# MUSCLE CATABOLISM IN CANCER AND ITS ATTENUATION BY EICOSAPENTAENOIC ACID.

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# THESIS SUMMARY

This work examines skeletal muscle catabolism in cancer and its attenuation by Eicosapentaenoic Acid (EPA).

In vivo studies in mice bearing a cachexia inducing murine colon adenocarcinoma - MAC16, demonstrated an elevation in the gastrocnemius muscle in the activity and expression of regulatory components of the ubiquitin-proteasome proteolytic pathway. This was accompanied by an accelerated loss of muscle tissue correlating with an increase in overall weight loss, all of which were attenuated by prior daily dosing with EPA. Recently a proteolysis inducing factor (PIF) has been isolated from the MAC16 tumour, and from the serum and urine of cachectic cancer patients. Previous studies have shown that PIF induces protein degradation in vitro, and that this is possibly mediated through 15-hydroxyeicosatetraenoic acid (15-HETE), a metabolite of the n-6 polyunsaturated fatty acid- arachidonate. Employing the murine myoblast cell line C2C12, it was shown that both PIF and 15-HETE increased protein degradation and expression of proteasome subunits, processes which were again attenuated by prior incubation in EPA. Similarly, in NMRI mice which had been fasted for 24hours, EPA and the lipoxygenase inhibitor CV-6504 (but not structurally related fatty acids) inhibited skeletal muscle proteolysis and expression of various proteasome subunits, showing that firstly, EPA may be anti-cachexic partly through its ability to influence 15-HETE production; and secondly that the effect is specific for EPA as other fatty acids had no effect. Previous studies have suggested the involvement of the signal transduction family NFκB in response to PIF in the liver. It has been demonstrated here that both PIF and 15-HETE increased nuclear translocation of NFκB in the skeletal muscle of tumour bearing mice and that EPA inhibited this process by its ability to prevent the degradation of the NFκB inhibitor protein IκB. When an NFκB inhibitor was added to C2C12 myotubes, prior to the addition of PIF, proteasome activity and protein degradation was inhibited, showing that NFκB is responsible for the increased proteasome activity and muscle catabolism induced by PIF.

Taken together this work suggests that 15-hydroxyeicosatetraenoic acid is the intracellular mediator for PIF induced protein degradation in skeletal muscle and that elevated muscle catabolism is accomplished through an increased functioning of the ubiquitin-proteasome pathway, a process possibly mediated through an NFκB dependent mechanism. The anti-cachectic (and possibly the anti-tumourigenic) effects of EPA appear to be achieved in part by its ability to inhibit the degradation of IκB and possibly by its ability to interfere with 15-HETE production.

Cachexia, Proteolysis, NFκB, Proteasome, 15-HETE

The gentleman fell silent for a while……

'I know you went to see that doctor yesterday… so how are you? What did the doctor tell you?'

'Idiot', snapped Ivan.

The Karamazov Brothers
Fyodor Dostoevsky

I would like to thank Professor M.J. Tisdale for allowing me to pursue this research, my colleagues in Cancer Biochemistry particularly, Drs. Smith, Field and Islam-Ali and Ms J Khal for their practical assistance and advice, and last but not least I should also like to thank Jon Charlton and my family for their continued moral (not to mention financial!) support.

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"**Cachexia**/ke'keksia/n. Also Anglicized as **cachexy**/-'keksi/. M16. [Fr.*Cachexie*] or late L cachexia f. Gk *kakhexia*, f. *kakos* bad + *hexis* habit. 1. Med. A condition of weakness and wasting due to severe chronic illness. M16.

The New Oxford English Dictionary
Oxford University Press, 1993.

## 1) Cachexia – An Introduction

Cachexia is derived from the Greek "Kakos" meaning bad and "Hexis" meaning condition or state of being. More recently cachexia has been defined as "a progressive wasting" (Lindsey 1986), "a progressive nutritional deterioration" (Puccio and Nathanson 1997), or as "the mechanism by which a cancer-bearing patient develops anorexia and a progressive wasting diathesis leading to body compositional changes associated with a severely malnourished state" (Langstein and Norton 1991). Cachexia can arise from a number of conditions. The most common causes are malignancy, sepsis and burns but it also found in AIDS, cystic fibrosis, myocardial infarct, Crohn's disease and some mental disorders.

The prevalence of the condition is variable, its aetiology multifactorial and different between cancer type and individual. It encompasses a wide range of metabolic, endocrine and cytokine related abnormalities that may differ from case to case, but the single unifying feature of this disorder is always a marked depletion of host tissue resulting in progressive weight loss or wasting. The myriad other symptoms can include anorexia, diminished nutritional intake, early satiety, asthenia, anaemia, oedema, easy fatigue, impaired immune function, water/electrolyte imbalance, apathy, torpor, anxiety and poor performance status (reviewed in Fearon 1991).

Why is understanding the mechanisms underlying cachexia important? Primarily because it is one of the main causes of death and morbidity amongst cancer patients. Moreover, as cancer is ever increasingly seen as a chronic disease and as the profile of western population shifts evermore toward the aged, without intervention the incidence of cachexia can only increase. In order to answer the question more fully consider the prevalence of this disorder and its serious impact on morbidity and mortality.

## 1.1 Statistics and Prevalence

As far back as 1932 Warren reported that the cause of death in 22% of cancer patients was cachexia, and its prevalence is now estimated to range from 8-84% depending on the tumour type (Tisdale 1991). In some cases weight loss occurs before the diagnosis has been made and has in fact been the initiating factor in individuals seeking medical attention. Indeed half of all cancer patients have symptoms and signs of cachexia at the time of diagnosis (DeWys et al 1980). An important point to note is that various neoplasms evolve different degrees of cachexia. In one study, 31-40% of cases of favourable subtypes of non-Hodgkins lymphoma, breast cancer, non-lymphocytic leukaemia and sarcoma demonstrated significant weight loss; 48-61% weight loss frequency was seen in unfavourable non-Hodgkins lymphoma, colon, prostate and lung cancer. Patients with pancreatic and gastric cancer showed the highest incidence of 83 – 87% weight loss. (Reviewed in DeWys et al 1980). These latter results are supported by those of an earlier study which also showed an occurrence of 84% weight loss in cases of stomach adenocarcinoma and the highest incidence in gastrointestinal and lung carcinomas (Strain 1979). This trend is reflected also in the percentage weight loss seen at the time of initial diagnosis for the various tumour types, ranging from 36% weight loss in 289 cases of breast cancer to 83% weight loss at the time of diagnosis in 111 and 179 cases of pancreas and lung cancer respectively (DeWys et al 1980). Significantly Brennan (1977) has suggested that the individual cannot normally survive greater that 30% weight loss below normal

In 1991 it was estimated that there were approximately 1-2 million cases of cachexia per annum in the U.K. (Stock 1991) and that one half to two thirds of individuals with cancer are cachectic at some point, this figure being a total of two thirds at death. (Morrison et al 1976). Approximately 50% of untreated cancer patients have lost some weight at the time of presentation and approximately 33% have lost greater than 5% in the preceeding six months (DeWys et al 1980).

Although weight loss is not the only component of cachexia it is highly correlated to clinical outcomes. It is associated with a poor prognosis (shorter survival time) and poor response to chemotherapy, particularly in cases of breast, colon, non-small cell lung cancer and acute lymphocytic leukaemia (DeWys et al 1985). Perhaps more importantly, percentage weight loss is particularly linked to mortality outcomes. In one study, DeWys et al (1980) showed that cancer patients with weight loss had a significantly reduced survival following treatment over those who presented with no weight loss. DeWys and colleagues also went on to suggest that nearly ever patient who dies from cancer will develop weight loss.

Current thinking suggests that cachexia could arise as a consequence of maladaptive metabolism including anorexia and/or increased metabolic expenditure; abnormal carbohydrate, protein or lipid metabolism and/or circulating factors which are produced by the host or by the tumour. The various arguments are shown in the diagram overleaf (adapted from Lindsey et al 1986) and are discussed in turn.

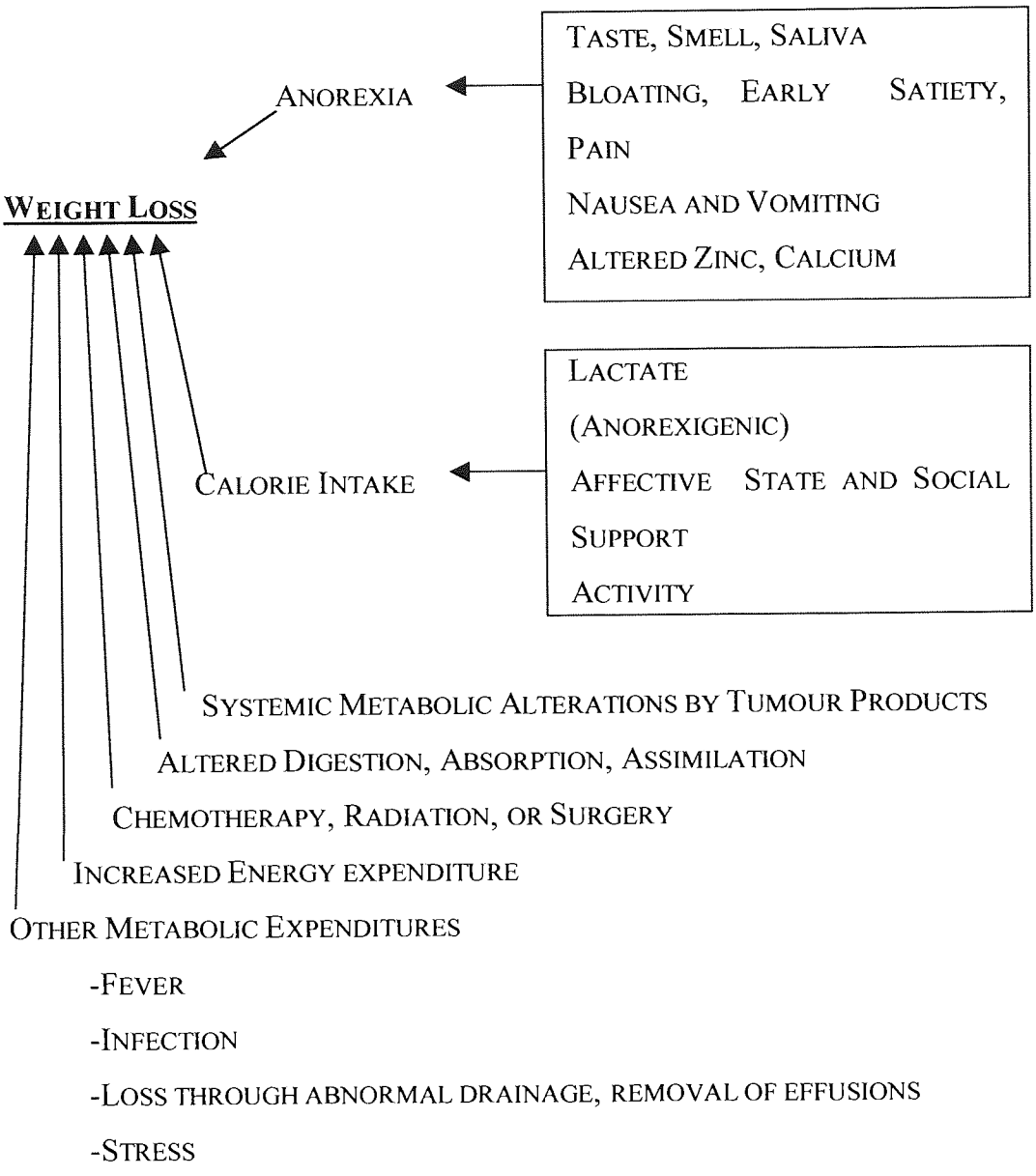



Figure 1). Factors Influencing Weight Loss in Cancer Cachexia (Adapted from Lindsey et al 1986)

## 1.2 Negative Energy Balance

A large negative energy balance (where energy output inappropriately exceeds energy input), is commonly seen in cachectic cancer patients. This situation could result either from an increase in energy expenditure, or from a decrease in energy intake. Some of the possible causes of the latter are listed in the table below adapted from Fearon (1992).

