Mechanism of muscle protein degradation in cancer cachexia

María José Lorite

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Summary

A protein-mobilising factor of estimated molecular weight 24 KDa (p24) was purified both from the cachexia-inducing MAC 16 tumour and the urine of cachectic cancer patients by a combination of ammonium sulphate precipitation and affinity chromatography using a monoclonal antibody developed against the murine material. Administration of p24 to non tumour-bearing mice caused a decrease in body weight 24 h after the first injection, which was attenuated by prior treatment with the monoclonal antibody. Loss of body weight was accompanied by an accelerated loss of skeletal muscle protein, as determined by the release of tyrosine from this tissue. This was associated with an increased release of PGE_2 and both protein degradation and PGE_2 release were attenuated by the monoclonal antibody. Loss of protein mass arose from both a decrease in the rate of protein synthesis and an elevation of protein breakdown; the latter due to an activation of the ubiquitin-proteasome proteolytic system.

In isolated muscle, p24 was capable of promoting protein breakdown and this was also associated with increased PGE_2 levels. Both tyrosine and PGE_2 release, were inhibited by PGE_2 inhibitors and a specific inhibitor of $cPLA_2$. When added to muscle cells in culture, p24 caused an elevation in the rates of total and myofibrillar protein breakdown and a depression in the rate of protein synthesis which was inhibitable by short-term incubation with insulin, suggesting that p24 may inhibit protein synthesis by causing an arrest in the translational process.

KEY WORDS: PROTEIN-MOBILISING FACTOR, PROTEIN SYNTHESIS, PROTEIN BREAKDOWN, PGE_2, UBIQUITIN-PROTEASOME PROTEOLYTIC PATHWAY.

A mis padres

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Abbreviations

AA	arachidonic acid
$AACOCF_3$	arachidonyltrifluoromethyl ketone
aFGF	acidic fibroblast growth factor
AIDS	acquired immunodeficiency syndrome
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP-1	activator protein-1
APF-I	active polypeptide factor from fraction I
APPs	acute phase proteins
APPR	acute protein phase response
APS	ammonium persulphate
AQM	acute quadriplegic dystrophy
ATP	adenosine triphosphate
BCAAs	branched-chain amino acids
BCE cells	bovine capillary endothelial cells
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
C2	proteasome subunit C2
C3	proteasome subunit C3
C5	proteasome subunit C5
C8	proteasome subunit C8
C9	proteasome subunit C9
CAF	calcium-activated factor
cAMP	cyclic AMP
CANP	calcium-activated neutral protease
CHO cells	chinese hamster ovary cells
CKII	casein kinase II

CLP	caecal ligation and puncture
$cPLA_2$	cytosolic phospholipase A_2
COX	cyclo-oxygenase
CRE	cAMP response element
CREM	cAMP response element modulator
CRF	chronic renal failure
CRP	C-reactive protein
CTP	cytidine triphosphate
CV-6504	2, 3, 5-trimethyl-6-(3-pyridylmethyl)-1, 4-benzoquinone
DAG	diacylglycerol
dbt-cAMP	dibutyryl-cAMP
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DHA	docosahexaenoic acid
DMD	Duchenne-muscular dystrophy
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DRB	5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	dithiothrietol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
E-64	trans-epoxysuccinyl-L-leucylamido-3-methylbutane
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EDL	extensor digitorum longus
EF	elongation factor
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N',

	N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
FCS	foetal calf serum
FFA	free fatty acids
GC	gas chromatography
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GlcN	glucosamine
GLUT-1	glucose transporter-1
GLUT-4	glucose transporter-4
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HA	hyaluronan
Hb	haemoglobin
HC3	human proteasome subunit C3
HCR	haemin-controlled repressor
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IF	initiation factor
IFN-γ	interferon-γ
IGF-1	insulin growth factor-1
IGFBP5	insulin growth factor binding protein 5
IgG2a	Immunoglobulin G2a
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-8	interleukin-8

ip	intraperitoneal
iv	intravenous
Ks	fractional rate of protein synthesis
α-LA	α-lactalbumin
LBM	lean body mass
LIF	leukaemia-inducing factor
LPA	lysophosphatidic acid
LPL	lipoprotein lipase
LPS	lipopolysaccharide
mAb	anti-p24 monoclonal antibody
MAC	murine adenocarcinoma
MAP kinase	mitogen-activated protein kinase
MCT	medium-chain triglycerides
MEK	MAP kinase inhibitor
3-MH	3-methylhistidine
MLPLI	melanoma-derived lipoprotein lipase inhibitor
MOPS	3-[N-morpholino]propanesulphonic acid
MS	mass spectroscopy
NFκB	nuclear factor κB
NPT	neopterin
NSAIDs	non-steroidal anti-inflammatory drugs
ODC	ornithine carboxylase
OPD	o-phenylenediamine
p24	24 KDa protein-mobilising factor
PAGE	polyacrylamide gel electrophoresis
PA-Ag	preabsorbing antigen
PBS	phosphate buffered saline
PCA	perchloric acid
PG	prostaglandin

PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGN	proteoglycan
PA	phosphatidic acid
PA	plasminogen activator
PGH_2	prostaglandin endoperoxide
PHAS-1	phosphorylated heat and acid stable protein-1
PI	phosphatidylinositol
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulfonylfluoride
PNGase F	peptide N-glycosidase
PP2A	protein phosphatase 2A
PUFAs	polyunsaturated fatty acids
PVP	polyvinylpyrrolidone
QS	Q-sepharose
RIA	Radioimmunoassay
REE	resting energy expenditure
S6	ribosomal subunit S6
SDS	sodium dodecyl sulphate
sICAM-1	soluble intercellular adhesion molecule-1
$sPLA_2$	secretory phospholipase A_2
Si	specific radioactivity of intracellular free pool phenylalanine
Sr	specific radioactivity of protein bound phenylalanine
SRE	serum response element
SRF	serum response factor

sTNFr55	soluble tumour necrosis receptor 55
sTNFr75	soluble tumour necrosis receptor 75
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TNF-α	tumour necrosis factor alpha
TPA	tetradecanoylphorbol-13-acetate
TPN	total parenteral nutrition
TRE	TPA response element
Tris	Trizma base
Tween 20	polyoxyethylene-sorbitan
UB	ubiquitin
UBPs	ubiquitin carboxy-terminal hydrolases
UTR	5'-untranslated region
WBPT	whole-body protein turnover
YAH	Yoshida ascites hepatoma

Chapter 1
Introduction

1.1. Cancer cachexia.

Cachexia is a syndrome of severe wasting whose most obvious clinical manifestation is the progressive weight loss which is often accompanied by anorexia. This condition occurs in some diseases such as congestive heart failure (Anker *et al*, 1997), rheumatoid arthritis (Taylor *et al*, 1996), acquired immunodeficiency syndrome (AIDS) (Von Roenn *et al*, 1992) and cancer amongst others, and it has been proposed as one of the major single causes of death (Warren, 1932 and Harnet, 1952).

In cancer, weight loss appears as one of the major prognostic factors together with the type of tumour, stage of the disease and performance status (DeWys, 1980). The frequency of weight loss ranges from 30-40% in patients with favourable non-Hodgkin's lymphoma, breast cancer, acute nonlymphocytic leukaemia and sarcomas, to 50-60% in patients with unfavourable non-Hodgkin's lymphoma, colon, prostate and lung cancer, and 85% of patients with pancreatic or gastric cancer. In the latter group, the extent and severity of cachexia are most dramatic, with patients having lost 15% of their pre-illness stable weight by the time of diagnosis and up to 25% by the time of death (Wigmore *et al*, 1997).

Loss of weight correlates with decreasing performance status and a lower quality of life (DeWys, 1980 and Ovesen *et al*, 1993) and those cancers associated with greater degrees of weight loss appear to have poorer prognosis and shorter survival rates. Furthermore, within a group of patients with the same type of malignancy, those with the greater weight loss show a lower response rate to chemotherapy and a shorter median survival (DeWys, 1986 and Costa 1977). In addition, a study carried out by Holter and Fisher (1977) showed that in a group of surgically-treated patients with gastrointestinal cancers, those who had not lost any weight had the lowest incidence of major complications.

Despite its elevated rate of occurrence, the mechanisms underlying the cachexia syndrome are still in debate since the 'apparent malnutrition' of these patients cannot simply be explained by one single cause. It is widely accepted that cachexia arises from a negative energy balance, but controversy exists as to whether this results from an insufficient nutrient intake or an elevated resting energy expenditure (REE) or a combination of both (section 1.2).

Anorexia is undoubtedly one of the clinical features of cachexia which can occur as an early manifestation of the disease or may appear as the malignant neoplasm grows and spreads. Cancer patients exhibit symptoms of early satiety and prolonged sense of fullness as a result of atrophied mucosal membranes and chemoreceptors associated with the ingestive apparatus (responsible for generating signals which control the appetite centres in the hypothalamus), and delayed stomach emptiness. Production of anorectic substances by the tumour, changes in the sense of smell and taste and learned food aversions are also important contributing factors to the development of anorexia (Theologides, 1974; DeWys, 1979 and Bernstein 1986). In addition, the psychological and emotional reactions to the disease and the side effects of radiotherapy and/or chemotherapy in some cases, also potentiates an already depressed appetite (Theologides, 1979).

However, the decreased food intake alone does not account for the progression of cachexia since hyperalimentation of the cancer patient can neither be sustained for long periods (Brennan and Burt, 1981) nor does it reverse the condition (Fearon and Carter, 1988), and in some instances it has been shown to be detrimental due to an enhancement of tumour growth. This has also been confirmed by the use of experimental models where forced feeding, paired feeding and caloric restriction experiments have shown that a decreased nutrient intake is not entirely responsible for such weight loss.

There are very few studies in which a beneficial effect of total parenteral nutrition (TPN) has been reported. No success has been achieved with regard to an increased response to

chemotherapy or survival rate. Only in patients undergoing surgical treatment has TPN support been shown to reduce the risk of operative and post-operative complications and, in those who succeeded in eating within 8-9 days post-operatively, the mortality rate has been found to be greatly reduced (Hyltander *et al*, 1994). Amelioration of anorexia, however, has been reported following administration of branched-chain amino acids (BCAAs) which, by providing a low tryptophan:BCAAs ratio, reduce the amount of tryptophan directed to the brain and consequently the precursor pool for the synthesis of the anorexia-inducing neurotransmitter, serotonin (Cangiano *et al*, 1996).

The induction of metabolic derangements in order to provide nutrients for tumour growth has been hypothesised as the cause for an increased caloric expenditure which, together with a decreased caloric intake, may result in a state of caloric deficit (Theologides, 1974 and Costa, 1977). Indeed, cancer patients present important aberrations with regard to the metabolism of carbohydrates, lipids and proteins, which are also frequently accompanied by changes in the content of intracellular and extracellular water, vitamins and enzymes, electrolyte and acid-base disturbances and changes in hormonal patterns (Theologides, 1979). However, there are some discrepancies as to whether there is a direct cause-effect between such metabolic alterations and an elevated REE and thus, while many investigators have found increases in REE in cancer patients, others have failed to do so (section 1.2). Also, although tumours show special nutrient requirements which are usually derived from the diet and the host, the notion of tumours acting as nitrogen traps - one of the hypotheses which might explain the existent negative nitrogen balance (Theologides, 1974 and Brennan and Burt, 1981) - is debatable since the tumour mass very rarely exceeds 10% of the weight of the cancer patient (Costa, 1977).

1.2. Body composition, energy expenditure and host metabolism.

1.2.1. Body composition and energy expenditure.

One of the hallmarks of cachexia is the loss of lean body mass, which unlike in starvation, contributes to the loss of body weight as much as the fat compartment and thus, there is

more wasting of muscle in a cancer patient than in a normal person for a given degree of weight loss (DeWys, 1985). This is one of the key points to consider in the management of this disease since preservation of total body protein is essential for maintaining vital functions in the cancer patient, including their immunological competence and respiratory homeostasis. Protein depletion contributes significantly to an overall morbidity and a shorter survival and renders patients less capable of withstanding surgery and/or treatment (Costa, 1977).

Indeed, nitrogen balance and whole body protein flux studies have shown that cancer patients are in a state of nitrogen deficit which correlates with the loss of body weight. Attempts to reverse this nitrogen-deficient state by means of TPN have shown a partial restoration of the rates of protein synthesis and degradation (Brennan and Burt, 1981), although the majority of the total weight gain has been shown to be consequence of the accumulation of fat and water (Cohn, 1986). In a group of post-operative patients, a better nitrogen balance was achieved following infusion of BCAAs, which was accompanied by an improvement in weight loss and even weight gain in some cases (Freund *et al*, 1979).

Under conditions of acute starvation, the organism undergoes a mechanism of adaptation in which energy consuming processes are shut down. This response includes the preservation of muscle mass and total body nitrogen by inhibiting gluconeogenesis and loss of urinary urea, and this is accompanied by an increased utilisation of ketone bodies as the source of energy (Brennan, 1977). This conversion to a 'fat fuel economy' by peripheral tissues, including the brain, consequently leads to a decrease in oxidative metabolism and to a much lower metabolic rate, which contrasts with the elevated REE found in cancer patients where this adaptive response appears to be impaired (Waterhouse, 1974 and Warnold *et al*, 1978).

In a group of 200 patients with a variety of tumour types, 26% were shown to be hypermetabolic, 41% normometabolic and 33% hypometabolic (Knox *et al*, 1982) and, in a

separate study, it was found that the tumour type was a major determinant for the elevation in energy expenditure (Fredrix *et al*, 1991). This research group found that in 104 patients with gastric and colorectal cancer, the REE was not different from control values, in contrast with the 47 patients with non-small cell lung cancer were it was significantly elevated. Furthermore, one year after tumour resection, lung cancer patients with no recurrence of the disease had significantly lower REE than those with unsuccessful treatment. One and a half years after tumour resection, patients with gastric and colorectal cancer -on the other hand- had unaltered REE regardless or not of recurrence. Some authors have also proposed that the presence of secondary metastases, and not the primary tumour, are responsible for the elevation in REE (Macfie *et al*, 1982).

In a study which included 202 subjects Hyltander *et al* (1991), also by means of indirect calorimetry, found that cancer patients had a much higher REE when compared to either weight losing or weight stable controls. It was also observed that such elevation in REE was independent of malnutrition (in agreement with Warnold *et al*, 1978), and irrespective of weight loss since some cancer patients who had not lost any weight also had an elevated rate of fat oxidation. The authors therefore concluded that an elevated adrenergic state was responsible for the elevated energy expenditure in the cancer patient and the fact that it preceded the weight loss points towards the cause rather than the consequence of cachexia. This assumption was supported by the elevated adrenaline levels found in these cancer patients and by the reduction of the REE by 10% following treatment with the β-adrenergic antagonist propranolol (Hyltander *et al*, 1993).

In contrast, others have suggested that despite the appearance of metabolic disturbances, it is the inadequate food intake what determines the progression of cachexia and that the metabolic state of the cancer patient neither determines the stage of the disease nor it is much different from that of a healthy individual (Mullen, 1994). These differences, however, may be accounted for by the sensitivity of the methods employed or by the lack

of a homogeneous group of patients and/or the choice of proper age-, sex- and weight-matched controls.

1.2.2. Carbohydrate metabolism.

Cori cycle activity and gluconeogenesis -two very highly energy demanding processes- have both been shown to be significantly enhanced as demonstrated by the incorporation of their respective ^{14}C-labelled precursors, lactate and alanine, into glucose which is then directed to the tumour (Waterhouse, 1974; Holroyde *et al*, 1975 and Waterhouse *et al*, 1979). It has been estimated that the increased Cori cycle activity could account for up to 10% of the energy expenditure (Young, 1977) which, together with an elevated oxygen consumption have both been shown to correlate with the upregulation of this pathway (Wesdorp, 1986). In addition, administration of exogenous glucose has been shown to be used for fatty acid synthesis rather than for Krebs cycle oxidation, a metabolic pathway which also has a considerable ATP requirement (Waterhouse, 1981). Increased gluconeogenesis has also been demonstrated in experimental models (Shapot and Blinov, 1974; Roh *et al*, 1984 and Argiles and Lopez-Soriano, 1991).

In addition to increased Cori cycle activity, cancer patients have also been found to have increased rates of glucose production, oxidation and turnover, accompanied by elevated levels of blood lactate and energy expenditure (Holroyde *et al*, 1975). Both overnight fasting glucose and basal insulin levels were normal, but when these patients were challenged by a glucose load, an impaired glucose tolerance was observed on the basis of its delayed clearance from the blood (Holroyde *et al*, 1984). The authors proposed that this was due to a reduced insulin secretion, although insulin resistance (as determined by the observed decreased uptake by peripheral tissues) could also be responsible for this situation of glucose intolerance.

In skeletal muscle, one of the most important organs for the disposal of glucose, a lowered capacity for its metabolism originates with the presence of malignancy (Lundholm *et al*,

1981). Insulin resistance in humans has been shown to be associated with a relative increase in percentage of insulin-insensitive fibres (type 2b or fast-twitch glycolytic) with respect to insulin-sensitive fibres (type 1 or slow-twitch oxidative and type 2a or fast-twitch oxidative/glycolytic) (Storlien *et al*, 1996). This is in agreement with the observed decrease in type 2a fibres reported in *musculus semitendinosus* of cancer patients by Lundholm *et al* (1981).

In an experimental model of cancer anorexia and cachexia, the effects of spontaneous physical exercise appeared to be beneficial at the early stages of the disease despite an elevated food intake (Daneryd *et al*, 1990), a finding that further supports the importance of the muscle fibre composition in the improvement of insulin action.

Besides exercise, and may be more importantly, peripheral insulin sensitivity is also influenced by the composition of dietary fat. A high proportion of n-3 polyunsaturated fatty acids (PUFAs) in plasma and membrane lipids has been shown to correlate with a better insulin sensitivity in humans (Vessby *et al*, 1994) and animals (Behme, 1996 and Luo *et al*, 1996). With regard to skeletal muscle, slow-twitch oxidative muscles such as soleus have been shown to have a greater ability than EDL (fast-twitch glycolytic muscle) to alter the content of n-3 fatty acids in their membrane phospholipids in response to a n-3 PUFA-rich diet (Ayre and Hulbert, 1996). Thus, the atrophy of oxidative fibres seen in cancer patients (which in addition have a higher content of both glucose transporters, GLUT-1 and GLUT-4, Johannsson *et al*, 1996) and the development of insulin resistance, clearly suggests a role for the composition of membrane phospholipids in the development of this disease and its contribution to cachexia.

1.2.3. Lipid metabolism.

Increased free fatty acid (FFA) and glycerol turnover rates were found in close association with the elevated REE levels of hypermetabolic patients with cancer, indicating a predominant state of hydrolysis with little re-esterification (Legaspi *et al*, 1987).

On the other hand, others have suggested that loss of body fat in cancer cachexia is due to a reduced rate of lipogenesis rather than augmented lipolysis, since no difference was found in the glycerol turnover rate of cancer patients in comparison with controls (Jeevanandam *et al*, 1986). In fact, glycerol turnover was slightly diminished due to its reduced clearance rate from circulation, a factor which would influence the rate of lipid synthesis. The rates of glucose and FFA oxidation, although slightly higher than in controls, were not substantially increased in a group of cancer patients under basal conditions (Waterhouse, 1974). However, in the presence of an exogenous load of glucose, cancer patients failed to suppress the elevated rate of fatty acid oxidation, unlike starved individuals whose rate of FFA oxidation is normalised following glucose administration.

In addition, insulin resistance has also been suggested to play a role in the suppression of lipogenesis due to an abnormal glucose regulation (Young, 1977). Incorporation of n-3 PUFAs into membrane phospholipids -as for skeletal muscle- has also been shown to improve the action of insulin in rat epididymal adipocytes, which was reflected in the higher glucose transport, oxidation and incorporation into total lipids (Luo *et al*, 1996).

Whereas glycerol can enter the gluconeogenic pathway in order to generate glucose for the tumour, it has been estimated that only 1% of FFAs is taken up by the tumour and the rest may be incorporated into hepatic glycerophospholipids and excreted as lipoproteins (Lundholm *et al*, 1981) or be directly oxidised. Therefore, the degree of hyperlipidemia may also be influenced by the capacity of muscles to oxidise fatty acids (depends in turn on muscle fibre type distribution).

Hypertriglyceridaemia is also a common alteration of lipid metabolism, which generally arises from an inhibition of lipoprotein lipase (LPL) activity in host body tissues. This is the key enzyme for the hydrolysis and plasma elimination of exogenous and endogenous triglycerides, which is considered the first step in dietary fat utilisation.

A significant reduction in LPL activity has been found in adipose tissue of tumour-bearing mice, which was associated with a decline in lipid synthesis and an elevated rate of lipolysis (Thompson *et al*, 1981). Hypertriglyceridaemia, and decreased adipose LPL and liver fatty acid synthase activities have been found in tumour-bearing rats (Lanza-Jacoby *et al*, 1984). Furthermore, changes in LPL activity have been shown to correlate negatively with tumour growth and positively with decrease of body fat mass of tumour-bearing rats following body composition analysis (Lanza-Jacoby *et al*, 1984). Depletion of carcass lipid in mice has been shown to be dependent on the type of tumour rather than the tumour burden and, indeed, small tumours have proved to have major cachectic effects on their hosts (Hollander *et al*, 1986). This further rules out the idea of nutrient sequestration by the tumour and supports a role for the production of lipolytic factors responsible for the increased lipolytic activity (section 1.3).

Fatty acid oxidation in experimental models of cachexia, has been shown to be suppressible by ketone bodies incorporated in the diet (Sidiqui and Williams, 1989) and, in humans, hyperketonaemia is known to play an important role in protein conservation, by replacing glucose as fuel for the brain and in reducing the availability of alanine for gluconeogenesis under conditions of negative caloric intake (Sherwin *et al*, 1975). Tumours are thought to be incapable of using ketone bodies as a source of energy (section 1.4), but a recent report has suggested that some types of tumours may be able to use these for lipid synthesis (Hildebrandt *et al*, 1995).

1.2.4. Protein metabolism.

It is generally accepted that one of the causative factors of cachexia is the inability to meet the very high metabolic demands imposed on the host by the presence of the tumour. The diminished, or even unaltered nutrient intake in some cases, becomes insufficient to provide energy for such processes but, unlike in starvation, cancer patients fail to adapt to an energy conservation state. Whole-body protein turnover (WBPT) and hepatic protein synthesis, some of the more energy-demanding processes in the organism, are not only allowed to

continue at their normal rates but, in fact, they are both considerably increased (Emery *et al*, 1984).

Increased rates of protein synthesis and degradation were found in a group of patients with leukaemia, as measured by the rate of excretion of urinary nitrogen (ammonia and urea) following a single dose of ^{15}N-glycine (Kien and Camita, 1983). Using the same method, WBPT was reported to be elevated by 35% in a group of patients with benign disease (Jeevanandam *et al*, 1984). An elevated rate of whole body protein turnover has also been found in cachectic patients with several types of carcinoma of the bronchus and hypernephroma by means of the ^{13}C-leucine enrichment method, which showed a depressed rate of protein synthesis for skeletal muscle despite a whole body elevated rate (Emery *et al*, 1984). The elevation in the rate of whole body protein synthesis is clearly an indication of an impaired adaptive response which, under a situation of undernutrition, would normally repress any cost effective metabolic process.

In a group of patients with lung carcinoma, the rate of WBPT as determined by ^{13}C-leucine flux studies was shown to be significantly higher than in the control group. Interestingly, the elevated rates of protein turnover did not significantly correlate with the weight loss and, even more, the REE (expressed per kilogram of lean body mass, LBM) was not different from the controls (Melville *et al*, 1990). Despite the enhancement of the WBPT rate, a contribution of only 3% to the total REE was estimated by the authors, which would not have been detected by means of any indirect calorimetry method.

In agreement with the above, another study has also shown that in a group of patients with lung or colon cancer, irrespective of weight loss, WBPT was significantly elevated and that this did not correlate with an increased REE. In fact, there was no significant difference between the REE in cancer patients and the weight stable non-cancer controls (Fearon *et al*, 1988b), indicating that the idea of cancer patients losing weight through an elevation of REE or WBPT is an oversimplification. As for the study carried out by Melville *et al*

(1990), the contribution of an elevated protein synthesis towards the REE was estimated to be around 8%, which again would have been undetectable.

These two studies are therefore in disagreement with that carried out by Hyltander *et al* (1991) (section 1.2.1). However, even if a normal rate of energy expenditure is considered in the cachectic patients, this would be inappropriate in the semistarving cancer host since the normal adaptive response to reduction in energy intake is a decrease in energy expenditure.

One of the theories which have been put forward as the cause of cachexia, is the competition for nutrients between the tumour and the host (Stein, 1978). Tumour cells display a preferential uptake for some amino acids (Wiseman and Ghadially, 1955; Lazo, 1981 and Kallinowski *et al*, 1987) and, indeed, tumours have been shown to be in positive nitrogen balance (Carrascosa *et al*, 1984) unlike their tumour-bearer hosts. Whether this avidity for certain amino acids is responsible for the negative nitrogen balance, seems quite unlikely since the tumour only represents a very small proportion of the total body weight. However, it may be possible that the removal of some amino acids from the circulation could influence the rate of protein synthesis in tissues such as skeletal muscle (Stein, 1978). This would potentiate an already existent catabolic state, due to an elevated demand for alanine in order to serve gluconeogenic purposes and, together with an uncontrolled oxidation of BCAAs (Williams and Matthaei, 1981), would largely account for the muscle wasting and negative nitrogen balance.

Ketone bodies are known to inhibit both the oxidation of BCAAs in muscle and the release of alanine from this tissue (Buse *et al*, 1972) and, during starvation, they replace glucose as the energy source. Since vasopressin suppresses the oxidation of long chain fatty acids and ketone production (Williamson *et al*, 1980), and its increased secretion appears to be related to the metabolic stress induced by the tumour (Williams and Matthaei, 1981), this has also been proposed as one of the mechanisms involved in the development of cachexia.

A ketogenic diet containing 70% medium-chain triglycerides supplemented with 3-hydroxybutyrate, was shown to be beneficial in patients with malignant disease who had lost 32% of their body weight (Fearon *et al*, 1988a). The induction of ketosis reduced blood glucose, lactate and pyruvate levels and there was a tendency for sparing alanine. Even more, the patients entered a state of positive nitrogen balance which was accompanied by a significant weight gain. However, despite the reduction of gluconeogenesis, the ketotic state was not responsible for the improved nitrogen status, since patients receiving a normal diet exhibited similar rates of protein synthesis and degradation, suggesting that the positive nitrogen balance might have been the consequence of a better nutritional status.

1.3. Mediators of cachexia.

1.3.1. Tumour-mediated host inflammatory response.

1.3.1.1. The acute protein phase response.

The presence of a tumour, as it happens under situations of trauma or sepsis, induces the development of an inflammatory response which involves the production of cytokines and acute phase proteins. The latter, at least in part, may account with the elevated rate of hepatic protein synthesis and the excessive depletion of skeletal muscle which serves as a source of amino acids for the synthesis of these proteins. This is supported by body composition experiments which have shown the dramatic loss of the lean body mass compartment in response to inflammation (Watson and Sammon, 1980).

In the study by Fearon *et al* (1988b) it was shown that the rate of protein turnover was 20% greater in patients with a positive acute protein phase response (APPR) than in those without. However, a correlation between APPR and REE does not seem straightforward, since the development of this response has been associated with increases in REE in some cases, but not in others. Thus, whilst Fearon *et al* (1988) reported no changes in the REE in a group of patients with lung or colon malignancies, a group of 87 patients with primary non-small cell lung cancer not only showed an elevated REE, but also this correlated with weight loss (Staal-van den Brekel *et al*, 1995). Increased REE has been reported in patients

with pancreatic cancer regardless of the presence of APPR, although it was much higher in those with a positive response (Falconer *et al*, 1994). Furthermore, the weight loss and the reduction of lean body mass were both greater in those patients with a positive APPR, although the difference was not statistically significant.

The pro-inflammatory cytokines tumour necrosis alpha (TNF-α) and interleukin-6 (IL-6) have each been implicated as potential mediators of the inflammatory response associated with cachexia. Support to this idea comes from a study by Wigmore *et al* (1995), where a group of patients with pancreatic cancer and elevated REE were treated with ibuprofen. The results showed that, following treatment with the anti-inflammatory drug, REE decreased significantly and, in 9 out of 10 patients, C-reactive protein (CRP) levels were decreased.

In the study carried out by Staal-van den Brekel *et al* (1995), the plasma levels of soluble TNF-receptor (sTNFr) 55, sTNFr 75, soluble intercellular adhesion molecule (sICAM)-1 and the acute phase proteins, lipopolysaccharide (LPS)-binding protein and CRP, were all significantly increased suggesting that TNF-α could be one of the mediators of the inflammatory response. However, TNF-α levels were undetectable in both cancer and control subjects in contrast with interleukin-6 (IL-6) which was detected in 41 of the 87 cancer patients. Similarly, Falconer *et al* (1994) were unable to detect TNF-α in the plasma of patients with either a positive or a negative APPR, although the former group exhibited higher -but not significant- IL-6 levels. However, the production of TNF-α and IL-6 was significantly greater in the isolated peripheral blood mononuclear cells from patients with a positive APPR, indicating that the local rather than the systemic cytokine production may be important in regulating the APPR.

A significant positive correlation between APPR and IL-6 was also shown in 6 patients with hepatic metastasis secondary to colon cancer (3 of them also had detectable serum levels of TNF-α) (Fearon *et al*, 1991) and, *in vitro*, hepatocyte CRP production has been

shown to be stimulated in response to IL-6, interleukin-8 (IL-8) and the supernatants of several pancreatic cell lines (Wigmore *et al*, 1994). Furthermore, the stimulation of CRP by the different pancreatic cell lines was inhibited to various degrees with anti-IL-8 or anti-IL-6 antibodies.

Nevertheless, administration of ibuprofen to a group of patients with colonic adenocarcinoma and multiple hepatic metastases, while being effective with regard to the attenuation of the APPR and WBPT, did not significantly alter the levels of IL-6 or TNF-α (Preston *et al*, 1995). Although in apparent disagreement, this finding may just emphasise that the production of acute phase proteins is regulated locally by these cytokines.

Despite the elevated production of acute phase proteins, the hepatic fractional synthetic rate in patients with a positive APPR has been shown to be decreased, suggesting that the synthesis of structural hepatic proteins is depressed and that the WBPT is prioritised towards the export of secretory proteins. Furthermore, it also suggests that increases in hepatic mass associated with malignancy may be the outcome of a decreased protein breakdown rather than and increased rate of protein synthesis (Fearon *et al*, 1991).

Dietary fish oil supplementation for 3 months (18% eicosapentaenoic acid, EPA, and 12% docosahexaenoic acid, DHA) has been shown to produce an important change in body weight (from -2.9 kg/month to +0.3 kg/month) in cachectic patients with pancreatic cancer undergoing clinical trials in Edinburgh (Wigmore *et al*, 1996). Body weight gain, which was due to an increase in fat and protein content, was accompanied by a reduction in acute phase protein production and the stabilisation of REE. It appears that part of the anticachectic effect of fish oil, resides in the ability of EPA to inhibit the APPR via suppression of IL-6 (Wigmore *et al*, 1997) and, in addition, EPA has also been found to have an antitumour effect against pancreatic cancer cells *in vitro* (Lai *et al*, 1996).

1.3.1.2. The role of cytokines.

1.3.1.2.1. Tumour necrosis factor-α (TNF-α).

In addition to their association with the production of acute phase proteins (APPs) and inflammatory response, the ability of cytokines to inhibit LPL activity and induce some of the cachectic parameters, has prompted the investigation into their role as possible causative agents of this disease. The first indication of a cytokine being involved in cachexia comes from experiments involving *Trypanosoma cruzi*, where infected rabbits developed a severe weight loss. The high levels of 'cachectin' (later identified as TNF-α, Beutler *et al*, 1985) found in the blood of these animals was postulated to be responsible for the disease. Even more, anti-TNF-α antibody, but not anti-IL-6 or anti-interferon (IFN)-γ antibodies, has been shown to attenuate the weight loss induced by this infectious agent (Truyens *et al*, 1995).

The role of this cytokine has been further supported by *in vitro* experiments which have shown recombinant TNF-α to be able to inhibit both LPL activity and mRNA in adipocytes (Zechner *et al*, 1988). In addition, Hauner *et al* (1995) reported a 400% increase in lipolysis in fat cells, which may be partly due to a reduced glucose transport, as measured by the decrease in GLUT 4 protein and mRNA levels. TNF-α has also been shown to induce insulin resistance *in vitro* by inhibiting the insulin-induced receptor autophosphorylation, possibly through the activation of a tyrosine phosphatase (Kroder *et al*, 1996).

However, a direct role for TNF-α in the development of cachexia *in vivo* is not so clear. In human studies, elevated circulating levels of the cytokine have been found in some cases (Balkwill, 1985 and Knapp *et al*, 1991), but not in others (Scuderi *et al*, 1987 and Socher *et al*, 1987) and in animals, despite the elevated levels of TNF-α associated with some tumour models, the induction of weight loss appears to be associated with the development of anorexia.

Chinese hamster ovary (CHO) cells which had been transfected with human TNF-α , produced an extensive degree of anorexia and weight loss in nude mice, the latter owing to the depletion of fat and muscle tissue (Oliff *et al*, 1987). Elevated TNF-α levels have been found in rats bearing the Yoshida AH-130 hepatoma, which were decreased after administration of anti-TNF-α antibody. However, no attenuation of either weight loss or anorexia was achieved (Costelli *et al*, 1993). Similarly, only partial reversal of the condition was observed following administration of the antibody to rats bearing the MCG 101 sarcoma or the Lewis lung carcinoma, where hypoalbuminaemia, anaemia and signs of an ongoing APPR were still evident (Sherry *et al*, 1989).

In addition, despite the induction of loss of muscle protein *in vivo*, this situation has not been reproduced *in vitro* (Moldawer *et al*, 1987).

Some investigators have suggested the possibility that part of the effects of TNF-α may be mediated through the release of adrenocortical hormones since, for instance, elevated corticosterone levels have been found together with an overproduction of TNF-α in tumour-bearing rats. However, adrenalectomy, despite correcting for hypertriglyceridaemia and reducing corticosterone levels, did not improve cachexia (Tessitore *et al*, 1994) and muscle hypercatabolism has been shown not to be inhibited by a glucocorticoid antagonist (Llovera *et al*, 1996). Others have proposed prostaglandin E_2 (PGE_2) as the mediator, but indomethacin was found not to have an effect on the reversal of cachexia (Costelli et al, 1993) or in the prevention of weight loss (Mahony and Tisdale, 1989).

1.3.1.2.2. Interleukin-6 (IL-6).

As discussed in section 1.3.1.1, elevated levels of this cytokine have been found in patients with metastatic disease who had elevated WBPT rates and a positive APPR (Fearon *et al*, 1991). Increased production of IL-6 has also been reported in patients with ovarian (Scambia *et al*, 1995), lung (Scott *et al*, 1996) and pancreatic cancer (Falconer *et al*, 1994).

However, in the latter group, no correlation between IL-6 and REE or APPR was found. Furthermore, ibuprofen decreased both APPR and WBPT, while having no effect on the levels of IL-6 (Preston *et al*, 1995).

In the colon 26 cachexia model, higher levels of serum IL-6 have been found to correlate with increasing weight loss, which mainly arises from a massive depletion of fat (85%) (Tanaka *et al*, 1990). Furthermore, both IL-6 and weight loss have both been reversed following tumour resection (Strassman *et al*, 1992a) and anti-IL-6 antibody treatment (Strassman *et al*, 1993b).

An elevated production of IL-6 has also been found in the colon 26 cell line, where this process has been shown to be stimulated by IL-1 (Strassman *et al*, 1992b). This is also confirmed by the fact that high affinity receptors for IL-1 have been found in the tumour and that intratumoral injection of a receptor antagonist reduces both the serum concentration of IL-6 and the loss of fat and muscle (Strassman *et al*, 1993a).

However, doubts about the role of IL-6 in cachexia arose from the development of two clones from colon 26, namely clone 20 and clone 5. The former has been shown to produce anorexia when inoculated in the host (Soda *et al*, 1994), although administration of an anti-IL-6 antibody did not completely reverse the weight loss (Yasumoto *et al*, 1995 and Fujimoto-Ouchi *et al*, 1995). In clone 5, no IL-6 was detected at the tumour site (Yasumoto *et al*, 1995) and injection of recombinant IL-6 to mice inoculated with this clone failed to induce cachexia but elevated serum levels remained high(Soda *et al*, 1995).

Finally, IL-6 has also been shown to be incapable of promoting muscle protein breakdown in vitro (Garcia-Martinez *et al*, 1994).

1.3.1.2.3. Interferon-γ (IFN-γ).

Neopterin (NPT) is produced by activated macrophages in response to IFN-γ from activated T cells, so NPT elevation demonstrates activation of the cellular immune system or increased endogenous IFN-γ production. Increased NPT has been reported in cancer patients where the levels of this factor have been shown to correlate with severe malnutrition and decreased serum tryptophan (IFN-γ induces the activation of the tryptophan degrading enzyme, indoleamine 2, 3-dioxygenase) (Iwagaki *et al*, 1995a). Even more, a high plasma NTP:CRP ratio was found in a group of cachectic patients, in contrast with patients with infectious diseases where both parameters were elevated (Iwagaki et al, 1995b), suggesting that IFN-γ may be the determinant of cachexia.

In animals, the weight loss and cachexia induced by the Lewis lung tumour has been shown to be antagonised by anti-IFN-γ antibody treatment (Matthys *et al*, 1991a). When nude mice were inoculated with CHO cells previously transfected with IFN-γ, they developed severe cachexia, although part of the weight loss was due to an anorectic effect. Pretreatment with anti-IFN-γ antibody prevented this effect, but proved ineffective when administered after transplantation (Matthys *et al*, 1991b).

Anti-IFN-γ antibody treatment has been shown to partially block the MCG 101-induced cachexia but this effect was only short-lived (Lanstein *et al*, 1991). The same group was also able to induce cachexia in rats following chronic administration of IFN-γ , but this was only achieved at sub-lethal doses.

1.3.1.2.4. Interleukin-1 (IL-1).

Administration of recombinant IL-1 into mice has been shown to induce weight loss and anorexia (Moldawer *et al*, 1988). In MCG 101 sarcoma-bearing rats, high levels of IL-1 and TNF-α have been detected at the tumour site but these appeared to be no different to those found in other body tissues (Lonnroth *et al*, 1990). Furthermore, neither neutralising antibodies against the IL-1 receptor (Gelin *et al*, 1991) nor an IL-1 receptor antagonist

(Costelli *et al*, 1995b) have been shown to have an effect on the attenuation of cachexia. Additionally, IL-1, as for the other cytokines, is also incapable of inducing muscle catabolism *in vitro* (Moldawer *et al*, 1987).

In conclusion, there is compelling evidence to suggest a role for cytokines in the development of cachexia, however, the results show that the action of one single cytokine is unlikely to account for all the cachectic parameters. A more complex system appears operative, since not only does the production of cytokines seem to be regulated by one another (Strassman *et al*, 1992a and b), but additionally, the cytokine network might act in conjunction with the endocrine system as judged by the abnormal levels of some hormones (Knapp *et al*, 1991 and Tessitore *et al*, 1993b). In any case, the results suggest that cytokines may act as the mediators or may be together with as yet unidentified factors, perhaps of tumour origin.

1.3.2. Tumour-derived catabolic factors.

Although the malignant state induces a state of high metabolic stress, the nutrient demand of the tumour alone does not account for the complete wasting of the bearer host. In some cases, the loss of body weight appears prior to the detection of the disease and, even when the presence of the tumour is clearly evident, the tumour mass only represents a small fraction of the total body weight, suggesting that additional factors are also involved.

In 1962, Costa and Holland suggested the presence of an unidentified lipolytic factor associated with Krebs-2 carcinoma. Transplantation of this tumour into mice, caused an extensive loss of fat mass which, in the first stage of the disease, was reduced by 50% when the tumour was still barely detectable and food intake was normal. Furthermore, this was shown to be reproducible by using non-viable preparations of the tumour.

Kitada *et al* (1980) found that, following injection of serum from AKR mice with thymic lymphoma into normal mice, a massive fat mobilisation was induced comparable to that

seen in the tumour-bearing mice. A lipid mobilising factor of apparent molecular weight 5 KDa produced by the tumour appeared to be responsible for this effect (Kitada *et al*, 1981), although aggregation into higher molecular fragments was shown to be essential for its biological activity (Kitada *et al*, 1982).

A lipolytic factor of 75 KDa (toxohormone-L) was purified from the ascites fluid of mice with Sarcoma 180 following ammonium sulphate precipitation and a combination of anion-exchange and gel exclusion chromatography (Masuno *et al*, 1981). This substance was also found in the ascitic fluid of patients with hepatoma and Grawitz's tumour.

A melanoma-derived lipoprotein lipase inhibitor (MLPLI), which has been found to be identical to leukaemia-inducing factor (LIF), has been isolated from two human melanoma cell lines (SEKI and G361) which have the ability to induce severe cachexia in nude mice The 40 KDa protein has been shown to inhibit LPL in adipocytes, for which it is thought to play a role in the development of the disease (Mori *et al*, 1989 and Mori *et al*, 1991) and it appears to be distinct from TNF-α (Kawakami *et al*, 1991). A lipid-mobilising factor of apparent molecular weight 6 KDa, which also appears to be immunochemically and biochemically distinct from TNF-α, has been purified from the human melanoma cell line A375 (Taylor *et al*, 1992).

An elevated lipolytic activity was found to be associated with the serum and urine of both cancer cachectic patients (Groundwater et al, 1990) and mice bearing the cachexia-inducing murine adenocarcinoma (MAC) 16 tumour (Beck *et al*, 1990 and Beck and Tisdale, 1991). The human material, which appears to be identical to that obtained from the tumour-bearing mice (40 KDa), has been found capable of inducing weight loss and extensive fat depletion when injected into obese mice (Hirai *et al*, 1997).

A proteolysis-inducing factor, closely associated with the material mentioned above, has also been purified from both murine and human sources and appears to have an apparent

molecular weight of 24 KDa (Todorov *et al*, 1996a). The ability of this material to induce skeletal muscle protein breakdown both *in vitro* and *in vivo* , will be the subject of discussion of this thesis.

Up to this date, there are no other reports in the literature concerning the identification of tumour-derived proteolytic factors capable of initiating muscle protein breakdown *in vitro*. There is, however, one study which has suggested the presence of an unidentified proteolytic factor in the serum of cancer patients with weight loss, capable of eliciting protein mobilisation from isolated rat diaphragm muscle (Belizario *et al*, 1991).

1.4. The MAC 16 cachexia model.

The choice of the MAC 16 as a model to study cachexia has the advantage of resembling the human condition in that it is a slow growing tumour capable of inducing extensive weight loss at very low tumour burdens, unlike many experimental models where the symptoms of cachexia appear once the tumour mass accounts for a considerable proportion of the total body weight. In addition, it provides an excellent model for studying the effects of lipid and protein mobilising factors since the development of cachexia takes place without an alteration in food or water intake and, therefore, the effects elicited in the host can exclusively be ascribed to the presence of the tumour.

1.4.1. Weight loss.

The MAC 16 tumour was originally developed by Double *et al* (1975), by injecting NMRI mice repeatedly with 1,2-dimethylhydrazine. Following this treatment, multiple colon tumours were induced and transplanted subcutaneously in the flank of recipient animals, which after serial transplantations, yielded 5 relatively stable, well-differentiated tumour lines. At less than 1% of the host weight, this tumour induces a considerable degree of weight loss (without a reduction in food or water intake) and, as the tumour mass grows, weight loss can increase up to 33% in females and 20% in males. The proportion of

carcass lipid varies inversely with the tumour size and, on a percentage basis, the decrease of carcass fat is greater than that of lean body mass (Bibby *et al*, 1987).

TNF-α appears not to be responsible (at least directly) for the induction of this syndrome in mice bearing the MAC 16 tumour. The development of weight loss following administration of this material into NMRI mice, seems to correlate with a reduction in the food and water intake, indicating the anorectic effect of the cytokine (Mahony *et al*, 1988). Furthermore, body composition experiments have shown the effect of TNF-α to be due to a dehydration effect since the water compartment was the only one affected (Mahony and Tisdale, 1989).

A high fat ketogenic diet in which up to 80% of the energy was supplied as medium chain triglycerides (MCT), was shown to have a profound effect in the reduction of weight loss induced by the MAC 16 tumour and was accompanied by an increase in the carcass fat and non-fat compartments (Tisdale *et al*, 1987). The attenuation of weight loss was the consequence of both an improved nitrogen balance (Beck and Tisdale, 1989) and an antitumour effect of the diet due to the inability of the MAC 16 tumour to metabolise ketone bodies (Tisdale and Brennan, 1986). Dietary supplementation of fish oil (19% EPA and 13% DHA) has also been shown to have an antitumour as well as an anticachectic effect (Tisdale and Dhesi, 1990), which will be further discussed in next section.

1.4.2. Metabolic alterations.

In vitro, MAC 16 cells displayed an increased rate of glucose consumption, lactate production and CO_2 production from both glucose and palmitate (Tisdale and Brennan, 1986). Tumour growth *in vivo*, has been shown to be accompanied by progressive hypoglycaemia (McDevitt and Tisdale, 1992) and a reduction in the plasma insulin levels (Bibby *et al*, 1987).

Plasma levels of FFA have been shown to be low, though transiently increased when animals have lost approximately 2 g of body weight (Bridon *et al*, 1991), probably as the consequence of an increased tumour utilisation (Hudson and Tisdale, 1994). Adipose LPL activity remains high up to a weight loss of 2.5 g and could account for the increased plasma levels of FFA at this point, which coincides with the period of maximum production of lipid-mobilising activity by the tumour (Groundwater *et al*, 1990). Although LPL activity appears to be depressed in adipose tissue at greater degrees of weight loss (5-6 g), no hypertriglyceridaemia is observed, which could be explained by the elevated LPL activity of other organs such as heart (Bridon *et al*, 1991).

Increased fatty acid oxidation has been ascribed to this model (Mulligan *et al*, 1992) and, indeed, the loss of body fat in mice bearing the MAC 16 tumour appears to arise solely from an increased lipolysis rather than a reduced rate of lipogenesis. There is no indication of lipogenesis being inhibited (Mulligan and Tisdale, 1991) and the activity of host liver fatty acid synthase has been shown to be increased (Tisdale and Leung, 1988). Furthermore, lipogenesis has been shown to be increased at the expense of glucose utilisation, rendering the host with a reduced amount of utilisable energy from a given dietary intake. This, however, does not appear to be specific to the cachectic state but is more related to the presence of the tumour since increased lipogenesis was also found in the MAC 13 model. Animals bearing the MAC 13 tumour also have a higher daily energy intake, which may be used to compensate for this extra energy requirement. MAC 16 mice, on the other hand, do not change their food intake and therefore part of their weight loss may be the result of their inability to make up for this wasting of energy.

An increase REE has been reported for these animals after the third and fourth weeks of tumour transplantation (Plumb *et al*, 1991), although the elevated REE was not the cause for the loss of body weight since this happened prior to the elevation of REE.

With regard to protein metabolism, little is known about the mechanisms behind the wasting of skeletal muscle. It appears that a combination of a depressed synthesis rate and increased proteolysis are responsible for the loss nitrogen and body weight (Beck and Tisdale, 1989 and Smith and Tisdale, 1993a). As for the elevated protein breakdown, PGE_2 has been proposed as one of the factors mediating this process (Smith and Tisdale, 1993b).

Administration of EPA to mice bearing the MAC 16 tumour has been found to have both an antitumour and an anticachectic effect (Beck *et al*, 1991). Part of the anticachectic effect of EPA arises from its ability to inhibit the rate of protein degradation in skeletal muscle, presumably by interfering with the synthesis of PGE_2. In addition, EPA has also been shown to inhibit lipolysis (Tisdale and Beck, 1991), an effect which resides in the ability of this PUFA to inhibit cAMP production by adenylyl cyclase *via* upregulation of G_i and downregulation of G_s protein isoforms (Price, 1997). In contrast with this model, EPA has failed to prevent cachexia and inhibit tumour growth in cachectic rats bearing the rapidly growing Yoshida-130 tumour (Costelli *et al*, 1995a).

1.5. Aims and objectives.

The aim of this work is to contribute towards the elucidation of the mechanisms controlling the process of skeletal muscle protein loss in cachexia. For such purpose, the MAC 16 tumour will be used as the source for isolation of a putative cancer cachectic factor the ability of which to induce protein breakdown will be studied using *in vivo* as well as *in vitro* muscle preparations. A detailed examination of the main proteolytic systems present in skeletal muscle will be reviewed, as well as their contribution towards the abnormally elevated rate of proteolysis elicited by this protein-mobilising factor (p24). Finally, the use of an *in vitro* cell system is suggested as a new model for studying the mechanisms of muscle protein turnover as determined by the reproducible effects of p24 in this cell line.

Chapter 2
Materials and Methods

2.1. Animals.

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

Fragments of the MAC 16 tumour excised from donor animals with established weight loss were implanted into the flanks of NMRI mice (20-25 g) by means of a trocar as described (Beck and Tisdale, 1987). Tumours were excised from mice with weight loss between 20 and 25% and used to purify the proteolytic factor.

Blood was obtained by cardiac puncture under total anaesthesia, and the animals were killed immediately by cervical dislocation.

2.2. Cell culture.

The C_2C_{12} mouse myoblast cell line was chosen as one of the models for studying protein synthesis and degradation rates *in vitro.* Cells were grown in 60 x 15 mm petri dishes in 3 ml of DMEM supplemented with 12% FCS, 1% non-essential amino acids and 1% penicillin-streptomycin in a humidified atmosphere of O_2: CO_2 (95: 5) at 37 ^{0}C. All the experiments carried out on myoblasts were performed in the subconfluent state.

Myotubes were grown when confluent myoblasts were fused by changing the medium to DMEM supplemented with 10% horse serum, 1% non-essential amino acids and 1% penicillin-streptomycin.

2.3. Chemicals.

Amersham Intl. Bucks., UK.

[α-^{32}P]dCTP

ECL Western blotting analysis system

Megaprime DNA labelling system

L-[4-^{3}H]-Phenylalanine

L-[2, 6-^{3}H]-Phenylalanine

[5, 6, 8, 11, 12, 14, 15-^{3}H (N)]-Prostaglandin E_2

Protein rainbow markers

Amicon LTD Gloucs., UK.

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

YM membranes: 10,000 cut-off

Microcon concentrators

Appligene-Oncor, Durham, UK.

Phenol (water saturated)

Bio-Rad Laboratories Ltd, Herts., UK.

Acrylamide

Ammonium persulphate

Bio-Rad protein reagent

N,N'-Methylene-Bis acrylamide (Bis-acrylamide)

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Calbiochem-Novabiochem Ltd., Beeston, Nottingham, UK.

Arachidonyltrifluoromethyl ketone ($AACOCF_3$)

Costar, Cambridge, MA, USA.

Polyvinylchloride ELISA plates

Fisons laboratory supplies, Loughborough, UK.

Chloroform

1,2-Dichloroethane

Ethanol

Glacial acetic acid

Hydrochloric acid

Isopropanol

Magnesium chloride

Nitric Acid

Optiphase Hisafe 3

Perchloric acid

Sodium carbonate

di-Sodium hydrogen orthophosphate dihydrate

Sodium dihydrogen orthophosphate

Gelman Sciences, Northampton, UK.

Acrodisc 0.2 μm syringe filters

Gibco BRL Life Technologies, Paisley, Scotland, UK.

