total IκBα in C2C12 myotubes transfected with PKR mutant plasmids upon treatment with PIF or Ang II.** PIF (A) or Ang II (B) were incubated with pcDNA3 (PC), wildtype PKR (WT), PKRΔ6 (Δ6) or non-transfected myotubes (C2) for 0.5h. Actin was used as a loading control. A densitometric analysis of total IκBα is shown underneath where ± represents the SEM of 3 replicate blots. Differences from control are shown as c:  $p < 0.001$ .



Figure 4.18: **DNA binding activity of NF-κB in murine myotubes transfected with PKR mutant plasmids upon treatment with PIF or Ang II.** An EMSA was performed to determine the DNA binding of NF-κB in murine myotubes transfected with pcDNA3 (PC), wildtype PKR (WT), PKRΔ6 (Δ6) or non-transfected myotubes (C2) incubated with PIF (A) or Ang II (B) for 0.5h. A densitometric analysis of DNA bound NF-κB is shown underneath where ± represents the SEM of 2 replicate blots. Differences from control are shown as b:  $p < 0.01$ , c:  $p < 0.001$ .



A



B

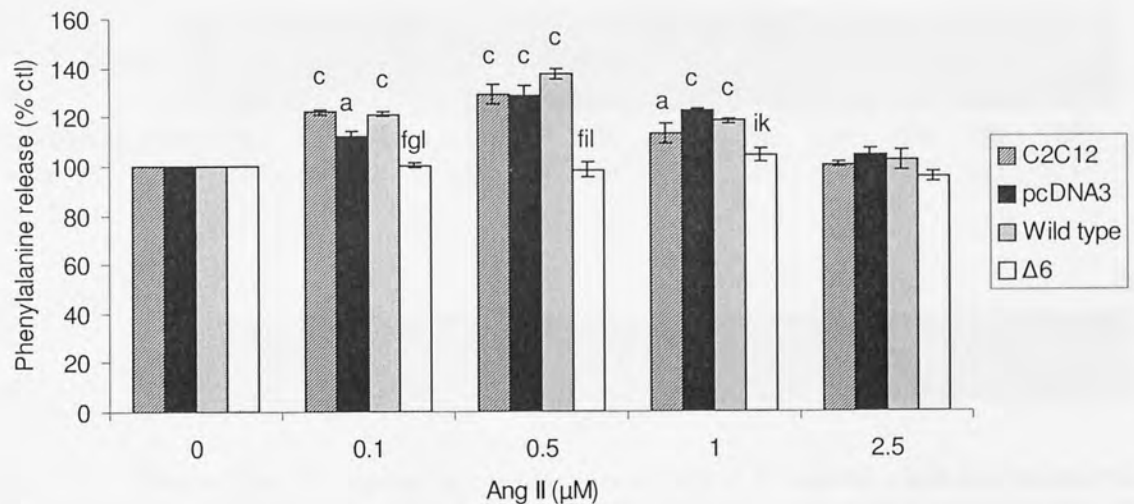


Figure 4.19: **Protein degradation in murine myotubes transfected with PKR mutant plasmids upon treatment with PIF or Ang II.** PIF (A) or Ang II (B) were incubated with murine myotubes transfected with pcDNA3(-), wildtype PKR, PKR $\Delta$ 6 or non-transfected myotubes for 24h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from non-transfected cells are shown as e:  $p < 0.01$ , f:  $p < 0.001$ , differences from pcDNA3(-) h:  $p < 0.01$ , i:  $p < 0.001$  and differences from wildtype k:  $p < 0.01$ , l:  $p < 0.001$

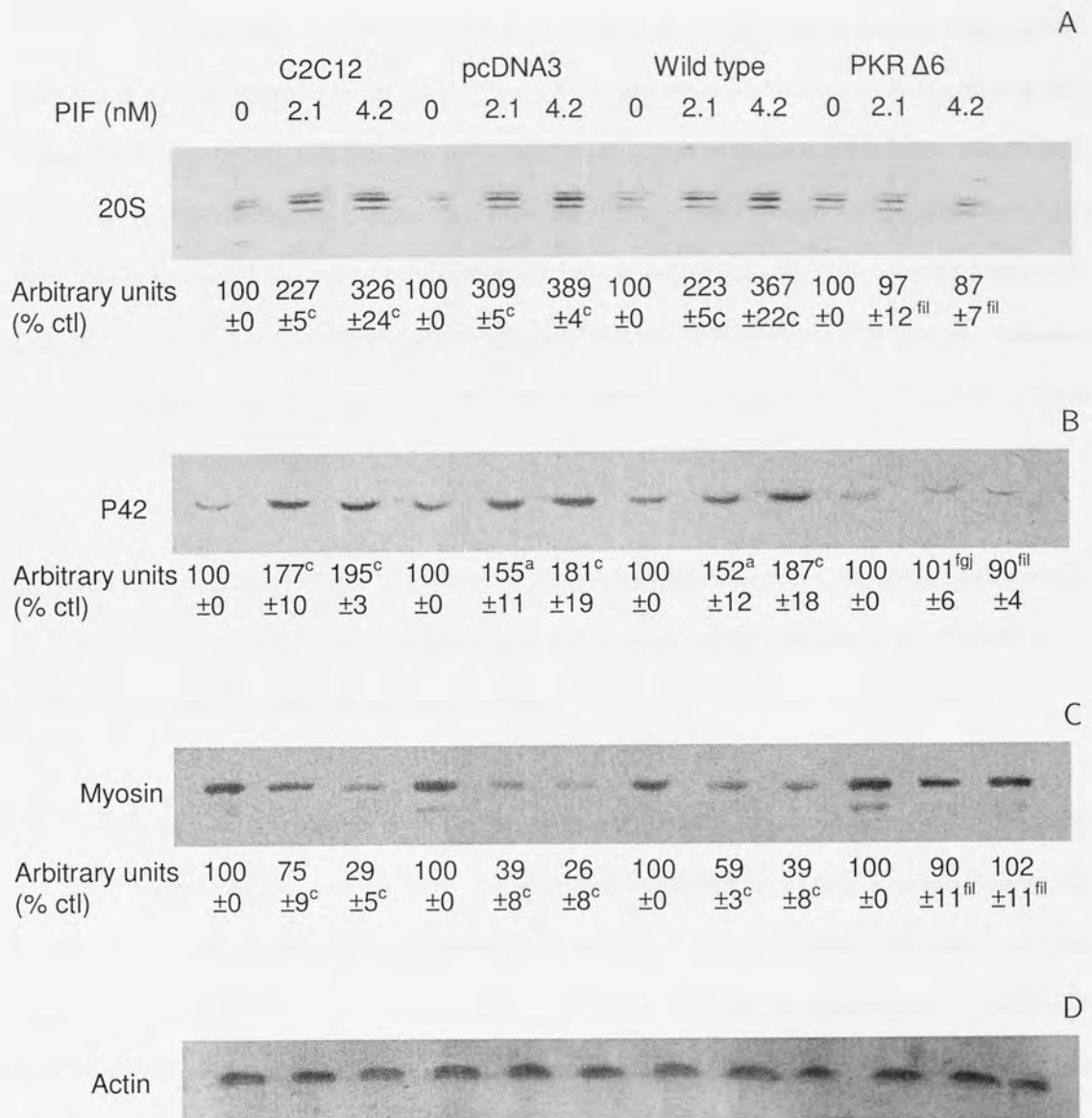


Figure 4.20: **Western blot of proteasome subunits and myosin in murine myotubes transfected with PKR mutant plasmids upon treatment with PIF.** Murine myotubes were incubated with PIF for 24h and western blotted for the 20S proteasome  $\alpha$ -subunit (A), P42 (B), myosin (C) and an actin loading control (D) in murine myotubes transfected with pcDNA3 (lanes 4-6), wildtype PKR (lanes 7-9) and PKR $\Delta$ 6 (lanes 10-12) in comparison with non-transfected myotubes (lanes 1-3). A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Differences from control are shown as a:  $p < 0.05$ , c:  $p < 0.001$ , while differences from non-transfected cells are shown as f:  $p < 0.001$ , differences from pcDNA3(-) g:  $p < 0.05$ , i:  $p < 0.001$  and differences from wildtype l:  $p < 0.001$

In the presence of PIF, chymotrypsin like enzyme activity was increased in pcDNA3, PKR wildtype and non-transfected cells similarly, while in PKR $\Delta$ 6 myotubes there was a lack of proteasome activity (Figure 4.16). PIF and Ang II induced degradation of I $\kappa$ B $\alpha$  (Figure 4.17) accompanied by nuclear localization of NF- $\kappa$ B (Figure 4.18) in pcDNA3, wildtype and non-transfected myotubes but not in PKR $\Delta$ 6 cells. Protein degradation was increased when myotubes were treated with PIF and Ang II (Figure 4.19) and the expression of proteasome subunits 20S and P42 were elevated while the myosin levels were reduced in the presence of PIF (Figure 4.20) in the non-transfected control cells, pcDNA3 and PKR wildtype cells. However there was no alteration in protein degradation, proteasome subunit expression or myosin levels in PKR $\Delta$ 6 myotubes when incubated with catabolic agents.

These results confirm that activation of PKR by autophosphorylation results in increased protein degradation through upregulation of ubiquitin proteasome activity and subunit expression, via degradation of I $\kappa$ B $\alpha$ , resulting in NF- $\kappa$ B activation and DNA binding.

MAC16 tumours have been evidenced to produce the cachexia inducing sulphated glycoprotein PIF, which leads to skeletal muscle atrophy once the tumour is implanted into NMRI mice [187]. Previous experiments with PIF in vitro, in this chapter, have demonstrated its ability to induce a depression in protein synthesis through the upregulation of PKR activity and subsequent induction of eIF2 $\alpha$  phosphorylation. Since MAC16 tumours have been shown to grow slowly and induce cachexia with very small tumour burdens [26] this suggests that perhaps the same mechanisms activated in the murine skeletal muscle cell line in response to PIF is also upregulated in the MAC16 tumour cell line, offering an explanation as to the extraordinary slow growth of the MAC16 tumour in vivo. Thus the MAC16 and histologically similar MAC13 tumour cells were subjected to a protein synthesis assay (method described in Section 2.3.15).



Figure 4.21: **Protein synthesis in MAC16 and MAC13 cells in vitro over a 4h time period.** Synthesis in the MAC16 cells was reduced in comparison with the MAC13 cells. Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences from the MAC16 tumour cells are shown as c:  $p < 0.001$

Protein synthesis in MAC16 cells was dramatically reduced compared to MAC13 cells (Figure 4.21). It is possible that the PKR/eIF2 $\alpha$  pathway is activated in the MAC16 cells by PIF as is observed in murine myotubes (Figure 4.3A and 4.4A), since PIF has been shown to be present in tumour tissue [187]. To determine whether the depression of protein synthesis was as a result of PKR activation, the cells were treated with an increasing concentration of PKR inhibitor. The cell growth was calculated as the number of cells present after 48h incubation with the PKR inhibitor (see Section 2.3.4 for method used).

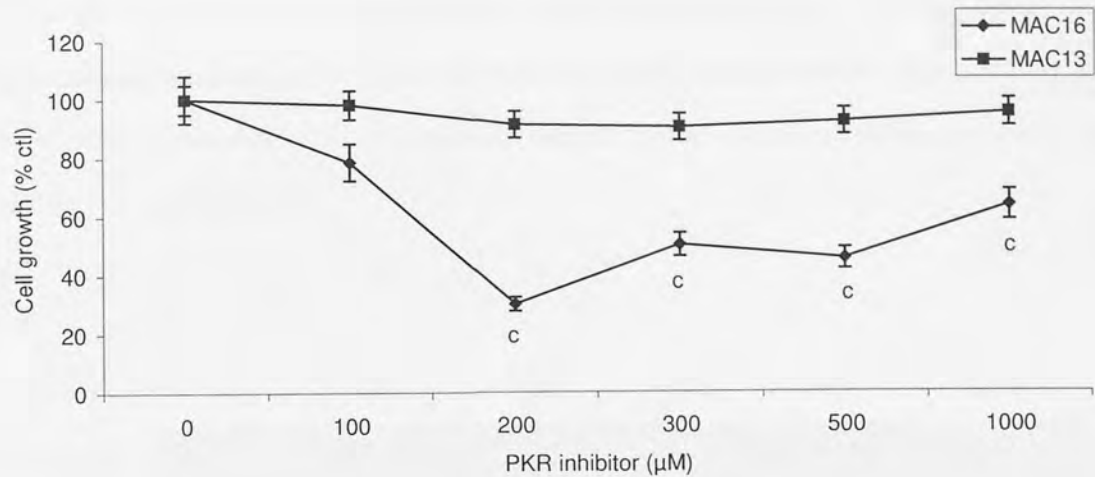


Figure 4.22: **Effect of increasing concentrations of the PKR inhibitor on growth of the MAC16 and MAC13 tumour cells in vitro.** The PKR inhibitor reduced the MAC16 cell number without affecting MAC13 cell growth. Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences are shown as c:  $p < 0.001$

Treatment with the PKR inhibitor had the effect of reducing cell growth of MAC16 cells with a maximum effect at 200nM, with higher concentrations producing less effective inhibition, while it had no effect on the growth of the MAC13 tumour cells, even at a concentration up to 1000nM (Figure 4.22). This suggests that PKR activation may be important in the development of MAC16 tumours.

In addition to inhibiting protein synthesis, PKR has also been shown to restrict cellular proliferation through interaction with p53, enhancing transcriptional activity of this stress responsive tumour suppressor [217]. This is in contradiction to the results presented above which show inhibition, rather than activation of PKR, results in a reduction of tumour cell growth. However this effect may be specific to MAC16 tumour cells since they form a tumour when implanted into mice which presents with an unusual growth rate [26]. This slow rate of growth may be advantageous to the tumour by reducing the nutritional requirements and thus having a protective effect on tumour development.

Since treatment of MAC16 cells with the PKR inhibitor produced inhibition of cell growth (Figure 4.22),

the effect of the inhibitor on phosphorylated and total PKR was determined in both MAC16 and MAC13 cells, to see if this effect correlated with inhibition of PKR autophosphorylation. The PKR inhibitor reduced 20S proteasome expression in C2C12 murine myotubes when co-treated with PIF (Figure 4.10A), so the tumour cells were also assayed for 20S expression using the western blotting technique (see Section 2.3.13 and 2.3.21 for method used).

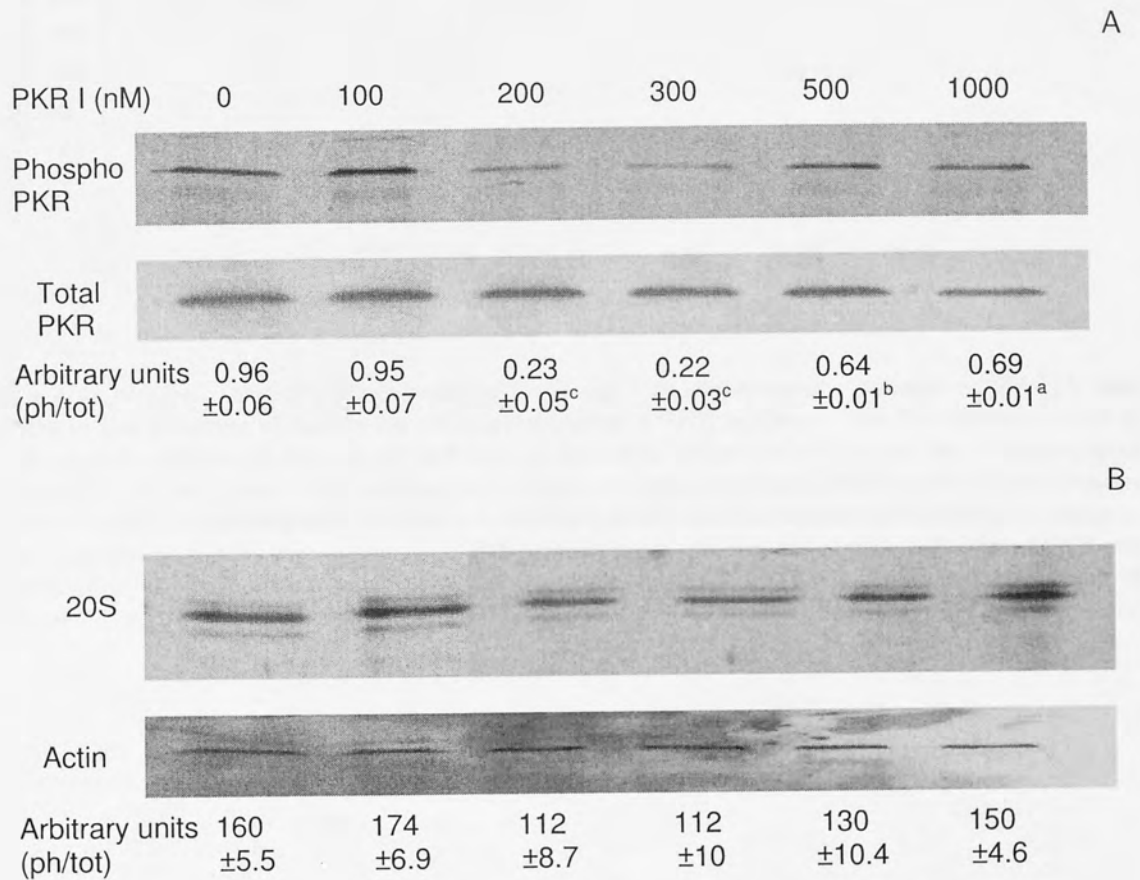


Figure 4.23

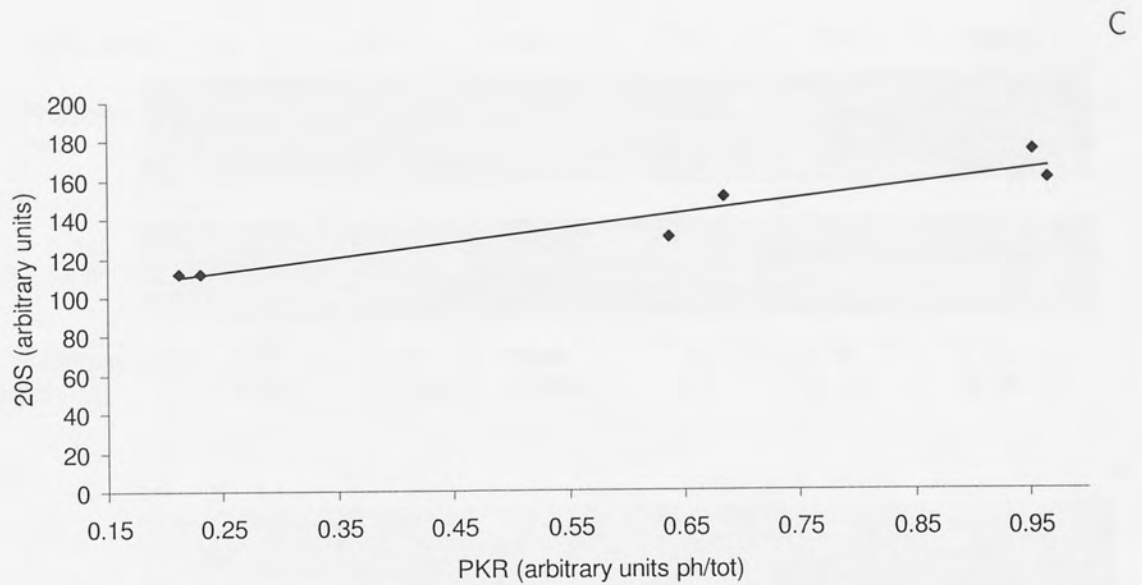


Figure 4.23: **Western blot of phosphorylated PKR and 20S proteasome  $\alpha$ -subunit in MAC16 cells in vitro in the presence of increasing concentrations of a PKR inhibitor.** The PKR inhibitor (PKR I) was incubated with MAC16 cells for 48h and western blotted for phospho PKR (A) and the 20S proteasome  $\alpha$ -subunit(B). A blot of total PKR was used to normalize the phosphorylated protein and actin was used as a loading control. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. The relationship between expression of 20S proteasome  $\alpha$ -subunits and autophosphorylated PKR was determined using the Pearsons linear correlation statistical analysis (C) (n=3). Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$ , c:  $p < 0.001$ , and the correlation coefficient is 0.957



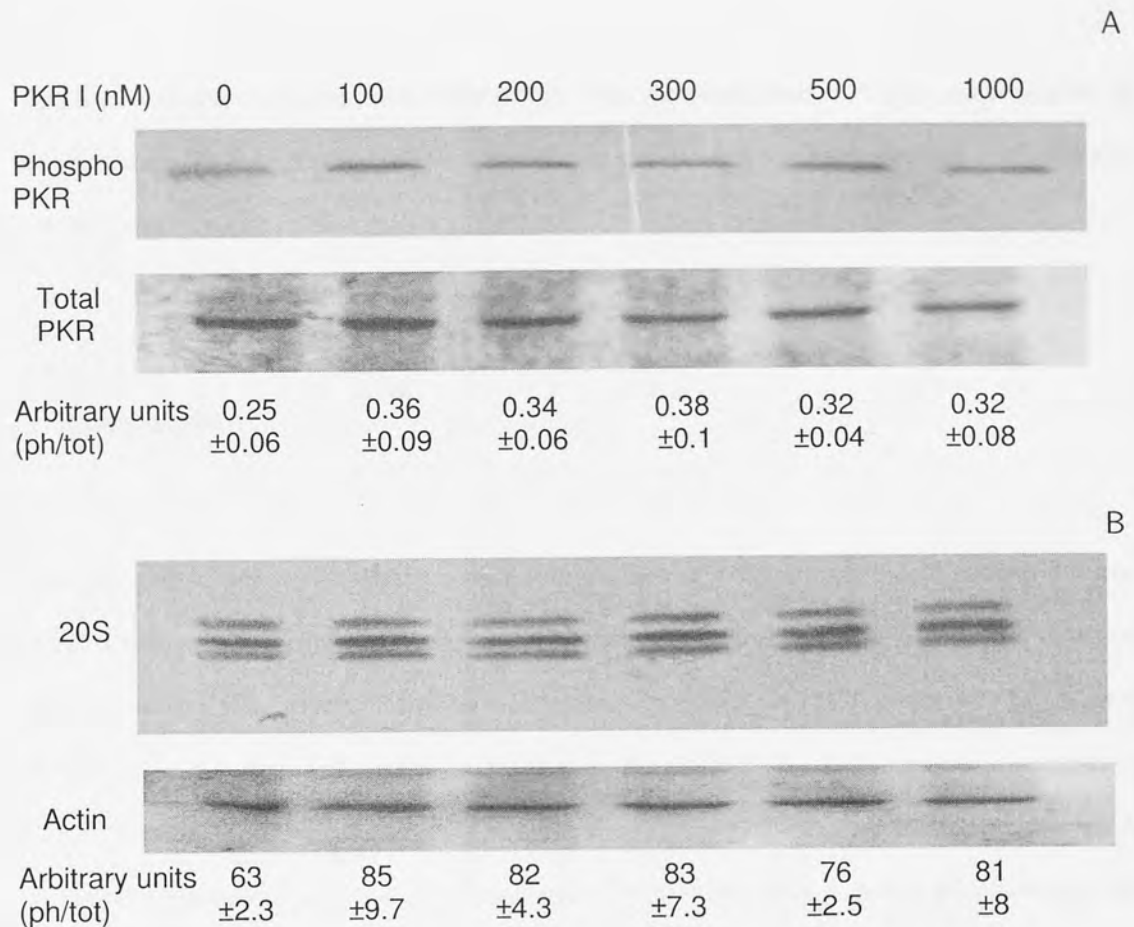


Figure 4.24: **Western blot of phosphorylated PKR and 20S proteasome  $\alpha$ -subunit in MAC13 cells *in vitro* in the presence of increasing concentrations of a PKR inhibitor.** The PKR inhibitor (PKR I) was incubated with MAC13 cells for 48h and western blotted for phospho PKR (A) and the 20S proteasome  $\alpha$ -subunit(B). A blot of total PKR was used to normalize the phosphorylated protein and actin was used as a loading control. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. No significant changes were observed

The PKR inhibitor attenuated autophosphorylation of PKR and 20S expression in MAC16 cells with a maximum effect between 200 and 300nM, whilst at higher concentrations it was less effective (Figure 4.23). There was no effect of the PKR inhibitor on the low levels of autophosphorylation of PKR and 20S in MAC13 cells (Figure 4.24).

There was a linear correlation (correlation coefficient 0.957) between phosphorylation of PKR and expression of the 20S proteasome (Figure 4.23), with the different concentrations of the PKR inhibitor (Figure 4.23C),

suggesting that expression of the 20S proteasome may also be controlled by PKR in MAC16 cells.

The results of this chapter suggest that inhibition of PKR phosphorylation may be a useful method of reducing the muscle wasting effects of PIF and Ang II in cancer patients displaying cachexia and could also have an inhibitory effect on tumour growth.

### 4.3 Discussion

The activation of PKR has been linked to several diseases, such as myotonic dystrophy [184] and rheumatoid arthritis [70], where the pathogenic hallmarks of cachexia, including muscle wasting and muscular weakness, are evident. The atrophy of skeletal muscle mass is generally seen as a combination of increased protein degradation and decreased protein synthesis, where the elevated degradation is a result of the transcriptional activator NF- $\kappa$ B, upregulating target genes such as the ubiquitin proteasome pathway, which targets myofibrillar proteins for proteasomal degradation. The loss of muscle protein is exacerbated by the lack of protein synthesis to repay the muscle fibre debt.

Both catabolic agents, PIF and Ang II, examined in this chapter have been linked to cancer cachexia in previous studies, and have both been demonstrated to cause an increased protein degradation and decreased protein synthesis in the C2C12 murine cell line. PIF and Ang II upregulated proteasome activity and protein degradation at the same concentrations as those decreasing protein synthesis but rates of protein synthesis were affected at an earlier time period of 4-6h while protein degradation did not occur until much later (24h). Protein synthesis is not inhibited by PIF through a block in amino acid uptake into the cells or by a drop in ribosome number, since 85% RNA is ribosomal [4] and PIF had no effect on total cellular RNA levels (see Chapter 3 for these results).

Alterations in protein degradation and synthesis were originally believed to be segregated since treatment of mice bearing the MAC16 tumour with EPA blocked the degradation of skeletal muscle but had no

effect on the rate of protein synthesis which remained depressed [162]. However this study puts both processes at different stages of a pathway activated by a common progenitor. This is substantiated by a report which shows HMB to inhibit both PIF induced depression in protein synthesis and increased protein degradation [57]. Since PKR can not only influence protein synthesis, through eIF2 $\alpha$  phosphorylation, but also upregulate protein degradation and cell death, via NF- $\kappa$ B activation, this makes it the perfect candidate.

Other studies have also linked PKR to the reduction in protein synthesis and increase in protein degradation by other agents. For example the inhibition of protein synthesis in apoptosis by TNF $\alpha$  has been associated with PKR activation and eIF2 $\alpha$  phosphorylation, while PKR has been shown to mediate degradative pathways in chondrocytes by TNF- $\alpha$  in osteoarthritis models [70]. Activation of PKR by extended CUG repeats in myotonic dystrophy is associated with the reduced rates of protein synthesis in these patients compared to healthy subjects or patients with other forms of muscular dystrophy and this is attributed to its ability to phosphorylate eIF2 $\alpha$  [184]. PKR could also affect protein synthesis in a manner independent of its ability to phosphorylate eIF2 $\alpha$ , by its interaction with the PP2A regulatory subunit B56 $\alpha$ . Overexpression of B56 $\alpha$  causes an upregulation of protein synthesis and PKR inhibits this effect probably by phosphorylating B56 $\alpha$  [212]. However this pathway was not studied and may prove interesting to examine further.

To confirm the role of PKR in phosphorylation of the eIF2  $\alpha$ -subunit and subsequent depression in protein synthesis, an inhibitor of PKR was employed and myotubes transfected with wildtype overexpressing PKR and catalytically inactive PKR $\Delta$ 6 mutant plasmids. Although PERK and GCN2 are plausible alternatives for phosphorylation of the eIF2  $\alpha$ -subunit, the PKR inhibitor and a dominant negative mutation of PKR strongly attenuated eIF2 $\alpha$  phosphorylation and consequent depression in protein synthesis seen in response to both PIF and Ang II, suggesting that the participation of PERK and GCN2 might be minimal in this experimental model.

The elevated protein degradation seen upon treatment of murine myotubes with PIF and Ang II was inhibited in the presence of the PKR inhibitor and in the myotubes transfected with the dominant negative PKR $\Delta$ 6 variant. Both agents utilise the ubiquitin proteasome pathway to increase protein degradation,

thus the attenuation of PKR activation by the PKR inhibitor and PKR $\Delta$ 6 variant had the effect of inhibiting protein degradation, via blocking the induction of the ubiquitin proteasome pathway, by antagonising NF- $\kappa$ B nuclear localisation. These results demonstrate that PIF requires functional PKR pathway to reduce protein synthesis and increase protein degradation in murine myotubes.

There is some controversy involving the contribution of eIF2 $\alpha$  phosphorylation to the activation of NF- $\kappa$ B. Some studies suggest that the phosphorylation of eIF2 $\alpha$  is a separate event to the release of NF- $\kappa$ B in response to PKR autophosphorylation [86, 54], while others imply that eIF2 $\alpha$  phosphorylation is critical for the activation of NF- $\kappa$ B, where one study reported that phosphorylation of the  $\alpha$ -subunit of eIF2 is fundamental to the process by which many stress signals activate NF- $\kappa$ B [88]. There are four eIF2 $\alpha$  kinases that co-ordinate the phosphorylation of eIF2 $\alpha$ , depending on the origin of the stress signal. PERK is involved in the stress response to impaired ER function, HRI is activated by oxidative and heat stress, GCN2 is activated by nutritional stress and PKR controls antiviral defence. Only PKR has been demonstrated to have a eIF2 $\alpha$  independent mechanism of activating NF- $\kappa$ B and is thought to physically associate with IKK to induce I $\kappa$ B phosphorylation and ubiquitin mediated degradation [88]. However other reports dispute this, noting that phosphorylation of I $\kappa$ Bs by PKR is indirect [217] and thus eIF2 $\alpha$  phosphorylation may be involved. For example a mutant eIF2 $\alpha$  which mimics a phosphorylated serine was sufficient to induce apoptosis. Also a requirement for inhibition of new protein synthesis is frequently observed for TNF $\alpha$  induced apoptosis, and inhibition of protein synthesis itself is sufficient to induce apoptosis in some systems [170].

Moreover Donze et al [54] showed that activation of NF- $\kappa$ B by PKR actually precedes eIF2 $\alpha$  phosphorylation, where cell survival is activated through NF- $\kappa$ B signalling, and apoptosis is then induced by eIF2 $\alpha$  phosphorylation. Since treatment with PIF has been shown to activate cell death it is possible that this sequence of events is that which occurs in response to PIF and possibly Ang II. To support this observation the results in this chapter do show a chronological induction of I $\kappa$ B $\alpha$  phosphorylation and degradation with consequent NF- $\kappa$ B release and DNA binding at approximately 30 min after exposure to PIF or Ang II. This is followed by PKR and eIF2 $\alpha$  phosphorylation after 2h incubation with both agents. This follows a similar time line as demonstrated by Donze et al after HeLa cells were infected with vesicular stomatitis

virus, where NF- $\kappa$ B activation occurs before PKR phosphorylation is detectable, while PKR and eIF2 $\alpha$  phosphorylation rise in parallel much later [54]. It is possible that phosphorylation of eIF2 $\alpha$  by PKR leads to translational inhibition which counteracts the protective effect of NF- $\kappa$ B dependent transcription of genes encoding proteins that prevent apoptosis, and instead selectively enhances the translation of mRNAs encoding proapoptotic functions [170].

The mechanisms by which PIF and Ang II might activate PKR are unknown. PKR can be activated in response to a diverse range of cellular stresses by many different stimuli such as heparin. PIF is a polyanionic agent similar to heparin and thus may be able to activate PKR directly [59]. Evidence shows that calcium release is involved in PKR activation in Alzheimer's disease, since activation of PKR and eIF2 $\alpha$  by  $\beta$ -amyloid (A $\beta$ ) peptides was significantly reduced in the presence of the intracellular calcium chelator BAPTA/AM [40]. Furthermore, as well as participating in the execution phase of apoptosis, caspase 3 and caspase 8 are also involved in A $\beta$  peptide neurotoxicity by activating PKR. Calcium release and caspase 8 activation are believed to be upstream of caspase 3 induced A $\beta$  peptide triggered PKR activation [174]. PIF has also been shown to stimulate apoptosis and activate caspase 3 and 8 in murine myotubes thus providing an alternative mechanism for the activation of PKR by PIF [164]. However another study puts caspase 8 downstream of PKR activation, suggesting the existence of a positive feedback loop in the regulation of PKR activation [86]. PIF induced caspase 3 activation may be responsible for the initial step in myofibrillar proteolysis to produce monomeric myosin, which the ubiquitin proteasome can degrade, since it is unable to break down the myosin when it is contained in actomyosin or myofibrils. In fact, limited actomyosin cleavage by caspase 3 yields a 125% increase in protein degradation by the ubiquitin proteasome system [55].

Activation of PKR by caspases may be through cleavage as well as phosphorylation. Cleaved PKR is fully capable of phosphorylating eIF2 $\alpha$  because the proteolysis of PKR by caspases release the kinase domain from the N-terminal regulatory domain [174]. It is suggested that caspase mediated activation of PKR may have evolved to provide the cell with a by-pass mechanism to fight virally and cellularly encoded PKR inhibitors [158]. eIF2 $\alpha$  can reduce global rates of protein synthesis as a result of its cleavage. In A $\beta$  peptide treated neurons there was a large portion of cleaved eIF2 $\alpha$ . It has been suggested that its cleavage causes



functional changes of the eIF2 $\alpha$  complex, which can no longer stimulate protein synthesis [174]. This cleavage also occurs as a result of caspase 3 activation, which can be upregulated by PIF [164]. Despite that, this study does not examine the cleavage of PKR or eIF2 $\alpha$ , although it may be an additional way to inhibit global protein translation and worth investigating further as a component of the PIF induced inhibition of protein synthesis.

A cellular protein activator of PKR (PACT) has been discovered, which activates PKR in the absence of dsRNA [136]. PACT has been implicated in the activation of PKR and subsequent proteoglycan release from cartilage that occurs in arthritic disease. Release of the naturally occurring sphingolipid C<sub>2</sub> ceramide in response to the proinflammatory cytokine TNF $\alpha$  is believed to stimulate PACT and subsequent PKR phosphorylation in rheumatoid arthritis and osteoarthritis [70]. Angiotensin is known to induce fluxes in intracellular calcium concentrations and stimulate the production of ceramide [21], both of which have been linked to PKR phosphorylation and activation, suggesting possible methods for Ang II to affect PKR activity. Alternatively, the activation of PKR may be modulated by cellular inhibitors that are regulated by external stimuli. For example, it was recently shown that ubiquitously expressed PKR inhibitor P58 [120] is inhibited by hsp40. Thus PIF or Ang II may affect inhibitors such as P58 to induce PKR activation [170]. However further studies are required to determine the exact methods by which PIF and Ang II alter PKR phosphorylation and whether PKR cleavage is involved.

PKR activation is also likely to be involved in MAC16 tumour cell growth and proliferation since the PKR inhibitor was effective at reducing MAC16 cell growth compared to the MAC13 cells. Both tumour cell types are from adenocarcinomas of the large bowel in mice, induced by prolonged administration of 1,2-dimethylhydrazine [49], but the MAC16 induces cachexia [24], while the MAC13 does not once implanted into NMRI mice. The results are surprising because numerous studies indicate that PKR is a tumour suppressor. One possible link between PKR and tumour growth involves activation of NF- $\kappa$ B. Activation of NF- $\kappa$ B has been connected with tumour cell survival and proliferation, as well as invasion and angiogenesis [92]. NF- $\kappa$ B has been reported to be constitutively activated in a number of tumour types including colorectal carcinoma [99] and pancreatic adenocarcinoma [203]. The PKR inhibitor reduced 20S expression in correlation with inhibition of PKR phosphorylation demonstrating a direct relationship

between the levels of phosphorylated PKR and expression of the 20S proteasome  $\alpha$ -subunits. In this chapter, upregulation of 20S has been shown in murine myotubes in response to NF- $\kappa$ B nuclear localisation upon PIF or Ang II treatment. This suggests that activation of the transcriptional activator NF- $\kappa$ B, is occurring in MAC16 tumour cells, possibly through the production of catabolic mediators like PIF or Ang II, as is observed in murine myotubes, and that PKR is involved.

## 4.4 Conclusion

The study confirmed the importance of PKR and eIF2 $\alpha$  phosphorylation in the depression of protein synthesis by both PIF and Ang II and provides a mechanism for activation of the ubiquitin-proteasome proteolytic pathway and increased muscle protein degradation, through activation of NF- $\kappa$ B, by a PKR-dependent process. This is strong evidence of a direct relationship between the depression of protein synthesis in vitro by PIF and Ang II, through activation of PKR, eIF2 $\alpha$  phosphorylation and enhanced degradation of the myofibrillar protein myosin, through activation of NF- $\kappa$ B, resulting in an increased expression and activity of the ubiquitin proteasome proteolytic pathway. PKR may also be upregulated in MAC16 tumours since a PKR inhibitor was effective in attenuating phosphorylation of PKR and expression of the 20S proteasome, and thus may be involved in tumour growth, since the PKR inhibitor also inhibited growth of MAC16 cells in vitro. This suggests that inhibition of PKR may be an effective step in combating skeletal muscle atrophy in cancer cachexia or other wasting conditions [59] and may be a useful therapeutic target against MAC16 tumour development, which would ultimately combat the pathogenesis of cachexia by targeting the source of the disease.



## Chapter 5

# Effect of PKR/eIF2 $\alpha$ Activation on Catabolic Processes In Vivo

### 5.1 Introduction

In both cachectic cancer patients and in vivo models of cachexia, such as mice bearing the MAC16 tumour, a specific atrophy of skeletal muscle mass is observed, which leads to asthenia, impairment of respiratory function and eventually death through hypostatic pneumonia [211]. Muscle growth is a major determinant of performance. Skeletal muscle (see diagram in Figure 5.1) is a unique tissue composed of bundles of myofibres with various contractile and metabolic properties bound together by collagen tissue. Each cell (fibre) consists of a membrane, many scattered nuclei lying under this membrane, and cytoplasm containing thousands of myofibrils. The number and size of the myofibers are the main determinants of muscle mass. Myofibrils consist of myosin and actin proteins that are arranged in sarcomeres. Contraction occurs when actin slides towards the centre of the myosin scaffold [125, 131].

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Figure 5.1: **The internal organization of a skeletal muscle fibre.** Actin forms the bulk of the thin filaments, while the myosin forms the thick filaments. Diagram modified from [117]

The bulk of muscle proteins (50-70%) exist in actomyosin complexes or myofibrils [55] and in different catabolic disorders, such as cancer cachexia, skeletal muscle is lost due to selective degradation of myosin, which is released from the muscle and degraded by the proteasome, while the actin content remains stable [3].

In the previous chapter (Chapter 4) both PIF and Ang II were demonstrated to activate PKR leading to eIF2 $\alpha$  phosphorylation and NF- $\kappa$ B mobilization in vitro, resulting in an inhibition of protein synthesis and an increase in protein degradation. Since C2C12 murine myotubes are a good representation of murine muscle it is likely that the mechanisms activated in vitro will mimic those seen in the MAC16 in vivo model as PIF and Ang II are believed to be the cachectic factors involved in inducing weight loss in MAC16

tumour bearing mice. To support this statement a study has implicated the phosphorylation of PKR and subsequent activation of eIF2 $\alpha$  in the cachexia syndrome seen in burned rats [91], while another study has shown the involvement of PKR and eIF2 $\alpha$  activation in myotonic dystrophy [184], which is characterised by delayed muscle maturation and skeletal muscle wasting. PKR is an interesting candidate because it is significantly expressed in skeletal muscle and activates some of the same components as PIF.

Few studies of PKR's role in cancer have been performed, and these reports generally imply that PKR demonstrates tumour suppressor activity since NIH-3T3 fibroblasts were transformed by dominant negative catalytically inactive PKR [100, 123, 14], while a decreased PKR expression and activity has been associated with a subset of human leukemias [19]. Also increased expression of PKR has been shown to correlate with better patient prognosis for certain tumour types [95]. More recent studies have shown that the role of PKR as a tumour suppressor remains enigmatic [20] because two lines of mouse PKR knockouts have been generated and characterised, neither demonstrating an increase in cancer incidence [213, 1]. Furthermore, mutational inactivation of PKR has not to date been reported in any human tumours [95]. PKR expression was frequently noted in intrahepatic cholangiocarcinoma and was correlated with good differentiation [183], while experiments examining cancers of the colon, breast, liver, head and neck have also shown a positive correlation between increasing PKR expression and more well-differentiated tumours, where PKR expression might even have tumour promoting properties. PKR autophosphorylation and the phosphorylation of its substrate eIF2 $\alpha$  were elevated in both breast and colon carcinoma cells when compared to surrounding non-transformed tissue. It has even been suggested that the high activity of PKR in these cancer cells may activate proto-oncogenes through such pathways that are required for the induction and sustenance of cell proliferation. However it is unknown as to whether an increased PKR activity is as a pro-neoplastic agent, or as a response to early changes of cancer formation. PKR, because of its ability to phosphorylate eIF2 $\alpha$ , is a potent inhibitor of protein synthesis; therefore it would seem likely that upregulation of PKR in tumour cells would lead to slow growth and tumour development. However the rate of protein synthesis of carcinoma cell lines were shown not to be affected by high PKR activity since levels of eIF2B were high enough to overcome the adverse effects on protein synthesis. This evidence provides examples of cancer cells in which PKR does not seem to function as a tumour suppressor. From this evidence it is likely that the function of PKR varies according to the type of cancer in question [94, 95].

In order to test the hypothesis that activation of PKR is responsible for the muscle atrophy seen in cancer cachexia the present study investigates the effect of a small molecule ATP-site directed inhibitor of PKR, 8-[1-(1H-imidazol-4-yl) meth-(Z)-ylidene]-6,8-dihydro-thiazolo[5,4-e]indol-7-one [84] on the development of cachexia in the MAC16 model, with particular reference to its effect on protein synthesis and degradation in skeletal muscle. The relative amounts of activated PKR and eIF2 $\alpha$ , as well as the expression and activity of proteasome subunits were examined, while the effect of the PKR inhibitor on the NF- $\kappa$ B transcriptional apparatus was determined. To further correlate the autophosphorylation of PKR with cancer cachexia the levels of phosphorylated PKR and eIF2 $\alpha$  in muscle excised from cachectic cancer patients were studied.

## 5.2 Results

The data shown in Chapter 4 demonstrate a link between autophosphorylation of PKR with a depression of protein synthesis and increase in protein degradation upon stimulation by catabolic agents PIF and Ang II *in vitro*. An increased protein degradation and reduced protein synthesis have been associated with the loss of muscle mass experienced by cancer patients with cachexia and other types of wasting diseases. Therefore we studied the effector components PKR, eIF2 $\alpha$  and NF- $\kappa$ B target genes, to deduce whether the mechanisms activated in response to PIF and Ang II in murine myotubes were also upregulated in mice bearing the cachexia inducing MAC16 tumour.

Slices of gastrocnemius muscle from mice bearing the MAC16 tumour, demonstrating increasing weight loss, were assayed for PKR and eIF2 $\alpha$  phosphorylation using the western blotting method (see Section 2.3.13 and 2.3.21 for method used).

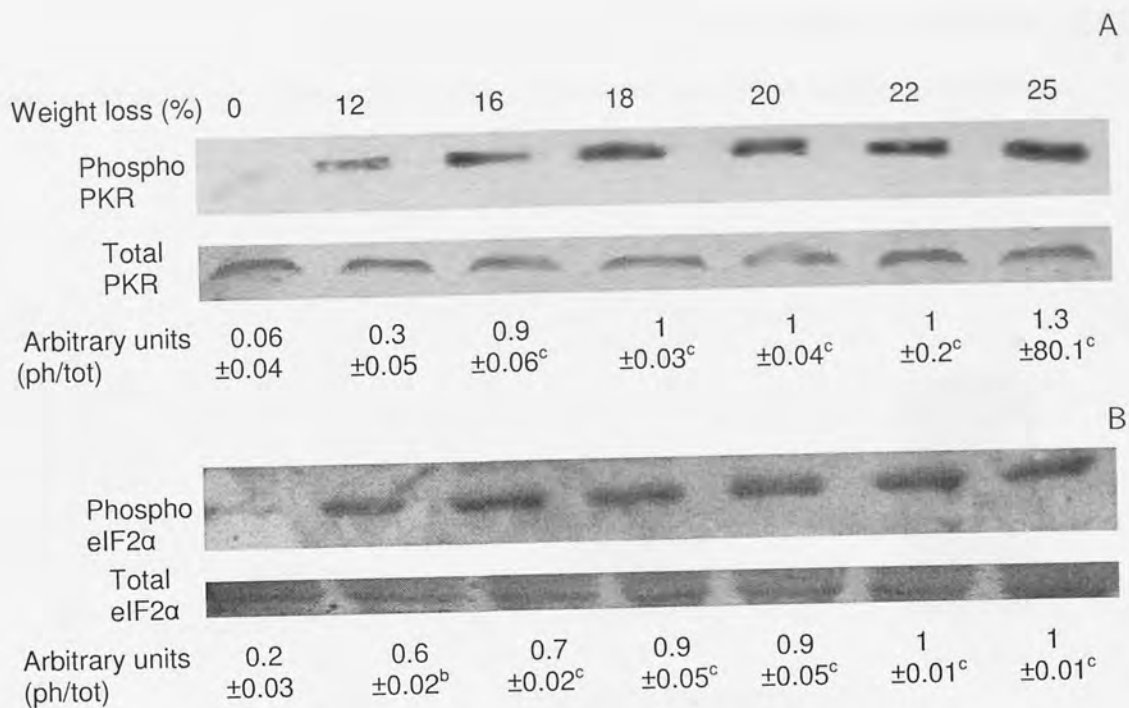


Figure 5.2: Western blot of phosphorylated PKR and eIF2 $\alpha$  in gastrocnemius muscle of mice bearing the cachexia inducing MAC16 tumour exhibiting different degrees of weight loss. Blots of phosphorylated PKR (A) and eIF2 $\alpha$  (B) were normalised against a blot of total PKR and eIF2 $\alpha$ . PKR and eIF2 $\alpha$  phosphorylation increased exponentially above 16 and 12% weight loss respectively. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from 0% weight loss control are shown as b:  $p < 0.01$ , c:  $p < 0.001$

Phosphorylation of PKR was significantly increased in the gastrocnemius muscle of weight losing mice bearing the MAC16 tumour, when weight loss exceeded 16% (Figure 5.2A). Activation of PKR was accompanied by a corresponding increase in eIF2 $\alpha$  phosphorylation (Figure 5.2B), which was significant above 12% weight loss. This suggests that the same events that were seen in murine myotubes in vitro are occurring in the skeletal muscle of cachectic mice and may be responsible for the depression of protein synthesis and increased protein degradation observed in the progression of cachexia.