Table 1) Causes of Reduced Food Intake in Cancer Patients
(Adapted from Fearon 1992)

- Tumour obstructing Gastro-Intestinal (GI) tract
- Radiotherapy or chemotherapy induced vomiting
- Altered taste sensitivity (increased serum calcium and lactic acid and zinc deficiency have been associated)
- Depression, stress, anxiety
- Oral ulceration or infection
- Learned food aversions (particularly associated with therapies)
- Atrophy of the GI tract (including decreased secretion and activity of GI enzymes)
- Altered host metabolism (e.g. lactic acidosis and hypercalcaemia)
- General debility and weakness
- Tumour Products

Although a decline in spontaneous intake is a major factor influencing the progression of cachexia, it is not thought to be a primary cause of the syndrome. Moreover provision of excess calories by total parenteral nutrition does not significantly improve weight gain or survival time (Heber 1993). Similarly several studies have shown that forced feeding, paired feeding and caloric restriction experiments in animal models cannot totally

account for the weight loss (reviewed in Tisdale 1993). The situation is complicated further because it is difficult to understand whether anorexia is a cause or effect of cachexia, since it may develop after weight loss has started to occur.

An increased energy expenditure on the other hand can be explained by the maladaptive metabolism seen in cachexia. In a normal starving individual, changes in caloric intake are normally succeeded by a fall in the metabolic rate allowing conservation of both energy and tissue. However energy balance in the cancer patient is negative and this adaptive response is absent. Progressive weight loss inevitably results from a prolonged negative energy balance and thus increased metabolic rate could be a significant contributor in many cases of cachexia.

The evidence for the role of metabolic rate and resting energy expenditure (REE) in cancer cachexia is confusing. In one study 26% of cancer patients were shown to have increased REE whilst 33% had decreased REE (Knox 1983). Similarly Dempsey et al (1984) in a study of 173 cancer patients showed that 36% had decreased REE and 22% had increased REE and that this distribution bore no correlation to weight loss. Hyltander et al (1991) demonstrated that cachectic cancer patients had a significant increase in REE compared to weight losing or weight stable controls. Interestingly it was also demonstrated that this correlated to an increased heart rate and that an elevated adrenergic state might be the likely explanation. Accordingly, many cancer patients show increased levels of catecholamine and adrenergic substance secretion compared to healthy starving individuals who usually show a decrease.

It appears that tumour type plays a role in abnormal metabolic rate and cachexia. For example patients with lung and pancreatic cancer show an increased REE compared to gastric and colorectal cancer patients who tend to show no difference over controls (Reviewed in Tisdale 1997)

The incongruity of results concerning the role of REE in cancer cachexia suggest that it is likely to play a role but that it cannot solely explain the phenomenon.

### 1.3. Changes in Carbohydrate Metabolism

Alterations occurring in carbohydrate metabolism in cachectic individuals include increased gluconeogenesis, glucose intolerance and decreased insulin sensitivity.

Due to poor vascularisation and subsequent hypoxia (some authors also argue altered enzyme profiles play a role (Weinhouse 1973) including increased lactate dehydrogenase activity (Holroyde 1975)) tumours tend to utilize anaerobic glycolysis (this being the only ATP generating pathway which does not require oxygen). As glucose is the only substrate for this pathway there is a greatly increased demand. This demand is met through gluconeogenic pathways. Several reports have demonstrated that the Cori Cycle, that is the gluconeogenic conversion of lactate to glucose is upregulated in cachectic cancer patients (Reviewed in Tisdale 1993 and Lindsey 1986).

This is problematic in that the Cori Cycle is particularly energy inefficient for the host. Lactate is recycled to glucose in the liver and kidney and the net loss in this activity is 4 ATP molecules per mole of glucose formed. It is thought that the most likely explanation for increased energy expenditure is increased Cori Cycle activity.

Furthermore, increased gluconeogenesis to provide glucose for tumour anabolism results in the production of nitrogenous waste via urea synthesis. Not only are these pathways less energy efficient for the host, further energy is then required for the elimination of nitrogen.

Cancer cachexia has also been associated with a catabolism favouring decreased insulin:glucagon ratio (Bartlett et al 1993). Briefly insulin favours

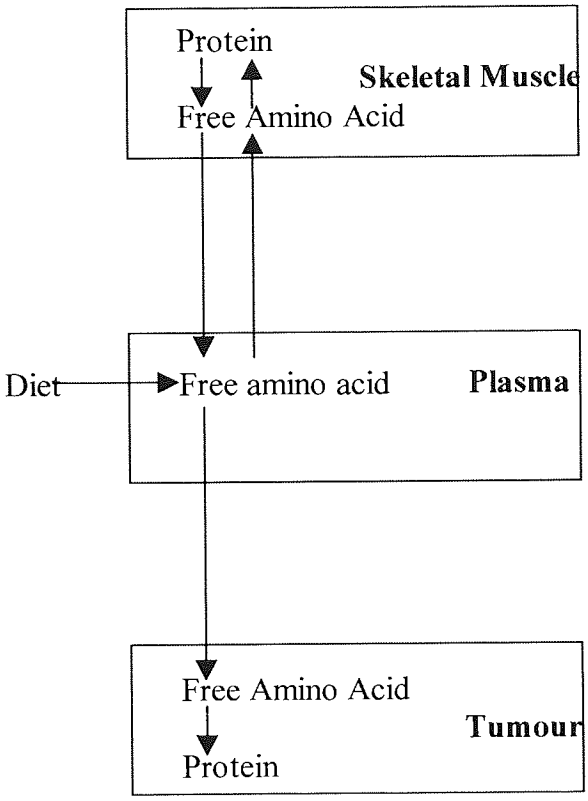
anabolism, functions to clear glucose thus precipitating hypoglycaemia. Several studies have shown an impaired glucose tolerance and decreased insulin sensitivity in cachectic individuals. During an i.v. challenge such people often still show reduced insulin levels (Lundholm 1981), suggesting that the sensitivity to glucose of pancreatic cells may be reduced. This decreased assimilation capacity of glucose and insulin responsiveness is a potential mechanism for cachexia.

### 1.4. Changes in Protein Metabolism

Normal protein metabolism is a balance obtained between protein synthesis and protein degradation. It is responsive to diet and cellular requirements and ultimately functions to maintain optimal skeletal and visceral muscle mass. Before considering the role of abnormal protein metabolism in cachexia it is necessary to look at the location of those amino acids involved. Amino acids exist as part of both fast and slow reacting pools. The first is formed by free amino acids, the reservoir being muscle, it is characterised by its high mobility and responsiveness to exogenous supply by diet or utilisation by cells. The slow reacting pool is formed by proteins mainly in skeletal muscle. Between these is the plasma pool, which functions as a bridge between the different cell populations (Reviewed in Lazo 1985). In the healthy individual protein synthesis and degradation is a balanced two way flux, however a growing tumour makes a demand on essential amino acids which depletes firstly the extracellular (plasma) pool and secondly the intracellular (muscle) pool. (See diagram overleaf, adapted from Lazo 1985).

Fig 2) The Flux of Essential Amino Acids Among Different Pools in an Adult Cancer Patient

(adapted from Lazo 1985)




The plasma pool can be depleted in a short time, the muscle pool sensing a demand responds by releasing amino acids from the muscle free intracellular pool. This is reflected firstly as a decrease in protein synthesis and later as an increased degradation. This results in altered amino acid ratios, satiety and negative nitrogen balance (reviewed in Lazo 1985).

Another drain on muscle amino acids is (as mentioned) that the tumour often requires glucose for anaerobic glycolysis and the body increases skeletal muscle breakdown to provide amino acids for gluconeogenesis. The healthy individual responds to chronic starvation by reducing muscle breakdown but in the cachectic patient this ability to conserve muscle is lost. The plasma amino acid profile reflects this situation in that there are decreases in the concentrations of gluconeogenic amino acids. Several studies have shown

that the tumour can have a specific amino acid requirement. Leucine requirements have been particularly well documented and it has been shown that the tumour can increase considerably the daily need for leucine and that this need correlates well with clinical deterioration (FAO/WHO 1973). Similarly requirements for Glutamine, isoleucine and valine have been shown to be enhanced (Holm 1995).

### 1.5 Changes in Lipid Metabolism

The major reservoir of calories in the body is adipose tissue, which consists of triglycerides. In the normal individual the first step in the mobilisation of this stored energy is lipase mediated hydrolysis to yield free fatty acids (FFA) and glycerol. These FFAs may either be reesterified within the adipocyte or released into the bloodstream where they are ultimately metabolised in the liver. Glycerol is released directly into the bloodstream due to the relative absence of glycerol kinase in the liver. The rate of glycerol appearance in plasma reflects net lipolysis. The glycerol released during lipolysis is primarily metabolised by the liver, either as a substrate for increased gluconeogenesis or in the synthesis of new triglycerides or phospholipids.

In cachectic individuals there is a decrease in total lipids which cannot be explained by reduced caloric intake or reversed by force feeding. In view of its high calorific content , fat is a vital fuel source when energy demands are high. Thus increased mobilisation of fat may be necessary to sustain the increased REE commonly seen. Interestingly it has been shown that if glucose is converted into fat and then utilised for energy, there is a large reduction in the amount of utilisable energy which may further contribute to the increased REE (Reviewed in Tisdale 1993).

Reasonably the mechanism underlying fat depletion should involve an imbalance between synthesis and degradation. Mulligan and Tisdale (1991) for example showed that there is an increased lipogenesis in kidney, liver and epididymal fat pads of mice bearing a cachexia inducing tumour. Similarly

the decreased insulin sensitivity which has been demonstrated in cachexia favours decreased lipid synthesis as do lowered levels of Lipoprotein Lipase (LPL), the enzyme responsible for the movement of triglycerides from the blood into adipocytes for lipid synthesis (Langstein and Norton 1991). Abnormalities in this enzyme appear to be central to the abnormal lipid metabolism seen in cachectic cancer patients

As tumours require fatty acids for oxidative metabolism, for incorporation into membrane phospholipids and as a source of biologically active eicosanoids and although some tumours have been reported to be able to synthesise fatty acids themselves, the general opinion is that the fatty acid requirement is met by the host (Spector 1975). It has long been suggested that the elevated lipolysis seen in cachexia is due to the lipid requirements of the tumour exceeding its biosynthetic capacity (McDevitt et al 1995).

Whatever the metabolic abnormalities, cachexia results in a progressive weight loss and significant changes in the relative contributions of body compartments.