Agarose

Bovine foetal calf serum (FCS)

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

Horse serum

Nunclon 60 x 15 mm petri dishes

Penicillin-Streptomycin

Phosphate buffered saline (without calcium and magnesium)

RPMI 1640 Tissue culture medium

Trypsin/EDTA

Hoefer Scientific Instruments, San Francisco, CA., USA.

Nitrocellulose membrane

Pharmacia Biotech, Herts., UK.

Ficoll (type 400)

Premier Beverages, Stafford, UK.

Marvel (fat-free dried milk)

Sigma Chemical, Dorset, UK.

Ammonium chloride

Ammonium sulphate

Aprotinin

ATP bioluminescent kit

Bovine Serum Albumin (BSA)

Bromophenol blue

Calcium chloride

Cycloheximide

Charcoal activated

Chloroquine

Dextran (D-70)

Dextran sulphate

Diethylpyrocarbonate (DEPC)

Dimethyl sulphoxide (DMSO)

Dithiothrietol (DTT)

D-Glucose

Eicosapentaenoic acid (EPA)

trans-Epoxysuccinyl-L-leucylamido-3-methylbutane (E-64)

Ethidium bromide

Ethylmaleimide

Ethylene Glycol-bis(β-aminoethyl ether) N,N,N',N'-Tetraacetic Acid (EGTA)

Ethylenediaminetetraacetic acid (EDTA)

Formaldehyde

Formamide

Guanidinium thiocyanate

Glycerol

Glycine

Hydrogen peroxide

Ibuprofen

Indomethacin

Isoamyl alcohol

Kodak Processing Chemicals: GBX Developer and Replenisher

Kodak Processing Chemicals: GBX Fixer and Replenisher

Kodak X-Omat A R film 35 cm x 43 cm

Kodak X-ray exposure holder 35.6 cm x 43.2 cm

Leupeptin

Magnesium sulphate

β-Mercaptoethanol

Methylamine

3-[N-Morpholino]propanesulphonic acid, (free acid) (MOPS)

1-Nitroso-2-naphthol

Non-essential amino acids

Pepstatin

o-Phenylenediamine

Phenol/chloroform/isoamyl alcohol (25:24:1)

Polyoxyethylenesorbitan

Phenylalanine

Phenylmethylsulfonylfluoride (PMSF)

Polyoxyethylene-sorbitan (Tween 20)

Potassium chloride

Potassium dihydrogen phosphate

Prostaglandin E_2 antiserum

Protein A peroxidase

Proteinase K (from Tritirachium album)

Polyvinylpyrolidone (PVP)

Sarcosyl (N-Lauroylsarcosine sodium salt)

Sodium acetate

Sodium azide

Sodium chloride

Sodium bicarbonate

Sodium citrate

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Sodium nitrite

Sodium pyrophosphate

Sucrose

Trichloroacetic acid

Tripotassium citrate

Trizma base (Tris)

Xylene cyanol FF

OXOID, Basingstoke, Hamps., UK.

Phosphate buffered saline (PBS) tablets

Takeda Chemicals Ltd., Japan.

2,3,5-Trimethyl-6-(3-pyridylmethyl)-1,4-benzoquinone (CV-6504)

Whatman Intl., Maidstone, Kent, UK.

Cellulose nitrate filters (0.2 μm, 25 mm)

3MM Whatman paper

Gifts.

Monoclonal anti-proteolytic factor Antibody (mAb), developed by Dr P Todorov and Dr P Cariuk. C_2C_{12} mouse skeletal muscle cell line, probes for ubiquitin, ubiquitin-conjugating enzyme E_2, GAPDH and Ubiquitin Antibody, by Dr M Thompson, Rowett Research Institute, Aberdeen, Scotland. C9 proteasome subunit probe was supplied by Boehringer.

2.4. Buffers and solutions.

2.4.1. Affinity purification.

2.4.1.1. Anion-exchange buffer (QS 1)

Tris-HCl pH 8.0	10 mM
DTT	1.0 mM
PMSF	0.5 mM
EGTA	0.5 mM

2.4.1.2. PBS

1 tablet/100 ml distilled water

2.4.1.3. Elution buffer

Glycine-HCl pH 2.5	100 mM

2.4.1.4. Collecting buffer

Tris-HCl pH 8.0	1M

2.4.2. *Enzyme-Linked Immuno-Sorbent Assay (ELISA).*

2.4.2.1. Sodium carbonate/bicarbonate buffer pH 9.5.

Sodium carbonate	0.1 M
Sodium bicarbonate	0.1 M

2.4.2.2. Sodium citrate/phosphate buffer pH 5.0.

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

2.4.2.3. Wash buffer.

PBS + 0.15% Tween 20

2.4.2.4. Antibody dilution buffer.

PBS + 3% BSA

2.4.2.5. Substrate buffer.

Phosphate/citrate buffer pH 5.0

o-Phenylenediamine (OPD)	0.04%
Hydrogen peroxide	0.012%

2.4.3. *Tyrosine release assay.*

2.4.3.1. Krebs-Ringer bicarbonate buffer.

Sodium chloride	118 mM
Calcium chloride	2.0 mM
Potassium chloride	5.0 mM
Potassium dihydrogen orthophosphate	1.0 mM
Sodium bicarbonate	25 mM
Magnesium sulphate	1.0 mM

2.4.3.2. Cyclo-oxygenase and lipoxygenase inhibitors.

Ibuprofen	10 μM
Indomethacin	50 μM
CV-6504	10 μM

2.4.3.3. cPLA2 inhibitors.

$AACOCF_3$	50 μM

2.4.3.4. Cathepsin inhibitors.

Ammonium chloride	10 mM
Chloroquine	250 μM
Methylamine	10 mM
Leupeptin	30 μM

2.4.3.5. Calpain inhibitors.

E-64	100 μM

2.4.3.6. ATP-depleting agents.

Deoxyglucose	5 mM
Sodium azide	0.2 mM

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

2.4.4.1. Stock 1.

Acrylamide	44%
N,N'-Bis-methylene acrylamide	0.8%

2.4.4.2. Stock 2.

Acrylamide	30%
N,N'-Bis-methylene acrylamide	0.8%

2.4.4.3. Running buffer (10x).

Trizma base	0.25 M
Glycine	1.9 M
SDS	35 mM

2.4.4.4. Sample buffer.

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

2.4.5. Western blotting.

2.4.5.1. Running buffer.

Trizma base	0.25 M
Glycine	1.9 M
SDS	50 mM

For use:	10x Blotting buffer	10%
	Methanol	20%

2.4.5.2. Wash buffer.

PBS + 0.15% Tween 20

2.4.5.3. Antibody diluent.

PBS + 0.15% Tween 20

Marvel	1.5%

2.4.5.4. Blocking solution.

PBS + 0.15% Tween 20

Marvel	5%

2.4.6. Phenylalanine stock solution.

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

2.4.7. Extraction of chromosomal DNA and gel electrophoresis.

2.4.7.1. Lysis buffer.

Tris-HCl pH 9.0	0.5 M
EDTA	2 mM
Sodium chloride	10 mM
SDS	1%
Proteinase K	0.03%

2.4.7.2. 50x TAE buffer.

Tris-acetate	40 mM
EDTA pH 8.0	1 mM

For use: 1x TAE

2.4.7.3. Ethidium bromide dye.

Ethidium bromide	1%

2.4.7.4. Agarose gel

Agarose	1.2%

Agarose was melted in water by microwaving for 1 minute. Then the following were added:

50x TAE	2.2%
Ethidium bromide	3 μl of 1% solution

2.4.7.5. TE buffer

Tris-HCl pH 8.0	10 mM
EDTA	1 mM

2.4.8. RNA extraction, gel electrophoresis and Northern blotting.

2.4.8.1. DEPC water.

0.1% DEPC water was allowed to stir for 30 minutes before autoclaving.

Eppendorfs and corex tubes were treated with DEPC water.

2.4.8.2. Sodium citrate buffer pH 7.0.

Sodium citrate (citric acid trisodium salt)	0.75 M
DEPC	0.5%

2.4.8.3. Solution D (incomplete, stored for 3 months).

Guanidinium thiocyanate	6.3 M
Sodium citrate	0.04 M
Sarcosyl (N-Lauroylsarcosine sodium salt)	0.03 M

2.4.8.4. Solution D (complete, stored for 1 month).

Incomplete solution D

β-Mercaptoethanol	0.7%

2.4.8.5. Sodium acetate pH 4.0.

Sodium acetate	2 M
DEPC	0.05%

2.4.8.6. Sodium acetate pH 5.2.

Sodium acetate	3 M
DEPC	0.05%

2.4.8.7. Ethanol.

0.1% DEPC water	
Ethanol	70%

2.4.8.8. 5x MOPS

MOPS pH 7.0	0.2 M
Sodium acetate	0.03 M
EDTA (pH 8.0)	0.5 M

2.4.8.9. Agarose gel.

Agarose	1.2%

Agarose was melted in water by microwaving for 1 minute. Then the following were added:

5x MOPS buffer	20%
Formaldehyde	0.67%

2.4.8.10. Running buffer.

1x MOPS	
Formaldehyde	0.67%

2.4.8.11. Blotting buffer.

di-Sodium hydrogen orthophosphate dihydrate	0.25 mM
Sodium dihydrogen orthophosphate	0.25 mM

2.4.8.12. FFM buffer.

Formaldehyde	22.6%
Formamide	64.5%
5x MOPS	12.9%

2.4.8.13. RNA loading dye (6x dye)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Ficoll	15%

2.4.8.14. Ethidium bromide dye.

Ethidium bromide	10 μl of 1% solution
Distilled water	150 ml

2.4.8.15. 5x P.

BSA	1%
PVP	1%
Ficoll	1%
Tris-HCl pH 7.5	250 mM
Sodium pyrophosphate	0.5%
SDS	5%

2.4.8.16. 20x SSC.

tri-Sodium citrate	0.3 M
Sodium chloride	3 M

2.4.9. Isolation and determination of ubiquitin conjugates.

2.4.9.1. Homogenising buffer

Tris-HCl pH 7.4	50 mM
Supplemented with:	
Sucrose	0.25 mM
EDTA	5 mM
PMSF	1 mM
Ethylmaleimide	5 mM
Pepstatin	1 μM
Leupeptin	1 μM
Aprotinin	0.1 TIU/ml
SDS	1%

2.4.9.2. SDS-PAGE and Western blotting.

As described in 2.4.4 and 2.4.5

2.5. Methods.

2.5.1 Determination of protein concentration.

Protein concentrations were measured by using Bio-Rad reagent (200 μl). The samples (diluted to 800 μl with water), were read at 595 nm and the protein concentration calculated according to the formula shown below which was derived from a BSA standard calibration curve.

Protein (μg/μl) = absorbance (595 nm)/ 0.053 *

* where 0.053 represents the gradient of the BSA standard curve.

2.5.2. *Determination of RNA concentration.*

RNA was measured by the method originally described by Munro & Fleck (1969) and calculated using the modified formula of Asford & Pain (1986):

$$\text{RNA } (\mu g) = [\text{Abs (260 nm)} \times 32.9] - [\text{Abs (232 nm)} \times 6.11] \times \text{vol (ml)}$$

2.5.3. *Immunoaffinity purification of the protein-mobilising factor from MAC 16 tumours.*

Tumours were weighed, homogenised thoroughly in Q-Sepharose buffer (QS1 buffer), and centrifuged at 4000 g in a Heraeus Megafuge 1.0 for 20 minutes. The pellet and the top fat layer from the supernatant fraction were both discarded, and the supernatant was then analysed for protein content and the total volume recorded.

Ammonium sulphate (38% w/v) was gradually added to the homogenate whilst stirring in the cold room. Once all the ammonium sulphate had been added, it was covered with foil and allowed to stir overnight to equilibrate.

After centrifugation at 4000 g for 20 minutes, the supernatant was dialysed against the same volume of PBS in an amicon filtration cell using a 10,000 cut-off membrane, and the total volume was reduced to 20-25 ml. The sample was centrifuged at 4000 g for 20 minutes, and after measuring the volume and protein content it was loaded onto an affinity column which was prepared by coupling the monoclonal antibody isolated from the splenocytes of MAC 16 tumour-bearing mice to an Affi-Gel Hz matrix. The sample was circulated through the affinity column at a flow rate of 0.1 ml/min overnight in the cold room.

To remove the unbound material, the column was washed with PBS for 3 hours at a rate of 0.1 ml/min and the sample finally eluted with 0.1 M Glycine-HCl buffer pH 2.5 at a flow

rate of 1.0 ml/min into tubes containing 0.5 ml 1 M Tris-HCl buffer pH 8.0, and 3 ml fractions were collected.

2.5.4. *Immunoaffinity purification of the protein-mobilising factor from human urine.*

Urine from cachectic patients with pancreatic cancer was centrifuged at 16,000 g at 4 ^{0}C for 15 minutes in a MSE Hi-Spin 21 centrifuge and the volume reduced to 500 ml using an amicon filtration cell with a 10,000 cutoff membrane.

Ammonium sulphate precipitation (80% w/v) and equilibration of the sample was carried out in the same way as for the tumour homogenate. After centrifugation at 16,000 g at 4 ^{0}C for 25 minutes the pellet was resuspended in distilled water and washed with the same volume of PBS three times using an amicon filtration cell until the volume was finally reduced to 20-25 ml. The sample was circulated through the affinity column and eluted as previously described.

2.5.5. *Detection of immunoreactive affinity material by ELISA.*

An aliquot (50 μl) from each fraction (10) was mixed with 0.1 M carbonate-bicarbonate buffer pH 9.5 (50 μl) and incubated at 37 ^{0}C for 2 hours in a covered polyvinylchloride multiwell plate in order to immobilise the samples. The contents were then removed and the wells washed three times with PBS-Tween 20 (200 μl). In order to block non-specific sites, PBS-Tween-3% BSA (200 μl) was added to each well and incubated at 37 ^{0}C for 30 minutes. The blocking solution was removed and the wells washed three times with PBS-Tween 20 (200 μl).

Samples were incubated at 37 ^{0}C for 1 hour in the presence of the monoclonal Antibody (100 μl of a 10 μg/ml solution) and PBS-Tween-3% BSA (100 μl) was added to the negative control samples. The contents of the wells were removed and another three washes carried out with PBS-Tween 20 (200 μl).

After incubation with protein A peroxidase (100 μl of a 0.25 μg/ml solution) at 37 ^{0}C for 30 minutes and three washes with PBS-Tween 20 (200 μl), the substrate (0.04% o-phenylenediamine in sodium phosphate-citrate buffer pH 5.0- 0.012% hydrogen peroxide) was added to each well (100 μl). Immunoreactive affinity material was detected when the yellow coloured product of the reaction developed after 15-30 minutes. The reaction was stopped by adding 0.2 M sulphuric acid (50 μl) and the samples read at 492 nm in a microtitre plate reader (Anthos Labtec Instruments).

2.5.6. Detection of immunoreactive affinity material by Western blotting.

2.5.6.1. SDS-PAGE.

Affinity purified material was electrophoresed by SDS-PAGE using a 15% denaturing polyacrylamide gel which was prepared as follows:

	Running gel	Stacking gel
1.5 M Tris-HCl pH 8.8	3.70 ml	
0.5 M Tris-HCl pH 6.8		1.50 ml
Stock 1 (2.4.4.1)	3.75 ml	
Stock 2 (2.4.4.2)		1.00 ml
Deionised water	3.95 ml	3.20 ml
10% SDS	0.30 ml	0.06 ml
TEMED	0.03 ml	0.016 ml
10% APS	0.04 ml	0.02 ml

The samples were corrected for protein (20 μg) and mixed 1:1 with sample buffer in a final volume of 40 μl. After boiling for 5 minutes, samples were loaded on the gel and electrophoresed at 180 V until the dye had reached the bottom of the gel.

2.5.6.2. Western blot.

Proteins were transferred onto a nitrocellulose membrane (180 V for 1 h) and left in a 5% marvel solution overnight. After washing (3 x 5 min) in blocking-washing solution, the membrane was incubated with the biotinylated mAb (10 μg/ml) for 1 h in a shaker at room temperature. The antibody was removed and the membrane washed (1 x 15 min and 2 x 5 min). The membrane was incubated with streptavidin-HRP (500-fold dilution of the original stock) for 1 h, washed (3 x 10 min) and developed by the ECL Western Blotting detection System according to the manufacturer's instructions.

2.5.7. Measurement of total protein breakdown in skeletal muscle.

2.5.7.1. In vitro tyrosine release assay.

Soleus muscles from normal NMRI mice were ligated by the tendons, dissected out virtually intact, and placed in ice-cold isotonic saline. These were then quickly ligated to stainless steel supports under slight tension which resembled that observed at resting length *in vivo* in order to prevent contraction and thus improve protein balance and energy status.

The muscles were pre incubated in a shaking water bath at 37 ^{0}C in RPMI 1640 medium without phenol red and in the presence of the proteolytic factor and normal mouse serum (7% in the incubation medium, final volume 3 ml) for 30 min. After this time they were rinsed three times, the medium replaced by Krebs-Henseleit buffer (3 ml) supplemented with glucose (6 mM), BSA (0.12%), and cycloheximide (0.5 mM), and an incubation was carried on for a further 1.5 h. At all times, the muscles were gassed with a mixture of O_2 and CO_2 (19:1).

Muscles were blotted and weighed, and the incubation medium removed (2 ml) and deproteinised with 30% TCA (200 μl). The samples were centrifuged in a Heraeus benchtop Megafuge 1.0 at 2800 g for 10 min and the supernatant (2 ml) analysed for tyrosine content (Waalkes and Udenfriend, 1957).

To the deproteinised fractions, 1-nitroso-2-naphthol reagent (1ml), and nitric acid (1ml) were added, mixed, and incubated at 55 ^{0}C for 30 min. After this time, samples were allowed to cool for 10 min and dichloroethane (5 ml) was added. The tubes were shaken and centrifuged at 2800 g for 10 min, and the fluorescence in the supernatants measured in a Perkin Elmer Luminiscence Spectrometer LS50 (460 nm excitation and 570 nm emission).

2.5.7.2. Ex vivo tyrosine release assay.

NMRI female mice were injected intravenously with the affinity purified proteolytic material (30-40 μg, 4 x 100 μl). Injections were given at 10.00 am, 12.30 pm, 3.00 pm, and 5.30 pm and the soleus muscles were removed 24 h after the first injection. The muscles were incubated for 2 h in Krebs-Henseleit buffer and the amount of tyrosine released into the medium was determined as described in 2.5.7.1.

For the determination of the proteolytic systems involved in muscle proteolysis, soleus muscles were quickly and gently removed with intact tendons from either p24-treated mice 24 h after the first injection or tumour-bearing mice, and incubated under conditions that either block or activate different proteolytic systems (Tiao *et al*, 1994).

To study the role of lysosomal proteolysis, muscles were incubated in medium with and without the lysosomal inhibitors ammonium chloride (NH_4Cl), chloroquine, methylamine and leupeptin. The three former agents inhibit lysosomal protein breakdown by raising the intralysosomal pH (chloroquine also inhibits cathepsin B) whereas the latter is a microbial peptide aldehyde that inhibits cathepsins B, H and L directly. They are all irreversible inhibitors except for NH_4Cl (Seglen *et al* , 1979).

The role of calcium-dependent proteolysis was tested by incubating muscles in calcium-containing and calcium-free medium in the presence of trans-epoxysuccinyl-L-leucylamido-3-methylbutane (E-64). This agent is a microbial peptide inhibitor which, in the presence

of calcium, inhibits calpain irreversibly by alkylating the sulphydryl group of the catalytic site cysteine to form thioesters. The inhibitory effect of E-64 is therefore exclusive to calpain since the other serine proteases lack an active site cysteine. All muscles were also incubated with methylamine in order to inhibit basal lysosomal proteolysis so that any difference between muscles incubated with or without E-64 reflected calcium-dependent proteolysis.

The role of energy-dependent proteolysis was investigated by incubating muscles in medium depleted of ATP for which glucose was omitted and replaced by 2-deoxyglucose (2-DG) and sodium azide (an inhibitor of oxidative phosphorylation) in order to block ATP production. All muscles were incubated in calcium-free medium containing methylamine, insulin and the branched-chain amino acids Leu, Ile and Val, to inhibit protein breakdown through the other two pathways.

Muscles were removed from the incubation medium, frozen in liquid nitrogen and stored at -70 ^{0}C until analysis. ATP levels were determined by means of an ATP-bioluminescent assay following the manufacturer's instructions.

2.5.8. Prostaglandin E_2 Radioimmunoassay (RIA).

From the incubation medium described above (2.5.7.1. & 2.5.7.2.), a small aliquot (100 μl) was used for analysis of PGE_2 by means of radioimmunoassay (RIA). The samples were mixed with ^{3}H- PGE_2 (100 μl, 0.1 μCi) and PGE_2 antiserum (100 μl; a dilution, which gave 40-50% binding was used) in Eppendorf tubes, vortexed and incubated in a water bath at 37 ^{0}C for 1 h. After this time, the samples were kept at 4 ^{0}C for 5 minutes and a mixture of ice cold dextran-charcoal was added (500μl) and allowed to stand for 15 min at 4 ^{0}C. The tubes were centrifuged in a refrigerated microfuge at high speed for 10 min. The supernatants were transferred into scintillation vials and counted in Hi-Safe II scintillation fluid (3 ml).

The PGE_2 content of the samples was determined from a PGE_2 calibration curve which was prepared on the same day of the experiment.

2.5.9. Measurement of the in vivo protein synthesis and degradation rates in skeletal muscle.

Mice were given one intraperitoneal injection of 0.25 ml physiological saline containing 0.4 mM L-[4-^{3}H]-phenylalanine (1.5 Ci/mmol), and intravenous injections of affinity purified material (30-40 μg, 4 x 100 μl as described in 2.5.7.2, controls were injected with PBS), 24 hours before the beginning of the experiment. In those experiments carried out in the presence of the monoclonal antibody, the animals were injected ip (0.8 mg, 2 x 100 μl/ mouse) 24 hours beforehand.

The muscles were removed and incubated in RPMI 1640 medium (3 ml) for 2 hours under the conditions previously described in 2.3.6. At the end of this incubation the muscles were blotted and weighed before being homogenised in 2% $HClO_4$ (4 ml).

The samples were centrifuged in a Heraeus Megafuge 1.0 at 2800 g for 15 minutes and the supernatants were converted to a pH close to 6 by the addition of saturated potassium citrate (1 to 5 ml). Insoluble potassium perchlorate was removed by centrifugation at 2800 g for 25 minutes and 1 ml of the supernatants were diluted (1:1) and added to scintillation fluid (10 ml) for the measurement of the intracellular free pool of ^{3}H-phenylalanine.

The precipitates from the original incubation were washed three times with 2% $HClO_4$ (4 ml), and hydrolysed in 6M HCl (5 ml) at 110 ^{0}C in sealed glass tubes for 24 h. The hydrolysates were evaporated to dryness and the respective residues were dissolved in 10 ml of distilled water. Aliquots (1 ml) were mixed with scintillation fluid and counted for protein-bound radioactivity.

Rates of protein synthesis were calculated by dividing the specific radioactivity of protein-bound ^{3}H-phenylalanine by the radioactivity present in the supernatant fraction, and rates of protein degradation were calculated by dividing the amount of radioactivity released into the incubation medium by the specific radioactivity of protein-bound ^{3}H-phenylalanine.

2.5.10. Measurement of synthesis rates in other tissues.

After the mice were injected in the same way as described in 2.5.8, heart, liver, spleen, and kidneys were removed, weighed and homogenised in 2% $HClO_4$ (4 ml). The same procedure as for skeletal muscle (2.5.9) was followed for determining the synthesis rates.

2.5.11. Plasma amino acid composition.

Blood samples obtained by cardiac puncture were centrifuged at 13,000 g in a microfuge for 5 min at room temperature in order to isolate the plasma, which was sent to Alta Bioscience, University of Birmingham, for amino acid analysis.

2.5.12. Measurement of the rate of protein synthesis in C_2C_{12} myoblasts and myotubes.

Protein synthesis was measured as previously described by Southorn & Palmer (1990), by adding 10 μl per ml of medium of a stock solution of phenylalanine (75 μmoles, containing 50 μCi of L-[2,6-^{3}H]-phenylalanine) during the last 60 min of the incubation with the proteolytic factor.

The incubation was terminated by washing the cells three times with ice-cold PBS (1 ml) which were then incubated at 4 ^{0}C with 0.2M perchloric acid (PCA) (1ml) for 20 min. After this time it was replaced by 0.3 M NaOH (1 ml) for 30 min, and the dishes were transferred to an incubator at 37 ^{0}C for 20 min.

The samples were transferred to test tubes and a further 1ml of 0.3M NaOH was used to rinse the dishes. In order to precipitate cellular protein, 2M PCA (0.5 ml) was added to the

samples and left on ice for 20 min. After this time, the samples were centrifuged at 3,000 g for 10 min in a bench top Heraeus centrifuge. The supernatant was used for measuring RNA content (2.5.2).

The pellet was dried with a tissue and resuspended in 0.3M NaOH (1 ml). One aliquot (20 μl) was used to measure protein concentration and another aliquot (0.5 ml) was placed in a scintillation vial and mixed with scintillation fluid (8 ml) to measure protein-bound radioactivity. The specific radioactivity was then calculated in dpm/μg of protein and this value was used to estimate the protein synthesis rates (Morrison, 1995).

2.5.13. Measurement of total protein breakdown in C_2C_{12} myoblasts.

C_2C_{12} myoblasts were labelled for 24 hours with phenylalanine (10 μl per ml of medium of a stock solution, 75 μmoles, containing 50 μCi of L-[2,6-^{3}H]-phenylalanine). After labelling, the cells were washed and incubated in fresh medium (3 ml) in the presence of the proteolytic factor and cycloheximide (1 μM) for the required time and the amount of radioactivity released into the medium was measured. Protein-bound phenylalanine was determined as described before (2.5.12) and the rate of proteolysis calculated by dividing the radioactivity released into the incubation medium by the protein-bound radioactivity.

2.5.14. Measurement of myofibrillar protein breakdown in C_2C_{12} myotubes.

When myotubes were fully differentiated the dishes were washed in serum-free DMEM and incubated in the same medium (2.5 ml) in the presence of the proteolytic factor for 48 h. Media were collected and analysed for 3-MH by GC-MS analysis (Thompson *et al*, 1996)

2.5.15. Isolation and analysis of chromosomal DNA from C_2C_{12} myoblasts.

A standard DNA isolation procedure was followed in order to extract DNA from C_2C_{12} myoblasts. After incubation of the cells with the proteolytic factor, the medium was removed and the cells washed with PBS. They were then trypsinised and centrifuged at 1,000 g for 5 min. The pellet was washed with PBS (10 ml), centrifuged, and incubated in lysis buffer (300 μl) at 50 ^{0}C overnight.

Nucleic acids were extracted twice with phenol/chloroform (1:1 v/v) and once with chloroform, and finally incubated with RNAase A (300 μg/ml) at 37 ^{0}C for 30 minutes.

In order to precipitate the DNA, 3M sodium acetate (0.1 x volume) and 100% ethanol (2 x volume) were added to the samples which were incubated either at 4 ^{0}C overnight or at -20 ^{0}C for 30 minutes. After a high speed spin (13,000 g) in a Heraeus microfuge for 10 min, the ethanol was removed and replaced by 70% ethanol (300 μl). The samples were centrifuged once more and the ethanol removed. The pellets were left to dry at room temperature for 10 min and resuspended in TE buffer (20-40 μl). The samples were electrophoresed using a 1.2% agarose gel for 1 h at 100 V.

2.5.16. Isolation of RNA from tissue for Northern blot analysis.

Tissues were rapidly dissected and frozen in liquid nitrogen until required. They were then homogenised (100 mg approx) in corex tubes containing solution D (3 ml), and 2 M sodium acetate pH 4.0 (0.3 ml), water saturated phenol (3 ml), and chloroform-isoamyl alcohol 49:1 (0.6 ml) were added. The tubes were vortexed for 10 s and left on ice for 15-30 min.

Samples were centrifuged at 10,000 g for 20 min at 4 ^{0}C in a Jouan MR 1822 centrifuge and the upper aqueous phase containing the RNA was transferred into fresh corex tubes to which isopropanol (3 ml) was added and carefully mixed. The samples were kept at -20

^{0}C for 1 h and after this time centrifuged again. The RNA pellets were transferred into eppendorf tubes and isopropanol (0.6 ml) was added and gently mixed. At this point, the samples could be left at -20 ^{0}C for either 1 h or overnight.

After RNA precipitated, the samples were centrifuged at 14,000 g for 15 min at 4 ^{0}C. The pellets were resuspended in 70% ethanol (300 μl), centrifuged at 14,000 g for 2 minutes and the ethanol removed. After 20-30 min at room temperature, the pellets were dried. DEPC water (30 μl) was added to the samples which were frozen at -20 ^{0}C overnight.

Samples were defrosted and heated at 65 ^{0}C for 10 min to solubilise the pellet. To a small aliquot (2 μl), DEPC water was added (498 μl) in order to estimate the RNA which was read 260 nm. A 280 nm reading was also taken to determine the purity of the sample and the volume of RNA required for loading on the gel (10 μg RNA) was calculated according to the formula:

$$\text{Vol } (\mu l) = 10/ \text{Abs } (260 \text{ nm}) \times 10$$

FFM buffer (15.5 μl) was added to the samples which were heated at 65 ^{0}C for 15 min. The dye was added (2 μl) and the samples electrophoresed on a 1.2% agarose gel at 70 V for about 2 h.

To visualise the RNA bands, the gel was removed, washed with distilled water and then left in ethidium bromide for 30 min. After his time, the gel was washed again in distilled water for another 30 min and finally photographed.

2.5.17. Northern blot and hybridisation.

Northern blot experiments were carried out in collaboration with Dr M Thompson. mRNAs from gastrocnemius and soleus muscles, heart and liver were analysed for the expression of ubiquitin (Ub), E2 (ubiquitin-conjugating) enzyme which is thought to be the

rate limiting step in the pathway, and the C9 proteasome subunit. All membranes were hybridised for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as an internal standard.

2.5.17.1. Northern blot.

The RNA was transferred onto a Genescreen membrane overnight by capillary action as depicted below:

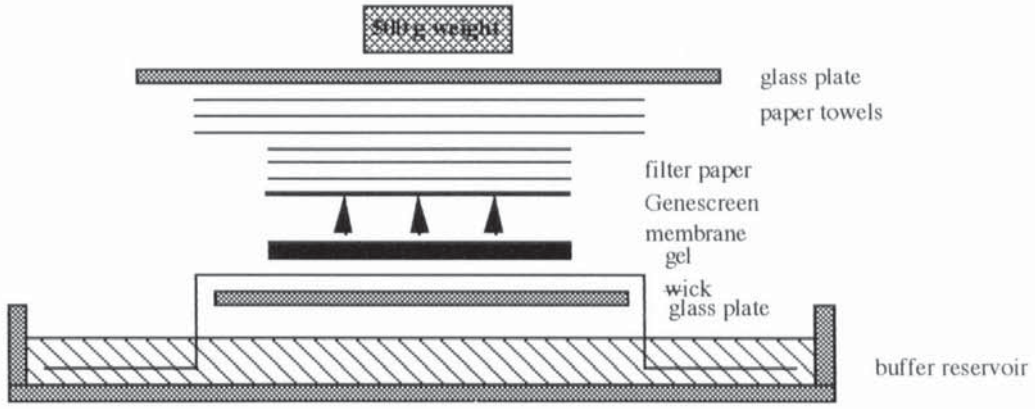

Figure 2.1. RNA transfer by Northern blotting.

The RNA was cross-linked using a Spectrolinker XL-1000 UV crosslinker (120,000 $mJ/cm^{2)}$ and the membrane allowed to dry.

2.5.17.2. Pre-hybridisation.

A pre-hybridisation mix was prepared with 50% dextran sulphate (2 ml), 5x P buffer (2 ml), formamide (5 ml) and sodium chloride (0.58 g), which were vortexed, warmed at 42 ^{0}C for 15 min and vortexed again for further 10 min. This solution was combined with 1 ml of salmon sperm DNA (1 mg/ml), which had previously been boiled and placed on ice for 10 and 5 min, respectively. The membrane was then pre-hybridised at 42 ^{0}C overnight.

2.5.17.3. Probe labelling and hybridisation.

The probes for Ub (Wing & Goldberg, 1993), E_2 (Wing & Banville, 1994) and C9 (supplied by Boehringer) were labelled using the Amersham Megaprime DNA labelling system, according to the manufacturer's instructions. Briefly, the probe (made up to a volume of 30 μl) was boiled for 5 min and allowed to cool at room temperature for about 10 min. Multiprime reaction buffer (10 μl), ^{32}P dCTP (1 μCi, 3000 Ci/mmol) and Klenow enzyme (2 μl) were added, gently mixed and incubated at 37 ^{0}C for 10 min. The probe was then boiled for 3-4 min, left on ice for 5 min and added (50 μl) to the hybridisation bottle containing the pre-hybridised membrane which was hybridised at 42 ^{0}C overnight.

On the following day, the DNA probe and dextran sulphate mixture were removed from the hybridisation bottle and the membrane was washed twice with 2x SSC buffer (100 ml). The membrane was then incubated twice at 65 ^{0}C with either 100 ml 2x SSC buffer-0.5% SDS (2 x 30 min for Ub and 2 x 45 min for C9) or 1x SSC-1% SDS (2 x 1 h for E_2 or GAPDH).

A final wash with 0.1x SSC (100 ml) was carried out at room temperature for 5 minutes to remove the SDS and the membranes were sealed in a plastic bag. They were then scanned with a Packard Instant Imager system and finally subjected to autoradiography.

2.5.18. Isolation and determination of ubiquitin conjugates by Western blotting.

The gastrocnemius muscles were removed 24 h after the first injection of affinity purified material (2.5.7.2) and homogenised in 2 ml of ice-cold 50 mM Tris-HCl buffer. The homogenates were then centrifuged at 4 ^{0}C for 20 min at 15,000 g and the supernatants analysed for protein content. Samples were electrophoresed using a 15% denaturing polyacrylamide gel as described in 2.5.6.1.

Proteins were transferred onto a nitrocellulose membrane (180 V for 1 h) and left in a 5% marvel solution overnight. After washing (3 x 5 min) in blocking-washing solution, the membrane was incubated with the ubiquitin antibody (10 μg/ml) for 1 h in a shaker at room temperature. The antibody was removed and the membrane washed (1 x 15 min and 2 x 5 min). The membrane was incubated with protein A peroxidase (1000-fold dilution of the original stock) for 1 h and washed (3 x 10 min). Streptavidin-horseradish peroxidase conjugate (1000-fold dilution) was added and the membrane developed by the ECL Western Blotting detection System according to the manufacturer's instructions. Densitometric analysis was carried out by using an UVP White/UV transilluminator.

Chapter 3
Induction of a cachexia state by p24. Effect on weight loss and muscle protein catabolism.

3.1. Introduction.

3.1.1. Isolation of p24.

The presence of circulatory catabolic factors associated with the MAC 16 model was first suggested by Beck and Tisdale (1987). A lipid/protein-mobilising activity was found to be associated with the MAC 16 tumour extracts as measured by the release of free fatty acids (FFA) and amino acids from fat and muscle tissues, respectively. Such activity was not found in the non-cachexigenic MAC 13 and MAC 15 tumours, indicating that such effect on fat and muscle catabolism was specific for the cachexia-inducing tumour. Evidence was provided not only for the production, but also for the secretion of this factor(s) into the circulatory system since lipolysis and proteolysis were both enhanced following treatment with serum from tumour-bearing animals. As for the tumour extracts, no evidence of activity was found to be associated with the serum from either MAC 13 or MAC 15 mice.

The lipid-mobilising factor associated with the MAC 16 tumour homogenates was identified by McDevitt *et al* (1995) by means of several chromatographic methods which included ion exchange (Mono Q), exclusion (Superose) and hydrophobic (C_8) chromatography. By using this approach, a negatively charged molecule of apparent molecular weight 24 KDa was isolated, which was immunoreactive to the MAC 16 monoclonal antibody (mAb). The mAb was isolated by fusing myeloma cells with the splenocytes from tumour-bearing mice which had developed a suspected immunological resistance to the tumour as judged by the delay in the onset of weight loss (Todorov *et al*, 1996b). Aliquots of this cell suspension were grown in 96-well plates and used to detect

those hybridomas which reacted positively to the antigen (obtained from Superose fractions of lipid-mobilising activity) by ELISA.

Surprisingly, preincubation of the lipid-mobilising factor with the mAb, did not prevent the induction of lipolysis in isolated murine adipocytes. In contrast, the proteolytic activity associated with the serum of MAC 16 mice (Smith and Tisdale, 1993a) was completely abolished by this treatment.

The mAb was used to purify the antigen by means of affinity chromatography and, following elution with glycine buffer, ELISA-positive fractions were identified and Western blotted. Two immunoreactive bands of estimated molecular weight 69 and 24 KDa were found, which were the main protein bands detected by silver stain after affinity chromatography (Todorov *et al*, 1996b). When added in the presence of serum from non tumour-bearing mice, this material was capable of initiating muscle protein breakdown in isolated gastrocnemius muscle, an effect which could be blocked by the MAC 16 mAb but not by a nonspecific IgG2a mAb (same subclass as the MAC 16 mAb) (Todorov *et al*, 1996b). Further fractionation of the affinity purified material was achieved using reverse phase hydrophobic chromatography with a C_8 column. Two peaks at 31.5-33.5 min (57.5-62% acetonitrile) and 42 min (90% acetonitrile) retention times were obtained, which yielded the same 24 KDa and 69 KDa immunoreactive bands by SDS-PAGE (McDevitt, 1996). When given intravenously to recipient animals, this material caused a significant weight loss as well as an increased release of tyrosine from an *in vitro* preparation of gastrocnemius muscle (Todorov *et al*, 1996b).

Subsequent fractionation of the remaining affinity-circulated material by the method described by McDevitt *et al* (1995) resulted in the isolation of a peptidic factor which appeared to be different to the immunoreactive material. Such factor retained lipolytic activity after Q-Sepharose anion exchange, Superdex gel exclusion and anion exchange

HPLC chromatography and displayed an estimated molecular weight of 40-43 KDa as determined by SDS-PAGE and gel exclusion chromatography (Khan, 1996).

Therefore, two separate factors appear to be responsible for the different lipolytic and proteolytic activities displayed by the MAC 16 tumour homogenates (Beck and Tisdale, 1987), which originally were thought to be the same since they co-purified (McDevitt *et al*, 1995).

The same or a very similar immunoreactive material was also isolated from the MAC 13 tumour and the serum from tumour-bearing animals, which showed a very similar protein profile, although these were four- and 2.6-fold less immunoreactive than the corresponding MAC 16 tumours and sera (McDevitt, 1996). In addition, metabolic labelling studies during which MAC 16 and MAC 13 cells were labelled with [^{35}S]-sulphate and [6-^{3}H]-glucosamine (GlcN), showed the affinity purified material to be present in the medium of MAC 16 cultures but not in the MAC 13 nor in the cells (Todorov *et al*, 1997). It has therefore been suggested that such antigen might also be produced by the MAC 13 tumour, but unlike the cachexia-inducing MAC 16 model, it is either not secreted into the circulatory system for unknown reasons or may be rapidly destroyed (McDevitt, 1996). This model awaits further characterisation.

3.1.2. Structural elucidation of p24.

N-terminal amino acid analysis of the 24 KDa protein after HPLC revealed the following sequence: N-YDPEAAS^{7}APGS^{11}GN^{13}PS^{15}HEA(S)(H), which did not match any of the proteins in the Swissprot and Genbank databases. However, it showed a high degree of similarity with a Streptococcal preabsorbing antigen (PA-Ag) found in the Pir 3 data base for uncloned proteins and in the absence of any microbiological contamination in the tumours (McDevitt, 1996). Such PA-Ag has an estimated molecular weight of 43 KDa and is involved in the pathogenesis of acute post-streptococcal glomerulonephritis (Yoshizawa *et al*, 1992).

N-terminal amino acid analysis of the 69 KDa protein showed the 24 KDa to be associated with mouse albumin. Interestingly, whether in the serum or on the surface of cells, albumin has been shown to bind heparin and other heparinoids and of particular interest is its ability to bind bacterial liposaccharide (LPS) and peptidoglycan (PGN) (Dzrarski, 1994).

Further attempts to obtain additional information on the sequence of this protein failed in response to proteolytic digestion by trypsin, pronase, chymotrypsin or V8 protease, indicating that such portion could be the proteolytic core of the molecule. The N-terminal amino acid sequence indicated four possible sites for glycosylation, ie, Asn 13 (potential site for N-glycosylation, as for the consensus sequence Asn-X-Ser/Thr) and Ser 7, Ser 11 and Ser 15 (potential sites for O-glycosylation, in particular the presence of Ser 11 within a Gly rich region is suggestive of a glycosaminoglycan [GAG] binding site). Indeed, the presence of carbohydrate moieties in the affinity purified material was confirmed by the use of a digoxigenin glycan detection kit (Todorov *et al*, 1996b) and treatment of the affinity purified material with PNGase F, O-glycosidase, and sulphatase destroyed its biological activity, which seemed to be unaffected by treatment with neuraminidase or trypsin. Similarly, treatment with these enzymes inhibited antibody binding activity and immunological activity was completely abolished by periodate (Todorov *et al*, 1997). These results demonstrate the presence of N- and O-linked carbohydrate chains, which appear as the biological and antigenic determinants of the molecule. Furthermore, antigen reactivity was reduced by treatment with chondroitinase ABC, but was unaffected by treatment with endo-β-galactosidase, indicating that the immunological determinants reside in sulphated oligosaccharide chains and that the material is either a glycoprotein or a proteoglycan.

Simultaneous labelling of the MAC 16 cells 24 KDa antigen with ^{35}S and ^{3}H resulted in the retention of both radiolabels after affinity purification and recirculation of the same material on the affinity column following treatment with PNGase F gave two bands of 14 KDa and

10 KDa both of which also contained ^{35}S and ^{3}H. Digestion of the doubly labelled 24 KDa material with O-glycosidase and fractionation on a Sephadex column gave three bands of radioactivity corresponding to 14 KDa, 6 KDa and 4 KDa. The former two bands contained both labels whilst the latter only contained ^{3}H. Treatment of the material of 14 KDa produced from PNGase F digestion with O-glycosidase converted it into two fractions corresponding to 6 KDa and 4 KDa. Despite the absence of sulphated residues, the 4 KDa material was found to be more acidic than the sulphated chains of 6 KDa and 10 KDa as determined by elution with sodium chloride from a DEAE-cellulose column. Treatment with phosphatase revealed not only that phosphate residues were responsible for the negative charge in this part of the molecule, but also that these may be important antigenic determinants since such treatment reduced the antibody binding activity by 53%. Additional evidence on the presence of phosphate residues was provided by labelling the 24 KDa material with [^{32}P]-orthophosphate which showed the radiolabel to be confined to the same 4 KDa fragment. Extensive chemical deglycosylation was achieved with anhydrous trifluoromethanesulphonic acid which yielded a fragment of approximately 4 KDa.

Overall, these studies suggest a model for the 24 KDa material (which will be referred to as p24) consisting of a central polypeptide core of 4 KDa and a short oligosaccharide chain containing GlcN and with phosphate residues (4 KDa fragment), one O-linked sulphated oligosaccharide chain containing GlcN (6 KDa fragment), and one N-linked sulphated oligosaccharide chain also containing GlcN (10 KDa fragment) (Todorov *et al*, 1997). The apparent discrepancy between the estimated molecular weight of the whole molecule and those of the individual fragments, could be due to the microheterogeneicity provided by the carbohydrate moieties, which might affect the electrophoretic mobility of the molecule and therefore provide wrong estimations of molecular weights. The fact that neither the O- nor the N-linked chain were cleaved into disaccharides by chondroitinase ABC, suggests that the material is a sulphated glycoprotein rather than a proteoglycan as previously suggested.

3.1.3. Biological functions of GAGs.

GAGs constitute the most important feature in the structure of many proteoglycans (PGNs) and glycoproteins which are very diverse in structure and function. Proteoglycans are major components in connective tissues, extracellular matrices (ECMs) and are present on the surface of many cells. Aggrecan appears to be the predominant proteoglycan in cartilage, to which it confers its unique gel-like properties and its resistance to deformation, essential for distributing the load in weight-bearing joints (Lodish *et al*, 1995). Hyaluronan (HA), is the only extracellular oligosaccharide that is not covalently linked to a protein, although it is also a major structural component of proteoglycans which are found in many ECMs, particularly cartilage. The HA molecule binds a large amount of water forming a viscous hydrated gel which forms ECMs and surrounds migrating and proliferating cells. Such is the case of myoblasts, which bear an HA-rich coat that prevents them from fusing while in the undifferentiated state (Lodish *et al*, 1995).

Cell surface PGNs are thought to anchor cells to matrix fibres (heparan sulphate chains bind to type I, III and V collagen and to fibronectin). They are also found attached to the surface of epithelial cells, eg. syndecan and fibroglycan, whose GAG chains are bound to serine residues in the core protein as they are in the extracellular proteoglycans (Lodish *et al*, 1995).

GAGs have also been implicated in the modulation of cell growth for their ability to bind growth factors. In human cultured fibroblasts, heparin, heparan sulphate and dermatan sulphate inhibited the degradation of insulin-like growth factor-binding protein-5 (IGFBP5). Heparin increased the amount of intact IGFBP5 in the medium and, consequently, enhanced the cellular growth in response to IGF-1 by two different mechanisms. First, by binding directly to IGFBP5 and therefore inducing a conformational change which made the molecule less susceptible to degradation and, secondly, by inactivating the protease which is responsible for its proteolytic cleavage. For the latter, the presence of the O-sulphated groups in the 2 or 3 carbon position was essential

since neither desulphated heparin nor N-resulphation had any effect in the inhibition of IGFBP5 degradation. In addition, other GAGs which do not contain O-sulphated groups linked to iduronic acid, such as hyaluronic acid, keratan sulphate and chondroitin sulphate-A and -C, showed no inhibitory activity (Takami *et al*, 1994). Similarly, heparin also potentiates aFGF-induced neurite outgrowth in rat pheochromocytoma (PC12) cells by prolonging the biological half-life of aFGF (Damon *et al*, 1989).

In cultured bovine capillary endothelial (BCE) cells, bFGF has been shown to bind heparan sulphate which protected it from proteolytic degradation by plasmin, therefore rendering the cells capable of inducing plasminogen activator (PA) and collagenase production. Addition of heparinase before addition of plasmid abolished such effect (Saksela *et al*, 1988).

Alterations in the content and composition of the various types of GAG have been found to occur in a variety of neoplasms where, generally, the content of chondroitin sulphate and hyaluronic acid is increased and that of heparan sulphate decreased in comparison with normal tissue. In normal rectum, heparan sulphate is the predominant GAG while the other types of GAG (hyaluronic acid, dermatan sulphate and keratan sulphate) occur in smaller amounts. In contrast, in human rectum carcinoma this situation appears to be reversed and, additionally, the GAG content was seen to be increased 2 fold compared to non-neoplastic tissue (Tsara *et al*, 1995).

In metastatic mouse melanoma cells, a cell surface heparan sulphate PGN has been shown to initiate cell adhesion to the synthetic peptide FN-C/HII (a heparan-binding domain of fibronectin), which is inhibited following treatment with heparitinase. The PGN, which contains a core protein of 63 KDa and a less prevalent 32 KDa peptide, is an integral plasma membrane protein that is attached by linkage to phosphatidylinositol (PI), suggesting a role for PI-associated signal transduction pathways in mediating melanoma cell adhesion to this defined heparin binding ligand (Drake *et al*, 1992). Another PI-anchored protein is Thy-1, a 25 KDa glycoprotein present in neurons, thymocytes and

peripheral T cells. In murine thymocytes as well as in other transfected cell lines, the concentration of intracellular calcium increased rapidly after cross-linking of Thy-1 to two mAbs (Kroczek *et al*, 1986).

Finally, the major transplantation antigens are also cell-surface glycoproteins composed of a single polypeptide chain non-covalently associated with a molecule of β2-microglobulin (Kress *et al*, 1983). The surface presentation of these antigens is essential both for their role in inducing allograft rejection and for their involvement in the associative recognition of viral and tumour antigens.

3.1.4. Aims of the study.

The following experiments aim to establish a role for p24 in the development of cachexia in the MAC 16 model, since this is the material that appears to be responsible for this wasting syndrome in the tumour-bearing mice. Its effect on body weight and muscle protein metabolism will be studied, and also the influence that the monoclonal antibody raised against p24 exerts on both. The ability of this material to stimulate protein breakdown *in vitro* will also be investigated, as well as the potential role for PGE_2 in the mechanism of action of p24.

3.2. Results.

An immunoreactive material was isolated from the MAC 16 tumour extracts by means of affinity chromatography using the monoclonal antibody which had been isolated from the splenocytes of cachectic animals with delayed weight loss (figure 3.1). This material, which was found in the albumin-bound and unbound forms (24 and 69 KDa bands) (figure 3.2), was capable of eliciting weight loss in recipient animals 24 h after the first intravenous injection. p24 also induced a reduction in the concentration of many plasma amino acids such as threonine, serine, proline, glycine, methionine, leucine, lysine tryptophan, histidine and particularly alanine which was decreased by 40% (table 3.1). The same effect was obtained with a similar immunoreactive material which was isolated from the urine of weight losing cancer cachectic patients. The weight loss caused by the mouse or human material, which reached between 5-8% of total body weight at 24 h, were both attenuated when animals were pre-treated with the monoclonal antibody (mAb) developed against the mouse material (table 3.2).

Skeletal muscle from p24-weight losing animals exhibited an elevated release of tyrosine, which was used as a marker for total protein breakdown. In addition, the amount of PGE_2 released into the incubation medium was also increased. Tyrosine and PGE_2 levels were both reduced in muscles from animals which had been pre-treated with the mAb previous to p24 administration (figures 3.3 and 3.4).

Loss of skeletal muscle protein was due to both a depression in the rate of protein synthesis and an elevated proteolysis rate both of which were found to be close to control values in mAb-treated mice which were injected with the mouse material (figures 3.5 and 3.6). Human p24 caused a significant reduction in the rate of protein synthesis in muscle and heart while it increased the hepatic rate (table 3.3).

Administration of p24 to non tumour-bearing animals did not cause significant changes in the protein and RNA contents of soleus muscle or in any of the other tissues examined,

with the exception of spleen. In spleen there was a considerable reduction in the amount of protein and RNA, the latter being significantly reduced, $P< 0.05$ (figures 3.7 and 3.8). MAC 16 mice, however, had a significantly lower RNA content for all of the tissues examined despite the absence of any significant changes in protein, although there was a non-significantly lowering of the mean values in spleen and soleus (figures 3.7 and 3.8).

In addition to its effects in *vivo*, p24 was also capable of initiating protein catabolism in isolated soleus muscle which again was found to be associated with increased tyrosine levels and both were blocked by the use of NSAIDs such as indomethacin and ibuprofen, and a $cPLA_2$ inhibitor, the arachidonic acid analogue $AACOCF_3$ (table 3.4).