To investigate whether PKR was involved in the pathogenesis of cachexia, mice implanted with the MAC16 tumour were administered a PKR inhibitor at two doses (1 and 5mg/kg<sup>-1</sup>) (see Section 2.3.1 and table 2.1 for procedure). The dose levels were chosen based on the effective dose in vitro (used in Chapter 4) and by using a dose range finding assay to determine toxicity. Protein synthesis (Section 2.3.16 for method) and

degradation (determined by release of tyrosine shown in Section 2.3.20) were measured in these animals. Change in body weight and tumour volume over the 5 day treatment period were calculated and body composition at the end of the treatment span was measured (see Section 2.3.3 for method used).

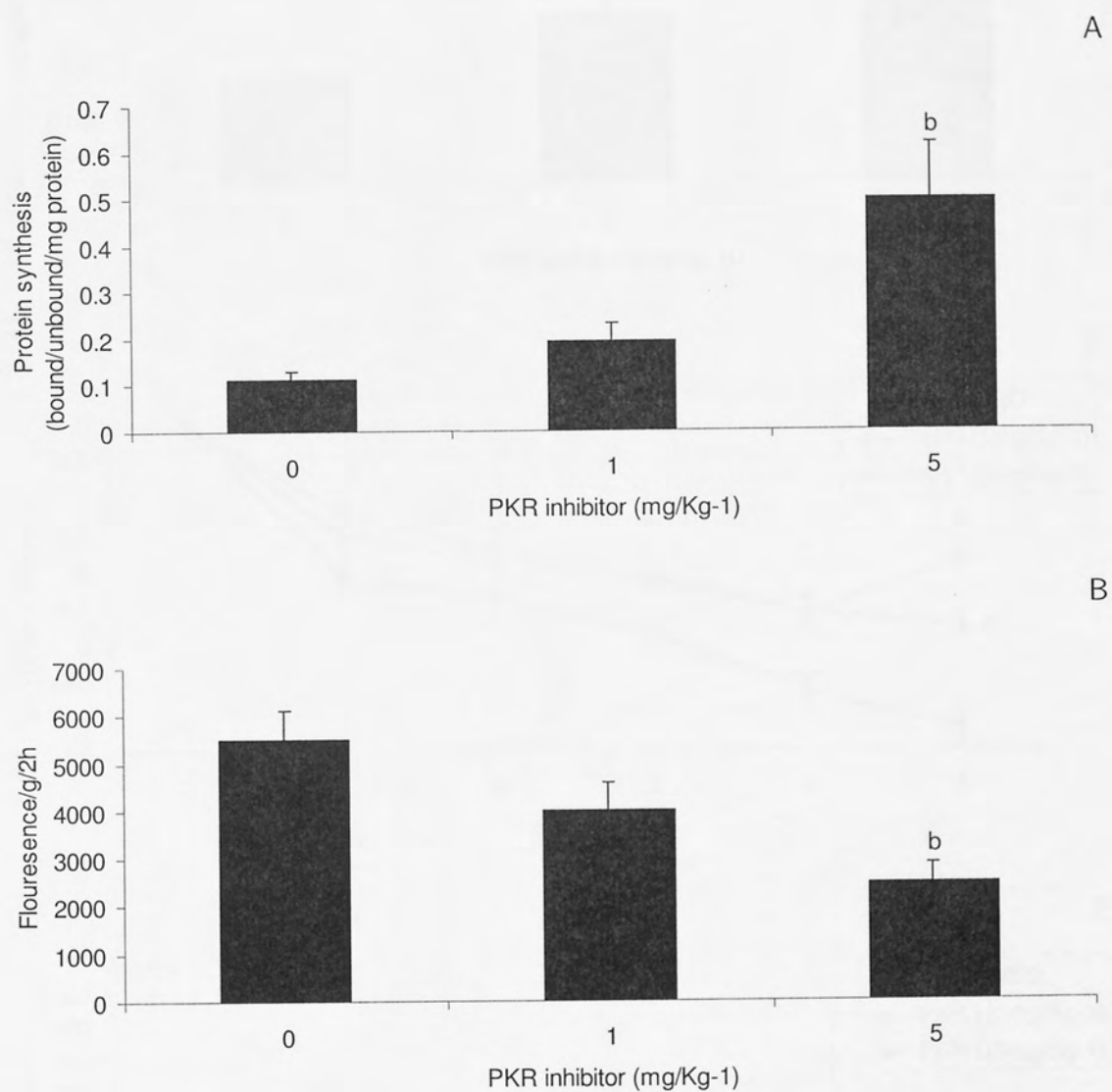
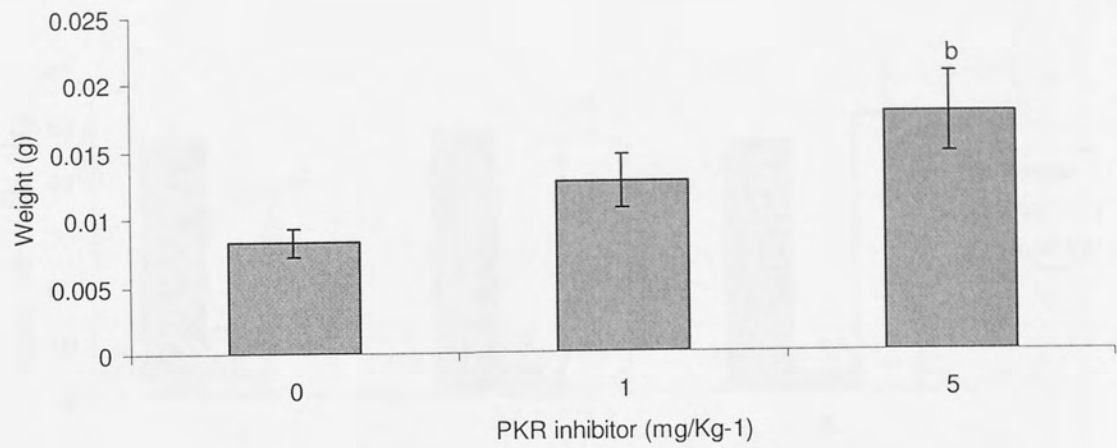
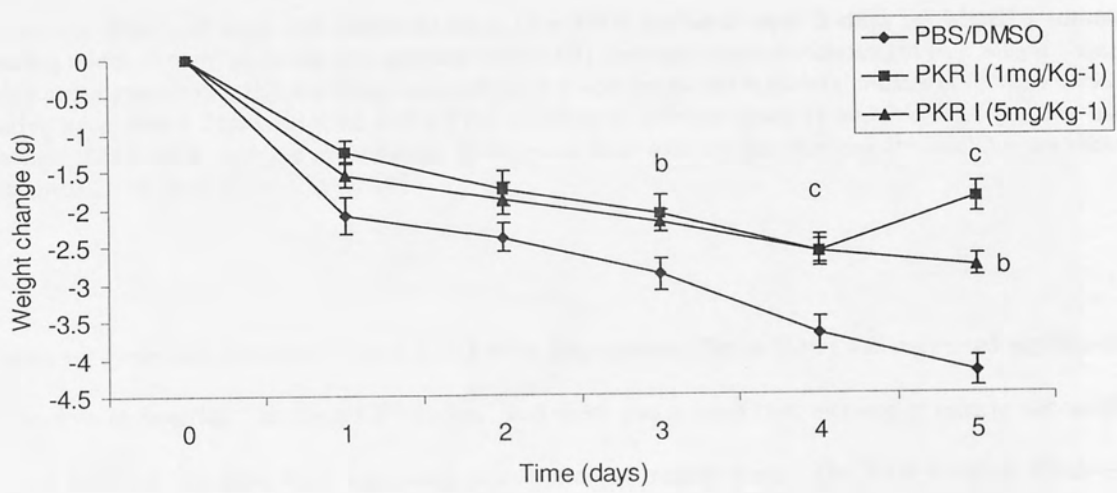


Figure 5.3

C



D



E

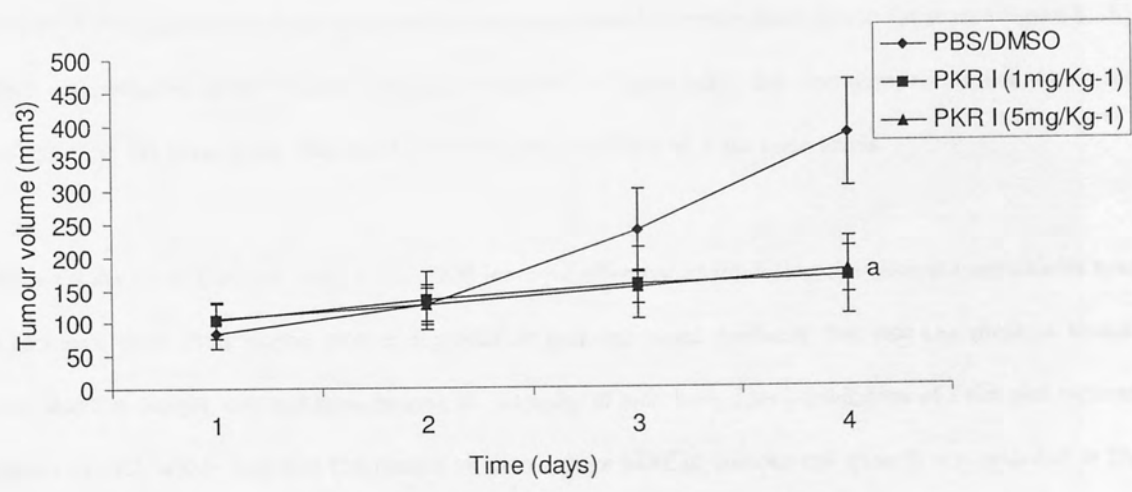


Figure 5.3



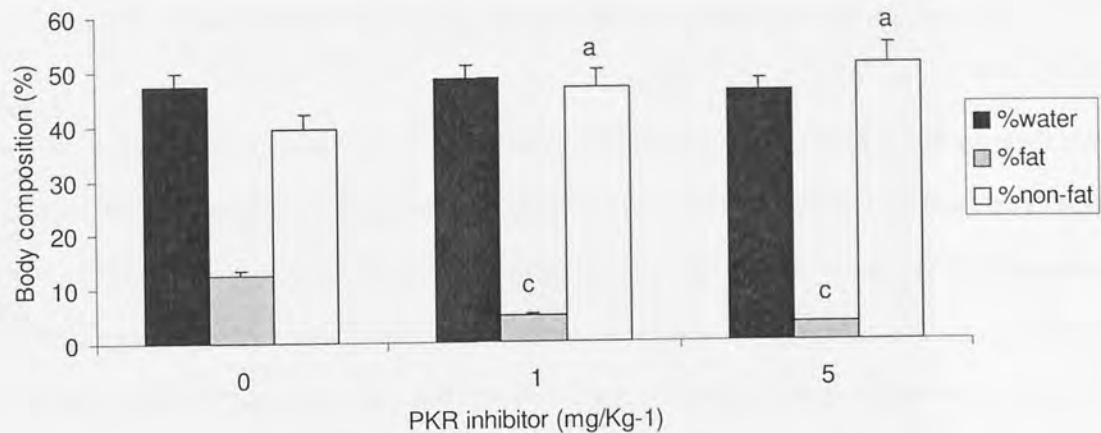


Figure 5.3: **Effect of daily s.c administration of a PKR inhibitor over 5 days on MAC16 tumour bearing mice.** Protein synthesis (A), tyrosine release (B), average soleus muscle weight (C), weight change (D), tumour growth rate (E) and body composition (F) were measured in skeletal muscle of MAC16 tumour bearing mice after 5 days treatment with a PKR inhibitor at different doses (1 and 5mg/kg<sup>-1</sup>). Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences from animals not receiving the inhibitor are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$

Protein synthesis was increased (Figure 5.3A) while degradation (Figure 5.3B) was decreased significantly at the doses of 5mg/kg<sup>-1</sup> of the PKR inhibitor, and there was a significant increase in muscle wet weight (Figure 5.3C) at this dose level suggesting preservation of muscle mass. The PKR inhibitor effectively attenuated both the decrease in body weight (Figure 5.3D) and tumour growth (Figure 5.3E) over the 5 day treatment administration. Body composition analysis showed a preservation of non-fat mass (Figure 5.3F), which was reflected in the muscle bulk gain observed in Figure 5.3C, but also demonstrated a significant depression in fat mass upon treatment with the PKR inhibitor at both dose levels.

These results show that not only is the PKR inhibitor effective at inhibiting the normal mechanisms seen in cachexia, such as increased protein degradation and decreased synthesis, but also can preserve muscle bulk, stabilize weight loss and even reverse the atrophy of lean body mass. Inhibition of PKR also reduced tumour growth which supports the results in vitro, where MAC16 tumour cell growth was retarded in the presence of the PKR inhibitor. This suggests that activation of PKR is involved in the pathogenesis of cachexia, probably by catabolic mediators such as PIF, cytokines or Ang II, and that tumour growth may

also be dependent on this process. This is supported by cell cycle studies conducted on PKR deficient cell lines that indicate that PKR is important for the normal passage of cells through the G1/S phase of the cell cycle [216]. These observations suggest a role for PKR as a positive growth regulator [94].

However, it is important to note that the depression of tumour growth may not be as a direct result of PKR inhibition. Protein breakdown is also reduced by treatment with the PKR inhibitor resulting in a diminished release of amino acids from skeletal muscle. As discussed in Section 1.1 the tumour has been suggested to constitute a metabolically active organ requiring its own sustenance and causing muscle protein breakdown to provide a source of nutrients. Thus the inhibitory effect on tumour growth may be through starvation of the tumour of essential nutritional components [159].

To determine whether the depression of protein degradation in MAC16 tumour bearing mice treated with the PKR inhibitor (Figure 5.3B) was as a result of an inhibition of proteasome activity and subunit expression, chymotrypsin like activity was measured (see Section 2.3.6 for method), 20S and P42 subunit expression was determined and myosin levels quantified using western blotting (see Section 2.3.13 and 2.3.21).

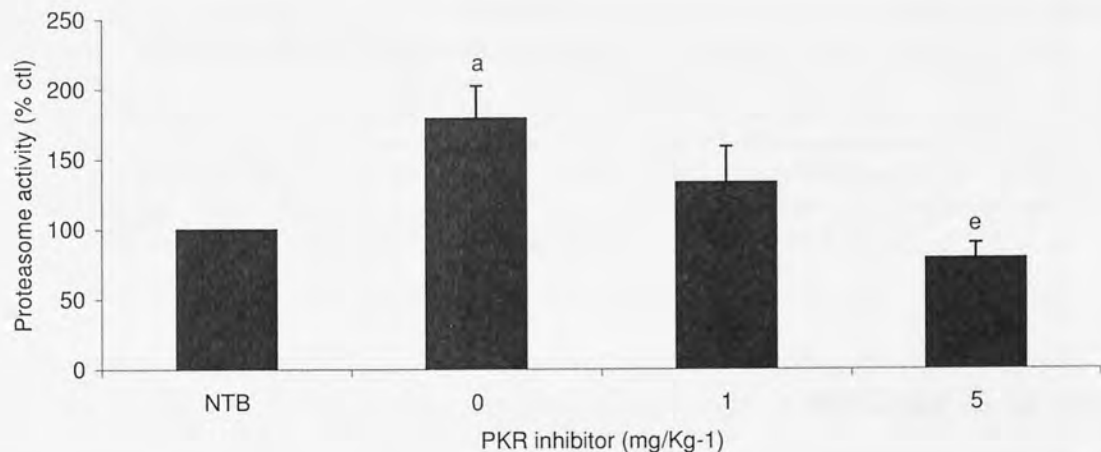


Figure 5.4: **Effect of a PKR inhibitor on proteasome activity in gastrocnemius of MAC16 tumour bearing mice.** The PKR inhibitor was administered daily by s.c injection over a period of 5 days at 1 and 5mg/kg<sup>-1</sup>, where only 5mg/kg<sup>-1</sup> was effective at reducing proteasome activity significantly. Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences from animals not receiving the inhibitor are shown as a:  $p < 0.05$ , while differences from non-tumour bearing (NTB) animals are shown as e:  $p < 0.01$

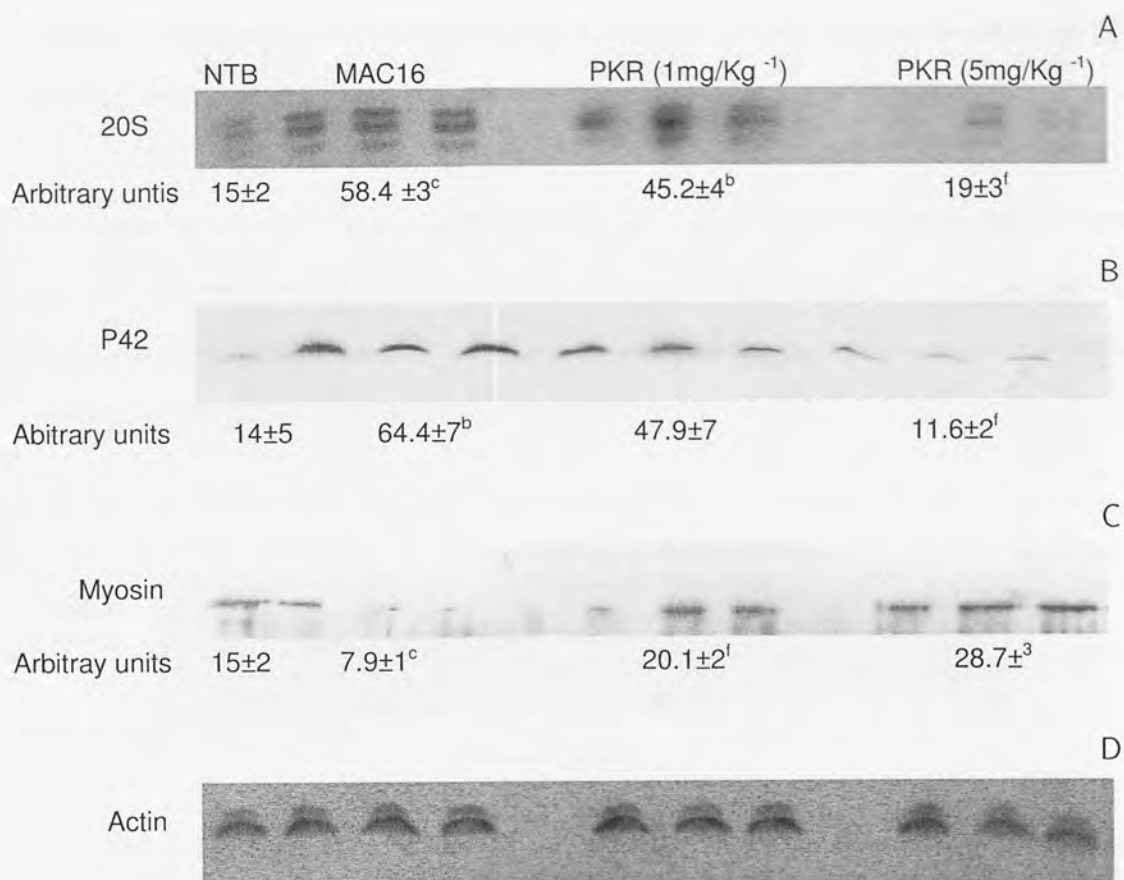


Figure 5.5: **Western blotting for proteasome subunits and myosin in gastrocnemius muscle of mice bearing the MAC16 tumour after treatment with the PKR inhibitor.** Western blots were performed to detect the  $\alpha$ -subunit of the 20S proteasome (A), P42 (B), myosin (C) and actin loading control (D) in gastrocnemius muscle of mice bearing the MAC16 (lanes 2-4) tumour after 5 days treatment with the PKR inhibitor (PKR I) 1mg/kg<sup>-1</sup> (lanes 5-7) and 5mg/kg<sup>-1</sup> (lanes 7-10), in comparison with non-tumour bearing controls (NTB) (lane 1). A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Differences from animals not receiving the inhibitor are shown as b:  $p < 0.01$ , c:  $p < 0.001$ , while differences from non-tumour bearing animals are shown as f:  $p < 0.001$ .

The functional activity of the 20S proteasome in gastrocnemius muscle of MAC16 mice was significantly reduced to levels comparable to non-tumour bearing animals when treated with 5mg/kg<sup>-1</sup> PKR inhibitor (Figure 5.4). This reflected the change observed in protein degradation in the same experimental animals (Figure 5.3B), and also that of the expression of proteasome 20S  $\alpha$ -subunit (Figure 5.5A) and P42 (Figure 5.5B) expression, which were also reduced to levels seen in non-tumour bearing controls at a dose level of 5mg/kg<sup>-1</sup> PKR inhibitor. The myofibrillar protein myosin was reduced in MAC16 tumour bearing mice. However, treatment with the PKR inhibitor restored the myosin content to levels not dissimilar to non-

tumour bearing controls. This effect was significant at both 1 and 5mg/kg<sup>-1</sup> PKR inhibitor. Pro-cachectic factors appear to have a high degree of specificity when it comes to myofibrillar protein downregulation [3], suggesting that PKR activity is directly involved in the catabolic mechanisms of cachectic factors.

To establish that the increase in proteasomal expression and activity in gastrocnemius of MAC16 mice was as a result of nuclear localisation of NF- $\kappa$ B, and whether the attenuation of this functional activity was through inhibition of PKR-induced NF- $\kappa$ B migration, an EMSA was performed to measure the DNA binding activity of NF- $\kappa$ B (see Methods Section 2.3.7).

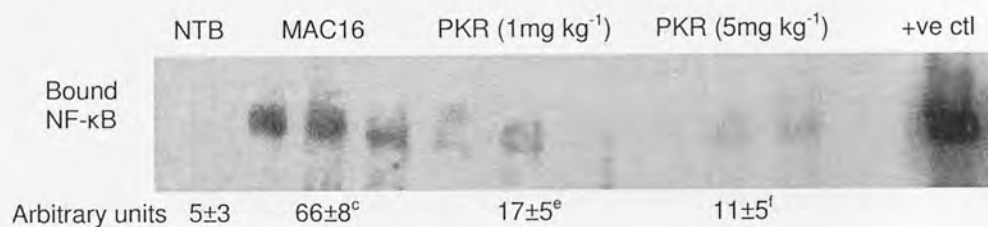


Figure 5.6: **DNA binding activity of NF- $\kappa$ B in gastrocnemius muscle of mice bearing the MAC16 tumour after treatment with a PKR inhibitor.** An EMSA was performed to determine DNA binding of NF- $\kappa$ B in gastrocnemius muscle of mice bearing the MAC16 (lanes 2-4) tumour after 5 days treatment with the PKR inhibitor (PKR I) 1mg/kg<sup>-1</sup> (lanes 5-7) and 5mg/kg<sup>-1</sup> (8-10), in comparison with non-tumour bearing controls (NTB) (lane 1). A positive control is shown in lane 11. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 2 replicate blots. Differences from animals not receiving the inhibitor are shown as c:  $p < 0.001$ , while differences from non-tumour bearing animals are shown as e:  $p < 0.01$ , f:  $p < 0.001$

There was a significant increase in DNA binding activity in skeletal muscle of MAC16 tumour bearing mice compared with non-tumour bearing control animals, which was attenuated by administration of the PKR inhibitor at both 1 and 5mg/kg<sup>-1</sup> to levels similar to control. These results suggest that inhibition of PKR autophosphorylation reduces protein degradation in MAC16 tumour bearing mice by attenuating NF- $\kappa$ B activation and subsequent induction of proteasome expression.

In the previous chapter (Chapter 4) inhibition of PKR and consequent eIF2 $\alpha$  phosphorylation was adequate in attenuating the depression of protein synthesis in response to the catabolic stimulators PIF and Ang II. To determine whether eIF2 $\alpha$  phosphorylation was associated with reduced protein synthesis in MAC16

tumour bearing mice, and whether PKR inhibition was effective at reducing this phosphorylation and restoring protein synthesis, the levels of autophosphorylated PKR and phospho eIF2 $\alpha$  were assayed in the same animals used in Figure 5.3, using the western blotting method (Method Section 2.3.13 and 2.3.21).

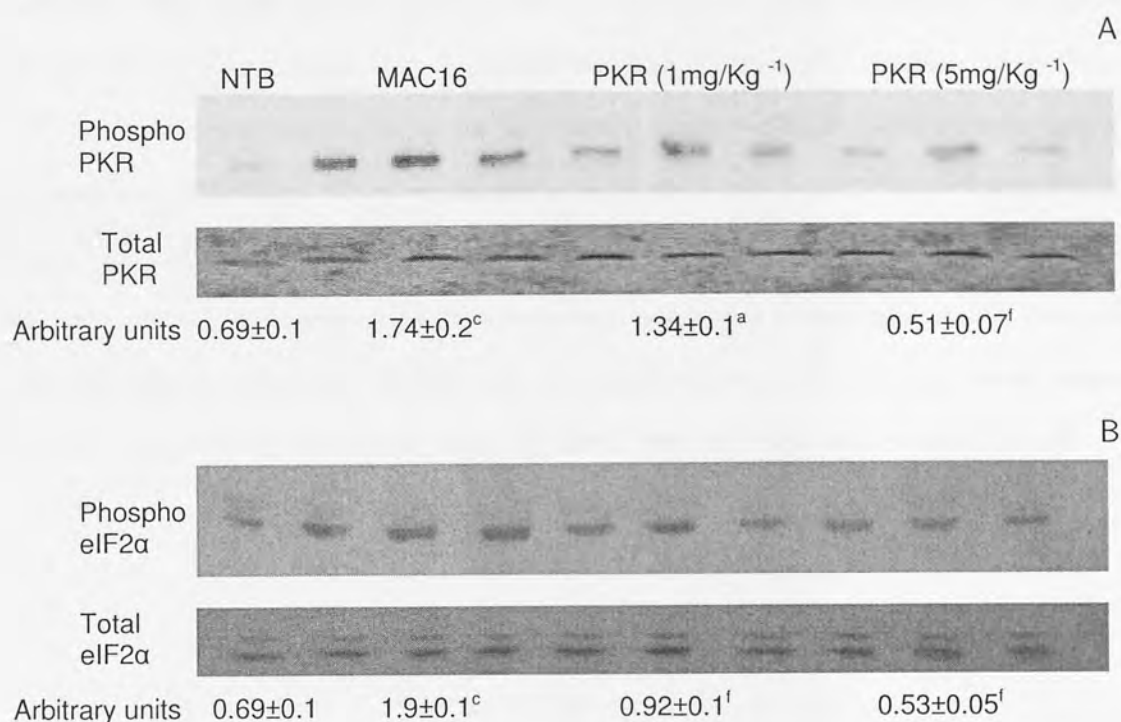


Figure 5.7: **Western blotting for the phosphorylated form of PKR and eIF2 $\alpha$  in gastrocnemius muscle of mice bearing the MAC16 tumour after treatment with a PKR inhibitor.** The phosphorylated forms of PKR (A) and eIF2 $\alpha$  (B) were analysed in gastrocnemius muscle of mice bearing the MAC16 tumour (lanes 2-4) after 5 days treatment with the PKR inhibitor (PKR I) 1mg/kg<sup>-1</sup> (lanes 5-7) and 5mg/kg<sup>-1</sup> (lanes 8-10), in comparison with non-tumour bearing controls (NTB) (lane 1). Blots of total PKR and eIF2 $\alpha$  were used to normalize the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate blots. Differences from animals not receiving the inhibitor are shown as b:  $p < 0.01$ , c:  $p < 0.001$ , while differences from non-tumour bearing animals are shown as f:  $p < 0.001$

Both PKR (Figure 5.7A) and eIF2 $\alpha$  (Figure 5.7B) phosphorylation were increased in the muscle of MAC16 tumour bearing mice compared to the non-tumour bearing control. Treatment with the PKR inhibitor reduced this effect maximally at 5mg/kg<sup>-1</sup> where levels of these phosphorylated proteins was comparable to that seen in non-tumour bearing control animals. The changes in PKR and eIF2 $\alpha$  phosphorylation paralleled the decrease in protein synthesis suggesting that phosphorylation of the  $\alpha$ -subunit of eIF2 is responsible for decreasing protein synthesis in skeletal muscle of mice bearing the cachexia inducing MAC16 tumour,

and that inhibition of PKR autophosphorylation may be a useful target in attenuating muscle atrophy in cancer cachexia.

The previous results suggest that the activation of PKR can stimulate protein degradation, and depress protein synthesis in skeletal muscle through phosphorylation of the translation initiator eIF2 on the  $\alpha$ -subunit. To assess whether these mediators are important in muscle wasting in cancer patients, levels of the phospho forms of PKR and eIF2 $\alpha$  were assayed in rectus abdominus muscle of weight losing patients with oesophago-gastric cancer by western blotting (described in Methods Section 2.3.13 and 2.3.21). The muscle content of the myofibrillar protein myosin was also investigated to determine whether there was a relationship between changes in PKR and eIF2 $\alpha$  phosphorylation and muscle atrophy. Muscle biopsies from healthy subjects undergoing elective surgery for hernia served as weight stable healthy controls.



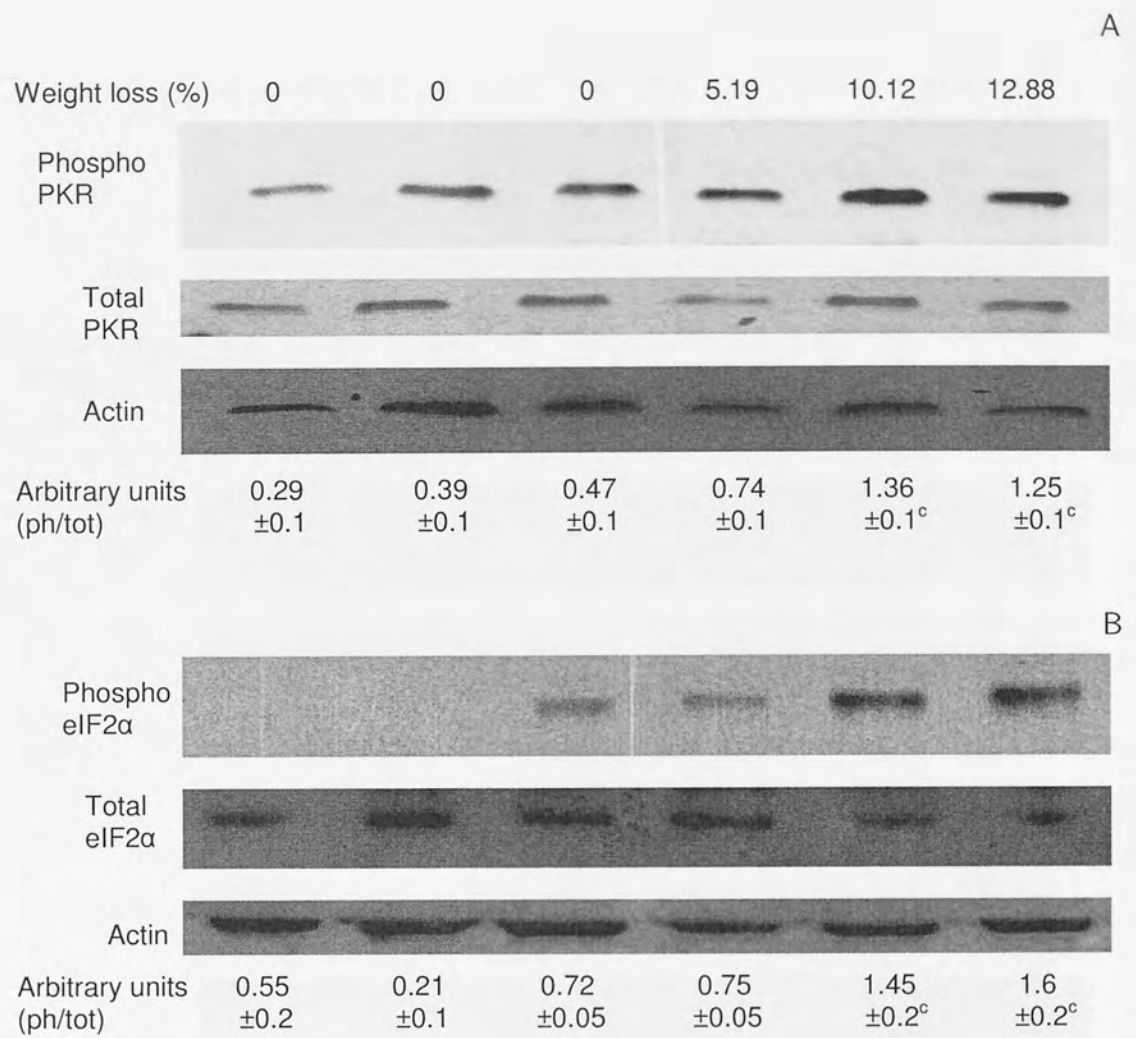


Figure 5.8: Western blots of phospho PKR and eIF2α in comparison with total PKR and eIF2α in rectus abdominus muscle. Phospho PKR (A) and eIF2α (B) in comparison with total PKR and eIF2α were analysed in rectus abdominus muscle as a function of weight loss. Actin was used as a loading control. Each lane represents muscle from an individual patient. A densitometric analysis is shown underneath where ± represents the SEM of 3 replicate blots. Differences from healthy controls (0% weight loss) are shown as c: p<0.001



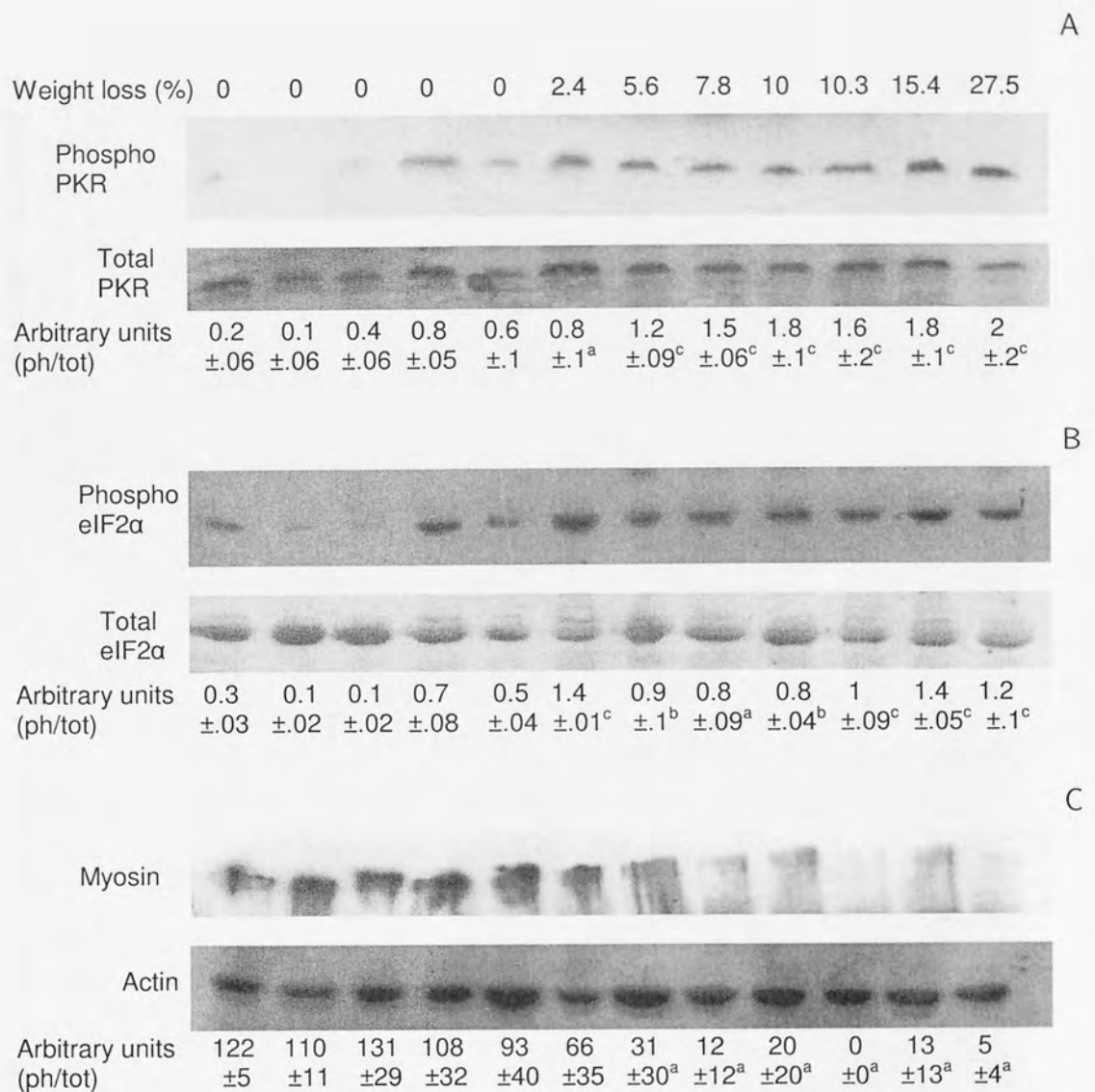


Figure 5.9: **Western blots of phospho PKR and eIF2α and myosin in comparison with total PKR and eIF2α in rectus abdominus muscle.** Phospho PKR (A) and eIF2α (B) and myosin (C) in comparison with total PKR and eIF2α were analysed in rectus abdominus muscle as a function of weight loss. Actin was used as a loading control. Each lane represents muscle from an individual patient. A densitometric analysis is shown underneath where ± represents the SEM of 3 replicate blots. Differences from healthy controls are shown as a: p<0.05, b: p<0.01 or c: p<0.001

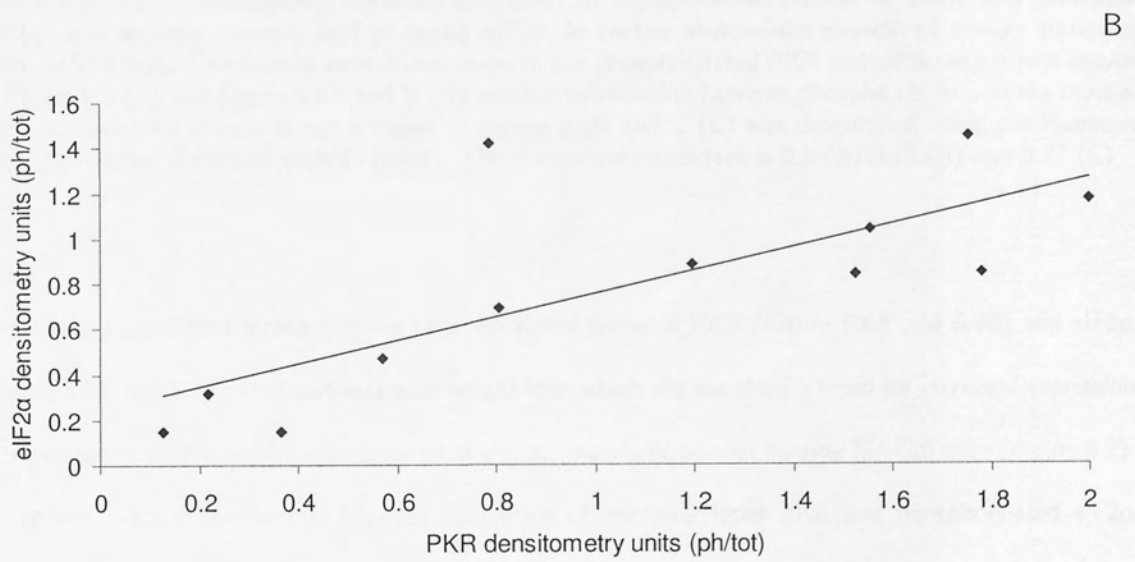
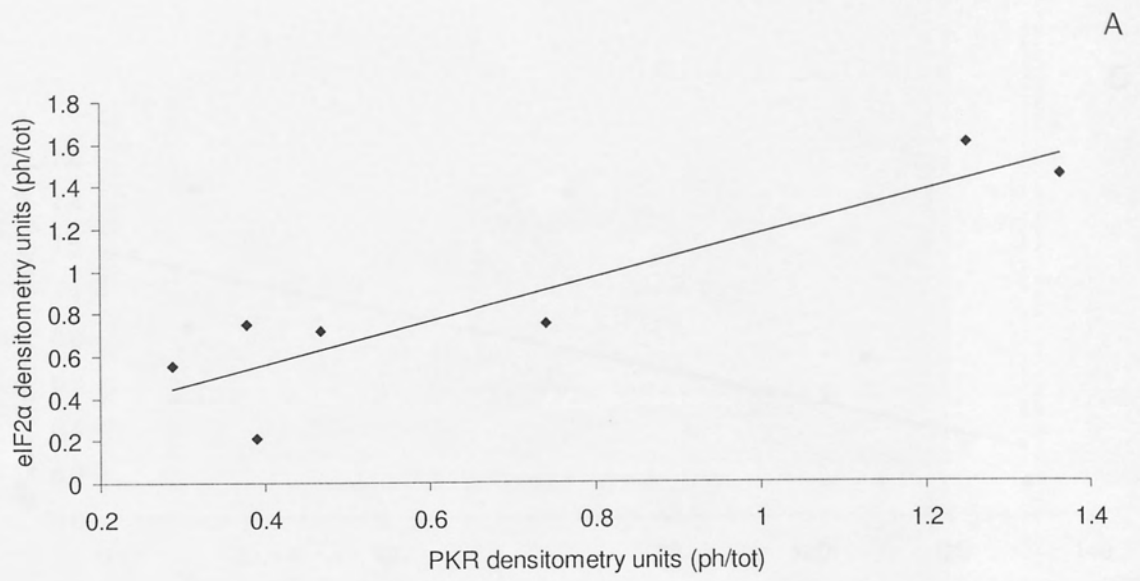


Figure 5.10

C

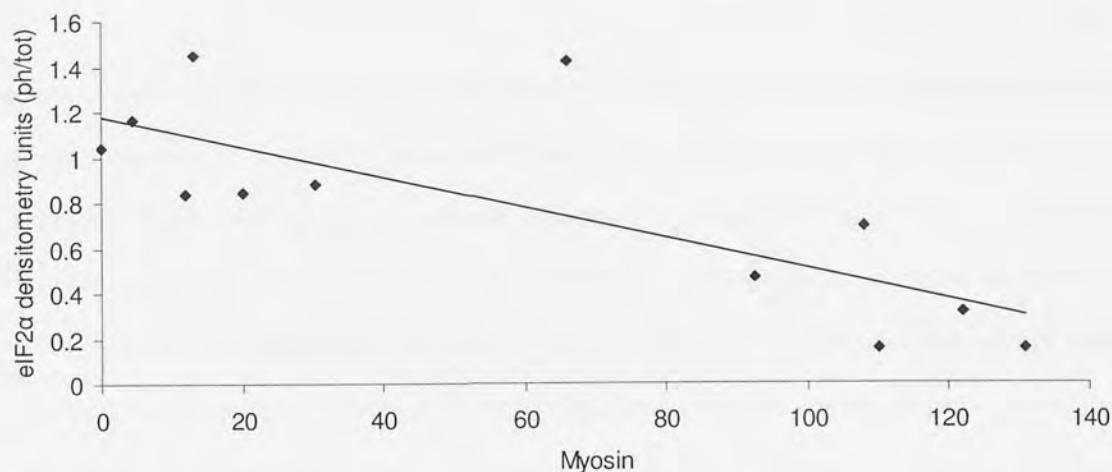


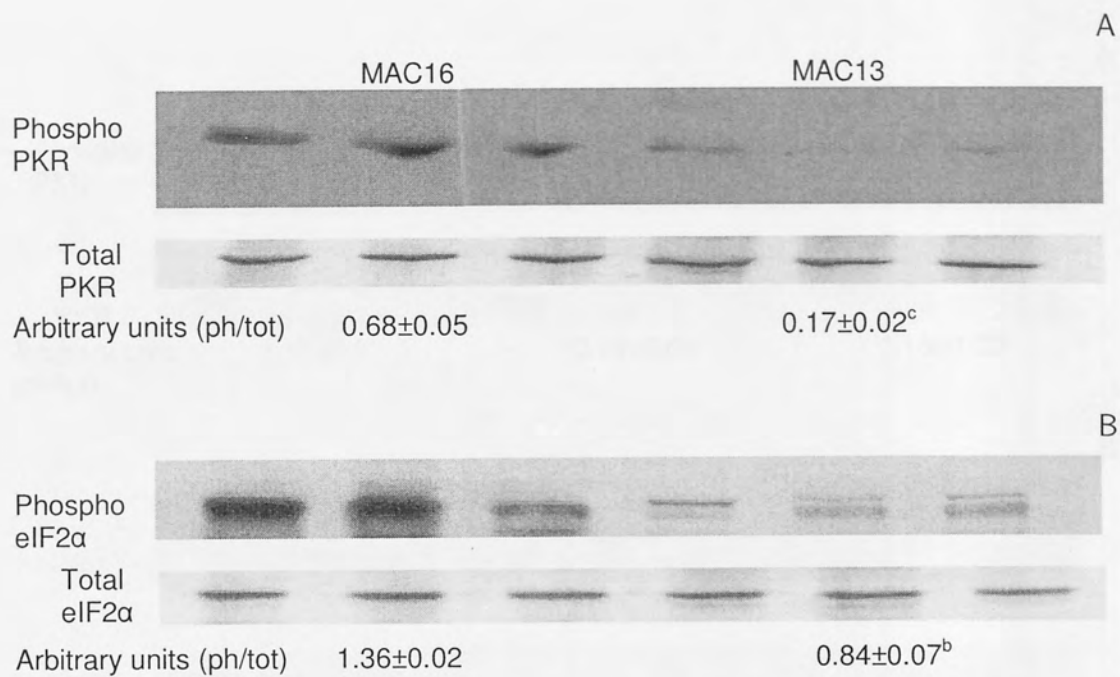
Figure 5.10: **The relationship between the level of autophosphorylation of PKR and phospho eIF2 $\alpha$ , and myosin content and phospho eIF2 $\alpha$  in rectus abdominus muscle of cancer patients with weight loss.** Correlations were drawn between the phosphorylated PKR and eIF2 $\alpha$  in patients shown in Figure 5.8 (A) and Figure 5.9A and B (B) and the relationship between phospho eIF2 $\alpha$  and the myosin content in skeletal muscle samples shown in Figure 5.9B and C (C) was determined using the Pearson's linear correlation statistical analysis ( $n=3$ ). The correlation coefficient is 0.9 (A) 0.75 (B) and 0.77 (C)

There was a significant increase in the phosphorylated forms of PKR (Figure 5.8A and 5.9B) and eIF2 $\alpha$  (Figure 5.8B and 5.9B) in all patients with weight loss, which did not show a trend for increased expression with increasing weight loss as was observed in skeletal muscle of tumour bearing MAC16 mice (Figure 5.2). There was a linear relationship between expression of phosphorylated PKR and phosphorylated eIF2 $\alpha$  (correlation coefficient 0.9 Figure 5.10A and 0.76 Figure 5.10B), suggesting that phosphorylation of eIF2 on the  $\alpha$ -subunit arose from autophosphorylation of PKR.