### 1.6 Changes in Body Composition

The adaptive response to starvation in the normal individual is to preserve skeletal muscle. This is accomplished by replacing the initial gluconeogenic and muscle catabolic response with a breakdown of adipose tissue. Moley (1987) showed that in cases of simple starvation only a very small amount of skeletal muscle is lost and that over three quarters of the weight lost is from fat depletion. In cancer cachexia however, skeletal muscle loss is equalled by adipose tissue loss and it is not possible to reverse cachectic weight loss by increasing caloric intake as is the case in simple starvation (Reviewed in Tisdale 1997). Smith and Tisdale (1993) found that as weight loss increases the size of the individual compartments decreases, although again with the exception of fat, the relative contribution of each remains the same.

Preston et al (1987) showed that in lung cancer patients with severe weight loss there was an 85% fall in total body fat which clearly reflected a prolonged negative energy balance. Secondly there was a 75% fall in skeletal muscle protein mass although the non-muscle compartment was preserved. Thus cachexia is associated with a disproportionate decrease in the levels of adipose tissue and a decrease in muscle tissue proportional to overall weight loss. This would differentiate cancer cachexia from simple starvation. The relative changes seen in cachexia in various body compartments are well demonstrated in the following table, adapted from Fearon (1992).

Table 2) Body composition of a group of lung cancer patients (n=6) who had lost 30% of their pre-illness weight (Fearon 1992).

| Body Compartment | Controls (kg) | Cancer (kg) |
|---|---|---|
| Fat | 17.3 | 3.1 |
| Non muscle protein | 8.3 | 8.1 |
| Muscle protein | 2.8 | 0.7 |
| Intracellular Water | 19.1 | 12.9 |
| Extracellular Water | 15.1 | 17.5 |
| Minerals | 3.0 | 2.6 |

### 1.7. Cytokine involvement

A number of factors have been implicated as mediators of cachexia. In particular the cytokines TNFα (Mahony et al 1988), IL-6 (Strassman et al 1992) and γ-interferon (Matthys et al 1991) have been shown to produce some of the symptoms in experimental animals. Beutler and Cerami (1986) transfected Chinese hamster ovary cells with the TNFα gene producing a syndrome resembling cachexia in which marked adipose depletion was evident whilst Costelli et al (1993) demonstrated that TNFα can stimulate

muscle protein degradation and that this is decreased by prior administration of an anti- TNFα antisera, (although, importantly, this had no significant effect on weight loss). Similarly, Starnes et al (1988) showed that administration of recombinant TNFα to human cachectic cancer patients caused an increase in whole body protein turnover. However, several studies have failed to detect elevated TNFα levels or to correlate levels to cachexia in in vivo or in vitro models (Tisdale 1997, Costelli 1993) and the evidence establishing a role for these cytokines in clinical cancer cachexia is also lacking.

In vivo studies have shown that TNFα and IL-6 cannot induce cachexia directly (McDevitt et al 1995) unlike lipolytic and proteolytic factors isolated from cancer patients and tumour models (discussed in chapter 2). Instead it is thought that the cachectic effect of these cytokines arises in part from an inhibition of lipoprotein lipase (LPL). As mentioned, this is the key enzyme for the hydrolysis of circulating triacylglycerol, thus inhibiting LPL would prevent the extraction of non-esterified fatty acids from plasma lipoproteins by adipocytes into the tissues for storage resulting in a net flux of lipid into the circulation (reviewed in McDevitt et al 1995).

## 2. Evidence for the Existence of Humoral Mediators of Cachexia

The first suggestion that cachexia might be mediated by a tumour product came in 1966 when Costa and Holland showed that it could be induced by non-viable preparations of Krebs-2 carcinoma cells. (Although as early as 1949 Nakahara and Fukuoka identified a circulating toxic substance produced by cancer tissue which they postulated might alter metabolism). Kitada et al (1980) provided further support for the idea of humoral mediation by demonstrating serum from lymphoma bearing AKR mice caused lipolysis when injected into healthy recipients, similarly in parabiotic rats, weight loss is observed in the non tumour bearing rat, even in the absence of metastases (Norton et al 1985).

### 2.1 Lipolysis

A number of tumours have a limited ability to synthesise fatty acids and obtain a substantial amount from the host (Spector and Burns 1987). Such fatty acids are incorporated into the complex lipids of the tumour cell, including phospholipids needed for membrane synthesis and eicosanoid formation. Kitada et al (1980) used 1-$^{14}C$ labelled linoleic acid implanted into AKR mice and found that in non-tumour bearing animals, fat was mobilised and appeared largely as respiratory $CO_2$ whereas in tumour bearing animals fat appeared largely in the tumour. This combined with the observations that basal lipolytic rates are often elevated by as much as 200-300% in cancer patients (Kralovic et al 1977) suggests that lipolysis is clearly significant and thus a factor which can elevate lipolysis may be important for the growth and reproduction of neoplastic cells.

Several groups have isolated factors from tumour cells which are capable of causing lipolysis in vitro and in vivo. Masuno et al (1981 and 1984), have described a 75kDa protein purified from the ascites of cachectic hepatoma patients and DDK mice with sarcoma 180, which they called toxohormone-L, and which causes lipid mobilisation when injected into recipient animals. Similarly Kitada et al (1980) demonstrated that a 5kDa inactive protein,

which was not a lipase and which aggregated to a larger molecule when active, was present in the serum of AKR mice bearing a thymic lymphoma and that it was capable of inducing lipolysis in rat adipocyte suspensions. Activity was also observed in AKRXDBA/2 lymphoma and in transplanted lymphomas from a Friend-virus induced erythroleukaemia cell line in DBA/2 mice, and the material (which could not be detected in normal thymus, spleen liver or other tissues) caused a massive lipid mobilisation (Kitada et al 1982).

Beck and Tisdale (1987) first provided evidence that a circulating factor produced by the cachexia inducing murine adenocarcinoma -MAC16 may be responsible for the lipolysis seen [*]. Beck and Tisdale (1987) showed that there was a direct correlation between tumour burden and lipolysis which could not be explained by direct competition of the tumour for host nutrients. No elevation in TNFα was observed suggesting that this cytokine was not responsible and neither cyclooxygenase, lipoxygenase and trypsin inhibitors nor phenylmethylsulfonylfluoride reversed the lipolysis suggesting that the effect was not due to a prostaglandin nor to non-specific proteolysis. Similarly, that lipolysis did not respond to propanalol indicates that it is distinct from adrenaline (which is normally produced during starvation to mobilise host lipids).

An in vitro assay designed to measure glycerol release in isolated murine adipocytes showed that both the serum and urine of cancer patients with cachexia had an increased lipolytic ability that correlated linearly with the severity of weight loss. This was paralleled by a corresponding rise in serum

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[*] The MAC16 tumour was originally derived from colon tumours induced by dimethylhydrazine in NMRI mice (Double, Ball and Cowen 1975). It is a good model of human cachexia since it induces weight loss when the tumour burden comprises less than one per cent of total body mass. In male NMRI mice transplanted with fragments of the MAC16 tumour, weight loss begins when the tumour is 0.3% of body mass and reaches 30% when the tumour mass is 3% of the host (Beck and Tisdale 1987). Weight loss occurs without a reduction in caloric or fluid intake

activity from MAC16 bearing NMRI mice, however no difference in this lipolytic activity was observed in control animals, control patients or patients with weight loss from Alzheimer's disease (Groundwater et al 1990). This study also showed that the factor was conserved across species as the human material stimulated lipolysis in murine adipocytes.

That fact that the anti-lipolytic hormone insulin and the ketone body β-hydroxybutyrate suppress lipolysis induced by the factor demonstrate that it is still subject to physiological control (Groundwater et al 1990).

That this might be a novel factor was supported by the observation (unlike those reports of Masuno et al (1984)) that no change in food and water intake was observed and (unlike Kitada et al (1982)), in that trypsin digestion had no effect. The finding that the two related adenocarcinomas which do not exhibit elevated lipolysis (MAC13 and MAC15a) do not contain the factor (Beck and Tisdale 1987) suggested that the factor was specifically responsible for lipolysis.

Using exclusion and reverse phase hydrophobic chromatography, Beck et al (1990) isolated the material (which exhibited identical chromatographic characteristics and molecular weight) from both the urine and serum of cachectic cancer patients, however it could not be purified from controls or under conditions of starvation, again showing that the material was specific to the induction of cachexia.

Subsequently the material named Lipid Mobilising Factor (LMF) has been purified from the serum (Beck et al 1992) and urine (Beck and Tisdale 1991) of cachectic cancer patients and from the tumour and serum of MAC16 but not MAC13 bearing mice (Beck and Tisdale 1987). It is a glycoprotein characterised by a negative pH, thus distinguishing it from naturally occurring lipolytic hormones which are all positively charged. LMF is heat and alkali labile, is thought to contain sulfate residues and does not contain triglyceride lipase activity (McDevitt et al 1995).

Mulligan and Tisdale (1991) demonstrated that not only is total weight loss proportional to tumour burden or LMF, but so to is percentage contribution of body fat. This study showed that there was a preferential conversion of lipids to glucose, that is to say lipogenesis was increased in both MAC16 and MAC13 animals, possibly resulting in loss of utilisable carbohydrate energy thereby increasing the overall energy requirements in the tumour bearing state and leading to further catabolism of host tissues. However elevated lipogenesis has not been seen by other workers (Jeevanandam et al 1986) and the fact that the non-cachexic MAC13 tumour also increased lipogenesis may suggest that this attribute may not be specific to the cachectic state but more related to the presence of certain tumours.

Normally, lipolysis is exerted through the binding of a hormone to its receptor. The intracellular mediator cAMP is formed in response to the activation of adenylate cyclase and activates a cAMP dependent protein kinase A, which in turn reversibly phosphorylates a single serine residue on hormone sensitive lipase. Lipolysis induced by LMF also results in elevation of cAMP levels and hormone sensitive lipase (Tisdale and Beck 1991), a process which is effectively inhibited by the polyunsaturated fatty acid-Eicosapentaenoic Acid (discussed in detail in following chapters) both in vivo and in vitro. The exact molecular mechanism of this interaction is unknown but thought to involve guanine nucleotide triphosphate binding proteins (GTP), and possibly be due to the inhibition of GTP mediated activation of adenylate cyclase (Hirai et al 1998)

Further information on the mechanism of action of LMF was provided by Khan and Tisdale (1999) who showed that the induction of lipolysis by LMF was attenuated by the adenylate cyclase inhibitor $MDL_{12330A}$ and the protein kinase A inhibitor H8, further suggesting that cAMP was the intracellular mediator of induction. That this response was affected by GTP and propanalol further indicated G proteins and adrenoceptors were involved.

More recently it has been shown that LMF is homologous to the plasma protein Zn-$\alpha$2-glycoprotein in amino acid sequence, electrophoretic mobility

and immunoreactivity, and that both cause lipolysis in isolated adipocytes with a comparable dose-response profile (Hirai et al 1998). Both have also been shown to possess the same chymotrypsin digestion pattern (Todorov et al 1998), the biological activity of both is completely destroyed by freezing and both can be detected at high levels in the urine of cachectic cancer patients (Todorov et al 1998). Furthermore treatment of genetically obese (ob/ob) mice with LMF caused a 19% reduction in fat over 160hours with no change in water or non-fat carcass mass. That a polyclonal antisera to Zn-α2 can neutralize in vitro lipolysis induced by LMF further suggests that the two may be the same, however it is not clear how a large acidic protein can stimulate adenylate cyclase (as LMF does) as other polypeptides having a similar role are small and basic. Elevated oxygen consumption in brown adipose tissue also demonstrated an increase in thermogenesis and pharmacological studies have indicated that the β-3 adrenoceptor is involved in this process (Hirai et al 1998). Further support for the notion was provided by the observation that Zn-α2 mRNA (as measured by competitive PCR) was only observed in those tumours capable of producing a decrease in carcass lipid (Hirai et al 1998).