3.2.1. Purification of p24.

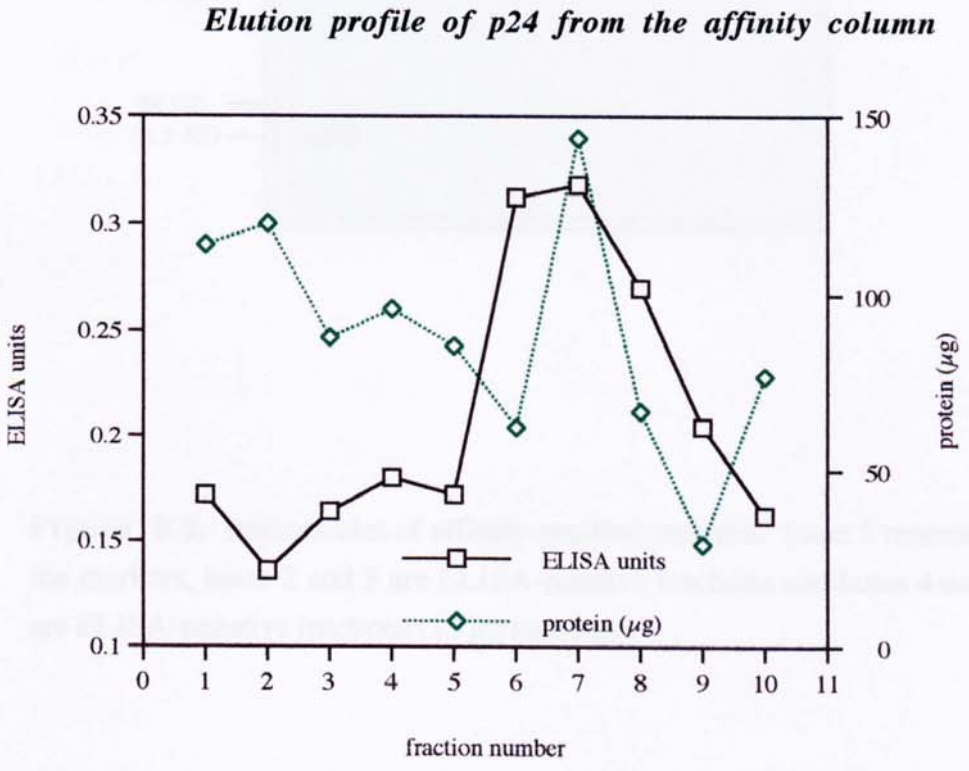

Figure 3.1. Representative elution profile of p24. p24 was circulated overnight on the affinity column at a flow rate of 0.1 ml/min, washed with PBS for 3 h at a flow rate of 0.1 ml/min and eluted with 0.1 M Gly-HCl buffer pH 2.5 at a flow rate of 1.0 ml/min. Samples were neutralised in 1 M Tris-HCl buffer pH 8.0 and ten 3 ml-fractions were collected.

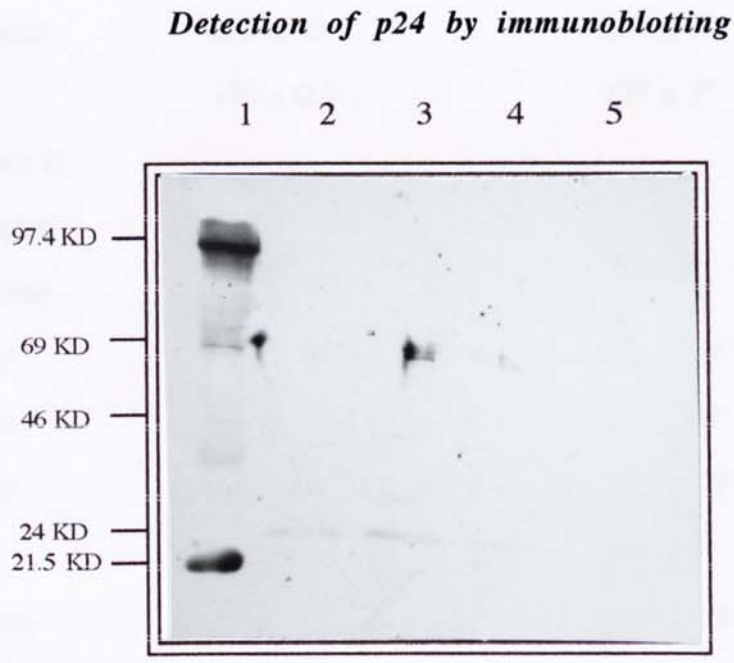

Figure 3.2. Immunoblot of affinity-purified material. Lane 1 represents the markers, lanes 2 and 3 are ELISA-positive fractions and lanes 4 and 5 are ELISA-negative fractions (15 μg loadings).

3.2.2. *Effect of p24 on body weight and amino acid plasma composition.*

Effect of p24 on plasma amino acid composition (nmol/ml)

	Control	p24
Aspartate	17.7 ± 4.3	13.0 ± 2.5
Threonine	200 ± 10	157 ± 7*
Serine	160 ± 0.0	127 ± 7*
Glutamic &		
Asparagine	170 ± 98	117 ± 3
Glutamine	413 ± 40	313 ±12
Proline	130 ± 6	66 ± 0.8**
Glycine	263 ± 13	200 ±10*
Alanine	630 ± 45	370 ± 10*
Valine	220 ±30	183 ± 7
Cysteine	2.5 ± 0.3	1.5 ± 0.9
Methionine	57 ± 1.5	41 ± 0.9**
Isoleucine	95 ± 5	69 ± 5*
Leucine	147± 6	107 ± 3*
Tyrosine	58 ± 0.7	53 ± 4
Phenylalanine	83 ± 6	72 ± 2
Lysine	320 ± 12	247 ± 15*
Tryptophan	110 ± 0	95 ± 2.5*
Histidine	69 ± 2	48 ± 2**
Arginine	21.6 ±18	106 ± 29

Table 3.1. Effect of p24 on the plasma amino acid concentration of NMRI mice 24 h after treatment with p24. Results expressed as mean ± sem, where n = 4 mice. Statistical analysis was performed by Student's unpaired t-test, where (*) $P< 0.05$ and (**) $P< 0.01$.

Total body weight change(g)			
		Number of mice	p
Control	-0.240 ± 0.143	50	
Mouse p24	-1.551 ± 0.118	40	<0.001
Ab-treated (mouse p24)	-0.060 ± 0.239	12	<0.001
Human p24	-1.632 ± 0.381	11	<0.001
Ab-treated p24 (human p24)	0.017 ± 0.162	4	<0.01

Table 3.2. Effects of mouse and human p24 (30-50 μg) on body weight loss 24 h after the first injection. These were injected into the tail vein of female NMRI mice respectively at 1.5-h intervals (total of 4 injections). Control animals received PBS (iv), while the antibody-treated groups received the monoclonal antibody (ip) 24 h before the first injection of the affinity-purified material. Results expressed as mean ± sem. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24).

3.2.3. *Effect of p24 on muscle protein metabolism.*

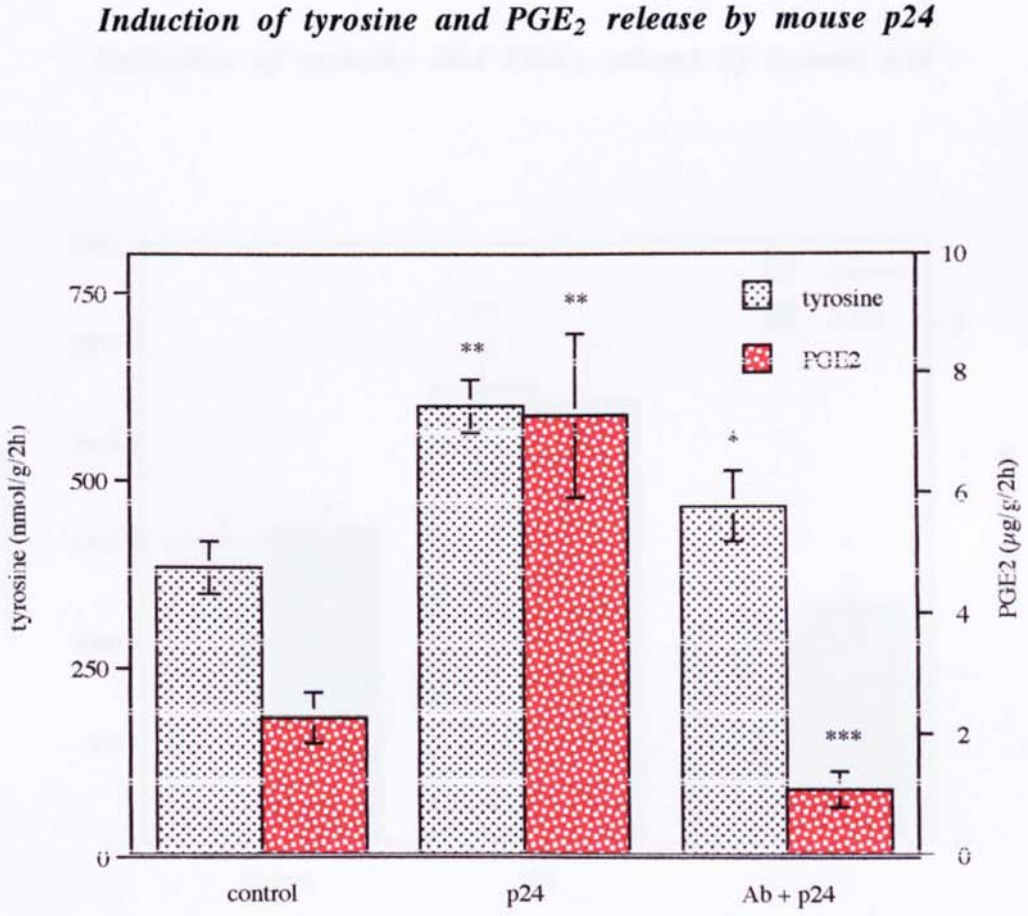

Figure 3.3. Induction of tyrosine and PGE_2 release from soleus muscle *ex vivo* 24 h after the first injection (iv) with mouse p24. Control mice were injected with PBS (iv) and the Ab-treated group received the mAb (ip) 24 h before the first injection of p24. Results are expressed as mean ± sem, where n = 3 mice. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24), where (*) $P< 0.05$, (**) $P< 0.01$ and (***) $P< 0.001$.

Induction of tyrosine and PGE_2 release by human p24

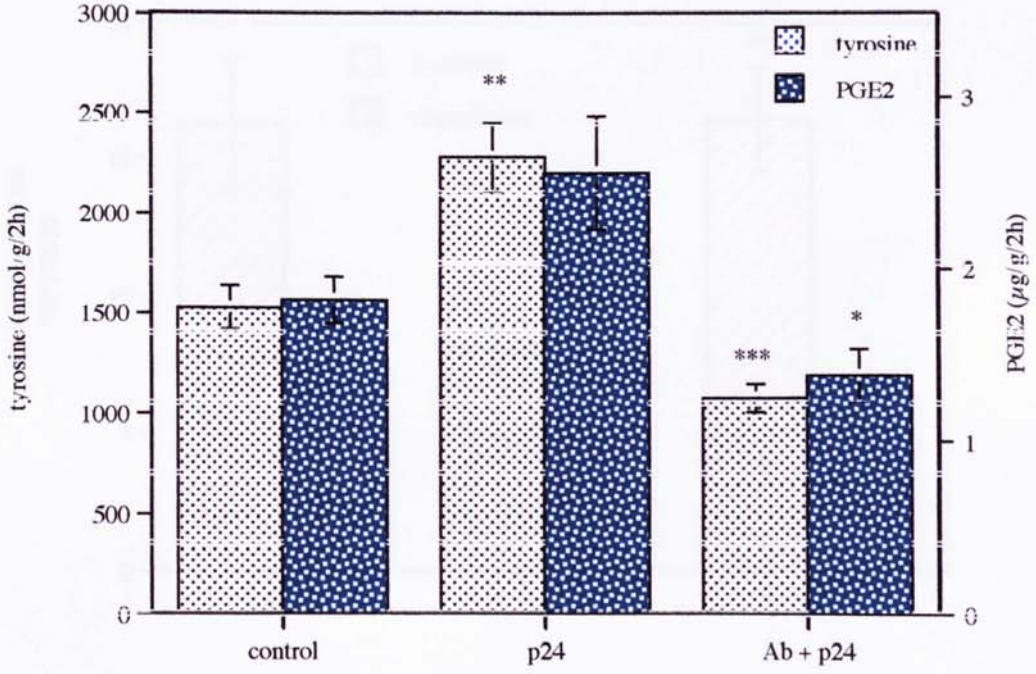

Figure 3.4. Induction of tyrosine and PGE_2 release from soleus muscle *ex vivo* 24 h after the first injection (iv) with human p24. Control mice were injected with PBS (iv) and the Ab-treated group received the mAb (ip) 24 h before the first injection of p24. Results are expressed as mean ± sem, where n = 3 mice. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24), where (*) $P< 0.05$, (**) $P< 0.01$ and (***) $P< 0.001$.

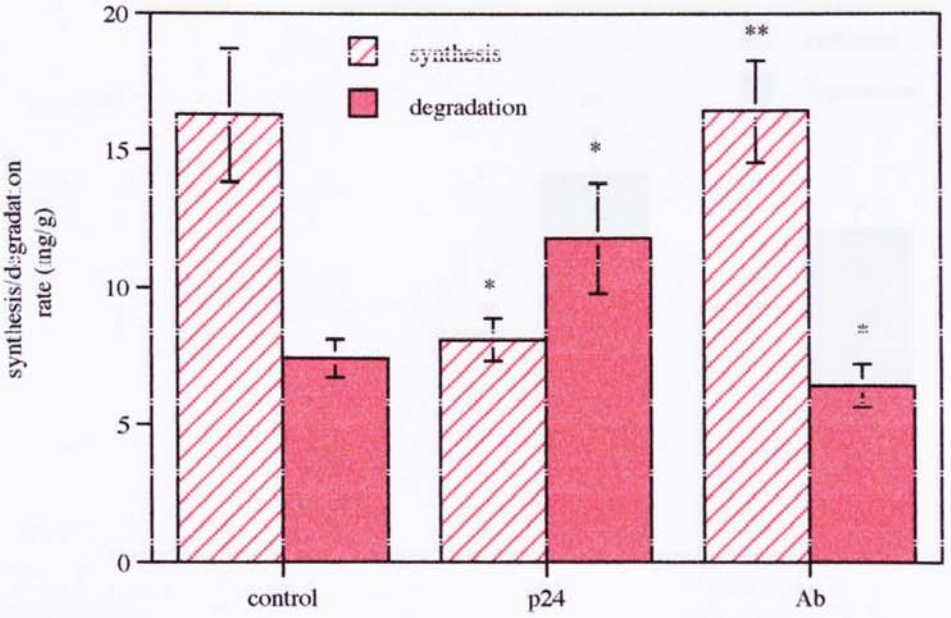

Figure 3.5. The effect of mouse p24 and the mAb on the rates of protein synthesis and degradation of gastrocnemius muscle. Mice were treated as previously described and pre-injected with 0.4 mM L-[4-^{3}H]phenylalanine (1.5 Ci/mmol) (ip) 24 h before treatment with p24. Results are expressed as mean ± sem, where n = 4 mice for each treatment, except for controls where n = 3 mice. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24), where (*) $P < 0.05$ and (**) $P < 0.01$.

Effect of human p24 on skeletal muscle protein metabolism

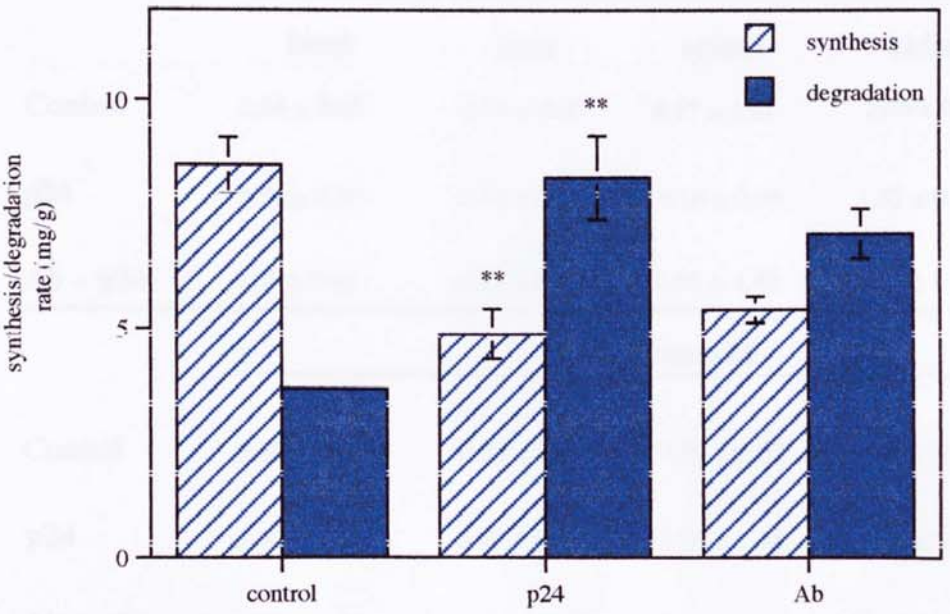

Figure 3.6. The effect of human p24 and the mAb on the rates of protein synthesis and degradation of gastrocnemius muscle. Mice were treated as previously described and pre-injected with 0.4 mM L-[4-^{3}H]phenylalanine (1.5 Ci/mmol) (ip) 24 h before treatment with p24. Results are expressed as mean ± sem, where n = 4 mice for each treatment, except for controls where n = 3 mice. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24), where (**) $P < 0.01$.

3.2.4. Effect of p24 on protein metabolism of other host tissues.

Effect of p24 on the rate of protein synthesis (mg/g)				
	mouse p24			
	heart	liver	spleen	kidney
Control	2.54 ± 0.65	0.16 ± 0.02	4.17 ± 1.31	2.06 ± 0.54
p24	2.54 ± 0.70	0.14 ± 0.02	4.16 ± 0.96	1.52 ± 0.56
Ab + p24	3.67 ± 0.63	0.15 ± 0.02	3.83 ± 1.42	2.19 ± 0.50
	human p24			
Control	8.89 ± 1.82	0.38 ± 0.03	7.59 ± 2.07	1.49 ± 0.57
p24	3.90 ± 0.38 *	0.49 ± 0.01 **	7.96 ± 1.18	3.72 ± 0.61
Ab + p24	5.32 ± 1.58	0.45 ± 0.07	7.97 ± 0.94	2.60 ± 0.16

Table 3.3. Effects of mouse and human p24 on the protein synthesis rates of host tissues. Mice received same treatment as in figures 3.5 and 3.6, respectively. Results are expressed as mean ± sem, where n = 4 mice for each treatment, except for controls where n = 3 mice. Statistical analysis was performed by Student's unpaired t-test (p24 vs control and Ab-treated vs p24), where (*) $P < 0.05$ and (**) $P < 0.01$.

Effect of p24 and the MAC 16 tumour on the protein content of host tissues

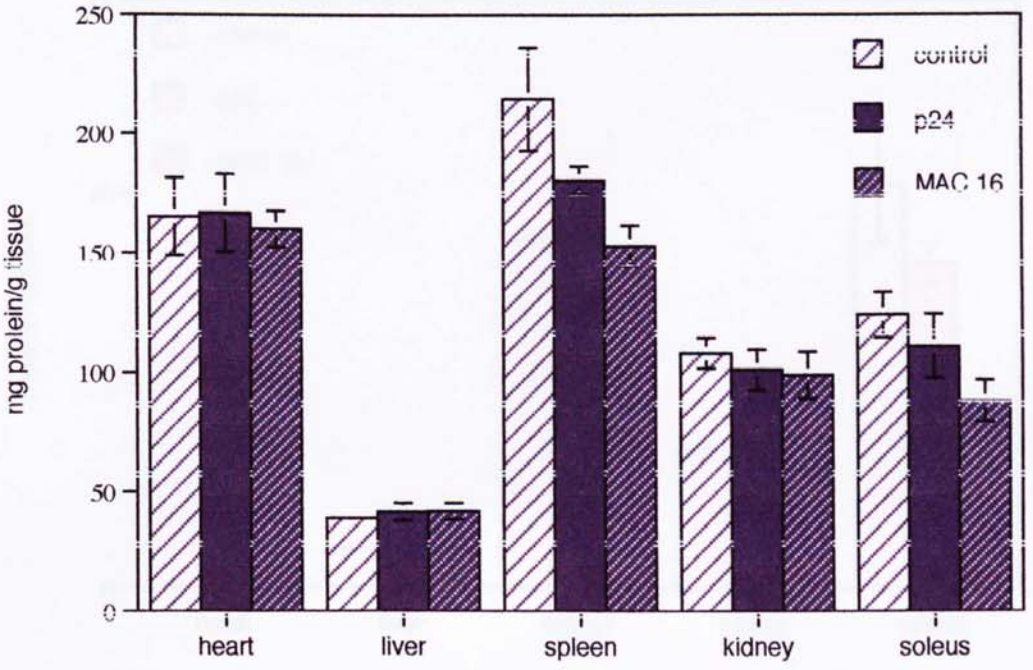

Figure 3.7. Total tissue protein content. Mice treated with p24 lost 1.4 g over 24 h and tumour-bearing animals lost 5 g over 17 days. Tissues were homogenised in 0.2 M PCA, centrifuged and redissolved in 0.6 M NaOH. Results are expressed as mean ± sem, where n = 5 mice.

Effect of p24 and the MAC 16 tumour on the RNA content of host tissues

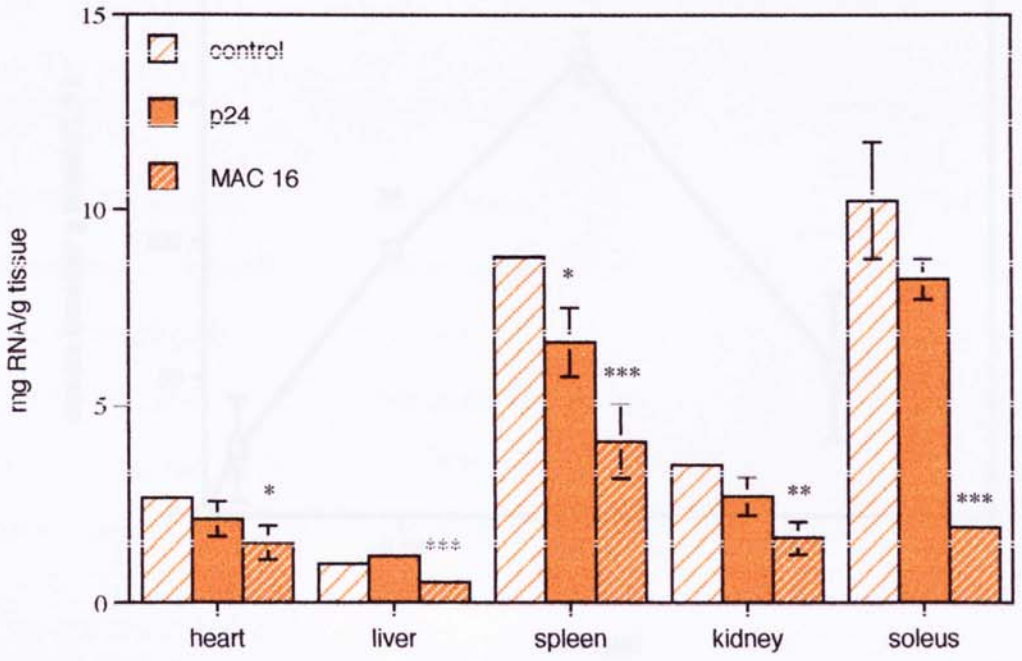

Figure 3.8. Total tissue RNA content. Mice received the same treatment as in figure 3.7. Protein extracts were deproteinised with 2M PCA, centrifuged and the RNA measured. Results are expressed as mean ± sem, where n = 5 mice. Statistical analysis was performed by Student's unpaired t-test (p24 and MAC 16 vs control, respectively), where (*) $P< 0.05$, (**) $P< 0.01$ and (***) $P< 0.001$.

3.2.5. *Effect of p24 on protein mobilisation in vitro.*

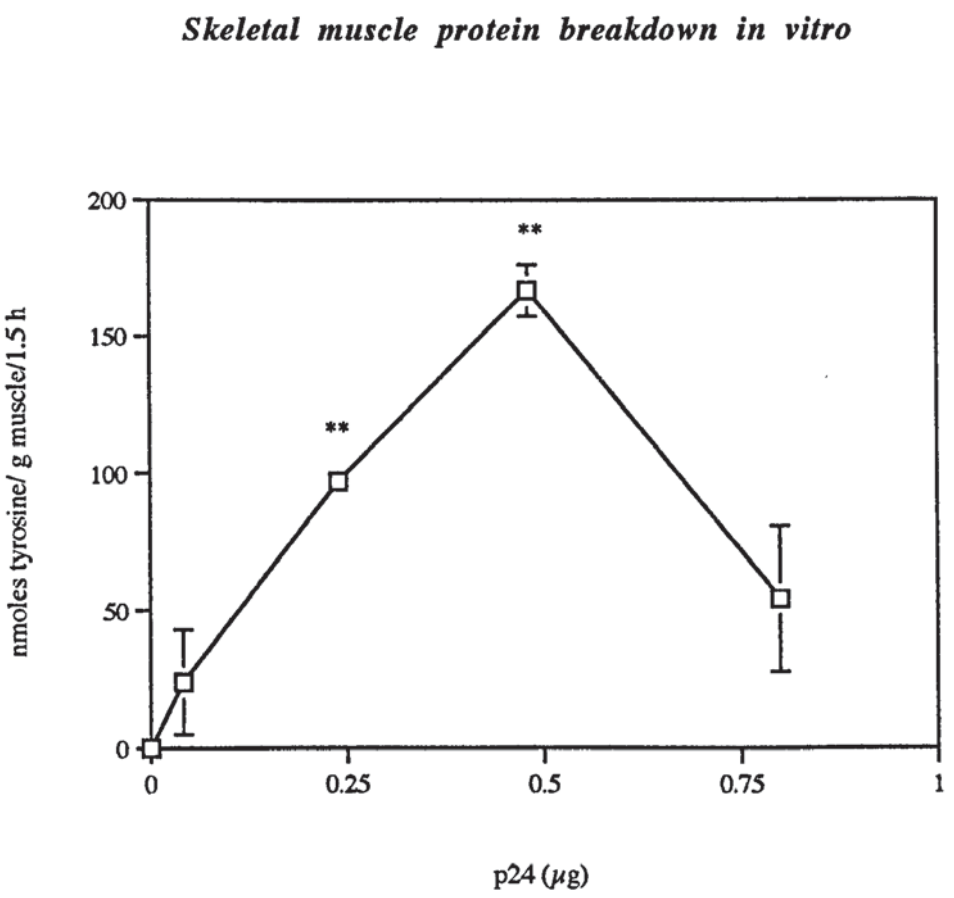

Figure 3.9. Induction of skeletal muscle protein breakdown *in vitro* as measured by tyrosine release from soleus muscle. Muscles were pre-incubated for 30 min in the presence of serum with or without p24 (basal values subtracted from total amounts). Media were then replaced and a further 1.5 h-incubation was carried out. Results are expressed as mean ± sem, where n = 3 muscles and each experiment was carried out at least twice. Statistical analysis was performed by Student's unpaired t-test, where (**) $P < 0.01$ vs zero control.

Inhibition of protein breakdown in vitro

Treatment	Inhibition of tyrosine release	Inhibition of PGE_2 release
$AACOCF_3$ (50 μM)	below basal *	99%
Indomethacin (50 μM)	below basal *	below basal *
Ibuprofen (10 μM)	78%	below basal *
CV6504 (10 μM)	below basal *	below basal *
EPA (1.25 g/Kg)	79%	below basal *
mAb (10μg/ml)	below basal *	-

* (> 100 % inhibition)

Table 3.4. Inhibition of tyrosine and PGE_2 release induced by p24 *in vitro*. Muscles were incubated as previously described in figure 3.2. All inhibitors were present in pre-incubation and incubation media, except for EPA which was administered *in vivo* 24 h before the start of the experiment and the mAb which was only present in the pre-incubation medium. The results are representative of at least 2 experiments, where n = 3 muscles.

3.3. Discussion.

3.3.1. Effect on body weight.

p24 was purified both from animal and human sources by immunoaffinity purification using a mouse monoclonal antibody (mAb). Mouse p24 was isolated from the MAC 16 tumours of cachectic animals, whereas the human material was extracted from the urine of cachectic patients with pancreatic cancer and established weight loss. Such immunoreactive material was not present in the urine of 12 normal subjects tested and, in addition, patients losing weight through conditions other than cancer showed no evidence for the presence of this factor (Cariuk *et al*, 1997). The N-terminal amino acid sequence was identical for both and showed no homology with any of the reported cytokines (Todorov *et al*, 1996a and Cariuk *et al*, 1997).

After fractionation on the affinity column (figure 3.1), immunoreactivity of this affinity-purified material was tested by ELISA and Western blotting. ELISA-positive fractions were pooled and concentrated by Amicon filtration against PBS before being subjected to SDS-PAGE. Immunoblots revealed 2 bands of relative molecular mass 69 and 24 KDa, respectively, which corresponded to the albumin-bound and free forms of p24 (Todorov *et al*, 1996a) (figure 3.2).

Administration of the affinity purified material into non-tumour bearing mice caused a rapid and significant weight loss 24 h after the first injection, in contrast with PBS-injected controls (table 3.1). p24 was given in four intravenous injections since the pharmacokinetics of the ^{125}I-labelled material indicated a rapid disappearance from the blood of these animals (Lorite *et al*, 1997). Neither the peptide portion of the molecule nor the non-immunogenic fractions from the reverse-phase column had been shown to produce a change in body weight (Todorov *et al*, 1996a).

The p24-induced weight loss was mainly due to loss of lean body mass as determined by body composition analysis, and both appeared to be specific for this material since it was

suppressed in animals treated with the mAb previous to p24 administration. In addition, no changes in water or food intake were associated with the weight loss, excluding the possibility of a toxic effect (Cariuk *et al*, 1997 and Lorite *et al*, 1997).

There was a significant decrease in blood glucose levels as well as the plasma levels of some amino acids, which was particularly pronounced for Thr, Ser, Pro, Gly, Met, Ile, Leu, Lys, Trp, His and Ala (table 3.2). Of particular importance is the reduction by 40% in the concentration of Ala, which is the main entry point for liver gluconeogenesis, and the decrease in the concentration of Trp which has been proposed as a tumour-marker in the progression of cachexia in humans (Iwagaki *et al*, 1995) and has also been found at low concentrations in the Walker 256 carcinosarcoma-bearing rats (Kraus *et al*, 1979). Gly, as opposed to starvation was not increased, in fact, its concentration was also significantly reduced.

Todorov *et al* (1996a) found no alteration in plasma triglyceride levels, unlike the effect observed with cytokines (where they are elevated, Beutler and Cerami, 1986 and Norton *et al*, 1987), although these appeared to be decreased in animals injected with human p24 (Cariuk *et al*, 1997). Similar changes in amino acid plasma composition and glucose had been found in cachectic mice bearing the MAC 16 tumour (Beck and Tisdale, 1989 and McDevitt and Tisdale, 1992). Human studies have shown that the concentrations of most plasma amino acids, in particular the concentrations of gluconeogenic amino acids, are decreased and that this is particularly true for tumours that induce cachexia in the host (Clarke *et al*, 1978).

Overall, injection of p24 elicited a cachectic-like state which resembled that produced by the tumour (Beck and Tisdale, 1987) which was largely due to loss of lean body mass.

3.3.2. Effect on muscle protein metabolism.

The weight loss induced by p24 was found to be associated with increased total protein breakdown as determined by the release of tyrosine from soleus muscle 24 h after p24 administration (figures 3.3 and 3.4). Tyrosine has been extensively used as a marker of net protein breakdown (ie, the balance between protein synthesis and protein degradation) because it is present in all proteins, it rapidly equilibrates between intracellular pools and the medium, it can easily be measured fluorometrically and in skeletal muscle is neither synthesised nor degraded. However, it does not distinguish between the breakdown of total and myofibrillar proteins. It is widely accepted that insulin, amino acids and agents that inhibit lysosomal protein breakdown can attenuate the degradation of soluble but not that of myofibrillar proteins.

3-Methylhistidine (3-MH) is a post-translationally modified amino acid which is only present in actin and myosin. The use of 3-MH as an indication of muscle protein breakdown over the tyrosine method has the advantage that this modified amino acid cannot be reutilised after its release and therefore, there is no need to block protein synthesis by means of cycloheximide. However, in whole-body experiments, and even in muscle preparations, the contribution of 3-MH from cytoskeletal actin of cells other than muscle makes measurement from muscle complicated. In addition, its determination in *in vitro* muscle preparations is even more difficult because of the interference from other amino acids which are present at much higher concentrations. A recent approach has recently been developed by Thompson *et al* (1996b), which allows the measurement of 3-MH released from cells in culture, grown under normal conditions without the manipulation of the incubation medium or any of its components. This method will be further discussed in chapter 5.

Goodman (1987) found that treatment of muscles with agents that specifically blocked the release of 3-MH from myofibrillar proteins by 21-35%, did not affect the release of tyrosine, indicating that changes in myofibrillar protein turnover were not reflected in total

cell proteolysis. Soluble and myofibrillar proteins are independently regulated and this will be further discussed in chapter 4.

The weight loss and the increased tyrosine levels associated with it after treatment with p24 were both attenuated with the mAb raised against the mouse material (figures 3.3 and 3.4), which shows that these effects are not only specific to such material, but also that both mouse and human p24 might be operating through similar mechanisms in the wasting process. Therefore, the elucidation of the mechanism of action of p24 in the MAC 16 model might shed some light in the understanding of this wasting condition in humans.

Increased protein loss occurred through a depression in protein synthesis and an increase in degradation in gastrocnemius muscle which, in the case of mouse p24, were both prevented in mice which had been pretreated with the mAb (figure 3.5). Human p24 caused a similar effect on the protein synthesis and degradation rates, although the antibody did not prevent the increased catabolic state of muscle despite its effect in the attenuation of weight loss (figure 3.6).

Protein synthesis and degradation were simultaneously measured in the same animals by the ^{3}H-phenylalanine flooding dose technique. However, other available methods could have provided more reliable estimates of both protein synthesis and degradation rates. The choice of this method might have resulted in an underestimation of both synthesis and degradation rates, since the long period of time between the single injection of the label and the termination of the experiment (50 h, ie, 24 h labelling, 24 h treatment with p24 and 2 h incubation), might have led to the measurement of only a residual fraction of the labelled precursor.

The choice of the flooding dose over the continuous injection technique for measuring protein degradation has the advantage of overcoming the dilution of the specific activity resulting from the degradation of unlabelled protein which, by using this method, is

minimised. Additionally, all of the possible precursor pools become similarly labelled, since the contribution from the intracellular and the extracellular amino acid pools to tRNA varies (Hider *et al*, 1971 and Li *et al*, 1973). However, the rapid equilibration of phenylalanine with the intracellular pool should have been taken into account in the design of the experiment and the administration of this amino acid should have been carried out at the same time as the injection of p24 in order to shorten the duration of the experiment. But even this length of time could have proved too long, since some studies in which protein synthesis was measured indicate that injection of phenylalanine (iv) 10 or 20 minutes just before the termination of the experiment was suitable in order to get equilibration of this amino acid (Emery *et al*, 1984 and Reeds *et al*, 1985). Therefore, since the effect of p24 was studied over a 24 h period, it may be that the continuous injection technique would have been more suitable, although it should also be taken into account that p24 is administered intravenously and therefore, the combination of the two would have undoubtedly caused to much stress for the animals.

Finally, due to the effect of p24 on protein degradation, subsequent amino acid release might have led to the reincorporation of these into protein synthesis. Therefore, separate experiments should have been carried out for measuring these rates individually. For synthesis, the above method employed by Garlick *et al* (1983) and Reeds *et al* (1985) could have been used or, alternatively, the rate of protein synthesis in muscle should have been measured *in vitro* in the presence of labelled phenylalanine following the *in vivo* treatment (Palmer, 1987).

Development of protein metabolic perturbations in skeletal muscle in response to p24 have previously been reported (Todorov *et al*, 1996a) and indeed, changes in protein muscle metabolism in tumour-bearing animals are well documented. Whether due to a decrease in the rate of protein synthesis or an increased rate of protein degradation or both, the imbalance between the two consequently leads to loss of nitrogen and increased muscle wasting.

In the MAC 16 model, protein depletion appears to be associated with depressed synthesis and increased proteolysis rates (Smith and Tisdale, 1993a), both of which were evident with a 5% weight loss and reached statistical significance when weight loss was 16-20% and above. In the Ehrlich ascites tumour model, protein synthesis was significantly suppressed even when the tumour mass was barely detectable (days 2-4) and it continued until day 9, whilst an elevation in protein degradation occurred after day 6 (Lopes *et al*, 1989). Pain *et al* (1984) also reported a depression in protein synthesis in the same murine model. Tessitore *et al* (1987) reported an elevated muscle protein degradation rate and no changes in the synthesis rate for rats bearing the Yoshida AH-130 hepatoma, whilst Strelkov *et al* (1989) reported a depressed rate of protein synthesis in addition to the enhanced proteolysis. Tayek *et al* (1988) also found a reduction in the rate of protein synthesis in rats bearing this tumour. Lack of evidence for elevated protein breakdown in skeletal muscle was found in mice bearing the MCG 101 sarcoma (Svaninger *et al*, 1983), and in rats bearing the Morris hepatoma 7777 the tumour significantly decreased muscle protein synthesis as well as protein degradation, which were reversed following tumour excision (Le Bricon *et al*, 1996). In contrast, enhanced proteolysis has been reported for the same tumour model by Strelkov *et al* (1989). Emery *et al* (1982) showed that the muscle wasting seen in cachectic mice bearing the XK1 tumour was due to both an increased in myofibrillar degradation and decreased protein synthesis. However, it was later reported that the decrease in protein synthesis was more important, since the observed increase in 3-MH concentration did not take into account the contribution made by other organs such as gut, particularly under circumstances of reduced food intake (Emery *et al*, 1984 and Rennie and Millward, 1983). Muscle protein synthesis and whole-body growth have both been reported to be significantly reduced in rats bearing the Walker 256 carcinosarcoma (Tayek *et al*, 1986) and the Novikoff hepatoma (Tayek *et al*, 1988).

The heterogeneity of these reports may be due to differences in tumour types or may simply reflect differences in methodology. In any case, the cause of enhanced proteolysis in skeletal muscle has only started to be understood recently with the discovery of the

ubiquitin-dependent proteolytic system, and the better understanding of how the different proteolytic systems work and how they get activated under different nutritional and hormonal conditions (this will be analysed in more detail in chapter 4). In contrast, alterations in protein synthesis have long been known to be largely caused by defects in ribosome activity.

In rats bearing the Walker 256 Carcinoma, a comparison of the incorporation of ^{3}H-lysine into protein by gastrocnemius polysomes isolated from control and tumour-bearing rats showed that such incorporation was reduced in the weight losing tumour-bearing rats (Goodlad and Clark, 1972). Further studies showed that such reduction was due to a defect in translation, as demonstrated by the formation of peptidyl-puromycin complexes, which indicated some kind of defect in either translocation or peptidyl transferase activity in the preparation from the tumour-bearing rats. However, the latter was ruled out since interchangeability between the 60S subunits (where the peptidyl transferase activity resides) did not alter their ability to synthesise polyphenylalanine, indicating that it was due to a defect in the 40S ribosomal subunit (Clark and Goodlad, 1975). In cachectic mice bearing the XK1 tumour the depression of protein synthesis in muscle was due to a reduction in both RNA content and RNA activity (Emery *et al*, 1984).

Changes in ribosome activity have also been reported in rat muscle in response to fasting (Henshaw *et al*, 1971). The protein synthesis rate of fasted animals was inhibited due to a decrease in ribosome content and polyribosome activity, as well as a reduction in the amount of ribosomes incorporated in polyribosomes (synthetically active units).

Administration of glucocorticoids caused a decline in the protein synthesis rate of rat skeletal muscle, where an accumulation of the 60S and the 40S ribosomal subunits was observed, suggesting a defect in peptide-chain initiation (Rannels *et al*, 1978b). It has been proposed that defects in the initiation of protein synthesis are due to phosphorylation of the initiation factor, eIF-2, and of peptides of the 40S ribosomal subunit which causes the

disruption of the ternary complex formed by GTP and Met-tRNA$_f$. A haemin-controlled repressor (HCR) with cAMP-independent protein kinase activity was isolated from rabbit reticulocytes and their cell-free lysates, which was responsible for the inhibition of protein synthesis in the absence of haemin (Kramer *et al*, 1976 and Ranu *et al*, 1976). HCR was shown to be capable of phosphorylating eIF-2 and other proteins associated with the 40S ribosomal subunit and an antibody raised against it neutralised the kinase activity as well as its inhibitory effect on haemin synthesis (Kramer *et al*, 1976).

In skeletal muscle from starved rats, depressed protein synthesis was associated with loss of eIF-2 activity, possibly due to the action of a cAMP-independent protein kinase (Rannels *et al*, 1978a). The reduction in protein synthesis induced by sepsis has also been shown to be due to an inhibition of peptide chain initiation at the level of eIF-2, whose restoration to control values was achieved following treatment with the interleukin-1 receptor antagonist IL-1ra (Vary *et al*, 1996).

Finally, the accelerated muscle catabolism seen in rats after endotoxin treatment appeared to be the consequence of both increased muscle protein breakdown and reduced synthesis, the latter due to a decrease in the translational activity (Jepson *et al*, 1986).

3.3.3. Effect on the protein synthesis rates of host tissues.

There was no significant depression in protein synthesis in other host organs at dose levels that produced a depression of protein synthesis in skeletal muscle with mouse p24 (table 3.3) and, in a separate experiment, p24 did not cause significant reductions in either protein or RNA content of host tissues except for the RNA content of spleen (figures 3.7 and 3.8). In mice bearing the MAC 16 tumour, however, the RNA content of all tissues examined was significantly lower than in non tumour-bearing animals.

In contrast, human p24 caused a significant depression in the rate of protein synthesis in heart (table 3.3) suggesting that, although the action of p24 appears to be specific to

skeletal muscle, it might also affect cardiac muscle to some extent. In fact, despite the absence of any significant changes in the rate of protein synthesis in heart, mouse p24 was shown to be capable of activating the expression of one of the components of the ubiquitin pathway *in vivo* (this will be further discussed in chapter 4).

No significant changes were found in protein synthesis for liver in the case of mouse p24 (this also correlates with the lack of activation of the ubiquitin system which will be discussed in chapter 4), although it was significantly increased in response to human p24. As for skeletal muscle, the liver response to tumour growth appears to be dependent upon the type of tumour. Thus, protein synthesis rates have been reported to be increased (Tayek *et al*, 1988; Lopes *et al*, 1989 and Pain *et al*, 1984), unchanged (Tayek *et al*, 1988) or depressed (Emery *et al*, 1984). Tessitore *et al* (1987) found no changes in protein synthesis in the early stages of tumour growth which was elevated at later stages, following a similar pattern to protein degradation, although it was not sufficient to balance the enhanced proteolysis. In contrast, LeBricon *et al* (1996) found an increased protein deposition in the liver of cachectic tumour-bearing rats.

3.3.4. Muscle protein breakdown in vitro.

The choice of soleus as a model to study muscle protein degradation was made because, in contrast with gastrocnemius, this muscle can be dissected end to end and is thin enough to permit oxygenation by diffusion and substances introduced in the surrounding medium gain ready access to the muscle cells.

p24 was capable of direct induction of protein breakdown in isolated soleus muscle (figure 3.9). There was a linear increase in the release of tyrosine in response to increasing concentrations of this material, which reached a maximum at a concentration of 0.5 μg (per 3 ml of medium) and then decreased with higher concentrations. Interestingly, the same effect was found when isolated gastrocnemius from normal animals was incubated with the serum of MAC 16 mice with increasing weight loss (Smith and Tisdale, 1993b). Induction

of muscle protein degradation *in vitro* by p24 had previously been reported (Todorov *et al*, 1996 and Todorov *et al*, 1997). Further evidence indicating that the systemic effect of the tumour on the host is mediated by circulatory factors is provided by a study carried out by Belizario *et al* (1991), where the plasma from cachectic cancer patients elicited an increased protein catabolism in rat diaphragm and soleus muscle, which was not seen with serum from healthy subjects.

Since control muscles were also incubated in the presence of serum, all increases in protein breakdown above basal values in treated muscles are exclusively due to the action of p24. The extent to which protein degradation was stimulated, however, does not probably reflect a true *in vivo* situation because of the inherent catabolic state of the isolated muscle. Isolation of muscle itself leads to a significant reduction in the rate of protein synthesis (Palmer *et al*, 1981) and additional factors such as the lack of oxygen and nutrients might cause net protein balance to become more negative. In addition, the inclusion of cycloheximide in the incubation medium may also potentiate this effect.

Indeed, *in vitro* muscle preparations have been shown to be largely influenced by the presence of anabolic agents in the incubation medium. In a study carried out by Fulks *et al* (1975) insulin was found not only to stimulate protein synthesis, but also to inhibit protein degradation. Glucose potentiated both effects but in the absence of insulin it only inhibited proteolysis. The branched-chain amino acids Leu, Ile and Val have also been shown to produce similar effects to those of insulin. Others have found similar results on the influence of branched-chain amino acids, but have failed to establish a role for insulin on the degradation rate of muscle proteins (Lundholm *et al*, 1981). Mitch and Clark (1984) reported the ability of Leu to stimulate muscle protein synthesis and inhibit degradation, but no effects were ascribed to Ile and Val. Tischler *et al* (1982) found both stimulation of protein synthesis and inhibition of proteolysis in diaphragm muscles from fed and fasted rats by exogenous Leu. Glutamine has also been reported to inhibit protein degradation, with no additive effects in the presence of insulin also an inhibitor of protein degradation

(McLennan *et al*, 1988). A recent study carried out in humans has also pointed out the importance of plasma amino acids as anabolic agents in addition to insulin and IGF-1 (Svanberg et al, 1996).

It also appears that muscles incubated under slight tension are able to maintain a better protein balance. It has been shown that by incubating muscles at resting length, overall protein balance is improved largely by the reduction in proteolysis. Enhanced proteolysis in unrestrained muscles appears to be due to the activation of calcium-dependent proteases and, in addition, these muscles have also been shown to keep lower ATP and phosphocreatine levels (Baracos and Goldberg, 1986).

3.3.5. Increased tyrosine and prostaglandin E_2 (PGE_2) levels.

Increased tyrosine release from soleus muscles *ex vivo* (figures 3.5 and 3.6) and *in vitro* (figure 3.9) following treatment with p24 were found to be associated with elevated PGE_2 production. The PGE_2 content in gastrocnemius muscle was shown to be significantly increased after incubation with serum from cachectic mice bearing the MAC 16 tumour (Smith and Tisdale 1993b). Both indomethacin and the polyunsaturated fatty acid eicosapentaenoic acid (EPA) inhibited the rise in PGE_2 and tyrosine, suggesting a role for PGE_2 in the degradation of muscle protein in cachectic animals. In contrast, EPA had no effect on protein synthesis *in vivo* (Beck *et al*, 1991) and in a study carried out by Palmer and Wahle (1987) neither EPA nor docosahexaenoic acid (DHA) were shown to have any effect on protein synthesis in the skeletal muscles of fasted rabbits. In addition, $PGF_{2\alpha}$ levels were reduced, indicating some type of competition between the ω3 fatty acids and the PG synthesising enzymes of the '2 series'.

Elevated tyrosine and PGE_2 release in response to p24 were both inhibited in *ex vivo* experiments using muscles from mice which had been pretreated with the mAb and whose weight loss had been prevented by this treatment. *In vitro*, the inclusion of cyclooxygenase inhibitors such as ibuprofen, indomethacin, CV6504 and the $cPLA_2$

inhibitor $AACOCF_3$, also prevented these effects. In addition, muscles from mice which had previously been dosed with EPA, showed no elevation in either tyrosine or PGE_2 release (table 3.4). Altogether, the results suggest a role for PGE_2 in the degradation of skeletal muscle protein by p24.

The role of arachidonic acid and its metabolites in protein muscle metabolism has been studied extensively. The ability of human skeletal muscle to synthesise prostaglandins from an exogenous ^{14}C-labelled arachidonic acid (^{14}C-AA) was first reported by Berlin *et al* (1979). Rodemann and Goldberg (1982) showed a dramatic increase in protein breakdown in rat skeletal muscle following treatment with AA and that such response could be blocked by using cyclooxygenase inhibitors. In addition, AA caused a sharp rise in the production of PGE_2 and $PGF_{2\alpha}$, which was also inhibited by anti-inflammatory drugs. Of all the AA metabolites tested, which included several prostaglandins, thromboxanes and leukotrienes, only PGE_2 and $PGF_{2\alpha}$ showed an effect on protein degradation and synthesis respectively. These findings are in contrast with those found by Hasselgren *et al* (1990), who failed to show any effect of either AA or PGE_2 on the rate of total or myofibrillar protein breakdown. Furthermore, indomethacin -while inhibiting plasma PGE_2 levels *in vivo* and PGE_2 production in isolated muscle- had no effect on the proteolytic rates of either total or myofibrillar proteolysis.

The role of $PGF_{2\alpha}$ in promoting protein synthesis has also been reported by Reeds and Palmer (1983) who showed that the reduced rate of protein synthesis in muscles from fasted rabbits was also accompanied by a reduction in the levels of this prostaglandin. Addition of AA to the medium lead to an increase of both protein synthesis and $PGF_{2\alpha}$ production.

In the study carried out by Rodemann and Goldberg (1982), PGE_2 caused an increase in the rate of protein degradation for EDL, diaphragm and soleus between 20 and 40% (a 50% increase in protein synthesis was seen in soleus, although the net effect remained

catabolic). Furthermore, AA-stimulated proteolysis was inhibited by the lysosomal inhibitors, leupeptin and Ep-475, indicating that AA and PGE_2 stimulate muscle protein degradation by activating the lysosomal proteases.

In rats infected with *Streptococcus pneumoniae*, cathepsin B activity -which rose early in the course of infection- was prevented by administration of leupeptin. This lysosomal inhibitor also inhibited muscle atrophy and the reduction in tension associated with the disease, but it failed to prevent fever. Indomethacin was not only capable of preventing fever, but also the changes in muscle mass, force generating capacity and cathepsin B activity, suggesting that muscle wasting due to sepsis is at least in part due to the action of prostaglandins (Ruff and Secrist, 1984).

Several lines of evidence indicate that calcium is the trigger for the PGE_2-mediated lysosomal proteolysis. Not only isolated rat skeletal muscles responded to treatment with the calcium ionophores A23187 or ionomycin, but also removal of extracellular calcium reduced overall proteolysis as well as most of the activation by A23187 (Rodemann *et al*, 1982). Furthermore, an inhibitor of calpain activity completely inhibited the Ca^{2+}-activated protease without altering the ability of calcium to stimulate proteolysis. Additionally, leupeptin and Ep-475 blocked the stimulation of protein breakdown by calcium and even more, the increased production of PGE_2 and $PGF_{2\alpha}$ elicited by the muscle after treatment with A23187 was inhibited by indomethacin (Rodemann *et al*, 1982 and Baracos *et al*, 1986).

In addition to calcium, and maybe more importantly, the synthesis of PGE_2 is regulated first, by the release of AA from the plasma membrane phospholipids and secondly, by its conversion through the cyclooxygenase pathway. These two appear to be the rate-limiting steps of the reactions which are catalysed by phospholipase A_2 (PLA_2) and cyclooxygenase (COX), respectively. Although some arachidonate used for prostanoid formation may be derived from the sequential hydrolysis of phosphoinositides by phospholipase C (PLC)

and diacylglycerol (DAG) lipase (Neufeld and Majerus, 1983), the major sources, however, are probably the most abundant glycerophospholipids, phosphatidylcholine and phosphatidylethanolamine. PLA_2 mobilises AA from the sn-2 position of glycerophospholipids and is sensitive to inhibition by arachidonic acid (Lister *et al*, 1988). The enzyme exists in two forms, the low molecular weight (14 KDa) secretory PLA_2 ($sPLA_2$) and the high molecular weight (85 KDa) cytosolic PLA_2 ($cPLA_2$), and it is the latter that seems to be involved in inflammatory responses (Hulkower *et al*, 1992 and Angel *et al*, 1994). $cPLA_2$ has been shown to be more selective than $sPLA_2$ in releasing AA from mammalian membranes (Diez *et al*, 1994).