The increase in eIF2 $\alpha$  expression observed in human skeletal muscle, correlated inversely with levels of the myofibrillar protein myosin (Figure 5.10C, correlation coefficient 0.77), which decreased as weight loss increased (Figure 5.9). As previously reported [3] in skeletal muscle of mice bearing a cachexia-inducing tumour (C-26), myosin levels decreased, while actin levels remained constant. This has been attributed to specific targeting of myosin by the ubiquitin-proteasome pathway. This evidence suggests that PKR and

eIF2 $\alpha$  phosphorylation are involved in the muscle atrophy commonly observed in cachectic cancer patients. This suggests that muscle atrophy in cachectic cancer patients may also be responsive to inhibitors of PKR.

Previously in this chapter the growth rate of the MAC16 tumour in mice was reduced when the mice were administered a PKR inhibitor at two dose levels 1 and 5mg/kg<sup>-1</sup> over 5 days (Figure 5.3E). This substantiated results from the previous chapter (Chapter 4, Section 4.2, Figure 4.22), where MAC16 tumour cell growth rate was also reduced in the presence of the PKR inhibitor maximally at 200nM. To determine whether PKR and eIF2 $\alpha$  phosphorylation were upregulated in MAC16 tumours in vivo compared to histologically similar but non-cachexia inducing MAC13 tumours, western blot analysis was employed on both tumour types to measure levels of phospho PKR and eIF2 $\alpha$  (method described in Section 2.3.13 and 2.3.21). Since the PKR inhibitor was effective at attenuating PKR autophosphorylation in tumour cells in vitro, the phosphorylation state of PKR and eIF2 $\alpha$  were also examined in MAC16 tumours from the same mice treated with the PKR inhibitor as shown in Figures 5.3 to 5.7.



**Figure 5.11: Western blotting for phosphorylated PKR and eIF2 $\alpha$  in MAC16 and MAC13 tumours.** The expression of phospho and total forms of PKR (A) and eIF2 $\alpha$  (B) were analysed in MAC16 (lanes 1-3) and MAC13 (lanes 4-6) tumours. Blots of total PKR and eIF2 $\alpha$  were used to normalize the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the average SEM of triplicate bands on 3 replicate blots. Differences from the MAC16 tumour are shown as b:  $p < 0.01$ , c:  $p < 0.001$

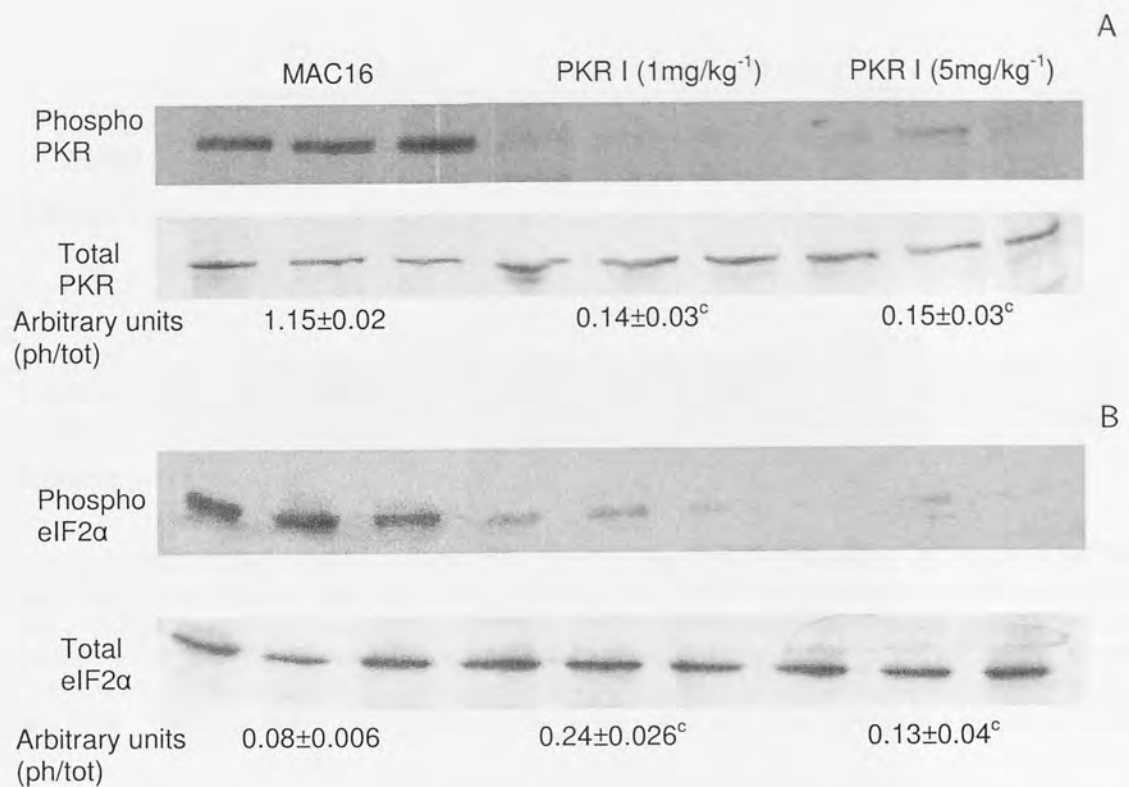


Figure 5.12: **Western blotting for phosphorylated PKR and eIF2 $\alpha$  in MAC16 tumours from NMRI mice.** The expression of phospho forms of PKR (A) and eIF2 $\alpha$  (B) were analysed in the MAC16 tumours from NMRI mice (lanes 1-3) administered the PKR inhibitor (PKR I) 1mg/kg<sup>-1</sup> (lanes 4-6) and 5mg/kg<sup>-1</sup> (lanes 7-9) daily by s.c. injection. Blots of total PKR and eIF2 $\alpha$  were used to normalize the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the average SEM of triplicate bands on 3 replicate blots. Differences from the control are shown as c:  $p < 0.001$ .

Elevated levels of both phospho PKR (Figure 5.11A) and eIF2 $\alpha$  (Figure 5.11B) were observed in the MAC16 tumour, but not in the MAC13 tumour. Treatment of mice bearing the MAC16 tumour with the PKR inhibitor caused a complete reduction of the elevated phosphorylation of both PKR (Figure 5.12A) and the  $\alpha$ -subunit of eIF2 (Figure 5.12B), irrespective of total levels of PKR and eIF2 $\alpha$ .

As mentioned in Chapter 4, Section 4.3 one of the functions of PKR is that it is capable of stimulating nuclear migration of NF- $\kappa$ B [217]. Thus a link between PKR and tumour growth may involve induction of NF- $\kappa$ B migration. Therefore to investigate this association an EMSA was performed to establish the DNA binding activity of NF- $\kappa$ B (see Methods Section 2.3.7 for procedure).



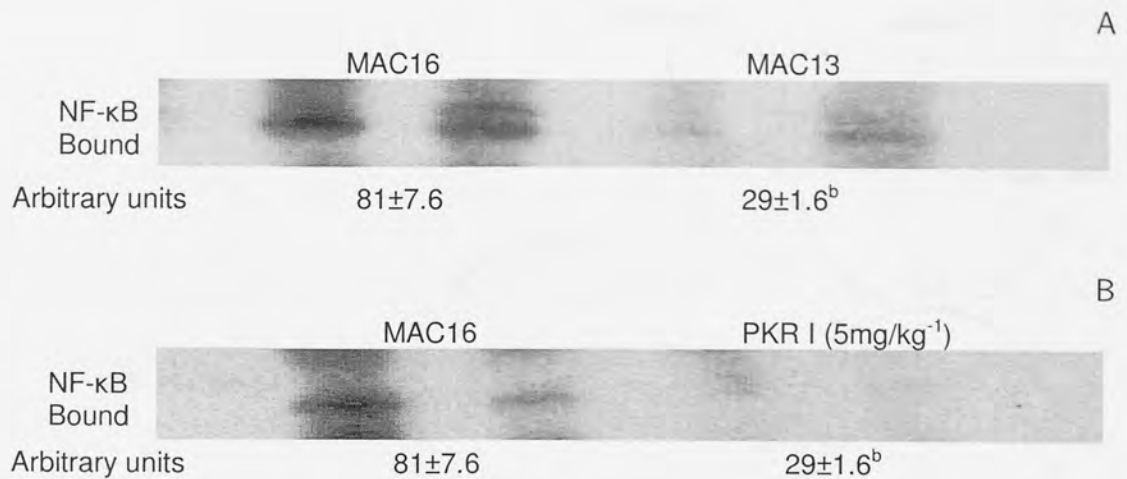


Figure 5.13: **DNA binding activity of NF-κB in MAC16 and MAC13 tumours and in the MAC16 tumour from mice treated with the PKR inhibitor.** An EMSA was performed to determine DNA binding of NF-κB in MAC16 (lanes 1-2) and MAC13 (lane 3-4) tumours (A) and in the MAC16 tumour from mice treated with the PKR inhibitor at 5mg/kg<sup>-1</sup> (lanes 3-4) for 4 days or solvent control (lanes 1-2) (B) as determined by EMSA. A densitometric analysis is shown underneath where ± represents the average SEM of duplicate bands on 3 replicate blots. Differences from the control are shown as b: p<0.01

High levels of NF-κB DNA binding activity in the MAC16 tumour was seen when compared to the MAC13 tumour. Treatment of mice bearing the MAC16 tumour with the PKR inhibitor attenuated constitutive activation of NF-κB in the tumour, suggesting that this activation is a result of PKR autophosphorylation.

The data produced above demonstrates that PKR autophosphorylation is involved in the pathogenesis of cachexia in MAC16 tumour bearing mice and identifies the possible role for PKR in the induction of muscle wasting and weight loss in cancer patients. Figure 5.14 is a representation of the proposed signalling events leading to skeletal muscle atrophy in cancer cachexia in response to the tumour specific catabolic factor PIF.

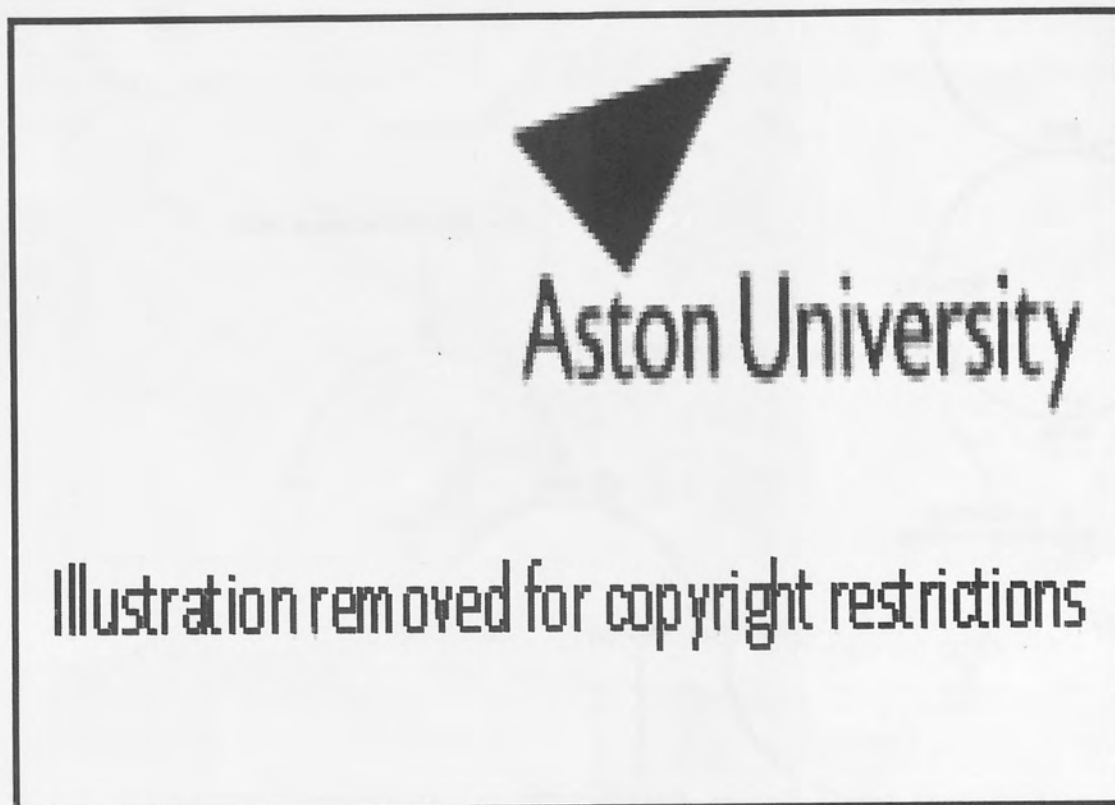


Figure 5.14: Summary of pathways leading to a depression in protein synthesis and an increase in protein degradation in skeletal muscle through activation of PKR. Kindly donated by Professor Mike Tisdale.

These results confirm the conclusions drawn from the previous chapter (Chapter 4) in that inhibitors of PKR may be useful therapeutic agents against muscle wasting and growth of tumours showing increased expression of PKR and constitutive activation of NF- $\kappa$ B.

## 5.3 Discussion

Previous chapters (Chapter 3 and 4) have demonstrated that the catabolic mediators PIF and Ang II are capable of inducing a depression in protein synthesis and elevation of protein degradation in C2C12 murine myotubes. Chapter 4 showed clear evidence that PKR mediated both pathways by increasing eIF2 $\alpha$  phosphorylation, to inhibit translation initiation and thus reduce overall protein synthesis, and by stimulating the DNA binding activity of NF- $\kappa$ B and subsequent upregulation of the ubiquitin proteasome pathway, resulting in an increased protein degradation. Inhibition of PKR using a low molecular weight inhibitor attenuated both the phosphorylation of the  $\alpha$ -subunit of eIF2, NF- $\kappa$ B nuclear migration and increased 20S proteasome activity and expression, which was paralleled by a reversal of the catabolic effects mediated by both PIF and Ang II.

Atrophy in skeletal muscle is an important prognostic factor in the overall survival of patients with cancer. Muscle wasting results in fatigue and a poor response to therapy. An attenuation of the underlying catabolism would improve and prolong patients survival and increase their susceptibility to treatment, increasing their recovery potential. Cachexia inducing murine and human tumours have been proven to produce PIF, which initiates catabolism of muscle protein both in vitro and in vivo through an increased protein degradation and decreased protein synthesis [211]. Since in vitro studies (Chapter 4) have determined that PIF stimulates PKR autophosphorylation to bring about catabolic processes, it is likely that this same process is occurring in vivo in response to PIF produced by the tumour. This was established in gastrocnemius muscle of mice bearing the MAC16 cachexia inducing tumour where a progressive increase in both PKR and eIF2 $\alpha$  phosphorylation at weight losses greater than 12% were observed. In this model there was a constitutive activation of PKR and phosphorylation of eIF2 $\alpha$ , which increased with rising weight loss, suggesting a constant stimulation, probably due to continuous tumour release of PIF, or stimulation of endogenous Ang II production. Therefore, if these results are taken into consideration it suggests that inhibition of PKR autophosphorylation may be a useful treatment to attenuate muscle atrophy in cancer patients, particularly for those with weight losses exceeding 12%. Other evidence shows that PKR is a downstream effector of other catabolic stimuli, such as the cytokines, like TNF $\alpha$  [86], which are believed to

be responsible for muscle wasting in disease states such as AIDS, sepsis [22] and rheumatoid arthritis [70]. Thus inhibition of PKR may also attenuate muscle loss in other cachexia presenting diseases.

To test this hypothesis the effect of a PKR inhibitor on muscle atrophy and wasting was investigated when administered to MAC16 tumour bearing cachectic mice. The results observed were promising, where muscle wet weight was conserved, probably through preservation of lean mass, although weight gain was not as pronounced as one would expect, because of a significant depression of carcass fat mass. Accordingly, activation of PKR has been shown to signal growth arrest of mouse fibroblasts and subsequent differentiation to adipocytes [136], thus loss of fat mass may be explained by a downregulation of this process by the PKR inhibitor. Furthermore, the regulation of fat metabolism can be controlled by mTOR and p70S6 kinase, where p70S6K mutant mice display reduced adipose tissue and decreased fat accumulation [209]. PKR has the potential to activate MK2 by a P38 MAPK mechanism [97], which can affect mTOR activity, demonstrating another plausible process for the reduced carcass fat mass observed upon the administration of a PKR inhibitor to MAC16 tumour bearing mice. However this is unlikely to occur as mTOR/p70S6K activity was diminished in muscle of MAC16 tumour bearing, weight losing mice (Chapter 6), while PKR phosphorylation was high (Figure 5.2A), suggesting that the two signalling pathways are not linked. However, it may be useful to determine whether the same events occur in adipose tissue of mice bearing the MAC16 tumour.

The stabilization of muscle weight was determined as a result of increased protein synthesis and decreased protein degradation related to the reduced PKR and eIF2 $\alpha$  phosphorylation, which was paralleled by a reduction in the ATP dependent ubiquitin proteasome subunit expression and activity. Activation of PKR and eIF2 $\alpha$  phosphorylation have both been deemed essential for mediating catabolic stimuli, via NF- $\kappa$ B, leading to apoptosis and for reducing protein synthesis in viral infected cells [170]. Thus it is likely that the activation of PKR and consequent phosphorylation of eIF2 $\alpha$  and NF- $\kappa$ B release is the core element regulating catabolic processes in skeletal muscle wasting of cachectic MAC16 tumour bearing mice in response to cachectic inducers such as PIF, Ang II and cytokines.

These same catabolic mediators have also been implicated in the pathogenesis of cachexia in human cancer

patients [189]. To determine the potential for PKR inhibition in combating cachexia in human wasting diseases, the phosphorylation state of PKR and the  $\alpha$ -subunit of eIF2 was assessed in rectus abdominus muscle from weight losing cancer patients. There was a parabolic relationship between levels of phospho PKR and eIF2 $\alpha$  and weight loss suggesting that phosphorylation of PKR led to phosphorylation of eIF2 $\alpha$ . There was a significant increase in the phosphorylated forms in all patients with weight loss while cancer patients with no weight loss, or weight gain showed the same low expression of phosphorylated PKR and eIF2 $\alpha$ , as non-weight losing healthy subjects. The levels of the myofibrillar protein myosin in skeletal muscle of cancer patients decreased as weight loss increased, which showed a linear relationship between myosin expression and the extent of phosphorylation of eIF2 $\alpha$ , suggesting that PKR and eIF2 $\alpha$  are important components of muscle depletion in cachectic cancer patients, probably via catabolic mediators such as PIF. Activation of PKR by PIF may be responsible for its ability to induce apoptosis in murine myotubes [164]. Increased apoptosis has also been observed in the skeletal muscle of rats bearing the cachexia-inducing Yoshida AH-130 ascites hepatoma [151] and in the early stage of weight loss in rabbits bearing the VX2 carcinoma [214], while new studies in humans confirm that apoptosis is present in human subjects undergoing cancer cachexia [34]. Thus activation of PKR might be expected to lead to increased apoptosis in the skeletal muscle of weight-losing cancer patients contributing to muscle atrophy. Phosphorylation of eIF2 $\alpha$  has also been shown to be responsible for the inhibition of protein synthesis in rat liver by vasopressin [96] and rat skeletal muscle by IL-1 [46]. However other causes of PKR and eIF2 $\alpha$  phosphorylation should be considered.

For example, most cancer patients are immobilized on hospital wards where infection by a host of diseases is a constant dilemma. To compound the problem many cancer patients are immunosuppressed due to their illness, chemotherapy and poor nutrition thus making them susceptible to contagion. Activation of PKR plays a central role in cytokine mediated antiviral response [20] and thus would be upregulated in many cancer patients due to hospital borne infections. For example, increases in viral envelope glycoprotein or endogenous long-terminal repeat sequence [126] might be responsible for the promotion of PKR autophosphorylation, directly through its dsRNA binding domain, or indirectly via the production of cytokines such as TNF $\alpha$  or IFN- $\gamma$ . Ceramide is an upstream mediator of the cellular activator of PKR (PACT). Diverse stimuli including chemotherapeutic drug treatment and irradiation promote the genera-



tion of ceramide [157], providing another possible stimulator of PKR phosphorylation. Accompanying the cachexia, cancer patients also have a nutrient deficit as a result of a combination of problems including anorexia, reduction in food intake due to factors such as physical obstruction of the gastrointestinal tract, vomiting and malabsorption associated with the cancer itself and the side effects of treatment [64]. During nutrient starvation cells need a strategy to synthesize essential proteins in the face of a limited nutrient supply. In nutrient limiting conditions, eukaryotic cells simultaneously decrease global rates of protein synthesis, and increase rates of protein degradation by an autophagic pathway, which can be activated via the phosphorylation of the  $\alpha$ -subunit of eIF2 by eIF2 $\alpha$  kinases. In yeast there is only one known kinase, GCN2, which phosphorylates eIF2 $\alpha$  in response to starvation. However PKR has also been observed to regulate autophagy in mammalian cells through its eIF2 $\alpha$  kinase activity. Furthermore, viral infection has also been shown to activate the autophagic pathway through autophosphorylation of PKR and eIF2 $\alpha$  phosphorylation [177].

A surprising observation was that PKR inhibition could affect tumour growth. Addition of the PKR inhibitor to cachexia inducing MAC16 tumour cells *in vitro* reduced their growth rate significantly compared to the MAC13 cells, which are histologically similar but do not induce cachexia when implanted into animals (Chapter 4). Similar results were seen *in vivo*, where MAC16 tumour bearing mice administered the PKR inhibitor demonstrated an inhibition of tumour growth. To determine whether this effect was as a result of alterations in PKR activity, the expression of PKR and its downstream effectors eIF2 $\alpha$  and NF- $\kappa$ B were examined in MAC16 tumours treated with the PKR inhibitor and in MAC13 tumours.

There was a constitutive activation of PKR, NF- $\kappa$ B and eIF2 $\alpha$  phosphorylation in the MAC16 tumours only and this was attenuated by the PKR inhibitor, suggesting that reduction in tumour cell growth arose from attenuation of PKR autophosphorylation and subsequent inhibition of NF- $\kappa$ B DNA binding activity and phosphorylation of the eIF2  $\alpha$ -subunit. The inhibition of tumour growth was unlikely to be responsible for the changes in protein synthesis and degradation in the mouse skeletal muscle however, since it occurred after the stabilisation of body weight.

It has been suggested that PKR is used as a signal transducer for NF- $\kappa$ B and IRF-1 dependent genes such

as ICAM, VCAM, E-selectin and INOS [102]. PIF has also been demonstrated to induce the expression of adhesion molecules ICAM and VCAM in vitro and thus may be utilising PKR as a mediator. Furthermore interference by PIF with adhesion molecules may interfere with syndecan expression, which is suggested to contribute to the pathogenesis/metastasis of tumours [204]. This positively correlates PKR activation by PIF to tumour cell proliferation. Additionally, if the rate of cell growth is unable to keep up with cell division, then cell proliferation cannot be sustained resulting in inevitable cell death. Thus inhibition of protein synthesis, which would occur in response to PKR activation, may have the effect of slowing cell growth and cell cycle progression to maintain cell mass and size between divisions and prevent cell death [65]. Finally, eIF2 $\alpha$  phosphorylation may be advantageous for the tumour because when the  $\alpha$ -subunit of eIF2 is phosphorylated it enhances the translation of specific mRNAs, which lead to amino acid biosynthesis possibly allowing the tumour cell to produce its own source of nourishment [145].

Increases in PKR expression and kinase activity have been documented in various other tumour types such as breast cancer [94] and colon cancer [95] where its presence has been associated with tumour promoting properties such as differentiation and growth of the tumour. A downstream target of PKR, NF- $\kappa$ B, is also known to be constitutively active in certain tumours and this has also been connected with tumour cell survival and angiogenesis. Thus inhibition of PKR autophosphorylation might be expected to, not only reduce PKR activation, but also downregulate nuclear binding of NF- $\kappa$ B in some human cancers, as it has been shown to do in both murine skeletal muscle and MAC16 tumour [58]. This would abrogate the tumours ability to proliferate and invade other tissues thus providing a strategy for combating tumour cell growth which exhibit upregulated PKR and NF- $\kappa$ B activity.

A role for PKR has been defined in the regulation of cellular responses to bulky adduct DNA damage, possibly by modulating DNA repair mechanisms. Thus PKR inhibition in tumours may be effective in combination therapy with other cancer drugs or chemotherapy since PKR deficient mouse embryonic fibroblasts demonstrate hypersensitivity to apoptosis when exposed to DNA damaging agents such as cisplatin, melphalen and UV radiation [20].

Although high PKR induced eIF2 $\alpha$  phosphorylation has been observed in mammary carcinoma cells, protein



synthesis was not inhibited. This was suggested to be as a result of correspondingly high levels of eIF2B, the initiation factor that is inhibited by eIF2 $\alpha$  phosphorylation [94]. However, protein synthesis was significantly reduced in MAC16 cells compared to MAC13 cells (Results Section 4.2 Figure 4.21) suggesting that eIF2B is not effective at compensating for increased eIF2 $\alpha$  phosphorylation in the MAC16 cachexia model. Elevated phosphorylation of the  $\alpha$ -subunit of eIF2 may explain the low rate of proliferation of some tumours which renders them insensitive to chemotherapy and radiation.

Previously PKR has been defined as a tumour suppressor. However, this role of PKR remains unclear in light of these results, and recent studies, which suggests a positive regulatory role of PKR in cancer progression and growth control of some tumour types [94, 95].

## 5.4 Conclusion

This study examines the effectiveness of a PKR inhibitor on skeletal muscle wasting and tumour growth in mice bearing the cachexia inducing MAC16 tumour and investigates the mechanism of muscle preservation and tumour growth inhibition by this PKR inhibitor. Inhibition of PKR was successful in stabilising weight and attenuating muscle atrophy in gastrocnemius muscle as well as reducing tumour growth through the inhibition of eIF2 $\alpha$  phosphorylation and nuclear binding of NF- $\kappa$ B. The PKR inhibitor may also be useful in combating cancer cachexia since elevation of both PKR and eIF2 $\alpha$  phosphorylation were observed in the rectus abdominus muscle of weight losing cancer patients compared to weight stable and healthy controls.

## Chapter 6

# Akt/mTOR Translational Control in Cancer Cachexia

### 6.1 Introduction

A vast array of pathways within mammalian cells serve to transduce signals from external stimuli to processes such as nutrient uptake, metabolism, gene transcription and mRNA translation. In most cases the transduction network consists of protein kinases, such as the serine/threonine kinase Akt, that serve to direct signals to the appropriate end [30].

Akt, also known as protein kinase B (PKB) is activated by a number of growth factors and cytokines and has the ability to regulate many cellular processes including mitogenesis, glucose metabolism and protein synthesis. Each of these processes are mediated by downstream effectors of Akt, including Bad, p21 and FKHR, which work together to promote the cell cycle and inhibit apoptosis, GSK3 and GLUT 4 for glucose metabolism, and mTOR or GSK3 $\beta$  for protein synthesis. The activation of Akt by growth factors and

cytokine treatment generally occurs via the phosphoinositide 3-kinase (PI3K) pathway. Upon stimulation, PI3K generates specific phosphoinositide lipids, which accumulate at the plasma membrane recruiting Akt through their affinity for pleckstrin homology domains. Akt undergoes phosphorylation at two residues, threonine 308 and serine 473. Phosphorylation at both residues is required for the activation. Akt can also be phosphorylated by Angiotensin II induced arachidonic acid release and production of Reactive oxygen species, which both lead to PI3K dependent activation of Akt [207, 122].

One of the best characterised downstream targets of Akt is mTOR. Akt can inactivate the upstream negative regulator of mTOR called tuberous sclerosis complex 2 (TSC2) by phosphorylation thereby activating mTOR. Akt does this in response to growth factors such as IGF-1 and insulin. Akt can also affect mTOR activity through its ability to modulate the level of ATP in cells. Akt can generate ATP by increasing glycolysis and oxidative phosphorylation. The effect of Akt on ATP levels causes a reduction in the AMP/ATP ratio and therefore reduces the activity of a protein called AMP activated protein kinase (AMPK). AMPK can impair the induction of mTOR activity through activation of its inhibitory regulator TSC2 (see figure 6.1 for diagram). One study suggests that the activation of mTOR by Akt-mediated inhibition of AMPK is the predominant pathway by which Akt activates mTOR [75].



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Figure 6.1: **Mechanism by which Akt regulates mTOR via inhibition of AMPK.** Akt inhibits AMPK preventing it from activating TSC2, which leads to the activation of mTOR. Diagram cited from [75]

As mentioned in Section 4.1, there are two major points where the regulation of translation initiation occurs. mTOR is involved in the second control point, which involves the binding of mRNA to the 43S preinitiation complex mediated by eIF4F, a complex of several subunits. During translation initiation the eIF4E/mRNA complex binds to eIF4G and eIF4A to form the active eIF4F complex allowing initiation to proceed [199].

Activation of mTOR results in phosphorylation of a variety of substrates including the inhibitory eIF4E binding protein (4E-BP1) and the ribosomal protein S6 protein kinase (p70S6K). Phosphorylation of both substrates requires the interaction of mTOR with the regulatory associated protein target of rapamycin (Raptor) and G $\beta$ L. Co-expression of eIF4E and p70S6K cooperatively increases cell size indicating that the two proteins operate independently to modulate cell growth. Activation of p70S6K occurs within minutes of mTOR activation. p70S6K phosphorylates ribosomal protein S6 which enhances translation of mRNA with an uninterrupted string of pyrimidine residues adjacent to the 5'-cap structure (TOP mRNA). TOP mRNA encode proteins responsible for increasing the long term capacity for synthesizing protein such as ribosomal proteins, translation elongation factors and poly(A) binding protein [30].

Phosphorylation of 4E-BP1 enhances the translation of mRNA with long highly structured 5'-untranslated regions, such as those encoding ornithine decarboxylase and cyclin D1, which are involved in the regulation of cell growth. eIF4G competes with 4E-BP1 for the same binding site, thus when 4E-BP1 is phosphorylated its affinity for eIF4E is reduced allowing eIF4G to bind. Once associated they form the active eIF4F translation initiation complex [30]. An increase in 4E-BP1 has been associated with the decreased protein synthesis in rats after infusion with Dexamethasone, which caused a loss of weight [109], while a reduction in the relative amount of eIF4G bound to eIF4E has been observed in sepsis, which is also associated with weight loss [199]. Formation of an active eIF4F complex can also be influenced by changes in either the phosphorylation state or availability of eIF4E. Phosphorylation of eIF4E has been suggested to stimulate translation rates via increased association with eIF4G and eIF4A and/or increased mRNA cap binding affinity [4].

Rates of protein synthesis can also be regulated at the elongation phase of translation. One factor that

regulates elongation is eEF2, which mediates the translocation step of elongation. eEF2 mRNA contains a 5' polypyrimidine tract, which suggests that it is translationally regulated by p70S6K [81]. p70S6K can also regulate eEF2 activity through phosphorylation and inactivation of an eEF2 kinase [135]. Phosphorylation of eEF2 results in the inhibition of elongation by decreasing its affinity for the ribosome. One study showed that protein synthesis can be depressed by an inhibition at the elongation phase of translation after hindlimb unloading. However since there was also a decreased p70S6K expression it is likely that changes in elongation reflects alterations in p70S6K activity [81].

Although all three proteins (4E-BP1, p70S6K and eEF2) can be regulated through mTOR, some evidence implies that they can also be activated through mTOR independent pathways in certain cell stress induced situations where they may instead be activated through the p38 MAP kinase pathway [135].

The eIF4F complex formation may hold important implications in cancer anorexia since phosphorylation of 4E-BPs in mouse skeletal muscle are diminished by starvation, which is paralleled by a dephosphorylation of p70S6K. Upon re-feeding these effects are reversed. However the phosphorylation state of eIF4E remains unchanged in response to fasting and re-feeding [76]. A depression in eIF4F stability will lead to a reduced capacity of the cell to synthesize proteins. Since anorexia and cancer cachexia often occur side by side this will result in a severe loss of skeletal muscle through an increase in protein degradation and depression in protein synthesis caused by catabolic factors such as PIF and cytokines, accompanied by a further loss of protein synthesis through starvation induced changes.

Many studies suggest that translation initiation is deregulated during tumorigenesis, and since PIF is a product of tumour growth it is possible that it may affect eIF4E. High expression of 4E-BP can induce apoptosis and target eIF4G for specific degradation [127], and since PIF has also been linked to apoptosis this suggests another pathway in which it can upregulate the cell death pathway.

Some studies suggest that inactivation of eIF4E, through sequestration by 4E-BP1, is a common cellular response to stress. PIF is considered as a stress inducer, and thus provides further evidence for its possible role in eIF4F assembly regulation. However, others argue that inhibiting eIF4F formation only has a small

effect on overall protein synthesis in the short term and since PIF causes a relatively acute depression in protein synthesis, that does not occur above 8 hours, this suggests the involvement of additional regulatory events in the PIF induced inhibition in protein synthesis, such as the activation of PKR and eIF2 $\alpha$  as discussed in Chapter 4 and 5, which are activated at earlier time points compared to eIF4F alterations [127].

The Akt/mTOR pathway is shown to be upregulated during hypertrophy and downregulated during muscle atrophy. Furthermore activation of the Akt/mTOR pathway and its downstream targets p70S6K and 4E-BP1 are believed to be involved in regulating skeletal muscle fibre size through an increase in protein synthesis initiation and elongation [29]. Since the major debilitating component of cachexia is the skeletal muscle wasting it is clearly important to examine the contribution of the Akt/mTOR pathway and its downstream effectors, since it is one of the major components affecting muscle mass in disuse atrophy [29], hindlimb unloading and denervation [81] as well as sepsis [105]. Some evidence suggests that maintenance of muscle weight in mature animals is not dependent on the Akt/mTOR pathway, which instead is involved in the recovery of weight after atrophy. This demonstrates the need for identifying the effect of Akt and its downstream effectors in cachexia, since it is a crucial pathway for regulating skeletal muscle hypertrophy, and may provide an important target for development of therapeutics for treating muscle protein loss [29], and encouragement of muscle growth.

As well as its effects on protein synthesis through translational control, Akt has also been reported to affect transcriptional activity through an effect on NF- $\kappa$ B. Indeed I $\kappa$ B kinase, the kinase involved in NF- $\kappa$ B activation, is a substrate of Akt suggesting that Akt can stimulate NF- $\kappa$ B activity. Although NF- $\kappa$ B can cause cell death through apoptosis, in many cases NF- $\kappa$ B activation promotes cell survival, such as through Akt, which is best known for its ability to inhibit cell death pathways by inhibiting proteins involved in apoptosis, such as GSK3, BAD, Forkhead and procaspase 9. Furthermore, other studies have found that Akt may actually be a down stream target of NF- $\kappa$ B, where NF- $\kappa$ B stimuli such as TNF $\alpha$  and LPS also stimulate Akt phosphorylation and activation [122]. Since the upregulation of NF- $\kappa$ B is evident after stimulation with the cachexia inducing factors, PIF and Ang II in C2C12 myotubes, it is possible that Akt phosphorylation is also altered, especially since Ang II has been shown to activate Akt via the release of arachidonic acid [207], suggesting that it may be a mediator in the progression of the cachectic state.



Activation of Akt could provide an alternative pathway for increased nuclear binding of NF- $\kappa$ B by PIF and Ang II. This pathway is utilised by both TNF- $\alpha$  and platelet derived growth factor (PDGF). Given the established role of Akt in the development of certain cancers such as ovarian cancer, prostate cancer and gastric adenocarcinomas it would seem appropriate to examine the role of Akt in mice bearing the MAC16 tumour [32, 149].

The aims of this study were to examine the potential role of PIF and Ang II in Akt signalling, eIF4F formation, p70S6 kinase and eEF2 phosphorylation and activation states in the C2C12 in vitro model of murine muscle, and to determine their involvement in muscular atrophy in the gastrocnemius muscle of MAC16 tumour bearing mice demonstrating weight loss. The results of this chapter should confirm or deny the involvement of Akt and/or its downstream targets in the catabolic processes induced by cachectic mediators, PIF and Ang II, and the importance of this signalling pathway in the pathogenesis of cachexia in vivo.

## 6.2 Results

A signalling pathway from PI3K to Akt/PKB is implicated in the regulation of diverse cellular processes, including protection from apoptosis in various cell types and protein synthesis in skeletal muscle and adipose tissue [79] through increases in translation initiation and elongation. Activation of Akt and its downstream targets are activated during muscle hypertrophy in vivo [29] and downregulated in some cancers presenting with cachexia.

In order to determine the effect of Akt expression on catabolic processes involved in cachexia, murine myoblasts were transfected with plasmids encoding a dominant negative Akt (DNAkt), with alanine residues substituted for threonine at position 308 and serine at position 473, or a constitutively active Akt (MyrAkt), containing the c-src myristoylation sequence fused in frame to the N-terminus of the wildtype Akt coding sequence [67]. The myoblasts were allowed to differentiate into myotubes before use (method described in

Section 2.3.18). As an extra control myotubes were transfected with the empty vector pcDNA3(-). Protein synthesis, protein degradation and proteasome activity (determined by measuring chymotrypsin like activity) was measured in Akt mutant murine myotubes incubated with PIF using the methods described in Methods Section 2.3.15, 2.3.14 and 2.3.6.

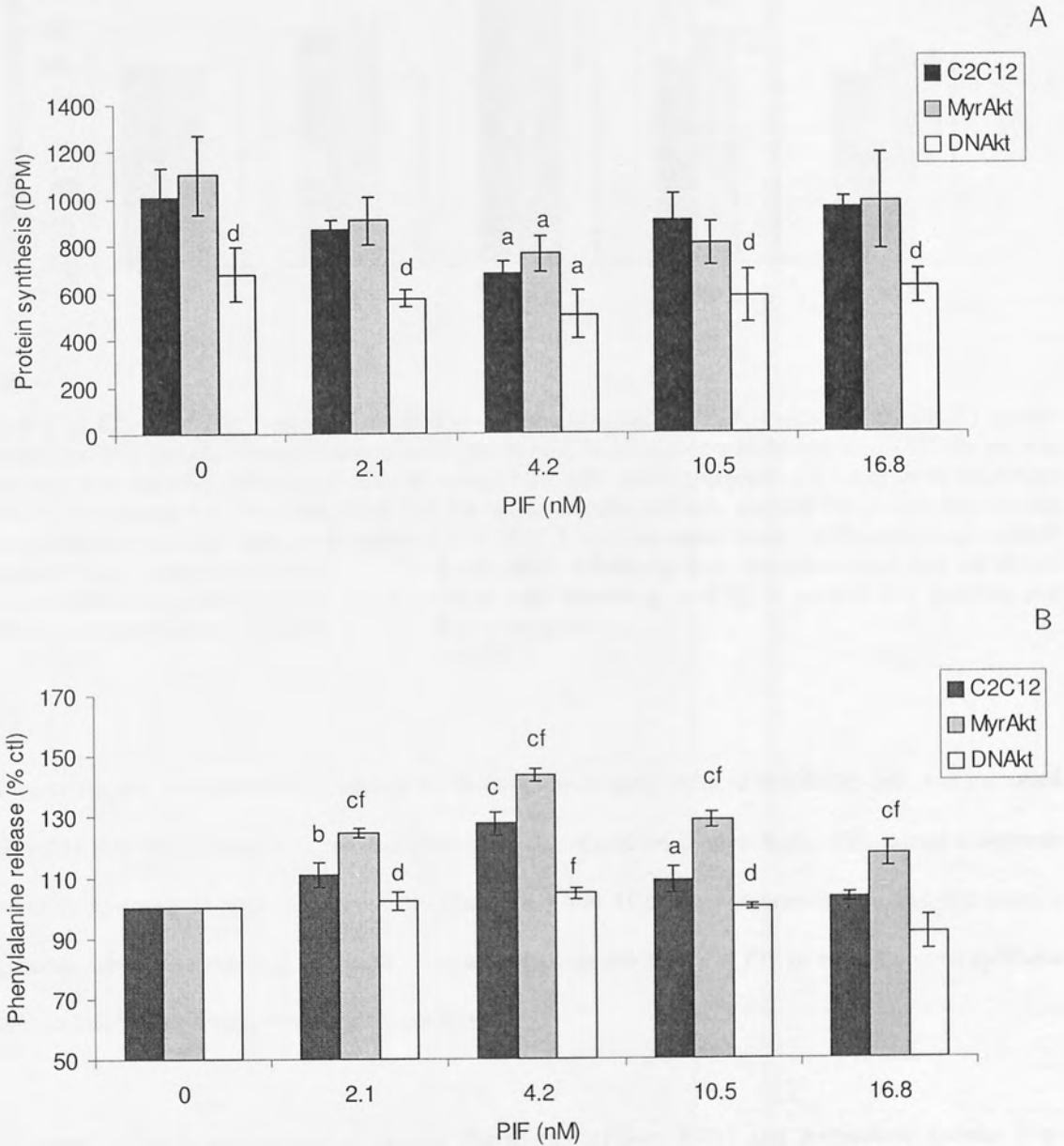


Figure 6.2

C

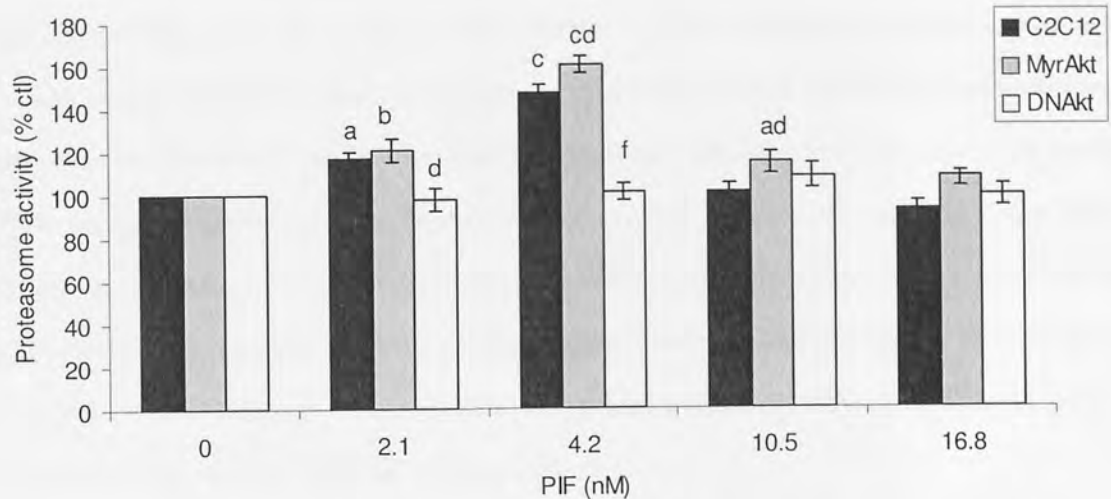


Figure 6.2: **Effect of Akt mutation on cell processes affected by PIF.** Protein synthesis (A) protein degradation (B) and chymotrypsin like activity (C) in murine myotubes transfected with wildtype myrAkt and dominant negative DNAkt plasmids in comparison with non-transfected myotubes upon treatment with PIF. Myotubes were incubated with PIF for 4h for protein synthesis and 24h for protein degradation and proteasome activity. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from non-transfected cells are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$ , differences from MyrAkt g:  $p < 0.05$ , h:  $p < 0.01$  or i:  $p < 0.001$  and differences from DNAkt j:  $p < 0.05$ , K:  $p < 0.01$  or l:  $p < 0.001$

As expected, the overall protein synthesis levels were significantly reduced in DNAkt cells and increased in MyrAkt cells in comparison to non-transfected murine myotubes (Figure 6.2A). PIF caused a decrease in protein synthesis as observed previously (Chapters 3 and 4) in the non-transfected, and Akt mutant myotubes, which was maximal at 4.2nM. This suggests that the ability of PIF to reduce protein synthesis does not involve the serine/threonine kinase Akt.