Recently it has been shown that LMF also functions to increase muscle mass in the in vitro muscle cell line C2C12 by increasing protein synthesis and decreasing proteasome mediated proteolysis (Islam-Ali and Tisdale 2001). Using a battery of COX/LOX inhibitors, involvement of cAMP and the β3 adrenergic receptor in this response was determined. As LMF enhanced protein synthesis in the tumour, it is also postulated that it may function as a tumour growth factor. By increasing protein synthesis in skeletal muscle, LMF may modulate the rate of loss of skeletal muscle and as such be antagonistic to tumour proteolytic factors which are discussed in the following chapter.

## 2.2 Proteolysis Inducing Factor (PIF)

The possibility of a circulating proteolytic factor in NMRI mice bearing the cachexia inducing colon adenocarcinoma MAC16, was identified when it was reported that serum from cachectic animals caused a significant decrease in protein synthesis (Smith and Tisdale 1993) and a massive increase in protein degradation (Smith and Tisdale 1993b) when added to isolated gastrocnemius muscle. Also, serum from mice with increasing levels of weight loss produced an increased protein degradation (as measured by tyrosine release) up to a weight loss of 20% (Smith and Tisdale 1993b).

Furthermore it was noted that mice bearing the MAC16 tumour and with established weight loss contained within their serum, antibodies that interacted with a 24kDa material which co-purified with a lipid-mobilising factor. These antibodies were not present in the serum of mice bearing the histologically identical but non-cachectic MAC13 tumour, suggesting that the antibodies were directed towards a factor involved in the induction of cachexia rather than the tumour itself (McDevitt et al 1995).

Similarly Cariuk et al (1997) isolated an antigen from the urine of cachectic cancer patients which when administered to mice caused a significant reduction in body weight ($P<0.005$) and fat and non-fat mass compared to controls and which was prevented by prior administration of the monoclonal antibody raised against murine PIF (see below). This material was not present in urine of normal subjects, those with non-cachectic cancers or those with weight loss from other causes (e.g., sepsis, burns, pancreatitis, multiple injuries, surgery, sleeping sickness and coeliac disease). A band of 24kDa was detected by Western analyses in the urine of cachectic cancer patients who had varying types of cancer including pancreatic, lung, colon, breast, rectal, liver, ovarian and cholangiocarcinoma suggesting that the single 24kDa factor

When purified PIF was injected into non tumour bearing mice, the effects were similar to those seen in animals bearing the MAC16 tumour, in that weight loss occurred without a reduction in food and water intake (possibly due to increased energy expenditure), there was a marked hypoglycaemia, and loss of adipose tissue was proportionally greater, effects which could be reversed by prior administration of the antibody (reviewed in Todorov et al 1996)

Although loss of adipose tissue may exceed loss of lean body mass, it is the latter which has the worst prognostic impact. Lorite et al (1997) showed that PIF caused a 50% decrease in protein synthesis and a 50% increase in protein degradation in isolated soleus muscle, although it was later discovered that the effects of PIF on protein synthesis in vitro appear to be transitory (Smith et al 1999), suggesting that the two effects are mediated by different mechanisms. It is the increase in protein degradation which is thought to be most causative of muscle wasting and therefore the most clinically significant.

### 2.2.1 PIF Structure.

Initial experiments showed that the factor was stable when heated to 60°C for five minutes and that it was not inhibited by phenylmethylsulfonyl fluoride suggesting that it was not a serine protease (Smith and Tisdale 1993b).

Todorov et al (1996b) fused splenocytes from MAC16 bearing mice with balb/c myeloma cells to produce a monoclonal antibody to the material which copurified with lipid-mobilizing factor and which has been subsequently identified as PIF. Western blotting demonstrated two bands of 69 and 24kDa. This monoclonal antibody did not neutralize lipolysis in vitro but did prevent protein degradation both in vitro and in vivo, indicating its specificity toward the proteolytic component. The bands isolated were further fractionated using a C8 hydrophobic reverse phase HPLC column and when injected into non tumour bearing mice, both were capable of inducing weight loss, as well as proteolyis in isolated gastrocnemius muscle, effects

which were blocked by injection or pre-incubation with the antibody. When sequenced, the 69 kDa band demonstrated the same amino acid sequence as the 24kDa band except that it also contained the sequence for albumin (shown below). The structure of this material is novel and distinct from recognised cytokines.

Y D P E A A S A P G S G N P S H E A (S) (A)

Fig 3) Amino Acid Sequence of the PIF Antigen

The amino acid sequence is identical for murine PIF and for human PIF obtained from a variety of neoplasms, including melanoma G361 which induces cachexia in nude mice. (Todorov et al 1999).

Although there is some homology with streptococcal pre-absorbing antigen, this showed no cross-reactivity with the antibody and the tumour was free from microbial contamination (Todorov et al 1996).

PIF contains carbohydrate, and lectin blotting shows a strong reaction with wheat germ and *Erythena crystagalli* agglutinins (Todorov et al 1996). This indicates that PIF is a glycoprotein or proteoglycan which binds strongly to albumin possibly through its carbohydrate residues. Further analysis showed that antigenic activity was destroyed by treatment with periodate, indicating that the carbohydrate moieties are in fact the antigenic determinant (Todorov et al 1997). Peptide N-glycosidase F (PNGase F) and endo-α-N-galactosaminidase (O-glycosidase) (but not neuraminidase or trypsin) reduced biological activity and antigenicity. Chondroitase ABC on the other hand completely destroyed the antigenicity of PIF. Taken together, these findings demonstrate that N- and O- linked sulphated oligosaccharide chains are both the antigenic and biological determinants of PIF

which was extensively glycosylated at Asn and Ser residues (Todorov et al 1996).

In 1997, Todorov and colleagues suggested a model for PIF based on their findings, this remains the favoured idea today and consists of a central polypeptide chain of 2kDa with attached phosphate residues or alternatively the phosphates may be attached to a short oligosaccharide which contains GLcN, an O-linked 6kDa GLc-N containing oligosaccharide and a 10kDa GLc-N containing N-linked oligosaccharide.

MAC16 cells produce PIF in vitro and it was originally interpreted that PIF was therefore a tumour derived factor. However, it seems unlikely that this protein would have no constitutive function. Accordingly Watchorn et al (2001) demonstrated that in fact the production of PIF peaks during E8 and E9 of gestation, suggesting a new constitutive function for PIF in embryonic development. It is likely that the increased expression seen in cachexia is an inappropriate expression by the tumour, or upregulation of what is a developmental factor.

A possible human homologue for PIF has recently been identified (Wang et al 2001) from a breast cancer library by using the available twenty amino acid sequence. This protein has been given the name human cachexia inducing protein (HCAP) and RT-PCR has detected expression of HCAP in Du 45 and LNCap cancer cell liners, the bone metastatic cell lines C4-2 and C4-2b although not in normal prostate tissues. Preliminary evidence has also suggested that (like PIF), HCAP has been detected in the urine from cachectic prostate cancer patients but not in the urine from non-cachectic cancer patients. However at this point it is still unknown whether PIF and HCAP are indeed the same molecule.

Before the evidence concerning the mechanism of degradation by PIF is considered, a review of skeletal muscle and its proteolysis will prove useful.

## 3. Proteolytic pathways in Skeletal Muscle

Skeletal muscle is composed of bundles of elongated, multinucleated cells called fibres which contain within their sarcoplasm thousands of myofibrils. These actin and myosin containing units (sarcomeres) provide the contractive force of muscle. The fibres do not divide themselves to produce new muscle, instead mononucleated satellite cells on the cell surface divide and stimulate the production of new actin and myosin. It is this accumulation of new myofibrils which results in an increase in muscle mass in response to exercise and it is their intracellular degradation which reflects muscle atrophy (Reviewed in Mitch and Price 2001).

The three main pathways involved in the degradation of skeletal muscle proteins are: -

1. Lysosomal proteases (including cysteine proteases) - The cathepsins
2. $Ca^{2+}$ dependent cysteine proteases - The calpains
3. ATP ubiquitin dependent proteolysis – The ubiquitin-proteasome

Degradation of extracellular proteins (e.g. hormones or phagocytosed bacteria) is typically mediated by endocytosis within lysosomes containing the four major proteases - cathepsins B, H, L and D, along with other acid hydrolases. Degradation of extracellular proteins constitutes the main proteolytic activity of the lysosomal system, although some cytosolic proteins can be degraded (either by direct transport into the lysosome or following incorporation into an autophagic vacuole which fuses with the lysosome). This system is thought to have a minor role in the turnover of cytosolic proteins and although enhanced lysosomal proteolysis has been reported in the muscle of cancer patients, it accounts for only a minor part of the degradation seen in catabolic states (reviewed in Lecker et al 1999).

The calcium dependent (ATP independent) cytosolic pathway involves the cysteine proteases termed calpains. Two isoforms have been identified -m and μ calpains, which differ in their affinities for calcium. It has been shown

that lysosomal proteolysis plays a minor role in protein breakdown, whilst the calcium dependent proteases may be qualitatively important for the degradation of crucial but quantitatively minor proteins (Reviewed in Attaix et al 1998).

Another important structurally related family of cytosolic cysteine proteases are the caspases or ICE (Interleukin-1β converting enzyme) –related proteases. These proteases cleave after aspartate residues and whilst they play a central role in apoptosis, they are not thought to be involved in the majority of protein degradation (Schutte et al 2000).

What is clear is that none of these pathways are responsible for the degradation of the major contractile myofibrillar proteins (actin and myosin) which make up the bulk of skeletal muscle. Instead these are degraded by the ubiquitin proteasome pathway which also plays a major role in the degradation of cytosolic proteins important in the cell cycle. This pathway has been found to play a significant role in muscle wasting. As such it has been implicated in both cachectic muscle catabolism (discussed in the following chapter) and tumour growth. It is the system primarily responsible for muscle cachexia in the MAC16 model utilised here.

## 3.1 The Ubiquitin Proteasome Pathway.

### 3.1.1 Proteasome Structure

The proteasome is a large multi-catalytic protease that degrades proteins into small peptides. It can be separated into two sub complexes - a catalytic 20S core and a regulatory particle.

The 20S core has a molecular mass of 700-750kDa and has a barrel shaped appearance. It is a 28mer of 14 different subunits arranged into a stack of two 7 membered outer α- and two 7 membered inner β- rings. The inner surface of the β rings contain the catalytically active sites but entry is

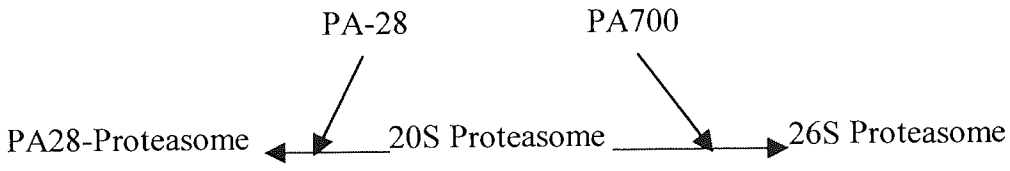
mediated through the α rings, the centres of which are normally almost closed. The catalytic activity of the proteasome is regulated by its subunit composition. Subunit variability may influence the rates and specificity of degradation. For example the three catalytic subunits β1 (Y or δ), β2 (Z) and β4 (X) can be replaced by the IFNγ inducible subunits LMP2, MECL1 and LMP7, resulting in a proteasome with altered catalytic activity. Proteasomes with these subunits, (unlike those with their constitutive counterparts), hydrolyse peptides with hydrophobic and basic residues in the P1 position (see table 3, pp40) more readily than those with acidic residues in the P1 (reviewed in DeMartino and Slaughter 1999).