Following its release from membrane phospholipids, arachidonic acid is converted to the prostaglandin endoperoxide PGH_2. This step is mediated by PGG/H synthase, an integral membrane protein which exhibits two distinct catalytic activities, a COX involved in PGG_2 formation and a hydroperoxidase mediating a net two-electron reduction of the 15-hydroperoxy group of PGG_2 to yield PGH_2. Both active sites are positioned on opposite sides of the haem prosthetic group, which explains why the cyclooxygenase, but not the hydroxyperoxidase activity, is specifically inhibited by aspirin (irreversible inhibitor) (Smith and Lands, 1971) and related non-steroidal anti-inflammatory drugs (NSAIDs) (ibuprofen, indomethacin and others which are reversible inhibitors) (Rome and Lands, 1975). There are two pharmacologically distinct cyclooxygenases on the basis of their sensitivity to inhibition by NSAIDs (Kalgutkar *et al*, 1996), COX-1 which is constitutively expressed in most tissues and is unaffected by steroids and the more recently discovered COX-2 (Kujubu *et al*, 1991), which is inducible by a variety of stimuli in the context of inflammation and is inhibited by glucocorticoids (Masferrer *et al*, 1992).

The anti-inflammatory activity of glucocorticoids has been shown to be due to their ability to inhibit prostaglandin production and they do so by inducing the synthesis of a group of proteins called lipocortins. These lipocortins have been shown to inhibit PLA_2 activity in various cell types by blocking the enzyme calcium-binding sites (Hirata *et al*, 1980 and

Rothhut *et al*, 1983). The insulin-antagonising action of glucocorticoids has also been shown to result from their ability to interact with prostaglandin production (Southorn, 1989 and Southorn and Palmer, 1990) and indeed, stimulation of muscle protein synthesis by $PGF_{2\alpha}$ has been shown to be inhibited following treatment with dexamethasone (Reeds and Palmer, 1984).

Stimulation of PLA_2 by a receptor-coupled G protein has been proposed as the mechanism of AA release from various cell types stimulated in various ways. Recent evidence suggests that protein kinase C (PKC) might be the link which, by phosphorylating the enzyme and in the absence of extracellular calcium, would allow translocation of $cPLA_2$ to the membrane, therefore enabling the hydrolysis of AA (Lloret *et al*, 1995 and Buhl *et al*, 1995). These experiments showed that intracellular calcium concentrations (micromolar levels) were enough to activate $cPLA_2$ and induce AA mobilisation and these were in agreement with the range required for PKC activation.

Whether this is one of the pathways involved in the mechanism of action of p24 is not known at the moment, although the inhibition of increased p24-induced proteolysis and PGE_2 production *in vitro* by the PLA_2 inhibitor, arachidonyltrifluoromethyl ketone ($AACOCF_3$), supports this idea. PLA_2 activity and PGE_2 production in muscle have both been found to be elevated in patients with muscular dystrophy where massive degeneration and loss of muscle activity takes place (Lindahl *et al*, 1995). The signal transduction pathways associated with PGE_2 and proteolysis in response to p24 deserves further investigation. Also, additional work is required in order to elucidate whether the recently isolated p24 receptor (Todorov, unpublished results) is a G-protein-coupled receptor.

In addition to their role in muscle protein degradation, prostaglandins have been reported as potential markers for tumour progression. The mouse fibrosarcoma, HSDM, was shown to produce large amounts of PGE_2 in cell culture as well as in the host animal, where it also induced a hypercalcemic effect and both were eliminated by indomethacin (Tashjian *et al*,

1974). The progression of cachexia in rats bearing the Yoshida ascites hepatoma AH130 seemed to correlate with the increased production of PGE_2 both by muscle and tumour and administration of naproxene inhibited weight loss by 64%, protein loss by 40% and tumour mass by 20% (Strelkov et al, 1989). In humans, increased levels of PGE_2 have been found in body fluids and tumour tissues from patients with malignant tumours. However, the biological significance of these findings has not yet been clearly established since its production does not always correlate with malignancy and aggressiveness. For instance, PGE_2 seems to play an important role in the growth of head and neck carcinomas (Klapan *et al*, 1992), whereas controversy exists as to whether it is a determinant factor in breast cancer (Bennett *et al*, 1977, Bishop et al, 1980, Rolland *et al*, 1980, Hendrick *et al*, 1988, Karmali *et al*, 1983 and Malachi *et al*, 1981). *In vitro*, 13762 rat mammary carcinoma cells have been shown to produce high levels of PGE_2 (as well as leukotriene LTB_4), which were reduced by the addition of indomethacin which also had an antiproliferative effect (Tripathi *et al*, 1996).

3.3.6. Role of cytokines in muscle breakdown.

Despite extensive research in this field, the role of cytokines on protein turnover is not completely clear. Most of this work has involved the use of TNF-α for which a role *in vivo* has emerged. However, *in vitro* attempts to reproduce its effects *in vivo* have generally failed. *In vivo*, acute treatment with TNF-α decreased the rate of protein synthesis in rat soleus muscle (22%) and caused an enhanced proteolytic rate in soleus (34%) and EDL (26%) as measured by tyrosine release (Garcia-Martinez *et al*, 1993). Similarly, Charters and Grimble (1989) found protein synthesis to be inhibited in rat skeletal muscle after treatment with recombinant TNF-α. In contrast, Tayek (1996) has reported that the acute administration of a subcutaneous dose of TNF-α had no effect on protein synthesis despite the significant drop in the total skeletal amino acid concentration. Muscle proteolysis was also enhanced by TNF-α which was synergistically augmented by the addition of interleukin-1 (IL-1) and both treatments correlated with an increased urinary nitrogen excretion (Flores *et al*, 1989). Goodman (1991) also reported an increased protein

breakdown as well as 3-MH following *in vivo* administration of TNF-α. *In vitro*, TNF-α has failed to affect skeletal muscle protein synthesis (Moldawer *et al*, 1987 and Oliff *et al*, 1987), although a recent report indicates that it is capable of activating the expression of some components of the ubiquitin system (Llovera *et al*, 1997).

Leukocytic pyrogen or IL-1, is responsible for signalling the hypothalamus to induce fever as well as for promoting protein catabolism during sepsis in order to provide amino acids for gluconeogenesis and synthesis of acute-phase proteins and immunoglobulins. IL-1 purified from human monocytes was shown to stimulate muscle protein degradation by increasing the production of PGE_2 in muscle tissue (Baracos *et al*, 1983). These changes resembled those obtained after infection with *E. Coli* or endotoxin (Goldberg *et al*, 1984). IL-1-induced proteolysis and PGE_2 production were both inhibited by indomethacin and Ep-475 (Goldberg *et al*, 1984). Increased tyrosine release from soleus and EDL were also seen after partially purified human monocyte preparations from septic patients were incubated with skeletal muscle *in vitro* or when administered *in vivo*. However, murine and human IL 1-α, human IL 1-β and human TNF-α failed to reproduce such effect (Moldawer *et al*, 1987). It has also been shown that IL-6 is not capable of promoting muscle protein breakdown *in vitro* (Garcia-Martinez *et al*, 1994).

These observations reflect that cytokines play an important role in the control of muscle protein metabolism, although the discrepancies found between *in vivo* and *in vitro* experiments suggest that cytokines may act as mediators rather than being the direct cause for the increased protein breakdown seen in skeletal muscle under different disease states.

Chapter 4
Mechanisms of intracellular protein breakdown in skeletal muscle. Effect of p24 on the activation of the different proteolytic pathways.

Skeletal muscle contains several proteolytic systems. The most extensively studied is the lysosomal pathway, which involves the cysteine proteases cathepsins B, H and L, and the aspartic protease cathepsin D. Lysosomes constitute major sites for the degradation of soluble and extracellular proteins whereas evidence suggests that myofibrillar proteins are degraded by a non-lysosomal ATP-ubiquitin-dependent pathway, which is also involved in the selective breakdown of abnormal and short-lived proteins.

A third and less studied proteolytic system is the Ca^{2+}-dependent cysteine proteases calpain I and II which are thought to play a role in the degradation of cytoskeletal but not myofibrillar proteins, although a clear physiological role for these enzymes has not yet been established.

4.1. Introduction.

4.1.1. The lysosomal system.

4.1.1.1. Link between intracellular proteolysis and lysosomal alterations. Effect of nutritional and hormonal factors in the autophagic response elicited by lysosomes.

The role of lysosomes in protein turnover has been extensively studied by Mortimore and co-workers, who first reported in 1970 the inhibition of proteolysis by insulin on perfused livers from fasted rats. Proteolysis measurements were obtained from the turnover of free valine after single pulse additions of L-1-^{14}C-valine to the perfusate, and from the release of label from livers previously labelled *in vivo* and then perfused with 15 mM unlabelled valine to minimise reincorporation. The amount of valine released was determined by

means of a chromatographic method which gave an increased proteolysis rate in the perfused livers 15-30 minutes after the start of the perfusion over the unperfused livers.

Insulin was shown to inhibit this response both *in vivo* and *in vitro*. In a separate study, the administration of glucagon during perfusion was shown to exert the opposite effect as insulin, whilst the addition of a complete mixture of amino acids mimicked the action of insulin in that it virtually abolished increased proteolysis upon perfusion (Mortimore *et al*, 1973).

These results are in agreement with those reported by Mortimore *et al* (1977), who showed that deprivation of amino acids induced a striking increase in autophagy which could be detected as early as 5 minutes and reached maximal intensity by 20 minutes. The magnitude of this response suggested that it could account for the increase in protein degradation that occurs in the absence of amino acids.

In a later study, Schworer *et al* (1981) examined the effect of a wide range of plasma amino acid concentrations (0-10 times (10X)) on liver perfusions. Both proteolysis and vacuole formation exhibited the same responses over the whole amino acid range, 1X concentrations were sufficient to suppress both, and at 4X and above maximal suppression was achieved.

Osmotic sensitivity as measured by the increase in lysosomal enzyme markers (free acid phosphatase and N-acetyl-β-D-glucosaminidase) has been shown to be spontaneously increased during perfusion of livers from normal fed rats to a degree comparable to that observed after large doses of glucagon *in vivo* but, unlike the mechanism of action of this hormone, no changes in the tissue levels of cAMP were observed in these experiments (Neely *et al*, 1974). This alteration was also prevented and reversed by insulin and amino acid mixtures during perfusion, whose inhibitory effect appeared to be additive.

Further evidence supporting the role of lysosomes as the site of endogenous proteolysis was provided by Neely *et al* (1974) who found that, in prelabelled rat liver perfusates, most of the radioactivity was associated with the lysosomal fraction which also coincided with the lysosomal marker enzyme peak and was separate from the mitochondria. In addition, a pool of acid soluble radioactivity (TCA radioactivity) was found in close relationship with the lysosomal fraction and the peak was distinct from that of the radioactive protein pool. Moreover, experiments where unlabelled lysosomal fractions were combined with the supernatants from prelabelled livers, no TCA radioactivity was found to be associated with the lysosomal fraction after subsequent density gradient centrifugation, whereas the opposite mixture revealed a radioactive peak associated with this fraction, thus indicating that the labelled protein substrate was contained within the lysosomes prior to homogenisation.

As well as increased sensitivity to osmolarity, lysosomes have been shown to undergo changes in density and size. Thus, using a different approach (Neely *et al*, 1977), lysosomal density was determined by the distribution of lysosomal marker enzymes after centrifugation in linear sucrose gradients, which revealed lysosomes of control perfused livers to be associated with the higher density sucrose fractions . This heavier lysosomal fraction disappeared following the addition of insulin and amino acids, respectively, resulting in a single enzyme peak similar to that found in unperfused livers. Enlarged lysosomes from perfused livers were also detectable by electron microscopy. Ward *et al* (1977) found the presence of higher density lysosomal fractions upon perfusion which correlated with the lysosomal marker enzyme activity peak, both of which could be inhibited by including insulin and amino acids during the treatment. In perfused livers which had been previously labelled *in vivo* , the specific radioactivities of proteins in the dense lysosomal peak were 2-fold higher than those found for the mitochondrial and supernatant fractions. Although the respective additions of insulin and amino acids lowered the specific radioactivity associated with the heavier lysosomal fraction, the radioactivities measured after suppression were found to be higher than one would expect

from the labelling of endogenous lysosomal proteins, indicating that full suppression was not achieved despite the absence of any clearly detectable morphological changes. Increased lysosomal density has also been shown in liver perfusates in their autophagic response to glycogen (Schworer *et al*, 1979).

In contrast with the above studies, others have failed to report any uptake of proteins by lysosomes (Huisman *et al*, 1974).

Finally, inhibitor studies have shown that deprivation-enhanced proteolysis in perfused rat livers can also be inhibited by agents which specifically block lysosomal protein breakdown. This is the case of pepstatin, a specific inhibitor of lysosomal cathepsin D, which was shown to inhibit the degradation of intracellular proteins by 50% upon perfusion (Dean, 1975). Other agents that inhibit lysosomal activity, such as leupeptin (an inhibitor of cathepsin B) and antipain (an inhibitor of cathepsins A and B) have been reported to inhibit the degradation of normal proteins but not abnormal proteins (Knowles and Ballard, 1976). Chemical compounds such as ammonium chloride, methylamine and chloroquine have also been shown to inhibit the lysosomal pathway by raising the lysosomal pH (Seglen *et al*, 1979).

Extensive work on this field therefore provides enough evidence on the role of lysosomes on intracellular protein degradation and outlines the importance of the nutritional status on the activation and regulation of this proteolytic system.

Despite the widely accepted role of lysosomes in the degradation of cytoplasmic constituents and deprivation-induced proteolysis, the proposal of the lysosomal pathway as a general mechanism for the turnover of long-lived proteins is not so clear. Mortimore and Ward (1981) reported the use of a common intralysosomal proteolytic pathway for both basal and deprivation-induced proteolysis. However, the existence of an alternative pathway was suggested earlier by Knowles and Ballard (1976) who found that, in contrast

with the degradation of normal (arginine-containing) proteins, abnormal (canavanine-containing) proteins were not subject to regulation by insulin and lysosomal inhibitors.

4.1.1.2. Role of lysosomes in myofibrillar protein breakdown.

Although recent evidence points towards an active role of the ATP-ubiquitin-dependent system in the degradation of myofibrils, lysosomes - or at least their proteolytic enzymes- have been shown to be able to degrade these *in vitro*.

Cathepsins B and D purified from muscle degraded isolated myofibrillar proteins, as determined by SDS-PAGE, although insoluble native myosin was not degraded to the same extent as the soluble denatured form (Bird *et al*, 1980). The proteolytic activity displayed by both enzymes was inhibited by leupeptin and pepstatin, respectively.

In monkey skeletal muscle, a cathepsin B activity was purified which was capable of breaking skeletal and cardiac muscle myofibrils (Hirao *et al*, 1984), and cathepsin B purified from rat liver has been shown to degrade myosin, actin, tropomyosin and troponin T, but not actin (Noda *et al*, 1981).

Rabbit skeletal muscle cathepsins have been found to have different affinities for different myofibrillar proteins. Thus, cathepsin B is different from cathepsin L in that it does not degrade α-actinin, and from cathepsin D in that it degrades actin but not α-actinin or tropomyosin. They also differ in their optimum pHs for the hydrolysis of myosin, actin and troponin, which were around 3 for cathepsin D, and around 4 for cathepsin L, while it was around 5 for cathepsin B. The ability of cathepsin B to cause myofibrillar breakdown was greater than that of cathepsin D, but lower than that of cathepsin L (Matsukura *et al*, 1981; Matsumoto *et al*, 1983). In addition, cathepsin L caused a greater change in the ultrastructure of the Z- and M-lines than cathepsin B (Matsukura *et al*, 1984).

The kinetics of the degradation of myofibrils and isolated myofibrillar proteins by rabbit skeletal muscle cathepsin B has been investigated by Matsuishi *et al* (1992). In myofibrils, after 2 h of incubation at 37 ^{0}C, two degradation products (180 and 150 KDa) corresponding to the myosin heavy chain were identified by SDS-PAGE the intensity of which increased up to 8 h and decreased after 24 h incubation. Maximal activity was achieved at pH 5.1. Microscopy studies revealed considerable damage made to the Z-line, the H-zone and the I-band. Rabbit skeletal muscle cathepsin B also degraded isolated heavy myosin heavy chain, actin and troponin, but not α-actinin or tropomyosin.

However, despite the ability of purified lysosomal enzymes to break myofibrils *in vitro*, the results from these studies cannot be interpreted within a physiological context. Lowell *et al* (1986) studied the role of lysosomes in the degradation of skeletal muscle myofibrils by measuring the release of 3-methylhistidine (3-MH) from perfused muscles of starved and fed rats. After brief starvation, a considerable increase in myofibrillar proteolysis was observed with respect to total protein breakdown as measured from both tyrosine and 3-MH release. The addition of lysosomal inhibitors to the perfusion medium (ammonium chloride, chloroquine and leupeptin), only inhibited the release of tyrosine whilst that of 3-MH remained unaffected. These experiments provide good evidence about the existence of two independently regulated pathways that operate in the breakdown of myofibrillar and non-myofibrillar proteins in skeletal muscle.

Despite their independent regulation, evidence has been provided for the association of these two systems under certain conditions. For instance, the stress-induced accelerated degradation of cellular proteins in autophagic vacuoles has been shown to be dependent on functional E1, the ubiquitin activating enzyme. The degradation of protein substrates by the ubiquitin system involves a cascade of reactions which start with the activation of ubiquitin by this enzyme (this will analysed in section 4.1.2). Increased proteolysis rates have been reported for both short- and long-lived proteins (Ciechanover *et al*, 1984 and Gropper *et al*, 1991) which were inhibited in the E1 thermolabile mutant cells at restrictive

temperatures. Heat-induced degradation of long-lived proteins was inhibited completely by ammonium chloride and chloroquine and, furthermore, exposure of the cells to starvation had no effect after inactivation of E1. In contrast, under the same conditions the degradation rate in the wild-type cells increased four fold. These results suggest that heat-induced enhanced degradation of cellular proteins in autophagic vacuoles and the formation of these vacuoles are dependent on the activity of E1 (Gropper *et al*, 1991).

The lysosomal and ubiquitin proteolytic systems have also been shown to be simultaneously involved in the endocytosis of cell surface receptors. Ste2 is an α-factor pheromone receptor present on the surface of *Saccharomyces cerevisiae*, whose endocytosis and degradation by the lysosomal system appear to be dependent upon ubiquitination of one lysine residue within the internalisation signal, SINNDAKSS (Hochstrasser, 1996). Furthermore, this modification depends on phosphorylation of the flanking sequences. This phosphorylation-dependent ubiquitination has also been shown for other proteins such as IκBκ (Hochstrasser, 1996).

4.1.2. The ATP-ubiquitin-proteasome-dependent system.

4.1.2.1. Isolation and ATP-dependence.

This system was first identified by Ciechanover and colleagues in 1978 who reported a factor isolated from reticulocyte lysate which degraded denatured globin in the presence of ATP. The system was resolved into 2 components: fractions I and II, according to their order of elution from DEAE-cellulose. Fraction II was found to have a neutral protease activity which was stimulated only slightly by ATP, whilst fraction I had no proteolytic activity but was capable of restoring ATP-dependent proteolysis when combined with fraction II.

This "active factor" from fraction I was found to be remarkably stable at high temperatures as it retained its proteolytic activity even following heating at 96 ^{0}C for 60 min. However, the fact that it was non-dialysable, precipitated by ammonium sulphate and its activity could

be destroyed completely by treatment with proteolytic enzymes, indicated a polypeptide structure.

The same group (Hershko *et al*, 1980) found that this heat-stable polypeptide (APF-I) of the reticulocyte proteolytic system was capable of forming covalent modifications with proteins in an ATP-requiring reaction. APF-I formed multiple conjugates with lysozyme, globin and α-lactalbumin, respectively, as shown by comigration of label from each upon gel electrophoresis. Analysis of the ratio of APF-I to lysozyme radioactivities and of the molecular weights of the bands demonstrated that these corresponded to conjugates of APF-I with the substrate protein.

The results also suggested the possible involvement of an amidase as part of this system, as removal of ATP after formation of the ^{125}I-labelled APF-I caused regeneration of APF-I.

4.1.2.2. The ubiquitin cycle.

Ubiquitin has been identified as a 76-amino acid residue protein which is highly conserved in eukaryotes. Degradation of intracellular proteins via the ubiquitin system involves a multistep process in which ubiquitin is covalently linked to the protein substrate in an ATP-dependent reaction by means of an isopeptide bond between the carboxy-terminal group of ubiquitin and the ε–amino group of lysine side chains in the target protein. Following ubiquitin conjugation, the protein is selectively degraded by the 26S proteasome and ubiquitin is released for further use.

The ubiquitin-conjugating system is made up of 3 separate enzymatic components which come into play in an ordered and sequential manner. First, ubiquitin is adenylated by the ubiquitin-activating enzyme (E1) and then transferred to a thiol group for covalent linkage as an enzyme-ubiquitin thioester. This is followed by a trans-esterification to the ubiquitin-conjugating enzyme (E2) which can either transfer ubiquitin directly *in vitro* (Hilt and Wolf, 1992 and Hochstrasser, 1996) to a target protein or together with a companion

protein termed ubiquitin-ligase (E3), which appears to be the general rule *in vivo*. Following several rounds of this cycle, the multiubiquitinated protein is then degraded by the 26S proteasome and an isopeptidase, which releases intact ubiquitin from the proteolysed protein and closes the cycle (figure 4.1).

Figure 4.1. The ubiquitin cycle (from Argiles and Lopez-Soriano, 1996).

A structural motif (UBA domain) has recently been reported by Hofmann and Bucher (1996) which appears to be commonly shared by all known E2 proteins, several E3 proteins and most ubiquitin carboxy-terminal hydrolases (UBPs), suggesting a role for the UBA domain in binding to ubiquitin domains.

The ability of ubiquitin to activate a substrate protein appears to be independent of its interaction with the proteasome, since the use of synthetic peptides has no effect on the proteolytic activity of the proteasome. Therefore, it has been suggested that ubiquitin may act as a chaotropic polypeptide inducing the unfolding of proteins and exposing certain residues which act as recognition signals for the proteasome (Wenzel & Baumeister, 1993).

In fact, recent evidence has been provided for the assistance of the molecular chaperone Hsc 70 in the conjugation and subsequent degradation of certain protein substrates by the ubiquitin system (Bercovich *et al*, 1997). Hsc 70, whose activity was dependent upon K^+ concentration, appeared to be indispensable in the conjugation and degradation of actin, α-crystallin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-lactalbumin and histone 2A, although it did not substitute for E3. Additionally, immunodepletion of the protein from the reticulocyte lysate by using a monoclonal antibody, resulted in the inhibition of both conjugation and degradation. Furthermore, the chaperone was found to be necessary for the formation of high molecular mass ubiquitin conjugates, but not for the formation of low molecular adducts of Hsc 70-dependent proteins. Likewise, the formation of low molecular mass conjugates is also E3-independent and only requires E1 and E2.

It has been proposed that Hsc 70 acts by unfolding the substrate protein in order to expose the E3 recognition domain. Interestingly, all three Hsc 70-independent substrates (lysozyme, BSA and oxidised RNase A) are primary 'N-end rule' proteins that are recognised and targeted via direct binding of their free N-terminal and 'destabilising' amino

acid residue (the N-end rule mechanism will be further discussed in section 4.1.2.4), and whose recognition domain is possibly exposed and directly recognised by E3α.

4.1.2.3. The role of the α-amino group in multiubiquitination of substrate proteins.

The presence of a free exposed terminal amino group in the substrate protein appears to be an absolute requirement for proteolysis by the ubiquitin system.

Studies where selective chemical modifications were made to the α-NH_2-terminal group of globin and lysozyme showed that these prevented their conjugation to ubiquitin and subsequent degradation by the reticulocyte system (Hershko *et al*, 1984). Furthermore, addition of α-NH_2 groups to α-NH_2-blocked proteins through the introduction of polyalanine side chains increased their activity as substrates for degradation.

Conjugation by ubiquitin of ε-NH_2 groups of lysine residues was also inhibited in α-NH_2-blocked proteins and, on the other hand, when ε-NH_2 groups were blocked but the α-NH_2-terminal group was free, degradation could still take place but at a reduce rate. Chau *et al* (1988) also showed that for degradation to take place, multiubiquitination of the substrate protein was required since the use of mutant ubiquitin (Lys 48 to Arg substitution) prevented both the formation of ubiquitin-ubiquitin isopeptide bonds and subsequent degradation of the protein.

Inhibition of multiubiquitination of proteins has also been studied by Hershko and Heller (1985), who examined the effect of pluriubiquitination of lysozyme on the ability of the 26S proteasome to degrade ubiquitin conjugates. Elimination of multiubiquitination by means of chemical methods produced a 50% reduction in proteolysis.

Taken together, these data provide important information about how the ubiquitin proteolytic system works. First, they show that the formation of high molecular weight ubiquitin-protein conjugates (containing multiple molecules of ubiquitin) is prevented when

the α-NH_2 group is blocked. Second, that the conjugation of ubiquitin to the NH_2 terminus precedes ε-NH_2 conjugation in the formation of high molecular weight ubiquitin-protein conjugates and, third, that only high molecular weight ubiquitin-protein conjugates are susceptible to degradation by the 26S proteasome.

4.1.2.4. The N-end rule and the role of arginine-tRNA.

In ATP-ubiquitin-dependent proteolysis, ubiquitination of the substrate protein is decisive for subsequent breakdown. The enzyme involved in this step is ubiquitin protein ligase E3, which contains the protein-substrate binding site and has specificity for proteins with free α-amino groups. The nature of this free α-amino group is what determines the half-life of proteins.

Gonda *et al* (1989) proposed that a single-residue code, the so-called N-end rule, operates in an *in vitro* system derived from mammalian reticulocytes in a hierarchical manner. Amino-terminal Glu and Asp (and also Cys in reticulocytes) are secondary destabilizing residues in that they are destabilizing through their ability to be conjugated to primary destabilizing residues such as Arg (Ferber and Ciechanover, 1986 and Ciechanover *et al*, 1985). Amino-terminal Gln and Asn are tertiary destabilizing residues in that they are destabilizing through their ability to be converted, via selective deamidation, into secondary destabilizing residues Glu and Asp.

Indeed, proteins with acidic amino termini have been shown to require tRNA for their degradation through the ubiquitin system. The conjugation of Asn/Gln and Asp/Glu (and also of Cys in reticulocytes) to Arg is carried out by a specific class of enzymes, aminoacyl-tRNA-protein transferases.

Ferber & Ciechanover (1987) showed that ubiquitin-dependent degradation of bovine α-lactalbumin (bovine α-LA) was inhibited in the presence of ribonuclease (RNase) and that addition of tRNA to the system restored the proteolytic activity after inhibition of the

enzyme. Furthermore, degradation of human α-LA, which contains lysine at the amino terminus instead of glutamic acid, was found not to be affected by RNase treatment.

The same group reported the presence of this substrate-specific amino-terminal modifying activity in crude reticulocyte fraction II, where they showed that about 90% of the arginylation of the exogenous substrate occurred at the amino-terminal position.

Elias and Ciechanover (1990), by using a chemical cross-linking technique, have demonstrated that proteins with acidic amino terminal positions do not bind (E3) without prior modification of this residue by the addition of Arg, whilst proteins which contain a basic residue in this position are able to bind the ligase without any modifications. Neutralisation of the carboxyl group of N-terminal acidic groups generated substrates able to bind E3 without modification, suggesting the presence of a negatively charged binding site in E3.

4.1.2.5. Isopeptidases.

The importance of a free α-amino terminal group in the conjugation of ubiquitin to a substrate protein has been outlined above. However, high molecular weight ubiquitin-protein conjugates are still subject to regulation before the substrate protein is targeted to the 26S proteasome for degradation, and it appears that a group of enzymes called isopeptidases are responsible for the reversal of ubiquitination.

Jonnalagadda *et al* (1989) identified and partially purified 2 different classes of enzymes from reticulocytes by dye-ligand-sepharose chromatography, those that specifically split N^{ε}-ubiquitin-ubiquitin, ie. multiubiquitin chains (Ub-Ubase) or N^{ε}-ubiquitin-protein bonds (Ub-Xase).

Agell *et al* (1991) reported a 160 KDa isopeptidase from yeast that breaks the isopeptide bond from polyubiquitin peptides translated from mRNA, and Matsui *et al* (1982) isolated

a 38 KDa isopeptidase both from a hamster cell line (DON) and a mouse cell line (L929) that was capable of cleaving protein A24 into histone 2A and ubiquitin *in vitro* stoichiometrically as identified by 2-dimensional gel electrophoresis and ^{3}H-residue labelling. This enzyme had previously been reported by Andersen *et al* (1981) who found this enzyme to be present in rat liver nuclei and to be responsible for the disappearance of A24 during mitosis.

A different type of isopeptidase activity has recently been shown to be present in the 26S proteasome complex (Lam *et al*, 1997). This enzyme has been found to be tightly associated with PA700 (a 19S complex) and its function appears to be the removal of one ubiquitin at a time from the distal end of the targeted polypeptide chain. It is therefore suggested that, by processing only from the distal end of a polyubiquitin chain, this enzyme may edit all or most of the polyubiquitin degradation signal from a poorly ubiquitinated conjugate, thereby precluding its degradation. Thus, this isopeptidase could be responsible for directing protein degradation towards highly ubiquitinated substrates and therefore preventing the destruction of poorly ubiquitinated or erroneously 'tagged' proteins.

4.1.2.6. The 26S proteasome.

The 26S proteasome is a large proteolytic complex of about 1700 KDa. Electron microscopy images reveal a central proteolytic core, the 20S proteasome, and 2 additional substructures, the 19S cap complexes, which are attached at both ends of the 20S proteasome.

The 19S cap complexes, which have ATPase activity, are composed of at least 15 different subunits with molecular masses ranging from 25 KDa to 110 KDa that associate with the 20S proteasome in an ATP-dependent manner. They are thought to be responsible for the recognition of ubiquitinated proteins as well as for the unfolding and transport of substrate proteins to the proteolytically active 20S core.

The 20S proteasome has been found in the cytoplasm and nucleus of all eukaryotes investigated so far, and also in some Archaebacteria. The 20S proteasome of *Thermoplasma acidophilum* has been shown to have a great degree of similarity with its eukaryotic counterpart in that they share the same quaternary structure (Hilt & Wolf, 1996).

X-ray crystallography studies have revealed a large cylindrical particle (700 KDa) made of a stack of 4 rings each of which contain 7 subunits. There are 2 types of subunits, α and β, which form the 2 outer and the 2 inner rings, respectively, leaving a central cavity which is exclusively formed by β-subunits. The α-subunits in the outer rings, which are thought to be enzymatically inactive, leave a very narrow opening therefore providing restricted access to the central cavity and preventing the cleavage of native proteins. A combination of immunoelectron microscopy and chemical crosslink experiments have demonstrated an identical arrangement for the human 20S proteasome (Kopp *et al*, 1997).

Only denatured or partially unfolded proteins have access to the proteolytic core of the 20S proteasome, which appears to be a threonine protease where the enzymatic activity is provided by a catalytic tetrad consisting of the hydroxyl group of Thr1, ε-amino group end of Lys33, and the carboxyl group of Glu17, as well as the α-amino group of Thr. The nucleophilic nature of Thr1 is enhanced by the interaction with the basic amino group of Lys33, which is in turn oriented by the carboxyl group of Glu17. This resembles the classic catalytic triad of serine proteases (Thr is used instead of a Ser, Lys replaces a His, and a Glu substitutes for an Asp), where the amino group of Thr1 plays the role of proton acceptor (Lys is unlikely to be directly involved in proton shuttle), thus converting the classical triad into a tetrad (Wlodawer, 1995).

The central cavity of the 20S proteasome is believed to contain 14 independent active sites with the closest distance between them of about 28 Å, which corresponds to the length of a extended peptide chain of 7-8 amino acids. Therefore, providing that the lifetime of the acyl-intermediate is sufficiently long, the bound substrate might find another active site

before being released from the first one, leading to a second cut 7-8 amino acids from the first cut (Wlodawer, 1995).

4.1.2.7. Cellular functions of the 26S proteasome.

Beside degradation of multiubiquitinated proteins, the 26S proteasome is also involved in degradative processes which are ubiquitin-independent and ATP-independent.

Ornithine carboxylase (ODC), a key enzyme in polyamine biosynthesis, is the most rapidly turned over mammalian enzyme. Its degradation occurs through an ATP and antizyme-dependent pathway which is totally independent of ubiquitin (Murakami *et al*, 1992). It has been suggested that the presence of structural domains (including PEST sequences), might get exposed upon binding to the antizyme and that these regions might ultimately be recognised by the 26S proteasome. In a study by Rogers *et al* (1986), PEST regions (proline, glutamic acid, serine and threonine) were found in the amino acid sequences of ten proteins whose intracellular half-lives were less than 2 h. In 35 proteins with intracellular half-lives between 20 and 200 h, only three contained a PEST region. An inverse correlation between the presence of these regions and protein stability was found, suggesting that PEST sequences may result in the rapid degradation of the proteins containing them.

Another case of ubiquitin-independent proteolysis has been reported by Pacifici *et al* (1993). They induced partial unfolding of haemoglobin (Hb) by exposure of the protein to hydroxyl radicals (·OH). This oxidatively modified Hb exhibited increased proteolytic susceptibility during incubation with red blood cell lysates, cell-free extracts, fraction II, a 40-80% ammonium sulphate-precipitated fraction, and a purified proteasome preparation (670 KDa). Partial denaturation is thought to cause exposure of hydrophobic amino acid groups which may interact in an ATP-independent manner with the 26S proteasome. In addition, highly denatured and covalently linked Hb molecules (produced at high ·OH/Hb ratios), were poorly degraded at all stages of purification, possibly due to the larger size of

these cross-linked tetramers which are unable to fit the proteasome active sites. Therefore, it appears that the 26S proteasome may have a more general function by recognising degradation signals other than ubiquitination.

A role for the proteasome in the control of cellular events such as cell progression, cell cycle and transcription has also been proposed. Evidence has been provided for the ubiquitination and subsequent proteasome destruction of the tumour-suppressor protein p53, whose intracellular levels (as well as the presence of ubiquitin-p53 conjugates) were shown to rise upon inhibition of the proteasome (Huibregtse and Howley, 1996).

Proteasome-mediated degradation of certain transcription factors such as c-Fos, c-Jun, NFκB and IκB have been reported (Ishida *et al*, 1995 and Jariel-Encontre *et al*, 1995) and it also seems to be required for degrading cyclins in order for cells to exit from mitosis (Ganoth *et al*, 1995 and Seufert *et al*, 1995).

Finally, the proteasome has recently been implicated in the degradation of misfolded or unassembled proteins from the endoplasmic reticulum, whose degradation has been shown to be blocked by proteasome inhibitors (Werner *et al*, 1996 and Wiertz *et al*, 1996).

4.1.2.8. Role of the ATP-ubiquitin-proteasome system in myofibrillar protein breakdown.

The existence of at least two independent proteolytic pathways in muscle was suggested by Lowell *et al* (1986a) who, in a study using perfused muscle from starved rats, showed that the increased 3-MH levels associated with food deprivation were not regulated by agents that inhibit lysosomal proteolysis. Thus, whereas the addition of these inhibitors to the perfusion medium decreased tyrosine release by 25-35%, no effect was found on the release of 3-MH from myofibrillar proteins, indicating that the two pathways are regulated by different mechanisms.

This independent regulation for both types of proteins has also been studied by Goodman (1987), who found that treatment of muscles with agents that inhibit 3-MH release had no effect on the attenuation of total proteolysis. Others have proposed that the ubiquitin system is the main proteolytic system in muscle responsible for both the degradation of myofibrillar as well as soluble proteins (Solomon and Goldberg, 1996).

There are many reports in the literature that link the increased proteolysis rates seen under different muscle wasting conditions to the activation of the ATP-ubiquitin system (see section 4.1.4). However, only a few have related such a response to the specific breakdown of myofibrillar proteins. Tiao *et al* (1994) were able to show that, in response to sepsis, the breakdown of myofibrillar proteins was the major contributor to total proteolysis (myofibrillar protein breakdown increased 438%, whilst non-myofibrillar protein breakdown only increased 9%), and that this response was associated with the increased expression of the 2.4 Kb ubiquitin transcript. More recently, the same group (Tiao *et al*, 1997) have provided conclusive evidence for the link between increased myofibrillar protein breakdown, the energy-dependent pathway and the activation of the ubiquitin degradative system.

Further support for the hypothesis that the ATP-ubiquitin system may be involved in the degradation of myofibrillar proteins comes from experiments which have shown the presence of ubiquitin-protein conjugates to be confined to the Z-discs of myofibrils (Riley *et al*, 1988; Laub and Jennissen, 1991 and Hilenski *et al*, 1992). Furthermore, the use of proteasome inhibitors has been shown to inhibit the degradation of these proteins using *in vitro* preparations of atrophying rat skeletal muscles (Tawa *et al*,1997).

4.1.3. The calpain system.

The calpain system is composed of a group of Ca^{2+}-dependent proteases (calpains), which differ in their respective requirements for calcium (millimolar (m) and micromolar (μ) concentrations, respectively) and the endogenous inhibitor calpastatin. Despite the early

discovery of this system in relation to myofibrillar protein turnover (Reville *et al*, 1976) and its identification in all vertebrate cells examined so far, a clear physiological role for this ubiquitous system has not yet been established. In general, calpains catalyse the cleavage of native proteins into large polypeptide fragments, but no further proteolytic action has been ascribed. Calpains have been found to be involved in the activation of some enzymes such as protein kinase C (PKC) (Melloni *et al*, 1986), cleavage of hormone receptors and other membrane proteins, remodelling of the cytoskeleton (Glasser and Kosower, 1986) and muscle regeneration following various kinds of injury (Moraczewski *et al*, 1996).

4.1.3.1. Isolation and calcium requirement.

m-calpain was first reported by Busch *et al* (1972) and later on by Reville *et al* (1976) as a Ca^{2+}-activated Z-disk-removing activity (CAF) associated with crude muscle extracts of porcine muscle. This Z-disk-removing activity was purified by several chromatographic methods (Dayton *et al*, 1976a), all of which yielded a single peak, and at all stages of purification this activity coeluted together with Ca^{2+}-activated proteolytic activity (as measured against casein and myofibrils). Purified CAF migrated as a single band when it was subjected to polyacrylamide gel electrophoresis (PAGE), but SDS-PAGE identified 2 subunits of estimated 80 and 30 KDa, respectively, which were found to be present in equimolar ratios.

Purified CAF from porcine skeletal muscle was found to be optimally active on either myofibril or casein substrates at 1 mM Ca^{2+}. No CAF activity was detected in the presence of other divalent ions (Co^{2+}, Cu^{2+}, Ni^{2+} and Fe^{2+}) which, in the presence of 1 mM Ca^{2+}, had an inhibitory effect. Mn^{2+}, Mg^{2+}, Ba^{2+} and soybean trypsin inhibitor had no effect on CAF activity, whereas iodoacetate inhibited it irreversibly (Dayton *et al*, 1976b).

The 30 KDa subunit is identical in m- and μ-calpains and shows a high degree of homology with other mammalian calpains sequenced so far. It is encoded by a single gene in humans and its C-terminal contains a calmodulin-like domain.

The 80 KDa subunits are products of different genes which encode a protein with 4 different domains. Domain II contains the active site presumably formed by a Cys and a His (similar to the active site found in other proteases), and domain IV contains a calmodulin-like domain with four potential Ca^{2+}-binding sites (Ohno *et al*, 1990).

A third type of Ca^{2+}-dependent protease, a 94 KDa n-calpain, has only been found in skeletal muscle. It shows significant sequence homology with both human μ-type (54%) and m-type (51%) large subunits, and contains four domains similar to those in the 80 KDa subunits of m- and μ-calpains (Sorimachi *et al*, 1989). This enzyme is thought to be the same as the high m-calpain reported by Wolfe *et al* (1989), which shows a very high Ca^{2+}-requirement, suggesting the possibility that the high m-calpain is an extracellular 74-76 KDa protease secreted after cleavage of the 94 KDa precursor.

It remains unclear how many Ca^{2+} atoms calpains bind since its determination is impeded by the involvement of calcium in the autolysis of these enzymes. Despite the prediction of eight binding sites from their amino acid sequences, m-calpains have been shown to bind five or six (two on the small subunit and three or four on the large subunit), and μ-calpains four (two on the small subunit and two on the large) Ca^{2+} atoms per molecule, respectively (Minami *et al*, 1987; Zimmerman & Schlaepfer, 1988 and Coolican *et al*, 1986).

4.1.3.2. Calpastatin.

In addition to calcium, calpain activity is regulated by a natural inhibitor protein, calpastatin. Suzuki *et al* (1988) identified 2 types of inhibitors from mammalian sources: liver and erythrocyte types. Despite having different molecular weights (107-170 KDa and 68-70 KDa, respectively), they were found to share the same properties in that they were capable of inhibiting more than one molecule, indicating the presence of multidomains for inhibition. Thus, one mole of liver type inhibitor was able to inhibit 4-10 moles of Ca^{2+}-activated neutral protease (CANP), whereas the erythrocyte type inhibited 3-5 moles of CANP.

Calpastatin activity has also been purified from rat skeletal muscle (Pontremoli *et al*, 1991) by means of ion exchange chromatography. Two peaks were obtained which yielded two forms of the enzyme with very similar molecular masses (105 KDa), but with different inhibitory efficiencies with respect to calpains. Thus, the predominant calpastatin form (calpastatin I) showed maximum efficiency for μ-calpain, whilst the less abundant calpastatin form (calpastatin II) was more active against m-calpain.

In mouse skeletal muscle, calpastatin was identified as a 70 KDa band and, using combined light microscope and immunochemical techniques, it was shown to be localised under the sarcolemma, corresponding to surface cytoplasmatic sites where dramatic changes of calcium concentrations occur during muscle activity. Another important site was the nucleus, probably in order to protect some oncogenes or kinases from calpain activity (Fumagalli *et al*, 1996).

4.1.3.3. Role of calpains in myofibrillar protein breakdown.

Dayton *et al* (1976b) reported the ability of purified CAF to remove the Z-disks, the 400 Å periodicity associated with troponin in the I band and to degrade M lines, but it did not cause any ultrastructurally detectable effects when incubated with myofibrils.

Goll *et al* (1991) have shown that both m- and μ-calpain are capable of completely removing Z-disks in skeletal muscle by releasing α-actinin from intact myofibrils. They do not seem, however, to be involved in the actual degradation of this myofibrillar protein since released α-actinin was able to bind pure F-actin, in the same way that untreated native α-actinin does. Furthermore, calpains did not degrade purified α-actinin as indicated by SDS-PAGE and N- and C-terminal analysis of calpain-treated and untreated α-actinin and actin.

These findings are in agreement with the notion that calpains are not directly involved in the degradation of myofibrillar proteins but, by cleaving at specific sites, they may enable other

proteases to initiate degradation of the actomyosin molecules. Solomon and Goldberg (1996), compared the rates of degradation of myofibrillar proteins both free and in the form of multicomponent complexes by the ATP-ubiquitin-proteasome system. The study showed that the purified components were more easily ubiquitinated and degraded by the proteasome than the intact myofibrils, indicating that specific interactions between myofibrillar proteins might protect them from ubiquitin-dependent degradation, with the dissociation from the contractile filaments probably being the rate-limiting step in their degradation.

4.1.3.4. Regulation of calpain activity.

It has been proposed that m- and μ-calpains are present as inactive proenzymes or zymogens in the absence of calcium and that they become autocatalytically activated at the cell membrane in its presence (Suzuki *et al* 1988).

CANP is thought to be inactive unless calcium is increased to micromolar levels in a process called autolysis, which greatly increases calcium sensitivity of both m- and μ-calpains by modifying the N-terminal regions of both subunits. Pontremoli *et al* (1990) purified a protein activator of estimated molecular mass 40-45 KDa from rat skeletal muscle, which was shown to activate m-calpain by reducing its calcium requirement by 50 fold. This activator, which was specific for m-calpain, was also capable of modulating calpastatin activity by suppressing its inhibitory activity on the proteinase. Michetti *et al* (1991) showed that the mechanism of activation of the enzyme involved the formation of an equimolar complex which resulted in the conversion of the native 80 KDa catalytic subunit of calpain into the autolysed 75 KDa form. The activator itself was found to be resistant to digestion by m-calpain, whilst it increased the degradation rate of the inhibitor calpastatin by the proteinase.

Although this type of modification appears not to have an effect on the activity and calcium sensitivity of the small subunit, its N-terminal region (in particular domain V which is rich

in Gly residues) seems indispensable for the interaction with phospholipid. Phosphatidylinositol has been found to be the most effective phospholipid to lower the calcium concentration, [Ca^{2+}], required for this process (Suzuki *et al*, 1988 and Cong *et al*, 1989), and so it allows autolysis to take place at physiological concentrations. Because the calcium concentration required to initiate autolysis is very similar to that needed for proteolytic activity (Cong *et al*, 1989), precautions must be taken when interpreting the results since the concentrations chosen to avoid autolysis are usually lower than those required for maximal calpain activity.

Other work disagrees with the role of membrane phospholipids in the regulation of calpain activity. Immunolocalisation studies have failed to show preferential binding of calpains and calpastatin to the plasma membrane (Kumamoto *et al*, 1992; Yoshimura *et al*, 1986 and Kitahara *et al*, 1986). Furthermore, calpains have been shown to be associated with proteins and not phospholipids at the plasma membrane (Inomata *et al*, 1989).

An alternative mechanism for the regulation of calpain activity has been suggested by Goll *et al* (1992), which involves the interaction of calpain with Ca^{2+} and calpastatin, and is based on the order of its increasing [Ca^{2+}] requirement. First, translocation of μ-calpain to subcellular organelles takes place (0.5-2 μM Ca^{2+}) and a subsequent increase in intracellular [Ca^{2+}], which induces proteolytic activity, would result in conformational changes that leave the active site open. If calpastatin is already bound to calpain when Ca^{2+} binds, opening of the active site will result in its inactivation, but in the absence of calpastatin, further binding of Ca^{2+} to calpain initiates autolysis. If [Ca^{2+}] is maintained, calpain will continue to autolyze in the absence of calpastatin until its activity is destroyed.

However, neither of the two models offers a solution to the problem that a non-physiological [Ca^{2+}] is required *in vitro* for this process to take place, where only the first step is likely to occur at physiological concentrations. It has been proposed (Goll *et al* 1992) that the presence of an unidentified activator (a kinase or phosphatase) in the cells

could increase the affinity of the Ca^{2+}-binding sites which would make calpains responsive to fluctuations in $[Ca^{2+}]$ in the physiological range.

Pontremoli *et al* (1992) have reported that the two calpastatin species isolated from rat skeletal muscle correspond to the phosphorylated and the dephosphorylated states of a single calpastatin isozyme form. The experiments suggest the implication of a kinase/phosphatase system where phosphorylation of calpastatin I leads to a decrease in the inhibitory efficiency versus μ-calpain and in an increased inhibition of m-calpain, which can be restored following dephosphorylation of calpastatin II by alkaline phosphatase.

In addition, recent evidence suggests the possible involvement of μ-calpain in the activation of m-calpain (a 'calpain cascade') which would overcome the much higher Ca^{2+} requirements for this enzyme (Tompa *et al*, 1996)

4.1.4. Activation of the different proteolytic pathways under various muscle wasting conditions.

4.1.4.1. Cachexia.

The fast-growing AH-130 Yoshida ascites hepatoma (YAH) has been shown to cause a pronounced decrease in body and muscle weights in recipient rats as early as 4-7 days after transplantation. This weight loss imposed by the tumour, appears to be the consequence of enhanced protein breakdown, which occurs through the activation of the ubiquitin pathway (Llovera *et al*, 1994). No change was observed in the activities of lysosomal cathepsins (apart from a transient effect on cathepsin L after 4 days of transplantation which was absent at day 7), in fact, they were decreased. In contrast, both ubiquitin mRNA expression (2.4 and 1.2 Kb transcripts) and free and conjugated ubiquitin were markedly increased in gastrocnemius muscle of tumour-bearing rats. In a separate study, clenbuterol was shown to effectively prevent the enhanced proteolysis associated with this model, by blocking the activation of the ubiquitin proteolytic pathway (Costelli *et al*, 1995).

Llovera *et al* (1995) also investigated the contribution of the other proteolytic systems to overall protein breakdown using *in vitro* preparations of skeletal muscle from AH-130 Yoshida rats. They found no difference in the lysosomal and calcium components between control and tumour-bearing animals, whereas the presence of ubiquitin conjugates was significantly elevated in the latter group.

Baracos *et al* (1995) have reported a small contribution of the lysosomal pathway in the same model. However, this does not seem enough to account for the enhanced proteolysis associated with skeletal muscle of YAH rats. Indeed, inhibition of this system and the calpains had no effect on proteolysis, whilst inhibition of ATP production abolished it. Accordingly, increased mRNA expression was found for ubiquitin (2.5 Kb and 1.2 Kb), and proteasome subunits C2 (1.3 Kb), C3 (1.0 Kb), C8 (1.1 Kb) and C9 (1.34 Kb).

Temparis *et al* (1994) also reported that in the atrophying muscles of Yoshida sarcoma-bearing rats, increased proteolysis could not be prevented when muscles were incubated under lysosomal and calcium inhibitory conditions. Furthermore, the mRNA expression for the lysosomal enzymes was no different from control muscles. Interestingly, however, there was increased expression of m-calpain mRNA despite its lacking contribution to total protein breakdown. The inclusion of ATP-depleting agents in the incubation medium, in contrast, caused a significant reduction in proteolysis which was accompanied by increased mRNA levels for ubiquitin (2.6 and 1.2 Kb), E2 (1.8 and 1.2 Kb) and the two proteasome proteolytic core subunits C8 (1.1 Kb) and C9 (1.3 Kb).

4.1.4.2. Sepsis and cytokines.

In a septic rat model induced by caecal ligation and puncture, a catabolic state occurred through a reduction in the protein synthesis rate (25%) and an increase in the total (50%) and myofibrillar protein (440%) degradation rates in EDL. These changes in both synthesis and degradation rates led to a pronounced muscle wasting state which was accounted for by the activation of the ubiquitin proteolytic system as determined by

inhibitor studies and Northern and Western blot analysis, respectively (Tiao *et al*, 1994). Using the same model, Garcia-Martinez *et al* (1995) reported similar results in the mRNA expression for ubiquitin in gastrocnemius muscle. They also studied the effects of *in vivo* administration of tumour necrosis alpha (TNF-α) and interleukin-1 (IL-1), respectively, since there is evidence that endotoxins might exert their effects through the action of these cytokines. TNF-α treatment was found to increase the expression of both of the ubiquitin transcripts (2.4 and 1.2 Kb), whereas IL-1 did not. TNF-α has been associated with an increased muscle proteolysis both *in vivo* and, more recently, it has been shown to be capable of directly activating the ubiquitin proteolytic system using *in vitro* preparations of rat soleus muscle (Llovera *et al*, 1997).

In a septic model induced by intravenous injection of *E. Coli* (Voisin *et al*, 1996), three different phases were observed in the rats. Muscle wasting occurred during the acute and chronic phases, whereas in the late septic phase, muscle mass stabilisation was reached. Muscle catabolism in the acute phase was due to a decrease in the rate of protein synthesis, whereas in the chronic phase an additional enhanced proteolysis was observed whose subsequent suppression characterised the late septic phase.

mRNA expression of the different proteolytic enzymes was in accordance with the stage of the disease. Thus, during the periods of significant muscle wasting, increased mRNA expression was found for cathepsin B, m-calpain, ubiquitin, C8 and C9 (interestingly, E2 remained unchanged). On the other hand, no difference was observed between the gene expression of muscles from late septic rats and those from pair-fed controls. Despite the contribution from the lysosomal and calcium dependent pathways to the increased rate of proteolysis seen in acute and chronic septic phases, their inhibition did not suppress it whereas ATP depletion did, indicating that the ATP-dependent pathway was mainly responsible for such an increase.