PIF caused a significant increase in protein degradation (Figure 6.2B) and proteasome activity (Figure 6.2C), as noted in previous chapters (Chapters 3 and 4), and this increase was augmented in the MyrAkt myotubes above non-transfected control, and depressed significantly below control in the DNAkt cells. This is surprising since Akt has been associated with anti-apoptotic survival pathways [122] with the ability to attenuate mechanisms that induce muscle atrophy and preserve muscle fibre size [29]. These

results demonstrate a contradictory role for Akt in murine myotubes, where degradative processes are activated in response to PIF, possibly through an Akt mediated process.

Both PIF and Ang II have the ability to activate NF- $\kappa$ B to induce upregulation of proteasome activity. Although initially believed to operate as components of distinct signalling pathways, several studies have shown that the NF- $\kappa$ B and Akt signalling pathways converge [122]. Akt has been reported to regulate NF- $\kappa$ B activity through its ability to phosphorylate I $\kappa$ K $\alpha$ , which stimulates the release of NF- $\kappa$ B and its migration to the nucleus [175]. To determine the involvement of Akt in the regulation of NF- $\kappa$ B in response to PIF, the DNA binding activity of the transcription factor was determined (see Method Section 2.3.7 for procedure) in pcDNA3 control cells and myotubes containing MyrAkt and DNAkt plasmids, in the presence and absence of PIF and the PI3-kinase inhibitor LY294002.

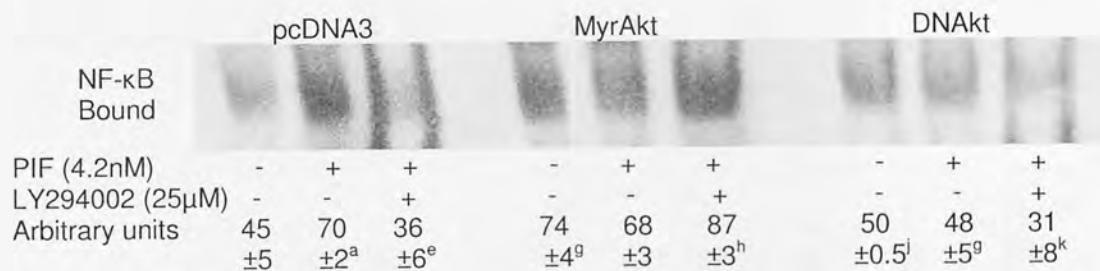


Figure 6.3: **DNA binding activity of NF- $\kappa$ B in murine myotubes transfected with Akt mutant plasmids upon treatment with PIF and LY294002.** An Emsa was performed to determine DNA binding of NF- $\kappa$ B in murine myotubes transfected with pcDNA3(-), wildtype MyrAkt and dominant negative DNAkt plasmids when treated with PIF and LY294002 (100 $\mu$ M). Myotubes were incubated with LY294002 for 2h then a further 0.5h with PIF. Error bars represent  $\pm$ SEM of 2 replicate blots. Differences from control are shown as a:  $p < 0.05$ , while differences in the presence of PIF are shown as e:  $p < 0.01$ , differences from corresponding pcDNA3(-) g:  $p < 0.05$ , h:  $p < 0.01$  and differences from corresponding MyrAkt j:  $p < 0.05$ , K:  $p < 0.01$

PIF induced nuclear migration of NF- $\kappa$ B in myotubes containing empty plasmid (pcDNA3(-)), but not in those containing DNAkt. In addition, in myotubes containing MyrAkt there was constitutive upregulation of NF- $\kappa$ B, and LY294002 had no effect on nuclear binding of NF- $\kappa$ B in the presence of PIF (Figure 6.3).

These results suggest that protein degradation through a PIF induced increase in proteasome activity

requires the activation of Akt, which involves nuclear localisation of NF- $\kappa$ B through a PI3K mediated process.

mTOR is activated by the small GTPase, Rheb, which is inhibited by its GAP protein tuberous sclerosis complex 2 (TSC2) that heterodimerizes with TSC1. TSC2 is an upstream negative regulator of mTOR [75]. Activation of Akt inhibits the ability of TSC2 to act as a Rheb-GTPase activating protein (GAP), allowing Rheb-GTP levels to rise, causing activation of mTOR. This has two primary downstream targets, p70S6 kinase and the 4E-BP, phosphorylation of which results in translation initiation and accelerated protein synthesis [103]. To determine whether this pathway was important in protein degradation (Method Section 2.3.14), myotubes were treated with the mTOR inhibitor rapamycin (25ng/ml), either before, or at various times after the addition of PIF (4.2nM), and the activation state of mTOR and it's downstream effector p70S6 kinase were studied by western blotting (Methods Section 2.3.13 and 2.3.21) for their phosphorylated forms after incubation with PIF (4.2nM) and Ang II (0.5 $\mu$ ).

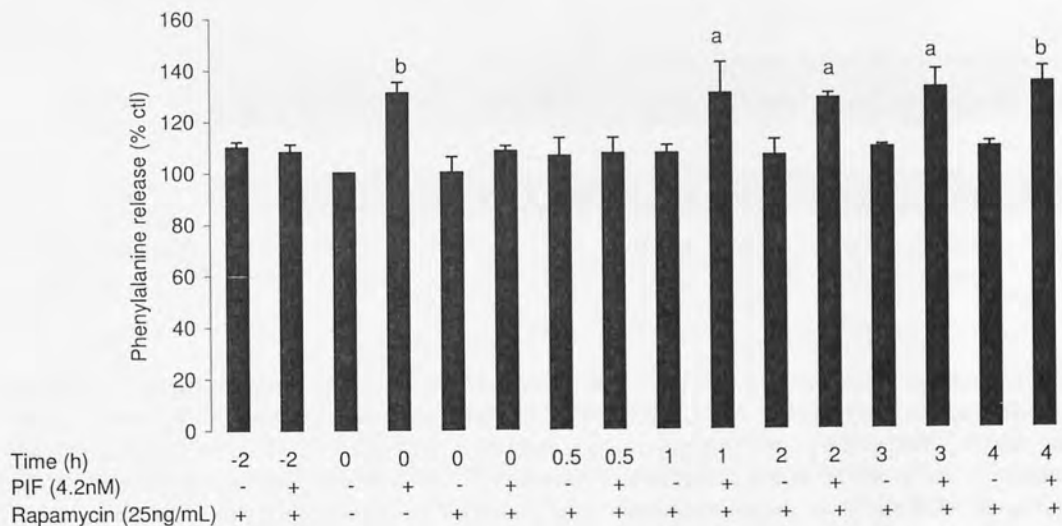


Figure 6.4: **Protein degradation in murine myotubes in response to PIF and rapamycin.** Rapamycin (25ng/mL) was added either 2h before or at 0.5h, 1h, 2h or 4h after PIF (4.2nM). Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from PIF alone are shown as f:  $p < 0.001$

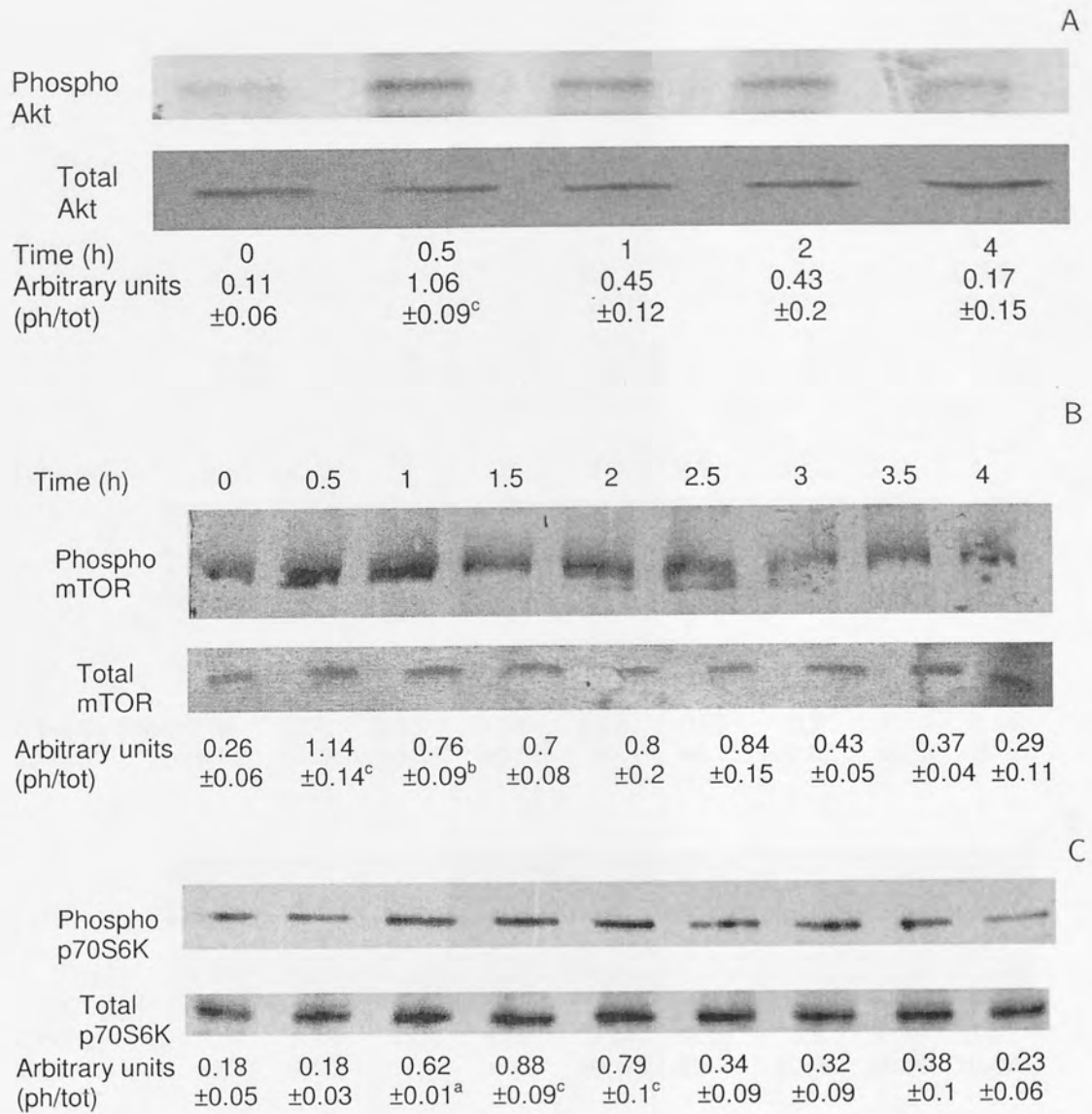


Figure 6.5: **Western blotting for phosphorylated Akt, mTOR and p70S6K in murine myotubes treated with PIF.** Phosphorylated forms of Akt (A), mTOR (B) and p70S6K (C) were analysed in murine myotubes treated with PIF (4.2nM) over a 4h time course. Blots of total Akt/mTOR/p70S6K were used to normalise the phosphorylated proteins. Densitometric analysis is shown below where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$



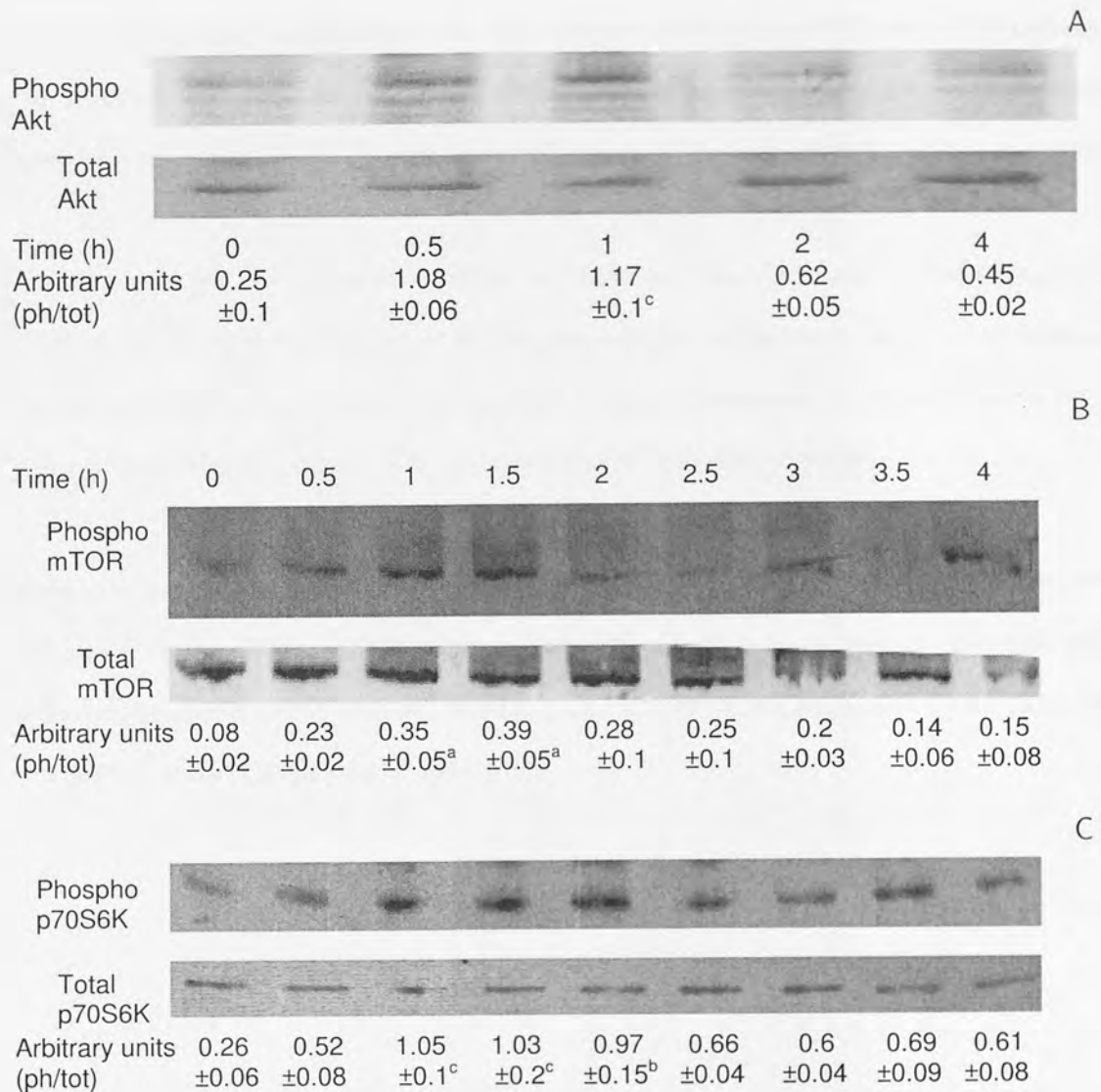


Figure 6.6: **Western blotting for Akt, mTOR and p70S6K in murine myotubes treated with Ang II.** Phosphorylated forms of Akt (A), mTOR (B) and p70S6K (C) were analysed in murine myotubes treated with Ang II (0.5 $\mu$ M) over a 4h time course. Blots of total Akt/mTOR/p70S6K were used to normalise the phosphorylated proteins. Densitometric analysis is shown below where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$

Addition of rapamycin either 2h before or up to 0.5h after addition of PIF completely attenuated protein degradation, whereas addition at later times had no effect (Figure 6.4). This is likely to occur through inhibition of mTOR activation by Akt since rapamycin is only effective during the time course at which Akt (Figure 6.5A) and subsequent mTOR (Figure 6.5B) phosphorylation is upregulated in response to PIF. This was followed by induction of p70S6K phosphorylation (Figure 6.5C), which was maximal between 1

and 2h after addition of PIF. The stimulation of Akt, mTOR and p70S6K by Ang II (Figure 6.6) followed a comparative, albeit slightly delayed time course. These results suggest that the PI3K/Akt/mTOR pathway may be important for a short burst of protein synthesis, which would be required for the synthesis of proteasome subunits, ubiquitin and ubiquitin liigase (E3) involved in the degradation of myofibrillar proteins.

The protein kinase Akt is a downstream effector of phosphoinositide-3-OH kinase (PI3K) [75], and a potential role for ROS in the stimulation of Akt has been indicated in response to Ang II in a PI3-kinase dependent manner [207], thus marking PI3K and ROS as potential mediators for Akt activation by PIF, since Ang II has comparative effects to PIF on several catabolic signalling pathways.

To investigate some of the mechanisms involved in the activation of Akt in response to PIF, murine myotubes were incubated with the PI3K inhibitor, LY294002, a tyrosine kinase inhibitor, Genestein, and a reactive oxygen species (ROS) inhibitor, Vitamin E, and assayed for the active state of Akt using the western blotting method (Section 2.3.13 and 2.3.21).

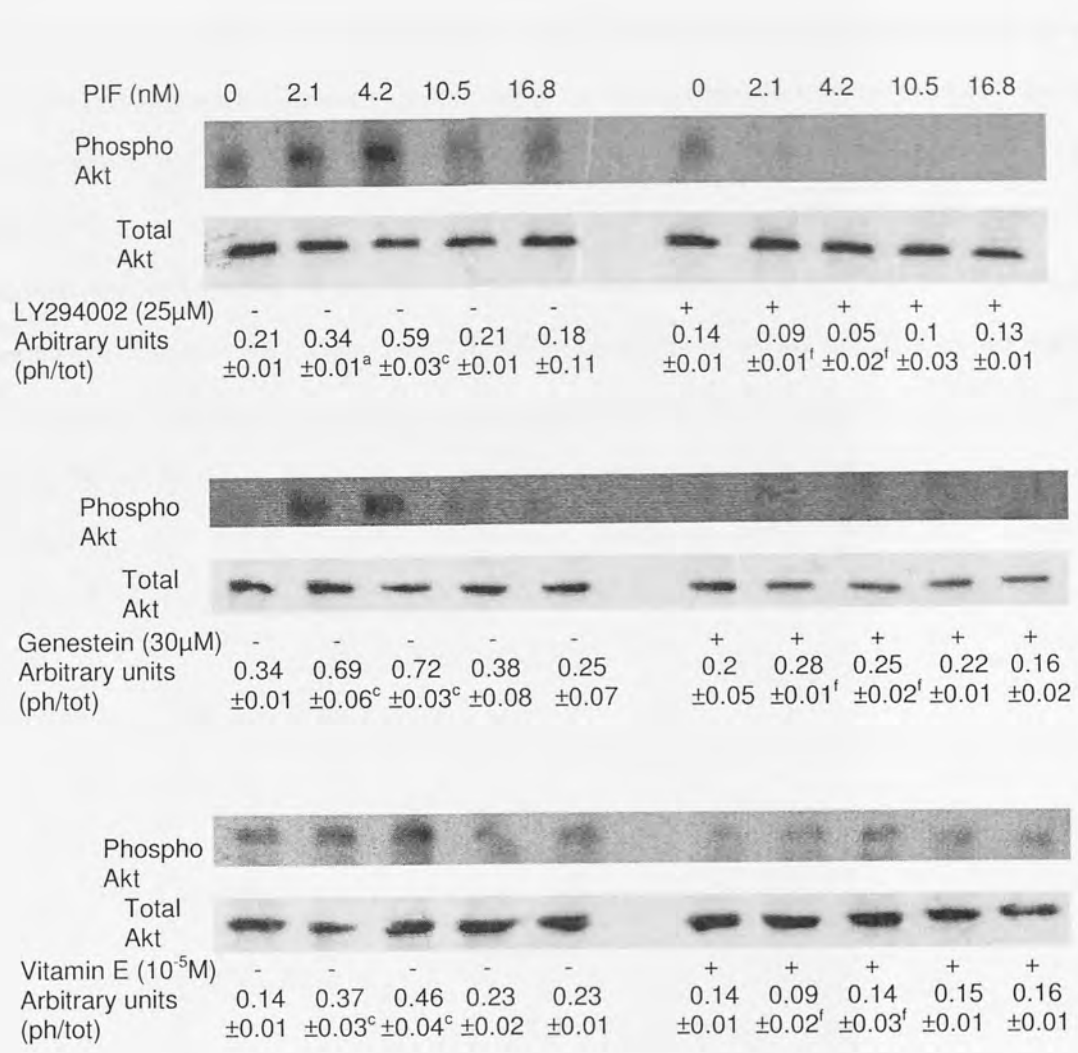


Figure 6.7: **Western blotting for phosphorylated forms of Akt in murine myotubes treated with PIF in the presence of inhibitors.** Phosphorylated forms of Akt were analysed in murine myotubes treated with PIF (0.5h) in the presence of LY294002 (25µM) (A), Genestein (30µM) (B) and Vitamin E (10<sup>-5</sup>M) (C). Inhibitors were incubated with myotubes for 2h prior to addition of PIF. Blots of total Akt were used to normalise the phosphorylated proteins. A densitometric analysis is shown below where ± represents the SEM of 3 replicate experiments. Significant differences from control are shown as a: p<0.05, c: p<0.001, while differences in the presence of inhibitor is shown as f: p<0.001

The activation of Akt was increased in response to PIF, determined by elevated phosphorylation which was maximally significant at 4.2nM (Figure 6.7). All three inhibitors attenuated the phosphorylation of Akt by PIF at all concentrations, suggesting that PIF stimulates the activation of Akt possibly through a tyrosine kinase receptor or second messenger, involving the generation of reactive oxygen species, which involve a PI3-kinase dependent mechanism.

Cachexia is a hallmark of many disease states with sepsis being one of them. Sepsis induces profound alterations in whole body protein metabolism and muscle protein wasting, resulting from a global decrease in protein synthesis and an increase in protein degradation, as is observed in cancer cachexia. The sepsis induced inhibition of protein synthesis results from a defect in translational efficiency, which has been determined to arise from diminished assembly of the active eIF4F complex [199, 197]. Since the presenting symptoms of muscle wasting are the same in sepsis and cancer cachexia it is likely that similar mechanisms are involved. Therefore the components of the eIF4F complex were studied in C2C12 murine myotubes upon treatment with the catabolic factor of cancer cachexia, PIF. Murine myotubes were incubated with PIF over 4h and the m<sup>7</sup> cap binding protein eIF4E was purified using m<sup>7</sup>GTP sepharose (see Section 2.3.12 for method). eIF4E and its associated proteins were analysed by western blotting using the methods described in Section 2.3.13 and 2.3.21.

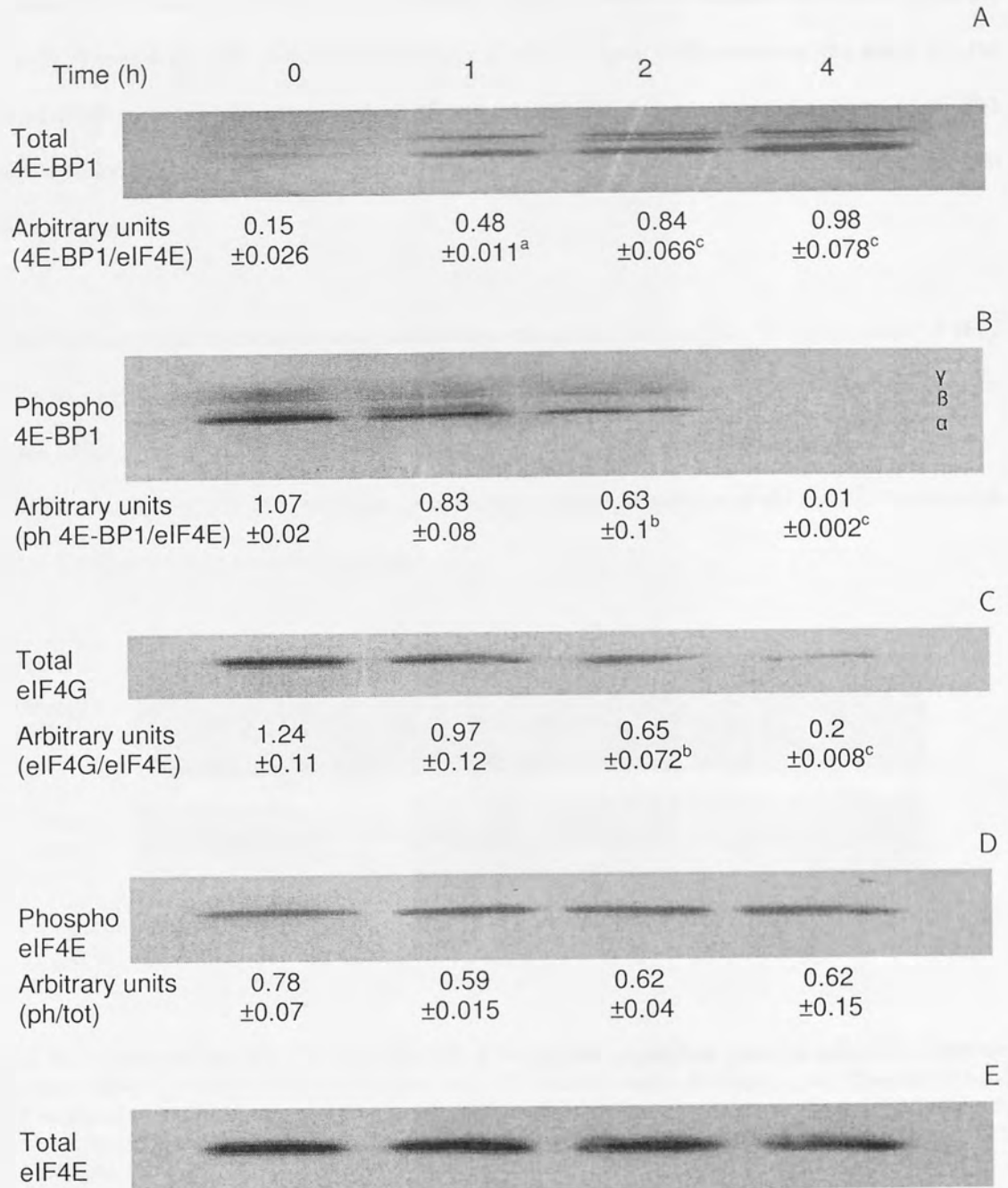


Figure 6.8: **Western blotting for components of the translation initiation complex eIF4F in murine myotubes treated with PIF.** The expression of the translation initiation complex proteins 4E-BP1 (A), phospho 4E-BP1 (B), eIF4G (C) and phospho eIF4E (D) purified by m<sup>7</sup>GTP sepharose in association with eIF4E (E) were analysed in murine myotubes treated with PIF (4.2nM) over a 4h time course. A densitometric analysis is shown below where ± represents the SEM of 2 replicate blots. Significant differences from 0h control are shown as a: p<0.05, b: p<0.01 or c: p<0.001

Exposure to PIF caused a decrease in the assembly of the active eIF4F complex, which was significant above 2h (Figure 6.8). The phosphorylation state of eIF4E (Figure 6.8D) remained the same, but the total eIF4G (Figure 6.8C) associated with eIF4E was reduced, probably as a result of a decreased 4E-BP1 phosphorylation (Figure 6.8B), and increased association with eIF4E (Figure 6.8A), sequestering eIF4E in an inactive complex.

Another component of the translational machinery is elongation factor eEF2. Phosphorylation of eEF2 inhibits its activity, apparently by inhibiting its ability to interact with the ribosome. Given that cellular stresses inhibit translation and PIF can be considered a stress factor it was important to study whether treatment of myotubes with PIF could elicit an increase in the phosphorylation of eEF2 [135]. See Methods Section 2.3.13 and 2.3.21 for procedure used.

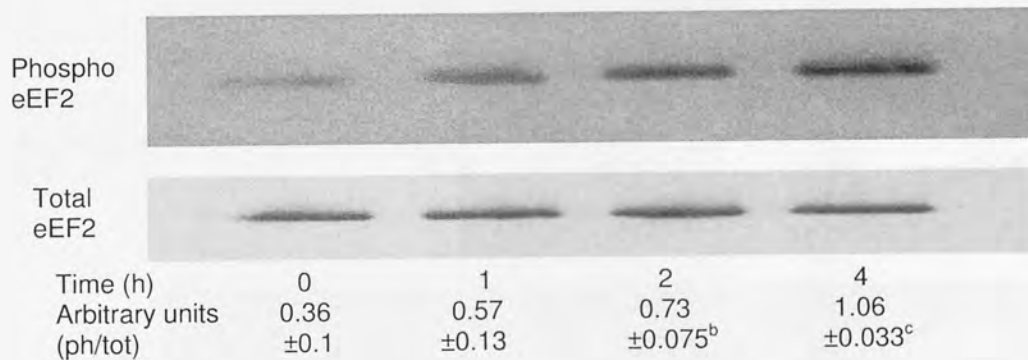


Figure 6.9: **Western blotting for phospho eEF2 in murine myotubes treated with PIF.** Phospho eEF2 was analysed in murine myotubes treated with PIF (4.2nM) over a 4h time course. The blot for total eEF2 was used to normalise the phosphorylated protein. A densitometric analysis is shown below where ± represents the SEM of 3 replicate blots. Significant differences from the 0h control are shown as b:  $p < 0.01$  or c:  $p < 0.001$

An increase in eEF2 phosphorylation was observed in response to PIF (Figure 6.9), which would result in a depression of mRNA elongation and halt translation progression.

It is unlikely that the Akt/mTOR pathway is responsible for the alterations in the eIF4F complex and eEF2 phosphorylation in vitro, since the time courses do not correlate. In muscle from mice bearing the



cachexia inducing colon-26 (C-26) tumour, an increase in p70S6 kinase phosphorylation was observed with no change in Akt activity [2], thus the mechanisms *in vitro* may not necessarily reflect those that occur *in vivo*, although it should be noted that Akt phosphorylation may be transient and thus not detected *in vivo* for this reason. An increased phosphorylation of p70S6K was observed after hindlimb unloading in the soleus muscle of rats at a time point where the rate of protein synthesis was inhibited [81]. Accordingly, to investigate the activation state of p70S6 kinase and that of its upstream activator mTOR in skeletal muscle of mice bearing the MAC16 tumour, gastrocnemius muscle from MAC16 tumour bearing mice was analysed using the western blotting technique (see Method Section 2.3.13 and 2.3.21 for procedure).

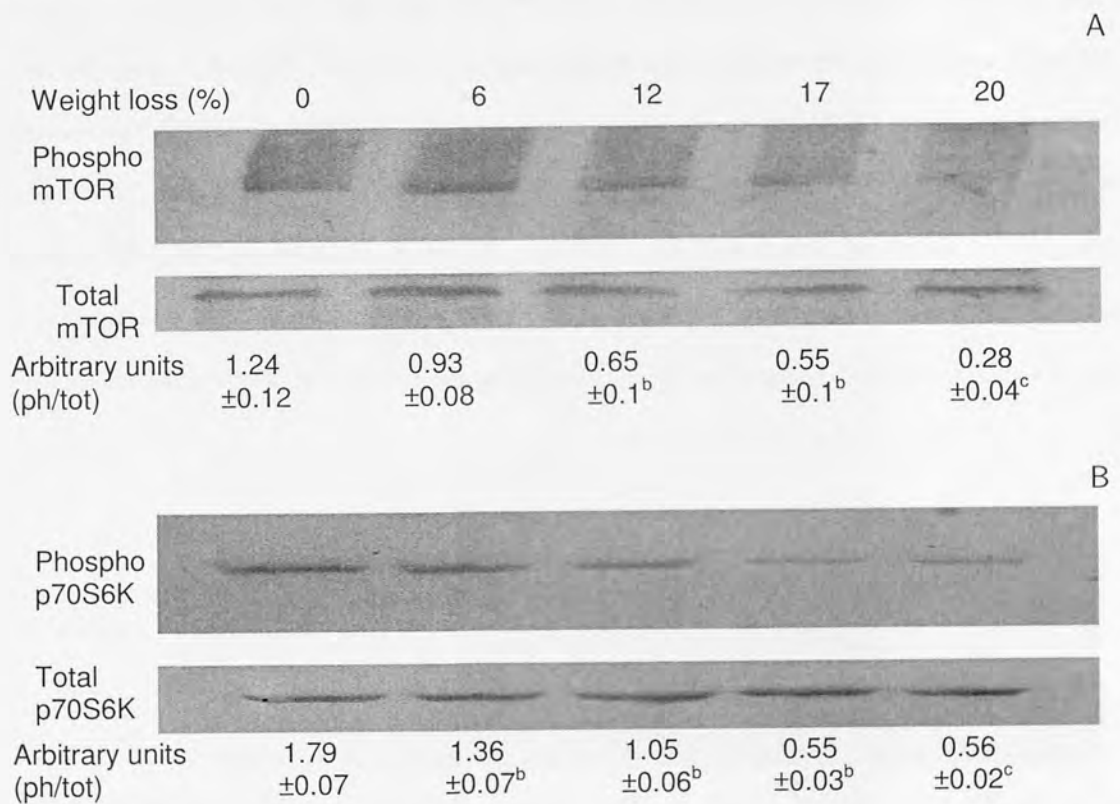


Figure 6.10: **Western blotting for mTOR and p70S6K in gastrocnemius muscle from MAC16 tumour bearing mice.** The phosphorylated forms of mTOR (A) and p70S6K (B) were analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour demonstrating weight loss. Blots for total mTOR and p70S6K were used to normalise the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate experiments. Significant differences from 0% weight loss control are shown as b:  $p < 0.01$  or c:  $p < 0.001$

Both mTOR (Figure 6.10A) and p70S6 kinase (Figure 6.10B) phosphorylation decreased incrementally as weight loss increased. Although this may occur in response to PIF, produced by the MAC16 tumour, other

mechanisms should not be ruled out, especially since myotubes only showed a transient rise in mTOR and p70S6 kinase when treated with PIF (Figure 6.5B and C). For example cachectic muscle from mice implanted with C-26 tumours displayed a pronounced increase in p70S6 kinase activity that was suggested to occur due to the accumulation of amino acids released from catabolised protein [2]. In patients with cancer cachexia a decrease in plasma concentrations of BCAA has been observed [185]. Since accumulation of amino acids has been implied to cause an increase in p70S6 kinase phosphorylation it may be that a loss of circulating amino acids results in a decrease in p70S6 kinase and mTOR activity.

mTOR plays an important role in regulating mRNA translation by inhibiting the activity of eEF2 [135]. eEF2 can also be regulated by p70S6 kinase which phosphorylates and inactivates the eEF2 kinase. Therefore the changes in mTOR and p70S6 kinase activity in the skeletal muscle of MAC16 tumour bearing mice may have a consequential effect on eEF2 activation. It has been suggested that the rapid decrease in protein synthesis observed in the soleus muscle after hindlimb unloading is due to inhibition of the elongation phase of translation [81]. To determine this effect the levels of eEF2 phosphorylation were determined in gastrocnemius muscle of MAC16 tumour bearing mice with weight loss (method described in Section 2.3.13 and 2.3.21).

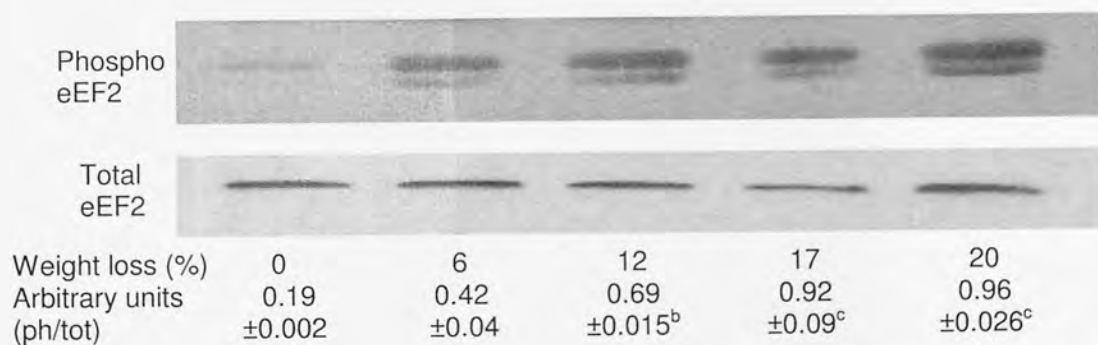


Figure 6.11: **Western blotting for phospho eEF2 in gastrocnemius muscle from MAC16 tumour bearing mice.** The phosphorylated form of eEF2 was analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour demonstrating weight loss. The blot of total eEF2 was used to normalise the phosphorylated protein. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate experiments. Significant differences from 0% weight loss control are shown as b:  $p < 0.01$  or c:  $p < 0.001$

Phosphorylation of eEF2 results in its inactivation halting elongation and deregulating protein synthesis. The above result shows a five-fold increase in phosphorylation of eEF2 (Figure 6.11), with no change in the total amount, indicating that global protein synthesis in skeletal muscle of mice bearing the MAC16 tumour, was likely reduced through a decrease in translation initiation and elongation.

Stimulation of active eIF4F complex assembly can also occur as a result of mTOR activation and was observed in murine myotubes in response to PIF, thus it seemed likely that eIF4F formation would also be affected in MAC16 tumour bearing mice as was eEF2 activity. To test this hypothesis, the effect of weight loss on eIF4E availability for active eIF4G.eIF4E complex formation in gastrocnemius muscle from MAC16 tumour bearing mice, was measured by using m<sup>7</sup> sepharose to purify the eIF4E m<sup>7</sup> cap binding protein and proteins associated with it (see Section 2.3.12, 2.3.13 and 2.3.21 for methods used).

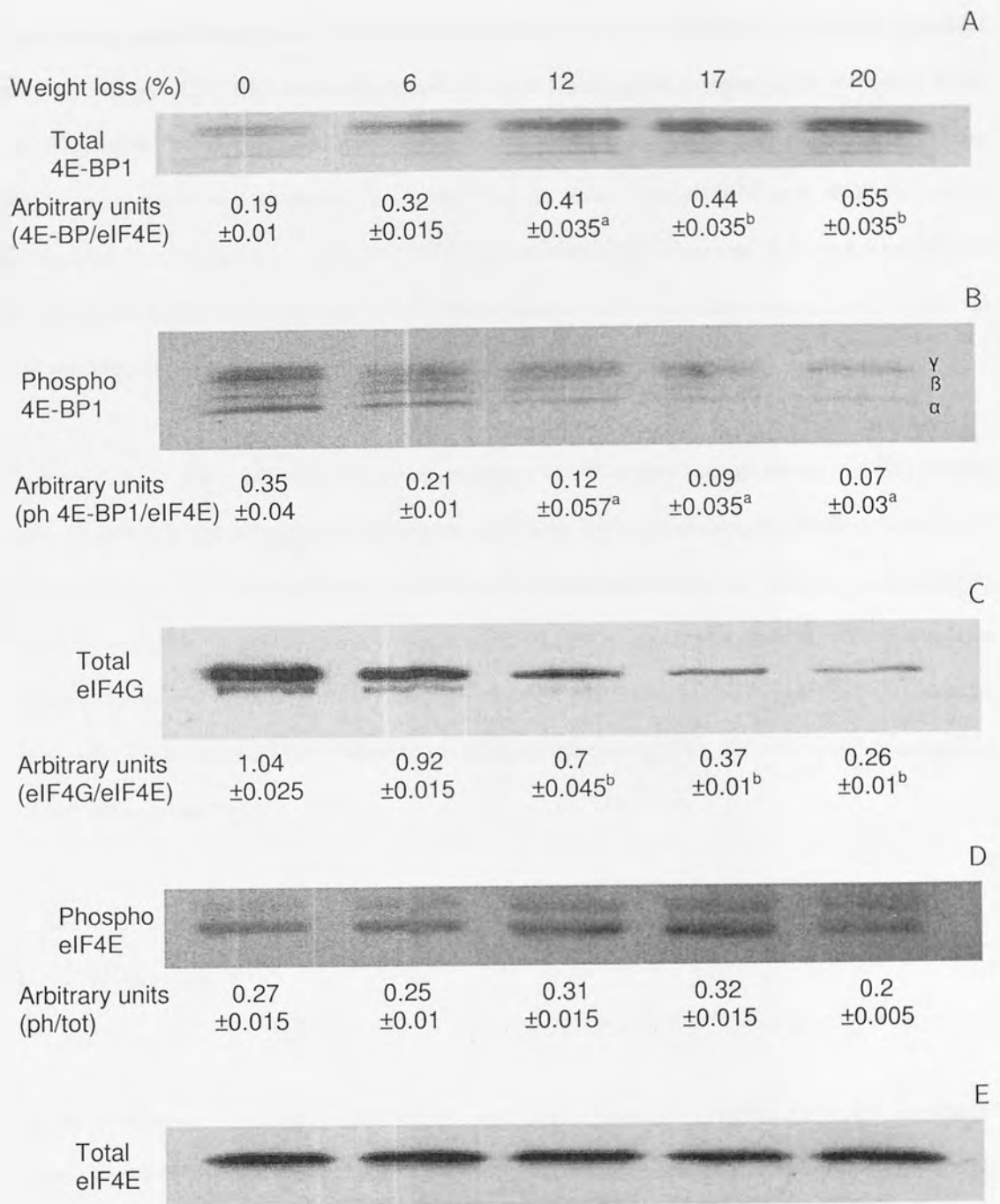


Figure 6.12: **Western blotting for components of the translation initiation complex eIF4F in gastrocnemius muscle from MAC16 tumour bearing mice.** The translation initiation complex proteins 4E-BP1 (A), phospho 4E-BP1 (B), eIF4G (C) and phospho eIF4E (D) purified by m<sup>7</sup>GTP sepharose in association with eIF4E (E) were analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour demonstrating weight loss. A densitometric analysis is shown underneath where ± represents the SEM of 2 replicate experiments. Significant differences from 0% weight loss control are shown as a: p<0.05 or b: p<0.01

These results confirm that formation of the eIF4F active complex is attenuated in skeletal muscle of MAC16 tumour bearing, weight losing mice. There was a progressive increase in the amount of 4E-BP1 associated with eIF4E (Figure 6.12A) with increasing weight loss, and a progressive decrease in the formation of the active eIF4G.eIF4E complex (Figure 6.12C), which showed an 80% decrease in animals with a weight loss of 20%, compared with non weight losing animals. The decrease in the availability of eIF4E for binding eIF4G resulted from hypophosphorylation of 4E-BP1 (Figure 6.12B). There was a five-fold reduction in 4E-BP1 phosphorylation in animals with a 20% weight loss compared with those without weight loss. No change was observed in the levels of eIF4E phosphorylation (Figure 6.12D).

The evidence implies that eIF4F and eEF2 can be regulated by PIF *in vitro* through an Akt/mTOR/p70S6K independent method. The *in vivo* model demonstrated that the same mechanistic alterations occurred in response to weight loss, or as a contributing factor towards it, which may or may not involve mTOR/p70S6K. Thus the depression in protein synthesis in response to cachectic stimulators, such as PIF, may involve the deregulation of the protein synthetic machinery namely the eIF4F, p70S6K and eEF2 components, resulting in the inhibition of mRNA binding to the 43S preinitiation complex and subsequent depression of translation initiation and protein synthesis.

### 6.3 Discussion

A transient stimulation of protein synthesis by Akt was shown to have an important role in the degradative pathways induced by PIF via the activation of NF- $\kappa$ B and increased proteasome activity through a PI3-kinase dependent process, which is possibly required for synthesis of proteasome subunits, as well as for the synthesis of the E3 ligases, MuRF1 and atrogin-1/MAFbx. This finding may seem contradictory to previous reports that show increased PI3K/Akt activity is associated with inhibition of muscle catabolism. However, the inhibition of protein degradation through PI3K suppression has been observed in other studies. For example Blommaert et al [28] showed a reduced basal protein breakdown in isolated hepatocytes incubated with the PI3K inhibitor, LY294002, while Fang et al [63] observed that LY294002 inhibited protein

breakdown in muscles from rats with catabolic conditions including fasting, sepsis and dexamethasone treatment. The authors of these reports interpreted this inhibition as evidence that PI3K activity is required for intracellular autophagy, an important pathway of intracellular protein degradation [63]. One study observed that p70S6 kinase, a downstream target of mTOR, is transiently upregulated during autophagy, which was suggested as a requirement for protein synthesis in the expansion of the preautophagolysosome vesicle and for the maturation of autophagolysosomes. Since the proteasome and lysosome are both proteolytic systems it seems likely that they will have some similar mechanisms of activation [141].

TNF $\alpha$  is a proinflammatory cytokine which mediates diverse pathological processes such as cancer cachexia, sepsis and inflammation in autoimmune disease. Although TNF $\alpha$  has been shown to inhibit protein synthesis through PKR activation and consequent eIF2 $\alpha$  phosphorylation, one study showed that TNF $\alpha$  could also stimulate protein synthesis in cardiac myocytes through a PI3K mediated pathway, which resulted in Akt activation. This activation was transient [79]. Both PIF and TNF $\alpha$  have been linked with cancer cachexia, and since PIF is able to activate PKR it is likely that other mechanisms in the TNF $\alpha$  signalling cascade may also be regulated by PIF. This substantiates the results shown in Figure 6.5A where PIF transiently activated Akt in vitro.