Whilst the 20S proteasome can be activated in vitro, it is thought that its activity in vivo depends upon its interaction with other subunits. A 700kDa activator called 19S, the μ-particle or PA700 can bind to both end of the 20S core, conferring both ubiquitin and ATP dependence on protein degradation, the complex is now called a 26S proteasome. The 19S particle is divided into two domains. A base consisting of six ATPases of the AAA family and of three additional proteins, and an eight-subunit lid. The 20S proteasome can break down denatured proteins in the absence of ATP, therefore one anticipated function of 19S is as a protein unfoldase catalysing conformational changes in the substrate, utilizing ATP hydrolysis to control access to the active sites. It has also been suggested that 19S may enhance proteolysis by allosterical activation of catalytic sites (reviewed in Hochstrausser 1995). 19S confers ubiquitin specificity to the proteasome because it serves as the recognition component of the polyubiquitin chain, it also has isopeptidase activity thought to be important in disassembly of polyubiquitin chains and whilst it utilises polyubiquitin to select most proteins for proteasomal degradation it can also recognise some non-ubiquitinated proteins (the most well known example being ornithine decarboxylase (ODC) (Attaix et al 1998).

Similarly, though less well studied, the 28kDa proteasome activator – PA28 or 11S can bind to 20S (see figure 4) in mammalian cells. It is composed of

two 28kDa subunits α and β which form a heterohexameric or heteroheptameric ring shaped molecule. 11S activates the hydrolysis of short peptides, but it does so in the absence of ATP or any other known co-factors. This probably explains its inability to unfold large peptides which are likely to require ATP hydrolysis. It is thought that 11S probably activates the proteasome in a manner comparable to that of 19S by opening the catalytic channel of 20S and unfolding/translocating short peptides. The physiological role of PA28 is unclear, although a postulated role is in antigen processing and the immune response (reviewed in Kornitzer and Chiechenover 2000).

Fig 4) Interaction of the 20S Proteasome with Alternative Regulatory Subunits.




### 3.1.2 Proteasome Activity

Work with fluorogenic substrates and inhibitors has defined five distinct specificities for the proteasome (see table 3), crystal structure and biochemical data suggest that each activity is related to a specific β subunit. These subunits contain N-terminal threonines as catalytic nucleophiles confirming the novel nature of the proteasome (reviewed in Tanahashi et al 1999 and Chiechanover 1994).

Table 3) The Preferred P1 Residues for the Variable Activities of the Proteasome.

| Proteasome Activity | Preferred residues in the P1 position |
|---|---|
| Chymotrypsin-like | Tyr or Phe (large and hydrophobic) |
| Trypsin-like | Arg or Lys (basic) |
| Post-glutamyl hydrolase | Glu (acidic) |
| BrAAP | Branched chain |
| SNAAP | Small and neutral |

### 3.1.3 The Ubiquitin System

Ubiquitin (ub) is a 76kDa protein which is conjugated to other peptides to form an amide (isopeptide) bond between the C-terminal (Gly 76) residue of ubiquitin and ε-amino group of a lysine residue in an acceptor protein. Ub is activated by an ub activating enzyme (E1) coupling ATP hydrolysis to the formation of a thioester bond between Glyc 76 and a cysteine residue of E1. It is them transesterified from E1 to a cysteine residue on the ub conjugating enzyme E2 and from there to a lysine residue on the target protein as shown in the diagram 5 overleaf (reviewed in Varshavsky 1997).

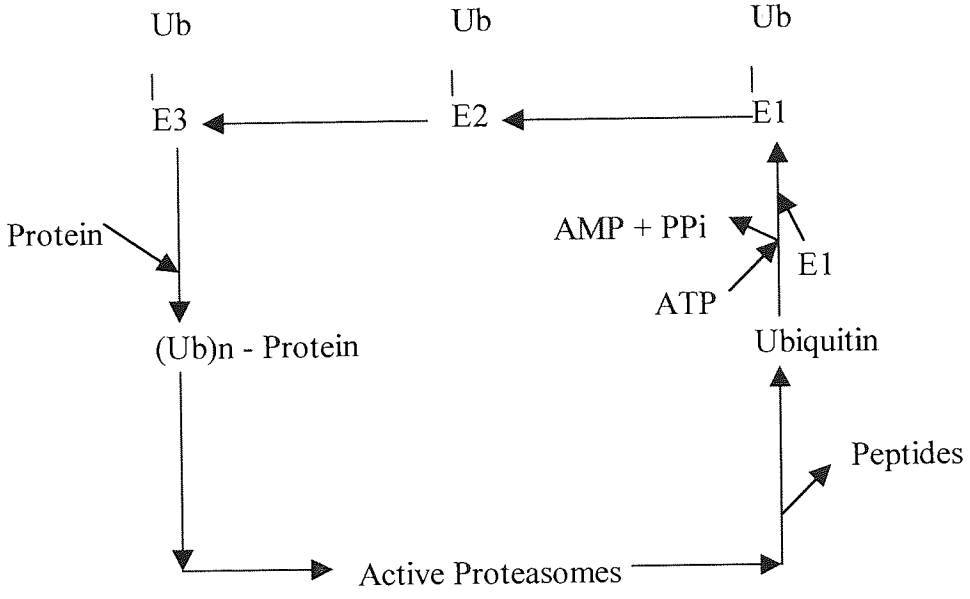


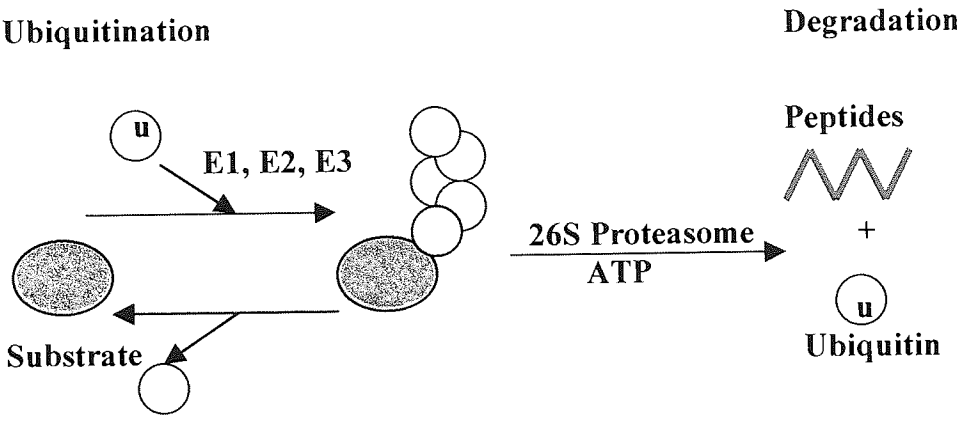

Fig 5) The Degradation of Proteins via Ubiquitin Conjugation.

The target protein is selected through the interaction of a derivative signal with the E3 enzyme (reviewed in Varshavsky 1997). Proteins can be multi-ubiquitylated, with many ubiquitin moieties attached as chains or trees.

The covalent bond between ubiquitin and other proteins can be cleaved by one of a large family of ub-specific processing proteases. Cleavage of ubiquitin removes the derivative signal and serves as another site in which the system can be regulated. The degradation signals themselves can either be active constitutively or conditionally. In eukaryotes the N-end rule pathway is part of the ubiquitin system. This has been reviewed by Lecker et al (1999) but briefly states that the in vivo half life of a protein depends upon the identity of its N-terminal residue. It has also been suggested that PEST motifs (sequences rich in Pro, Glu, Ser and Thre) function as degradation signals. It is pertinent to point out that Ub does however, have functions not associated with proteasomal degradation. Multiubiquitin chains linked to the cytosolic face of transmembrane receptors signal them for endocytosis (and not necessarily degradation), whilst compartmentalised proteins can also be tagged with ub, signalling them for degradation via non proteasomal

pathways (Reviewed in Varshavsky 1997). The relationship between ubiquitylation and the proteasome is shown below.

Fig 6) Simplified View of Protein Ubiquitination and Degradation




### 3.2 Proteolytic pathways in Cancer Cachexia

The ubiquitin proteasome pathway has been implicated in muscle catabolism occurring in a wide range of conditions including diabetes (Merforth et al 1999, Lecker et al 1999), AIDS (Llovera et al 1998); sepsis (Voisin 1996, Hasselgren 1999, Fischer et al 2000), starvation (Medina et al 1991, Wing and Goldberg 1993) and denervation atrophy (Medina et al 1991). However the largest body of evidence concerns the role of the proteasome in tumour growth and cachexia.

Research has suggested that the ubiquitin proteasome pathway is the major pathway of muscle protein loss in both normal and cachectic states (Llovera et al 1994; Lorite et al 1998; Solomon et al 1998; Wing and Banville 1994).

Llovera et al (1994) demonstrated that whilst lysosomal proteolysis was not involved in the protein degradation observed in the gastrocnemius muscles of rats bearing the AH-130 Yoshida ascites hepatoma, the ubiquitin pools (both free and conjugated) were markedly altered as a result of tumour burden. This was associated with a (>500%) increased ubiquitin expression

compared to controls. This study also found elevated levels of TNFα which it suggested might be involved in the activation of the proteasome system in this model.

Again, Temparis et al (1994) demonstrated that in the *extensor digitorium longus* and *tibialis anterior* muscles of rats bearing the Yoshida sarcoma protein degradation was significantly depressed. Inhibitors of lysosomal and calcium dependent proteolysis did not attenuate the increased proteolysis and cathepsin B and B+L activities were unchanged. In contrast ATP depletion almost totally suppressed the increased protein breakdown. Additionally mRNA levels for ubiquitin, $E2_{14k}$ and the C8 and C9 subunits of the proteasome were increased in the atrophying muscles, again suggesting that the ubiquitin proteasome pathway is mainly responsible for the muscle atrophy in Yoshida sarcoma-bearing rats.

Combaret et al (1999) demonstrated that inhibition of muscle atrophy in sarcoma bearing rats was mediated through the nonlysosomal calcium dependent proteolytic pathway, this occurred through suppression of the enhanced expression of ubiquitin as well as $E2_{14k}$ and the C2 proteasome subunit in muscle in cancer bearing rats. The mRNA levels for the ATPase subunit MSS1 of the 19S complex increased in cancer cachexia, in contrast with the activation of other regulatory subunits.

The cachexia inducing colon tumour C-26 was used to create the cell line R-1, capable of inducing cytokine independent muscle and adipose loss in the presence of unchanging food intake, when injected into recipient animals. Lazarus et al (1999) showed that this was associated with a significant degradation of protein and an increased level of ubiquitin conjugation in the muscle. The latter was prevented by the proteasome inhibitor - lactacystin, although this had no effect on proteolysis. Similarly several markers of the pathway most noticeably $E2_{14k}$ expression were unaffected. This latter finding is in conflict with other workers who have found that the levels of ubiquitin protein conjugates (Llovera et al 1994 and Lorite et al 1998), and

$E2_{14k}$ (Lorite et al 1998; Medina et al 1995) in particular have been elevated in cachexia.