Finally, a 3-4 fold increase in the expression of the ubiquitin gene and one of the 20S proteasome subunits (HC3) have been found in human septic muscle, concomitant with increased tissue levels of phenylalanine and 3-methylhistidine (Tiao *et al*, 1997).

4.1.4.3. Starvation and denervation atrophy.

Rats deprived of food for 24-48 h exhibited increased mRNA expression for ubiquitin and the proteasome subunits C2, C3, C5, C8 and C9 in soleus and EDL (despite net loss of total muscle RNA), which returned to normal levels 24 h later upon refeeding. No changes in the expression of other proteolytic enzymes were found. Enhanced proteolysis, which rose and fell in response to these changes, was attributed to skeletal muscle since other organs (apart from heart) were not affected (Medina *et al*, 1995). The absence of apparent changes in any of the components of the ubiquitin system in liver, in which overall proteolysis also rises upon starvation, agrees with previous findings supporting a major role for the lysosomal apparatus in the control of protein degradation (Wing *et al*, 1991). In addition, the increase in ubiquitin conjugates was concomitant with the increase in both mRNA expression and proteolysis (Wing *et al*, 1995).

Identical results regarding the activation of the ubiquitin-proteasome-dependent pathway were obtained for denervated soleus muscle (Medina *et al*, 1995 and Wing *et al*, 1995).

4.1.4.4. Glucocorticoids in fasting, ageing and chronic renal failure.

Breakdown of muscle tissue is one of the earlier adaptations to fasting where glucocorticoids are essential in the activation of proteolysis. The degradative systems on which glucocorticoids exert their effects have been investigated by Wing and Goldberg (1993) who found increased proteolysis in EDL muscles from fasted rats due to the activation of the lysosomal and ATP-ubiquitin-dependent systems after one day of food deprivation. No changes in proteolysis were observed in the fasted adrenalectomized rats which, after administration of dexamethasone (DEX), exhibited the same response as the fasted controls. Glucocorticoids appear to be responsible for the activation of the ATP-

ubiquitin-dependent pathway (ubiquitin mRNAs are up-regulated) and subsequent myofibrillar breakdown, whilst insulin deprivation leads to the enhanced lysosomal activity. Interestingly, insulin has been shown to suppress the increase in mRNA expression of E2 in L6 myotubes (Wing and Banville, 1994 and Wing and Bedard, 1996), although it did not show any effect on the expression of ubiquitin or any of the proteasome subunits (Wing and Bedard, 1996).

In insulinopenic rats, activation of the ubiquitin-proteasome pathway was concomitant with increases in muscle protein degradation which was inhibited by a proteasome inhibitor and by blocking the synthesis of ATP, but not by agents that suppress lysosomal or calcium-activated proteases (Price *et al*, 1996).

The calpain system has also been shown to be upregulated in skeletal muscle of fasted rabbits (Ilian and Forsberg, 1992). There was a 3-4 fold increase for m-, μ-calpain and calpastatin mRNAs and, in addition, the expression of cathepsin D and the proteasome subunit C2 were also elevated.

Many disease states result in hypersecretion of glucocorticoids in ageing, which finally leads to muscle wasting. Dardevet *el al* (1995) found that adult and old rats responded in different ways in response to DEX treatment. Muscle wasting occurred more rapidly and the recovery of muscle mass was more delayed in old rats than in adults. In adult rats, muscle protein catabolism occurred through an increase in the rate of protein breakdown as a consequence of the activation of the ubiquitin-proteasome proteolytic pathway (increases in ubiquitin, E2, C2 and C9 transcripts were reported). However, a lack of activation of this system was found in old rats (only ubiquitin expression was elevated) where wasting of muscle tissue was due to a depression in the rate of protein synthesis. mRNA levels for cathepsin D and m-calpain were found to be high in both adult and old rats.

Chronic renal failure (CRF) is another disease associated with negative nitrogen balance and loss of muscle tissue, where glucocorticoids have been implicated as the mediators in the proteolytic response to acidification. In a study carried out by Isozaki *et al* (1996), acidification of the medium or addition of DEX separately, did not increase proteolysis in BC_3H1 myocytes grown in 1% medium (ie, under conditions which reduce the influence of other glucocorticoids as well as other hormones and cytokines). Acidification of the medium, however, caused an increase in mRNAs for ubiquitin and the C2 proteasome subunit which was significantly enhanced by the addition of DEX. The use of the steroid-receptor antagonist RU 486 resulted in the blockage of the response to DEX, but the mRNA levels for these genes remained elevated indicating that although acidosis can itself have a direct effect on the activation of the ubiquitin-proteasome pathway, glucocorticoids are required for increased proteolysis.

Similar results have been obtained by Bailey *et al* (1996) who reported increased mRNA levels for ubiquitin, C3 and C9 in epitrochlearis muscle from acidotic rats. They showed that the proteasome inhibitor MG 132 was capable of eliminating this response and, in addition, neither inhibitors of the lysosomal nor those of the calpain system stopped CRF-induced proteolysis whereas ATP-depletion did.

4.1.4.5. Other conditions.

A co-ordinated activation of the lysosomal, calcium- and ATP-ubiquitin-dependent proteinases has also been found for unweighted soleus muscle (Taillandier *et al*, 1996). Indeed, hindlimb suspension led to muscle atrophy and loss of protein, mainly due to an elevation in protein breakdown to which the ubiquitin system was the main contributor (the other two pathways only accounted for 18% of total proteolysis) and is therefore thought to be responsible for the wasting seen in unweighted rats.

Changes in ubiquitin levels have also been found in human biceps muscle following high force eccentric exercise. Both free and conjugated ubiquitin levels were elevated in

response to exercise-induced muscle damage as determined by SDS-PAGE (Thompson and Scordilis, 1994).

Patients with severe head trauma injuries who exhibited negative nitrogen balance and increased whole body and myofibrillar protein breakdown were used in a study performed by Mansoor *et al* (1996) who showed that muscle wasting resulted from the simultaneous stimulation of the ATP-ubiquitin-dependent proteolytic pathway with either the lysosomal or the calcium system or both. The high circulating levels of cortisol and/or IL-1β and/or interleukin-6 (IL-6) could have accounted for the observed changes.

Finally, Duchenne-muscular dystrophy (DMD) and acute quadriplegic myopathy (AQM) are the only muscle wasting conditions reported so far where, in contrast with the studies mentioned above, the calpain system seems to play a major role. DMD in humans and in *mdx* mice results from a defect in the dystrophin gene. It has been suggested that the lack of this protein causes sarcolemmal defects that influence leak channels, leading to increased protein degradation and a decrease in maximal active tension (Dupont-Versteegden and McCarter, 1992). Indeed, dystrophic muscles from *mdx* mice have been shown to have elevated intracellular calcium concentrations ($[Ca^{2+}]_i$) and higher rates of protein degradation both at rest and during stimulation (Turner *et al*, 1980). Furthermore, the mRNA expression of m-calpain, but not that of the components of the ubiquitin-proteasome pathway, has been reported to be elevated in dystrophic muscles (Combaret *et al*, 1996).

Calpain activity has been found to be higher in muscles from AQM patients and it appears to be responsible for the degradation of muscle proteins, namely myosin and actin, which finally leads to loss of muscle fibres and profound muscle weakness. Accordingly, calpain mRNA expression was found to be high whilst that of ubiquitin was only slightly increased in a few atrophic fibres (Schowalter and Engel, 1997).

4.1.5. Aims of the study.

The following experiments will investigate the effect of p24 and the MAC 16 tumour on the activation of the different proteolytic pathways in skeletal muscle. The gene expression of some of the components of the ubiquitin-proteasome degradative system will be analysed as well as the presence of ubiquitin conjugates.

4.2. Results.

Administration of p24 elicited the activation of an ATP-dependent proteolytic pathway in skeletal muscle of NMRI females 24 h after the first injection (weight loss 1.4 g). ATP depletion caused a significant reduction in the degradation of total muscle protein, whilst inhibitors which blocked the lysosomal or calcium-dependent pathways had no effect on the release of tyrosine from soleus muscles (figure 4.2). This inhibition of proteolysis was consistent with the lower ATP content of those muscles which were incubated under depleting conditions in contrast with control muscles which were incubated in ATP-containing medium (table 4.1).

A co-ordinated activation of the lysosomal and ATP proteolytic pathways was responsible for the increased protein breakdown associated with skeletal muscle of cachectic animals bearing the MAC 16 tumour (weight loss 5 g 17 days after tumour transplantation) (figure 4.3). Total protein breakdown was inhibited when soleus muscles were incubated with the inhibitors of the lysosomal or ATP-dependent pathways, suggesting the ability of the tumour to activate the degradation of myofibrillar as well as non-myofibrillar proteins in the process of wasting.

Further investigation into the mechanism of action of p24 revealed that, 24 h after the first injection, this material was capable of significantly inducing the mRNA expression of ubiquitin (Ub), E2 and C9 in gastrocnemius muscle. The 1.2 and 1.8 Kb transcripts of E2 (figure 4.4) and the 1.2 and 2.4 Kb transcripts of Ub (figure 4.5) were significantly increased by 81%, 31%, 67% and 61%, respectively, as well as that of the 1.3 Kb transcript of the C9 subunit (121%), which was determined in a separate experiment (figure 4.6). The expression of the 1.2 Kb transcript of E2 was also significantly increased in heart tissue (12%) (figure 4.7).

The MAC 16 and MAC 13 tumours caused marked increases in the expression of the 1.2 Kb transcript of E2 and C9 (figures 4.8 and 4.9), although these were more pronounced for the MAC 16 tumour (120 vs 22.5% and 203 vs 58%, respectively).

In accordance with the increased expression of some of the components of the ubiquitin-proteasome system, the amount of ubiquitin-protein conjugates was also significantly increased both in gastrocnemius muscles from p24-treated and mice bearing the MAC 16 tumour (14% and 42%, respectively) (figure 4.10).

Inhibition of the p24-induced proteolysis by inhibitors of the three main proteolytic systems

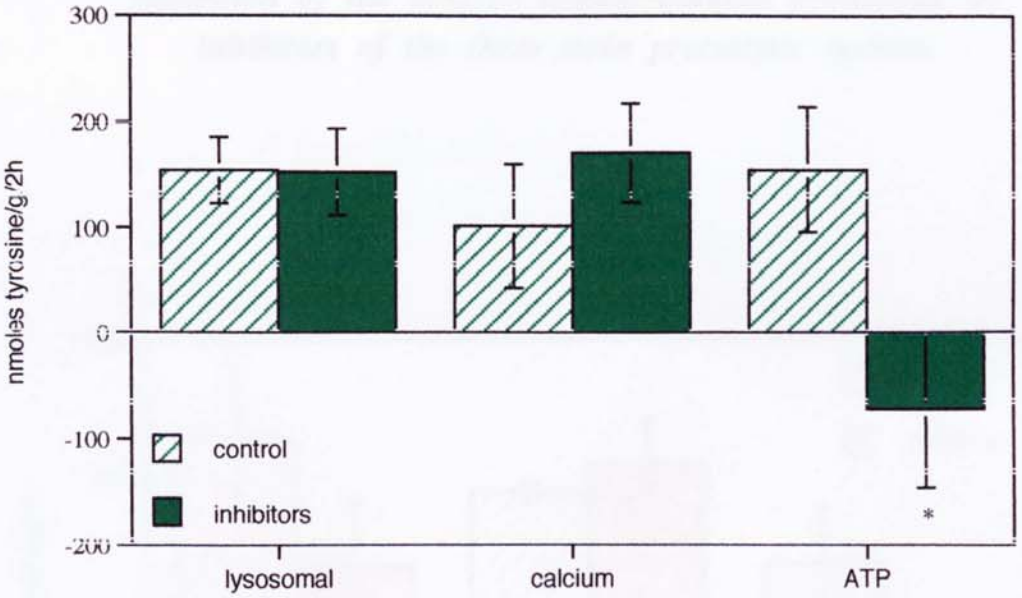

Figure 4.2. Inhibition of total protein breakdown as measured by tyrosine release after a 2 h incubation. Muscles previously pre-incubated for 45 min (lysosomal- and calcium-dependent pathways) and 1 h (ATP-dependent pathway). Media replaced thereafter and all muscles incubated in Krebs buffer supplemented with glucose (except for the ATP pathway) and cycloheximide. Inhibitors present throughout. Results are expressed as mean ± sem, where n = 4 muscles. Statistical analyses were performed by Student's unpaired t-test, where (*) $P < 0.05$.

ATP content in energy-depleted and control soleus muscles

Treatment	arbitrary light units
+ATP	0.023 ± 0.009
-ATP	0.006 ± 0.001

Table 4.1. Reduction in the ATP content of muscles after energy depletion. Results expressed as mean ± sem, where n= 4 muscles.

Inhibition of the MAC16 tumour-induced proteolysis by inhibitors of the three main proteolytic systems

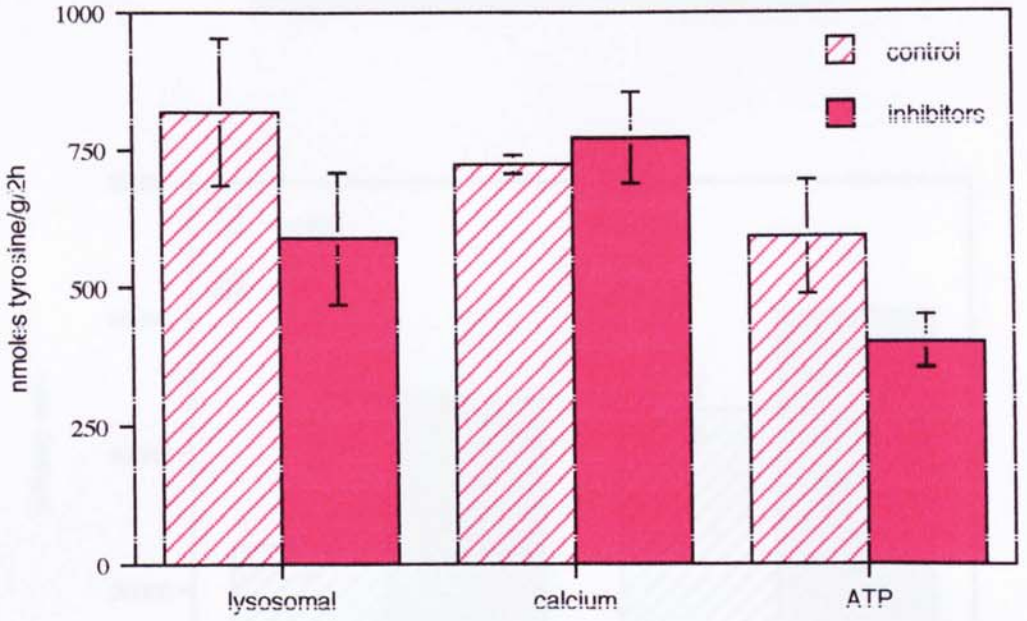

Figure 4.3. Inhibition of total protein breakdown as measured by tyrosine release after a 2 h incubation. All muscles were incubated in Krebs buffer supplemented with glucose (except for the ATP pathway) and cycloheximide. Inhibitors present throughout. Results are expressed as mean ± sem, where n = 4 muscles. Statistical analyses were performed by Student's unpaired t-test.

E2 expression in gastrocnemius muscle following treatment with p24 in vivo

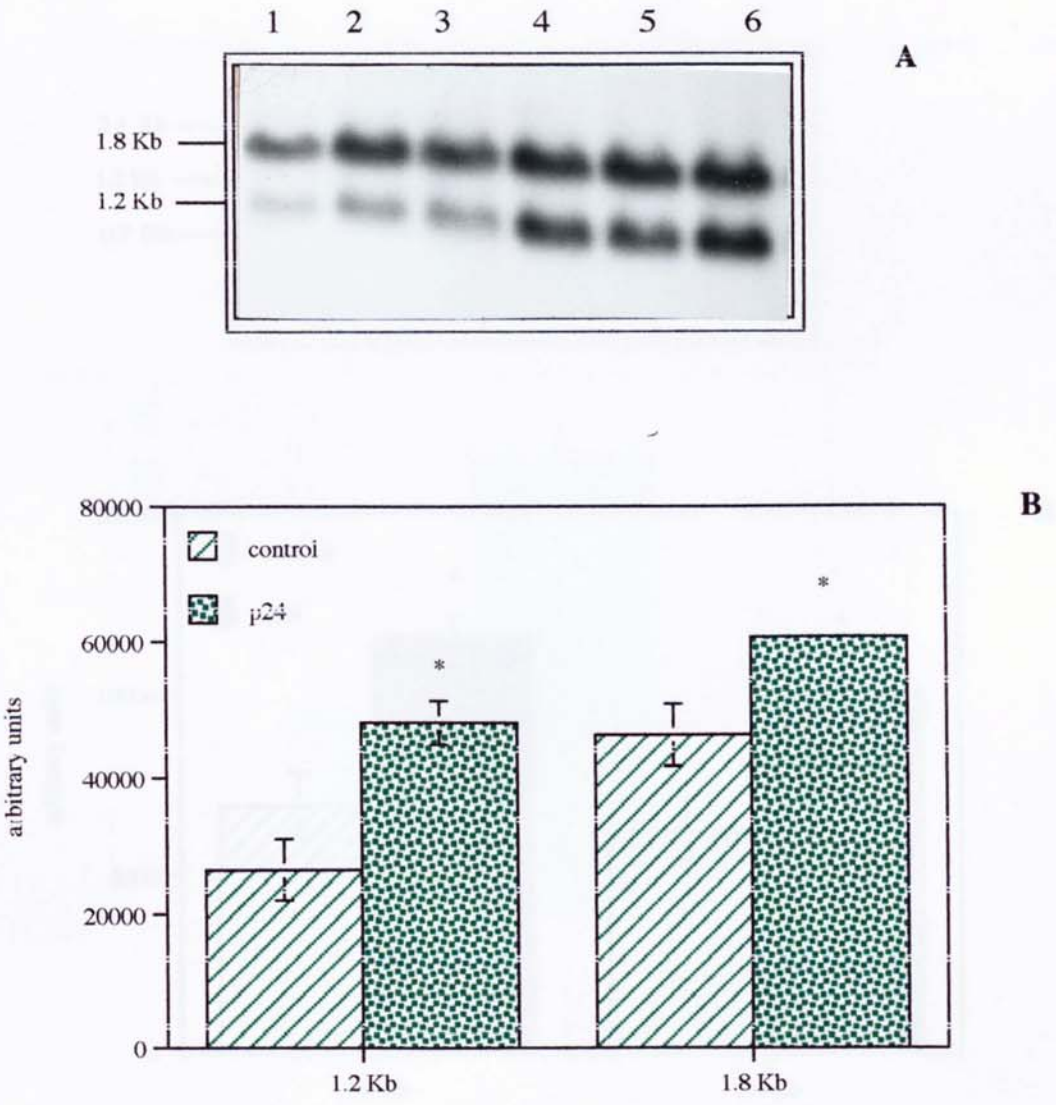

Figure 4.4. (A) Effect of p24 on the expression of E2 mRNA 24 h after the first injection. Lanes 1, 2 and 3 are control muscles and lanes 4, 5 and 6 are p24-treated muscles. (B) Results are expressed as mean ± sem and statistical analyses were performed using the unpaired Student's t-test, where n = 3 muscles and (*) $P < 0.05$.

Ubiquitin expression in gastrocnemius muscle following treatment with p24 in vivo

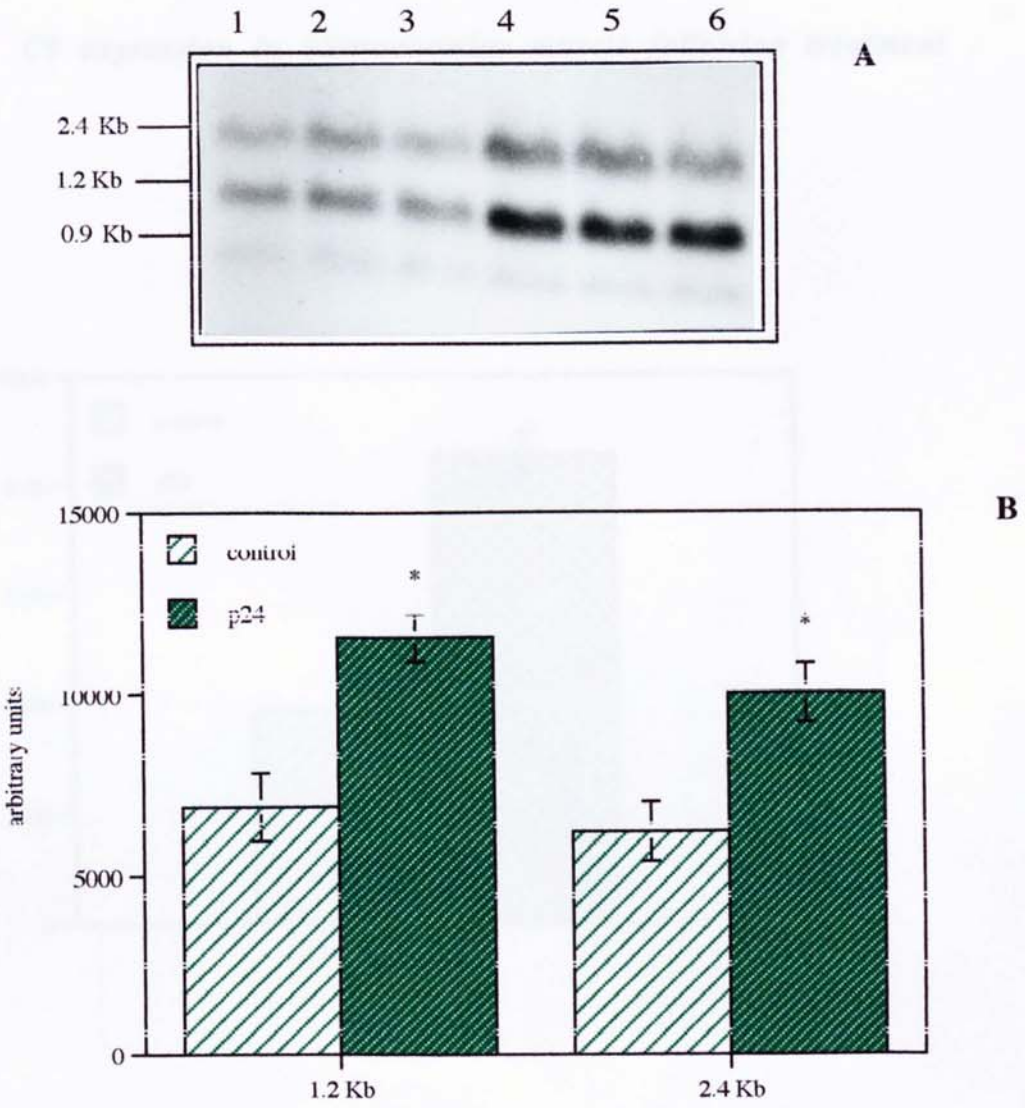

Figure 4.5. (A) Effect of p24 on the expression of Ub mRNA 24 h after the first injection. Lanes 1, 2 and 3 are control muscles and lanes 4, 5 and 6 are p24-treated muscles. (B) Results are expressed as mean ± sem and statistical analyses were performed using the unpaired Student's t-test, where n = 3 muscles and (*) $P < 0.05$.

C9 expression in gastrocnemius muscle following treatment with p24 in vivo

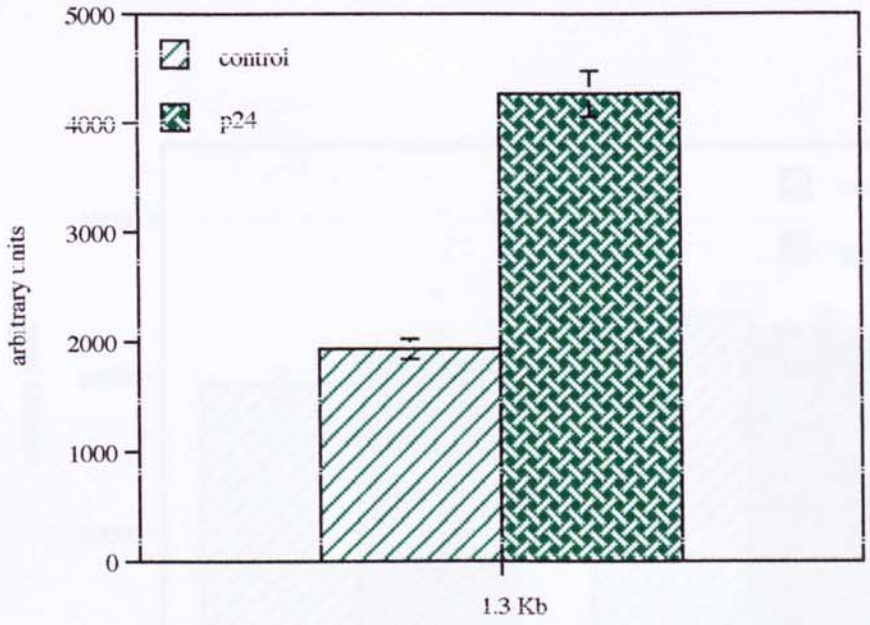

Figure 4.6. Effect of p24 on the expression of C9 mRNA 24 h after the first injection. Results are expressed as mean ± sem and statistical analyses were performed using the unpaired Student's t-test, where n = 3 muscles.

E2 expression in heart following treatment with p24 in vivo

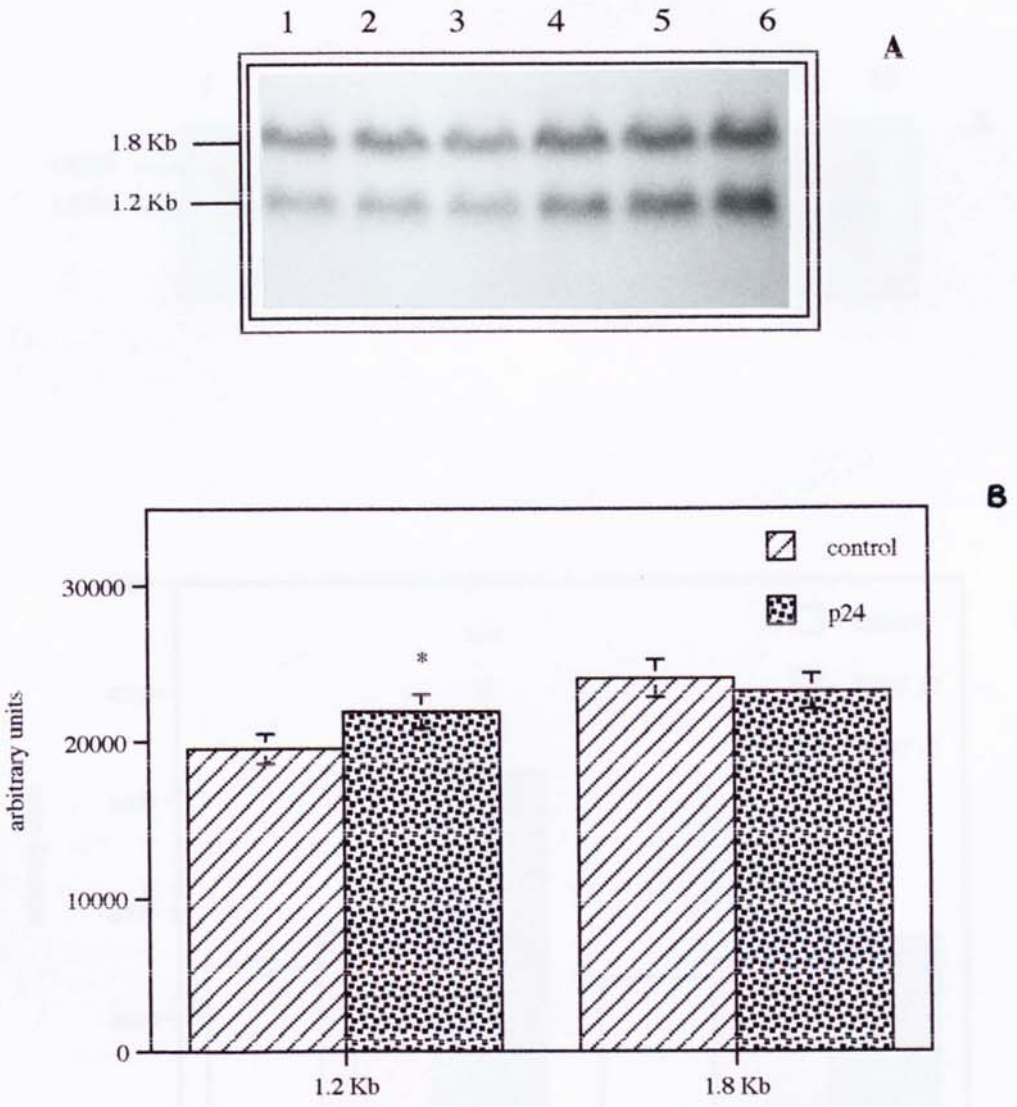

Figure 4.7. (A) Effect of p24 on the expression of E2 mRNA 24 h after the first injection. Lanes 1, 2 and 3 are control samples and lanes 4, 5 and 6 are p24-treated samples. (B) Results are expressed as mean ± sem and statistical analyses were performed using the unpaired Student's t-test, where n = 3 muscles and (*) $P < 0.05$.

E2 expression in gastrocnemius muscles from mice bearing the MAC16 and MAC 13 tumours

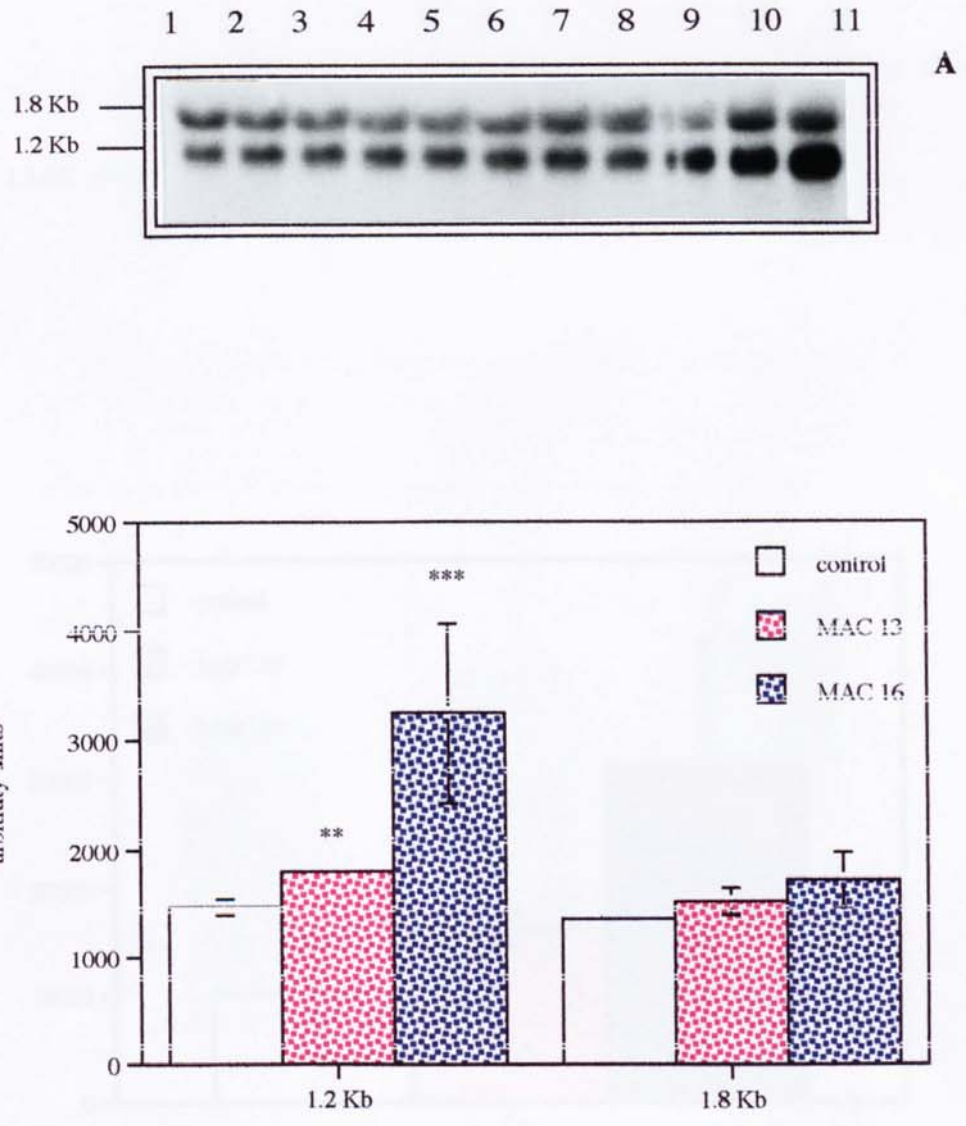

Figure 4.8. (A) Effect of the MAC 16 and MAC 13 tumours on the expression of E2 mRNA 2 weeks after transplantation. Lanes 1 to 4 are control muscles, 5 to 8 are MAC 13 muscles and 9 to 11 are MAC 16 muscles. (B) Results are expressed as mean ± sem, where n = 4 muscles (except for MAC 16 where n = 3). Statistical analyses were performed by unpaired Student's t-test, where (**) $P < 0.01$ and (***) $P < 0.001$.

C9 expression in gastrocnemius muscles from mice bearing the MAC16 and MAC 13 tumours

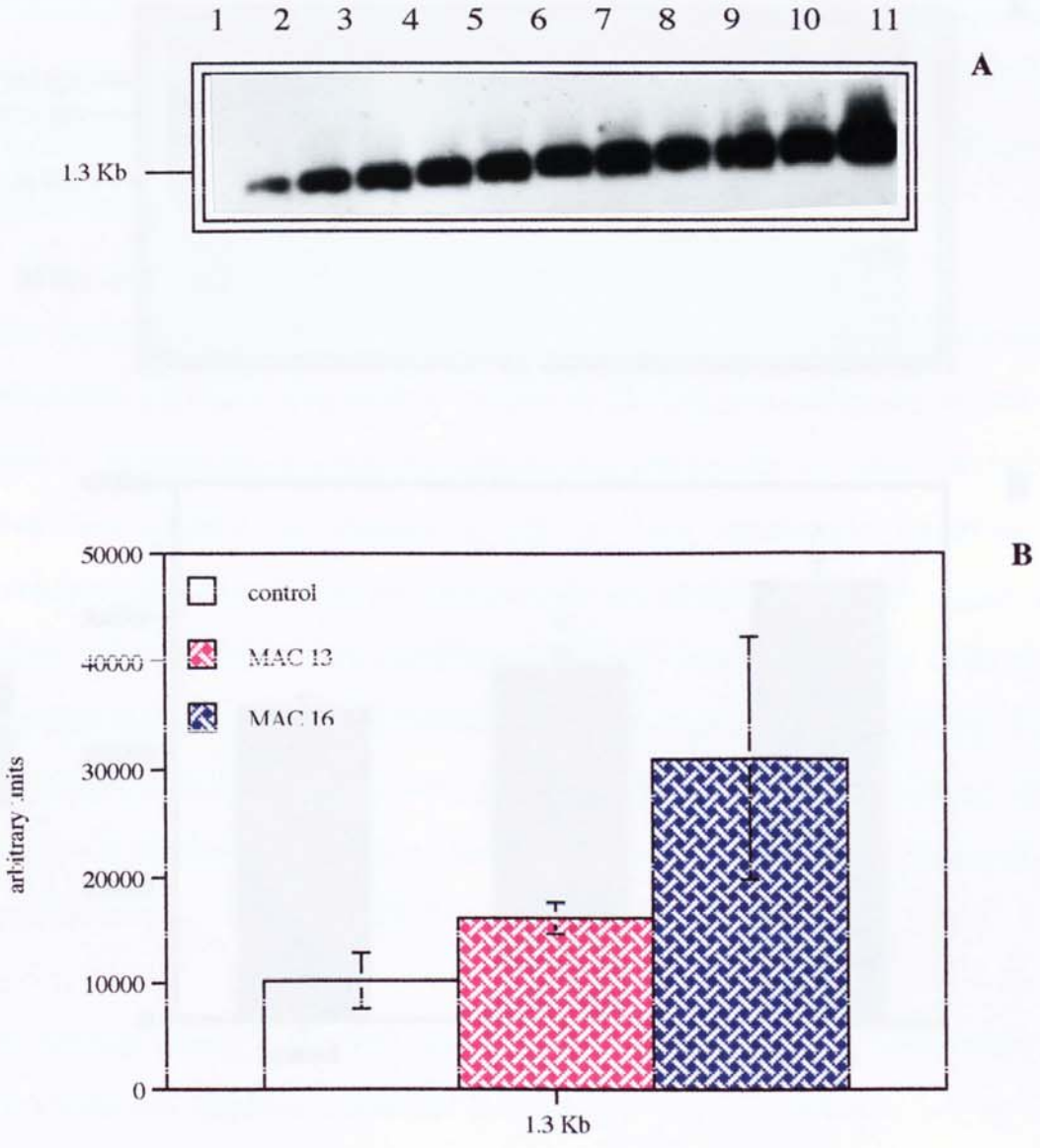

Figure 4.9. (A) Effect of the MAC 16 and MAC 13 tumours on the expression of C9 mRNA 2 weeks after transplantation. Lanes 1 to 4 are control muscles, 5 to 8 are MAC 13 muscles and 9 to 11 are MAC 16 muscles. (B) Results are expressed as mean ± sem, where n = 4 muscles (except for MAC 16 where n = 3). Statistical analyses were performed by unpaired Student's t-test.

Ubiquitin-protein conjugates in gastrocnemius muscles from MAC 16 and p24-treated mice

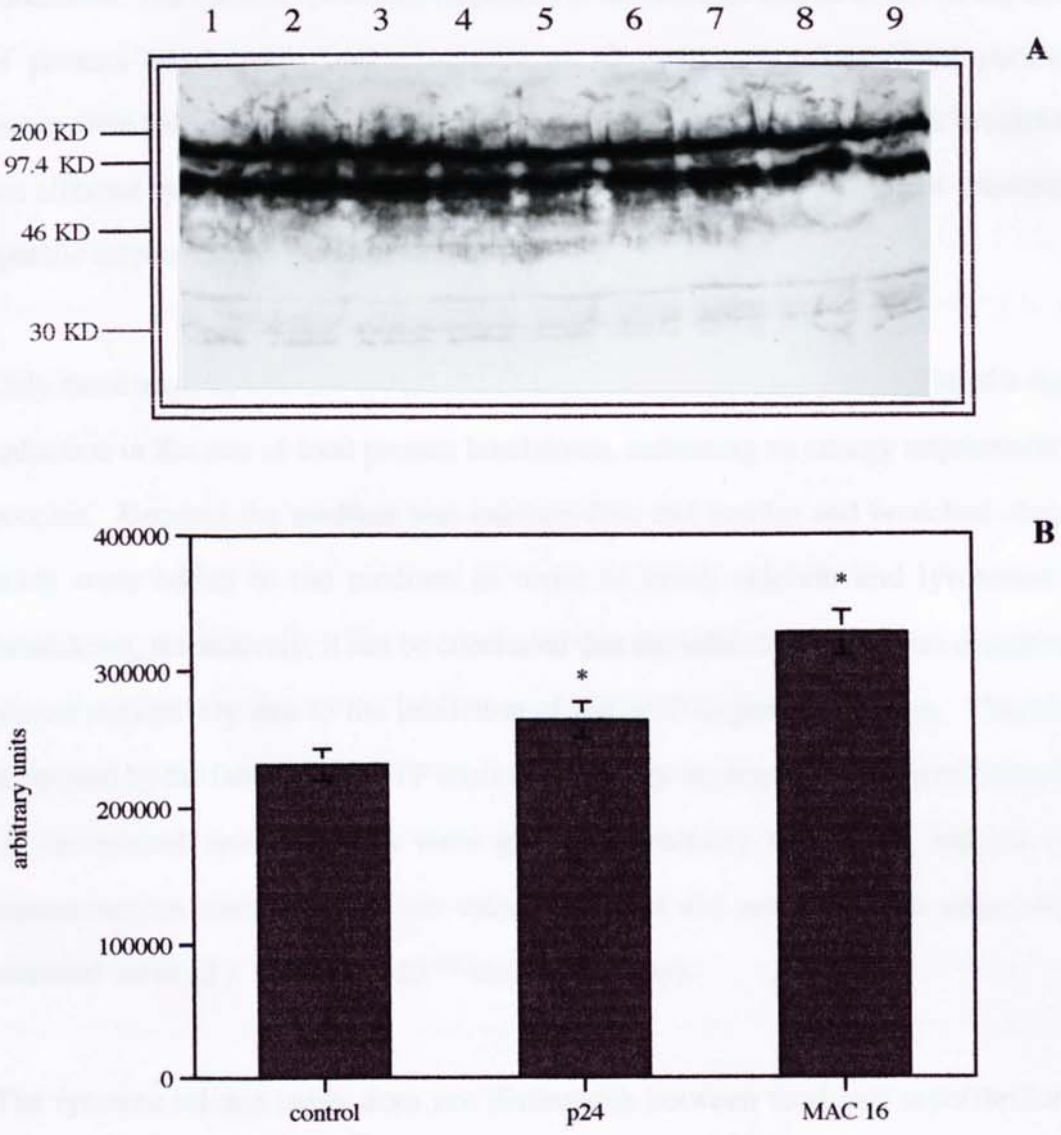

Figure 4.10. Effect of p24 and the MAC 16 tumour on the formation of ubiquitin-protein conjugates. (A) Lanes 1 to 3 are control muscles, 4 to 6 are p24-treated muscles and 7 to 9 are MAC 16 muscles (15 μg loadings). (B) Results are expressed as mean ± sem and statistical analyses were performed using the unpaired Student's t-test, where n = 3 muscles and (*) $P < 0.05$.

4.3. Discussion.

Administration of p24 caused an increase in total protein breakdown as measured by tyrosine release from soleus muscle, which could only be inhibited under energy-depleting conditions. Addition of lysosomal inhibitors to the medium had no effect on the attenuation of protein breakdown, indicating that an alternative non-lysosomal pathway was responsible for the increased protein degradation. The increased protein breakdown was not affected by incubation of muscles in calcium-free medium and in the presence of the specific calpain inhibitor, E-64.

Only those muscles which were incubated in ATP-depleted medium, exhibited a significant reduction in the rate of total protein breakdown, indicating an energy requirement for this process. Because the medium was calcium-free and insulin and branched-chain amino acids were added to the medium in order to block calcium and lysosomal protein breakdown, respectively, it can be concluded that the inhibition of protein degradation was almost exclusively due to the inhibition of the ATP-dependent system. This is further supported by the fact that the ATP content for energy-depleted muscles was lower than that of undepleted muscles (data were given in arbitrary light units instead of actual concentrations since the very low values obtained did not match the sensitivity of the standard curve (2×10^{-9} to 2×10^{-12} moles per assay).

The tyrosine release assay does not distinguish between total and myofibrillar protein breakdown and therefore, additional 3-MH measurements could have proved useful in these experiments in order to determine the influence of the different proteolytic pathways in the degradation of both soluble and myofibrillar proteins. However, despite the uncertainty about the contribution of myofibrillar protein breakdown to total proteolysis, the fact that none of the inhibitors of the other two pathways had any influence on the attenuation of protein degradation, in contrast with the response to energy depletion, indicates that the early metabolic changes imposed by p24 are likely to be due to its ability

to activate the ATP-dependent-proteolytic system which is responsible for the breakdown of myofibrillar proteins in skeletal muscle (Goodman, 1987 and Lowel *et al* , 1986a)

The mechanism of action of p24 on the degradation of skeletal muscle protein and the ATP dependence of this process were further investigated by Northern and Western blot analyses. Northern blots showed mRNA levels for ubiquitin and the ubiquitin-conjugating enzyme (E2) to be significantly increased in gastrocnemius muscle 24 h after treatment with p24. Expression of the 2.4 Kb and 1.2 Kb ubiquitin transcripts increased by 61 and 67%, respectively, and those of E2 by 31 and 81% (1.8 and 1.2 Kb), and in a separate experiment, upregulation of the proteasome subunit C9 mRNA was also found (increased by 121%).

Despite the observed changes in total proteolysis in response to energy-depleting conditions, no detectable changes in the expression of these genes were found in soleus muscle (not shown) which could have been due to the low amount of RNA which was recovered. Alternatively, an early activation of gene expression before changes in energy-dependent protein breakdown are detectable is also possible (Tiao *et al*, 1997).

As for soleus muscle, no changes were observed in the expression of these genes in liver (not shown). However, a small but significant increase was observed for the E2 1.2 Kb transcript in heart muscle (12%), suggesting that p24 might also be targeting heart muscle as well as fast-twitch skeletal muscle through the activation of the same proteolytic pathway.

Muscle homogenates were subject to SDS-PAGE and subsequent Western blot analysis which revealed several bands corresponding to the different molecular weight ubiquitin-protein conjugates. Overall, the presence of ubiquitin conjugates was significantly higher in p24-treated muscles than in controls (14% increase), although no drastic changes were observed between individual bands. Maybe, in view of the magnitude of the change in

mRNA expression for some of the components of the ubiquitin pathway, a more pronounced increase in ubiquitin conjugates might have been expected . However, in addition to the inherent lower sensitivity of the method, these results can also be explained by the fact that no fractionation between sarcoplasmic and myofibrillar proteins was carried out and therefore, despite the preferential targeting of myofibrillar proteins for degradation, this might have been masked by the presence of other soluble protein conjugates present in the whole muscle preparations.

The increased total protein breakdown associated with soleus muscle from tumour-bearing mice, was partially blocked under conditions which inhibited the lysosomal and ATP-dependent pathways where total proteolysis was reduced by 28 and 32%, respectively. Unlike soleus muscles from p24-treated animals, total proteolysis was not significantly inhibited by depleting the medium of ATP. However, this could have been due to the presence of residual ATP since no pre-incubation was carried out in this case. Unfortunately, ATP levels were not measured on these samples to confirm this.

In addition, the contribution from the lysosomal pathway to total protein breakdown might have obscured the higher proportion of myofibrillar protein breakdown. Indeed, significant changes were observed in the levels of ubiquitin-conjugates of gastrocnemius muscles from MAC 16 mice.

The presence of the MAC 16 and MAC 13 tumours elicited the activation of the ubiquitin-proteasome system in their respective hosts, as determined by the increased expression of E2 (1.2 Kb) and C9, although this was more dramatic in the case of the cachexia-inducing MAC 16 tumour.

Although these data clearly suggest that the ATP-ubiquitin-proteasome proteolytic pathway might be responsible for the muscle wasting associated with this cachectic model, an association between myofibrillar protein breakdown and the activation of this system has

not yet been made. First, technical difficulties may be overcome by the use of a more suitable muscle than gastrocnemius for the muscle incubation assay, for which EDL could be a good candidate. However, as for soleus muscle, only very small quantities of mRNA can be obtained and therefore a large number of animals would be required. Secondly, the release of 3-MH from the muscle preparation would provide the link between myofibrillar protein breakdown and the activation of this proteolytic system.

Chapter 5
Investigation into the mechanism of action of p24 on protein turnover of cultured muscle cells.

5.1. Introduction.

5.1.1. Regulation of protein synthesis.

5.1.1.1. Regulation of protein synthesis at the translational level.

The process consists of three different stages: initiation, elongation and termination, all of which are regulated by protein factors which ultimately modulate the rate of protein synthesis. The control of protein synthesis by these factors is mainly determined by their phosphorylation states and, in particular, those controlling the peptide-chain initiation stage have received much of the attention on this field since it appears to be the main site of regulation by hormones and growth factors. Long-term effects have been shown to involve changes in the number of translation factors and ribosomes, whilst short-term effects are known to be mainly due to changes in activity due to phosphorylation events. In other words, there appear to be two distinct components which affect translation: one is the capacity for translation which reflects the number of synthetically active ribosomes, the increase of which requires the synthesis of new proteins and rRNA, and the second is the efficiency of translation which relates to the translational machinery and activity of initiation factors.

5.1.1.1.1. Capacity of translation.

In cultured chick embryo fibroblasts grown in the presence of 4% foetal calf serum, Dephilip *et al* (1979) showed that treatment with insulin for 30 min stimulated the incorporation of [^{3}H]leucine and [^{3}H]adenine into total cell protein and 28S rRNA, respectively. Hesketh *et al* (1986) studied the response of quiescent 3T3 cells to insulin and serum repletion after growing in media containing 4% foetal calf serum. Addition of insulin caused an increase in protein synthesis after both short- and long-term incubations,

although the mechanism of these responses was different in each case on the basis of their inhibition by actinomycin D. The inhibitor had no effect on the stimulation of protein synthesis to insulin at 1 h, whilst it abolished this response at longer term incubations (at 1-3 h and 7-8 h). Serum repletion had the same effect on protein synthesis as the later component of the insulin response, ie, it increased ribosome production, while the immediate response to insulin involved an increase in the number of ribosomes incorporated into polysomes, which are the synthetically active units. Furthermore, downregulation of protein kinase C (PKC) by treatment with dibutyrylphorbol ester completely blocked the immediate response to insulin (1 h) but had no effect on the response to serum or the later response to insulin. In conclusion, insulin stimulated protein synthesis both at the translational and transcriptional levels and the former event appeared to be dependent upon PKC.

These findings were further confirmed by inhibitor studies which showed that the stimulation of protein synthesis by insulin after 1 h was not attenuated by α-amanitin (an inhibitor of mRNA and tRNA synthesis), 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (a mRNA synthesis inhibitor) or actinomycin D (an inhibitor of rRNA). In contrast, by 2-3 h protein synthesis stimulation was sensitive to all three RNA inhibitors, showing the need for both new rRNA and mRNA (Bardozc and Hesketh, 1989).

5.1.1.1.2. Efficiency of translation.

5.1.1.1.2.1. Regulation at the initiation stage.

As mentioned earlier, the increase in the efficiency of translation involves stimulation of the activity of initiation factors (IFs), usually through changes in their phosphorylation states. The best and most studied example is eIF-2, which catalyses the binding of Met-tRNA to the ribosome in a GTP-dependent manner. eIF-2·GDP is released from the ribosome and, in order to bind another Met-tRNA, GDP must be exchanged by GTP in a step which is carried out by eIF-2B (Redpath and Proud, 1994) (figure 5.1).

The activity of eIF-2B has been found to be decreased in skeletal muscle extracts from starved and diabetic rats, which could not be attributed to any changes in the phosphorylation state of the α subunit of eIF-2 (Jeffrey et al, 1990). The activity of eIF-2B was found to be markedly reduced in gastrocnemius and psoas muscles from diabetic rats compared to control muscles and was restored following treatment with insulin *in vivo* for 2 h. This diabetes-induced impairment in peptide-chain initiation was specific for fast-twitch muscle, since the activity of eIF-2B remained unchanged in soleus and heart (Kimbal and Jefferson, 1988). Likewise, a decreased activity for eIF-2B has also been found in tissues from old rats in comparison with younger rats. The amount of eIF-2 was decreased in general and this loss was shown to be due to a decrease in the efficiency of translation of the eIF-2α mRNA with age rather than to a change in transcription (Kimbal *et al*, 1992). In 3T3 cells where protein synthesis was shown to be stimulated by insulin, whole serum, phorbol esters and epidermal growth factor (EGF), all these increases correlated with an enhanced activity for eIF-2B, whilst no changes in the level of phosphorylation for eIF-2α were found (Welsh and Proud, 1992).