As discussed in Chapter 7 the generation of reactive oxygen species (ROS) may be involved in the catabolic signalling pathways of both PIF and Ang II. Vitamin E, a ROS inhibitor, was effective at attenuating expression of activated Akt in response to PIF suggesting that ROS production may be part of the process which results in Akt phosphorylation. This is substantiated by evidence that Akt can be activated by ROS as a downstream component of Ang II signalling [207]. Alternatively, Akt/mTOR may be stimulated as an indirect result of PKC activation by PIF and Ang II. It was suggested that PIF activated PKC may be involved in the phosphorylation of proteasome subunits, increasing their catalytic activity [211]. Accordingly, a PKC-dependent TSC2 regulatory role has been proposed which mediates mTOR activation [209]. PKC was upregulated at 30min in response to PIF [211], which supports the previous argument, since the PIF induced Akt/mTOR activation demonstrated in this chapter was only transient.

The state of mTOR and p70S6K phosphorylation was downregulated in weight losing MAC16 mice sug-



gesting that the Akt/mTOR pathway is inhibited as a long term effect of PIF exposure to skeletal muscle, or as a result of a decrease in circulating nutritional stimulators of the pathway such as insulin and branched chain amino acids, which has been noted in cachectic cancer patients [55, 185]. Of note, downregulation of the Akt pathway has also been observed in skeletal muscle atrophy [51]. TOR signalling promotes anabolic processes and antagonises catabolic processes [209], thus a loss of mTOR function would leave the cell vulnerable to proteolytic attack by catabolic factors such as PIF, Ang II or cytokines. Autophagy may be an important factor to consider in the changes observed in mTOR and p70S6K in response to nutritional depletion. Like the proteasome, the lysosome is a powerful hydrolytic mechanism in eukaryotic cells. The lysosome is responsible for the autophagic process of the cell in response to a number of diverse stimuli including starvation or therapeutic treatment, and has been linked to several diseases which display features of cachexia such as cancer, cardiomyopathy, Alzheimer's, Parkinson's and Huntingdon's. Although p70S6 kinase is required for maximal activation of autophagy, both mTOR and p70S6K are switched off once autophagy commences. Furthermore, energy depletion in cachectic mice would result in elevated AMP/ATP ratio and in muscle, the AMP-dependent kinase (AMPK) negatively modulates protein synthesis by impairing the mTOR dependent signals to p70S6 kinase and 4E-BP1 [141]. mTOR signalling is also downregulated in response to DNA damage and reducing conditions [209], both of which are increased in response to oxidative stress, which has been linked to many muscle wasting diseases [128] and to PIF induced catabolic processes (see Chapter 7 for details). Thus the generation of reactive oxygen species (ROS) may be responsible for the sustained reduction of mTOR activity and its downstream effectors.

Another downstream effector of mTOR, eEF2, may also be activated in response to changes observed in mTOR and p70S6 kinase phosphorylation. Activity of eEF2 kinase is normally fully dependent on Ca/Cam [146]. A possible mechanism of PIF induced effects is through the release of calcium (see Chapter 7 for details). Thus a rise in calcium levels may increase the negative regulation of the eEF2 kinase, providing an mTOR independent pathway of controlling eEF2 activity in catabolic processes. Furthermore, one study [126] showed differential expression of eEF2 in the MAC16 tumour compared to the MAC13 tumour, where eEF2 expression was 4-fold lower in MAC16 tumour cells. The MAC13 and MAC16 tumours are quite distinct in their abilities to induce weight loss. MAC16 tumours induce substantial weight loss, without alterations in caloric intake, when implanted into NMRI mice, while mice implanted with MAC13

tumour cells maintain a stable weight. Therefore some of the changes in eEF2 may be tumour specific and play a role in the aetiology of cancer cachexia in the MAC16 tumour model [126].

Assembly of the active eIF4F complex was reduced and phosphorylation of the elongation factor eEF2 was increased in murine myotubes when dosed with PIF, and in the skeletal muscle of mice bearing the MAC16 tumour when weight loss exceeded 20%. The inhibition of eIF4F complex formation was associated with hypophosphorylation of 4E-BP1 and inactivation of eIF4E through sequestration by 4E-BP1 and a resultant decreased association with eIF4G. Formation of the active eIF4F complex is required for binding mRNA to the 43S preinitiation complex and commencement of translation initiation, while the eEF2 is responsible for translation elongation. Thus weight loss in MAC16 mice might, at least in part, be associated with diminished mRNA translation and elongation and consequent decline in protein synthesis.

mTOR is an upstream regulatory component of eIF4F formation and eEF2 phosphorylation, thus at least in vivo the changes observed in eIF4F and eEF2 may be initiated through an mTOR dependent pathway. However it is unlikely that this occurs in the murine myotubes since the transient rise in Akt/mTOR signalling did not reflect the phosphorylation changes observed in the eIF4F complex or eEF2. Therefore it is likely that PIF has additional mechanisms of action on the protein synthetic machinery independent of Akt/mTOR which regulates the eIF4F triad and eEF2 activity in vitro.

Cellular stress induced by agents such as arsenite and hydrogen peroxide have been shown to cause profound inhibition of protein synthesis accompanied by dephosphorylation of 4E-BP1 and increased binding of this repressor protein to eIF4E, inhibition of the p70S6 kinase and alterations in eEF2 activity. Although all three proteins can be regulated through mTOR, evidence implies that in response to some cellular stresses mTOR function is not affected and that stress induced responses may be regulated through a P38 MAP kinase dependent pathway [135].

Another translational repressor, PKR, has been demonstrated to activate the P38 MAP kinase pathway [161]. PIF and Ang II might be considered stress inducing agents and have been shown to upregulate PKR autophosphorylation (Chapter 4), suggesting an alternative mechanism for the regulation of eIF4F,

p70S6K and eEF2 in conditions of PIF/Ang II induced cellular stress. Accordingly a requirement for PKR for the association of 4E-BP1 with eIF4E after DNA damaging etoposide treatment indicates that PKR could be involved in the regulation of 4E-BP1 phosphorylation in response to other forms of cellular stress [86].

The serine/threonine kinase, PKR can regulate translation initiation by phosphorylating the  $\alpha$ -subunit of eIF2 which is responsible for regulating the binding of met-tRNA to the 40S ribosome. Once phosphorylated on its  $\alpha$ -subunit eIF2 becomes a negative regulator of translation, inhibiting further rounds of initiation and thus depressing rates of protein synthesis [147]. Recently a novel role for PKR has been described in the control of translation initiation by regulation of the mRNA binding step to the 43S ribosome. It does this by phosphorylating B56 $\alpha$ , which is a regulatory subunit of PP2A. PKR phosphorylation of B56 $\alpha$  causes an increase in PP2A activity. PP2A has been previously reported to regulate different proteins involved in translation control including 4E-BP1 and eEF2 [212].

The modifications in the protein synthetic machinery associated with the actions of PIF may not be a direct effect. Apoptosis appears to play a role in PIF mediated effects as discussed in Section 1.3, where the progress of apoptosis can be characterised by the cleavage and activation of certain caspases. PIF has been demonstrated to upregulate the phosphorylation of PKR/eIF2 $\alpha$  (Chapter 4, Section 4.2) and downregulate the binding of eIF4G to eIF4E (Section 6.2). Apoptosis associated modifications include the specific fragmentation of protein eIF4G, eIF4B and alterations in the state of phosphorylation of initiation factors eIF2 $\alpha$ , eIF4E and 4E-BP1. Caspases have been shown to induce irreversible cleavage of eIF4G, cause the loss of p70S6K activity and increase binding of 4E-BP1 to eIF4E as well as induce PKR activation and eIF2 $\alpha$  phosphorylation. This may provide a common event that links the activation of PKR/eIF2 $\alpha$  and the destabilization of the eIF4F complex by PIF [127], since activation of caspases-3 has been shown to be an initial step in the loss of muscle proteins through the ubiquitin-proteasome pathway and is known to be activated by PIF in murine myotubes, together with caspases-8 and -9 [55, 164]. Apoptotic events have been described in different experimental models of skeletal muscle atrophy such as ageing, denervation, unloading, burn injury, and cancer. In addition, evidence of increased skeletal muscle apoptosis has been demonstrated in humans affected by chronic heart failure [31]. Apoptosis obviously plays an important role in the pathogenesis of cachexia and may be involved in the depression of protein synthesis observed in

atrophy muscle as well as in the stimulation of degradative pathways that leads to muscular wasting in cachexia.

## 6.4 Conclusion

PIF induced protein degradation in wild-type myotubes with a parabolic dose-response curve, while myotubes containing the constitutively active MyrAkt showed an enhanced response to both agents, and those containing the dominant negative DNakt showed a reduced protein degradation. A similar effect was observed on the induction of chymotrypsin-like enzyme activity probably through the activation of NF- $\kappa$ B through a PI3K dependent mechanism. However, neither Akt mutants affected the ability of PIF to induce protein synthesis depression.

Protein degradation induced by PIF was attenuated by rapamycin, a specific inhibitor of mammalian target of rapamycin (mTOR), when added before or up to 30min after addition of PIF. PIF induced transient stimulation of Akt, mTOR and p70S6 kinase between 30min and 2h after addition. Activation of Akt by PIF was inhibited by PI3K, tyrosine kinase and ROS inhibitors. These results suggest that PIF induces activation of Akt through induction of PI3K activity that involves tyrosine kinase and reactive oxygen species. In addition, activation of Akt may allow for a transient specific synthesis of ubiquitin ligases and proteasome subunits through the Akt/mTOR/p70S6K pathway.

PIF induced an incremental decrease in eIF4F active complex formation in murine myotubes which was also observed in weight losing MAC16 tumour bearing mice due to hypophosphorylation of 4E-BP1. This may be due to a reduction in the phosphorylation of the mammalian target of rapamycin (mTOR), which may also be responsible for the decreased phosphorylation of the 70kDa ribosomal S6 kinase (p70S6K). There was also an increase in the phosphorylation of the elongation factor eEF2 in myotubes and skeletal muscle. Thus weight loss in MAC16 mice is associated with a depression of protein synthesis via decreased binding of mRNA to the 43S preinitiation complex and a decrease in translation elongation.

## Chapter 7

# Effect of ROS and $\text{Ca}^{2+}$ Release in the Pathogenesis of Cachexia

### 7.1 Introduction

#### 7.1.1 Oxidative Stress

Dysregulation of the oxidative balance has been linked with the pathogenesis of many chronic diseases such as Duchenne dystrophy, infectious diseases like sepsis and HIV, diabetes, Alzheimer's and cancer, all of which exhibit muscle wasting and loss of body weight as a clinical feature of disease progression [128].

Oxidative stress is due to an imbalance of oxidant production to antioxidant production which is normally under tight control. The source of oxidants is mostly derived from enzymatic chemical reactions that produce superoxide anion, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), or nitric oxide (NO). These species undergo conversion



to highly reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS can damage critical cellular components which may mark myofibrillar protein for degradation or stimulate expression and activity of skeletal muscle protein degradation pathways. For example direct application of  $H_2O_2$  to C2C12 myotubes increases expression of E2<sub>14k</sub>, atrogin and MuRF1 which are E3 ligases involved in adding ubiquitins to target proteins [128]. Furthermore, both ROS [128] and RNS [12] have been shown to activate the ubiquitin proteasome proteolytic pathway through the upregulation of NF- $\kappa$ B mobility, which would lead to the muscle atrophy observed in the chronic diseases mentioned previously.

This mechanism has been demonstrated [71], where mild oxidative stress increased protein degradation in skeletal muscle by causing an upregulation of the major components of the ubiquitin proteasome pathway. ROS has also been shown to alter contractile regulation by reducing muscle force, which may contribute to muscle weakness, which is common in chronic disease patients demonstrating weight loss through loss of muscle mass [15].

The pathological effects of oxidative stress on skeletal muscle may be mediated by multiple mechanisms. For example cytokines like IL-6, TNF- $\alpha$  and IFN- $\gamma$  may promote oxidative stress through activation of peripheral leukocytes which invade tissues and produce excess oxidants. Alternatively inflammatory cytokines can interact with muscle receptors to initiate catabolic stimuli and ROS may act as second messengers. Cytokines also have an effect on appetite (discussed in Section 1.7). Malnutrition results in a lack of antioxidants gained from feeding (discussed in Section 1.8), which would prevent the compensatory rise of antioxidants in response to oxidants, resulting in oxidative stress. Glucocorticoids, which are involved in starvation induced muscle atrophy and are elevated in sepsis, can also induce oxidative stress. External factors such as treatment of cancer with chemotherapy or radiation produces an increase in oxidative stress, both directly, and through nausea induced malnutrition, while inactivity or mobilization due to chronic illness leads to atrophy which is strongly linked to oxidative stress [128].

Cellular antioxidants consist of oxidant scavengers and antioxidant enzymes that convert free radicals to more benign molecules. Scavengers include vitamins C and E. These molecules are able to donate an electron and neutralize free radicals. Alternatively, thiol containing compounds such as glutathione



and thioredoxin are oxidized by free radicals and are subsequently converted to their reduced forms by glutathione reductase or thioredoxin reductase using NADPH as a cofactor [128].

ROS and RNS production appear to be important in the multistage processes of carcinogenesis and tumour progression, where they are mainly involved in DNA damage leading to mutations in tumour suppressor genes. Although antioxidant activity is upregulated in a number of cancer cells protecting them from oxidative stress, the host cells are more vulnerable to oxidant attack, accordingly signs of oxidative stress in animal models are evident in plasma and other tissues including the skeletal muscle [15, 128].

PIF has been shown to reduce the stability of the eIF4F complex of initiation factors by dephosphorylating 4E-BP1 and decreasing the amount of eIF4G associated with eIF4E as well as phosphorylating eEF2 (see Chapter 6). All these effects would result in the depression of protein synthesis and possible activation of apoptosis via the upregulation of 4E-BP1 activity. Both these instances would result in a loss of skeletal muscle mass. Oxidative stress has been demonstrated to increase binding of 4E-BP1 to eIF4E thus decreasing the association with eIF4G, while inhibiting the p70S6 kinase and phosphorylating the eEF2 [135]. PIF has also been shown to upregulate the expression of activated PKR and eIF2 $\alpha$  (see Chapter 4 and 5). Generation of ROS has been shown to contribute to NF- $\kappa$ B activation by the PKR pathway, where inhibition of PKR and its downstream molecules such as eIF2 $\alpha$  was observed upon treatment with the antioxidants, vitamin C and E. PKR may be influenced by ROS through the alteration of p38 MAPK which is a virus-independent activator of a cellular protein called PKR-activating protein or PACT [179]. These results imply that PIF and oxidative stress share a common mechanistic pathway and that the two pathways may coincide.

Antioxidants such as melatonin, an oxidant scavenger, stimulates the activity of glutathione peroxidase, SOD and catalase. It has been shown to reduce oxidative stress in diabetic patients and has anti-tumour effects that improve survival in patients with advanced cancer [128]. Other antioxidants such as vitamin E, A and C have been combined with anti-inflammatories to treat cachectic cancer patients. These patients showed a significant increase in lean body mass, a significant decrease in ROS, IL-6 and TNF, and improved quality of life [114]. These results may have an important impact on the development of future treatments

for chronic diseases associated with muscle atrophy and cachexia and may even have a preventative effect, where early intervention with antioxidants reduces the risk of developing Alzheimer's and treatment with an oxidant scavenger reduced tumour mass [128].

It is also important to note that generation of ROS is likely to be involved in the regulation of calcium controlled intracellular signalling pathways in muscle cells, since treatment of cells with H<sub>2</sub>O<sub>2</sub> induced a transient rise of intracellular calcium [62]. Calcium release has also been linked to various catabolic diseases and will be discussed in the next section (Section 7.1.2).

### 7.1.2 Calcium

The ubiquitin proteasome is believed to be the major proteolytic machinery involved in the breakdown of skeletal muscle proteins in the cachexia syndrome, which is frequently concomitant with many chronic diseases such as sepsis, AIDS and cancer [48]. The evidence is conflicting however, because it is mainly the myofibrillar proteins that are degraded in cachectic muscle, and the proteasome is unable to degrade intact myofibrils. This suggests that actin and myosin need to be detached from the myofibrils before they can be ubiquitinated and degraded by the proteasome. Some studies have shown the involvement of a calcium dependent proteolytic system [77]. Calcium dependent proteolysis relies on the activity of cysteine proteases called calpains (calcium activated neutral protease). Calpains are activated when intracellular calcium concentrations rise, inducing calpains to translocate to the plasma membrane where they are activated by calcium and phospholipids. Calcium binding is likely to cause a conformational change which allows the assembly of the catalytic active site [48]. Calpains are suggested to be involved in the physiological maintenance of skeletal muscle mass, exerting a regulatory influence rather than having a digestive function. Calpains are necessary to initiate degradation of myofibrillar proteins, while they are not involved in bulk degradation of sarcoplasmic or sarcolemmal proteins. However, since myosin is specifically degraded in the catabolic process observed in cachexia, this observation does not rule out a role for calpains in the pathogenesis of cachexia.

It is possible that, in cachexia, an early event in the breakdown of skeletal muscle involves the release of myofilaments from the myofibrils by a calcium dependent mechanism, which is followed by ubiquitination and degradation by the 26S proteasome [77]. Release of myofibrils may activate the proteasome through a production of substrates. Modulations in intracellular calcium concentrations have been linked to the atrophy of muscle in several diseases including muscular dystrophies [48], denervation atrophy, burn injury and sepsis. These observations are important from a clinical standpoint because they suggest that calcium antagonists may be a potential treatment for the prevention of muscle wasting in many catabolic conditions since, although not identical, the intracellular mechanisms and molecular regulation of muscle atrophy are similar in many of these conditions [77]. For example treatment of rats with calcium antagonists inhibited muscle proteolysis in various catabolic conditions and rats dosed with a substance that blocks calcium release from the sarcoplasmic reticulum prevented sepsis induced increase in muscle protein breakdown [121].

Increased cellular calcium levels exert numerous biological effects, not only affecting the activation of proteolytic enzymes such as the calpains, but also various kinases [121], such as protein kinase C, calcium/calmodulin dependent protein kinase IV (camK) [48] and PKR [144]. Thus, the involvement of calcium release in pathological diseases associated with muscle protein depletion may not solely involve the activation of calpains.

ROS/RNS generation and calcium release may provide a separate mechanism for myosin release and muscle wasting, alternatively both stimuli have been shown to activate PKR and therefore may be involved in the PIF induced activation of PKR, rather than being separate from it.

The aim of this study was to test the hypothesis that oxidative stress and calcium are involved in generation of the pathological symptoms of cachexia including an increase in protein degradation, an increase in protein synthesis and an upregulation of proteasomal activity. The involvement of the PKR/eIF2 $\alpha$  pathway was also tested in respect to calcium alterations.

## 7.2 Results

Muscle wasting in AIDS and cachectic cancer patients is closely associated with increased expression of two proinflammatory cytokines,  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$ . These cytokines can interfere with muscle growth and regeneration through the production of ceramide. Intracellular ceramide is elevated in chronic diseases, such as AIDS and cancer, and can be synthesized through a neutral sphingomyelinase (N-SMase) and/or an acidic sphingomyelinase (A-SMase) pathway [173]. Since PIF is a major stress factor involved in the genesis of cancer cachexia, and has been shown to have similar actions to cytokines and induce the production of cytokines itself [167], it is possible that it is also involved in the induction of ceramide production. To explore the possibility that PIF mediates some of its actions through the release of ceramide, murine myotubes were pre-treated with an N-SMase inhibitor, GSH and a A-SMase inhibitor, D609, and protein synthesis determined (Section 2.3.15 for method) in response to PIF.

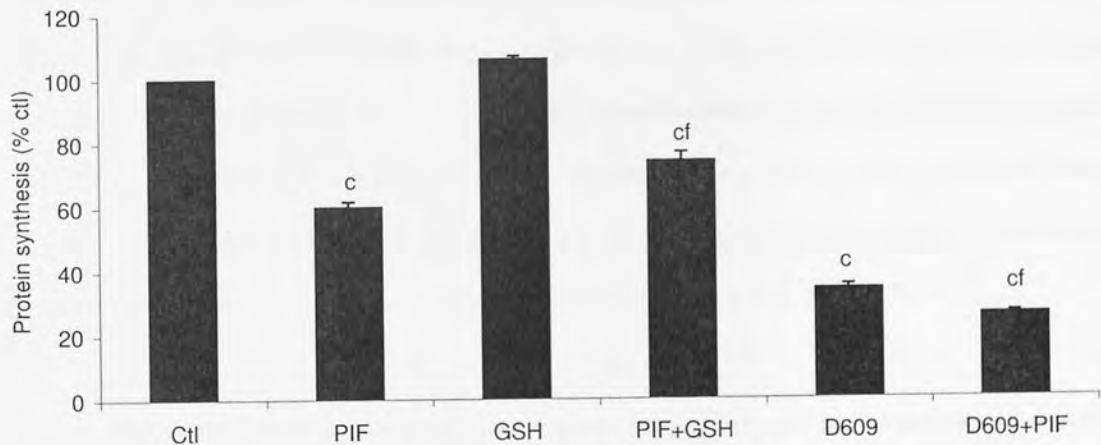


Figure 7.1: **Effect of glutathione and D609 on protein synthesis when co-treated with PIF.** Glutathione (GSH) (3mM) or D609 (50 $\mu\text{g}$ ) was added 2h prior to PIF (4.2nM), which was incubated with the myotubes for a further 4h. Error bars represent  $\pm\text{SEM}$  of 3 replicate experiments. Differences from control are shown as c:  $p < 0.001$  and differences from PIF alone f:  $p < 0.001$

As previously observed (Chapter 3), PIF caused a reduction in protein synthesis by approximately 40% (Figure 7.1). This was not reversed by the co-treatment with GSH or D609 implying that PIF does not

reduce protein synthesis through production of ceramide. Co-treatment of D609 and PIF had a cumulative effect causing a significant reduction in protein synthesis compared to PIF alone suggesting that the two agents affected two separate signalling pathways. However, pre-treatment with GSH increased protein synthesis in the presence of PIF significantly above PIF alone. Oxidative stress caused by generation of reactive oxygen species (ROS), is associated with an early and highly specific decrease in the antioxidant glutathione (GSH) [181]. Reports have shown that oxidative stress, GSH and N-SMase activity are tightly linked [173], suggesting that PIF may partially decrease protein synthesis through oxidative stress.

Production of free radicals has been proposed as a causative mechanism of cancer-induced cachexia. Oxidative cellular damage in rats bearing the ascites hepatoma Yoshida AH-130 has been associated with the process of muscle wasting, involving the reduction of muscular force and a ROS mediated upregulation of the ubiquitin proteasome pathway, resulting in an accelerated rate of protein degradation and muscle mass loss [15]. Bearing this in mind, it is possible that an increase in ROS production in mice bearing the cachexia inducing MAC16 tumour may be linked with muscle atrophy and weight loss. To explore this hypothesis, MAC16 tumour bearing mice were treated with a ROS inhibitor, vitamin E, over 4 days and the gastrocnemius and soleus muscle removed for experimentation (procedure described in Method Section 2.3.1). The effect of ROS inhibition on the catabolic mechanisms of cachexia in skeletal muscle of MAC16 tumour bearing mice were determined by measuring rates of protein synthesis, tyrosine release and muscle weight using the methods described in Methods Section 2.3.16 and 2.3.20.

ROS production appears to be important in tumour progression. Host cells are vulnerable to the oxidant attack as demonstrated in Figure 7.2A, B and C, where inhibition of ROS has a protective hypertrophic effect on skeletal muscle. However antioxidant activity is upregulated in a number of cancer cells protecting them from oxidative stress [15, 128]. Reversal of oxidative stress through the use of antioxidants was shown to have anti-tumour effects and improve survival of patients with advanced cancer [128], illustrating the importance of ROS production upon tumour growth. To examine the role of free radicals on tumourigenesis in the MAC16 cachexia model, tumour growth was measured over a 4 day period in mice bearing the MAC16 tumour treated with vitamin E.



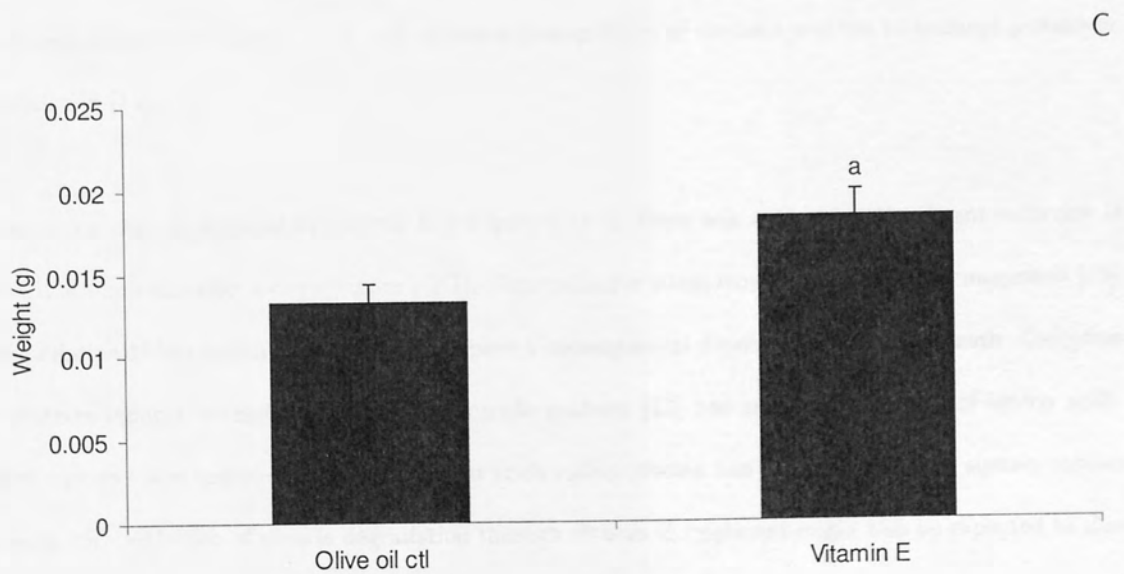
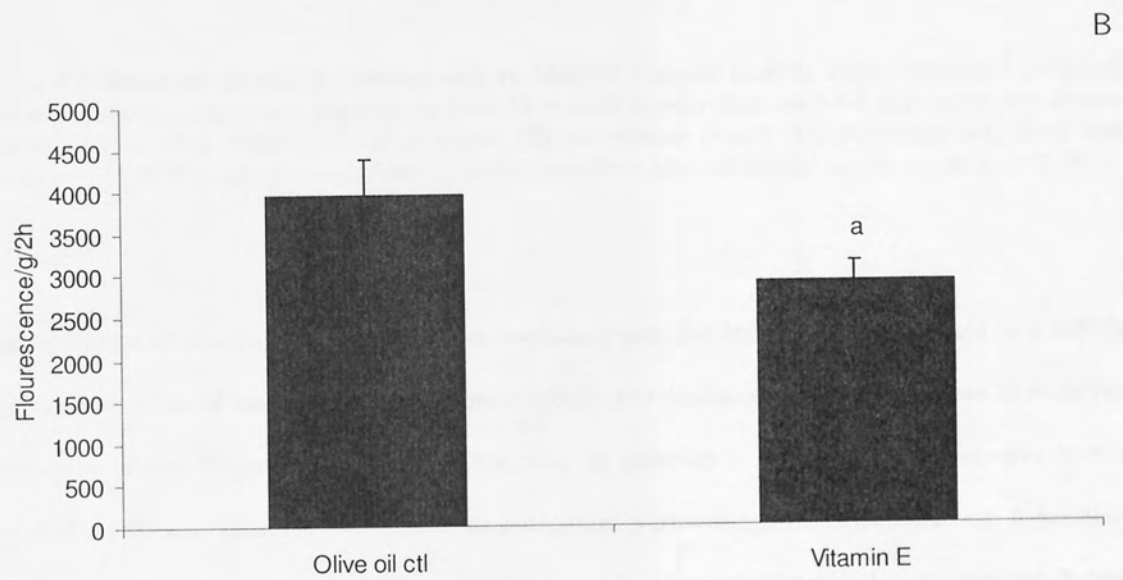
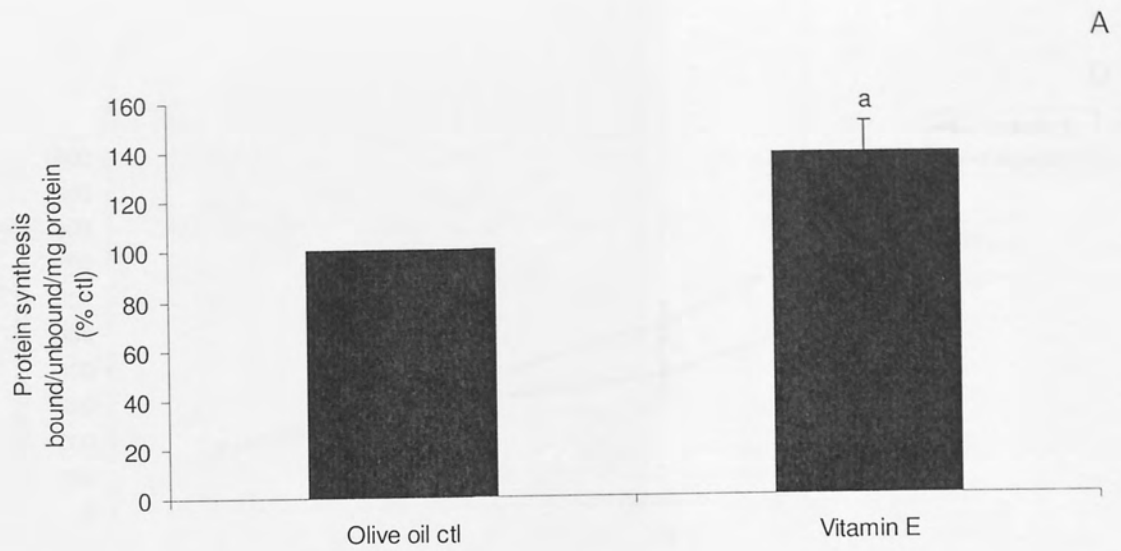


Figure 7.2



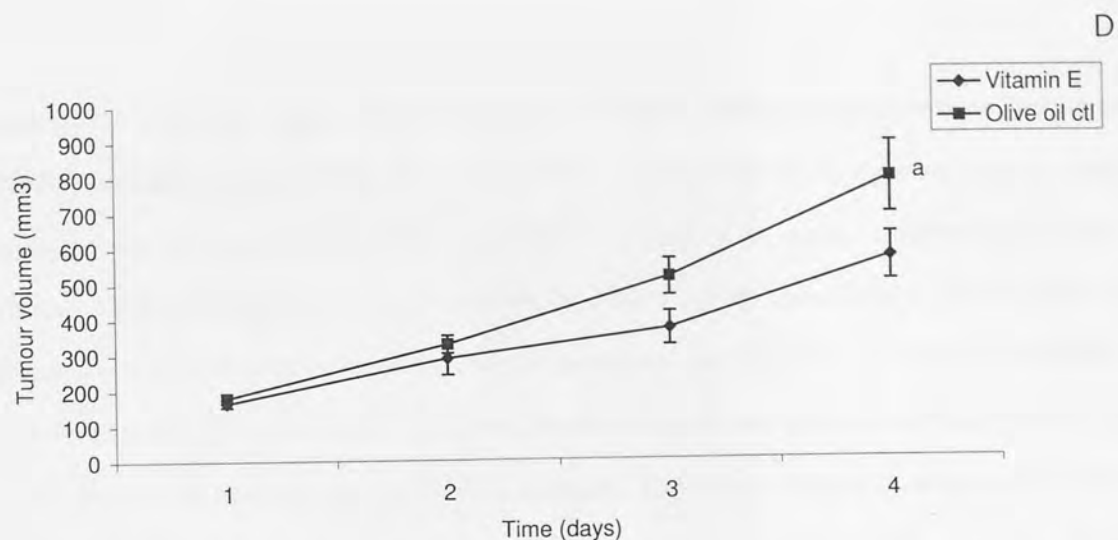


Figure 7.2: **Effect of vitamin E administered to MAC16 tumour bearing mice.** Vitamin E (1mg/kg) was administered daily by s.c. injection to MAC16 tumour bearing mice over a 4 day period and protein synthesis (A) tyrosine release (B), soleus weight (C) and tumour growth (D) were measured. Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences from olive oil control are shown as a:  $p < 0.05$

Administration of vitamin E to cachectic mice implanted with the MAC16 tumour resulted in a slightly significant elevation of protein synthesis and muscle bulk, and decreased protein degradation as measured by tyrosine release (Figure 7.2). This suggests that, as observed *in vitro*, catabolic mediators have a partial effect through generation of reactive oxygen species and production of oxidative stress. It has been hypothesized that muscle wasting in response to free radicals is generated by oxidative damage contributing to skeletal muscle dysfunction [128] and increased susceptibility of oxidised proteins to undergo proteolytic degradation [15].

As with the changes induced by vitamin E in Figure 7.2A-C, there was a small but significant reduction in MAC16 tumour size after 4 days (Figure 7.2D). Since oxidative stress may be involved in carcinogenesis [15], the inhibition of free radical production could have a consequential depression in tumour growth. Oxidation of proteins induces excessive proteolysis of muscle proteins [12] and subsequent release of amino acids. Some human colon tumours require the amino acids valine, leucine and isoleucine [185] to sustain tumour growth, thus inhibition of muscle degradation through vitamin E treatment might also be expected to slow

tumour progression.

Accumulation of reactive oxygen species and exposure of cells to oxidative stress eventually leads to an increased intracellular calcium [181]. This calcium influx was also observed in myotubes exposed to the superoxide anion hydrogen peroxide [62], suggesting that calcium is an important downstream mediator of oxidative stress. Oxidative stress has been shown to increase protein degradation in skeletal muscle by upregulating the major components of the ubiquitin proteasome pathway [15]. To elucidate whether the catabolic actions of PIF were mediated through an increase in calcium and increased proteasome proteolytic activity, the calcium chelator BAPTA/AM was employed. Expression of the proteasome subunits were determined by western blotting (described in Methods Section 2.3.13 and 2.3.21) and proteasome activity was established by measuring chymotrypsin like activity (see Section 2.3.6 for method) in murine myotubes incubated with PIF in the presence and absence of BAPTA/AM.

A

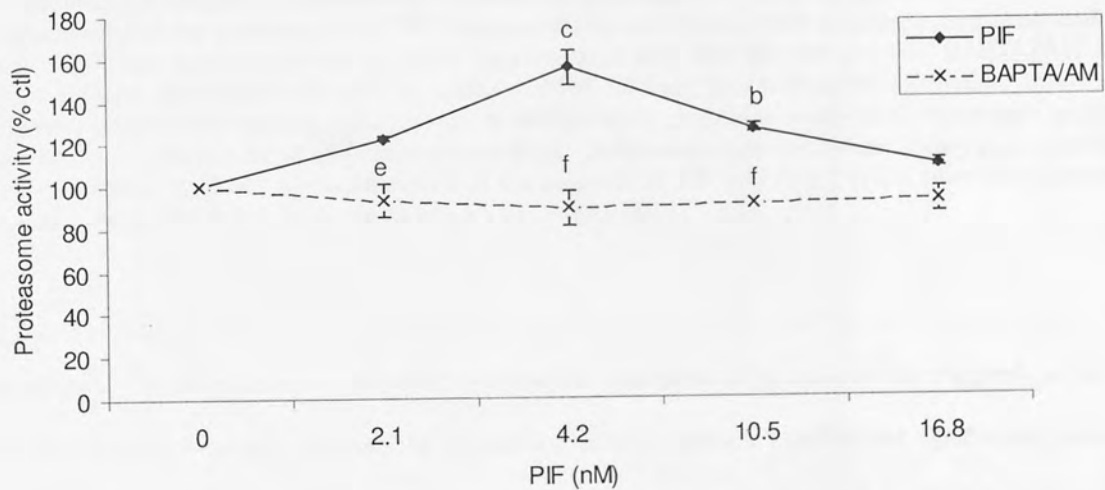


Figure 7.3

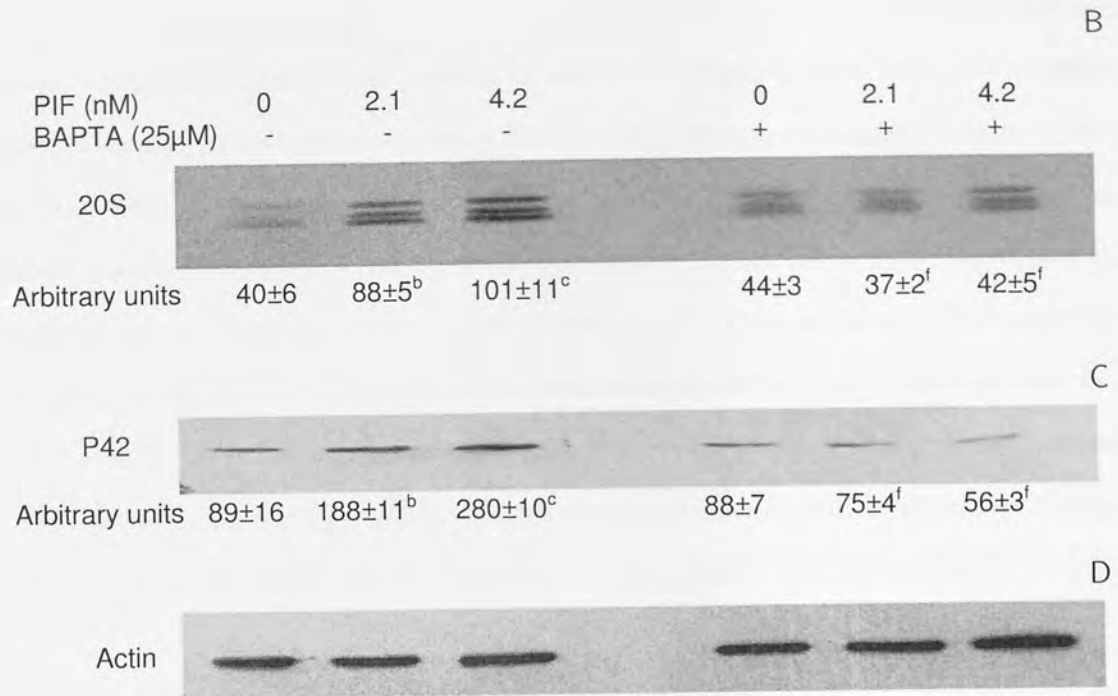


Figure 7.3: **Effect of the calcium chelator BAPTA/AM on proteasome activity and the expression of proteasome subunits in murine myotubes co-treated with PIF.** Chymotrypsin like activity (A) and the expression of the  $\alpha$ -subunit of the 20S proteasome (B) and P42 (C) with a corresponding actin loading control (D) was measured in murine myotubes co-treated with BAPTA/AM and PIF. BAPTA/AM was incubated with myotubes for 2h prior to addition of PIF followed by a further 24h incubation. Error bars represent  $\pm$ SEM of 3 replicate experiments. A densitometric analysis is shown underneath each western blot where  $\pm$  represent SEM of three replicate blots. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$  while differences in the presence of PIF and BAPTA/AM from corresponding treatment with PIF alone are shown as e:  $p < 0.01$ , f:  $p < 0.001$

Upregulation of the proteasome enzymatic activity and expression of its subunits are triggered by an influx of intracellular calcium, because the intracellular calcium chelator BAPTA/AM significantly reduced chymotrypsin like activity, 20S and P42 proteasome subunit expression (Figure 7.3). Apoptosis is an important factor in cancer cachexia and is associated with the muscle wasting observed. Programmed cell death involving calcium and subsequent activation of PKR has been observed in brains of Alzheimer's patients [174]. A requirement for PKR autophosphorylation in PIF induced proteasome activity was illustrated in previous chapters (Chapter 4) and the results of this chapter suggest that calcium may be the activating stimuli.

Protein synthesis was attenuated in rat livers perfused with medium containing vasopressin, and an inhibition of peptide chain initiation was implicated in this action by a calcium dependent mechanism. Inhibition of protein synthesis by vasopressin was proposed to occur at the formation of the pre-initiation complex through the phosphorylation and sequestration of the eIF2 $\alpha$  [96]. Other studies suggest that calcium influx occurs downstream of eIF2 $\alpha$  and can induce programmed cell death in neuropathological disorders, such as Alzheimer's and Parkinson's disease [40]. Phosphorylation of eIF2 $\alpha$  has been demonstrated previously to be an important contributing factor in the production of cancer cachexia (Chapters 4 and 5). A depression of protein synthesis and apoptosis are downstream events stimulated by eIF2 $\alpha$  phosphorylation and both can also be induced by calcium release. To examine the role that calcium plays in regulating protein synthesis and eIF2 $\alpha$  phosphorylation in response to PIF, murine myotubes were incubated with the calcium chelator BAPTA/AM and assayed using the methods described in Section 2.3.15 and 2.3.13 and 2.3.21.

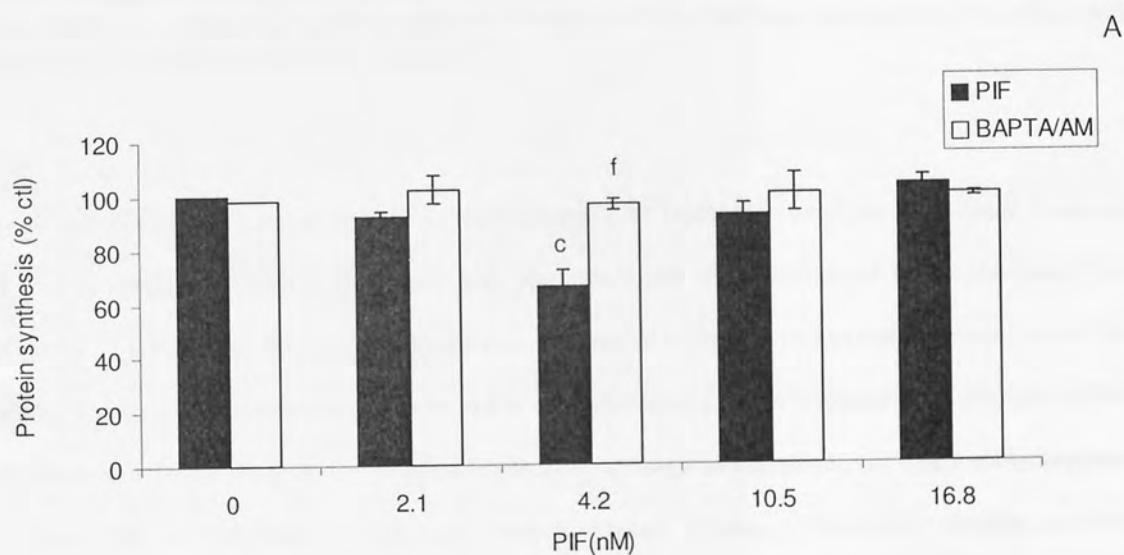


Figure 7.4

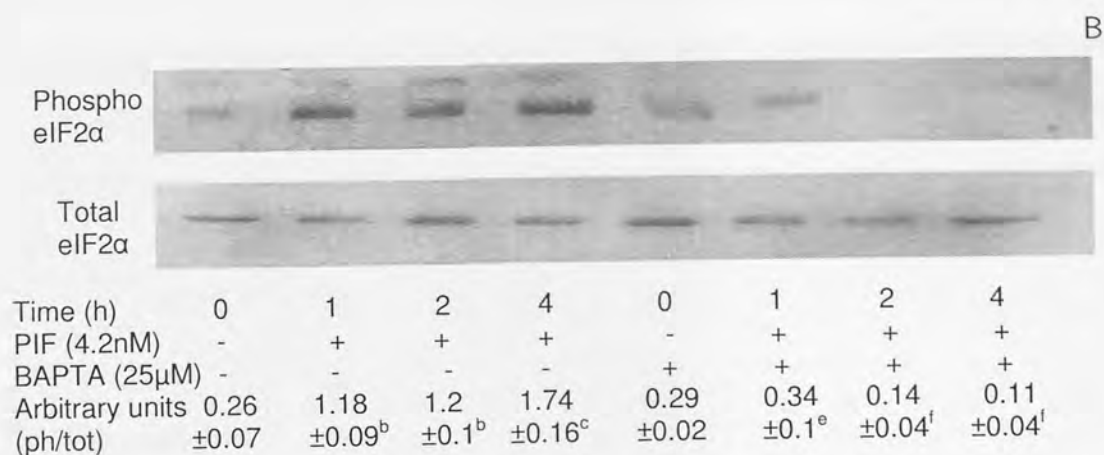


Figure 7.4: **Effect of the calcium chelator BAPTA/AM on protein synthesis and phosphorylated eIF2 $\alpha$  in murine myotubes in response to PIF.** The effect of BAPTA/AM (25 $\mu$ M) was analysed on protein synthesis (A) and phosphorylation of the  $\alpha$ -subunit of eIF2 with a corresponding eIF2 $\alpha$  total (which was used to normalise the phosphorylated protein) (B) in murine myotubes in response to PIF. BAPTA/AM was incubated with myotubes for 2h prior to addition of PIF followed by a further 4h incubation. Error bars represent  $\pm$ SEM of 3 replicate experiments. A densitometric analysis is shown underneath the western blot where  $\pm$  represent SEM of three replicate experiments. Differences from control are shown as b:  $p < 0.01$ , c:  $p < 0.001$  while differences in the presence of PIF and BAPTA/AM from corresponding treatment with PIF alone are shown as e:  $p < 0.01$ , f:  $p < 0.001$

Incubation of murine myotubes with the calcium chelator BAPTA/AM inhibited the PIF induced depression in protein synthesis (Figure 7.4A), which was associated with the inhibition of eIF2 $\alpha$  phosphorylation (Figure 7.4B). PKR was reported as the upstream activator of eIF2 $\alpha$ , which was itself activated by calcium release from the endoplasmic reticulum in brains of Alzheimer's patients in response to  $\beta$ -amyloid [174]. Therefore it is highly likely that PKR autophosphorylation was also the stimuli for eIF2 $\alpha$  phosphorylation in Figure 7.4B, since this has been proven in previous chapters (Chapter 4) in murine myotubes incubated with PIF. However the antibody for phospho PKR used previously was no longer commercially available and no other acceptable alternatives were identified, thus further analysis of PKR involvement was prohibited.