Comparable results have been found in human studies. Williams et al (1999) reported that the mRNA levels for ubiquitin and the 20S proteasome α- (HC3 and HC9) and β- (HC5 and HC7) subunits were 2-4 times higher in rectus abdominus muscle from patients with cancer compared to controls, reflecting the involvement of this pathway in cancer associated muscle catabolism across the species.

Baracos et al (1995) investigated the involvement of the various proteolytic pathways in rats implanted with the cachexia inducing Yoshida ascites hepatoma and showed that the 63-95% increase in protein degradation observed, was not affected by blocking the calcium dependent system whilst methylamine – an inhibitor of lysosomal function – reduced proteolysis by only 12% in cachectic rats. Thus whilst it seems that the lysosomal pathway was activated to a modest degree, ATP depletion resulted in a fall in the elevated proteolysis to control levels. Accordingly increased levels of ubiquitin conjugated proteins and mRNA levels of the proteasome subunits C2, C3, C6 and C9 were observed. (Typically heart and liver which did not exhibit weight loss show no changes in the levels of these mRNA species). This established that the accelerated muscle proteolysis in tumour bearing rats results from activation of the ubiquitin proteasome pathway. (Although it is possible that a coordinated stimulation of these two different proteolytic systems might serve to eliminate different classes of proteins). Interestingly these workers also showed that the prostaglandin synthesis inhibitor-naproxen, did not reverse the elevated ubiquitin mRNA levels, indicating that this mediator is not responsible for the elevated muscle catabolism. Correspondingly, Llovera et al (1994) showed that lysosomal enzyme activity (cathepsins B and B+L) was actually decreased in gastrocnemius muscles from rats bearing the Yoshida tumour, whilst free and conjugated ubiquitin pools were increased and ubiquitin mRNA levels were 500% greater in tumour bearing rats compared to controls. Like the findings of

Baracos et al (1995) this implicates the ubiquitin proteasome pathway in this model of cachexia. However unlike Baracos, the authors suggest that the high levels of TNF found in this model might contribute to the activation of this system, possibly by triggering enhanced ubiquitin gene expression.

Costelli et al (2001) specifically examined the role of the calcium dependent pathway in the skeletal muscle and heart of rats bearing the Yoshida sarcoma and found that whilst calpain levels were unchanged, levels of calpastatin (the natural inhibitor) declined, resulting in a progressively increasing imbalance in the calpain/calpastatin ratio. An observation which suggests that the calcium mediated pathway may play a greater role in cachexia than is suggested by the unchanging calpain levels frequently observed.

It is interesting to note that unlike other workers (Baracos et al 1995), Combaret and colleagues (1999) reported a decrease in the mass of liver, small intestine, kidney, heart and skin in rats bearing the Yoshida sarcoma. Whether the potential proteolysis of visceral muscle in this case is significant in terms of the active proteolytic mechanisms, is not known

An example of pharmacological manipulation of the ubiquitin proteasome pathway in cachexia, was provided by Combaret et al (1999). Daily administration of the xanthine derivative – Pentoxifylline (PTX) prevented muscle atrophy in Yoshida sarcoma bearing rats. E64 and methylamine, inhibitors of lysosomal and calcium dependent proteolysis did not suppress the increased protein breakdown and had no effect upon cathepsin D or m-calpain mRNA levels. In contrast PTX suppressed the activation of nonlysosomal calcium independent pathway and reduced the expression of multiple components of the ubiquitin proteasome pathway including $E2_{14k}$, C2 and other proteasome subunits. The effects upon muscle mass were not mediated as a consequence of reduced tumour mass, since this remained unaffected. Again the authors postulated (but have not proven) that since PTX prevents TNFα transcription (and that because anti-TNF antibodies can

sometimes block increased proteasome subunit expression), this cytokine may be involved in this model.

Likewise, Temparis et al (1994) showed that cathepsin B and B+L activities, cathepsin mRNA levels were unaffected, and that inhibitors of calcium and lysosomal dependent proteolysis did not prevent increased proteolysis in the extensor digitorum longus and tibialis anterior muscles of Yoshida sarcoma bearing rats. On the other hand, levels of C8 and C9 proteasome subunits and the $E2_{14k}$ ubiquitin conjugating enzyme were elevated and ATP depletion almost completely suppressed the elevated proteolysis.

Llovera et al (1995) used the Yoshida model and demonstrated a 30%+ loss of muscle mass in the gastrocnemius and EDL muscles, which could not be prevented by methylamine or the calcium ionophore A12387. ATP depletion did, however, abolish the elevated proteolysis, demonstrating once again that the ATP ubiquitin dependent pathway was responsible for the excessive muscle wasting seen.

To summarise, table 4 (adapted from Lecker et al 1999) demonstrates the current evidence implicating the ubiquitin proteasome pathway in models of cachexia.

Table 4) Experimental Observations in Models of Muscle Wasting.
(adapted from Lecker et al 1999)

| |
|---|
| Increased ATP dependent proteolysis in skeletal muscles |
| Increased susceptibility of proteolysis to proteasome inhibitors |
| Lack of susceptibility to calpain and cathepsin inhibitors |
| Increased ubiquitin content of muscle |
| Increased content of ubiquitin protein conjugates |
| Elevated ubiquitin mRNA |
| Elevated $E2_{14k}$ mRNA |
| Elevated Proteasome subunit mRNA |
| General lack of elevation of cathepsin and/or calpain mRNA |
| Increased ubiquitin conjugation to muscles |

### 3.2.1 Mechanism of Protein Degradation by PIF

The point that a circulating factor might be responsible for the skeletal muscle catabolism seen in MAC16 bearing animals was made nearly two decades ago and the process of its discovery is discussed in chapter 2.2. However the mechanisms through which this proteolysis inducing factor – PIF effects protein degradation is still a subject of investigation.

It has now been repeatedly demonstrated that the central action of PIF is to massively increase protein degradation both in vitro (e.g. Smith and Tisdale 1993) and in vivo (e.g. Beck et al 1991 and Cariuk et al 1997), in a direct manner, not associated (in vivo) with a reduction in food intake. Accordingly NMRI mice administered PIF show depressed plasma levels of several amino acids (threonine, serine, proline, glycine, alanine, methionine, isoleucine, leucine, lysine, tryptophan and histidine) reflecting the increase in muscle catabolism (Lorite et al 1997).

Although some in vivo studies have noted a depression in protein synthesis (Smith and Tisdale 1993b and Lorite et al 1997),it appears to be only transiently affected in vitro (Smith et al 1999).

Many tumours have an increased specific requirement for glucose, and the increased gluconeogenic demand can often result in increased energy expenditure. This in turn could result in a negative energy balance which could further contribute to weight loss (Cariuk et al 1997).

Rodemann and Goldberg (1982) have shown that the polyunsaturated fatty acid - arachidonate is capable of stimulating protein degradation in skeletal muscle and it was originally thought that skeletal muscle catabolism might be the indirect result of arachidonic acid release during lipolysis. It has been shown that PIF can induce protein degradation in a dose dependent manner in C2C12 myoblasts, with a maximum at a concentration of 4nM. It also produces an increased release in arachidonate from pre-labelled cells, which is rapidly metabolised to prostaglandins and hydroxyeicosatetraenoic acids

(see chapter 4 for further discussion). The increased degradation induced by PIF is associated in particular with elevated 15-hydroxyeicosatetraenoic acid and prostaglandin $E_2$ production. That proteolysis can be suppressed by the cyclooxygenase inhibitor indomethacin, suggested that prostaglandin metabolites may be directly involved in the mechanism (Smith et al 1999 and Smith and Tisdale 1993).

Lorite et al 1998 investigated the role of the three main proteolytic systems in MAC16 tumour bearing mice and in NMRI mice treated with purified PIF. Combinations of methylamine, calcium depletion+E64 and ATP depletion, were used to inhibit lysosomal, calcium dependent and ATP dependent functions respectively. Tyrosine release in soleus muscle demonstrated that in MAC16 mice compared to healthy controls, lysosomal proteolytic activity and levels of Cathepsins B and L were elevated. Similarly in calcium blocking conditions, a significant reduction in protein degradation occurred suggesting that both these pathways play a role. However the non-lysosomal ATP dependent pathway appeared to play the major role in the excessive proteolysis of skeletal muscle seen in mice bearing the MAC16 tumour. Significantly, in animals treated with PIF only the ATP dependent non-lysosomal pathway was activated in soleus muscle, with no contribution from either of the two other major pathways. Western analyses also showed a 42% elevation in the levels of ubiquitin conjugates in MAC16 mice and a significant increase in PIF treated mice. These results, taken together, suggest that the ATP-ubiquitin dependent pathway is the primary event in the degradation of skeletal muscle by PIF.

Recently Lorite et al (2001) demonstrated that the weight loss induced by intravenous administration of PIF to healthy mice (8.2% after 24 hours) was accompanied by increased ubiquitin (64% for the 1.2kb transcript and 70% for the 2.4kb transcript), E2 (83% for the 1.2kb transcript and 31% for the 1.8kb transcript), and C9 proteasome subunit (approximately200%) mRNA levels. Cellular levels of 20S and 19S subunits were detected. Similarly increased protein degradation induced in vitro by addition of PIF, can be

attenuated by the proteasome inhibitors MG115 and lactacystin, confirming that PIF acts directly to stimulate the proteasome pathway in muscle cells.

Although the involvement of the ubiquitin proteasome pathway in PIF induced protein catabolism is paramount, Belizario et al (2001) also examined the role of the caspases in MAC16 and MAC13 bearing mice and found that the activities of caspases-1, 8, 3, 6 and 9 were increased by 84%, 98%, 151%, and 177% respectively, in the gastrocnemius muscle of MAC16 compared to MAC13 tumour bearing mice. There was also a dual pattern in poly-ADP-ribose polymerase fragmentation possibly suggesting that apoptosis is involved in the degradation process. It is possible that caspases act to initiate apoptosis, and the fragments are then degraded further via the ubiquitin proteasome pathway. However it is not known whether the caspase activity was even related to cell death, particularly given that there was no evidence of DNA fragmentation.

Therefore whether the caspase family - and how the proteasome family – are involved in the specific actions of PIF remains to be determined

### 3.3 Proteolytic Pathways and Tumour Growth

It is well known that proteases are involved in the progression of apoptosis and as the following examples indicate, inhibitors of the ubiquitin proteasome pathway can function as anti-tumour agents.

Cell cycle progression requires degradation of key regulatory proteins such as cyclins, cyclin dependent kinase inhibitors and anaphase inhibitory proteins (Meng et al 1999). These processes are mediated by the ubiquitin proteasome pathway. Accordingly, the attention of much research has been the role of the proteasome in apoptosis and proteasome inhibitors as anti-tumour agents

Exposure of cultured rodent fibroblasts and human lymphoblasts to benzoyloxycarbonyl-leucyl-leucyl-phenylalaninal (Z-LLF-CGO), a peptidyl aldehyde inhibitor of the proteasome, resulted in induction of apoptosis in a rapid dose-dependent fashion. Moreover when these cells were transformed with ras or myc they were up to 40 fold more susceptible to apoptosis. In in vivo studies, single doses of Z-LLF-CGO to nude mice bearing Burkitt's lymphoma tumours, significantly delayed tumour progression, implicating the proteasome in the growth of c-myc and ras mutated tumours (Orlowski et al 1998).