It has been proposed that changes in eIF-2B activity may be mediated by the action of casein kinase II (CK II). CK II has been found to be significantly reduced in fast-twitch skeletal muscle but not in cardiac muscle of diabetic rats, which may explain the reduced activity of eIF-2B in the former tissue but not in the latter. In addition, $NADP^+$ inhibited eIF-2B *in vitro* and this was reversed following addition of an equimolar amount of NADPH. Furthermore, an elevated NADPH/$NADP^+$ ratio was found in heart suggesting that NADPH may also prevent inhibition of eIF-2B *in vivo,* whilst it remained unchanged for fast-twitch skeletal muscle (Karinch *et al*, 1993). Dholakia and Wahba (1988) also found an increase in eIF-2B activity upon phosphorylation by CK II and inactivation by glycogen synthase kinase-3 (GSK-3)-induced phosphorylation has been reported by Welsh and Proud (1993). GSK-3 in turn, has been found to be inhibited by stimulation of mitogen-activated protein (MAP) kinase and $p90^{rsk}$ (through phosphorylation) upon treatment with insulin (Welsh *et al*, 1994).

Defects in translation at the level of the formation of ternary complexes have also been reported for skeletal muscle extracts from starved and diabetic rats. These were measured by assaying the incorporation of [^{35}S]methionyl-tRNA^{fMet} into [^{35}S]methionyl-tRNA^{fMet}. 40S-ribosomal subunit initiation complexes, which were 30-40% less active when compared to those derived from fed and insulin-maintained controls, respectively. The activity of ternary complexes in muscles from diabetic rats was restored by previous treatment with insulin *in vivo* for 30 min (Harmon *et al*, 1984).

Defects in peptide-chain initiation in fast-twitch skeletal muscle from septic rats have also been shown to be due to a decrease in activity for eIF-2B. Furthermore, no changes in the phosphorylation pattern of eIF-2α nor in its content were observed. In contrast and in accordance with the physiological features of the disease, neither the extent of phosphorylation of eIF-2α nor the activity of eIF-2B were altered in the liver of septic rats compared with controls. In fact, the hepatic eIF-2 content increased through an enhanced rate of translation of eIF-2 mRNA (Vary *et al*, 1994).

Another site of regulation of peptide-chain initiation involves the major ribosomal protein S6. The efficiency of translation of globin mRNA was significantly stimulated in ribosomal subunits purified from rabbit reticulocytes following phosphorylation with mitogen-stimulated S6 kinase *in vitro*. In fact, the sites phosphorylated by the enzyme were identical to those observed *in vivo* in response to insulin and growth promoting agents, suggesting that synthesis of specific proteins may be modulated by the phosphorylation state of S6 (Palent and Traugh, 1987). Further studies have also shown that the initiation of protein synthesis was greatly enhanced by serum, epidermal growth factor (EGF), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and insulin, all of which stimulated phosphorylation of S6. Even more, those ribosomes containing highly phosphorylated forms of S6 were found to have a selective advantage in entering polysomes (Thomas *et al*, 1982).

Another important site for regulation of translational activity is the 5'-end cap-binding site of eukaryotic mRNAs. This cap structure is a modified guanosine moiety (7-methyl guanosine) which is linked to the next nucleotide by a 5'-5' triphosphate group and it appears to be essential for the alignment of the ribosome with the initiation codon. This step is aided by the protein factors: eIF-4α, eIF-4A, eIF-4γ and eIF-4B, from which only eIF-4α can bind directly to the cap structure. The former three factors form a complex termed eIF-4F to which eIF4-B binds in order to release eIF-4α and therefore allow its recycling (Redpath and Proud, 1994). eIF-4A, as part of this complex, is responsible for unwinding the mRNA in order to permit the scanning of the 5'-untranslated region (UTR) by which the 40S subunit locates the initiation codon.

Treatment with insulin has been shown to stimulate phosphorylation of all the group 4 initiation factors and S6 in 3T3-L1 quiescent starved cells (Morley and Traugh, 1990). Furthermore, a similar pattern of phosphorylation was achieved by 12-O-tetradecanoylphorbol-13-acetate (TPA). eIF-4α isolated from insulin- and TPA-treated cells showed the same phosphorylation pattern, whilst eIF-4γ isolated from insulin presented additional phosphorylation sites to those obtained following treatment with TPA. Even more, treatment of downregulated cells with insulin showed little or no stimulation by phosphorylation of eIF-4α whilst that of eIF-4γ (and also S6) was increased. This indicates that phosphorylation of eIF-4α is stimulated only upon activation of protein kinase C (PKC) while that of eIF-4γ occurs via TPA-dependent and insulin-dependent pathways (Morley and Traugh, 1990).

Recently, the activity of eIF-4α has also been shown to be regulated by phosphorylated heat and acid stable protein (PHAS-1) in response to insulin which, through activation of MAP kinase, resulted in its dissociation from eIF-4F and rendered a functional translation complex (Pause *et al*, 1994 and O'Brien, 1994).

The rate of cell growth in HeLa cells decreased when the cells were grown in unreplenished medium and this decrease was found to be associated with a decrease in both the relative abundance of most initiation factors and in the fraction of polysomal ribosomes. Serum stimulation of serum-depleted cells resulted in the recruitment of most inactive ribosomes and mRNAs into polysomes. In contrast, no recruitment of initiation factor mRNAs was observed except for eIF-4α whose synthesis was stimulated (Duncan and Hershey, 1985).

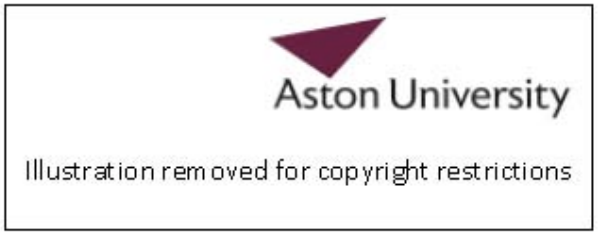

Figure 5.1. Regulation of translation (from Redpath and Proud, 1994).

5.1.1.1.2.2. Regulation at the elongation stage.

In contrast with the extensive regulation at the initiation stage, the role of elongation in the regulation of translation is less well known. Elongation factor-2 (eEF-2) mediates the translocation step which, following peptide bond formation, involves the transfer of the peptidyl-tRNA from the A- to the P-site of the ribosome, the movement of the deacylated tRNA from the P-site to the E-site and the movement of the ribosome relative to the mRNA by one codon. eEF-2 binds GTP and after translocation eEF-2·GDP is released from the ribosome. It has been demonstrated that eEF-2 activity is suppressed through phosphorylation by an eEF-2 kinase which leads to the cessation of translation (Redpath and Proud, 1993).

5.1.1.2. Regulation of protein synthesis at the transcriptional level.

In addition to transcriptional attenuation and mRNA stability, regulation of transcription is also carried out by the reversible phosphorylation of the factors controlling this process. There are three main routes by which the information is transferred from the cell surface to the nucleus (Edwards, 1994). The first, which is the most commonly used by growth factors, involve the activation of MAP kinases which translocate to the nucleus where they alter the phosphorylation state of transcriptional factors. The second one involves direct phosphorylation of a nuclear factor which is phosphorylated by a membrane receptor or associated kinase, leading to its translocation to the nucleus (eg. Stat91). Finally, the third pathway is engaged with the phosphorylation of inhibitor proteins which upon activation can release a transcription factor which subsequently translocates to the nucleus (eg. nuclear factor-κB, NF-κB).

The conversion points for all these signal transduction pathways are the so-called serum response element (SRE), TPA response element (TRE) and the cAMP response element (CRE). These promoter sites (and subsequently gene expression) are subject to regulation by transcription factors which are under the control of protein kinases whose activities, in turn, are regulated by hormones and growth factors.

One of the most studied transcription factors is activator protein-1 (AP-1), which binds to the TRE of several cellular and viral genes whose transcription is induced by TPA via PKC activation, although there is also evidence for PKC-independent pathway(s) (Angel and Karin, 1991). In addition, PKC also mediates the activation of other transcription factors which are distinct from AP-1 and which are involved in the recognition of other promoter sites such as the SRE of the c-*fos* gene (Hata *et al*, 1993).

AP-1 is a dimer formed by the association of the products of the c-*jun* and c-*fos* proto-oncogenes (c-Jun:c-Fos heterodimers), although the Jun proteins can also form homodimers (c-Jun:c-Jun). Dimerisation takes place through hydrophobic interactions between 'leucine zippers' and all possible complexes have very similar properties with regard to DNA recognition and interaction (which relies on a basic region localised immediately upstream to the leucine zipper), although the higher thermostability of the former accounts for their greater affinity (Angel and Karin, 1991).

In addition to their role in inducing the transcription of many genes, c-Jun and c-Fos also induce their own transcription after PKC activation and in the case of the c-*fos* promoter, several binding sites have been found which are the target of a wide range of stimuli. One of these binding sites is SRE which is the conversion point for many of the PKC-dependent and -independent signals triggered by growth factors. Function of this promoter requires the binding of a protein termed serum response factor (SRF) to which an additional protein, $p62^{TCF}$(ternary complex factor), binds to form a ternary complex which confers responsiveness to PKC-dependent signalling pathways. On the other hand, SRF on its own or in the presence of other unidentified factor(s) is responsible for PKC-independent modulation (Graham and Gilman, 1991).

Elk-1, which is immunologically related to HeLa cell $p62^{TCF}$, is a SRF accessory protein which has also been shown to have $p62^{TCF}$-like DNA binding properties. Phosphorylation of Elk-1 has been suggested to occur via activation of MAP kinases in response to growth

factors, an event which has no effect on the ability of the transcription factor to bind DNA but it potentiates the activity of a transcriptional activation function associated with the conserved Elk-1 C-terminal region (Marais *et al*, 1993).

Finally, the activity of AP-1 has also been shown to be modulated at the post-transcriptional level by means of phosphorylation/dephosphorylation events which may involve the action of glycogen synthase kinase-3 (GSK-3) and/or phosphatases (Angel and Karin, 1991) and by the interaction of AP-1 with other proteins distinct from the *jun* or *fos* families such as the glucocorticoid receptor (Jonat *et al*, 1990 and Schüle *et al*, 1990).

5.1.1.3. The role of second messenger signal transduction pathways in the control of protein synthesis in skeletal muscle.

The studies described above have outlined the importance of phosphorylation and dephosphorylation events in the control of protein synthesis. Since the phosphorylation state of protein factors controlling both translational and transcriptional processes appears to be crucial in determining the expression of proteins, the mechanisms by which these signal transduction events take place deserves further consideration.

Most extracellular stimuli interact specifically with their respective receptors at the plasma membrane level and it is this specific interaction that leads to the generation of second messenger molecules which are responsible for transducing this information within the cell. There are some exceptions such as in the case of glucocorticoid hormones which, due to their lipidic nature, are able to diffuse through membranes and can directly bind to their nuclear targets. In this chapter we are concerned with the molecular events modulating the rate of protein synthesis in skeletal muscle in response to hormones (mainly insulin) and growth factors.

A major source of second messengers is derived from phospholipid metabolism. Hydrolysis of membrane phospholipids, either generated upon receptor stimulation or

mechanical stimuli, leads to the production of diverse signalling molecules such as arachidonic acid (AA) and its metabolites, diacylglycerol (DAG), phosphatidic acid (PA) and lysophosphatidic acid (LPA). In addition to phospholipid-derived second messengers, alterations in the intracellular concentration of ions or cyclic nucleotides have also been shown to influence the rates of protein synthesis.

5.1.1.3.1. Role of prostaglandins in the stimulation of protein synthesis by insulin.

The stimulation of protein synthesis by insulin has been shown to correlate significantly with the release of $PGF_{2\alpha}$ from isolated muscles from fasted rabbits. The effect of insulin on protein synthesis was completely abolished by the cyclooxygenase inhibitors indomethacin and meclofenamate (Reeds and Palmer, 1983) and indomethacin has also been shown to block this stimulatory effect *in vivo* in response to insulin (which increased the plasma concentration of $PGF_{2\alpha}$) (Reeds *et al*, 1985), in L6 myoblasts (Palmer and Bain, 1989) and in isolated rabbit muscles in response to intermittent stretching (Palmer *et al*, 1983).

The use of cultured cells makes it possible to distinguish between translational and transcriptional effects of hormones on the rates of protein synthesis. Thus, in L6 myoblasts, insulin stimulated protein synthesis by 15% at 30 min and by 40% at 6h when RNA accretion occurred. Indomethacin inhibited the rate of protein synthesis both in the absence and in the presence of insulin and also the insulin-stimulated accretion of RNA. These results indicated that the effect of insulin on protein synthesis was mediated by eicosanoid production which affected both the translational efficiency and RNA accretion (Palmer and Bain, 1989) although the use of ibuprofen, a more selective cyclooxygenase inhibitor, showed no inhibition of RNA accretion in L6 cells in response to insulin (Thompson *et al*, 1993), excluding the possibility of prostaglandins controlling the transcriptional process.

However, these results do not exclude the role of arachidonate (AA) metabolism in mediating the transcriptional effect of insulin since indomethacin is also known to be a phospholipase A_2 (PLA_2) inhibitor (Kaplan et al, 1978). Furthermore, the PLA_2 inhibitors, dexamethasone and mepacrine, prevented the insulin-induced stimulation of protein synthesis in L6 myoblasts (as well as $PGF_{2\alpha}$ production) at 6 h to a extent similar to that achieved with the cyclooxygenase inhibitors (Southorn and Palmer, 1990). In fact, glucocorticoids have been shown to have opposite effects to those of insulin on protein synthesis in addition to their ability to induce insulin resistance (Southorn *et al*, 1990).

5.1.1.3.2. The role of protein kinases in the control of protein synthesis by insulin.

5.1.1.3.2.1. $p70^{s6k}$ and $p90^{rsk}$ S6 kinases.

The insulin-induced increase in the translational component of protein synthesis has been suggested to be due to phosphorylation of the major ribosomal protein S6 and, in Swiss 3T3 cells, $PGF_{2\alpha}$ has been shown to stimulate S6 phosphorylation (Pelech and Krebs, 1987). S6 can be phosphorylated by the 70 and 80 KDa ($p70^{s6k}$) and the 90 KDa ($p90^{rsk}$) S6 kinases. The activation of $p90^{rsk}$ involves mitogen-activated protein (MAP) kinases whilst those responsible for the activation of $p70^{s6k}$ are unknown.

The role of $p90^{rsk}$ in the activation of protein synthesis by insulin, however, is not so clear since much higher concentrations of insulin appear to be required to stimulate MAP kinase (100 nM) (Thompson *et al*, 1995, 1996a) than those needed to stimulate protein synthesis (below 1 nM) in L6 cells (Palmer and Bain, 1989 and Thompson *et al*, 1995). Additionally, rapamycin, an inhibitor of $p70^{s6k}$, attenuated the stimulation of protein synthesis in L6 (Thompson *et al*, 1995) and C_2C_{12} myoblasts in response to insulin (Palmer *et al*, 1997). Rapamycin has been shown to block both $p70^{s6k}$ activation and dephosphorylation of S6 with a subsequent decrease in the translational rate of protein synthesis in 3T3 cells (Jefferies *et al*, 1994).

Insulin also controls the activity of the mRNA cap-binding complex, eIF-4F, by regulating the phosphorylation state of PHAS-1 (see section 5.1.1.1.2). PHAS-1 has been shown to be a substrate for MAP kinase *in vitro*, unlike for the other two major insulin-stimulated protein kinases $p90^{rsk}$ and $p70^{s6k}$ (Haystead *et al*, 1994), and *in vivo* (Azpiazu *et al*, 1996) in response to insulin, which causes its dissociation from eIF-4E therefore relieving the inhibition of translation (Pause *et al*, 1994).

MAP kinases also regulate the activity of key enzymes in skeletal muscle. For instance, stimulation of glycogen synthesis in skeletal muscle results from the dephosphorylation and activation of glycogen synthase. This occurs by inhibition of glycogen synthase kinase-3 (GSK-3) through phosphorylation (Saito *et al*, 1994) which has been shown to be induced by insulin, insulin growth factor-1 (IGF-1) (Cross *et al*, 1994) and epidermal growth factor (Saito *et al*, 1994), and reversed by incubation with protein phosphatase-2A (PP2A) (Cross *et al*, 1994). Incubation of rat diaphragm muscles with insulin increased glycogen synthase activity by 2-fold as well as the activities of the $p90^{rsk}$ and $p70^{s6k}$ kinases, which were inhibited by a MAP kinase inhibitor (MEK) while having no effect on synthase activity. In addition, rapamycin inhibited the activation of both $p70^{s6k}$ and glycogen synthase (Azpiazu *et al*, 1996), suggesting a role for $p70^{s6k}$ in the stimulation of glycogen synthesis by insulin. In the study carried out by Cross *et al* (1994), in contrast, rapamycin also blocked the activation of $p70^{s6k}$ but it was found no effect on the inhibition of GSK-3 or the activation of $p90^{rsk}$ by IGF-1. Even more, the PI 3-kinase inhibitor wortmannin, prevented both the inactivation of GSK-3 and the activation of $p90^{rsk}$ and $p70^{s6k}$ by insulin and IGF-1, respectively, suggesting that $p90^{rsk}$ mediates the stimulatory effect of insulin of glycogen synthesis.

5.1.1.3.2.2. Protein kinase C (PKC).

Insulin promotes protein synthesis by stimulating both the translation and transcription processes. Prostaglandins (in particular $PGF_{2\alpha}$) appear to be involved in the rapid translational effect of the hormone, whilst an AA-sensitive pathway which implicates a

PKC isoform resistant to downregulation by TPA seems to be responsible for the stimulation of transcription (Thompson *et al* , 1993) (Figure 5.2). Thompson and co-workers found that the PKC inhibitor, Ro-31-8220, inhibited the ability of insulin to stimulate RNA accretion and protein synthesis at 6 h, while this was not prevented by downregulation of L6 cells with TPA. Immunoblotting of L6 PKC isoforms revealed the presence of α, δ, μ, ε and probably ι and ζ isoforms (Thompson *et al*, 1997). Since skeletal muscle and L8 skeletal muscle cells contain α, δ, ε and ζ isoforms, from which α, ε and ζ can be stimulated by AA, and downregulation with TPA removes α, δ and ε, the ζ isoform therefore appears as the candidate for mediating the insulin action (Thompson and Palmer, 1997).

Figure 5.2. Stimulation of protein synthesis by insulin (from Thompson and Palmer, 1997).

In skeletal muscle, Wahle *et al* (1987) found that TPA stimulated protein synthesis by 57%, however, indomethacin -at concentrations that block stimulation of protein synthesis by insulin and AA- did not fully reverse this effect, suggesting an alternative pathway in the stimulation of protein synthesis by TPA. PKC has been shown to stimulate protein synthesis in both L6 and C_2C_{12} myoblasts through its ability to stimulate phospholipase D (PLD) in response to TPA following a 90-min incubation . PLD activity was assessed by the intra- and extracellular release of [^{3}H]choline from L6 and C_2C_{12} cells and the release of [^{14}C]ethanolamine from L6 cells. Even more, a similar pattern was obtained with the addition of a highly purified exogenous source of PLD (Thompson *et al*, 1994; Morrison *et al*, 1995 and Thompson *et al*, 1997). In fact, TPA and PLD elicited similar responses regarding the stimulation of protein synthesis at 90 min (15 and 13%, respectively). The PKC inhibitor, Ro-31-8220, and downregulation of PKC (which removed α, ε and δ) in L6 myoblasts inhibited both PLD activity and stimulation of protein synthesis, suggesting that the stimulatory action of TPA on protein synthesis might be mediated through the α, ε and/or δ isoforms (Thompson *et al*, 1997).

A similar pathway to that employed by insulin might also be involved in the stimulation of protein synthesis by vasopressin, in that it enhanced in the rate of transcription and RNA accretion after 6 h. Downregulation with TPA did not abolish this response and yet it was completely blocked by the PKC inhibitors Ro-31-8220 and bisindolylmaleimide, rendering the PKC ζ isoform as the mediator of the vasopressin response (Thompson *et al*, 1997). As for TPA, vasopressin also stimulated PLD, although this enzyme activity was not responsible for the action of vasopressin on protein synthesis since the maximum stimulation of protein synthesis by vasopressin was much greater than by PLD after 6 h (30 and 12%, respectively). Furthermore, the EC_{50} for activation of protein synthesis was 10-fold greater than that for the activation of PLD (Thompson *et al*, 1994).

5.1.1.3.2.3. Cyclic adenosine 3'-5'-monophosphate (cAMP) and protein kinase A (PKA). Part of the anabolic effect of clenbuterol on muscle is due to its ability to increase protein synthesis but, unlike the effect of non-steroidal antiinflammatory drugs (NSAIDs) on the stimulation of protein synthesis by insulin, administration of fenbufen -while inhibiting $PGF_{2\alpha}$ production- did not prevent the clenbuterol-induced RNA and protein deposition in rat skeletal muscle (Palmer *et al*, 1990), indicating that alternative pathways are involved in the control of protein synthesis. This is further supported by the fact that TPA-induced stimulation of protein synthesis is not completely blocked by indomethacin (Wahle *et al*, 1987).

A cAMP-dependent kinase was shown to directly phosphorylate hepatic ribosomal S6 protein in both whole ribosomes and 40S ribosomal subunits (Wettenhall and Cohen, 1982 and Wettenhall and Morgan, 1984). cAMP and its analogue dibutyryl-cAMP, dbt-cAMP, were shown to stimulate both cAMP-dependent kinase activity and S6 phosphorylation in bovine anterior pituitary gland (Barden and Labrie, 1973) and, in rat skeletal muscle, cAMP stimulated the rate of protein synthesis (Lewis *et al*, 1982). Concomitant stimulation of S6 phosphorylation and translation by cAMP have indeed been reported in a number of mammalian (Keller *et al*, 1982 and Wettenhall and Howlett, 1973) and non-mammalian systems (Gressner and Van de Leur, 1980 and Glover, 1982).

In rat L6 myoblasts, dbt-cAMP stimulated protein synthesis at 90 min and 6 h and similar responses were achieved with forskolin, an agent that directly stimulates adenylate cyclase. The 90-min response was not affected by actinomycin D whilst TPA and insulin had additive effects to those of dbt-cAMP. Rapamycin, an inhibitor of $p70^{s6k}$, also had no effect on the attenuation of this response, however, immunoblotting of stimulated cell extracts revealed increased phosphorylation of the 42 and 44 KDa forms of MAP kinase, although the additive effects of TPA were not mediated at this level (Thompson *et al*, 1996a). These results suggest the existence of an alternative pathway controlling protein synthesis in skeletal muscle which involves phosphorylation of S6 by PKA, possibly

through the action of $p90^{rsk}$. Additionally, this route may also be involved in the activation of translation through the phosphorylation of initiation factors such as eIF-4E (Proud, 1994).

Regarding the role of this pathway in stimulation of transcription, dbt-cAMP (and forskolin) stimulated both RNA accretion and protein synthesis but, unlike at 90 min, insulin did not cause a synergistic effect and yet it elicited an additive effect on MAP kinase activation, supporting the notion that this enzyme is involved in the additive effects of insulin and cAMP on protein synthesis at 90 min (Thompson *et al*, 1996a). It has been suggested that stimulation of protein synthesis by this pathway may involve phosphorylation of cAMP-responsive element modulator, CREM, by PKA (Takahashi, 1993). Furthermore, deGroot *et al* (1994) have shown $p70^{s6k}$ to regulate the activity of CREM and also to phosphorylate the same serine residue as PKA in response to mitogenic stimulation with serum growth factors both *in vitro* and *in vivo*.

Finally, protease-activated kinase (a cAMP-independent kinase) phosphorylation of S6 and enhancement of translation has also been reported by Burkhard and Traugh (1983) who, in contrast, found a decrease in translation upon phosphorylation of S6 by a cAMP-dependent kinase. Furthermore, five phosphorylation sites were induced by protease-activated kinase II and two in response to cAMP-dependent kinase (one of them was common to both) (Perisic and Traugh, 1983). These results suggest that an alternative role of cAMP in the modulation of protein synthesis although, up to this time, this system has not been characterised in skeletal muscle.

5.1.2. Regulation of protein degradation and the possible role of second messenger signal transduction pathways.

The mechanisms which control the regulation of myofibrillar and non-myofibrillar protein breakdown have been discussed in detail in chapter 3. However, not so much is known about the cell signalling pathways which modulate these processes.

Regarding the degradation of myofibrillar proteins, the presence of phosphorylation sites in several proteasome subunits (Tanaka *et al*, 1990; Heinemeyer *et al*, 1994; Dubiel *et al*, 1992 and Arrigo and Mehlen, 1993) suggests the possibility of some type of short-term regulatory events which may take place through the activation of phosphorylation/dephosphorylation cascades. Furthermore, a cAMP-dependent protein kinase activity and a casein kinase II activity have been found to co-purify with different proteasome subunits (Pereira and Wilk, 1990 and Ludemann *et al*, 1993).

Long-term induced changes in the mRNA expression of lysosomal and calcium-dependent proteases and in particular that of the components of the ubiquitin-proteasome pathway have been discussed in chapter 4. Glucocorticoids have been shown to activate the expression of the ubiquitin-conjugating enzyme E2, and simultaneously stimulate the degradation of myofibrillar proteins in C_2C_{12} myotubes, as measured from the release of 3-MH into the incubation medium, a finding that for the first time provides a direct link between the glucocorticoid-induced myofibrillar protein degradation and the activation of the ubiquitin system (Thompson *et al*, 1996c). However, dexamethasone, which has also been shown to increase the rate of protein breakdown in L8 myotubes, did not have any effect on the expression of the C2 proteasome subunit although, interestingly, was associated with an increased expression for cathepsins B and D (Hong and Forsberg, 1995). The mechanism by which glucocorticoids influence the expression of some genes, including those encoding the ubiquitin system, has been proposed to be mediated by direct protein-protein interactions between the glucocorticoid receptor and transcription factors such as AP-1 (Jonat *et al*, 1990 and Schüle *et al*, 1990).

Finally, cytokines have also been proposed to play a role in the signalling pathways mediating the degradation of muscle proteins. An increased 26S proteasome activity (as well as cathepsins B and L activities) has been found to be associated with an enhanced transcription in C_2C_{12} myotubes following treatment with human recombinant IL-6 (Ebisui *et al*, 1995). In isolated skeletal muscle, human recombinant TNF-α has been shown to be

capable of directly inducing ubiquitin expression (Llovera *et al*, 1997) and myofibrillar protein breakdown (Goodman, 1991). A novel intracellular signalling pathway mediated by TNF, which involves the hydrolysis of sphingomyelin to ceramide with the subsequent stimulation of a ceramide-activated protein kinase, has been suggested to increase nuclear transcription by NF-κB in HL-60 cells (Yang *et al*, 1993). Furthermore, a protein phosphatase activity has also been shown to be stimulated by ceramide, supporting the notion of a new sphingomyelin-mediated signalling pathway (Dobrowsky and Hannun, 1992).

5.1.3. Aims of the study.

In an attempt to establish the exact mechanism of action of p24 on the protein turnover rates of skeletal muscle, the C_2C_{12} cell line will be used as the *in vitro* system to study the rates of both protein synthesis and degradation. The use of cultured cells will provide accurate and reliable measurements on the stimulation of these rates without the influence of external factors and the disadvantages of using animal models or *in vitro* muscle preparations.

5.2. *Results.*

p24 induced a significant alteration of the protein turnover of C_2C_{12} myoblasts by causing both an inhibition of the rate of protein synthesis and an enhancement of proteolysis. Protein synthesis was inhibited in a dose-dependent manner after a 90 min incubation with concentrations of the proteolytic factor which ranged from 0.05 to 0.25 μg of protein, reaching a maximal significant effect at 0.1 μg for which a 20% decrease was achieved (figure 5.3). Incubation of C_2C_{12} myoblasts with p24 for longer periods did not significantly affect the rates of protein synthesis although there was a slight tendency for its decrease at very low concentrations (0.05 μg). Incubation of C_2C_{12} myotubes with p24 did not affect the rate of protein synthesis at longer incubations (6 and 24 h) whilst after a 90 min incubation there was a small reduction at lower concentrations, again reaching a maximum effect at 0.1 μg although these reductions were not significantly different from the control values (figure 5.4).

The depression in the rate of protein synthesis was shown to be specific for the affinity purified glycoprotein since the inclusion of the monoclonal antibody (mAb) in the incubation medium not only inhibited this response completely but, in fact, it increased the rate of protein synthesis significantly (figure 5.5).

Treatment of myoblasts with insulin completely blocked the inhibition of protein synthesis by p24 (figure 5.6). Insulin was effective in suppressing the effect of p24 at concentrations as low as physiological levels (1 nM) and below (0.1 nM) and significant increases in the rate of protein synthesis were observed at all concentrations of insulin studied.

As for the muscle preparations (chapter 3), EPA had no effect on the restoration of protein synthesis rates (figure 5.7). At 10 μM and 50 μM, EPA was found to have a positive effect on the basal protein synthesis rate of C_2C_{12} myoblasts when incubated for 24 h,

while having a toxic effect at higher concentrations (not shown). However, these concentrations did not prevent the inhibition of protein synthesis by p24.

Increased proteolysis, as measured by phenylalanine release, was detected as early as 6 h after the addition of p24 to C_2C_{12} myoblasts, which reached significant levels at concentrations between 0.07 and 0.10 μg. Increased proteolysis rates were maintained significantly higher than the controls when C_2C_{12} myoblasts were incubated for longer periods of time (24 and 48 h) at concentrations corresponding to 0.01 and 0.10 μg. Above these concentration values, the rate of proteolysis was not different from control values or even became significantly inhibitory (figure 5.8).

In C_2C_{12} myotubes, p24 caused a significant elevation in the rate of myofibrillar protein breakdown (17%) as measured by 3-MH release after 48 h (figure 5.9).

Effect of p24 on the rate of protein synthesis of C_2C_{12} myoblasts

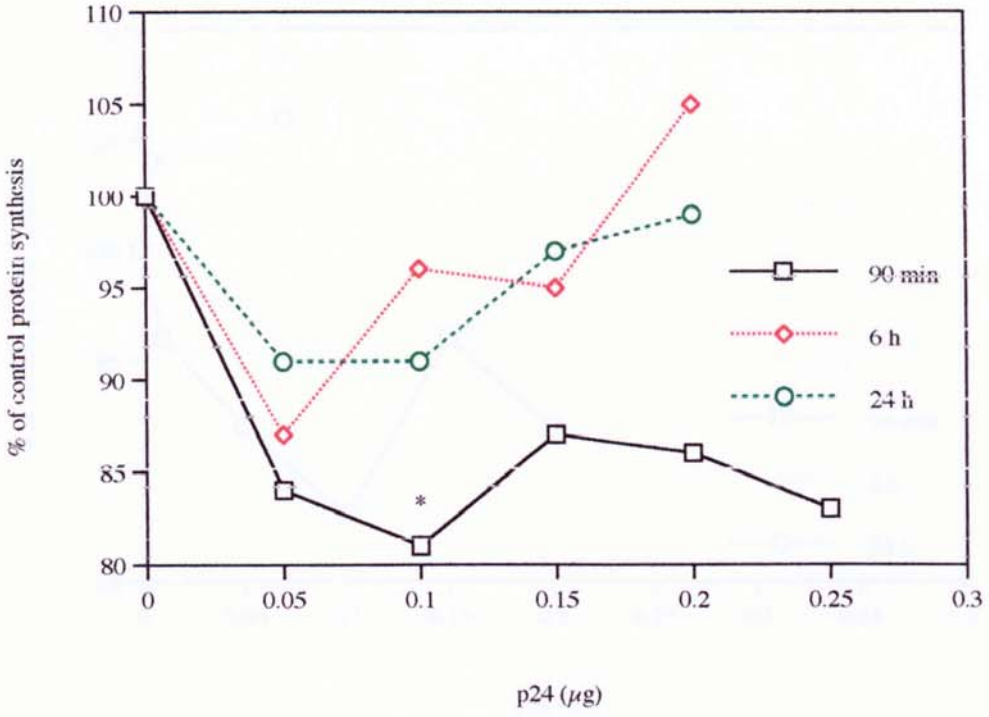

Figure 5.3. Effect of p24 on the rate of protein synthesis of C_2C_{12} myoblasts. L-[2,6-^{3}H]-Phenylalanine was included during the last hour of each incubation period and the synthesis rates were calculated as specific radioactivity (Sr), ie, dpm incorporated per μg of protein per hour. Each point represents the mean of 5 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test where (*) $P< 0.05$.

Effect of p24 on the rate of protein synthesis of C_2C_{12} myotubes

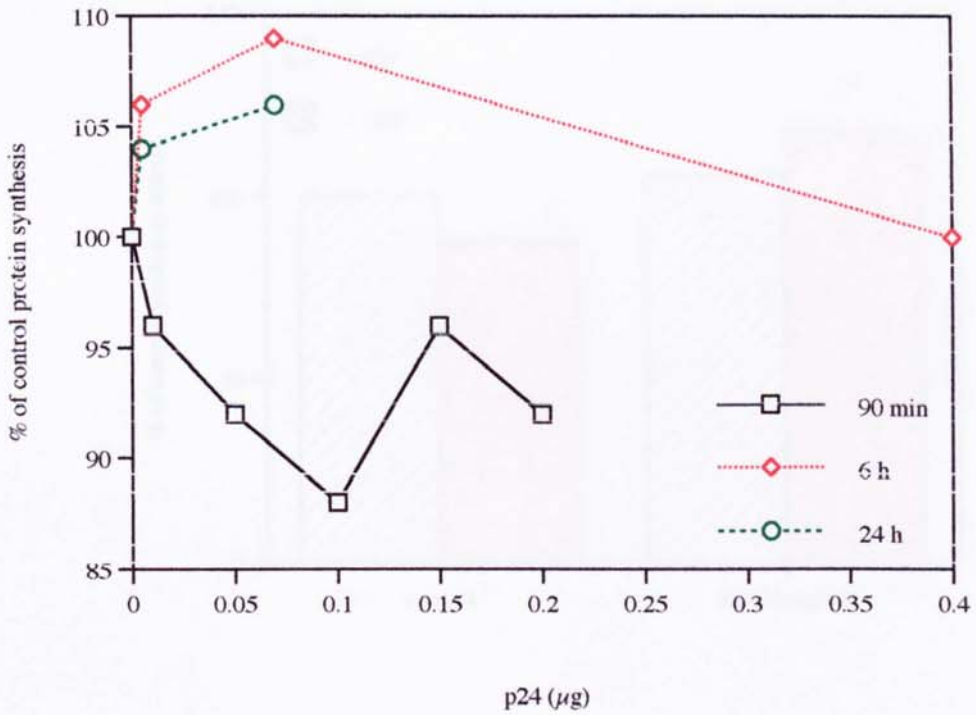

Figure 5.4. Effect of p24 on the rate of protein synthesis of C_2C_{12} myotubes. L-[2,6-^{3}H]-Phenylalanine was included during the last hour of each incubation period and the synthesis rates were calculated as specific radioactivity (Sr), ie, dpm incorporated per μg of protein per hour. Each point represents the mean of 5 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test.

Effect of the monoclonal antibody on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts

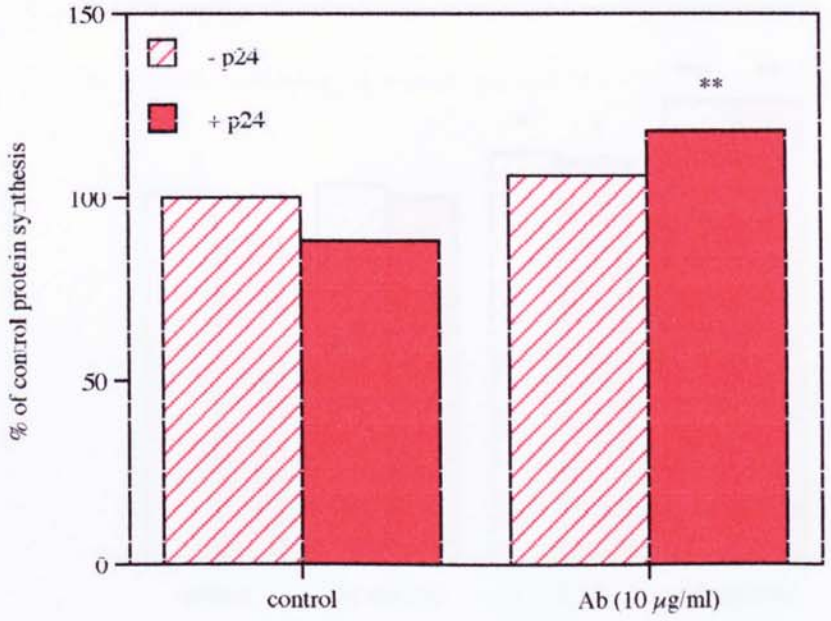

Figure 5.5. Effect of the monoclonal antibody (mAb) on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts. Cells were pre-treated with the mAb for 1 h and then incubated with p24 for 90 min. Protein synthesis rates were determined as previously described in figure 5.2 and each point represents the mean of 5 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test, where (**) $P< 0.01$.

Effect of insulin on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts

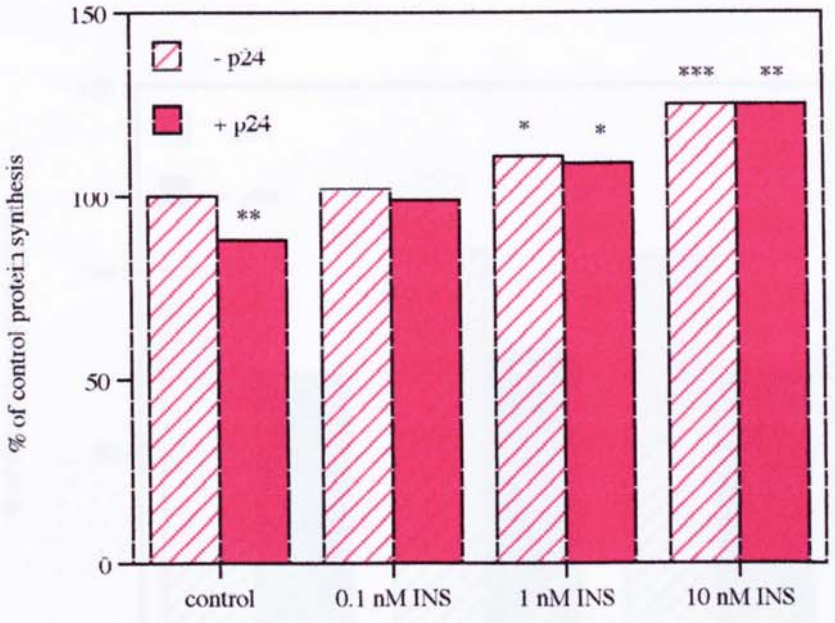

Figure 5.6. Effect of insulin on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts. Cells were pre-incubated with insulin for 1.5 h and p24 was then added for another 1.5 h. Protein synthesis rates were determined as previously described in figure 5.2 and each point represents the mean of 5 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test where (*) $P< 0.05$, (**) $P< 0.01$ and (***) $P< 0.001$.

Effect of EPA on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts

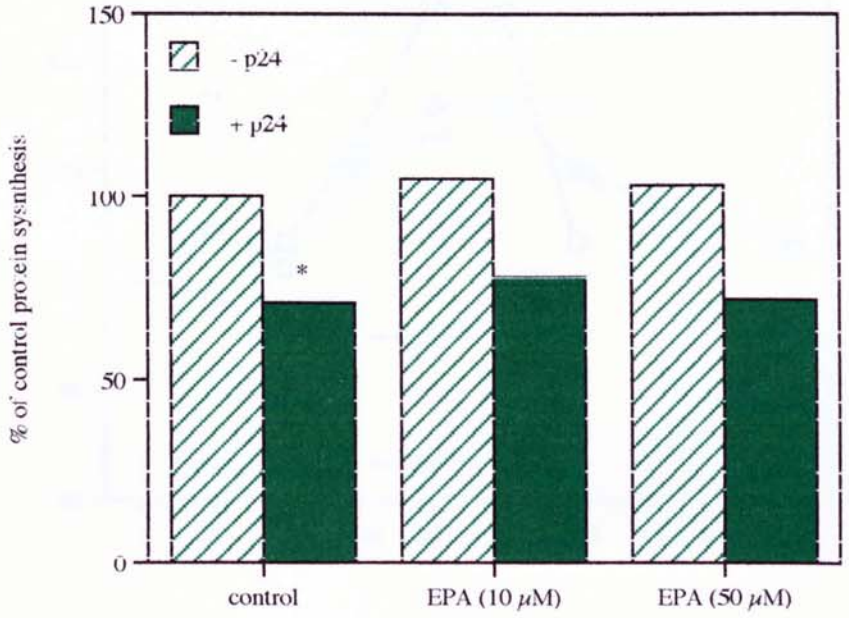

Figure 5.7. Effect of EPA on the p24-induced inhibition of protein synthesis of C_2C_{12} myoblasts. Cells were pre-treated with EPA 24 h before the start of the experiment and then incubated with p24 for 1.5 h. Protein synthesis rates were determined as previously described in figure 5.2 and each point represents the mean of 5 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test where (*) $P< 0.05$.

Effect of p24 on the rate of total protein degradation of C_2C_{12} myoblasts

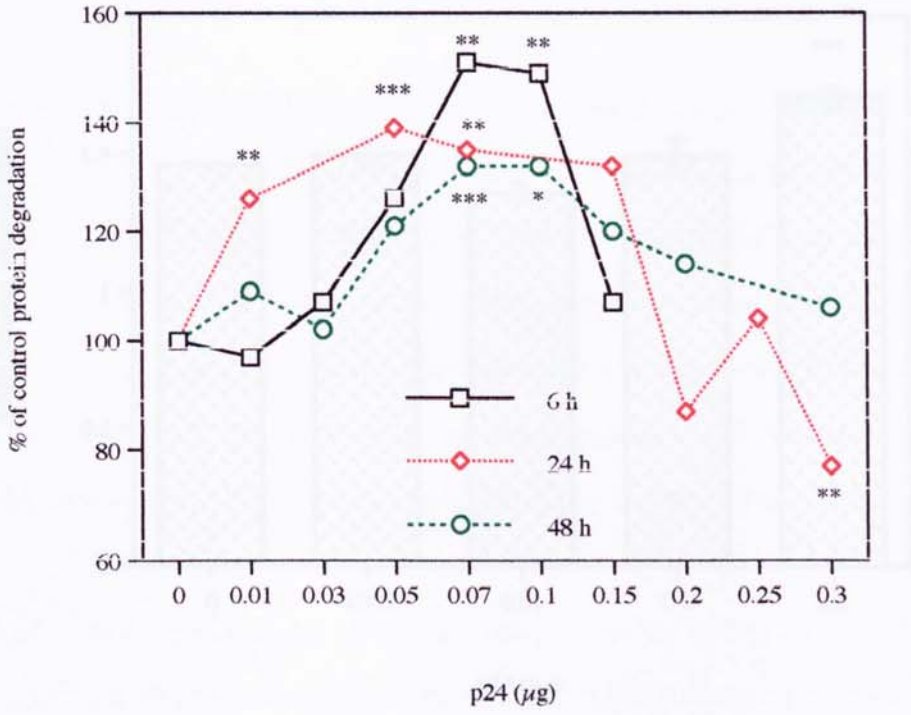

Figure 5.8. Effect of p24 on total protein degradation of C_2C_{12} myoblasts. Cells were labelled with L-[2,6-^{3}H]-Phenylalanine (^{3}Phe) 24 h before the start of the experiment, washed and incubated with p24 for different periods of time. Rates of protein degradation were calculated by dividing the amount of ^{3}Phe released into the incubation medium by the protein-bound ^{3}Phe. Each point represents the mean of 6 samples and each experiment was carried out at least twice. Statistical analysis was performed by the unpaired Student's t-test where (*) $P< 0.05$, (**) $P< 0.01$ and (***) $P< 0.001$.

Effect of p24 on myofibrillar protein breakdown of C_2C_{12} myotubes

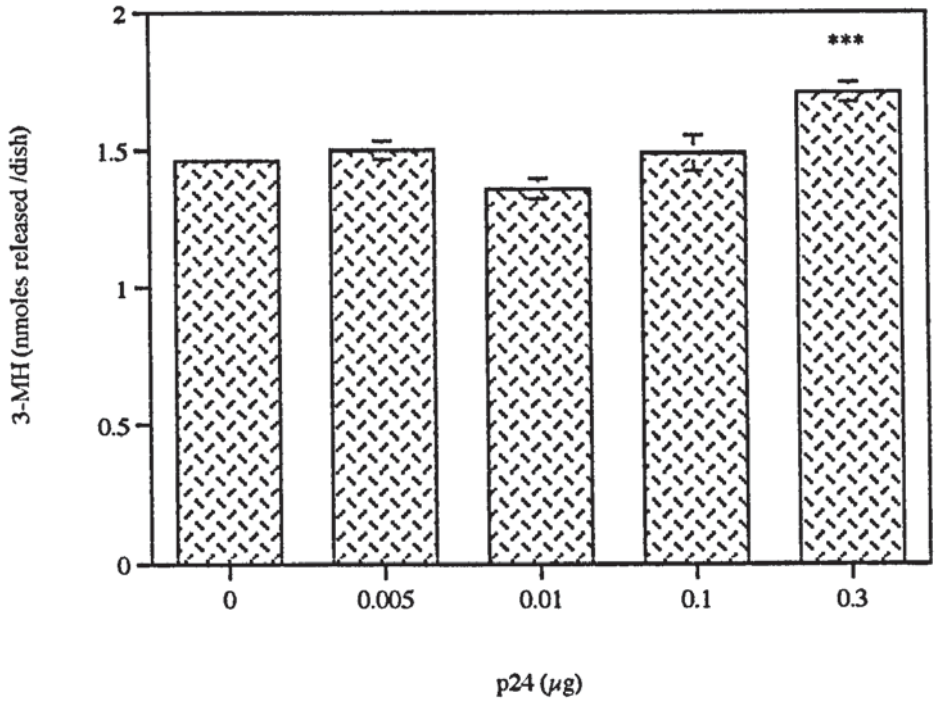

Figure 5.9. Effect of p24 on myofibrillar protein breakdown of C_2C_{12} myotubes. Cells were incubated with p24 for 48 h at the end of which the amount of 3-MH released into the incubation medium was determined by GC-MS analysis. Results are expressed as mean ± sem, where n = 5. Statistical analysis was performed by the unpaired Student's t-test where (***) $P < 0.001$.

5.3. Discussion.

The results presented in the previous section have shown that p24 is capable of increasing protein turnover in C_2C_{12} cells by causing both an inhibition of protein synthesis and increasing proteolysis. Rates of protein synthesis were calculated as Sr, ie, the specific radioactivity (dpm) incorporated per μg of protein per hour, as described by Southorn and Palmer (1990) and expressed as the percentage of control values. *In vivo*, fractional rates of protein synthesis are calculated according to the following equation:

$$Ks = Sr \times 100 / Si \times t,$$

where Ks represents the rate of protein synthesis expressed as a percentage of the protein synthesised per day, Sr is the specific radioactivity of the protein-bound phenylalanine (^{3}Phe), Si corresponds to the specific radioactivity of the intracellular free pool and t is the time of incorporation in days. However, unlike the *in vivo* situation, in cultured cells and *in vitro* muscle preparations, the Si values are considered to be constant since the precursor amino acid pool is virtually the same as the specific radioactivity of ^{3}Phe in the incubation medium and consequently, Ks is directly proportional to Sr (Morrison, 1995).

Inhibition of protein synthesis by p24 might result from its ability to block translation, since no significant changes were observed at 6 h or beyond which would have reflected changes at the transcriptional level. However, this cannot be completely ruled out since very low concentrations appeared to cause a small reduction in protein synthesis at longer incubation times (6 and 24 h). Significant reductions in the rate of protein synthesis were repeatedly achieved by incubating C_2C_{12} myoblasts with approx 0.1 μg of glycoprotein for 90 min, an effect which was specific to this material since it was completely abolished following pretreatment of the cells with the monoclonal antibody.

The role of p24 on the inhibition of translation is supported by the fact that pretreatment of cells with insulin for 90 min prevented any effect induced by the affinity purified material.

Furthermore, all concentrations of insulin used (0.1 nM to 10 nM), significantly increased the rates of protein synthesis in the presence of p24 at a concentration which maximally suppressed protein synthesis in the absence of the hormone. These results suggest that the interaction of p24 with its receptor may trigger a cascade of phosphorylation/dephosphorylation events which may finally result in the dephosphorylation of translation factors or the S6 ribosomal protein, although at the present moment this remains purely speculative. In order to elucidate whether p24 exerts its effect on the regulation of translation through the same signalling pathway as insulin, further experiments should test the ability of the hormone to reverse the changes imposed by this agent.

Unlike its effect on C_2C_{12} myoblasts, p24 did not significantly alter the rate of protein synthesis of C_2C_{12} myotubes, which suggest either uncoupling or downregulation of the receptor in this differentiation stage. Uncoupling of other receptors such as that for insulin has been shown to take place in C_2C_{12} cells upon fusion (Palmer *et al*, 1996).

Protein degradation rates increased by as much as 50% in response to p24 and, interestingly, maximal stimulation of proteolysis was achieved at concentrations of p24 (0.07-0.1 μg) which also produced maximal inhibition of protein synthesis (20%). In fully differentiated myotubes, p24 was capable of initiating myofibrillar protein breakdown, although a significant effect was observed at higher concentrations (0.3 μg) than those required to induce total protein breakdown in myoblasts. Further experiments are required in order to determine the optimal dose and time points for the action of p24 on the degradation of myofibrillar proteins.

In addition to the mechanism of action of p24 on protein turnover, the possibility of a p24-induced DNA damaged was also investigated by isolation of DNA from cells which had been exposed to p24 and electrophoresis of the nucleic material on an agarose gel in search for the presence of DNA fragmentation. DNA extracted from both adherent and non-

adherent cells showed no signs of fragmentation and no visible differences were observed with respect to DNA from control cells (results not shown). Since fragmentation of DNA is considered as one of the hallmarks of apoptosis, one could rule out the possibility of the activation of the machinery responsible for switching on the programmed cell death process in C_2C_{12} in response to treatment with p24. Indeed, DNA fragmentation and apoptosis (as determined by other techniques such as *in situ* end labelling of fragmented DNA and electron microscopy assays) are quite outstanding features of dystrophic *mdx* fibres from dystrophin-deficient mice (Sandri *et al*, 1995; Tidball *et al*, 1995 and Veal and Jackson, 1996).

In conclusion, the results presented in this chapter are in agreement with those found in skeletal muscle (chapter 3) of mice treated with p24, where a combination of both a depression of protein synthesis and increased proteolysis was shown, which appears to be cause for the muscle wasting seen in skeletal muscle of animals bearing the MAC 16 tumour. The use of this *in vitro* model has provided evidence for a mechanism of action of p24 on protein synthesis which may involve the induction of a peptide-chain initiation defect at the translational level, although this awaits further characterisation.

Chapter 6
Conclusion

In this study, a protein-mobilising factor of 24 KDa (p24) has been isolated from a cachexia-inducing tumour, which has the ability to induce a cachexia-like condition when injected into non tumour-bearing mice. This novel glycoprotein, which shows no similarity with any of the cytokines reported so far, has been found to have unique properties, in that not only can it induce weight loss in the absence of anorexia, but is also able to promote protein breakdown *in vitro* (Todorov *et al*, 1996a). Furthermore, a similar material has been found in the urine of weight-losing patients with cancer cachexia, also capable of inducing weight loss and producing similar effects when administered to NMRI mice (Cariuk *et al*, 1997). These findings clearly support a role for this proteolytic factor in the development of cachexia in both mice and humans, but may be more importantly, they also suggest that both factors may operate through a similar mechanism.

p24 induced a rapid weight loss 24h after the first injection which was largely due to the loss of lean body mass whilst having no effect on fat or water contents (Cariuk *et al*, 1997 and Lorite *et al*, 1997). This was reflected by an increased protein loss as measured from the release of tyrosine from skeletal muscle under conditions where reincorporation of this amino acid into protein synthesis was inhibited. The suitability of this assay as a measure of myofibrillar protein breakdown has been discussed in previous chapters, but nevertheless, it has provided a convenient and relatively quick and simple way for studying the effects of p24 on total muscle protein degradation.