The evidence suggests that PIF exerts its catabolic effects both in vitro and in vivo through a series of events that include the depletion of GSH, accumulation of ROS and eventual rise in intracellular calcium, which can increase protein degradation through an increase in the proteolytic activity of the ubiquitin proteasome pathway, either directly, or via PKR, and inhibit protein synthesis through phosphorylation of eIF2 $\alpha$ , which also may or may not involve PKR. An overproduction of oxidants induced by catabolic

mediators such as PIF would overwhelm the antioxidant defence mechanisms resulting in cell, organ and tissue damage and muscle wasting. The results show that by replenishing components of the antioxidant defence such as GSH and vitamin E the balance of oxidant to antioxidant may be restored to a level that can counterbalance and neutralise the free radicals, attenuate skeletal muscle atrophy and inhibit tumour growth.

### 7.3 Discussion

Underlying the pathogenesis of chronic disease is the state of oxidative stress which is often accompanied by muscle wasting, suggesting a causal relationship between the generation of free radicals and cachexia. It has been hypothesized that catabolic programmes leading to muscle wasting are mediated by oxidative stress and the ensuing release of intracellular calcium [128]. The results illustrated in this chapter substantiate this hypothesis to a degree in that treatment with ROS inhibitors, GSH and vitamin E, are partially effective at reversing the depression in protein synthesis by the catabolic mediator PIF, and attenuating muscle wasting in cachectic mice through an elevated protein synthesis and diminished protein degradation in skeletal muscle. Administration of vitamin E was also able to inhibit the progression of tumour growth, suggesting that the generation of ROS had a positive effect on tumourigenesis.

PIF is the product of the MAC16 cachexia-inducing tumour, which induces muscle atrophy through a depression in protein synthesis and an elevation of protein degradation. Recently Ang II has been shown to exert a similar effect, and the angiotensin-converting enzyme (ACE) inhibitor, imidapril, has been shown to attenuate the development of cachexia in MAC16 tumour bearing mice [152]. To emphasize the results of this chapter, Ang II can stimulate superoxide radicals through the activation of a membrane bound NAD(P)H oxidase in vascular smooth muscle cells. Superoxide conversion to hydrogen peroxide ( $H_2O_2$ ) was shown to be important to some of Ang II mediated effects [21]. Furthermore,  $H_2O_2$  can activate PKR [134], supplying a possible means by which Ang II could activate PKR. Although PIF and Ang II act through different receptors they both stimulate similar catabolic mechanisms [155], suggesting that PIF



has the potential to stimulate ROS production and PKR activation through a comparative method.

Free radicals are normally counterbalanced by antioxidative mechanisms but in cachectic states there is a diminished nutritional status and subsequent depletion of certain antioxidants, such as glutathione synthetase, which are produced from components in the diet, such as glucose [112]. Thus the levels of oxidants in cancer cachexia patients would outweigh the counter attack from neutralising antioxidants. This implies nutritional supplementation may be effective at combating muscle wasting as a result of oxidative stress. In fact a phase II study combining antioxidants with pharmaconutritional support in cachectic cancer patients caused an increase in body weight and lean body mass demonstrating the importance of oxidative stress in cancer related wasting. Moreover there was an increased expression of mRNA for the NADPH oxidase protein components, p47phox, p40phox and p67phox, together with a decrease in expression of superoxide dismutase in the skeletal muscle of weight losing mice bearing the MAC16 tumour. This suggests a potential increase in the production of superoxide with the development of cachexia, together with a reduced ability to detoxify it [152].

Reactive oxygen species can damage critical cellular components such as DNA. It should be noted that PKR is involved in the cellular response to genotoxic stress, where DNA damage by cisplatin, melphalen and UV radiation can induce its activation. PKR may regulate cellular response to DNA damage by regulating DNA repair mechanisms. MDA-protein adducts were markedly increased in the muscles of tumour induced cachectic animals through a ROS mediated mechanism, since the antioxidant D- $\alpha$ -tocopherol abrogated this increase and prevented muscle wasting [15]. PKR has been shown to have a function in regulating the cellular response to bulky-adduct inducing agents [20] and thus may be upregulated in response to MDA-protein adducts formed by free radical production. Although strong evidence has been presented for the involvement of PKR in muscle wasting (Chapters 4 and 5), this is unlikely to be the main activator of PKR phosphorylation since the attenuation of catabolic mechanisms in MAC16 mice treated with Vitamin E was only slightly significant, whereas inhibition of PKR by a PKR inhibitor (Chapter 5, Section 5.2) had highly significant effects in both MAC16 tumour bearing mice and murine myotubes treated with PIF. Moreover, in dendritic cells generation of ROS has been described as a consequence of PKR activation [179] rather than an activator of it. However Vit E can also be a pro-oxidant at high dosage [137] and thus it

is difficult to gauge its plasma levels correctly to induce anti-oxidant activity *in vivo*. Therefore vitamin E may be more effective than is demonstrated in this study, according an interest for further research into the use of vitamin E as an antioxidant and anti-tumour agent in MAC16 tumour bearing mice.

Oxidative stress is associated with muscle wasting in many catabolic conditions including arthritis, cancer, ageing, heart failure, infectious diseases and Alzheimer's [128], all of which have also been linked with an increased PKR activity [70, 58, 104, 59, 133, 219]. Therefore PKR may be a critical second messenger in oxidative stress induced muscle wasting. One study reported an inhibition of PKR and its downstream molecules such as eIF2 $\alpha$  in dendritic cells treated with vitamin C and E [179], suggesting that eIF2 $\alpha$  is also involved in PKR mediated signalling in response to ROS generation, which may explain the attenuation of protein synthesis depression in mice treated with vitamin E. Conversely, another eIF2 $\alpha$  kinase, HRI, can be activated directly by nitric oxide [97] and may serve to sense cytoplasmic oxidative stress and thus inhibit translation in response to ROS [145].

Treatment with vitamin E has been shown to inhibit the NF- $\kappa$ B pathway as well as intracellular ROS, PKR, eIF2 $\alpha$  and PKC [179], all of which have been implicated in the genesis of cachexia. Thus treatment with vitamin E can potentially attenuate most of the signalling pathways that have involvement in the induction of cachexia by both PIF and Ang II.

The PIF induced inhibition of protein synthesis was reversed in response to the calcium chelator BAPTA/AM, and this was linked with the inhibition of eIF2 $\alpha$  phosphorylation by BAPTA/AM. Depletion of calcium from the endoplasmic reticulum and the resultant rise in intracellular calcium levels correlates with inhibition of protein synthesis and increased eIF2 $\alpha$  phosphorylation in a PKR dependent manner [171]. Thus the increase in calcium and resulting depression of protein synthesis in response to PIF may be mediated through PKR.

Calcium influx is part of the signalling cascade activated by oxidative stress. eIF2 $\alpha$  phosphorylation can result in resistance to oxidative stress. The results shown in Chapter 4 demonstrate that eIF2 $\alpha$  phosphorylation was upregulated in MAC16 tumour cells. Tumour cells have resistance to oxidative stress, where

the presence of ROS may actually prove beneficial for tumour progression, since neutralisation of ROS by vitamin E treatment inhibits tumour growth. This evidence suggests that the tumour cells may actually upregulate eIF2 $\alpha$  as a protective mechanism, since it can regulate the major intracellular antioxidant, GSH [181]. eIF2 $\alpha$  may act as a switch which dictates whether a cell activates a survival response or follows a cell death pathway [54]. eIF2 $\alpha$  has been shown to protect nerve cells from oxidative stress induced cell death by inhibiting GSH depletion, and inhibiting the rise in ROS and intracellular calcium. In MAC16 tumours there is an upregulation of the PKR/eIF2 $\alpha$  and NF- $\kappa$ B pathway (Chapter 5) but no increase in proteasome subunit 20S (results not shown), as was seen in skeletal muscle cells in vitro and in vivo (Chapters 4 and 5). This suggests that a different mechanism works in the skeletal muscle which, rather than eIF2 $\alpha$ /NF- $\kappa$ B having an antiapoptotic effect, they stimulate degradation and apoptosis instead. An explanation for the difference observed might be that implantation of the tumour cells and effect of whole body processes affects the signalling pathways resulting in muscle protein breakdown to nourish the tumour and stimulate growth while protecting the tumour from the same proteolytic process. This points to a central role of eIF2 $\alpha$  as a translational switch in the control of oxidative stress [181].

One of the most characterised signalling pathways of both PIF and Ang II (linked to AT1 receptor activation) is that of the protein kinase C (PKC) pathway, involving stimulation of phospholipase C (PLC), with subsequent inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) generation, leading to the activation of PKC [167, 73]. Calcium mobilization is part of the signalling transduction cascade generated by IP3 [171], hence supplying a method by which both PIF and Ang II can stimulate a rise in intracellular calcium and consequent PKR activation. It is possible that the rise in intracellular calcium induces a unique dsRNA PKR activator. Increased cytosolic calcium may alter the secondary structure of some RNA molecules to become good PKR activators [171]. Moreover, ER calcium release has been shown to activate caspase 3 [174], which may be the intermediate effector linking PIF/Ang II induced calcium release to PKR activation.

As mentioned above, PKC is part of the signalling cascade activated by PIF. One study [220] showed a PKC activator to decrease levels of autophosphorylated PKR through proteasomal targeting and subsequent proteolysis in a manner that was deemed PKC dependent. This is contradictory, since both PKC and PKR are activated in response to PIF and Ang II. It is possible that PKR phosphorylation occurs prior to, or

concurrently with the activation of PKC. Moreover it was shown that autophosphorylated PKR in cells infected with a virus is relatively stable and resistant to proteolysis, which may protect PKR from PKC mediated downregulation [220]. However other studies have suggested that PKC is a substrate of PKR rather than vice versa [153]. It is obvious that further investigation is required to determine the placement of these components in the PIF/Ang II induced signalling cascade.

## 7.4 Conclusion

To determine whether ROS formation was important *in vivo*, in the development of cachexia, mice bearing the MAC16 tumour were treated orally every day with vitamin E (1mg kg<sup>-1</sup>) in olive oil. In comparison with olive oil controls, mice receiving vitamin E had a significant increase in protein synthesis together with a significant increase in soleus muscle wet weight. In addition there was a significant reduction in protein degradation in skeletal muscle. Furthermore mice receiving vitamin E showed a small, but significant, reduction in tumour growth rate compared with olive oil controls by day 4. These results suggest that ROS production is important in the development of cachexia and that therapeutic intervention with vitamin supplementation might be effective at reversing the skeletal muscle atrophy observed in cancer cachexia.

Inhibition of intracellular calcium release using the calcium chelator BAPTA/AM demonstrated that the phosphorylation and consequent depression in protein synthesis by PIF is mediated through a calcium dependent mechanism. Since both oxidative stress and calcium influx have been placed in the same catabolic signalling pathway it is likely that inhibition of either would prevent the PIF induced activation of PKR and subsequent eIF2 $\alpha$  phosphorylation and NF- $\kappa$ B DNA binding activity, which would in turn attenuate the ensuing muscle protein loss.

## Chapter 8

# Reversal of the Cachectic Effect by Anabolic Factors

### 8.1 Introduction

A depression in protein synthesis is a key regulatory component of skeletal muscle wasting in cachexia and is therefore an important therapeutic target for combating weight loss in patients with chronic catabolic diseases. Insulin, IGF and amino acids all lead to enhancements in protein synthesis in skeletal muscle [30], demonstrating their importance in alleviating the muscle atrophy associated with cachexia.

The synthesis of many mammalian proteins by the translational machinery is selectively regulated by mitogenic and nutritional stimuli. Regulation at the translational level allows a quick and readily reversible adaptation to ever changing physiological conditions. For example, upon a shortage of amino acid availability translation is rapidly arrested to avoid energy wastage, since most of the energy consumption during cellular growth is utilized for generating components of protein synthesis machinery [124].



Protein synthesis requires a correctly balanced mixture of amino acids, and a number of studies have noted widespread decreases in the plasma levels of free amino acids in patients with cachexia [130]. The maximum decreases are often found for the branched chain amino acids (BCAA) leucine, isoleucine and valine [16]. The BCAA, as well as being an integral component of skeletal muscle proteins, are uniquely able to initiate signal transduction pathways that modulate translation initiation [215].

Mammalian cells display a drop in protein synthesis upon depletion of medium amino acids, which is characterised by a loss of polysomes and an increased monomeric ribosome number, indicating a block in peptide chain initiation [76]. As mentioned previously (1.10) there are two major control points of translation initiation, the binding of the met-tRNA to the 40S ribosome and the binding of the mRNA to the 43S ribosome. The depletion of amino acids leads to the activation of GCN2, an eIF2 $\alpha$  kinase, which sequesters eIF2 $\alpha$  and eIF2B in an inactive complex preventing assembly of the 43S preinitiation complex. Amino acids are also positive regulators of mTOR signalling. Through mTOR activation amino acids can regulate the phosphorylation of p70S6 kinase, eEF2, 4E-BP1 and eIF4F complex assembly to promote protein synthesis [76] (see Section 1.10 and 6.1 for greater detail on these pathways). Amino acids indirectly modulate mTOR through tuberous sclerosis complex (TSC) 1 and TSC2, G $\beta$ L-raptor and/or RHEB [215]. Although other amino acids have been shown to increase signalling through mTOR, leucine is arguably the most potent of the amino acids in this process [97]. The exceptional potency of leucine could relate to the frequency of its utilization in protein synthesis and to the existence of multiple tRNAs for the amino acid arising from the 6-fold codon degeneracy [76]. Leucine has also been shown to induce protein synthesis and eIF4F formation in an mTOR independent manner [215], and since the ability of amino acids to control p70S6 kinase phosphorylation is only partially antagonized by rapamycin this suggests that p70S6K, at least in part, can also be regulated independently of mTOR [76].

Insulin and IGF-1 signalling to mTOR and the translational apparatus are believed to act through convergent pathways distinct from those controlled by amino acids [76]. Unlike amino acids, insulin and IGF-1 activate PI3K or Akt, which in turn phosphorylates and activates mTOR, leading to an upregulation of the active eIF4F complex and p70S6 kinase phosphorylation. Phosphorylation of Akt by growth factors also leads to the phosphorylation and inactivation of GSK3 which results in the dephosphorylation of the  $\epsilon$  subunit



of eIF2B, upregulating its activity and thus increasing the conversion of GDP to GTP on eIF2 $\alpha$  [30]. Circulating insulin and IGF levels are reduced in patients with cancer cachexia and some other catabolic conditions [25].

In Chapter 4, PIF was shown to activate PKR in vitro and in Chapter 5, PKR was also shown to be upregulated in gastrocnemius muscle of MAC16 tumour bearing mice with weight loss. It has been demonstrated that PKR can downregulate the gene expression of IGF-1 [37] and, in regard to the results mentioned above, this suggests that PIF may have a role in the downregulation of IGF in a PKR dependent manner. Some findings have identified a protein phosphatase called PP1<sub>c</sub> which modulates PKR activity via dephosphorylation and subsequent disruption of PKR dimers. There is evidence of the involvement of a cellular regulatory subunit of PP1<sub>c</sub> called glycogen-targeting subunit of PP1, termed PP1<sub>GL</sub>, which is expressed in heart and skeletal muscle.

Both PP1<sub>c</sub> and PP1<sub>GL</sub> are stimulated by insulin and some other growth factors [39], which implicates insulin in PP1<sub>c</sub> catalysed dephosphorylation of PKR. Interestingly, insulin has been found to reduce eIF2 $\alpha$  phosphorylation in chondrocytes possibly through the dephosphorylation of PKR [180]. Many of the signalling pathways regulated by amino acids overlap with those classically associated with cellular responses to hormones, such as insulin and IGF-1 [97], therefore the activation of PP1<sub>c</sub> by insulin may be mimicked by other nutrients suggesting a mechanism by which leucine can also affect the PKR/eIF2 $\alpha$  pathway.

Most treatments of cachexia have little effect on preventing muscle atrophy. Since anorexia accompanies cachexia and exacerbates the wasting syndrome, appetite stimulants such as megestrol acetate have been tried to attenuate weight loss. However these have not proved particularly effective in preserving lean body mass [128]. In contrast, nutritional supplementation with BCCA has been shown to be beneficial since they stimulate protein synthesis, inhibit protein degradation and are an important source of energy for skeletal muscle [128].

Mammals require an adequate supply of dietary essential amino acids (those that can not be synthesized de novo) to grow and thrive. Deficiency of one or more essential amino acids in the diet of rodents leads

to anorexia and loss of lean body protein, due to a depression in protein synthesis at the initiation stage of translation [7]. In animal models, administration of essential amino acids spare lean body mass during weight loss and promote muscle protein anabolism with ageing [128]. Leucine is believed to be the most effective among the branched chain amino acids to enhance protein synthesis in vitro, although isoleucine has also been shown to have a lesser effect [6]. Some studies have shown that leucine supplementation improves nitrogen balance, reduces skeletal muscle catabolism, increases skeletal muscle protein synthesis and maintains plasma amino acid concentrations [128], through augmentation of substrate availability [6] and activation of the mRNA binding step in translation initiation. Leucine has also been shown to stimulate insulin secretion, which would prove beneficial since circulating insulin levels are decreased in the cachectic syndrome [215]. In one study a leucine rich diet prevented the decrease in plasma insulin normally seen in tumour bearing rats [200]. The transient rise in leucine-induced serum insulin would facilitate the enhancement of protein synthesis by leucine [215].

In rat liver, a 10-fold increase in the concentration of all amino acids present in rat plasma results in an elevation of protein synthesis by 25%. However when leucine alone is increased in the liver the hepatic protein synthesis is reduced due to a eIF2B mediated reduction in overall protein synthesis even though eIF4F assembly and p70S6K phosphorylation are increased. It may be that cohort amino acids are required for a remaining signal necessary for activation of eIF2B and elevated protein synthesis [160]. It is important to note however, that the signalling pathways activated by leucine in muscle differ from those activated in liver, suggesting that the responses seen are tissue specific [215].

Dexamethasone represses protein synthesis in skeletal muscle associated with a decreased formation of the active eIF4F triad and increased assembly of the inactive 4E-BP1/eIF4E complex. Dephosphorylation of 4E-BP1 and p70S6K are also observed with dexamethasone treatment. However treatment of leucine to dexamethasone dosed rats reversed all these changes [98].

The expression of translation initiation factors eIF4E, eIF4G and p70S6 kinase increased in tumour bearing rats fed a leucine rich diet, as did protein synthetic rates, suggesting that oral administration of leucine induces protein synthesis in skeletal muscle through the activation of the eIF4F and p70S6 kinase pathway.

Since the leucine dependent stimulation of mRNA translation and protein synthesis was partly insensitive to rapamycin this suggests that leucine can increase protein synthesis through both an mTOR dependent and independent pathway [200]. In support of this, another study observed that leucine increased protein synthesis in diabetic rats without concomitant changes in the phosphorylation of 4E-BP1 or p70S6 kinase [5]. Another study identified that neither the phosphorylation of 4E-BP1, or p70S6K, nor enhanced binding of eIF4G to eIF4E associated with oral leucine administration, was sufficient to stimulate global rates of protein synthesis. Thus another mechanism must be involved in the effect, for example an increase in eIF2B phosphorylation or a depression in eIF2 $\alpha$  kinase activity [98].

In eukaryotic cells, leucine deprivation reduces translation by a mechanism involving phosphorylation of eIF2 $\alpha$  through the activation of GCN2 (discussed in Section 1.10). In nutrient limiting conditions eukaryotic cells decrease overall protein synthesis and increase rates of protein degradation by an autophagic pathway also involving eIF2 $\alpha$  and GCN2. PKR has also been known to functionally substitute for GCN2 in the control of starvation induced autophagy in yeast, raising the possibility that autophagy may be an evolutionary conserved mechanism of different eIF2 $\alpha$  kinase family members that have diverged to respond to different stimuli. Thus it is possible that leucine may affect other eIF2 $\alpha$  kinases [177] other than GCN2, when reintroduced after nutrient deprivation.

$\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB), a metabolite of leucine, has been shown to inhibit protein degradation and stimulate protein synthesis in the muscles of cachectic mice [163]. HMB, combined with L-arginine and L-glutamine, has undergone clinical evaluation in patients with cancer cachexia, and has been shown to stimulate an increase in body weight through an increase in lean body mass [119]. HMB has also been shown to increase body weight in human immunodeficiency virus (HIV)-infected patients with a weight loss of at least 5% [44].

Despite the above information, other studies have found no significant benefits with amino acid treatment [128]. For example administration of leucine to septic rats was unable to increase protein synthesis in gastrocnemius muscle. Sepsis markedly impaired the ability of leucine to stimulate mTOR and p70S6 kinase activation or eIF4F assembly [105].

Evidence suggests that anabolic stimuli have multiple recognition sites and multiple signalling pathways [97]. The aim of this study was to investigate a few of these pathways and evaluate their effect on the catabolic actions of PIF *in vitro*, and to determine the effect of nutritional supplementation on the catabolic pathways activated in MAC16 tumour bearing mice in specific regard to muscle protein preservation.

## 8.2 Results

Various forms of stress (trauma, thermal burn, sepsis, cancer etc) can affect the nitrogen balance and cause significant loss of body mass. The hypercatabolic state observed during those stresses is characterised by an increase of nitrogen loss but also by increased protein degradation and decreased protein synthesis. The amino acids freed by muscular proteolysis are mostly used by the liver to synthesise proteins involved in the inflammatory response, or utilised by the tumour in cases of cancer cachexia [89]. Branched chain amino acids (BCAA) have the unique ability to act as both, substrates of protein synthesis, and regulatory components of mRNA translation, thus marking them as important components in combating muscle wasting in cancer cachexia. Leucine independently stimulates protein synthesis in muscle. The remaining BCAAs, isoleucine and valine, are similar in structure to leucine, and like leucine, are degraded extensively in skeletal muscle. Therefore, dietary isoleucine or valine may also signal independently for enhanced rates of translation initiation in muscle. [4]. To determine the usefulness of BCAAs leucine, isoleucine and valine in the treatment of muscle wasting in cachexia, protein synthesis (Section 2.3.15) and degradation (Section 2.3.14) were measured in murine myotubes when co-treated with PIF, the catabolic agent purified from MAC16 cachexia inducing tumours (see Method Section 2.3.11 for purification protocol).

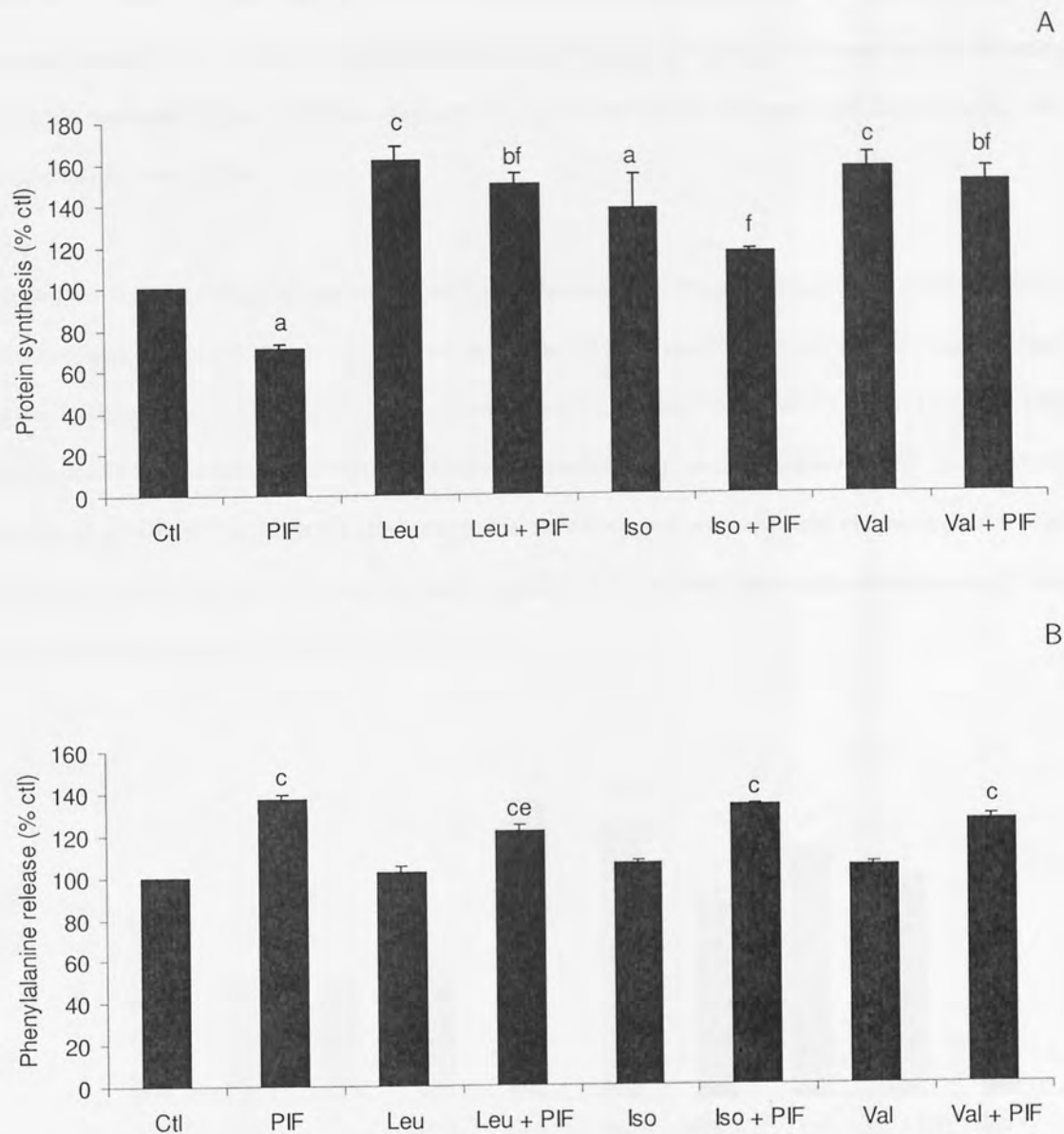


Figure 8.1: **Effect of branched chain amino acids (BCAA) on protein synthesis and protein degradation in murine myotubes when co-treated with PIF.** The branched chain amino acids (BCAA), leucine (Leu), isoleucine (Iso) and valine (Val) (2mM) were incubated with the murine myotubes for 2h prior to PIF, then PIF (4.2nM) was added and incubated for a further 4h for protein synthesis (A) or 24h for protein degradation (B). Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$  and differences from PIF alone e:  $p < 0.01$  or f:  $p < 0.001$

In an early study the BCAAs, leucine, isoleucine and valine, in combination with glucose, were shown to produce a stimulatory effect on protein synthesis in skeletal muscle of fasted rats. Other studies have shown that among the BCAAs, leucine alone is sufficient to enhance protein synthesis in isolated muscle



preparations as well as in perfused rat hindlimb preparations [97]. Although all three BCAAs incubated with murine myotubes in the presence of the catabolic factor PIF, were successful at attenuating the depression in protein synthesis (Figure 8.1A) and elevation of protein degradation (Figure 8.1B) in response to PIF, leucine was the most effective.

The protein anabolic actions of leucine are mediated by activation of intracellular signalling pathways, the elements of which are also stimulated in response to insulin. The amino acid and mitogen stimulated pathways appear to converge at the serine/threonine protein kinase mammalian target of rapamycin (mTOR) [105], which coordinates various cellular signalling mechanisms with energy sensing pathways [196]. To determine the role of mTOR activation in the stimulatory effects of mitogens and nutrients, murine myotubes were incubated in the presence of PIF, leucine, insulin and the mTOR inhibitor, rapamycin, and protein synthesis was measured (see Section 2.3.15 for method used).

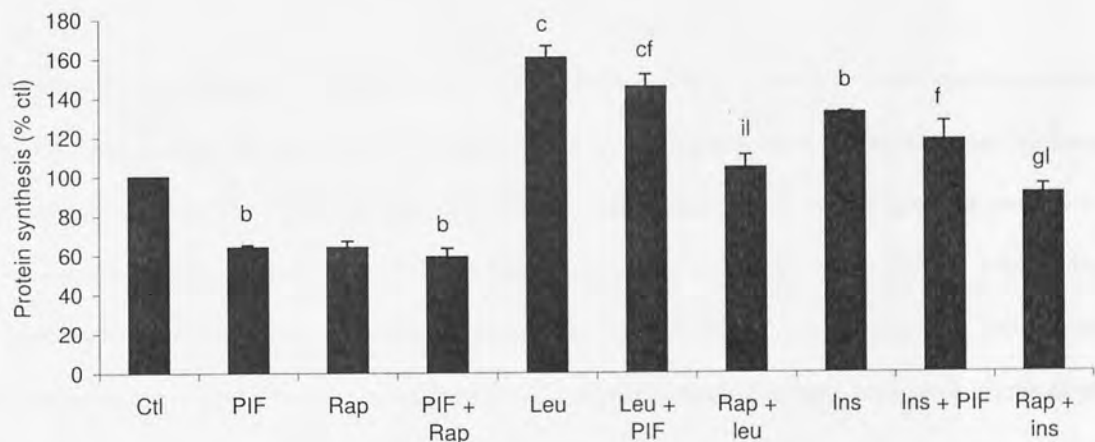


Figure 8.2: **Effect of leucine, insulin and rapamycin on protein synthesis in murine myotubes upon treatment with PIF.** Protein synthesis was measured in murine myotubes in the presence of leucine (Leu) (2mM), insulin (Ins) (1nM) and rapamycin (Rap) (25ng/mL) when co-treated with PIF (4.2nM). Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as b:  $p < 0.01$  or c:  $p < 0.001$ , differences from PIF alone f:  $p < 0.001$ , differences from rapamycin alone g:  $p < 0.05$ , i:  $p < 0.001$  or leucine/insulin l:  $p < 0.001$

The inhibition of protein synthesis in the presence of PIF was attenuated when myotubes were incubated with either leucine or insulin. However, when rapamycin was administered protein synthesis dropped



significantly, which was irreversible when co-treated with both leucine or insulin (Figure 8.2). This is evidence that PIF and rapamycin act via two distinct pathways. Although rapamycin did not completely abolish the stimulatory effect of the growth factors on protein synthesis, it did have a significant effect, suggesting that leucine and insulin affects synthesis mainly through an mTOR dependent mechanism.

BCAAs have been documented to enhance protein synthesis in association with an increased phosphorylation of mTOR and downstream targets p70S6 kinase and the eIF4F complex [97]. Phosphorylation of the inhibitory factor, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) results in an increased availability of eIF4E to form the active eIF4G.eIF4E complex, which enhances mRNA binding to the 43S preinitiation complex stimulating translation initiation. Phosphorylation and activation of p70S6K can regulate protein synthesis at the elongation stage through the phosphorylation and inactivation of an eEF2 kinase allowing de-phosphorylation of the elongation factor, eEF2 and resultant initiation of elongation by increasing its affinity for the ribosome [135].

$\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) is a metabolite of the amino acid leucine. HMB has been used as a supplement to enhance the effects of weight training on muscle mass and to minimise muscle damage during resistance training. HMB has also been shown to attenuate loss of body weight and preserve lean body mass in tumour bearing mice [192]. HMB was effective at increasing mTOR and p70S6 kinase phosphorylation in murine myotubes (see appendix A.2, Figure A.3C and D). PIF has been demonstrated previously to interfere with the formation of the eIF4F active complex and consequently diminish the binding of mRNA to the ribosome. To determine whether supplementation with HMB could reverse this effect and stimulate protein anabolism, murine myotubes were administered PIF and HMB. The phosphorylation state of 4E-BP1 and eIF4E was determined, while the association of eIF4G and 4E-BP1 with eIF4E was assayed by separating the m<sup>7</sup> binding protein, eIF4E, from the cell supernatant using m<sup>7</sup> GTP sepharose affinity purification (see Section 2.3.12 for method). Phosphorylation of the eEF2 was also examined using the remaining supernatant fraction from myotubes incubated with PIF and HMB (Section 2.3.21 for western blotting method).

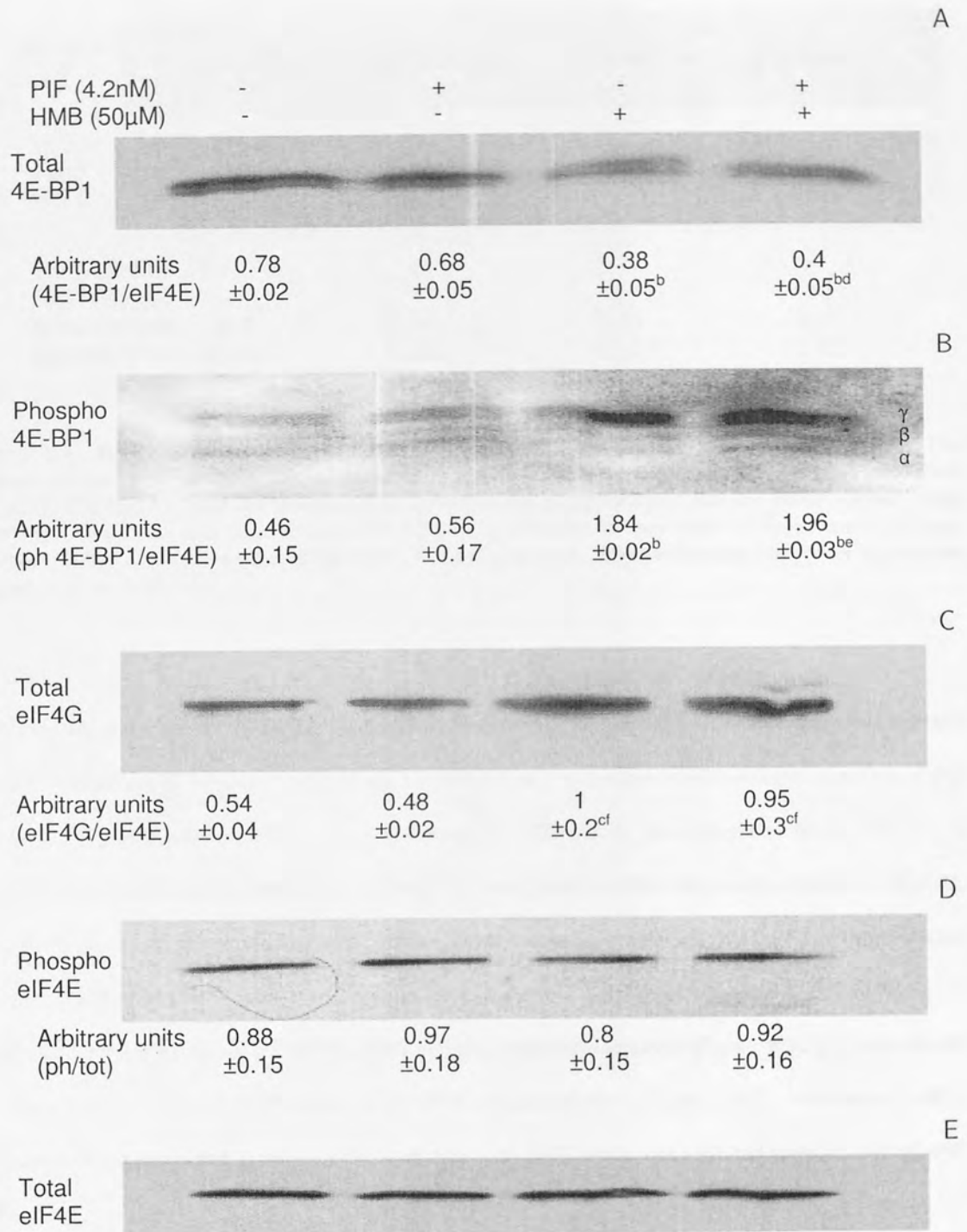


Figure 8.3: **Western blotting for components of the translation initiation complex eIF4F in murine myotubes treated with PIF and HMB.** The translation initiation complex proteins 4E-BP1 (A), phospho 4E-BP1 (B), eIF4G (C) and phospho eIF4E (D) purified by m<sup>7</sup>GTP sepharose in association with eIF4E (E) were analysed in murine myotubes treated with PIF (4.2nM) and HMB (50 $\mu$ M). HMB was incubated with myotubes for 2h prior to addition of PIF followed by a further 4h incubation. A densitometric analysis is shown below where  $\pm$  represents the SEM of 2 replicate blots. Significant differences from 0h control are shown as b:  $p < 0.01$  or c:  $p < 0.001$  while differences from PIF alone are shown as d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$

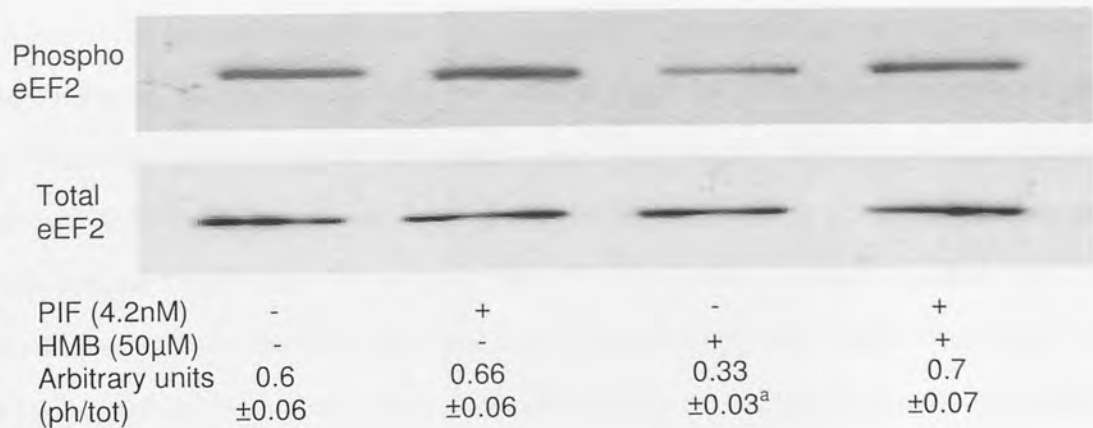


Figure 8.4: **Western blotting for eEF2 in murine myotubes treated with PIF and HMB.** The phosphorylated form of eEF2 was analysed in murine myotubes treated with PIF (4.2nM) and HMB (50μM). The blot for total eEF2 was used to normalise the phosphorylated protein. HMB was incubated with myotubes for 2h prior to addition of PIF followed by a further 4h incubation. A densitometric analysis is shown below where  $\pm$  represents the SEM of 3 replicate blots. Significant differences from control are shown as a:  $p < 0.05$

The translational repressor 4E-BP1 is regulated through mTOR and undergoes phosphorylation at multiple sites, which affects its function [146]. 4E-BP1 was resolved into multiple electrophoretic forms ( $\alpha$ ,  $\beta$  and  $\gamma$ ) on a 15% SDS polyacrylamide gel, representing the differentially phosphorylated forms, with the  $\gamma$  form having the slowest electrophoretic mobility. PIF alone had no effect on phosphorylation of 4E-BP1, but HMB alone and in combination with PIF produced a significant increase in 4E-BP1 phosphorylation (Figure 8.3B), and a 50% decrease in the amount of 4E-BP1 associated with eIF4E (Figure 8.3A). In addition the total eIF4G in the eIF4E.eIF4G complex was also increased (Figure 8.3C). There was no significant effect of any of the treatments on eIF4E phosphorylation, (Figure 8.3D). A decrease in eEF2 phosphorylation was noted in response to incubation with HMB which was inhibited when co-treated with PIF.

These results suggest that HMB may have the ability to stimulate protein synthesis through an increased binding of the mRNA to the ribosome and reverse some of the catabolic actions of PIF in this manner. Since HMB is a metabolite of leucine it is highly likely that they have comparative actions. Thus it is also probable that leucine can affect the eIF4F triad and eEF2 in the same way as HMB.

Although protein synthesis was attenuated when both leucine or insulin were co-treated with rapamycin in murine myotubes, the effect was not completely abolished (Figure 8.2). This suggests that both nutrients can affect another pathway separate from mTOR. There is evidence that some changes in the phosphorylation of 4E-BP1 are independent of mTOR [127]. This is substantiated by a report, which shows that leucine produces a stimulatory effect on global rates of protein synthesis in skeletal muscle through a rapamycin insensitive mechanism, the identity of which is unknown [97], while another study showed that leucine increased protein synthesis in diabetic rats without concomitant changes in the phosphorylation of 4E-BP1 or p70S6 kinase through an unknown mechanism [5]. Loss of body proteins in growing animals is due in large part to a depression in protein synthesis at the initiation stage of mRNA translation [7]. Changes in translation facilitated by amino acids or hormones can be mediated through the regulation of either the met-tRNA or mRNA binding steps [97]. It is possible therefore, that another regulatory step of translation is affected by leucine, such as the control of polysome formation, where there is an increase in the proportion of mRNA associated with the ribosome. This is controlled by the phosphorylation of the  $\alpha$ -subunit of eIF2. The reduction in skeletal muscle protein synthesis seen during cancer cachexia has been associated with a decrease in eIF2 $\alpha$ , while leucine treatment strongly enhanced eIF2 $\alpha$  expression [200].

Accordingly, to determine whether leucine administration induced alterations in eIF2 $\alpha$  expression and activity, murine myotubes were co-treated with PIF and leucine and assayed by western blotting (method described in Section 2.3.13 and 2.3.21). Since leucine has also been shown to induce insulin release, the effect of insulin was also determined on eIF2 $\alpha$  phosphorylation to examine the possibility that the effect of leucine is mediated through an insulin dependent manner.

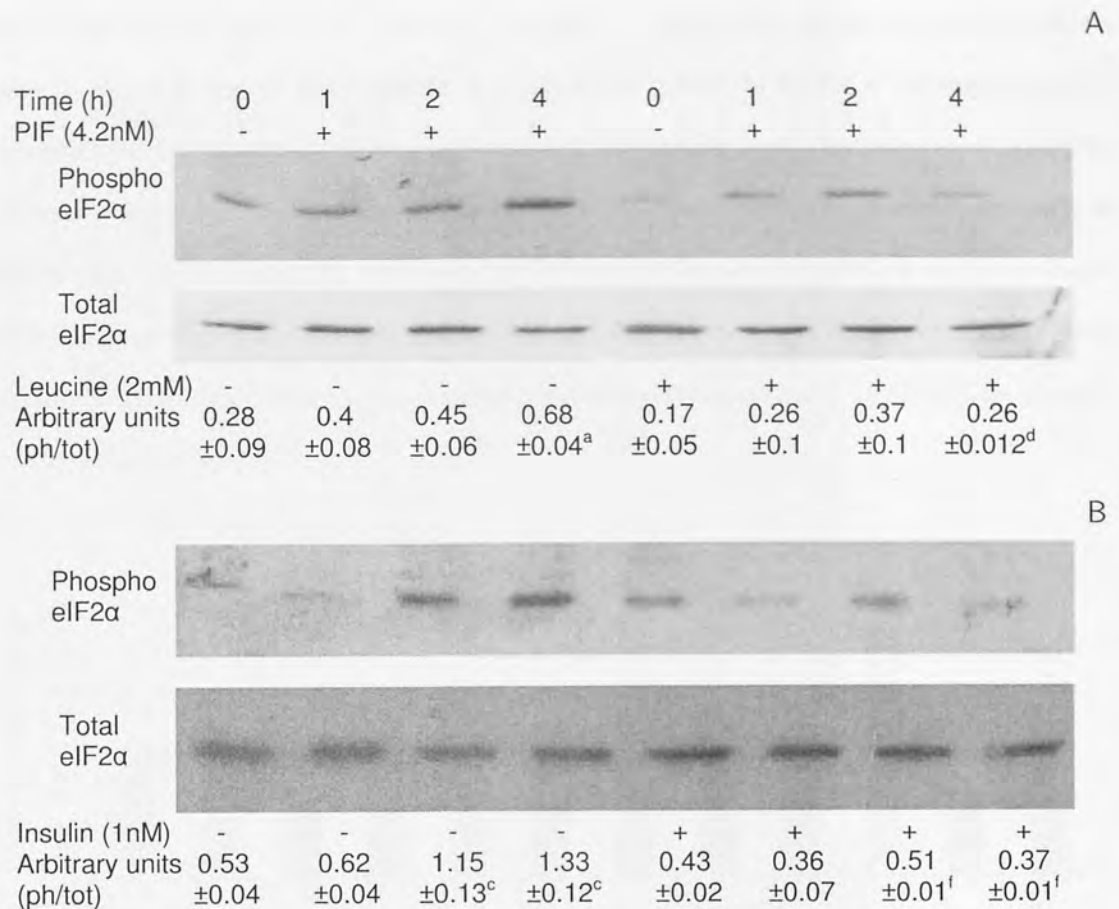


Figure 8.5: **Effect of leucine and insulin on phosphorylated eIF2 $\alpha$  in murine myotubes in response to PIF.** A western blot was performed to analyse the phosphorylated  $\alpha$ -subunit of eIF2 in murine myotubes upon co treatment with PIF (4.2nM) and leucine (2mM) (A) or insulin (1nM) (B) with a corresponding eIF2 $\alpha$  total (which was used to normalise the phosphorylated protein). leucine/insulin was incubated with myotubes for 2h prior to addition of PIF followed by a further 4h incubation. Error bars represent  $\pm$ SEM of 3 replicate experiments. A densitometric analysis is shown underneath the western blot where  $\pm$  represent SEM of three replicate experiments. Differences from control are shown as a:  $p < 0.05$ , c:  $p < 0.001$  while differences in the presence of PIF and leucine/insulin from corresponding treatment with PIF alone are shown as d:  $p < 0.05$ , f:  $p < 0.001$

Both leucine and insulin attenuate the upregulation of eIF2 $\alpha$  phosphorylation upon treatment of PIF (Figure 8.5), thus the effects of leucine may or may not be mediated by an upregulation of insulin secretion. Previously PIF has been shown to increase autophosphorylation of the double stranded RNA-dependent protein kinase (PKR), which is responsible for protein synthesis inhibition. Unfortunately the phospho PKR antibody used previously was not available commercially and no other suitable alternative could be found to replace it. Therefore the effect of leucine and insulin on PKR activation could not be tested in vitro. Previous studies have demonstrated that under conditions of nutrient deprivation the eIF2 $\alpha$

kinase termed general control nonderepressible 2 (GCN2) is activated, with subsequent protein synthesis depression [7]. It is possible that inhibition of GCN2 kinase activity by leucine in murine myotubes is responsible for the attenuation of eIF2 $\alpha$  phosphorylation. Furthermore, insulin has been shown to activate the protein phosphatase 1 which, in turn dephosphorylates PKR, allowing the commencement of translation initiation [180]. To investigate the involvement of PKR and eIF2 $\alpha$  phosphorylation in protein synthesis, murine myotubes were treated with PIF and supplemented with leucine and its metabolite HMB, or IGF-1 and insulin and co-treated with the eIF2 $\alpha$  dephosphorylation inhibitor salubrinal or a PKR inhibitor (method described in Section 2.3.15).