Meng et al (1999) used the selective 20S inhibitor eponomycin and demonstrated that the three major activities of the proteasome were inhibited inducing a spindle like cellular morphological change and apoptosis. Inhibition of the proteasome with eponomycin has also been shown to result in angiogenesis inhibition in the chick chorioallantoic membrane suggesting a role for the pathway in neovascularisation (Oikawa et al 1991).

Cells exposed to proteasome inhibitors have been noted to arrest at various points in the cell cycle, but those that seem to undergo apoptosis most readily appear to be traversing the G1-S boundary. As a result proteins that impact on this transition point, such as p53 and p27-kip1, have been suggested to be of importance in the mechanism of induction of apoptosis by proteasome inhibitors (reviewed in Orloeski et al 1998).

The boronic acid analogue proteasome inhibitor, PS-341, has exhibited substantial cytotoxicity against the human prostate cancer cell line PC-3. In vitro this agent resulted in substantial apoptosis and in vivo it caused a 60% or 70% decrease in tumour burden when injected i.v. or directly into the tumour (Adams et al 1991). Shinohara et al (1996) induced apoptosis in MOLT-4 and L5278Y cells with the proteasome inhibitor ZLLLa1. This effect was accompanied by an accumulation of p53, again suggesting that inhibition of the proteasome induces p53 dependent apoptosis and that the proteasome has a protective function. As

discovered that this too strongly induced apoptosis, suggesting the possibility that calpain activity may be involved. However ZLLa1 a strong calpain but poor proteasome inhibitor did not induce apoptosis in these cell lines and led the authors to postulate that apoptosis induction was mediated mainly by the proteasome.

The dipeptidyl proteasome inhibitor CEP1612 rapidly induced programmed cell death in human Jurkat cells, the prostate cancer PC-3 and breast cancer lines MDA-MB-231 and MCF-7, all of which either contain a mutant p53 gene and/or overexpress bcl-2. The process was p53 dependent and associated with accumulation of the cyclin dependent kinase inhibitors p21 and p27. It was also found that CEP1612 caused accumulation of p27 and induced apoptosis in SV-40 transformed fibroblasts, but not the parental normal fibroblasts (An et al 1998).

You et al (1999) showed that 5μM MG132 triggered apoptosis in PC3 human prostate cancer cells and also in human T-leukaemic cells. This was accompanied by Bcl-2 phosphorylation. The authors speculate that the significance of this may be that an accumulation of phospho-Bcl-2 might reflect an inhibition of its proteasome mediated degradation, and that the products of Bcl-2 might be key to its antiapoptotic function. Thus an inability to degrade Bcl-2 favours an apoptotic environment.

Whilst it seems there is some evidence for the involvement of the proteasome in apoptosis, it is conflicting and not well investigated and it seems that the role of the pathway could be cell system specific. It is well established that the main enzymes involved in apoptosis are the caspases (Reviewed in Solary et al 1998), although caspase activation is often a requirement in proteasome inhibitor induced apoptosis, suggesting proteasome involvement may be upstream of the caspases (An et al 1998). What is clearly suggested by current evidence is that proteasome inhibition has obvious consequences upon tumour growth and muscle catabolism. There is a large body of evidence which establishes a role for dietary fats in the progression and manipulation of tumour growth (including apoptosis) and cachexia. More

recent findings have suggested a novel role for dietary fats in the manipulation of the proteasome. Taken together the question which arises is whether dietary fatty acids can influence tumour growth and cachexia through a mechanism that is proteasome dependent.

# 4 A Review of Fatty Acid Structure, Nomenclature, Biochemistry and Function

Fatty acids are long chain hydrocarbons containing a methyl and a carboxyl terminus and which vary in their degree of saturation, ranging from fully saturated, to mono-unsaturated and polyunsaturated fatty acids (PUFAs). Fatty acids have numerical identifiers for example 18:2 Δ 9,12. The first number indicates the number of carbons in the chain, followed by the number of double bonds, the number following delta indicates the position of the double bonds counting from the first carboxyl carbon. The omega (ω or n) classification refers to the position of the omega group (double bond) relative to the methyl carbon, such that n-3 fatty acids are those which contain their first double bond 3 carbons away from the methyl end. Four major omega families exist in man n-3, n-6, n-7 and n-9. Of these, n-3 and n-6 are essential, in that they cannot be generated from precursors in humans and must be obtained from the diet.

Both experimental and epidemiological studies have shown that the intake of essential fatty acids can influence the development and metastatic potential of human cancers. Of key significance is the role of n-3 and n-6 fatty acids, but before this is examined, a review of their metabolism will be useful.

## 4.1 n-3 Versus n-6 Fatty Acids, a Biochemical Review

As an example of polyunsaturated fatty acid (PUFA) metabolism, consider the most physiologically active member – arachidonic acid (AA). AA is stored as phospholipid in cell membranes, upon stimulation it is freed through the action of various phospholipases (Figure 7).

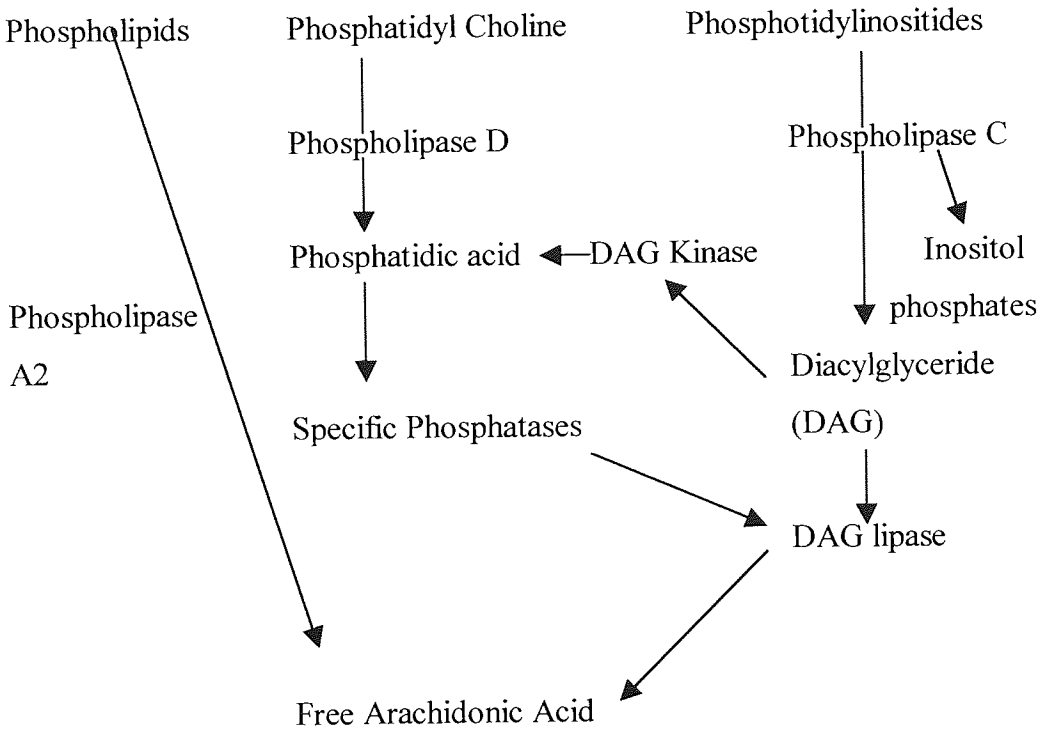



Figure 7) Pathways of Arachidonic Acid Release.

### 4.1.1 Cyclooxygenase Metabolism

Once freed arachidonic acid is metabolised mainly by the cyclooxygenase (COX) or lipoxygenase (LOX) pathways, the former leads to the production of prostaglandins (PG) $D_2$, $PGE_2$, $PGF_2\alpha$ as well as thromboxane $A_2$, $B_2$ and prostacyclin. Cyclooxygenase attaches molecular oxygen at C11 of AA, the molecule cyclises and a second molecular oxygen is attached at C15 leading to the production of the intermediate prostaglandin $G_2$ (see figure 8).

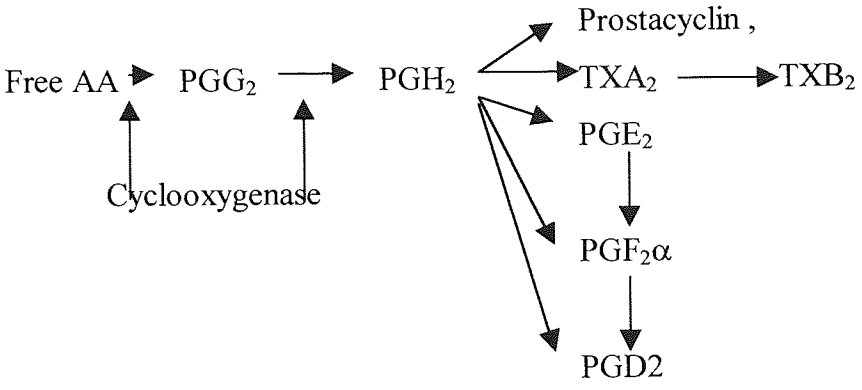



Fig 8) The COX Pathway for Arachidonic Acid Metabolism

(for explanation of abbreviations see text)

This prostaglandin endoperoxide can then be metabolised further to form thromboxanes (the major product of platelet AA metabolism, thromboxanes are potent vaso and smooth muscle constrictors, induce platelet aggregation and serotonin release); prostacyclin (a vasodilator) or other prostaglandins by the respective synthetase enzymes. These prostaglandins have various biological activities including platelet aggregation, peripheral vasodilaton, and pulmonary vaso- and broncho-constriction) (Reviewed in Needleman 1986). The main biological activities of arachidonic acid metabolites are listed in table 5.

Table 5) The Biological Effects of Various Arachidonic Acid Metabolites (adapted from Sigal 1991)

| AA METABOLITE | MAJOR BIOLOGICAL ACTIVITY |
|---|---|
| $PGD_2$ | Bronchoconstriction |
| $PGE_2$ | Broncho- vaso-dilation, increased epithelial chloride secretion |
| $TXA_2$ | Broncho, -vaso- constriction, increased epithelial chloride secretion |
| Prostacyclin | Vasodilation, increased vascular permeability, inhibited platelet aggregation |
| Leukotriene $B_4$ | Leucocyte migration, adhesion and activation |
| Leukotriene $S_4$, $D_4$, $E_4$, (SRS-A) | Broncho-, vaso-constriction, increased vascular permeability |

### 4.1.2 Lipoxygenase Metabolism

An alternative pathway for AA metabolism is the lipoxygenase pathway. These enzymes catalyse incorporation of molecular oxygen to yield a hydroperoxyeicosatetraenoic acid product (HPETE). The hydroxy group is then reduced resulting in formation of the corresponding and more stable monohydroxylated derivative- hydroxyeicosatetraenoic acid (HETE). There are three main isomers of HETE (corresponding to the position at which oxygen is inserted) and these are 5, 12 and 15 although other isomers including 8-, 9-, 11-, 19- and 20 have been detected (Spector et al 1988). The process is shown diagrammatically in figure 9 including those structures involved in the generation of 15-HETE.