Protein degradation, as determined by this method, was associated with an increased production of prostaglandin E_2 (PGE_2), which was released into the incubation medium and measured by radioimmunoassay. Both tyrosine and PGE_2 release were reduced in soleus muscles from mice whose weight loss had been attenuated by treatment with the

monoclonal antibody prior to p24 administration, and *in vitro*, both were inhibited by agents which block PGE_2 synthesis.

The role of PGE_2 in protein degradation has been extensively studied and appears to involve the activation of the lysosomal enzymes. Therefore in this model, it is possible that the production of p24 by the MAC 16 tumour may enhance the synthesis of PGE_2 and subsequently, promote the activation of this degradative pathway.

However, the lysosomal system alone does not appear to be responsible for the degradation of all proteins, since in soleus muscles from MAC16 mice protein breakdown was only partially blocked when the incubations were carried out in the presence of lysosomal inhibitors. In fact, in muscles incubated with lysosomal inhibitors and calcium-free medium, those which additionally were depleted of ATP showed a considerable reduction in the levels of tyrosine released into the medium, indicating that an ATP-dependent degradative pathway was operative. In contrast, no changes in total proteolysis were observed when calcium-dependent proteases were inhibited.

In agreement with the ATP-dependence of the process, a massive increase in the gene expression for some of the components of the ubiquitin-proteasome proteolytic system was found in gastrocnemius muscles from mice bearing the MAC 16 tumour. The up-regulation of the mRNA levels for the ubiquitin-conjugating enzyme (E2) and the C9 proteasome subunit, was in sharp contrast with the expression of these genes in muscles from non tumour-bearing controls. In addition, with the non cachexigenic MAC 13 tumour though also causing an up-regulation of the system, the effects were not as pronounced as those elicited by the MAC 16 tumour, indicating the basis for the increased muscle wasting associated with this cachexia-inducing model.

The elevated proteolysis associated with soleus muscle following *in vivo* treatment with p24, was found to be significantly inhibited when muscles were incubated in the presence

of ATP-depleting agents. Furthermore, protein degradation was not inhibited by inhibitors of either lysosomal or calcium-dependent enzymes, suggesting that p24 could be responsible for the activation of the ATP-ubiquitin system and the protein loss.

Indeed, p24 was shown to cause an up-regulation of the ubiquitin-proteasome pathway as determined by the increase in the mRNA expression of E2, ubiquitin and C9 in gastrocnemius muscle, which were all significantly different from the PBS-injected controls. In addition, p24 also produced a small but significant increase in the amount of E2 mRNA in heart tissue.

Furthermore, when muscles were analysed for the presence of ubiquitin conjugates, these were found to be significantly elevated in gastrocnemius both from MAC 16 and p24-treated mice.

In conclusion, these results provide enough evidence to suggest that the mechanism of muscle wasting in the MAC 16 model mainly results from the ability of p24 to activate the ATP-dependent ubiquitin-proteasome pathway, which not only has also been reported in other experimental models of cachexia (Llovera *et al*, 1994 and 1995 and Baracos *et al*, 1995), but also plays a major role in other muscle wasting conditions such as starvation, fasting, sepsis , denervation atrophy and many others (Wing *et al*, 1991 and 1995; Tiao *et al*, 1994 and 1997; Medina *et al*, 1995 and Wing and Goldberg 1993).

In addition to its influence on the rate of muscle protein breakdown, p24 was also found to inhibit protein synthesis in this tissue. Both the increased proteolysis and depressed synthesis rates were shown to be attenuated in mice which had been pretreated with the monoclonal antibody.

In the C_2C_{12} mouse skeletal muscle cell line, p24 was shown to produce an inhibition of protein synthesis following a short-time incubation (90 min), suggesting a blockage of the

translational process. This again appeared to be specific to the tumour glycoprotein since it was completely blocked by the inclusion of the monoclonal antibody in the incubation medium. Furthermore, the inhibition of the rate of protein synthesis was completely abolished by insulin, a translational activator, indicating that p24 may be acting through one of the signalling pathways leading to the phosphorylation and subsequent inactivation of initiation factors or ribosomal proteins, although this still remains to be further investigated.

Besides the inhibition of protein synthesis, p24 also produced an increase in both total and myofibrillar protein breakdown in these cells. The use of this cell line provides an excellent model to study the degradation of myofibrillar proteins since this can now be readily determined by measuring the amount of 3-methylhistidine released into the incubation medium. A recently developed technique which combines gas chromatography (GC) and mass spectroscopy (MS) (Thompson *et al*, 1996), allows the detection of this relatively less-abundant amino acid even in the presence of other amino acids at concentrations present in normal supplemented medium. Thus, the use of this method not only has the advantage of measuring myofibrillar protein breakdown from intact cells in culture, but in addition, it allows a correlation to be made on the effects of any catabolic agent to the activation of the ubiquitin system.

As for the influence of p24 on other body tissues, its effect appeared quite specific to skeletal muscle since no other organ seemed to be affected with regard to protein synthesis inhibition, except for heart where human p24 caused a significant reduction. Additionally, a small though significantly higher expression for E2 was also detected in this tissue, indicating that part of the total protein loss is also accounted for by the wasting of cardiac muscle.

Regarding the liver, no significant changes were detected in either protein synthesis or degradation, although there was a significant increase in the rate of protein synthesis in response to human p24. These results are in agreement with those in support of an

increased liver and WBPT rates and may explain the observed reduction in the concentration of most plasma amino acids.

From these, the significant decrease in the amount of alanine is particularly interesting since, additionally, it constitutes an important source for gluconeogenesis and if this process were increased in these animals, it would constitute a considerable drain of energy from the host. Therefore, this aspect needs to be further investigated since apart from satisfying the metabolic demands of the tumour, an increased glucose production could also explain the enhanced lipogenesis (Mulligan and Tisdale, 1991 and Tisdale and Leung, 1988) and elevated resting energy expenditure (Plumb *et al*, 1991) associated with this model.

In conclusion, this study has shown that a tumour-derived factor, with the ability to target skeletal muscle specifically, is capable of inducing a state of cachexia when injected into non tumour-bearing mice by promoting an accelerated loss of protein from this compartment. The mechanism behind this protein wasting appears to be a combination of both an increased proteolysis, which arises from the activation of the ubiquitin-proteasome proteolytic pathway, and a depression in the rate of protein synthesis perhaps as the result of a translational arrest.

Chapter 7
References

AGELL N, RYAN C & SCHLESINGER MJ (1991). Partial purification and substrate specificity of a ubiquitin hydrolase from *Saccharomyces cerevisiae*. *Biochemical Journal* 273: 615-620.

ANDERSEN MW, BALLAL NR, GOLDKNOPF IL & BUSH H (1981). Protein A24 lyase activity in nucleoli of thioacetamide-treated rat liver releases histone 2A and ubiquitin from conjugated protein A24. *Biochemistry* 20: 1100-1104.

ANGEL J, BERENBAUM F, LE DENMAT C, NEVALAINEN T, MASLIAH J & FOURNIER C (1994). Interleukin-1-induced prostaglandin E_2 biosynthesis in human synovial cells involves the activation of cytosolic phospholipase A_2 and cyclooxygenase-2. *European Journal of Biochemistry* 226: 125-131.

ANGEL P & KARIN M (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta* 1072:129-157.

ANKER SD, PONIKOWSKI P, VARNEY S, PEN CUA T, CLARK AL, WEBB-PEPLOE KM, HARRINGTON D, KOX WJ, POOLE-WILSON PA & COATS AJS (1997). Wasting as independent risk factor for mortality in chronic heart failure. *The Lancet* 349: 1050-1053.

ARAI T, ARAI A, BUSBY WH & CLEMMONS DR (1994). Glycosaminoglycans inhibit degradation of insulin-like growth factor-binding protein-5. *Endocrinology* 135 (6): 2358-2363.

ARGILES JM & LOPEZ-SORIANO FJ (1991). The energy state of tumour-bearing rats. *Journal of Biological Chemistry* 266: 2978-2982.

ARRIGO AP & MEHLEN (1993). HeLa cells proteasome interacts with leucine-rich polypeptides and contains a phosphorylated subunit. *Biochemical and Biophysical Research Communications* 194 (3): 1387-1393.

AYRE KJ & HULBERT AJ (1996). Dietary fatty acid profile influences the composition of skeletal muscle phospholipids in rats. *Journal of Nutrition* 126: 653-662.

AZPIAZU I, ALTIEL AR, DePAOLI-ROACH AA & LAWRENCE JC Jr (1996). Regulation of both glycogen synthase and PHAS-1 by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent rapamycin sensitive pathways. *Journal of Biological Chemistry* 271: 5033-5039.

BAILEY JL, WANG X, ENGLAND BK, PRICE SR, DING X & MITCH WE (1996). The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin-proteasome pathway. *Journal of Clinical Investigation* 97: 1447-1453.

BALKWILL F, BURKE F, TALBOT D, TAVERNER J, OSBOURNE R, NAYLOR S, DURBIN H & FIERS W (1985). Evidence for TNF/cachectin production in cancer. *The Lancet* 2: 1229-1232.

BARACOS VE, DEVIVO C, HOYLE DHR & GOLDBERG AL (1995). Activation of the ATP-ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. *American Journal Physiology* 268: E996-E1006.

BARACOS VE & GOLDBERG AL (1986). Maintenance of normal length improves protein balance and energy status in isolated rat skeletal muscles. *American Journal of Physiology* 251: C588-C596.

BARACOS V, GREENBERG RE & GOLDBERG AL (1986). Influence of calcium and other divalent cations on protein turnover in rat skeletal muscle. *American Journal of Physiology* 250: E702-E710.

BARACOS V, RODEMANN HP, DINARELLO CA & GOLDBERG AL (1983). Stimulation of muscle protein degradation and prostaglandin E_2 release by leukocytic pyrogen (interleukin-1). *The New England Journal of Medicine* 308 (10): 553-58.

BARDEN N & LABRIE F (1973). Cyclic adenosine 3'-5'-monophosphate dependent phosphorylation of ribosomal proteins from bovine anterior pituitary gland. *Biochemistry* 12: 3096-3102.

BARDOCZC S & HESKETH JE (1989). Effect of RNA synthesis inhibitors on insulin-induced protein synthesis by 3T3 cells. *International Journal of Biochemistry* 21 (11): 1265-1268.

BECK SA, MULLIGAN HD & TISDALE MJ (1990). Lipolytic factors associated with murine and human cancer cachexia. *Journal of the National Cancer Institute* 82: 1922-1926.

BECK SA, SMITH KL & TISDALE MJ (1991). Anticachectic and antitumour effect of eicosapentaenoic acid and its effect on protein turnover. *Cancer Research* 51: 6089-6093.

BECK SA & TISDALE MJ (1987). Production of lipolytic and proteolytic factors by a murine tumor-producing cachexia in the host. *Cancer Research* 47: 5919-5923.

BECK SA & TISDALE MJ (1989). Nitrogen excretion in cancer cachexia and its modification by a high fat diet in mice. *Cancer Research* 49: 3800-3804.

BECK SA & TISDALE MJ (1991). Lipid mobilising factors specifically associated with cancer cachexia. *British Journal of Cancer* 62: 846-850.

BEHME MT (1996). Dietary fish oil enhances insulin sensitivity in miniature pigs. *Journal of Nutrition* 126: 1549-1553.

BELIZARIO JE, KATZ M, CHENKER E & RAW I (1991). Bioactivity of skeletal muscle proteolysis-inducing factors in the plasma proteins from cancer patients with weight loss. *British Journal of Cancer* 63: 705-710.

BERCOVICH B, STANCOVSKI I, MAYER A, BLUMENFELD N, LASZLO A, SCHWARTZ AL & CIECHANOVER A (1997). Ubiquitin-dependent degradation of certain protein substrates *in vitro* requires the molecular chaperone Hsc 70. *Jounal of Biological Chemistry* 272: 9002-9010.

BERLIN T, CRONESTRAND R, NOWAK J, SONNENFELD T & WENNMALM A (1979). Conversion of arachidonic acid to prostaglandins in homogenates of human skeletal muscle and kidney. *Acta Physiologica Scandinavica* 106: 441-445.

BEUTLER B & CERAMI A (1986). Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* 320: 584-588.

BIBBY MC, DOUBLE JA, ALI SA, FEARON KCH, BRENNAN RA & TISDALE MJ (1987). Characterisation of a transplantable adenocarcinoma of the

mouse colon producing cachexia in recipient animals. *Journal of the National Cancer Institute* 78 (3): 539-545.

BIRD JW, CARTER JH, TRIEMER RE, BROOKS RM & SPANIER AM (1980). Proteinases in cardiac skeletal muscle. *Federation Proceedings* 39: 20-25.

BRENNAN MF (1977). Uncomplicated starvation *versus* cancer cachexia. *Cancer Research* 37: 2359-2364.

BRENNAN MF & BURT ME (1981). Nitrogen metabolism in cancer patients. *Cancer Treatment Reports* 65 (5): 67-78.

BRIDDON S, BECK SA & TISDALE MJ (1991). Changes in activity of lipoprotein lipase, plasma free fatty acids and triglycerides with weight loss in a cachexia model. *Cancer Letters* 57: 49-53.

BROSTOM MA, CHIN KV, CADE C, GMITTER D & BROSTOM CO (1987). Stimulation of protein synthesis in pituitary cells by phorbol esters and cAMP. *Journal of Biological Chemistry* 262: 16515-16523.

BUHL WJ, YU B & GEHRING U (1994). Signal transduction pathways leading to arachidonic acid release from neutrophilic HL-60 cells. The involvement of G protein, protein kinase C and phospholipase A_2. *Cellular Signalling* 7 (4): 351-364.

BURKHARD SJ & TRAUGH JA (1983). Changes in ribosome function by cAMP-dependent and cAMP-independent phosphorylation of ribosomal protein S6. *Journal of Biological Chemistry* 258: 14003-14008.

BUSCH WA, STROMER MH, GOLL DE & SUZUKI A (1972). Ca^{2+}-specific removal of Z-lines from rabbit skeletal muscle. *Journal of Cell Biology* 52: 367-381.

BUSE MG, BIGGENS SJ, FRIDERICI KH & BUSE JF (1972). Oxidation of branched-chain amino acids by isolated hearts and diaphragms of the rat. The effect of fatty acids, glucose and pyruvate respiration. *Journal of Biological Chemistry* 247: 8085-8096.

CANGIANO C, LAVIANO A, MEGUID MM, MULIERI M, CONVERSANO L, PREZIOSA I & ROSSI-FANELLI F (1996). Effects of administration of oral

branched-chain amino acids on anorexia and caloric intake in cancer patients. *Journal of the National Cancer Institute* 88 (8): 50.

CARIUK P, LORITE MJ, TODOROV PT, FIELD WN, WIGMORE SJ & TISDALE (1997). Induction of cachexia in mice by a product isolated from the urine of cachectic cancer patients. *British Journal of Cancer* 76: 606-613.

CARRASCOSA JM, MARTINEZ P & NUÑEZ DE CASTRO I (1984). Nitrogen movement between host and turnover in mice inoculated with Ehrlich ascitic tumor cells. *Cancer Research* 44: 3831-3835.

CHARTERS Y & GRIMBLE RF (1989). Effect of recombinant human necrosis factor α on protein synthesis in liver, skeletal muscle and skin of rats. *Biochemical Journal* 258: 493-497.

CIECHANOVER A, FINLEY D & VARSHAVSKI A (1984). Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. *Cell* 37: 57-66.

CIECHANOVER A, HOD Y & HERSHKO A (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic pathway. *Biochemical and Biophysical Research Communications* 81: 1100-1105.

CIECHANOVER A & SCHWARTZ AL (1989). How are substrates recognized by the ubiquitin-mediated proteolytic system?. *Trends in Biochemical Sciences* 14: 483-488.

CIECHANOVER A, WOLIN SL, STEITZ JA & LODISH HF (1985). Transfer RNA is an essential component of the ubiquitin- and ATP-dependent proteolytic system. *Proceedings of the National Academy of Sciences, USA* 82: 1341-1345.

CLARK C & GOODLAD GAJ (1971). Depletion of proteins of phasic and tonic muscles in tumour-bearing rats. *European Journal of Cancer* 7: 3-9.

CLARK CM & GOODLAD GAJ (1975). Muscle protein biosynthesis in the tumour-bearing rat. A defect in a post-initiation stage of translation. *Biochimica et Biophysica Acta* 378: 230-240.

CLARKE EF, LEWIS AM & WATERHOUSE C (1978). Peripheral amino acid levels in patients with cancer. *Cancer* 42: 2909-2913.

COHN SH (1986). Body composition of patients with malnutrition and cancer. *Clinics in Oncology* 5 (2): 263-275.

COMBARET L, TAILLANDIER D, VOISIN L, SAMUELS SE, BOESPFLUG-TANGUY O & ATTAIX D (1996). No alteration in gene expression of components of the ubiquitin-proteasome proteolytic pathway in dystrophin-deficient muscles. *FEBS Letters* 393: 292-296.

CONG JY, GOLL DE, PETERSON AM & KAPPRELL HP (1989). The role of autolysis in activity of the Ca^{2+}-dependent proteinases (μ-calpain and m-calpain). *Journal of Biological Chemistry* 264: 10096-10103.

COOLICAN SA, HAIECH J & HATHAWAY DR (1986). The role of subunit autolysis in activation of smooth muscle Ca^{2+}-dependent proteases. *Journal of Biological Chemistry* 261: 4170-4176.

COSTA G (1977). Cachexia, the metabolic component of neoplastic diseases. *Cancer Research* 37: 2327-2335.

COSTA G & HOLLAND JF (1962). Effects of Krebs-2 carcinoma on the lipide metabolism of male swiss mice. *Cancer Research* 22: 1081-1083.

COSTELLI P, CARBO N, TESSITORE L, BAGBY GJ, LOPEZ-SORIANO FJ, ARGILES JM & BACCINO FM (1993). Tumour necrosis factor-α mediates changes in tissue protein turnover in a rat cancer cachexia model. *Journal of Clinical Investigation* 92: 2783-2789.

COSTELLI P, GARCIA-MARTINEZ C, LLOVERA M, CARBO N, LOPEZ-SORIANO FJ, AGELL N, TESSITORE L, BACCINO FM & ARGILES JM (1995). Muscle protein waste in tumour-bearing rats is effectively antagonized by a β_2-adrenergic agonist (clenbuterol). *Journal of Clinical Investigation* 95: 2367-2372.

COSTELLI P, LLOVERA M, LOPEZ-SORIANO J, CARBO N, TESSITORE L, LOPEZ-SORIANO FJ, BACCINO FM & ARGILES JM (1995a). Lack of effect of eicosapentaenoic acid in preventing cancer cachexia and inhibiting tumor growth. *Cancer Letters* 71: 25-32.

COSTELLI P, LLOVERA M, LOPEZ-SORIANO J, CARBO N, GARCIA-MARTINEZ, LOPEZ-SORIANO FJ, & ARGILES JM (1995b). Interleukin-1 receptor antagonist (IL-1ra) is unable to reverse cachexia in rats bearing an ascites hepatoma (Yoshida AH-130). *Cancer Letters* 95: 33-38.

CROSS DAE, ALESSI DR, VANDENHEEDE JR, McDOWELL HE, SUNDAL HS & COHEN P (1994). The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor-1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochemical Journal* 303: 21-26.

DAMON DH, LOBB RR, D'AMORE PA & WAGNER JA (1989). Heparin potentiates the action of acidic fibroblast growth factor by prolonging its biological half-life. *Journal of Cellular Physiology* 138: 221-226.

DANERYD PLE, HAFSRÖM LS & KARLBERG IH (1990). Effects of spontaneous physical exercise on experimental cancer anorexia and cachexia. *European Journal of Cancer* 26 (10): 1083-1088.

DARVERET D, SORNET C, TAILLANDIER D, SAVARY I, ATTAIX D & GRIZARD J (1995). Sensitivity and protein turnover response to glucocorticoids are different in skeletal muscle from adult and old rats. *Journal of Clinical Investigation* 96: 2113-2119.

DAVIS RJ (1993). The mitogen-activated protein kinase signal transduction pathway. *Journal of Biological Chemistry* 268: 14553-14556.

DAYTON WR, GOLL DE, ZEECE MG, ROBSON RM & REVILLE WJ (1976a). A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry* 15: 2151-2158.

DAYTON WR, REVILLE WJ, GOLL DE & STROMER MH (1976b). A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterisation of the purified enzyme. *Biochemistry* 15: 2159-2167.

DEAN RT (1975). Direct evidence of importance of lysosomes in degradation of intracellular proteins. *Nature* 257: 414-416.

deGROOT RP, BALLON LM & SASSONE-CORSI P (1994). Positive regulation of the cAMP-responsive activator CREM by mitogen-induced gene expression. *Cell* 79: 81-91.

DEPHILIP RM, CHADWICK DE, IGNOTZ RA, LYNCH WE & LIEBERMAN I (1979). Rapid stimulation by insulin of ribosome synthesis in cultured chick embryo fibroblasts. *Biochemistry* 18: 4812-4817.

DE WYS WD (1979). Anorexia as a general effect of cancer. *Cancer* 43: 2013-2019.

DE WYS W (1985). Management of cancer cachexia. *Seminars in Oncology* 12 (4): 452-460.

DE WYS WD (1986). Weight loss and nutritional abnormalities in cancer patients: incidence, severity and significance. *Clinics in Oncology* 5 (2): 251-261.

DE WYS WD, BEGG C, LAVIN PT, BAND PR, BENNETT JM, BERTINO JR, CONEN MH, DOUGLAS JR HD & ENGSTRON PF (1980) Prognostic effect of weight loss prior to chemotherapy in cancer patients. *American Journal of Medicine* 69: 491-497.

DHOLAKIA JN & WAHBA AJ (1988). Phosphorylation of the guanine nucleotide exchange factor from rabbit reticulocytes regulates its activity in polypeptide chain initiation. *Proceedings of the National Academy of Sciences, USA* 85: 51-54.

DIEZ E, CHILTON FH, STROUP G, MAYER RJ, WINKLER JD & FONTEH AN (1994). Fatty acid and phospholipid selectivity of different phospholipase A_2 enzymes studied by using a mammalian membrane as substrate. *Biochemical Journal* 301: 721-726.

DOBROWSKY RT & HANNUN YA (1992). Ceramide stimulates a cytosolic protein phosphatase. *Journal of Biological Chemistry* 267: 5048-5051.

DOUBLE JA, BALL CR & COWEN PN (1975). Transplantation of adenocarcinomas of the colon in mice. *Journal of the National Cancer Institute* 54 (1): 271-275.

DRAKE SL, KLEIN DJ, MICKELSON DJ, OEGEMA TR, FURCHT LT & McCARTHY JB (1992). Cell surface phosphatidylinositol-anchored heparan sulfate

proteoglycan initiates mouse melanoma cell adhesion to a fibronectin-derived, heparin-binding synthetic peptide. *The Journal of Cell Biology* 117 (6): 1331-1341.

DUBIEL W, FERRER K, PRATT G & RECHSTEINER (1992). Subunit 4 of the 26S protease is a member of a novel eukaryotic ATPase family. *Journal of Biological Chemistry* 267: 22699-22702.

DUNCAN R & HERSHEY JWB (1985). Regulation of initiation factors during translational repression caused by serum depletion. *Journal of Biological Chemistry* 260: 5486-5492.

DUPONT-VERSTEEGDEN EE & McCARTER RJ (1992). Differential expression of muscular dystrophy in diaphragm versus hindlimb muscles of *mdx* mice. *Muscle and Nerve* 15: 1105-1110.

DZIARSKI R (1994). Cell-bound albumin is the 70-kDa peptidoglycan-, liposaccharide-, and lipoteichoic acid-binding protein on lymphocytes and macrophages. *The Journal of Biological Chemistry* 26: 20431-20436.

EBISUI C, TSUJINAKA T, MORIMOTO T, KAN K, ILJIMA S, YANO M, KOMINAMI E, TANAKA K & MONDEN M (1995). IL-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C_2C_{12} myotubes. *Clinical Science* 89: 431-439.

EDWARDS DR (1994). Cell signalling and the control of gene transcription. *TiPS* 15: 239-244.

ELIAS S & CIECHANOVER A (1990). Post-translational addition of an arginine moiety to acidic NH_2 termini of proteins is required for their recognition by ubiquitin-protein ligase. *Journal of Biological Chemistry* 265: 15511-15517.

EMERY PW, EDWARDS RHT, RENNIE MJ, SOUHAMI RL & HALLIDAY D (1984). Protein synthesis in muscle measured *in vivo* in cachectic patients with cancer. *British Medical Journal* 289: 584-586.

EMERY PW, LOWELL L & RENNIE MJ (1984). Protein synthesis measured *in vivo* in muscle and liver of cachectic tumor-bearing mice. *Cancer Research* 44: 2779-2784.

FALCONER JS, FEARON KCH, PLESTER CE, ROSS JA & CARTER DC (1994). Cytokines, the acute-phase response, and resting energy expenditure in cachectic patients with pancreatic cancer. *Annals of Surgery* 219 (4): 325-331.

FEARON KCH, BORLAND W, PRESTON T, TISDALE MJ, SHENKIN A & CALMAN KC (1988a). Cancer cachexia: influence of systemic ketosis on substrate levels and nitrogen metabolism. *American Journal of Nutrition* 47: 42-48.

FEARON KC & CARTER DC (1988). Cancer cachexia. *Annals of Surgery* 208 (1): 1-5.

FEARON KCH, HANSELL DT, PRESTON T , PLUMB JA, DAVIES J, SHPAIRO D & SHENKIN A (1988). Influence of whole body protein turnover rate on resting energy expenditure in patients with cancer. *Cancer Research* 48: 2590-2595.

FEARON KCH, McMILLAN DC, PRESTON T, WINSTANLEY FP, CRUIKSHANK AM & SHENKIN A (1991). Elevated circulating interleukin-6 is associated with an acute-phase response but reduced fixed hepatic protein synthesis in patients with cancer. *Annals of Surgery* 213 (1): 26-31.

FERBER S & CIECHANOVER A (1986). Transfer RNA is required for conjugation of ubiquitin to selective substrates of the ubiquitin- and ATP-dependent proteolytic system. *Jounal of Biological Chemistry* 261: 3128-3134.

FERBER S & CIECHANOVER A (1987). Role of arginine-tRNA in protein degradation by the ubiquitin pathway. *Nature* 326: 808-811.

FLORES EA, BISTRIAN BR, POMPOSELLI JJ, DINARELLO CA, BLACKBURN GL & IFSTAN NW (1989). Infusion of tumour necrosis factor/cachectin promotes muscle protein catabolism in the rat. *Journal of Clinical Investigation* 83: 1614-1622.

FREDRIX EWHM, SOETERS PB, WOUTERS EFM, DEERENBERG IM, von MEYENFELDT MF & SARIS WHM (1991). Effect of different tumor types on resting energy expenditure. *Cancer Research* 52: 6138-6141.

FREUND H, HOOVER HC, ATAMIAN S & FISCHER JE (1979). Infusion of the branched-chain amino acids in post-operative patients. *Annals of Surgery* 190 (1): 18-23.

FUJIMOTO-OUCHI K, TAMURA S, MORI K, TANAKA & ISHITSUKA (1995). Establishment and characterization of cachexia-inducing and -non-inducing clones of murine colon 26 carcinoma. *International Journal of Cancer* 61: 522-528.

FULKS RM, LI JB & GOLDBERG AL (1975). Effects of insulin, glucose and aminoacids on protein turnover in rat diaphragm. *The Journal of Biological Chemistry* 250: 290-298.

FUMAGALLI L, BUSINARO R, NORI SL, TOESCA A, POMPILI E, EVANGELISTI E, GIANNETTI S, IPPOLITI F & DE RENZIS G (1996). Protease inhibitors in mouse skeletal muscle: tissue-associated components of serum inhibitors and calpastatin. *Cellular and Molecular Biology* 42 (4): 535-546.

GARCIA-MARTINEZ C, LLOVERA M, AGELL N, LOPEZ-SORIANO FJ & ARGILES J (1995). Ubiquitin gene expression in skeletal muscle is increased during sepsis: involvement of TNF-α but not IL-1. *Biochemical and Biophysical Research Communications* 217 (3): 839-844.

GARCIA-MARTINEZ C, LOPEZ-SORIANO FJ & ARGILES JM (1993). Acute treatment with tumour necrosis factor-α induces changes in protein metabolism in rat skeletal muscle. *Molecular and Cellular Biochemistry* 125: 11-18.

GARCIA-MARTINEZ C, LOPEZ-SORIANO FJ & ARGILES JM (1994). Interleukin-6 does not activate protein breakdown in rat skeletal muscle. *Cancer Letters* 76: 1-4.

GELIN J, ANDERSSON C & LUNDHOLM (1991). Effects of indomethacin, cytokines and cyclosporin A on tumor growth and the subsequent development of cancer cachexia. *Cancer Research* 51: 880-885.

GLASER T & KOSOWER NS (1986). Fusion of rat erythrocytes by membrane-mobility agent A_2C depends on membrane proteolysis by a cytoplasmic calpain. *European Journal of Biochemistry* 159: 387-392.

GLOVER CVC (1982). Heat shock induces rapid dephosphorylation of a ribosomal protein in Drosophila. *Proceedings of the National Academy of Sciences, USA* 79: 1781-1785.

GOLDBERG AL, BARACOS V, RODEMANN P, WAXMAN L & DINARELLO C (1984). Control of protein degradation in muscle by prostaglandins, Ca^{2+} and leukocytic pyrogen (interleukin 1). *Federation Proceedings* 43 (5): 1301-1306.

GOLL DE, DAYTON WR, SINGH I & ROBSON RM (1991). Studies of the α-actinin/actin interaction in the Z-disk by using calpain. *Journal of Biological Chemistry* 266: 8501-8510.

GOLL DE, THOMPSON VF, TAYLOR RG & ZALEWSKA T (1992). Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin?, *Bioessays* 14 (8): 549-556.

GONDA DK, BACHMAIR A, WUNNING I, TOBIAS JW, LANE WS & VARSHAVSKY A (1989). Universality and structure of the N-end rule. *Journal of Biological Chemistry* 264: 16700-16712.

GOODLAD GAJ & CLARK CM (1972). Activity of gastrocnemius and soleus polyribosomes in rats bearing the Walker 256 carcinoma. *European Journal of Cancer* 8: 647-651.

GOODMAN MN (1987). Acute alterations in sodium flux *in vitro* lead to decreased myofibrillar protein breakdown in rat skeletal muscle. *Biochemical Journal* 247: 151-156.

GOODMAN MN (1991). Tumour necrosis factor induces skeletal protein breakdown in rats. *American Journal of Physiology* 260: E727-E730.

GRAHAM R & GILMAN M (1991). Distinct protein targets for signals acting at the c-*fos* serum response element. *Science* 251: 133-240.

GRESSNER AM & VAN de LEUR (1980). Interaction of synthetic polynucleotides with small rat liver ribosomal subunits possessing low and highly phosphorylated protein S6. *Biochimica et Biophysica Acta* 608: 459-468.

GROPPER R, BRANDT RA, ELIAS S, BEARER CF, MAYER A, SCHWARTZ AL & CIECHANOVER A (1991). The ubiquitin-activating enzyme, E1, is required for the stress-induced lysosomal degradation of cellular proteins. *Jounal of Biological Chemistry* 266: 3602-3610.

GROUNWATER P, BECK SA, BARTON C, ADAMSON C, FERRIER IN & TISDALE MJ (1990). Alteration in serum and lipolytic activity with weight loss in cancer cachectic patients. *British Journal of Cancer* 62: 816-821.

HARMON CS, PROUD CG & PAIN VM (1984). Effects of starvation, diabetes and acute insulin treatment on the regulation of polypeptide-chain initiation in rat skeletal muscle. *Biochemical Journal* 223: 687-696.

HARNET WL (1952). Cause of death in 11,074 patients. A Survey of Cancer. *British Empire Cancer Campaign* : 26.

HASSELGREN PO, ZAMIR O, JAMES JH & FISHER JE (1990). Prostaglandin E_2 does not regulate total or myofibrillar protein breakdown in incubated skeletal muscle from normal or septic rats. *Biochemical Journal* 270: 45-50.

HATA A, AKITA Y, SUZUKI K & OHNO S (1993). Functional divergence of protein kinase C (PKC) family members. *Journal of Biological Chemistry* 268: 9122-9129.

HAUNER H, PETRUSCHKE T, RUSS M, RÖHRIG J & ECKEL J (1995). Effects of tumour necrosis factor α (TNFα) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia* 38: 764-771.

HAYSTEAD TAJ, HAYSTEAD CMM, HU C, LIN TA & LAWRENCE JC Jr (1994). Phosphorylation of PHAS-1 by mitogen-activated protein (MAP) kinase. *Journal of Biological Chemistry* 269: 23185-23191.

HEINEMEYER W, TRÖNDLE N, ALBRECHT G & WOLF DH (1994). *PRE5* and *PRE6*, the last missing genes encoding 20S proteasome subunits from yeast? Indication for a set of 14 different subunits in the eukaryotic proteasome core. *Biochemistry* 33: 12229-12237.

HENSHAW EC, HIRSCH CA, MORTON BE & HIATT HH (1971). Control of protein synthesis in mammalian tissues through changes in ribosome activity. *The Journal of Biological Chemistry* 246: 436-446.

HERSHKO A, CIECHANOVER A, HELLER H, HAAS AL & ROSE IA (1980). Proposed role of ATP in protein breakdown: conjugation of proteins with

multiple chains of the polypeptide of ATP-dependent proteolysis. *Proceedings of the National Academy of Sciences, USA* 77: 1783-1786.

HERSHKO A, HELLER H, EYTAN E, KAKLIJ G & ROSE IA (1984). The role of the α-amino group of protein in ubiquitin-mediated protein breakdown. *Proceedings of the National Academy of Sciences, USA* 81: 7021-7025.

HESKETH JE, CAMPBELL GP & REEDS PJ (1986). Rapid response of protein synthesis to insulin in 3T3 cells: effects of protein kinase C depletion and differences from the response to serum depletion. *Bioscience Reports* 6 (9): 797-804.

HESKETH JE, McKENZIE N & CAMPBELL GP (1988). Increased protein synthesis response to insulin in fibroblasts treated with the diacylglycerol kinase inhibitor R59022. *FEBS Letters* 241 (1-2):115-118.

HILDEBRANDT LA, SPENNETTA T, ELSON C & SHRAGO E (1995). Utilisation and preferred metabolic pathway of ketone bodies for lipid synthesis by isolated rat hepatoma cells. *American Journal of Physiology* 269: C22-C27.

HILENSKI LL, TERRACIO L, HAAS AL & BORG T (1992). Immunolocalization of ubiquitin conjugates at Z-bands and intercalated discs of rat cardiomyocytes in vitro and in vivo. *Journal of Histochemistry and Cytochemistry* 40 (7): 1037-1042.

HILT W & WOLF DH (1992). Stress-induced proteolysis in yeast. *Molecular Microbiology* 6 (17): 2437-2442.

HIRAI K, HUSSEY HJ, BARBER MD, PRICE SA, ISHIKO O & TISDALE MJ (1997). Biological evaluation of lipid-mobilizing factor (LMF) isolated from the urine of cancer patients. Submitted for publication.

HIRAO T, HARA K & TAKAHASKI K (1984). Purification and characterisation of cathepsin B from monkey skeletal muscle. *Journal of Biochemistry* 95: 871-879.

HIRATA F (1987). Drugs that inhibit the activities or activation of phospholipases and other acylhydrolases. In: Handbook of eicosanoids: prostaglandins and related compounds, volume II: Drugs acting via the eicosanoids, *CRC press*, Florida.

HIRATA F, SCHIFFMANN E, VENKATASUBRAMANIAN K, SALOMON D & AXELROD J (1980). A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proceedings of the National Academy of Sciences, USA* 77: 2533-2536.

HOCHSTRASSER M (1996). Protein degradation or regulation: Ub the judge. *Cell* 84: 813-815.

HOLLANDER DM, EBERT EC, ROBERTS AI & DEVEREUX DF (1986). Effects of tumour type and burden on carcass lipid depletion in mice. *Surgery* 100 (2): 292-297.

HOLROYDE CP, GABUZDA TG, PUTNAN RC, PAUL P & REICHARD GA (1975). Altered glucose metabolism in metastatic carcinoma. *Cancer Research* 35: 3710-3714.

HOLROYDE CP, SKUTCHES CL, BODEN G & REICHARD GA (1984). Glucose metabolism in cachectic patients with colorectal cancer. *Cancer Research* 44: 5910-5913.

HONG DH & FORSBERG NE (1995). Effects of dexamethasone on protein degradation and protease gene expression in rat L8 myotube cultures. *Molecular and Cellular Endocrinology* 108: 199-209.

HUDSON EA & TISDALE MJ (1994). Alterations in plasma and tumour levels of fatty acids with weight loss in an experimental cachexia model. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 50: 229-234.

HUISSMAN W, LANTING L, BOUMA JMW & CRUBER M (1974). Proteolysis at neutral pH in a lysosomal-cytosol system cannot be attributed to the uptake of proteins into lysosomes. *FEBS Letters* 45: 129-131.

HULKOWER KI, HOPE WC, CHEN T, ANDERSON CM, COFFEY JW & MORGAN DW (1992). Interleukin-1β stimulates cytosolic phospholipase A_2 in rheumatoid sinovial fibroblasts. *Biochemical and Biophysical Research Communications* 184: 712-718.

HYLTANDER A, DROTT C, KÖRNER U, SANDSRÖM R & LUNDHOLM K (1991). Elevated energy expenditure in cancer patients with solid tumours. *European Journal of Cancer* 27 (1): 9-15.

HYLTANDER A, KÖRNER U & LUNDHOLM KG (1993). Evaluation of mechanisms behind elevated energy expenditure in cancer patients with solid tumours. *European Journal of Clinical Investigation* 23: 46-52.

HYLTANDER A, SANDSTRÖM R & LUNDHOLM M (1994). Nutritional support. *Handbook of supportive care in cancer* : 169-187.

ILIAN MA & FORSBERG NE (1992). Gene expression of m- and μ-calpain and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. *Biochemical Journal* 287: 163-171.

INOMATA M, HAYASHI M, NAKAMURA M, SAITO Y, KON K & KAWASHIMA S (1989). Properties of erythrocyte membrane binding and autolytic activation of calcium activated neutral protease. *Journal of Biological Chemistry* 264: 18838-18843.

ISOZAKI Y, MITCH WE, ENGLAND BK & PRICE SR (1996). Protein degradation and increased mRNAs encoding proteins of the ubiquitin-proteasome proteolytic pathway in BC_3H1 myocytes require an interaction between glucocorticoids and acidification. *Proceedings of the National Academy of Sciences, USA* 93: 1967-1971.

IWAGAKI H, HIZUTA A, TANAKA N & ORITA K (1995a). Decreased serum tryptophan in patients with cancer cachexia correlates with increased serum neopterin. *Immunological Investigations* 24 (3): 467-478.

IWAGAKI H, HIZUTA A, TANAKA N & ORITA K (1995b). Plasma neopterin/C-reactive protein ration as an adjunct to the assessment of infection and cancer cachexia. *Immunological Investigations* 24 (3): 479-487.

JARIEL-ENCONTRE I, MARTIN PM, CARILLO S, SALVAT C & PIECHACZYK M (1995). Ubiquitylation is not an absolute requirement for degradation of c-Jun protein by the 26S proteasome. *Journal of Biological Chemistry* 270: 11623-11627.

JEEVANADAM M, HOROWITZ GD, LOWRY SF & BRENNAN MF (1984). Cancer cachexia and protein metabolism. *The Lancet* 1: 1423-1426.

JEEVANADAM M, HOROWITZ GD, LOWRY SF & BRENNAN MF (1986). Cancer cachexia and the rate of whole body lipolysis in man. Metabolism 35 (4): 304-310.

JEFFERIES HBJ, REINHARD C, KOZMA SC & THOMAS G (1994). Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proceedings of the National Academy of Sciences, USA* 91: 4441-4445.

JEFFREY IW, KELLY FJ, DUNCAN R, HERSHEY JWB & PAIN VM (1990). Effect of starvation and diabetes on the activity of the eukaryotic initiation factor eIF-2 in rat skeletal muscle. *Biochimie* 72: 751-757.

JENNISSEN HP (1995). Ubiquitin and the enigma of intracellular protein degradation. *European Journal of Biochemistry* 231: 1-30.

JEPSON MM, PELL JM, BATES PC & MILLWARD DJ (1986). The effects of endotoxaemia on protein metabolism in skeletal muscle and liver of fed and fasted rats. *Biochemical Journal* 235: 329-336.

JOHANNSSON E, McCULLAGH KJA, HAN XX, FERNANDO PK, JENSEN J, DAHL HA & BONEN A (1996). Effect of overexpressing GLUT-1 and GLUT-4 on insulin- and contraction-stimulated glucose transport in muscle. *American Journal of Physiology* 271: E547-E555.

JONAT C, RAHMSDORF HJ, PARK KK, CATO ACB, GEBEL S, PONTA H & HERRLICH P (1990). Antitumour promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 62: 1189-1204.

JONNALAGADDA S, BUTT TR, MONIA BP & MIRABELLI CK (1989). Multiple (α-NH-ubiquitin) protein endoproteases in cells. *Journal of Biological Chemistry* 264: 10637-10642.

KALGUTKAR AS, CREWS BC & MARNETT LJ (1996). Kinetics of the interaction of nonsteroidal antiinflammatory drugs with prostaglandin endoperoxide synthase-1 studied by limited proteolysis. *Biochemistry* 35:9076-9082.

KALLINOWSKI F, RUNKEL S, FORTMEYER HP, FÖRSTER H & VAUPEL P (1987). L-Glutamine: a major substrate for tumour cells *in vivo* ?. *Journal of Cancer Research and Clinical Oncology* 113: 209-215.

KAPLAN I, KATIC V, CULO F & CUK V (1992). Prognostic significance of plasma prostaglandin concentration with heart and neck cancer. *Journal of Cancer Research and Clinical Oncology* 118: 308-313.

KARINCH AM, KIMBALL SR, VARY TC & JEFFERSON LS (1993). Regulation of eukaryotic initiation factor-2B activity in muscle of diabetic rats. *American Journal of Physiology* 264: E101-E108.

KAWAKAMI M, KONDO Y, IMAI Y, HASHIGUCHI M, OGAWA H, HIRAGUN A, AOTSUKA S, SHIBATA S, ODA T, MURASE T & TAKAKU F (1991). Suppression of lipoprotein lipase in 3T3-L1 cells by a mediator produced by SEKI melanoma, a cachexia-inducing human melanoma cell line. *Journal of Biochemistry* 109:78-82.

KELLER BT, LANDES GM & KITOS PA (1982). Evidence for more than one mechanism of action of the glucocorticoid hormones. *Biochimica et Biophysica Acta* 717: 228-235.

KHAN S (1996). Mechanistic studies on a putative cancer cachectic factor. *PhD thesis*, Aston University.

KIEN CL & CAMITA BM (1983). Increased whole-body protein turnover in sick children with newly diagnosed leukemia or lymphoma. *Cancer Research* 43: 5586-5592.

KIMBAL SR & JEFFERSON LS (1988). Effects of diabetes of guanine nucleotide exchange factor activity in skeletal muscle and heart. *Biochemical and Biophysical Research Communications* 156 (2): 706-711.

KIMBAL SR, VARY TC & JEFFERSON LS (1992). Age-dependent decrease in the amount of eukaryotic initiation factor 2 in various rat tissues. *Biochemical Journal* 286: 263-268.

KITADA S, HAYS EF & MEAD JF (1980). A lipid mobilizing factor in serum of tumour-bearing mice. *Lipids* 15: 168-174.

KITADA S, HAYS EF & MEAD JF (1981). Characterisation of a lipid mobilizing factors from tumours. *Progress in Lipid Research* 28:823-825.

KITADA S, HAYS EF, MEAD JF & ZABIN I (1982). Lipolysis in adipocytes by a protein from tumor cells. *Journal of Cellular Biochemistry* 20: 409-416.

KITAHARA A, TAKANO E, OHTSUKI H, KIRIHATA Y, YAMAGATA Y, KANNAGI R & MURACHI T (1986). Reversed distribution of calpains and calpastatin in human pituitary gland and selective localization of calpastatin in adrenocorticotropin-producing cells as demonstrated by immunohistochemistry. *Journal of Clinical Endocrinology and Metabolism* 63: 343-348.

KNAPP ML, AL-SHEIBANI S, RICHES PG, HANHAM IWF & PHILLIPS RH (1991). Hormonal factors associated with weight loss in patients with advanced breast cancer. *Annals of Clinical Biochemistry* 28: 480-486.

KNOWLES SE & BALLARD FJ (1976). Selective control of the degradation of normal and aberrant proteins in Reuber H35 hepatoma cells. *Biochemical Journal* 156: 609-617.

KNOX LS, CROSBY CO, FEURER ID, BUZBY GP, MILLER CL & MULLEN JL (1982). Energy expenditure in malnourished cancer patients. *Annals of Surgery* 197 (2): 152-162.

KOPP F, HENDIL KB, DAHLMANN B, KRISTENSEN P, SOBEK A & UERKVITZ W (1997). Subunit arrangement in the human 20S proteasome. *Proceedings of the National Academy of Sciences, USA* 94: 2939-2944.

KRAMER G, CIMADEVILLA JM & HARDESTY B (1976). Specificity of the protein kinase activity associated with the hemin-controlled repressor of rabbit reticulocyte. *Proceedings of the National Academy of Sciences, USA* 73 (9): 3078-3082.

KRAUSE R, JAMES JH, HUMPHREY C & FISCHER JE (1979). Plasma brain amino acids in Walker 256 carcinosarcoma-bearing rats. *Cancer Research* 39: 3065-3069.

KRESS M COSMAN D, KHOURY & JAY G (1983). Secretion of a transplantation-related antigen. *Cell* 34 : 189-196.

KROCZEK RA, GUNTER KC, GERMAIN RN & SHEVACH EM (1986). Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. *Nature* 332: 181-183.

KRODER G, BOSSENMAIER B, KELLERER M, CAPP E, STOYANOV B, MÜLHÖFER A, BERTI L, HORIKOSHI H, ULLRICH A & HARING H (1996). Tumour necrosis factor-α and hyperglycemia-induced insulin resistance. *Journal of Clinical Investigation* 97: 1471-1477.

KUMAMOTO T, KLEESE WC, CONG J, GOLL DE, PIERCE PR & ALLEN RE (1992). Localization of the Ca^{2+}-dependent proteinases and their inhibitor in normal fasted, and denervated rat skeletal muscle. *Anatomical Record* 232: 60-77.

LAI PBS, FEARON KCH, ANDERSON JD & CARTER DC (1996). Cell cycle arrest and induction of apoptosis in pancreatic cancer cells exposed to EPA *in vitro*. *British Journal of Cancer* 74: 1375-1383.

LANSTEIN HN, DOHERTY GM, FRAKER DL, BURESH CM & NORTON JA (1991). The roles of γ interferon and tumour necrosis factor-α in an experimental rat model of cancer cachexia. *Cancer Research* 51: 2302-2306.

LANZA-JACOBY S, LANSEY SC, MILLER EE & CLEARY MP (1984). Sequential changes in the activities of lipoprotein lipase and lipogenic enzymes during tumor growth in rats. *Cancer Research* 44: 5062-5067.

LANZA-JACOBY S, MILLER EE & ROSATO FE (1982). Changes in the activities of lipoprotein lipase and the lipogenic enzymes in tumor-bearing rats. *Lipids* 17 (12): 944-949.

LAUB M AND JENNISSEN HP (1991). Ubiquitination of myosin light chains. *Biological Chemistry Hoppe-Seyler* 372: 704.

LAZO PA (1981). Tumour induction of host leucine starvation. *FEBS Letters* 135 (2): 229-231.

LE BRICON T, CYNOBER L & BARACOS VE (1996). Protein Metabolism in cachectic tumour-bearing rats: effect of tumor excision. *Journal of Surgical Research* 60: 207-215.

LEGASPI A, JEEVANANDAM M, STARNES HF & BRENNAN MF (1987). Whole body lipid and energy metabolism in the cancer patient. *Metabolism* 36 (10): 958-963.

LEVISON MR, GROEGER JS, JEEVANANDAM M & BRENNAN MF (1988). Free fatty acid turnover and lipolysis in septic mechanically ventilated cancer-bearing humans. *Metabolism* 37 (7): 618-625.

LEWIS SEM, ANDERSON P & GOLDSPINK DF (1982). The effects of calcium on protein turnover in skeletal muscles of the rat. *Biochemical Journal* 204: 257-264.

LINDHAL M, BACKMAN E, HENRIKSSON KG, GOROSPE JR & HOFFMAN EP (1995). Phospholipase A_2 activity in distrophinopathies. *Neuromuscular Disorders* 5 (3): 193-199.

LISTER MD, DEEMS RA, WATANABE Y ULEVITCH RJ & DENNIS EA (1988). Kinetic analysis of the Ca^{2+}-dependent membrane-bound, macrophage PLA_2 and the effects of arachidonic acid. *The Journal of Biological Chemistry* 263: 7506-7513.

LLORET S, MARTINEZ J & MORENO JJ (1995). Influence of calcium and arachidonic acid mobilization by murine resident peritoneal macrophages. *Archives of Biochemistry and Biophysics* 323 (2): 251-257.

LLOVERA M, GARCIA-MARTINEZ C, AGELL N, LOPEZ-SORIANO FJ & ARGILES JM (1995). Muscle wasting associated with cancer cachexia is linked to an important activation of the ATP-dependent ubiquitin-mediated proteolysis. *International Journal of Cancer* 61: 138-141.

LLOVERA M, GARCIA-MARTINEZ C, AGELL N, LOPEZ-SORIANO FJ & ARGILES JM (1997). TNF can directly influence the expression of the ubiquitin-dependent proteolytic system in rat soleus muscle. *Biochemical and Biophysical Research Communications* 230 (2): 238-241.

LLOVERA M, GARCIA-MARTINEZ C, AGELL N, MARZABAL M, LOPEZ-SORIANO FJ & ARGILES JM (1994). Ubiquitin gene expression is increased in skeletal muscle of tumour-bearing rats. *FEBS Letters* 338: 311-318.

LLOVERA M, GARCIA-MARTINEZ C, COSTELLI P, AGELL N, CARBO N, LOPEZ-SORIANO FJ & ARGILES JM (1996). Muscle hypercatabolism during cancer cachexia is not reversed by the glucocorticoid receptor antagonist RU38486. *Cancer Letters* 96: 7-14.

LODISH H, BALTIMORE D, BERK A, ZIPURSKY SL, MATSUDAIRA P & DARNELL J (1995). Multicellularity: cell-cell and cell-matrix interactions. In: Molecular Cell Biology, third edition. *Scientific American Books*, New York.

LONNROTH C, MOLDAWER LL, GELIN J, LINDBLOM L, SHERRY B & LUNDHOLM (1990). Tumour necrosis factor-α and interleukin-1α production in cachectic, tumour-bearing mice. *International Journal of Cancer* 46: 889-896.

LOPES N, BLACK P, ASHFORD AJ & PAIN VM (1989). Protein metabolism in the tumour-bearing mouse. *Biochemical Journal* 264: 713-719.