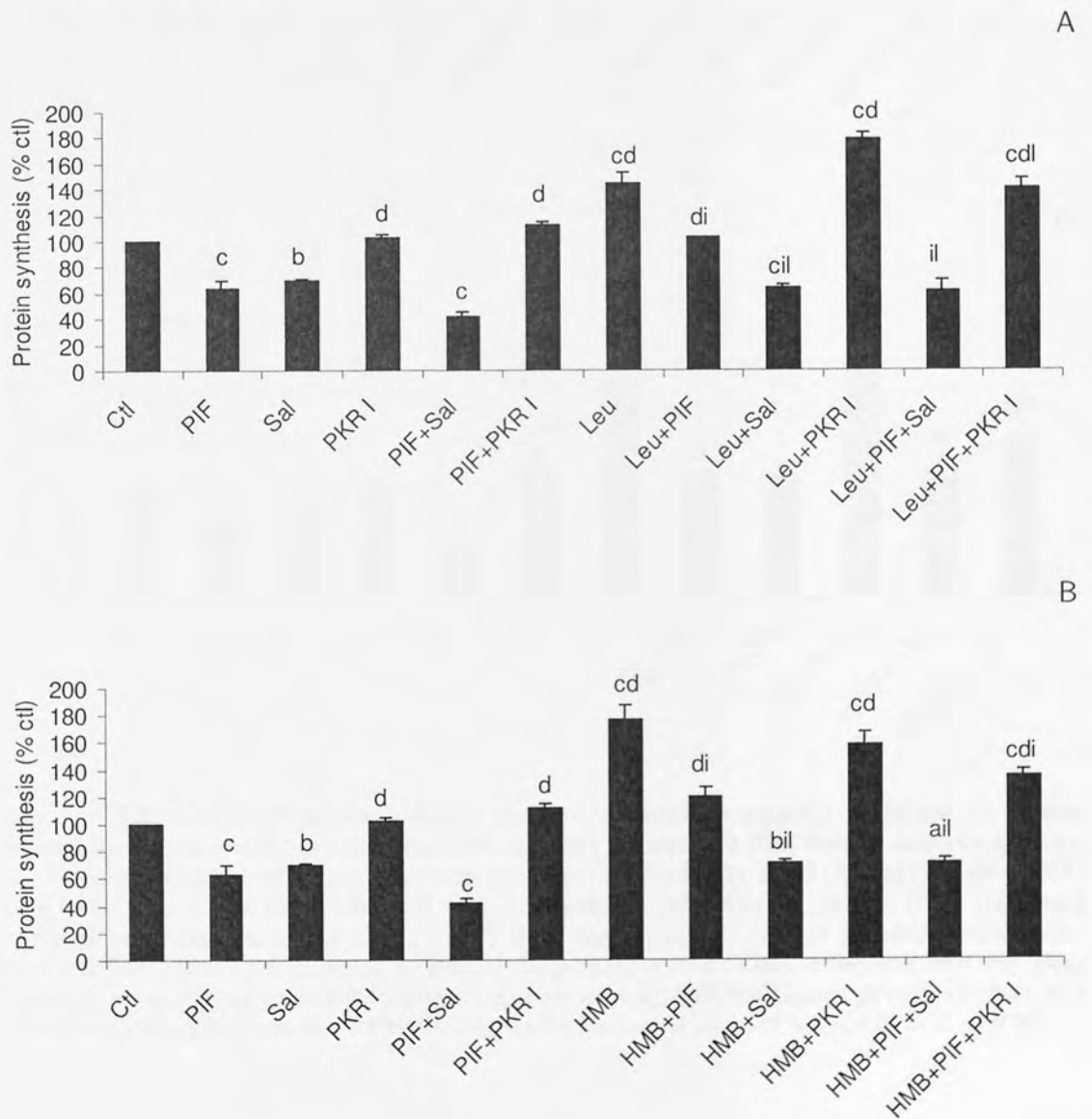


Figure 8.6



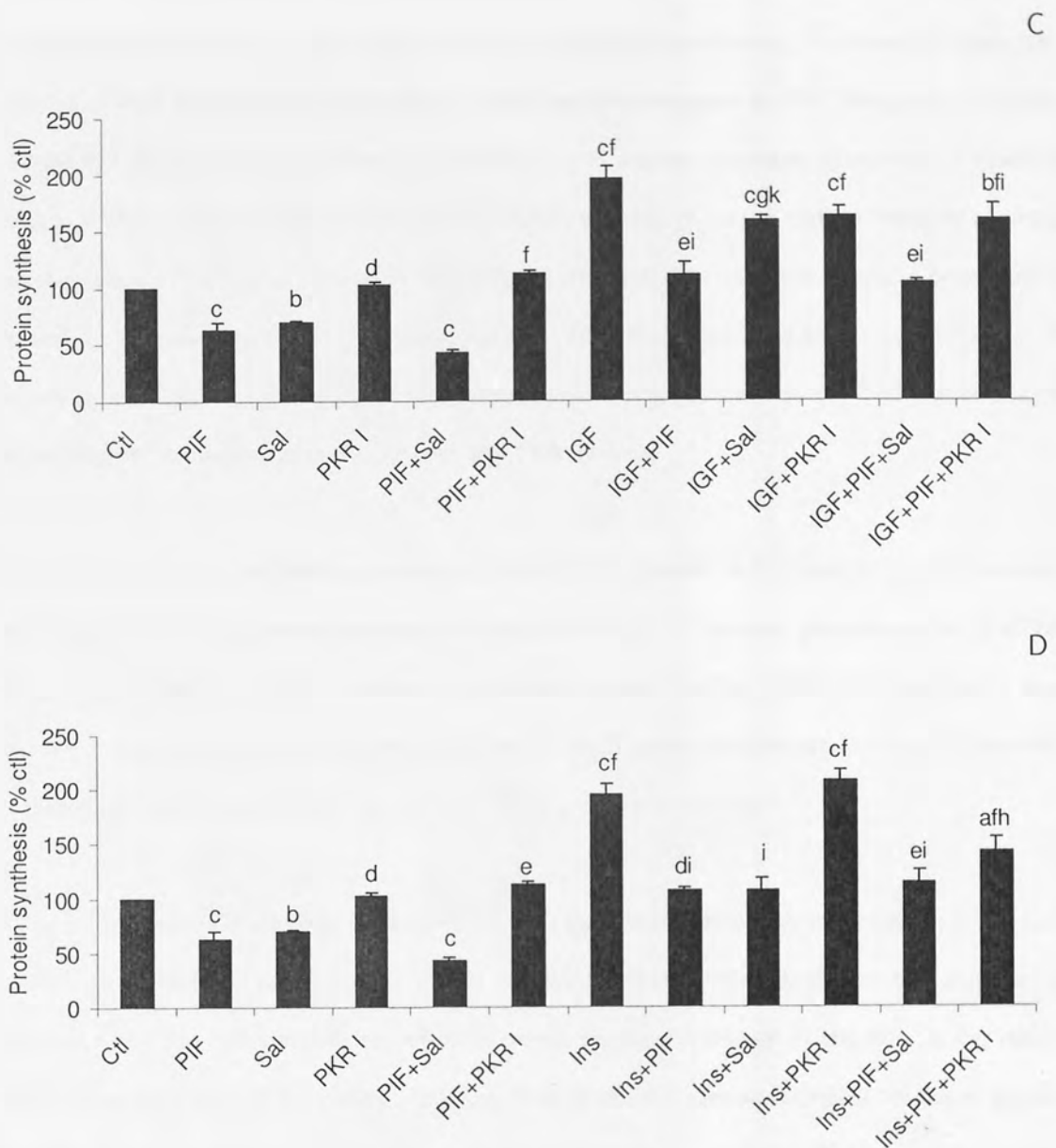


Figure 8.6: Effect of PKR phosphorylation and eIF2 $\alpha$  dephosphorylation inhibition on protein synthesis in murine myotubes incubated with anabolic factors and PIF. Protein synthesis was measured in murine myotubes when incubated with leucine (Leu) (2mM) (A), HMB (50 $\mu$ M) (B), IGF-1 (IGF) (100ng/mL) (C) or insulin (Ins) (1nM) (D), co-treated with PIF (4.2nM), PKR inhibitor (PKR I) (210nm) and the eIF2 $\alpha$  inhibitor, salubrinal (Sal) (15 $\mu$ M). Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , differences from PIF alone d:  $p < 0.05$ , e:  $p < 0.01$  or f:  $p < 0.001$ , differences from leucine/HMB/IGF/insulin alone h:  $p < 0.01$  or i:  $p < 0.001$  and differences from leucine/HMB/IGF/insulin co-treated with PIF k:  $p < 0.01$  or l:  $p < 0.001$

In a previous chapter (Chapter 4) PIF has been shown to inhibit protein synthesis in myotubes by PKR autophosphorylation with a consequential increase in eIF2 $\alpha$  phosphorylation. As shown in Figure 8.6, inhibition of PKR attenuated the depression in protein synthesis produced by PIF. Stimulation of protein synthesis by nutritional supplementation in the absence of PIF was also attenuated by salubrinal, a selective inhibitor of eIF2 $\alpha$  dephosphorylation, suggesting that this was due, at least in part, to inhibition of eIF2 $\alpha$  phosphorylation. This is substantiated by the ability of salubrinal to attenuate the stimulation of protein synthesis by all growth factors in the presence of PIF. This effect appears to be due to attenuation of the activity of PKR, since the ability of both nutrients and mitogens to reverse the inhibition of protein synthesis by PIF was enhanced in the presence of a PKR inhibitor.

Similar results were obtained with angiotensin II (Ang II) (see appendix A.2, Figure A.2), which has also been shown to attenuate protein synthesis in myotubes through an increased phosphorylation of eIF2 $\alpha$  (Chapter 4). As seen with PIF, nutritional supplementation with leucine, HMB, IGF-1 and insulin also attenuated the depression of protein synthesis induced by Ang II, where co-treatment with the PKR inhibitor broadened this differential, and this was reversed in the presence of salubrinal.

Of the BCAAs leucine is the most potent and thus the most important amino acid to study in terms of its effect on translational regulation and muscle cell growth [215]. One study showed that provision of leucine to fasted rats had a stimulatory effect on protein synthesis in skeletal muscle [97]. In this regard weight losing mice bearing the cachexia inducing MAC16 tumour were administered leucine as well as two other BCAA's isoleucine and valine to study the mechanism by which the BCAAs promotes muscle protein synthesis and to examine their use as an anti-cachexia treatment (see Methods Section 2.3.1, 2.3.16 and 2.3.20 for procedures used).

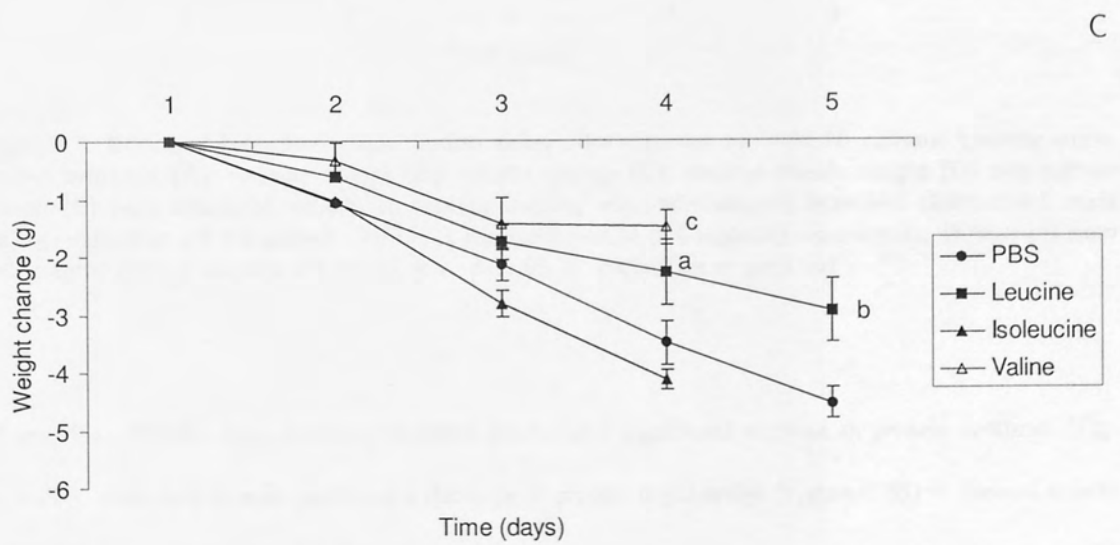
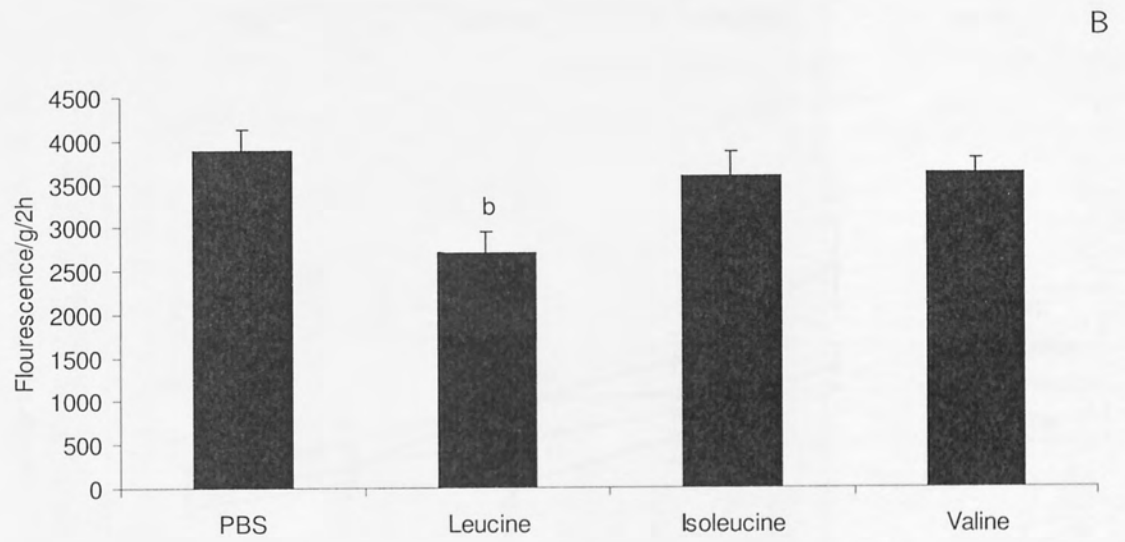
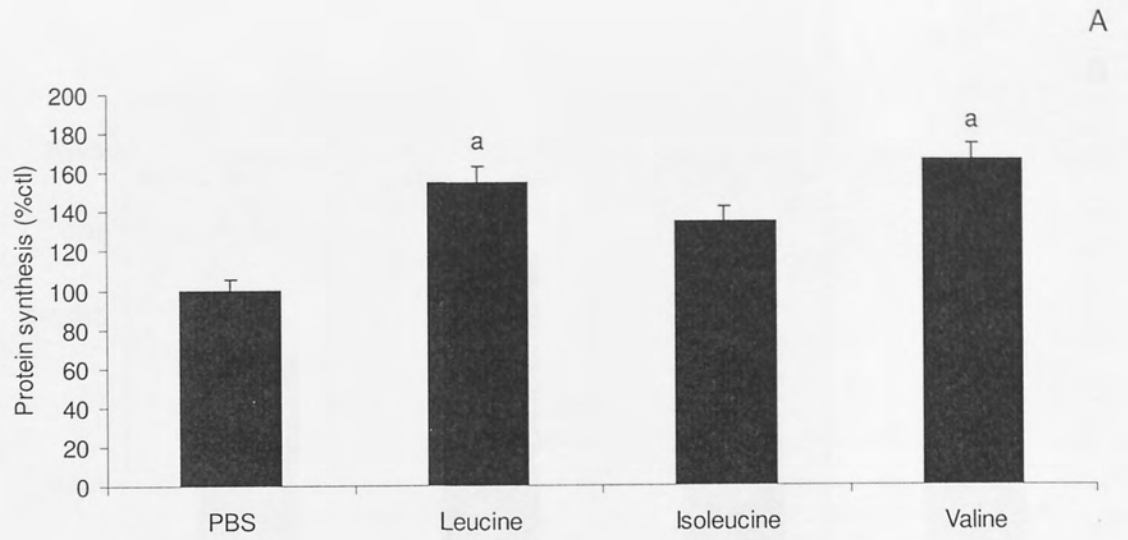
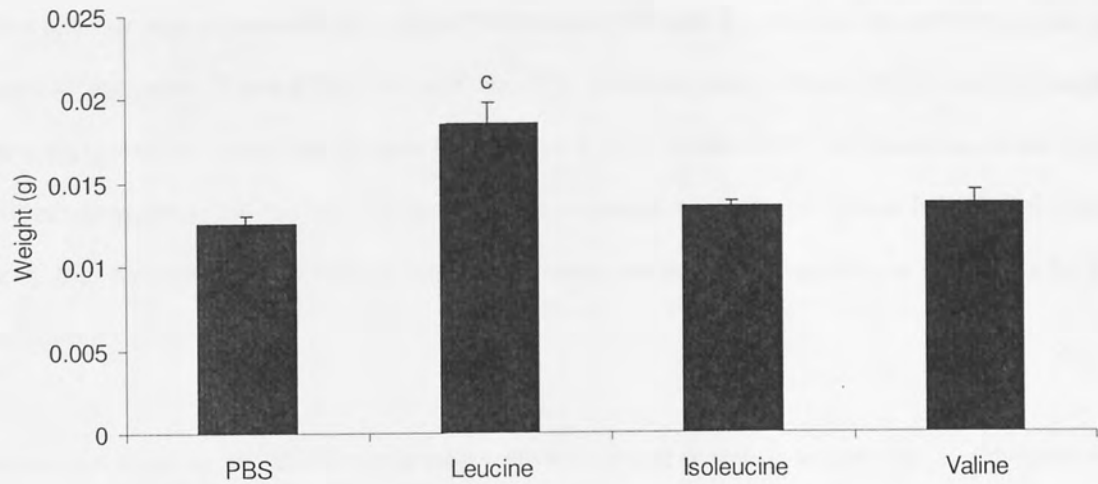


Figure 8.7

D



E

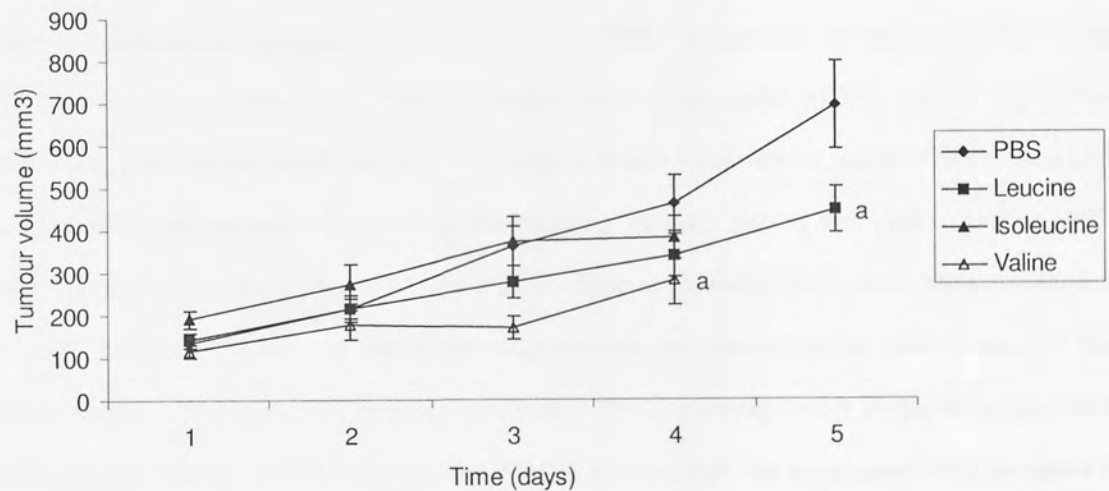


Figure 8.7: Effect of branched chain amino acids administered to MAC16 tumour bearing mice. Protein synthesis (A), tyrosine release (B), weight change (C), skeletal muscle weight (D) and tumour growth (E) were measured in MAC16 tumour bearing mice administered branched chain amino acids (1g/kg) daily over a 5 day period. Error bars represent  $\pm$ SEM of 6 replicate experiments. Differences from PBS treated control animals are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$

Of the three BCAAs, only leucine and valine produced a significant increase in protein synthesis (Figure 8.7A), while only leucine produced a decrease in protein degradation (Figure 8.7B) in skeletal muscle of MAC16 tumour bearing mice. Leucine and valine also caused a significant suppression of body weight

loss (Figure 8.7C), while only leucine caused an increase in soleus muscle wet weight (Figure 8.7D). Interestingly leucine and valine produced a small, but significant inhibition of tumour growth (Figure 8.7E), an effect also seen with a low molecular weight PKR inhibitor (Chapter 5). Leucine was demonstrated to reduce PKR activation (Figure 8.11A) and resultant eIF2 $\alpha$  phosphorylation (Figure 8.11B), possibly through the activation of the protein phosphatase PP1 (Figure 8.11C), of which PKR is a known substrate [180]. PKR autophosphorylation has been shown to have an important role in tumour growth (discussed in Chapter 5), thus the attenuation of PKR activation by leucine, and possibly valine, may be responsible for its anti-tumour effect.

Leucine was shown *in vitro* to increase protein synthesis, at least in part, in a rapamycin sensitive manner. The proportion of TOP mRNAs actively engaged in protein synthesis, i.e. the proportion associated with polysomes, is significantly lower than that of other ubiquitous mRNAs. Amino acid treatment has been shown to independently enhance the binding of TOP mRNAs through the regulation of p70S6K in an mTOR mediated mechanism [182]. The mammalian target of rapamycin, mTOR, and its downstream components, p70S6 kinase, eIF4F and eEF2 were studied to determine whether the same mechanisms were occurring *in vivo* in response to leucine supplementation. Western blotting was used to analyse p70S6 kinase phosphorylation along with eIF4E and its associated components to determine the active state of the eIF4F complex. The effect of leucine on elongation initiation was studied by blotting for eEF2 (see Method Section 2.3.12 and 2.3.21 for procedures used). These experiments were performed using skeletal muscle of mice bearing the cachexia inducing MAC16 tumour from the same experiment as shown in Figure 8.7.

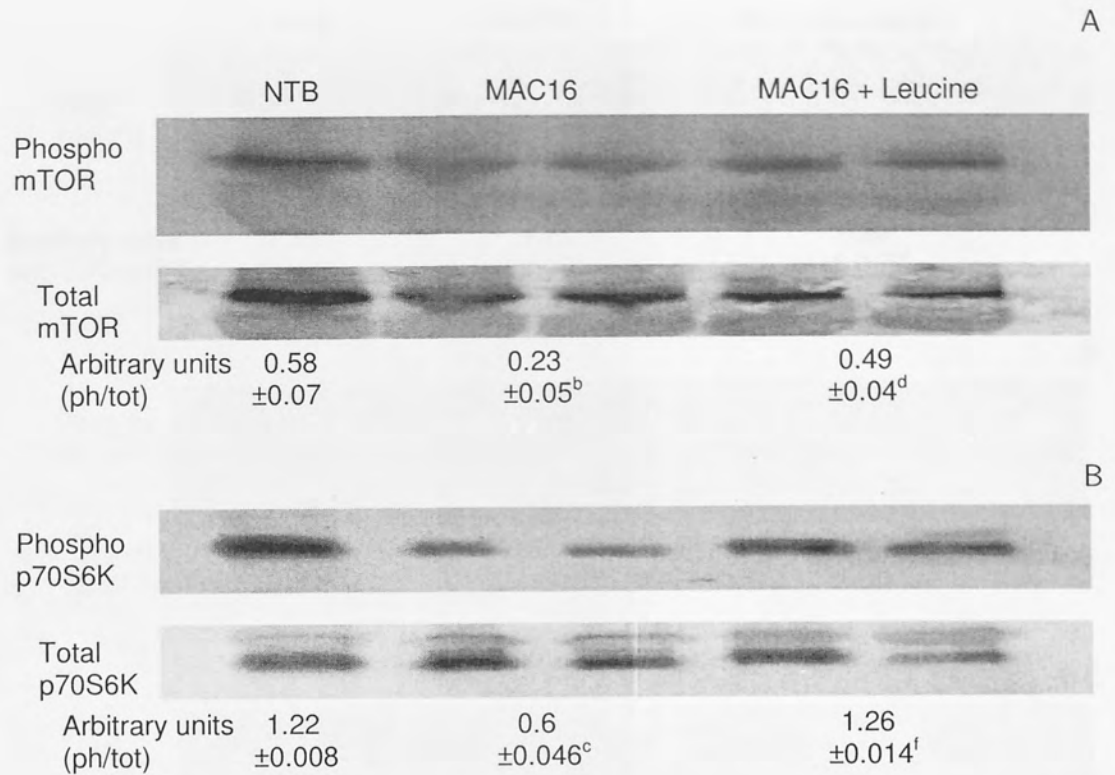


Figure 8.8: **Western blotting for mTOR and p70S6K in skeletal muscle from MAC16 tumour bearing mice treated with leucine.** The phosphorylated forms of mTOR (A) and p70S6K (B) were analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour treated with leucine (1g/kg) daily over a 5 day period. Blots for total mTOR and p70S6K were used to normalise the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate experiments. Significant differences from non tumour bearing control (NTB) mice are shown as b:  $p < 0.01$  or c:  $p < 0.001$ , while differences from MAC16 tumour bearing PBS treated animals is shown as d:  $p < 0.05$  or f:  $p < 0.001$



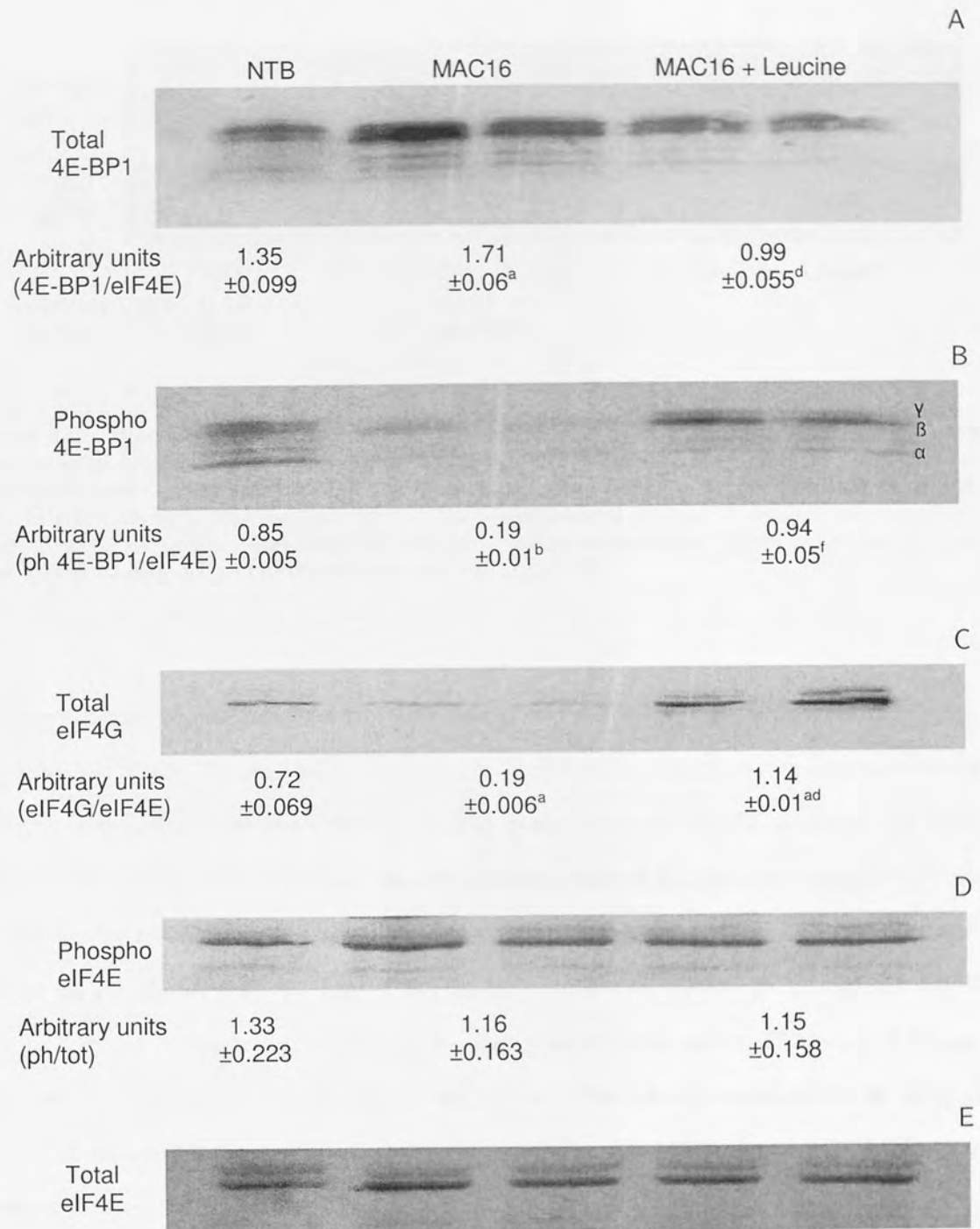


Figure 8.9: **Western blotting for components of the translation initiation complex eIF4F in skeletal muscle from MAC16 tumour bearing mice treated with leucine.** The translation initiation complex proteins 4E-BP1 (A), phospho 4E-BP1 (B), eIF4G (C) and phospho eIF4E (D) purified by m<sup>7</sup>GTP sepharose in association with eIF4E (E) were analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour treated with leucine (1g/kg) daily over a 5 day period. A densitometric analysis is shown underneath where ± represents the SEM of 2 replicate experiments. Significant differences from non tumour bearing control (NTB) mice are shown as a: p<0.05 or b: p<0.01, while differences from MAC16 tumour bearing PBS treated animals is shown as d: p<0.05 or f: p<0.001

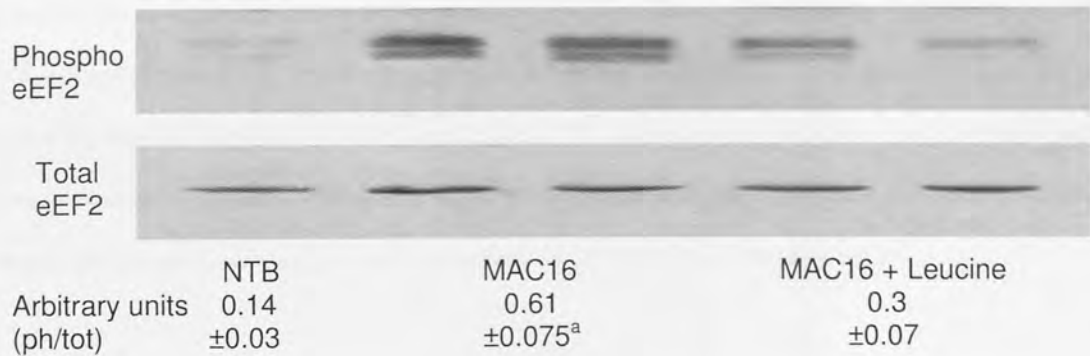


Figure 8.10: **Western blotting for eEF2 in skeletal muscle from MAC16 tumour bearing mice treated with leucine.** The phosphorylated form of eEF2 was analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour treated with leucine (1g/kg) daily over a 5 day period. The blot of total eEF2 was used to normalise the phosphorylated protein. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate experiments. Significant differences from non tumour bearing control (NTB) mice are shown as a:  $p < 0.05$

Leucine treatment of mice bearing the MAC16 tumour increased levels of phosphorylated mTOR (Figure 8.8A) and p70S6K (Figure 8.8B) in gastrocnemius muscle, up to values found in non-tumour bearing animals. Leucine treatment also caused a reduction in the amount of 4E-BP1 associated with eIF4E (Figure 8.9A), and an increase in the eIF4G.eIF4E complex (Figure 8.9C) above the value found in non tumour-bearing animals. This was due to hyperphosphorylation of 4E-BP1 (Figure 8.9B), without an effect on phosphorylation of eIF4E (Figure 8.9D). Leucine treatment also reduced phosphorylation of eEF2 (Figure 8.10), which may have arisen through the effect of p70S6 kinase on the eEF2 kinase, or through preferential upregulation of eEF2 due to its 5'-cap structure. These changes would explain the ability of leucine to stimulate protein synthesis in gastrocnemius muscle, probably through an mTOR dependent pathway.

It is possible that isoleucine and valine affect protein synthesis in a similar manner, since one study showed that isoleucine, to a lesser extent, also promoted eIF4E availability and facilitated hyperphosphorylation of p70S6 kinase [4], while valine stimulated phosphorylation of 4E-BP1 and p70S6K the least effectively [215]. The reduced ability of isoleucine and other BCAAs to activate translational machinery may indicate specific structural requirements of leucine for regulating signalling pathways that modulate protein synthesis [4].

Leucine was demonstrated to attenuate the increased phosphorylation of the  $\alpha$ -subunit of eIF2 in response to PIF *in vitro* (Figure 8.5A). Evidence suggests that this occurs through an inhibition of PKR activation since a PKR inhibitor reversed the inhibitory actions of PIF on protein synthesis alone and co-treated with leucine (Figure 8.6A). Previous chapters (Chapter 5) have also shown that PKR and eIF2 $\alpha$  phosphorylation increased incrementally with weight loss experienced by MAC16 tumour bearing mice.

To examine the role of nutritional supplementation on the formation of the 43S preinitiation complex and activation of the translational machinery *in vivo*, MAC16 mice were treated with leucine, or HMB (see Section 2.3.1 for animal studies) and levels of phosphorylated proteins were determined by western blotting (Methods Section 2.3.13 and 2.3.21). Compared with the widely studied mechanisms for the phosphorylation and activation of PKR, the mechanisms underlying the inactivation of the enzyme are largely uncharacterised. The Herpes simplex virus (HSV) encodes an inhibitor (ICP345) which binds to PP1 and blocks PKR translational repression [177]. Furthermore, expression of PP1 leads to reduced phosphorylation of PKR and its physiological substrate eIF2 $\alpha$  [180]. In regard to this evidence we chose to study PP1 as a possible regulatory point of PKR upon leucine stimulation in MAC16 tumour bearing mice.

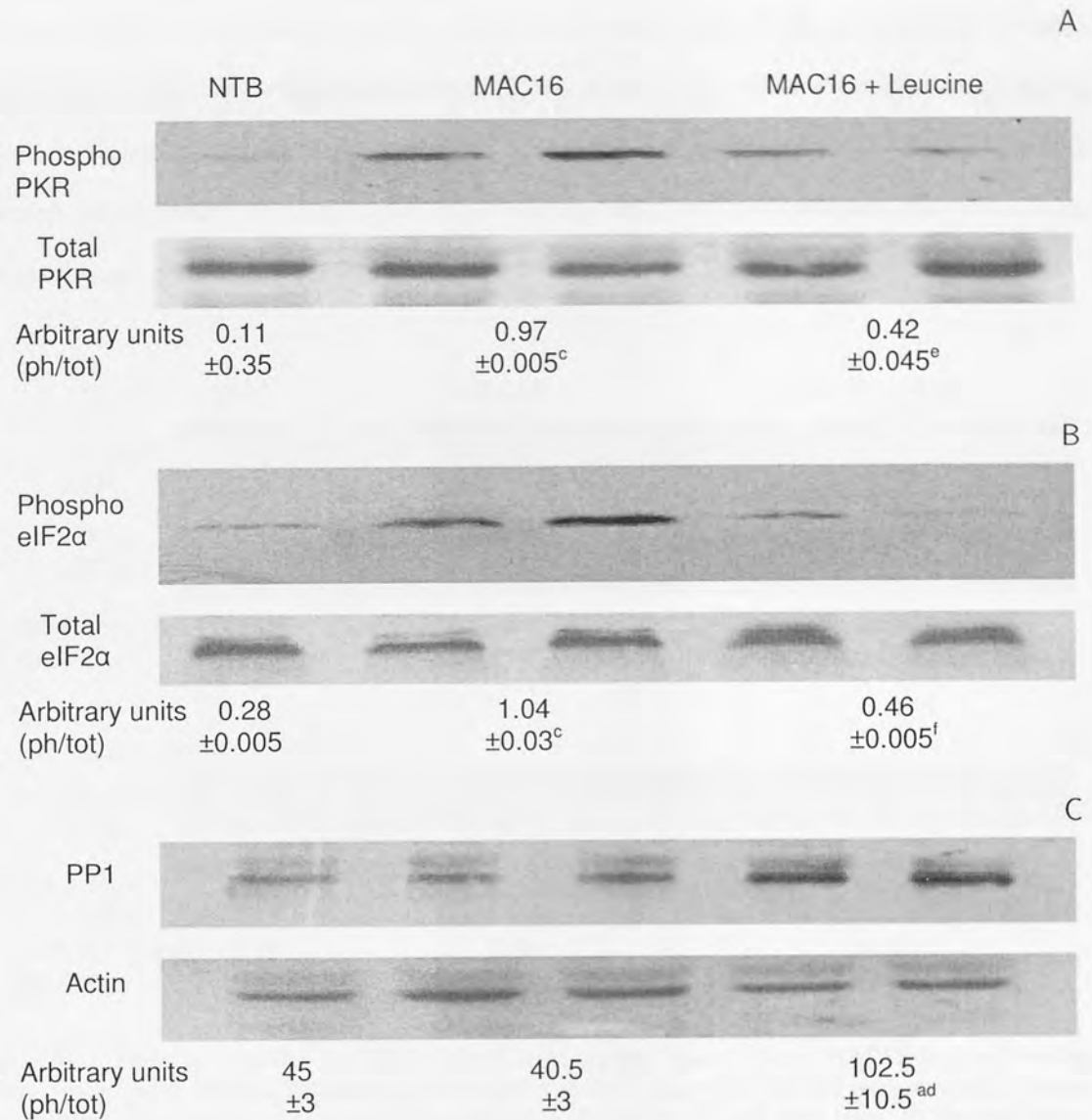


Figure 8.11: **Western blotting for PKR, eIF2α and PP1 in skeletal muscle from MAC16 tumour bearing mice treated with leucine.** The phosphorylated forms of PKR (A), eIF2α (B) and total PP1 (C) with a corresponding actin loading control were analysed in gastrocnemius muscle from mice bearing the cachexia inducing MAC16 tumour treated with leucine (1g/kg) daily over a 5 day period. Blots for total PKR and eIF2α were used to normalise the phosphorylated proteins. A densitometric analysis is shown underneath where ± represents the SEM of 3 replicate experiments. Significant differences from non tumour bearing control (NTB) mice are shown as a:  $p < 0.05$  or c:  $p < 0.001$ , while differences from MAC16 tumour bearing PBS treated animals is shown as d:  $p < 0.01$ , e:  $p < 0.01$  or f:  $p < 0.001$ .

The results shown in Figure 8.11A and B indicate that leucine attenuated the increased phosphorylation of both PKR and eIF2α, almost to values found in non-tumour-bearing animals. The decreased phosphorylation of PKR is accompanied by an increase in expression of PP1 (Figure 8.11C) in muscles of mice bearing the MAC16 tumour that were treated with leucine. PP1 has the ability to inhibit transphosphorylation

abilities of PKR, inhibiting dimer formation and kinase activation [180]. HMB was also shown to inhibit PKR (Figure 8.12A) and eIF2 $\alpha$  phosphorylation (Figure 8.12B) in the soleus of MAC16 tumour bearing mice (see Section 2.3.1 for animal experiment). Since HMB is a metabolite of leucine it is likely both nutrients act via similar mechanisms thus HMB may also affect PKR autophosphorylation through the upregulation of PP1 activity.

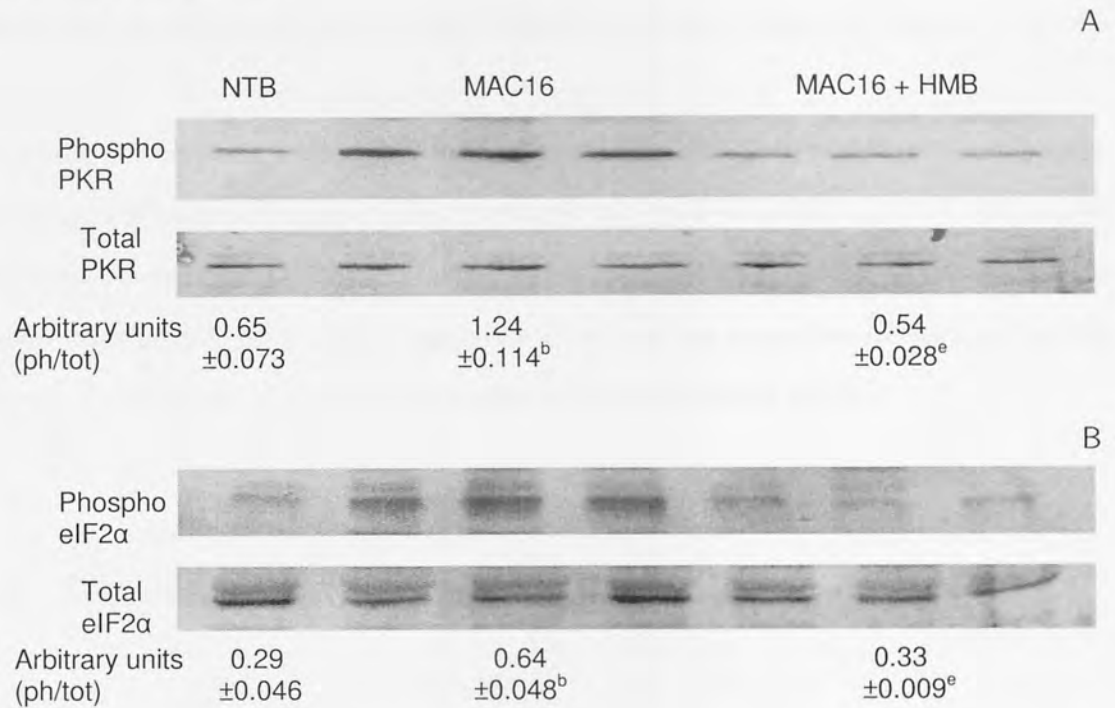


Figure 8.12: **Western blotting for PKR and eIF2 $\alpha$  in skeletal muscle from MAC16 tumour bearing mice treated with HMB.** The phosphorylated forms of PKR (A) and eIF2 $\alpha$  (B) were analysed in soleus muscle from mice bearing the cachexia inducing MAC16 tumour treated with HMB (0.25g/kg) over a period of 4 days. Blots for total PKR and eIF2 $\alpha$  were used to normalise the phosphorylated proteins. A densitometric analysis is shown underneath where  $\pm$  represents the SEM of 3 replicate experiments. Significant differences from non tumour bearing control (NTB) mice are shown as b:  $p < 0.01$ , while differences from MAC16 tumour bearing PBS treated animals is shown as e:  $p < 0.01$

Although both leucine and its metabolite, HMB, attenuate the phosphorylation of PKR and eIF2 $\alpha$  it is unlikely that they have an effect on basal levels of these proteins, since HMB was unable to alter either PKR or eIF2 $\alpha$  phosphorylation in vitro (see appendix A.2, Figure A.3A and B). It seems likely that treatment with leucine or HMB is only effective at reversing PKR and eIF2 $\alpha$  phosphorylation in the presence of a stimulatory factor such as PIF. The involvement of leucine in the phosphorylation state of eIF2 $\alpha$  remains debatable, although insulin has been shown to activate PP1 to dephosphorylate and thus

deactivate PKR. Therefore it is possible that the actions of leucine is modulated indirectly through a stimulation of insulin release. In account of these observations it is reasonable to suggest that the anabolic stimuli of leucine is mediated through a direct effect on the assembly of active eIF4F complex, and possibly via a secondary action on insulin release which deactivates PKR through PP1 phosphorylation. Moreover it is not implausible to suggest that leucine can modulate PP1 activity directly since there is an overlap in many nutrient and mitogen signalling pathways which share common mediators and actions.

The results suggest that nutritional supplementation, especially with leucine, HMB, IGF-1 and insulin, may provide a protective effect against muscle wasting in cachexia by inhibiting degradative processes and stimulating muscle protein anabolism. Leucine especially, seems a likely candidate for an anti-cachectic agent in cancer cachexia, since it can not only induce protein synthesis through translational and elongation pathways but also inhibits muscle protein degradation and has anti-tumour qualities.

### 8.3 Discussion

The amino acids represent a class of biologic molecules exerting dynamic and complex influences on highly disparate physiologic processes including protein degradation, and synthesis, pancreatic insulin and glucagon secretion and sensitization of tissues to the anabolic effects of insulin. Leucine has been shown to be particularly effective as regards to protein turnover, as a protein synthetic substrate, and a signal transduction modulator in the regulation of the translational machinery [160]. The protein anabolic actions of leucine are mediated by activation of intracellular signalling pathways, the elements of which are also stimulated in response to insulin and insulin-like growth factor (IGF) [105]. Hence the administration of leucine, or either of the two mitogens, would be expected to have very similar end results.

Hunger is not stimulated following weight loss in cancer cachexia as it is during starvation conditions. In fact food intake is normally decreased, which contributes to weight loss, hence insulin levels will be diminished and the essential amino acids normally gained from nutritional intake will be reduced. One



study showed a 49% loss of plasma insulin in the MAC16 cachexia model [26]. Increased appetite is thought to be mediated by falling levels of circulating insulin and leptin, as both hormones normally act on the CNS to inhibit feeding. However, even with the reduced levels of insulin observed in mice bearing the cachexia inducing MAC16 tumour, there was no stimulation of the neuropeptide Y neurons in the arcuate nucleus [26]. This suggests that supplementation with insulin would not be an effective hunger stimulant. However, supplementation with the mitogens and nutrients that are normally gained from feeding may serve to replenish the diminished caloric intake through hypophagia and thus stimulate some of the anabolic processes which are normally induced through feeding.

In starvation the stimulatory effect of leucine on protein synthesis has been attributed to hyperphosphorylation of 4E-BP1 and p70S6K, with little effect on the phosphorylation of eIF2 $\alpha$ , or on the activity of eIF2B [4]. The results of this chapter showed that, in cachexia, the mechanism appears to be similar to that seen in starvation, where addition of leucine and its metabolite  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) to C2C12 murine myotubes upregulated the activity of mTOR and its downstream effectors p70S6 kinase and the eIF4F triad. Activation of the eIF4F triad involves the hypophosphorylation and deactivation of the eIF4E repressor 4E-BP1, which induces the formation of the active eIF4E.eIF4G complex stimulating the binding of the mRNA to the ribosome and translation initiation. Hyperphosphorylation of p70S6 kinase leads to a preferential increase in translation of mRNAs that contain a 5-polypyrimidine tract, which codes for proteins that are generally involved in the translation apparatus such as eEF2 and PABP, which enhance the cells potential to synthesise protein [56]. Leucine starvation has been previously shown to increase phosphorylation of eEF2 in murine myotubes [178]. Treatment of cachectic mice with leucine decreased the phosphorylation of elongation factor 2 (eEF2) in gastrocnemius muscle, which would attenuate the depression of protein synthesis, since phosphorylation of eEF2 results in an inhibition of elongation by decreasing its affinity for the ribosome by ten to one hundred times [36]. The decrease in eEF2 phosphorylation by leucine may have been by a rapamycin sensitive pathway through the dephosphorylation of the inactivating eEF2 kinase. Cellular energy levels also regulate eEF2 in an mTOR independent manner. Thus leucine may interact with energy sensing pathways to change eEF2 activity [7].

A decrease in mTOR activity would worsen the wasting observed in cachexia since mTOR prevents turnover

of amino acid and glucose transporters and control trafficking of nutrient transporters, thereby promoting the uptake of nutrients such as glucose, amino acids, lipoprotein and iron. mTOR regulates nutrient response transcription programmes [209], suggesting a reason for the continued loss of weight despite normal feeding. Thus it is important to stimulate nutrient response pathways such as mTOR to restore the bodies ability to regulate nutritional input and maintain lean body protein.

The downregulation of active mTOR expression in murine skeletal muscle and its downstream effectors has been shown previously (Chapter 6). Administration of leucine to MAC16 tumour bearing mice was shown to reverse this inhibition, which was associated with an increase in protein synthesis and decrease in degradation as well as a preservation of muscle bulk. Autophagy may also play a role in the dephosphorylation of mTOR and its substrates. The inactivation of mTOR is essential to the induction of the autophagic response to nutrient starvation. An imbalance in amino acid concentration has been observed in cancer cachexia patients [185], suggesting that the autophagic response will be upregulated in the disease, exacerbating the wasting condition. Leucine has been shown to attenuate hepatic macroautophagic proteolysis induced by amino acid deprivation working synergistically with insulin, which enhances the efficacy of proteolytic inhibition by leucine [160]. This suggests that, regardless of the mechanism of muscle wasting, be it tumour specific, or as a result of autophagy, leucine has the ability to attenuate muscle atrophy in patients with cancer cachexia. Talloczy et al [177] showed that the eIF2 $\alpha$  kinase activity of PKR is involved in the stress induced translational regulation of autophagy. Autophagy is also activated in response to diminished nutritional stimuli and PKR was demonstrated to substitute for GCN2 in the translational control mechanism.

Additionally leucine was also shown to inhibit phosphorylation of eIF2 $\alpha$  through a direct effect on the activation of PKR by phosphorylation, which has been shown in previous chapters (6, 5 and 4), to be involved in the muscle wasting process activated by catabolic factors such as PIF and Ang II. Insulin and IGF-1 also showed effectiveness in attenuating the depression of protein synthesis by PIF in a PKR/eIF2 $\alpha$  dependent mechanism, while insulin had the ability to reduce eIF2 $\alpha$  phosphorylation stimulated in response to incubation with PIF in vitro. Phosphorylation of eIF2 on its  $\alpha$  subunit by PKR increases its affinity for the guanine nucleotide exchange factor, eIF2B, allowing eIF2 $\alpha$  to sequester eIF2B in an inactive complex,

inhibiting eIF2B stimulated GTP/GDP exchange on eIF2 and preventing new rounds of translation [37]. This evidence supports the claim made in this chapter that, nutrients can inhibit translational arrest through attenuation of PKR autophosphorylation induced by PIF. It may be that nutritional stimuli such as BCAA, HMB, insulin and IGF, inhibit the PIF induced inhibition of the 43S preinitiation complex formation by interfering with the PKR eIF2 $\alpha$  kinase activity and stimulates protein synthesis through the activation of the mTOR pathway, resulting in increased mRNA binding to the 43S ribosome. Evidence suggests that supplementation with either BCAA, IGF-1, insulin or a combination of the three addresses the underlying catabolic mechanisms of cachexia, reversing muscle wasting not only at the substrate level but at the translational level also.

The method by which BCAAs reduce protein degradation was not assessed in this chapter, although treatment of leucine, isoleucine and valine to murine myotubes, and administration of leucine to MAC16 tumour bearing mice, reduced protein degradation induced by catabolic stimuli. Previous studies have shown that HMB can inhibit PIF induced protein degradation by inhibition of PKC, with resultant stabilization of the cytoplasmic I $\kappa$ B/NF- $\kappa$ B complex preventing the movement of NF- $\kappa$ B into the nucleus with concomitant activation of gene expression of the proteasome [167]. Thus HMB attenuates the upregulation of the ATP-dependent ubiquitin proteasome proteolytic pathway stimulated by PIF. Being a metabolite of leucine, HMB has been demonstrated to have comparable effects to the BCAA, hence the BCAAs, leucine, isoleucine and valine may attenuate the rise in protein degradation induced by PIF through a similar means to HMB. Moreover, PIF and Ang II have been shown to activate protein degradation in a PKR dependent manner (Chapter 4 and 5), and since leucine was shown to reduce PKR phosphorylation it is possible that degradation was attenuated through PKR deactivation.

An upregulation of eIF2 $\alpha$  phosphorylation has been linked to the decreased protein synthesis and increased protein degradation with consequential loss of muscle mass in mice bearing the MAC16 cachexia inducing tumour (Chapter 5). Evidence suggests that autophosphorylation and subsequent activation of PKR by PIF is the determining factor for this event. The results of this chapter imply that leucine has the ability to decrease PKR phosphorylation and thus rescue cells from translation inhibition through the activation of PP1. Inhibition of PKR may also explain the small, but significant inhibition of tumour growth by

leucine, since inhibition of PKR by a low molecular weight inhibitor also inhibits tumour growth (result from Chapter 5). Leucine induces the release of insulin [97], which has been proven to activate PP1 [180], so it should be taken into consideration that insulin may be the effector for PP1 activation upon leucine administration. To support this, one study suggested that an increase in plasma insulin concentration above fasted values is required for the stimulation of muscle protein synthesis in response to oral leucine administration [97].

Additionally, tumour bearing rats fed leucine showed enhanced PKC expression [200]. One report demonstrated that PKC can inhibit PKR activation [220], which may provide an alternative mechanism by which leucine could inhibit PKR independent of insulin. Although autophosphorylated PKR has been shown to be resistant to downregulation by PKC [220], leucine may stimulate sustained PKC activation, unlike the transient stimulation observed in response to PIF [211], which would prevent activation of further PKR proteins.

Nutrients such as leucine and insulin can further affect translation initiation through alterations in the phosphorylation state of eIF2B $\epsilon$ . The best characterised mechanism of regulation of the guanine nucleotide eIF2B is the phosphorylation of eIF2 $\alpha$ . The  $\epsilon$  subunit of eIF2B is the substrate, at least in vitro, of glycogen synthase kinase (GSK-3), which can be phosphorylated and inactivated by insulin. This mechanism provides an alternative mechanism for the regulation of eIF2 $\alpha$  phosphorylation by mitogens [160].

Insulin has been shown to increase the bioactivity of IGF-1 [90]. For example, plasma IGF-1 concentrations are low in children with insulin-dependent diabetes mellitus, which can be restored to the normal range with insulin replacement [18]. This suggests that either insulin or IGF-1 supplementation will potentially have comparative effects, which is demonstrated in Figure 8.6A and B, where both hormones can increase protein synthesis in the presence of PIF, which is enhanced when co-treated with the PKR inhibitor, and diminished when incubated with the inhibitor of eIF2 $\alpha$  dephosphorylation. Insulin resistance has been demonstrated in both sepsis and burn injury which has been attributed to a postreceptor mechanism interference. However, IGF-1 sensitivity in skeletal muscle after burn injury [63] and sepsis [105] is maintained. This has important clinical implications because, although both insulin and IGF-1 have comparative signalling pathways and

effects, IGF-1 may be more effective than insulin in the treatment of muscle wasting in some catabolic conditions [63]. IGF-1, which has a primary structure similar to insulin [25], is important in skeletal muscle development, since it increases expression of myogenin and MyoD, which are critical muscle specific transcription factors required for myoblast differentiation and fusion into myotubes [173]. IGF-1 has also been shown to enhance protein synthesis and decrease protein degradation in skeletal muscle [83, 66].

Sepsis is a condition, like cancer, with characteristic muscle wasting due to an impairment of skeletal muscle protein synthesis, which results from a diminished translation initiation [105]. IGF-1 stimulated protein synthesis in septic rats through an increase in translation initiation through the Akt/mTOR signalling pathway [173]. Muscle wasting in sepsis, as well as cachectic cancer patients, is closely associated with increased expression of proinflammatory cytokines [105]. As both catabolic conditions present with severe muscle wasting linked with cytokine production it is likely that, since IGF-1 can stimulate muscle protein synthesis in septic rats [105], it will also be effective in reducing muscle wasting in cancer cachexia.

GH is released from the pituitary gland in a pulsatile manner, promotes growth directly in selected targets, or indirectly through the activation of the somatomedins and in particular IGF-1 [25]. This suggests that growth hormone will have a similar, and perhaps superior, positive influence on muscle wasting as IGF-1. Although used widely to treat weight loss in HIV-infected patients due to its ability to increase lean body mass and reverse negative nitrogen balance, little attention has been given to the beneficial effects it may have on cancer cachexia patients. However a small study did show that cancer patients administered growth hormone over a 3 day period showed an increase in plasma IGF-1 levels and a decreased urinary nitrogen loss [192].

In response to viral infection such as is seen in sepsis, PKR can be activated, possibly through the cytokine IFN- $\gamma$ , leading to reduced IGF-1 transcription. PKR is also upregulated in mice bearing the cachexia inducing MAC16 tumour and thus may be responsible for the disturbances in the GH-IGF1 axis observed in patients with cancer cachexia [25]. Furthermore GH resistance was observed in colorectal cancer patients with cachexia, which was reflected by decreased levels of IGF-1. This was reversed upon removal of the tumour indicating that changes in the GH/IGF-1 axis in colorectal cancer patients is likely caused by the



tumour rather than adaptations to malnutrition [25]. This supports the involvement of PKR, since it was demonstrated to be activated by the tumour specific factor, PIF. Therefore, as well as stimulating muscle development, IGF-1 administration may combat muscle wasting by inhibiting the autophosphorylation of PKR. This is substantiated by a report which showed that addition of IGF-1 to C6 cells blocked the induction of PKR protein by poly (IC) [37], while another study demonstrated that IGF-1 attenuated the ability of Ang II to induce PKR autophosphorylation through the activation of PP1 [153]. Moreover, IGF-1 has the ability to prevent Ang II induced muscle loss in mice [51]. Insulin supplementation may also prove effective, since it has the ability to activate some of the same anabolic signalling pathways as IGF-1, and has also been shown to inhibit PKR phosphorylation through PP1.

The results in this chapter support this evidence, where treatment of murine myotubes with IGF-1 or insulin increased protein synthesis and reversed the inhibition of protein synthesis by PIF, in a PKR dependent manner, since treatment with a PKR inhibitor increased the efficacy of this effect. It should be noted however that IGF-1 administration to animals is associated with tumour promoter properties and an increased metastatic potential of certain tumour types [87], suggesting that, at least in some cases, IGF-1 treatment would not be appropriate. Furthermore, GH administration is associated with an increased morbidity and mortality in critically ill patients, also limiting the potential usefulness of this hormone [176].

IGF-1 treatment has also shown effectiveness in muscle atrophy induced by other conditions such as starvation, glucocorticoid upregulation [51] and burn injury. IGF-1 inhibited burn induced muscle protein proteolysis and stimulated muscle protein anabolism through multiple mechanisms, including PI3K/Akt mediated inactivation of GSK3 $\beta$  and Foxo transcription factors [63]. Although these signalling pathways obviously play an important role it does not rule out the possibility that other mechanisms are involved as well. For example, as well as the inhibitory phosphorylation of GSK-3 $\beta$ , PKR activation has also been demonstrated to be upregulated in skeletal muscle of burned rats, which leads to muscle wasting [91]. Since IGF-1 has been shown to reduce PKR activation [37], it is possible that this mechanism is also involved in the IGF-1 induced block in protein breakdown in muscle from burned rats. This suggests that mechanisms similar to cancer cachexia are upregulated in other conditions where muscle atrophy occurs, and that inhibitors of PKR autophosphorylation or nutritional supplementation, which is effective in cancer



cachexia, may also be appropriate for reversing muscle wasting in other catabolic conditions. For example, muscle atrophy in congestive heart disease, has been linked to Ang II [132], while AIDS, parasitic infections and cancer, have been associated with  $TNF\alpha$ , both of which have been demonstrated to activate PKR [86].

As mentioned previously the amino acid levels in cancer cachexia patients is diminished [185], while imbalances in IGF-1 and insulin have been reported [25]. This disequilibrium in circulating growth factors and nutrients has been linked with diabetic cachexia. Diabetic cachexia results, in part, from net protein catabolism in which proteolysis exceeds protein synthesis, particularly in skeletal muscle, which adversely affects tissue repair, ensuing a susceptibility to injury or infection [160]. Neither are desirable conditions in cancer patients whose poor quality of life will be exacerbated, and recovery from therapy severely impaired. It has been proposed that treatment with BCAAs may serve to counteract the depressed rate of protein synthesis observed in diabetic cachexia as well as reverse the proteolytic processes [160]. Leucine administration would also stimulate an insulin release which might replace the insulin levels which are insufficient in diabetes. In this regard, leucine has obvious potential for treatment of several catabolic processes which underlay the muscle wasting observed in cancer cachexia patients. These findings provide rationale for developing new therapeutic strategies for skeletal muscle wasting conditions [51]

## 8.4 Conclusion

The branched chain amino acids (BCAAs) leucine and valine caused a significant suppression of the loss of body weight in mice bearing the MAC16 cachexia-inducing tumour, producing a significant increase in skeletal muscle wet weight, due to an increase in protein synthesis, and a decrease in degradation. The BCAAs also attenuated the depression of protein synthesis and increased protein degradation in response to PIF *in vitro*. Leucine, its metabolite HMB, insulin and IGF-1 attenuated the depression of protein synthesis in murine myotubes in response to PIF by an ability to attenuate phosphorylation of  $eIF2\alpha$  through attenuation of the activation of PKR. Leucine, and its metabolite HMB also attenuated the increased phosphorylation of PKR and  $eIF2\alpha$  in skeletal muscle of mice bearing the MAC16 tumour, and

this was attributed to an increased expression of protein phosphatase 1 (PP1). Treatment of MAC16 tumour bearing mice with leucine enhanced phosphorylation of mTOR, hyperphosphorylation of the translational repressor 4E-BP1, which increases the availability of the mRNA cap binding protein eIF4E for binding eIF4G and assembly of the initiation complex known as eIF4F. The eIF4F complex collectively serves to recognise, unfold, and guide the mRNA to the 43S ribosomal subunit. Leucine treatment also induced hyperphosphorylation of p70S6K, which may be responsible for the increased activity of eEF2 and also increase the synthesis of proteins involved in translation and ribosome biogenesis [4].

These changes would be expected to increase protein synthesis, while a reduction in the activation of PKR would be expected to attenuate the increased protein degradation and inhibit tumour growth. The evidence implies that nutritional supplementation may, at least partially, ameliorate cancer induced weight loss.

## Chapter 9

### Conclusions

Much evidence in this study indicates the modulation of the PKR/eIF2 $\alpha$  and eIF4F triad translational pathways, as well as the production of reactive oxygen species, and release of calcium in the muscle wasting disorder of cachexia (see Figure 9.1 for a diagram of the proposed signalling pathways involved in the genesis of muscle wasting by the catabolic agents PIF and Ang II). TNF $\alpha$  is a stress factor which has been associated with muscle wasting in a number of diseases, including cancer. Exposure of mammalian cells to TNF $\alpha$  inhibits overall translation and stimulates protein degradation and apoptosis, which is associated with the increased phosphorylation of PKR/eIF2 $\alpha$ , activation of NF- $\kappa$ B, and increased association of eIF4E with the inhibitory eIF4E-binding protein (4E-BP1) [86]. TNF $\alpha$  has also been shown to activate Akt [79]. All of these effects have been demonstrated in this study, and by others, to be regulated by both PIF and Ang II, both of which are considered stress factors of cancer cachexia [59]. Although this is a complex picture, it is not beyond reproach to assume that PIF/Ang II can mediate some of the same effects through the regulation of comparative signalling pathways to TNF $\alpha$ .

The current understanding in the academic community implies that signalling pathways involving PKR, which have a role in the muscle wasting of cachectic cancer patients, are also upregulated in other conditions

where muscle atrophy occurs. This suggests that inhibitors of PKR autophosphorylation, which is effective in animal cancer cachexia models, may also be a suitable therapeutic candidate for reversing weight loss and muscle atrophy in other catabolic conditions such as in Alzheimer's, Parkinson's, Huntington's [219], arthritic disease [70, 143, 202], burn injury's [91] and myotonic dystrophy [184], all of which have PKR implicated as an important pathogenic factor.

However, the meaning of these alterations is not completely obvious in regard to a causal relationship. It is tempting to present them as controlling events in the pathogenesis of cachexia, however there is a possibility that they are secondary adaptive responses to a chronic and devastating disease which involves sustained changes of many physiological pathways as a response to tumour burden as well as a direct effect of the tumour itself. Thus further studies would be advantageous to better examine the potential relationship between the production of PIF/Ang II and their involvement in the regulation of the mechanisms examined in this study in the genesis of cachexia and muscle wasting. A further requirement would be to clarify whether the mediators are acting in parallel or co-ordinately concur, and where the different signals ultimately converge to produce the hypercatabolic response [48]. An understanding of the complex molecular mechanisms underlying cachexia would allow the controlled interference of specific processes resulting in a possible reduction in the catabolic response in skeletal muscle. Such observations have important clinical implications since muscle wasting contributes to complications and early death of patients suffering from many diseases hallmarked by cachexia.

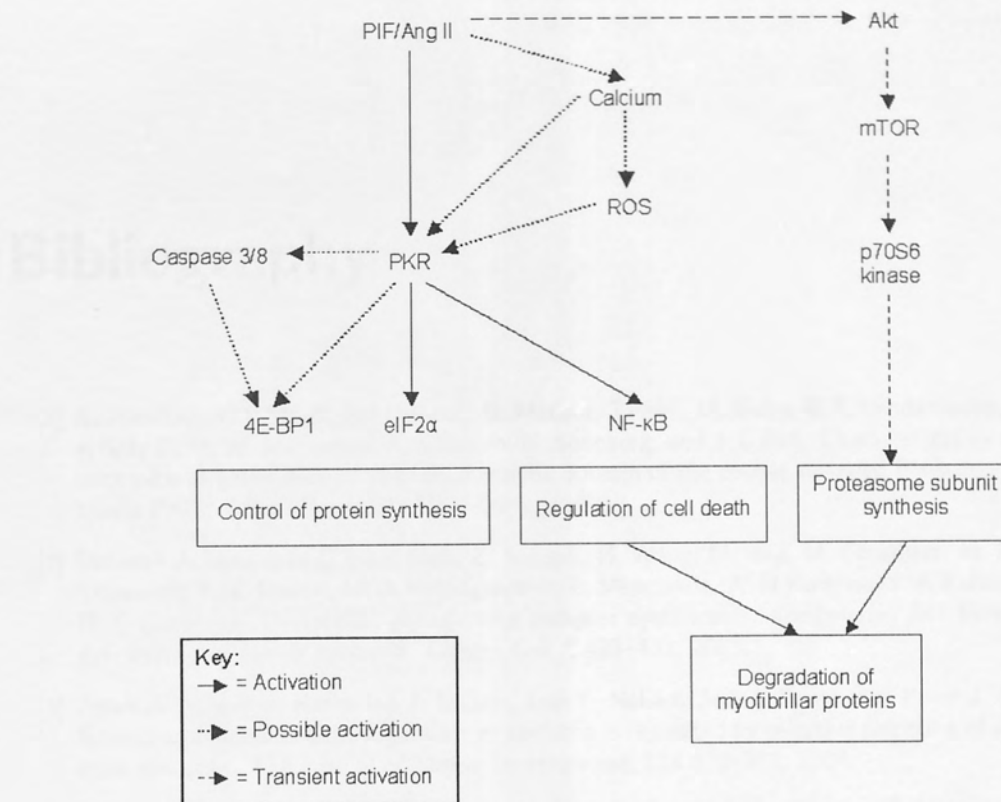


Figure 9.1: Outline of the proposed signalling pathways activated by catabolic agents in the pathogenesis of cachexia. Adapted from [86]

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## Appendices

# Appendices

## Additional Figures

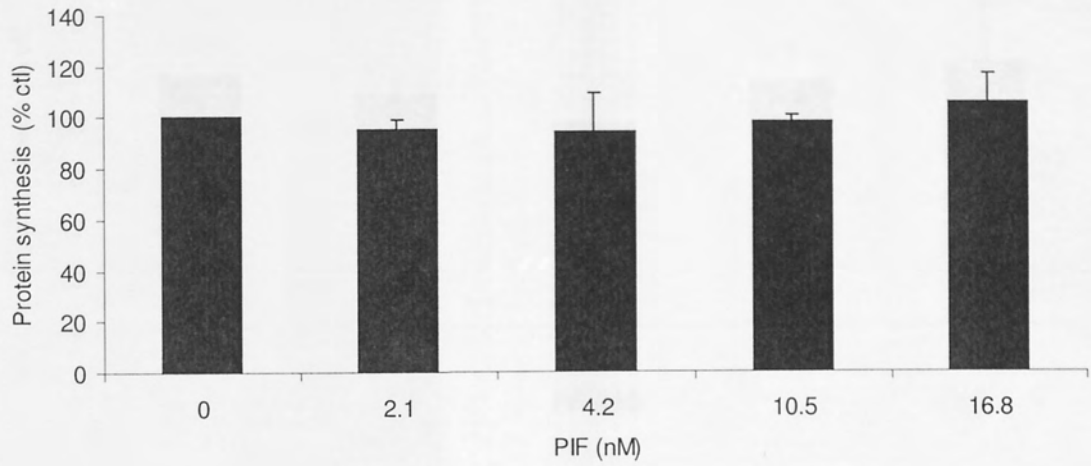
## A.1 Additional Figures for Chapter 3

### Appendix A

### Additional Figures

## A.1 Additional Figures for Chapter 3

A



B

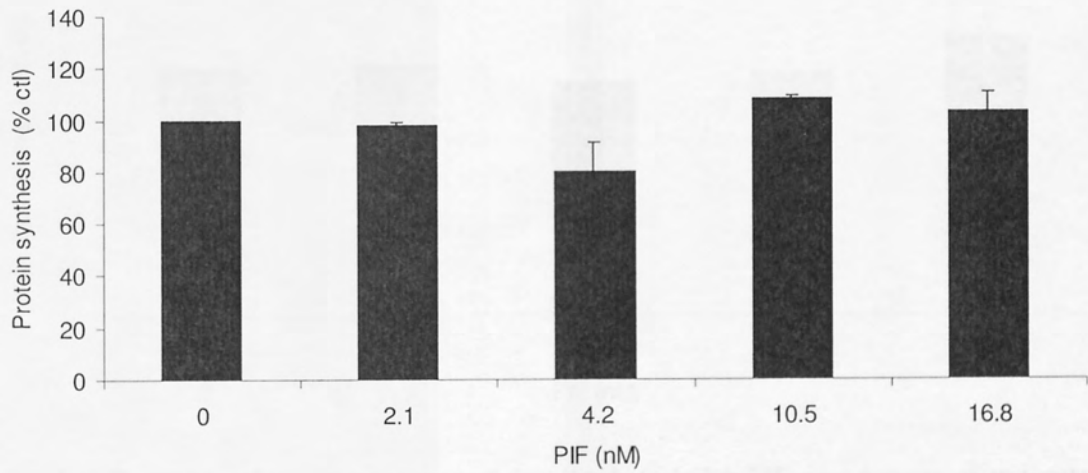


Figure A.1

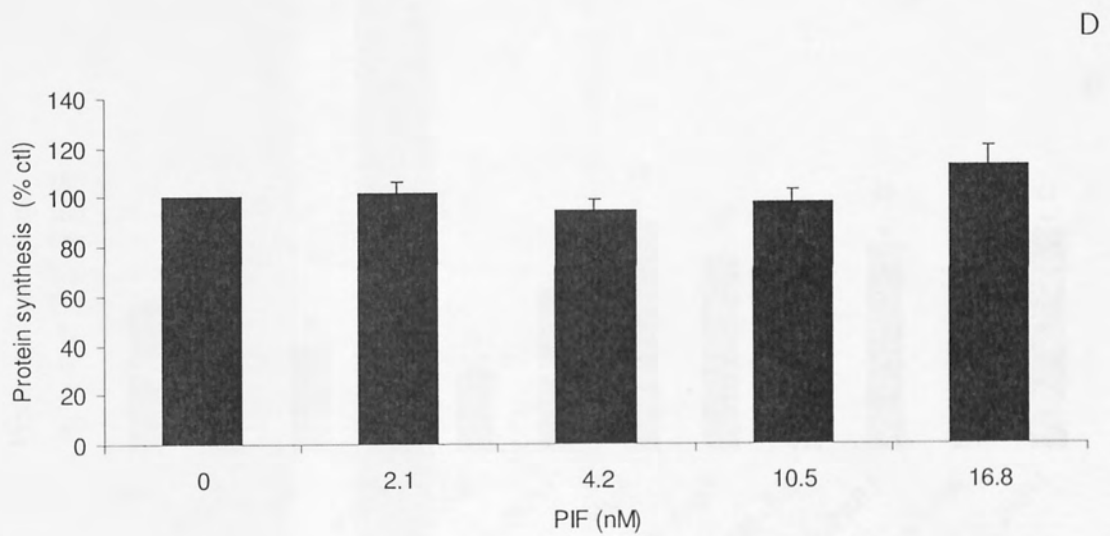
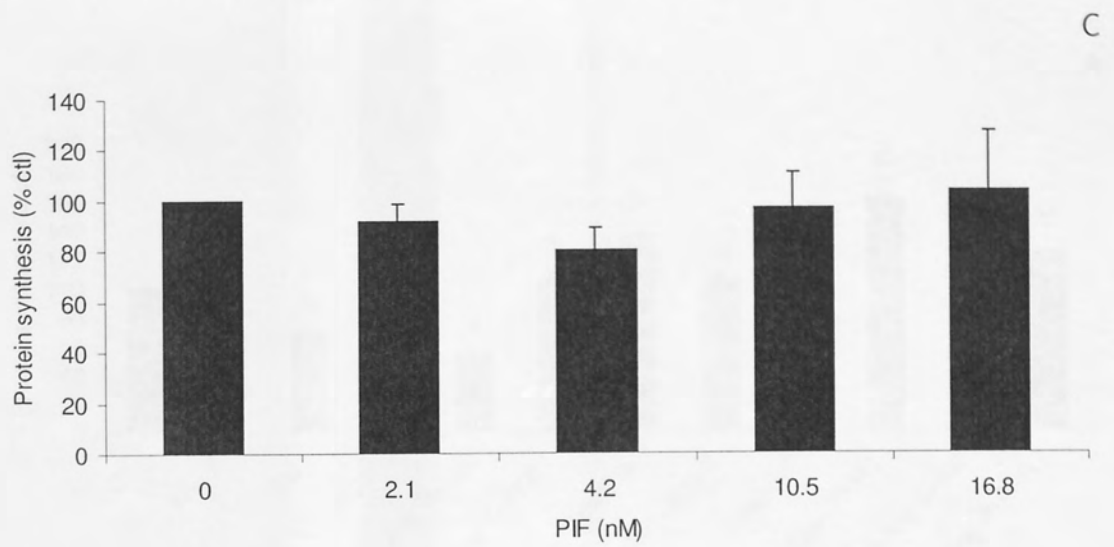


Figure A.1: **Protein synthesis in murine myotubes incubated with PIF over a range of concentrations and time periods.** PIF was incubated with the myotubes for 0.5h (A), 1h (B), 2h (C) and 24h (D). Error bars represent  $\pm$ SEM of 3 replicate experiments. No significant changes in protein synthesis were observed in response to PIF at these time points

## A.2 Additional Figures for Chapter 8

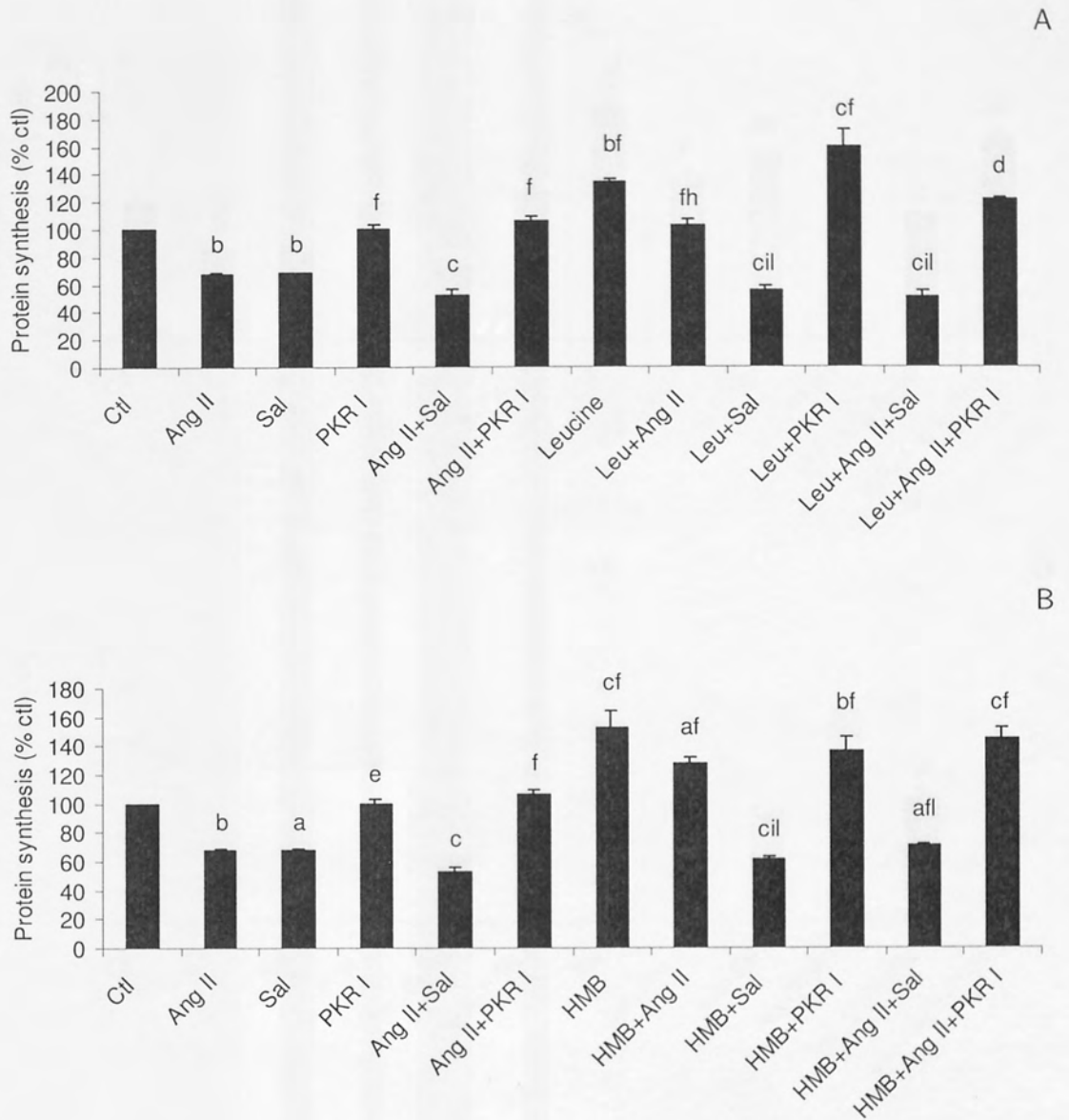


Figure A.2



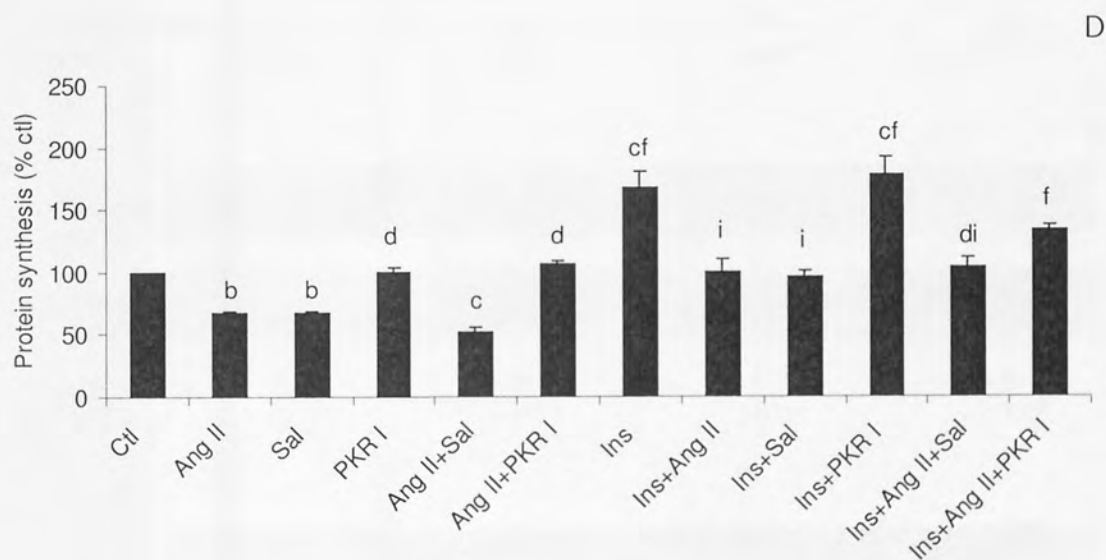
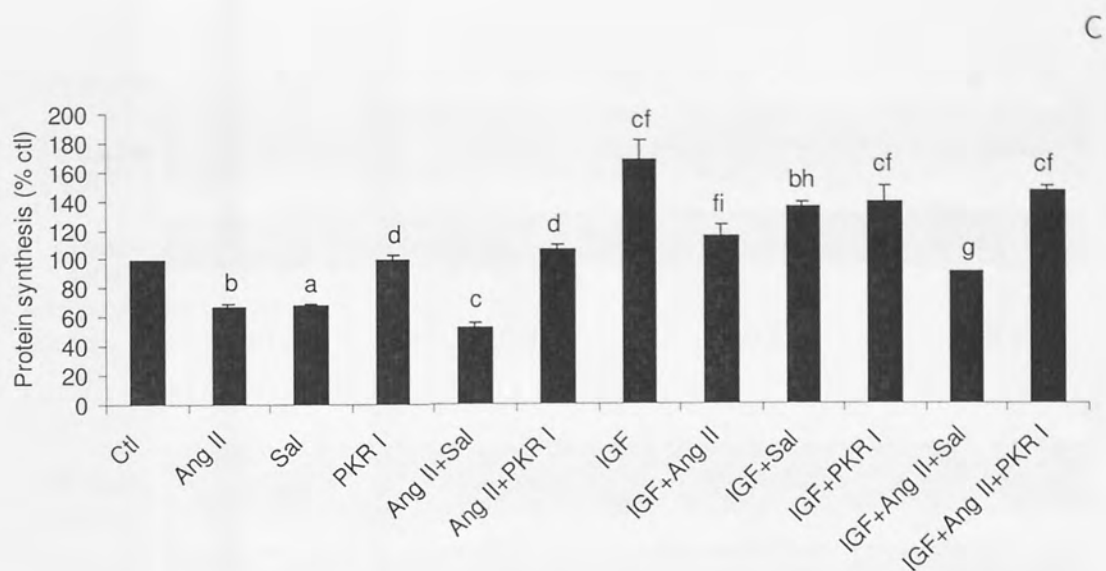


Figure A.2: **Effect of PKR phosphorylation and eIF2 $\alpha$  dephosphorylation inhibition on protein synthesis in murine myotubes incubated with anabolic factors and Ang II.** Protein synthesis was measured in murine myotubes when incubated with leucine (Leu) (2mM) (A), HMB (50 $\mu$ M) (B), IGF-1 (IGF) (100ng/mL) (C) or insulin (Ins) (1nM) (D), co-treated with Ang II (0.5 $\mu$ M), PKR inhibitor (PKR I) (210nm) and the eIF2 $\alpha$  inhibitor, salubrinal (Sal) (15 $\mu$ M). Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$ , b:  $p < 0.01$  or c:  $p < 0.001$ , differences from Ang II alone d:  $p < 0.05$ , e:  $p < 0.01$ , or f:  $p < 0.001$  differences from leucine/HMB/IGF/insulin alone g:  $p < 0.05$ , h:  $p < 0.01$ , or i:  $p < 0.001$  and differences from leucine/HMB/IGF/insulin co-treated with Ang II j:  $p < 0.05$  or l:  $p < 0.001$

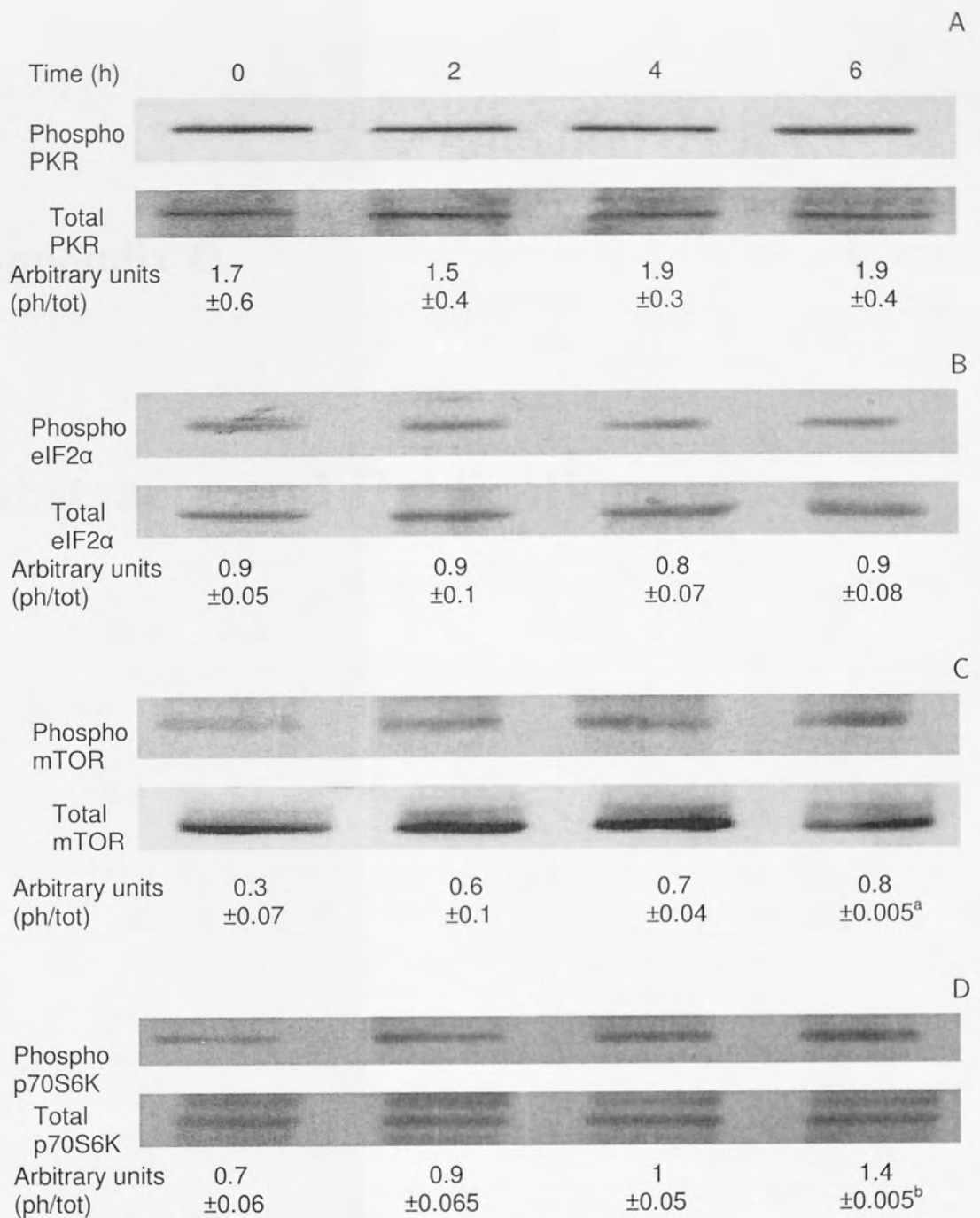


Figure A.3: **Effect of HMB on PKR,  $\alpha$ -subunit of eIF2, mTOR and p70S6K.** The phosphorylation of PKR (A),  $\alpha$ -subunit of eIF2 (B), mTOR (C) and p70S6K (D) with a corresponding PKR, eIF2 $\alpha$ , mTOR and p70S6K total (which was used to normalise the phosphorylated protein) was analysed by western blotting in murine myotubes treated with HMB (50 $\mu$ M). HMB was incubated with myotubes for 6h. Error bars represent  $\pm$ SEM of 3 replicate experiments. Differences from control are shown as a:  $p < 0.05$  or b:  $p < 0.001$

## Appendix B

## Abstracts and Publications

This section includes the following published articles:

**Skeletal muscle atrophy, a link between depression of protein synthesis and increase in degradation.**

H. L. Eley and M. J. Tisdale. *The Journal of Biological Chemistry*. Vol 282. pp7087-7097 (2007).

**Attenuation of muscle atrophy in a murine model of cachexia by inhibition of the dsRNA-dependent protein kinase.** H. L. Eley, S. T. Russell and M. J. Tisdale. *British Journal of Cancer*.

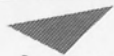
Vol 96. pp1216-1222 (2007).

**Mechanism of attenuation of angiotensin-II-induced protein degradation by insulin-like growth factor-1 (IGF-1).** S. T. Russell, H. L. Eley and M. J. Tisdale. *Cellular Signalling*. Vol 19. pp 1583-1595 (2007).

**Role of reactive oxygen species on protein degradation in murine myotubes induced by proteolysis-inducing factor and angiotensin II.** S. T. Russell, H. L. Eley and M. J. Tisdale. *Cellular Signalling*. Vol 19. pp1797-1806 (2007).

**Signalling pathways initiated by  $\beta$ -hydroxy- $\beta$ -methylbutyrate to attenuate the depression of protein synthesis in skeletal muscle in response to cachectic stimuli.** H. L. Eley, S. T. Russell, J. H. Baxter, P. Mukherji and M. J. Tisdale. *Am J Physiol Endocrinol Metab* (2007). Available via the Internet ahead of publication.

**Effect of branched-chain amino acids on muscle atrophy in cancer cachexia.** H. L. Eley, S. T. Russell and M. J. Tisdale. *Biochem J*. Vol 407. pp113-120 (2007).



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