LORITE MJ, CARIUK P & TISDALE MJ (1997). Induction of muscle protein degradation by a tumour factor. *British Journal of Cancer* 76: 1035-1040.

LOWELL BB, RUDERMAN NB & MN GOODMAN (1986). Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. *Biochemical Journal* 234: 237-240.

LUDEMANN R, LEREA KM & ETLINGER JD (1993). Copurification of casein kinase II with 20S, proteasomes and phosphorylation of a 30 KDa proteasome subunit. *Journal of Biological Chemistry* 268: 17413-17417.

LUNDHOLM K, EDSTRÖM S, EKMAN L, KARLBERG I & SCHERSTÈN T (1981). Metabolism in peripheral tissues in cancer patients. *Cancer Treatment Reports* 65 (5): 79-83.

LUNDHOLM K, EDSTROM S, EKMAN L, KARLBERG I, WALKER P & SCHERSTEN T (1981). Protein degradation in human skeletal muscle tissue: the effect of insulin, leucine, amino acids and ions. *Clinical Science* 60: 319-326.

LUO J, RIZKALLA SW, BOILLOT J, ALAMOWITCH C, CHAIB H, BRUZZO F, DESPLANQUE N, DALIX AM, DURAND G & SLAMA G (1996). Dietary (n-3) polyunsaturated fatty acids improve adipocyte insulin action and

glucose metabolism in insulin-resistant rats: relation to membrane fatty acids. *Journal of Nutrition* 126: 1951-1958.

MACFIE J, BURKINSHAW L, OXBY C, HOLMFIELS JHM & HILL GL (1982). The effect of gastrointestinal malignancy on resting metabolic expenditure. *British Journal of Surgery* 69: 443-446.

MACLENNAN PA, SMITH K, WERYK B, WATT PW & RENIE MJ (1988). Inhibition of protein breakdown by glutamine in perfused rat skeletal muscle. *FEBS Letters* 237: 133-136.

MAHONY SM, BECK SA & TISDALE MJ (1988). Comparison of weight loss induced by recombinant tumour necrosis factor with that produced by a cachexia-inducing tumour. *British Journal of Cancer* 57: 385-389.

MAHONY SM & TISDALE MJ (1989). Reversal of weight loss induced by tumour necrosis factor-alpha. *Cancer Letters* 45: 167-172.

MAHONY SM & TISDALE (1990). Metabolic effects of tumour necrosis alpha in NMRI mice. *British Journal of Cancer* 61: 514-519.

MAKI CG, HUIBREGTSE JM & HOWLEY PM (1996). *In vivo* ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Research* 56 (11): 2649-2654.

MANSOOR O, BEAUFRERE B, BOIRIE Y, RALLIERE C, TAILLANDIER D, AUROUSSEAU E, SCHOEFFLER P, ARNAL M & ATTAIX D (1996). Increased mRNA levels for components of the lysosomal, Ca^{2+}-activated, and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients. *Proceedings of the National Academy of Sciences, USA* 93: 2714-2718.

MARAIS R, WYNNE J & TREISMAN R (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* 73: 381-393.

MASFERRER JL, ZWEIFEL BS, MANNING PT, HAUSER SD, LEATHY KM, SMITH WG, ISAKSON PC & SEIBERT K (1994). Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proceedings of the National Academy of Sciences, USA* 91: 3228-3232.

MASUNO H, YAMASAKI N & OKUDA (1981). Purification and characterisation of a lipolytic factor (toxohormone-L) from cell-free fluid of ascites sarcoma 180. *Cancer Research* 41: 284-288.

MATSUI SL, SANDBERG AA, NEGORO A, SEON BK & GOLDSTEIN G (1982). Isopeptidase: a novel eukaryotic enzyme that cleaves isopeptide bonds. *Proceedings of the National Academy of Sciences, USA* 79: 1535-1539.

MATSUISHI M, MATSUMOTO T, OKITANI A & KATO H (1992). Mode of action of rabbit skeletal muscle cathepsin B towards myofibrillar proteins and the myofibrillar structure. *International Journal of Biochemistry* 24 (12): 1967-1978.

MATSUKURA U, MATSUMOTO T, TASHIRO Y, OKITANI A & KATO H (1984). Morphological changes in myofibrills and glycerinated muscle fibres on treatment with cathepsins D and L. *International Journal of Biochemistry* 16: 957-962.

MATSUKURA U, OKITANI A, NISHIMURO T & KATO H (1981). Mode of degradation of myofibrillar studies by an endogenous protease, cathepsin L. *Biochimica et Biophysica Acta* 662: 41-47.

MATSUMOTO T, OKITANI A, KITAMURA Y & KATO H (1983). Mode of degradation of myofibrillar proteins by rabbit muscle cathepsin D. *Biochimica et Biophysica Acta* 755: 76-80.

MATTHYS P, HEREMANS H, OPENDAKKER G & BILLIAU A (1991a). Anti-interferon-γ antibody treatment, growth of Lewis lung tumours in mice and tumour-associated cachexia. *European Journal of Cancer* 27 (2): 182-187.

MATTHYS P, DIJKMANS R, PROOST P, VAN DAMME J, HEREMANS H, SOBIS H & BILLIAU A (1991b). Severe cachexia in mice inoculated with interferon-γ-producing tumor cells. *International Journal of Cancer* 49: 77-82.

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

McDEVITT TM & TISDALE MJ (1992). Tumour-associated hypoglycaemia in a murine cachexia model. *British Journal of Cancer* 66: 815-820.

McDEVITT TM, TODOROV PT, BECK SA, KHAN SH & TISDALE MJ (1995). Purification and characterization of a lipid-mobilizing factor associated with cachexia-inducing tumors in mice and humans. *Cancer Research* 55: 1458-1463.

MEDINA R, WING SS & GOLDBERG AL (1995). Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy. *Biochemical Journal* 307: 631-637.

MELVILLE S, McNURLAN MA, CALDER AG & GARLICK PJ (1990). Increased protein turnover despite normal energy metabolism and responses to feeding in patients with lung cancer. *Cancer Research* 50: 1125-1131.

MELLONI E, PONTREMOLI S, MICHETTI M, SACCO D, SPARATORE B & HORECKER BI (1986). The involvement of calpain in the activation of protein kinase C in neutrophils stimulated by phorbol myristic acid. *Journal of Biological Chemistry* 261: 4101-4105.

MICHETTI M, VIOTTI PL, MELLONI E & PONTREMOLI S (1991). Mechanism of action of the calpain activator protein in rat skeletal muscle. *European Journal of Biochemistry* 202: 1177-1180.

MINAMI Y, EMORI Y, KAWASAKI H & SUZUKI K (1987). E-F hand structure-domain of calcium-activated neutral protease (CANP) can bind Ca^{2+} ions. *Journal of Biochemistry* 101: 889-895.

MITCH WE & CLARK AS (1984). Specificity of the effects of leucine and its metabolites on protein degradation in skeletal muscle. *Biochemical Journal* 222: 579-586.

MOLDAWER LL, ANDERSSON C, GELIN J & LUNDHOLM (1988). Regulation of food intake and hepatic protein synthesis by recombinant-derived cytokines. *American Journal of Physiology* 254: G450-G456.

MOLDAWER LL, SVANINGER G, GELIN J & LUNDHOLM (1987). Interleukin 1 and tumor necrosis factor do not regulate protein balance in skeletal muscle. *American Journal of Physiology* 253: C766-C773.

MORACZEWSKI J, PIEKARSKA E, ZYMOWSKA M & SOBOLEWSKA (1996). Activity of μ- and m-calpain in regenerating fast and slow twitch skeletal muscles. *Acta Biochimica Polonica* 43 (4): 693-700.

MORI M, YAMAGUCHI K & ABE K (1989). Purification of a lipoprotein lipase-inhibiting protein produced by a melanoma cell line associated with cancer cachexia. *Biochemical and Biophysical Research Communications* 160 (3): 1085-1092.

MORI M, YAMAGUCHI K, HONDA S, NAGASAKI K, UEDA M, ABE O & ABE K (1991). Cancer cachexia syndrome developed in nude mice bearing melanoma cells producing leukemia-inhibitory factor. *Cancer Research* 51: 6656-6659.

MORLEY SJ & TRAUGH JA (1990). Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3 and ribosomal protein S6 by insulin and phorbol esters. *Journal of Biological Chemistry* 265: 10611-10616.

MORRISON KS (1995). Cellular signalling in the regulation of skeletal muscle growth. *PhD thesis*, University of Aberdeen.

MORRISON KS, MACKIE SC, PALMER RM & THOMPSON MG (1995). Stimulation of protein synthesis and DNA synthesis in mouse C_2C_{12} satellite cells: evidence for phospholipase D-dependent and -independent pathways. *Journal of Cellular Physiology* 165: 273-283.

MORTIMORE GE, NEELY AN, COX JR & GUINIVAN RA (1973). Proteolysis in homogenates of perfused rat liver: responses to insulin, glucagon and aminoacids. *Biochemical and Biophysical Research Communications* 54: 89-95.

MORTIMORE GE & MONDON CE (1970). Inhibition by insulin of valine turnover in liver. *Journal of Biological Chemistry* 245: 2375-2783.

MORTIMORE GE & SCHWORER CM (1977). Induction of autophagy by aminoacid deprivation in perfused rat liver. *Nature* 270: 174-176.

MORTIMORE GE & WARD WF (1981). Internalization of cytoplasmic protein by hepatic lysosomes in basal and deprivation-induced proteolytic states. *Journal of Biological Chemistry* 256: 7659-7663.

MUKHERJEE AB, MIELE L & PATTABIRAMAN N (1994). Phospholipase A_2 enzymes: regulation and physiological role. *Biochemical Pharmacology* 48 (1): 1-10.

MULLEN JL (1994). Hypermetabolism and advanced cancer. *Annals of Surgery* 219 (4): 323-324.

MULLIGAN HD, BECK SA & TISDALE MJ (1992). Lipid metabolism in cancer cachexia. *British Journal of Cancer* 66: 57-61.

MULLIGAN HD & TISDALE MJ (1991). Lipogenesis in tumour and host tissues in mice bearing colonic adenocarcimonas. *British Journal of Cancer* 63: 719-722.

MURAKAMI Y, MATSUFUGI S, KAMEJI T, HAYASHI SI, IGARASHI K, TAMURA T, TANAKA K & ICHIHARA A (1992). Ornitine carboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* 360: 597-599.

NEELY AN, COX JR, FORTNEY JA, SCHWORER CM & MORTIMORE GE (1977). Alterations of lysosomal size and density during rat liver perfusion. *Journal of Biological Chemistry* 252: 6948-6954.

NEELY AN & MORTIMORE GE (1974). Localization of products of endogenous proteolysis in lysosomes of perfused rat liver. *Biochemical and Biophysical Research Communications* 59: 680-687.

NEELY AN, NELSON PB & MORTIMORE GE (1974). Osmotic alterations of the lysosomal system during rat liver perfusion: reversible suppression by insulin and aminoacids. *Biochimica et Biophysica Acta* 338: 458-472.

NEUFELD EJ & MAJERUS PW (1983). Arachidonate release and phosphatidic acid turnover in stimulated human platelets. *The Journal of Biological Chemistry* 258: 2461-2467.

NODA T, ISOGAI K, HAYASHI H & KATUNUMA N (1981). Susceptibilities of various myofibrillar proteins to cathepsin B and morphological alteration of isolated myofibrills by this enzyme. *Journal of Biochemistry* 90: 371-379.

NORTON JA, PEACOCK JL & MORRISON SD (1987). Cancer cachexia. *Critical Reviews in Oncology and Hematology* 7: 289-327.

O'BRIEN C (1994). Missing link in insulin's path to protein production. *Science* 266: 542-543.

OHNO S, MINOSHIMA S, KUDOH J, FUKUYAMA R SHIMIZU Y, OHMI-IMAJOH S, SHIMIZU N, & SUZUKI K (1989). Four genes for the

calpain family locate on four distinct human chromosomes. *Cytogenetics and Cell Genetics* 53: 225-229.

OLLIF A, DEFEO-JONES D, BOYER M, MARTINEZ D, KIEFFER D, VUOCOLO G, WOLFE A & SOCHER SH (1987). Tumours secreting human TNF/cachectin induce cachexia in mice. *Cell* 50: 555-563.

OVESEN L, HANNIBAL J & MORTENSEN EL (1993). The interrelationship of weight loss, dietary intake and quality of life in ambulatory patients with cancer of the lung, breast and ovary. *Nutrition and Cancer* 19 (2): 159-167.

PACIFI RE, KONO Y & DAVIES KJA (1993). Hydrophobicity as the signal for selective degradation of hydroxyl radical-modified hemoglobin by the multicatalytic proteinase complex, proteasome. *Journal of Biological Chemistry* 268: 15405-15411.

PAIN VM, RANDALL DP & GARLICK PJ (1984). Protein synthesis in liver and skeletal muscle of mice bearing an ascites tumor. *Cancer Research* 44: 1054-1057.

PALENT E & TRAUGH JA (1987). Phosphorylation of ribosomal protein S6 by cAMP-dependent protein kinase and mitogen-stimulated S6 kinase differentially alters translation of globin mRNA. *Journal of Biological Chemistry* 262: 3518-3523.

PALMER RM & BAIN PA (1989). Indomethacin inhibits the insulin-induced increases in RNA and protein synthesis in L6 skeletal muscle myoblasts. *Prostaglandins* 37 (2): 193-201.

PALMER RM, REEDS PJ, ATKINSON T & SMITH RH (1983). The influence of changes in tension on protein synthesis and prostaglandin release in isolated rabbit muscles. *Biochemical Journal* 214: 1011-1014.

PALMER RM, THOMPSON MG, KNOTT RM, CAMPBELL GP, THOM A & MORRISON KS (1997). Insulin and insulin-like growth factor-I responsiveness and signalling mechanisms in C_2C_{12} satellite cells: effect of differentiation and fusion. *Biochimica et Biophysica Acta* 1355 (2): 167-176.

PALMER RM & WAHLE KWJ (1987). Protein synthesis and degradation in isolated muscle. Effect of ω3 and ω6 fatty acids. *Biochemical Journal* 242: 615-618.

PAUSE A, BELSHAM GJ, GINGRAS AC, DONZE O, LIN TA, LAWRENCE Jr JC & SONENBERG N (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* 371: 762-767.

PEREIRA ME & WILK S (1990). Phosphorylation of the multicatalytic proteinase complex from bovine pituitaries by a copurifying cAMP-dependent protein kinase. *Archives of Biochemistry and Biophysics* 283 (1): 68-74.

PERISIC O & TRAUGH JA (1983). Protease-activated kinase II mediates multiple phosphorylation of ribosomal protein S6 in reticulocytes. *Journal of Biological Chemistry* 258: 13998-14002.

PLUMB JA, FEARON KCH, CARTER KB & PRESTON T (1991). Energy expenditure and protein synthesis rates in an animal model of cancer cachexia. *Clinical Nutrition* 10: 33-29.

PONTREMOLI S, MELLONI E, VIOTTI PL, MICHETTI M, SALAMINO F & HORECKER BL (1991). Identification of two calpastatin forms in rat skeletal muscle and their susceptibility to digestion by homologous calpains. *Archives of Biochemistry and Biophysics* 288: 644-652.

PONTREMOLI S, VIOTTI PL, MICHETTI M, SPARATORE B, SALAMINO F & MELLONI E (1990). Identification of an endogenous activator of calpain in rat skeletal muscle. *Biochemical and Biophysical Research Communications* 171: 569-574.

PRESTON T, FEARON KCH, McMILLAN DC, WINSTANLEY FP, SLATER C, SHENKIN A & CARTER DC (1995). Effect of ibuprofen on the acute-phase response and protein metabolism in patients with cancer and weight loss. *British Journal of Surgery* 82: 229-234.

PRICE SA (1997). The role of eicosapentaenoic acid in cancer cachexia. *PhD thesis*, Aston University.

PRICE SR, BAILEY JL, WANG X, JURKOVITZ C, ENGLAND BK, DING X, PHILLIPS LS & MITCH WE (1996). Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin-proteasome proteolytic

pathway by a mechanism including gene transcription. *Journal of Clinical Investigation* 98 (8): 1703-1708.

PROUD CG (1994). Turned on by insulin. *Nature* 371: 747-748.

RANNELS SR, RANNELS DE, PEGG AE & JEFFERSON LS (1978). Glucocorticoid effects on peptide-chain initiation in skeletal muscle and heart. *American Journal of Physiology* 235: E134-E139.

RANNELS DE, PEGG AE, RANNELS SR & JEFFERSON LS (1978). Effect of starvation on initiation of protein synthesis in skeletal muscle and heart. *Journal of Physiology* 235: E126-E133.

RANU RS, LEVIN DH, DELAUNAY J, ERNST V & LONDON IM (1976). Regulation of protein synthesis in rabbit reticulocyte lysates: characteristics of inhibition of protein synthesis by a translational inhibitor from heme-deficient lysates and its relationship to the initiation factor which binds Met-tRNA$_f$. *Proceedings of the National Academy of Sciences, USA* 73 (8): 2720-2724.

REDPATH NT & PROUD CG (1993). Purification and phosphorylation of elongation factor-2 kinase from rabbit reticulocytes. *European Journal of Biochemistry* 212: 511-520.

REDPATH NT & PROUD CG (1994). Molecular mechanisms in the control of translation by hormones and growth factors. *Biochimica et Biophysica Acta* 1220: 147-162.

REEDS PJ, HAY SM, GLENNIE RT, MACKIE WS & GARLICK PJ (1985). The effect of indomethacin on the stimulation of protein synthesis by insulin in young post-absorptive rats. *Biochemical Journal* 227: 255-261.

REEDS PJ & PALMER RM (1983). The possible involvement of prostaglandin $F_{2\alpha}$ in the stimulation of muscle protein synthesis by insulin. *Biochemical and Biophysical Research Communications* 116 (3): 1084-1090.

REEDS PJ & PALMER RM (1984). Changes in prostaglandin release associated with inhibition of muscle protein synthesis by dexamethasone. *Biochemical Journal* 219: 953-957.

REVILLE WJ, GOLL DE, STROMER MH, ROBSON RM & DAYTON WR (1976). A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Subcellular localization of the protease in porcine skeletal muscle. *Journal of Cell Biology* 70: 1-8.

RILEY DA, BAIN JL, ELLIS S & HAAS AL (1988). Quantitation and immunocytochemical localization of ubiquitin conjugates within rat red and white skeletal muscles. *Journal of Histochemistry and Cytochemistry* 36 (6): 621-632.

RODEMANN HP &D GOLDBERG AL (1982). Arachidonic acid, prostaglandin E_2 and $F_{2\alpha}$ influence rates of protein turnover in skeletal and cardiac muscle. *Journal of Biological Chemistry* 257: 1632-1638.

RODEMANN HP, WAXMAN L & GOLDBERG AL (1982). The stimulation of protein degradation in muscle by Ca^{2+} is mediated by prostaglandin E_2 and does not require the calcium-activated protease. *Journal of Biological Chemistry* 257: 8716-8723.

ROGERS S, WELLS R & RECHSTEINER M (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234: 364-368.

ROH MS, EKMAN L, JEEVANANDAM M & BRENNAN MF (1984). Gluconeogenesis in tumour-influenced hepatocytes. *Surgery* 96 (2):427-434.

ROME LH & LANDS WEM (1975). Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proceedings of the National Academy of Sciences, USA* 72: 4863-4867.

ROTHHUT B, RUSSO-MARIE F, WOOD J, DiROSA M & FLOWER RJ (1983). Further characterisation of the glucocorticoid-induced antiphospholipase protein "renocortin". *Biochemical and Biophysical Research Communications* 117: 878-884.

RUFF RL & SECRIST D (1984). Inhibitors of prostaglandin synthesis or cathepsin B prevent muscle wasting due to sepsis in the rat. *Journal of Clinical Investigation* 73: 1483-1486.

SAKSELA O, MOSCATELLI D, SOMMER A & RIFKIN DB (1988). Endothelial cell-derived heparan sulphate binds basic fibroblast growth factor and protects it from proteolytic degradation. *Journal of Cell Biology* 107: 749-751.

SCAMBIA G, TESTA U, PANICI PB, FOTI E, MARTUCCI R, GODDUCCI A, PERILLO A, FACCHINI V, PERSCHLE C & MANCUSO S (1995). Prognostic significance of interleukin-6 serum levels in patients with ovarian cancer. *British Journal of Cancer* 71: 354-356.

SCHWORER CM, COX JR & MORTIMORE GE (1979). Alteration of lysosomal density by sequestered glycogen during deprivation-induced autophagy in rat liver. *Biochemical and Biophysical Research Communications* 87: 163-170.

SCHWORER CM, SHIFFER KA & MORTIMORE GE (1981). Quantitative relationship between autophagy and proteolysis during graded aminoacid deprivation in perfused rat liver. *Journal of Biological Chemistry* 256: 7652-7658.

SCOT HR, McMILLAN DC, CRILLY CS & MILROY R (1996). The relationship between weight loss and interleukin 6 in non-small-cell lung cancer. *British Journal of Cancer* 73: 1560-1562.

SCUDERI P, LAM KS, RYAN KJ, PETERSEN E, STERLING KE, FINLEY PR, RAY CG, SLYMAN DJ & SALMON SE (1986). Raised antiserum levels of tumour necrosis factor in parasitic infections. *The Lancet* 2: 1364-1365.

SEGLEN PO, GRINDE B & SOLHEIM AE (1979). Inhibition of the lysosomal pathway of protein degradation in isolated rat hepatocytes by ammonia, methylamine, chloroquine and leupeptin. *European Journal of Biochemistry* 95: 215-225.

SEUFERT W, FUTCHER B & JENTSCH S (1995). Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature* 373: 78-81.

SHOWALTER CJ & ENGEL AG (1997). Acute quadriplegic myopathy: analysis of myosin isoforms and evidence for calpain-mediated proteolysis. *Muscle and Nerve* 20: 316-322.

SIDDIQUI RA & WILLIAMS JF (1989). The regulation of fatty acid and branched-chain amino acid oxidation in cancer cachectic rats: a proposed role for a cytokine, eicosanoid and hormone trilogy. *Biochemical Medicine and Metabolic Biology* 42: 71-86.

SHAPOT VS & BLINOV VA (1974). Blood glucose levels in gluconeogenesis in animals bearing transplantable tumors. *Cancer Research* 34: 1827-1832.

SHERRY BA, GELIN J, FONG Y, MARANO M, WEI H, CERAMI A, LOWRY SF, LUNDHOLM KG & MOLDAWER LL (1989). Anti-cachectin/tumour necrosis factor alpha antibodies attenuate the development of cachexia in tumour models. *FASEB Journal* 3: 1956-1962.

SHERWIN RS, HENDLER RG & FELIG P (1975). Effect of ketone infusions and nitrogen metabolism in man. *Journal of Clinical Investigation* 55: 1382-1390.

SHÜLE R, RANGARAJAN P, KLIEWER S, RANSONE LJ, BOLADO J, YANG N, VERMA IM & EVANS RM (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 62: 1217-1266.

SMITH WL & LANDS WEM (1971). Stimulation and blockade of prostaglandin biosynthesis. *Journal of Biological Chemistry* 246: 6700-6703.

SMITH K & TISDALE MJ (1993a). Increased protein degradation and decreased protein synthesis in skeletal muscle during cancer cachexia. *British Journal of Cancer* 76: 680-685.

SMITH KL & TISDALE MJ (1993b). Mechanism of muscle protein degradation in cancer cachexia. *British Journal of Cancer* 68: 314-318.

SOCHER SH, MARTINEZ D, CRAIG JB, KUHN JG & OLLIF A (1988). Tumour necrosis factor not detectable in patients with clinical cancer cachexia. *Journal of the National Cancer Institute* 80 (8): 595-598.

SODA K, KAWAKAMI M, KASHII A & MIYATA M (1994). Characterisation of mice bearing subclones of colon 26 adenocarcinoma disqualifies interleukin-6 as the sole inducer of cachexia. *Japanese Journal of Cancer Research* 85: 1124-1130.

SODA K, KAWAKAMI M, KASHII A & MIYATA M (1995). Manifestations of cancer cachexia induced by colon 26 adenocarcinoma are not fully ascribable to interleukin-6. *International Journal of Cancer* 85: 1124-1130.

SOLOMON V & GOLDBERG AL (1996). Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *Journal of Biological Chemistry* 271: 26690-26697.

SORIMACHI H, IMAJOH-OHMI S, EMORI Y, KAWASAKI H, OHNO S, MINAMI Y & SUZUKI K (1989). Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- an μ-types. Specific expression of the m-RNA in skeletal muscle. *Journal of Biological Chemistry* 264: 20106-20111.

SOUTHORN BG (1989). Interactive effects of glucocorticoids and insulin in the control of protein synthesis in rat skeletal muscle *in vivo* and in cultured myoblasts. *PhD thesis*, Rowett Research Institute, Aberdeen.

SOUTHORN BG & PALMER RM (1990). Inhibitors of phospholipase A_2 block the stimulation of protein synthesis by insulin in L6 myoblasts. *Biochemical Journal* 270: 737-739.

SOUTHORN BG, PALMER RM & GARLICK PJ (1990). Acute effects of corticosterone on tissue protein synthesis and insulin-sensitivity in rats in vivo. *Biochemical Journal* 272: 187-191.

STAAL-van den BREKEL A, DENTENER MA, SCHOLS AMWJ, BUURMAN WA & WOUTERS EFM (1995). Increased resting energy expenditure and weight loss are related to a systemic inflammatory response in lung cancer patients. *Journal of Clinical Oncology* 13 (10): 2600-2605.

STORLIEN LH, PAN DA, KRIKETOS AD, CONNOR JO, CATERSON ID, COONEY CJ, JENKINS AB & BAUR LA (1996). Skeletal muscle membrane lipids and insulin resistance. *Lipids* 31: S261-S265.

STRASSMAN G, FONG M, FRETER CE, WINDSOR S, D'ALESSANDRO F & NORDAN RP (1993b). Suramin interferes with interleukin-6 receptor binding *in vitro* and inhibits colon-26-mediated experimental cancer cachexia *in vivo*. *Journal of Clinical Investigation* 92: 2152-2159.

STRASSMAN G, FONG M, KENNEY JS & JACOB CO (1992a). Evidence for the treatment of interleukin-6 in experimental cancer cachexia. *Journal of Clinical Investigation* 89: 1681-1684.

STRASSMAN G, JACOB CO, EVANS R, BEALL D & FONG M (1992a). Mechanisms of experimental cancer cachexia: interaction between mononuclear phagocytes and colon-26 carcinoma and its relevance to IL-6 mediated cancer cachexia. *Journal of Immunology* 148 (11): 3674-3678.

STRASSMAN G, MASUI Y, CHIZZONITE R & FONG M (1993a). Mechanisms of experimental cancer cachexia: local involvement in colon-26 tumour. *Journal of Immunology* 150(6): 2341-2345.

STRELKOV AB, FIELDS ALA & BARACOS VE (1989). Effects of systemic inhibition of prostaglandin production on protein metabolism in tumor-bearing rats. *American Journal of Physiology* 257: C261-C269.

SUDAKIN V, GANOTH D, DAHAN A, HELLER H, HERSHKO J, LUCA FC, RUDERMAN JV & HERSHKO A (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Molecular Biology of the Cell* 6 (2): 185-197.

SUZUKI K, IMAJOH S, EMORI Y, KAWASAKI H, MINAMI Y & OHNO S (1988). Regulation of activity of calcium activated neutral protease: domain structure and activity regulation. *Advances in Enzyme Regulation* 27: 153-169.

SVANINGER G, BENNEGARD K, EKMAN L, TERNER M & LUNDHOLM (1983). Lack of evidence for elevated breakdown rate of skeletal muscles of weight-losing tumor-bearing mice. *Journal of the National Cancer Institute* 71: 341-346.

SVANBERG E, MOLLER-LOSWICK AC, MATTHEWS DE, KORNER U, ANDERSON M & LUNDHOLM K (1996). Effects of amino acids on synthesis and degradation of skeletal muscle protein in humans. *American Journal of Physiology* 271: E718-E724.

TAILLANDIER D, AUROUSSEAU E, SCHOEFFLER P, MEYNIAL-DENIS D, BECHET D, FERRARA M, COTTINS P, DUCASTAING A, BIGARD X, GUEZENNEC CY, SCHMID HP & ATTAIX D (1996). Coordinate activation of lysosomal, Ca^{2+}-activated, and ATP-ubiquitin-dependent proteinases in unweighted rat soleus muscle. *Biochemical Journal* 316: 65-72.

TAKAHASHI J (1993). Circadian clocks á la CREM. *Nature* 365: 299:300.

TANAKA Y, EDA H, TANAKA T, UDAGAWA T, ISHIKAWA T, HORI I, ISHITSUKA H, KATAOKA T & TAGUCHI T (1990). Experimental cancer cachexia induced by transplantable colon-26 adenocarcinoma in mice. *Cancer Research* 50: 2290-2295.

TANAKA K, FUJIWARA T, KUMATORI A, SHI S, YOSHIMURA T, ICHINARA A, TOKUNAGA F, ARUGA R, IWANAGA S, KAKIZUKA A & NAKANISHI S (1990). Molecular cloning of cDNA for proteasomes from rat liver: primary structure of component C3 with a possible tyrosine phosphorylation site. *Biochemistry* 29: 3777-3785.

TASHJIAN AH, VOELKEN EF, GOLDHARBER P & LEVINE L (1974). Prostaglandins, calcium metabolism and cancer. *Federation Proceedings* 33 (1): 81-86.

TAWA NE, ODESSEY R & GOLDBERG AL (1997). Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *Journal of Clinical Investigation* 100: 197-203.

TAYEK JA (1996). Effects of tumor necrosis factor alpha on skeletal muscle amino acid metabolism studied *in vivo*. *Journal of the American College of Nutrition* 15 (2): 164-168.

TAYEK JA, BLACKBURN GL & BRISTIAN BR (1988). Alterations in whole body, muscle, liver, and tumor tissue protein synthesis and degradation in Novikoff hepatoma and Yoshida sarcoma tumor growth studied in vivo. *Cancer Research* 48: 1554-1558.

TAYEK JA & BRASEL JA (1990). Effects of tumor necrosis factor α on skeletal muscle and Walker 256 carcinosarcoma protein metabolism studied *in vivo*. *Cancer Research* 90: 2765-2768.

TAYEK JA, ISTFAN NW, JONES CT, KAMAWY KJ, BRISTIAN BR & BLACKBURN GL (1986). Influence of the Walker 256 carcinosarcoma on muscle, tumor, and whole-body protein synthesis and growth rate in the cancer-bearing rat. *Cancer Research* 46: 5649-5654.

TAYLOR GA, CARBALLO E, LEE DM, LAI WS, THOMPSON MJ, PATER DD, SCHENKMAN DI, GILKESON GS, BROXMEYER HE, HAYNES BF & BLACKSHEAR PJ (1996). A pathogenetic role for TNFα in the syndrome of

cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity* 4: 445-454.

TAYLOR DD, GERCEL-TAYLOR C, JENIS LG & DEVEREUX DF (1992). Identification of a human tumor-derived lipolysis-promoting factor. *Cancer Research* 52: 829-834.

TEMPARIS S, ASENSI M, TAILLANDIER D, AUROUSSEAU E, LARBAUD D, OBLED A, BECHET D, FERRARA M, ESTRELA JM & ATTAIX D (1994). Increased ATP-ubiquitin-dependent proteolysis in skeletal muscles of tumour-bearing rats. *Cancer Research* 54: 5568-5573.

TESSITORE L, BONELLI G & BACCINO FM (1987). Early development of protein metabolic perturbations in the liver and skeletal muscle of tumour-bearing rats. *Biochemical Journal* 241: 153-159.

TESSITORE L, COSTELLI P, BONETTI G & BACINO FM (1993). Cancer cachexia, malnutrition and tissue protein turnover in experimental animals. *Archives of Biochemistry and Biophysics* 306 (1): 52-58.

THEOLOGIDES A (1974). The anorexia-cachexia syndrome: a new hypothesis. *Annals New York Academy of Sciences* 230: 14-22.

THEOLOGIDES A (1979). Cancer cachexia. *Cancer* 43: 2004-2012.

THOMAS G, MARTIN-PEREZ J, SIEGMANN M & OTTO AM (1982). The effect of serum, epidermal growth factor, prostaglandin $F_{2\alpha}$ and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. *Cell* 40: 235-242.

THOMPSON MG, ACAMOVIC F, MACKIE SC, MORRISON KS & PALMER RM (1993). Arachidonate activation of protein kinase C may be involved in the stimulation of protein by insulin in L6 myoblasts. *Bioscience Reports* 13: 359-366.

THOMPSON MP, KOONS JE, TAN ETH & GRIGOR MR (1981). Modified lipoprotein lipase activities, rates of lipogenesis and lipolysis as factors leading to lipid depletion in C57Bl mice bearing the preputial gland tumour, EST-586. *Cancer Research* 41: 3228-3232.

THOMPSON MG, MACKIE SC, MORRISON KS, THOM A & PALMER RM (1994). Stimulation of protein synthesis and phospholipase D activity by vasopressin and phorbol ester in L6 myoblasts. *Biochimica et Biophysica Acta* 1224: 198-204.

THOMPSON MG, MACKIE SC, THOM A, HAZLERIGG DG, MORRISON KS & PALMER RM (1996a). Cyclic AMP stimulates protein synthesis in L6 myoblasts and its effects are additive to those of insulin, vasopressin and 12-O-tetradecanoylphorbol-13-acetate. Possible involvement of mitogen activated protein kinase. *Biochimica et Biophysica Acta* 1311: 37-44.

THOMPSON MG, MACKIE SC, THOM A & PALMER RM (1997). Regulation of phospholipase D in L6 skeletal muscle myoblasts. *Journal of Biological Chemistry* 272: 10910-10916.

THOMPSON MG & PALMER RM (1997). Signalling pathways regulating protein turnover in skeletal muscle. In press.

THOMPSON MG, PALMER RM, THOM A, GARDEN K, LOBLEY GE & CALDER G (1996b). N^{τ}-methylhistidine turnover in skeletal muscle cells measured by GC-MS. *American Journal of Physiology* 270: C1875-C1879.

THOMPSON MG, PASCAL M, MACKIE SC, THOM A, MORRISON KS, BACKWELL FRC & PALMER RM (1995). Evidence that protein kinase C and mitogen activated protein kinase are not involved in the mechanism by which insulin stimulates translation in L6 myoblasts. *Bioscience Reports* 15: 37-46.

THOMPSON HS & SCORDILIS SP (1994). Ubiquitin changes in human biceps muscle following exercise-induced damage. *Biochemical and Biophysical Research Communications* 204 (3): 1193-1198.

THOMPSON MG, THOM A, PARTRIDGE K, CAMPBELL G, GARDEN K, CALDER G & PALMER RM (1996c). Regulation of the ubiquitin-proteasome system and myofibrillar protein degradation in C_2C_{12} skeletal muscle cells. *Biochemical society Transactions* 24 (4): 630S.

TIAO G, FAGAN JM, SAMUELS N, JAMES JH, HUDSON K, LIEBERMAN M, FISCHER JE & HASSELGREN PO (1994). Sepsis

stimulates nonlysosomal, energy-dependent proteolysis and increases ubiquitin mRNA levels in rat skeletal muscle. *Journal of Clinical Investigation* 94: 2255-2264.

TIAO G, HOBLER S, WANG JJ, MEYER TA, LUCHETTE FA, FISCHER JE & HASSELGREN PO (1997). Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. *Journal of Clinical Investigation* 99 (2): 163-168.

TIAO G, LIEBERMAN M, FISCHER JE & HASSELGREN PO (1997). Intracellular regulation of protein degradation during sepsis is different in fast- and slow-twitch muscle. *American Journal of Physiology* 272: R849-R856.

TISCHLER ME, DESAUTELS M & GOLDBERG AL (1982). Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle?. *Journal of Biological Chemistry* 257: 1613-1621.

TISDALE MJ & BECK SA (1991). Inhibition of tumour-induced lipolysis *in vitro* and cachexia and tumour growth *in vivo* by eicosapentaenoic acid. *Biochemical Pharmacology* 41 (1): 103-107.

TISDALE MJ & BRENNAN RA (1986). Metabolic substrate utilisation by a tumour cell line which induces cachexia *in vivo*. *British Journal of Cancer* 54: 601-606.

TISDALE, BRENNAN RA & FEARON KC (1987). Reduction of weight loss and tumour size in a cachexia model by a high fat diet. *British Journal of Cancer* 56: 39-43.

TISDALE MJ & DHESI JK (1990). Inhibition of weight loss by ω-3 fatty acids in a experimental cachexia model. *Cancer Research* 50: 5022-5026.

TISDALE MJ & LEUNG YC (1988). Changes in host liver fatty acid synthase in tumour-bearing mice. *Cancer Letters* 42: 231-235.

TODOROV P, CARIUK P, McDEVITT T, COLES B, FEARON K & TISDALE M (1996a). Characterization of a cancer cachectic factor. *Nature* 379: 739-742.

TODOROV PT, DEACON M & TISDALE MJ (1997). Structural analysis of a tumor-produced sulfated glycoprotein capable of initiating muscle protein degradation. *Journal of Biological Chemistry* 272: 12279-1288.

TODOROV PT, McDEVITT TM, CARIUK P, COLES B, DEACON M & TISDALE MJ (1996b). Induction of muscle protein degradation and weight loss by a tumor product. *Cancer Research* 56: 1256-1261.

TOMPA P, BAKI A, SCHAD E & FRIEDRICH P (1996). The calpains cascade. *Journal of Biological Chemistry* 271: 33161-33164.

TRIPATHI SC, VOSSELLER KA & McCORMICK DL (1996). Arachidonic acid metabolism and cell proliferation in rat mammary carcinoma cells treated with indomethacin. *Biochimica et Biophysica Acta* 1316: 5-7.

TRUYENS C, TORRICO F, ANGELO-BARRIOS A, LUCAS R, HEREMANS H, DE BAETSELIER P & CARLIER Y (1995). The cachexia associated with *Trypanosoma cruzi* acute infection in mice is attenuated by anti-TNF-α but not by anti IL-6 or anti-IFN-γ antibodies. *Parasite Immunology* 17: 561-568.

TSARA ME, PAPAGEORGACOPOULOU N, KARAVIAS DD & THEOCHARIS DA (1995). Distribution and changes of glycosaminoglycans in neoplasias of rectum. *Anticancer Research* 15:2107-2112.

TSURUMI C, ISHIDA N, TAMURA T, KAKIZUKA A, NISHIDA E, OKUMURA E, KISHIMOTO T, INAGAKI M, OKAZAKI K, SAGATA N *et al* (1995). Degradation of c-fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. *Molecular Biology of the Cell* 15 (10): 5682-5687.

TURNER PR, WESTWOOD T, REGEN CM & STEINHARDT RA (1988). Increased protein degradation results from elevated free calcium levels found in muscle from *mdx* mice. *Nature* 335: 735-738.

VARY TC, JURANSINKI CV, KARINCH AM & KIMBALL SR (1994). Regulation of eukaryotic initiation factor-2 expression during sepsis. *American Journal of Physiology* 266: E193-E201.

VARY TC, VOISIN L & COONEY RN (1996). Regulation of peptide-chain initiation in muscle during sepsis by interleukin-1 receptor antagonist. *American Journal of Physiology* 271: E513-E520.

VESSBY B, TENGBLAD S & LITHELL H (1994). Insulin sensitivity is related to the fatty acid composition of serum lipids and skeletal muscle phospholipids in 70-year-old men. *Diabetologia* 37: 1044-1050.

VOISIN L, BREUILLE D, COMBARET L, POUYET C, TAILLANDIER D, AUROUSSEAU E, OBLED C & ATTAIX D (1996). Muscle wasting in a rat model of long-lasting sepsis results from the activation of lysosomal, Ca^{2+}-activated, and ubiquitin-proteasome proteolytic pathways. *Journal of Clinical Investigation* 97: 1610-1617.

VON ROENN JH, ROTH EL & CRAIG (1992). HIV-related cachexia: potential mechanisms and treatment. *Oncology* 49 (Suppl 2): 50-54.

WAALKES TP & UDENFRIEND S (1957). A fluorometric method for the estimation of tyrosine in plasma and tissues. *Journal of Laboratory and Clinical Medicine* 50 (5): 733-736.

WAHLE KWJ, PALMER RM & REEDS PJ (1987). Phorbol ester stimulation of protein synthesis in muscle is only partially blocked by indomethacin. *Biochemical Society Transactions* 15 (6): 1123-1124.

WARD WF, COX JR & MORTIMORE GE (1977). Lysosomal sequestration of intracellular protein as a regulatory step in hepatic proteolysis. *Journal of Biological Chemistry* 252: 6955-6961.

WARNOLD I, LUNDHOLM K & SCHERSTEN T (1978). Energy balance and body composition in cancer patients. *Cancer Research* 38: 1801-1807.

WARREN S (1932). The immediate causes of death in cancer. *American Journal of Medical Science* 184: 610-615.

WATERHOUSE C (1974). How tumours affect host metabolism. *Annals New York Academy of Sciences* 230: 86-93.

WATERHOUSE C (1981). Oxidation and metabolic interconversion in malignant cachexia. *Cancer Treatment Reports* 65 (5): 61-66.

WATERHOUSE C, JEANPRETRE N & KEILSON J (1979). Gluconeogenesis from alanine in patients with progressive malignant disease. *Cancer Research* 39: 1968-1972.

WATSON WS & SAMMON AM (1980). Body composition in cachexia resulting from malignant and non-malignant diseases. *Cancer* 46: 2041-2046.

WELSH GI, FOULSTONE EJ, YOUNG SW, TAVARE SW & PROUD CG (1994). Wortmannin inhibits the effects of insulin and serum on activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochemical Journal* 303: 15-20.

WELSH GF & PROUD CG (1992). Regulation of protein synthesis in Swiss 3T3 fibroblasts. *Biochemical Journal* 284: 19-23.

WELSH GF & PROUD CG (1993). Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochemical Journal* 294: 625-629.

WENZEL T & BAUMEISTER W (1993). *Thermoplasma acidophilum* proteasomes degrade partially unfolded and ubiquitin-associated proteins. *FEBS Letters* 326: 215-218.

WERNER ED, BRODSKY JL & McCRACKEN AA (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proceedings of the National Academy of Sciences, USA* 93: 13797-13801.

WESDORP RIC (1986). Role of abnormal metabolism in the aetiology of cancer cachexia. *Clinics in Oncology* 5 (2): 307-315.

WETTENHALL REH & COHEN P (1982). Isolation and characterisation of cyclic AMP-dependent phosphorylation sites from rat liver ribosomal protein S6. *FEBS Letters* 140 (2): 263-269.

WETTENHALL REH & MORGAN FJ (1984). Phosphorylation of hepatic ribosomal protein S6 on 80 and 40S ribosomes. *Journal of Biological Chemistry* 259: 2084-2091.

WIERTZ EJHJ, TORTORELLA D, BOGYO M, YU J, MOTHES W, JONES TR, RAPOPORT TA & PLOEGH HL (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384: 432-438.

WIGMORE SJ, FALCONER JS, PLESTER CE, ROSS JA, MAINGAY JP, CARTER DC & FEARON KCH (1995). Ibuprofen reduces energy expenditure and acute-phase protein production compared with placebo in pancreatic cancer patients. *British Journal of Cancer* 72: 185-188.

WIGMORE SJ, FEARON KCH, MAINGAY JP & ROSS JA (1997). Downregulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. *Clinical Science* 92: 215-221.

WIGMORE SJ, PLESTER CE, RICHARDSON RA & FEARON KCH (1997). Changes in nutritional status associated with unresectable pancreatic cancer. *British Journal of Cancer* 75: 106-109.

WIGMORE SJ, ROSS JA, FALCONER JS, PLESTER CE, TISDALE MJ, CARTER DC & FEARON KCH (1996). The effect of polyunsaturated fatty acids on the progress of cachexia in patients with pancreatic cancer. *Nutrition* 12: S27-S30.

WIGMORE SJ, ROSS JA, FEARON KCH, MAINGAY JP & CARTER DC (1994). Pancreatic cancer cells stimulate the production of C-reactive protein in isolated hepatocytes via interleukin-6 or 8. *British Journal of Surgery* 81: 1814.

WILLIAMS JF & MATTHAEI KI (1981). Cancer induced body wasting: a review of cancer cachexia and a hypothesis concerning the molecular basis of the condition. *Asean Journal of Clinical Science* 2 (2): 158-167.

WILLIAMSON DH, ILIC V, TORDOFF AFC & ELLINGTON EV (1980). Interactions between vasopressin and glucagon on ketogenesis and oleate metabolism in isolated hepatocytes from fed rats. *Biochemical Journal* 186: 621-624.

WING SS & BANVILLE D (1994). 14-kDa ubiquitin-conjugating enzyme: structure of the rat gene and regulation upon fasting and by insulin. *American Journal of Physiology* 267: E39-E48.

WING SS & BEDARD N (1996). Insulin-like growth factor I stimulates degradation of an mRNA transcript encoding the 14 KDa ubiquitin-conjugating enzyme. *Biochemical Journal* 319: 455-461.

WING SS & GOLDBERG AL (1993). Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *American Journal of Physiology* 264: E668-E676.

WING SS, HAAS AL & GOLDBERG AL (1995). Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. *Biochemical Journal* 307: 639-645.

WISEMAN G & GHADIALLY (1955). Studies in amino acid uptake by RD_3 sarcoma cell suspensions *in vitro*. *British Journal of Cancer* IX (3): 481-485.

WLODAWER A (1995). Proteasome: a complex protease with a new fold and a distinct mechanism. *Structure* 3 (5): 417-420.

WOLFE FH, SATHE SK, GOLL DE, KLEESE WC, EDMUNDS T & DUPERRET SM (1989). Chicken skeletal muscle has three Ca^{2+}-dependent proteinases. *Biochimica et Biophysica Acta* 998: 236-250.

YANG Z, COSTANZO M, GOLDE DW & KOLESNICK RN (1993). Tumour necrosis factor activation of the sphyngomyelin pathway signals nuclear factor κB translocation in intact HL-60 cells. *Journal of Biological Chemistry* 268: 20520-20523.

YASUMOTO K, MUKAIDA N, HARADA A, KUNO K, AKIYAMA M, NAKASHIMA E, FUJIOKA N, MAI M, KASAHARA T, FUJIMOTO-OUCHI K, MORI K, TANAKA Y & MATSUSHIMA K (1995). Molecular analysis of the cytokine network involved in cachexia in colon 26 adenocarcinoma-bearing mice. *Cancer Research* 55: 921-927.

YOSHIMURA N, MURACHI T, HEATH R, KAY J, JASAND B & NEWMAN GR (1986). Immunogold electron-microscopic localization of calpain I in skeletal muscle of rats. *Cell and Tissue Research* 244: 265-270.

YOSHIZAWA N, OSHIMA S, SAGEL I, SHIMIZU J & TRESER G (1992). Role of a streptococcal antigen in the pathogenesis of acute poststreptococcal glomerulonephritis. *The Journal of Immunology* 148 (10): 3110-3116.

YOUNG VR (1977). Energy metabolism and requirements in the cancer patient. *Cancer Research* 37: 2336-2347.

ZECHNER R, NEWMAN JC, SHERRY B, CERAMI A & BRESLOW JL (1988). Recombinant human cachectin/TNF but not IL-1α down-regulates LPL mouse gene expression at the transcription level in mouse 3T3-L1 adipocytes. *Molecular and Cellular Biology* 8 (6): 2394-2401.

ZIMMERMAN UJP & SCHLAEPFER WW (1988). Activation of calpain I and calpain II; a comparative study using terbium as a fluorescent probe for calcium-binding sites. *Archives of Biochemistry and Biophysics* 266: 462-469.

Appendix

Publications

Papers

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

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

Mechanism of muscle protein degradation induced by a cancer cachectic factor. MJ Lorite, JA Summers, G Carling and MJ Tisdale (1997). Submitted for publication.

Abstracts

Induction of muscle protein degradation by a tumour product. BACR 37th Annual Meeting, Edinburgh, 1996.

Induction of protein degradation in skeletal muscle by a cachectic factor. AACR 88th Annual Meeting, San Diego, 1997.

INDUCTION OF MUSCLE PROTEIN DEGRADATION BY A TUMOUR PRODUCT.

M. J. Lorite*, P. Cariuk and M. J. Tisdale, Dept. of Pharmaceutical Sciences, University of Aston, Birmingham, B4 7ET.

An immunoreactive factor of Mr 24 kDa was purified from the cachexia-inducing MAC16 tumour using affinity chromatography with a mouse monoclonal antibody. Administration of this material to mice (n = 40) caused a decrease in body weight over a 24 h period (-1.55 ± 0.118 g, $p<0.0005$ from PBS controls, n = 50), which was attenuated by prior treatment with the monoclonal antibody (weight change -0.06 ± 0.239 g, $p< 0.0005$, n = 12, from treated animals). A similar weight change (-1.632 ± 0.381g, $p< 0.0005$, from PBS controls, n = 50) was produced by a similar immunoreactive factor of Mr 24 kDa isolated from the urine of cachectic pancreatic cancer patients. The weight change induced with the human factor was also attenuated by the mouse antibody (weight change 0.017 ± 0.163 g, $p< 0.01$, n = 4, from treated animals, n = 11).

In vitro studies showed the Mr 24 kDa material to cause an increased protein degradation in *soleus* muscle (as measured by tyrosine release) which was associated with increased levels of PGE_2. Both the elevated protein degradation and PGE_2 release were inhibited by the mouse monoclonal antibody.

Using the [^{3}H]-phenylalanine method to measure protein synthesis and degradation *in vivo*, weight loss induced by the Mr 24 kDa material was associated with a depression in protein synthesis rates for spleen, kidney, heart and *gastrocnemius* muscle, and an increase in proteolysis for *gastrocnemius* muscle. Protein degradation was reversed by prior antibody treatment. A similar effect was produced by the human Mr 24 kDa factor.

From these results it is concluded that the induction of cachexia in animals bearing the MAC 16 tumour is due to the changes imposed on the host by the presence of circulatory factors produced by the tumour, and that this immunoreactive factor might be one of the candidates for the induction of cachexia in the MAC 16 model. Finally, it is proposed that cachexia in humans might also be caused by the same species as that in the mouse.

INDUCTION OF PROTEIN DEGRADATION IN SKELETAL MUSCLE BY A CACHECTIC FACTOR. Lorite MJ*, Cariuk P, Tisdale MJ. Nutritional Biochemistry Group, Pharmaceutical Sciences Inst., Aston Univ., Birmingham, B4 7ET, UK

A proteoglycan of Mr 24 kDa (p24) isolated both from a cachexia-inducing tumour (MAC16) and from the urine of patients with cancer cachexia has been shown to induce a state of cachexia (weight loss approx. 10% in 24 h) when administered to non tumour-bearing mice. Body composition analysis showed a decrease in lean body mass (14%) without an effect on fat or water content. The effect of both the mouse and human p24 was attenuated in animals pretreated with a mouse monoclonal antibody. *In vivo*, weight loss induced by p24 was associated with depressed protein synthesis rates (50%) and increased proteolysis (50%) in *gastrocnemius* muscle, which was reversed by prior antibody treatment. *Ex vivo*, *soleus* muscle showed an increased protein degradation and PGE_2 production. Both effects were blocked by the mouse mAb. *In vitro* studies showed p24 to increase protein degradation in isolated *soleus* muscle which was associated with increased PGE_2. Both elevated protein degradation and PGE_2 release were inhibited by indomethacin and a $cPLA_2$ inhibitor. Experiments carried out on C_2C_{12} myoblasts showed p24 to cause a significant reduction in the rate of protein synthesis (60%) which was blocked by prior treatment with insulin and mAb. From these results it is concluded that induction of cachexia in animals bearing the MAC16 tumour is due to changes imposed on the host by the presence of circulatory factors produced by the tumour.