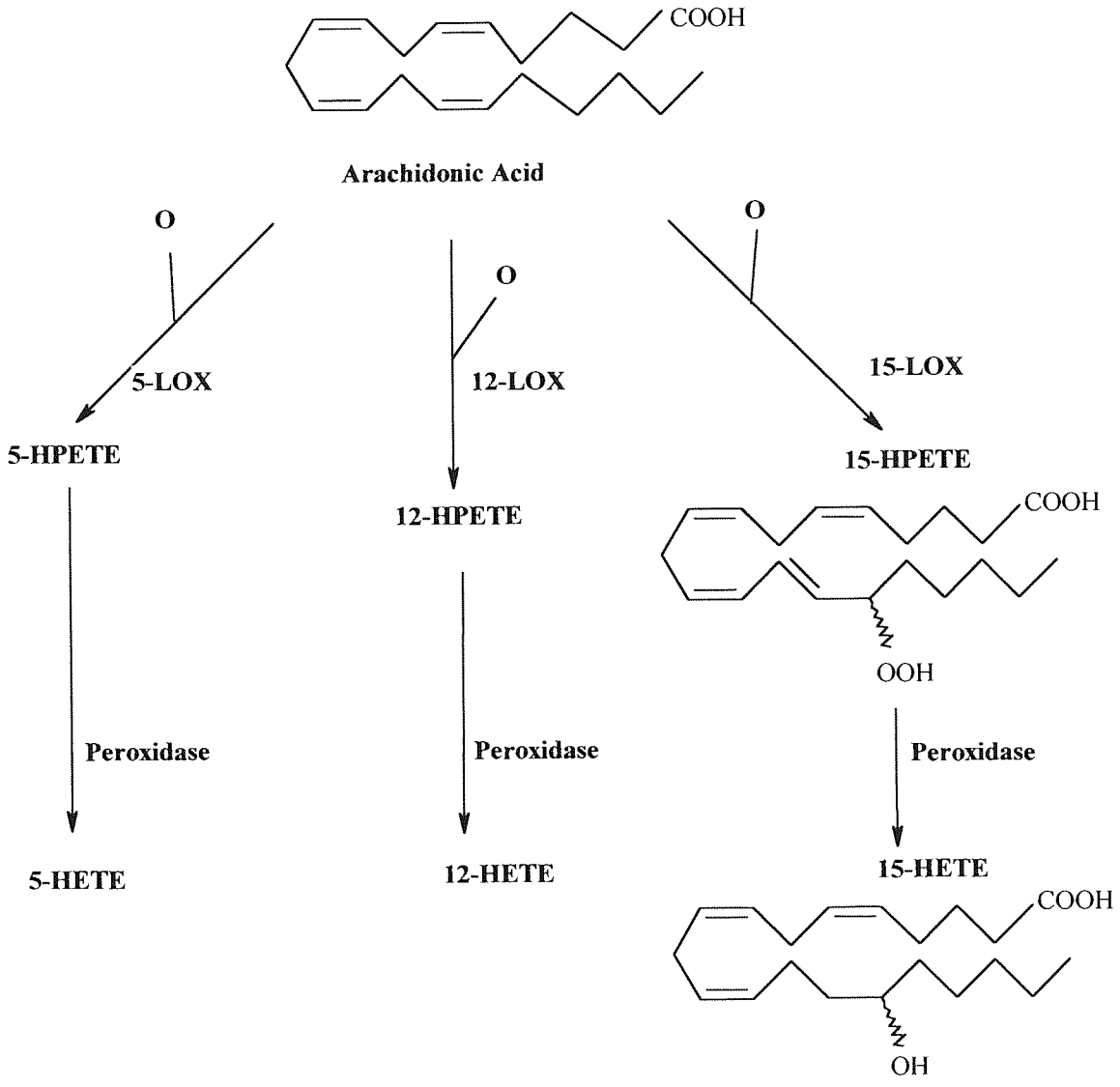



Figure 9) Simplified Diagram to show the Synthesis of the Major HETE Isomers

Originally HETEs were considered inactivation products of HPETEs which had no biological function, however it is now known that HETEs have a very significant biological impact (see table 6 overleaf) in chemotactic mediation, intracellular calcium concentration, regulation of prostaglandin to name a few (reviewed in Spector et al 1988)

Table 6) The Biological Effects of the Major HETE Isoforms

(Adapted from Spector et al 1988)

| Isoform | Major Biological Effects |
|---|---|
| **5-HETE** | Stimulates islet cells<br>Stimulates LH and FSH release<br>Mediates prolactin release<br>Inhibits phospholipase in human platelets<br>Stimulates PAF synthesis in human and rat neutrophils |
| **12-HETE** | Stimulates/inhibits islet cells<br>Stimulates cultured beta cells<br>Suppresses renin production<br>Inhibits COX in human and bovine endothelial cells<br>Inhibits $PGE_2$ in canine epithelium<br>Inhibits phospholipase in human platelets<br>Inhbits COX in murine macrophages |
| **15-HETE** | Inhibits islet cells<br>Suppresses renin production<br>Inhibits corticosterone production in response to ACTH<br>Stimulates LH and FSH release<br>Inhibits phospholipase in human platelets<br>Inhibits COX in mouse macrophages |

### 4.1.3 Epoxygenase metabolism

Arachidonic acid may also be metabolised via cytochrome P450, in the epoxygenase pathway. The characteristic reaction of this pathway is hydroxylation of arachidonic acid and its substrates, but P450 can dealkylate, deaminate, dehalogenate and epoxygenate giving rise to epoxyeicosatetraenoic acids (EETs) (Needleman 1986).

### 4.1.4 HETE Metabolites.

Leukotrienes and lipoxins represent the next stage in arachidonic acid metabolism. Leukotrienes are products of 5-HETE metabolism, and lipoxins of 15-HETE as represented in figure 10.

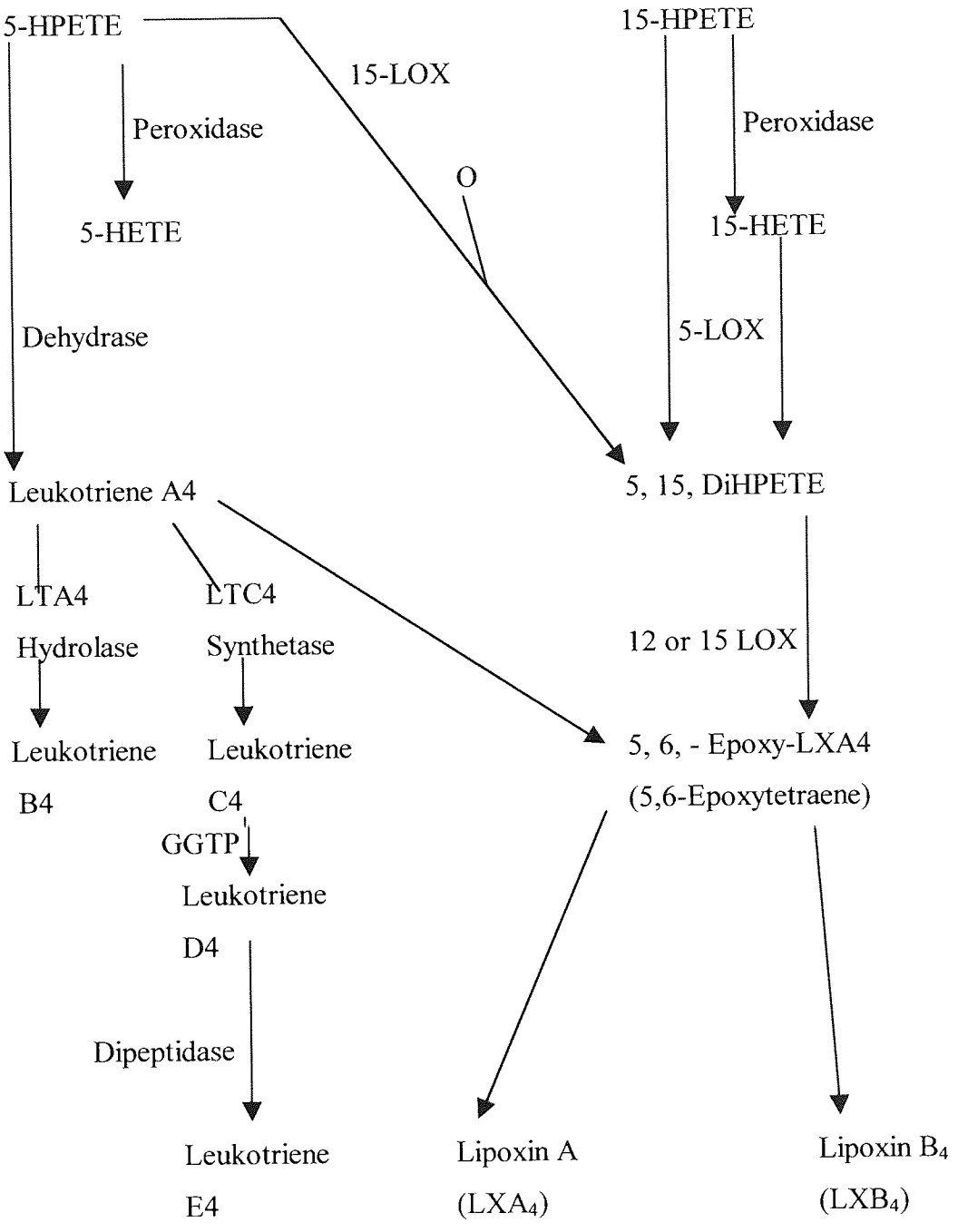
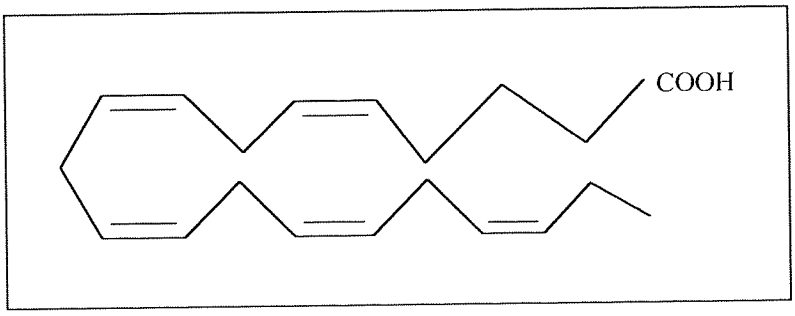



Figure 10) The Metabolism of HETEs to Lipoxins and Leukotrienes

The emphasis thus far has been on the metabolism of arachidonic acid, however metabolism of other fatty acids occurs via the same pathways, often in direct competition for the same enzymes. The other precursors of the biologically active 20 carbon eicosanoid products so far described are linoleic (n-6) , α-linolenic (n-3), eicosapentaenoic (n-3) and docosahexaenoic (n-3) acids. The relationship of these fatty acids and the structure of EPA are shown in figures 11 and 12. The most important of these fatty acids are linoleic and α–linolenic as there is some ability to desaturate and elongate these 'parent' n-6 and n-3 molecules to other PUFAs in the series.

The desaturation of fatty acids in mammalian cells involves the insertion of a double bond into the fatty acyl Co-A molecule. This transformation is catalysed by NADH and oxygen dependent microsomal desaturase enzymes. Elongation enzymes function to catalyse the insertion of two carbon units into the chain.

Figure 11) The Structure of Eicosapentaenoic Acid



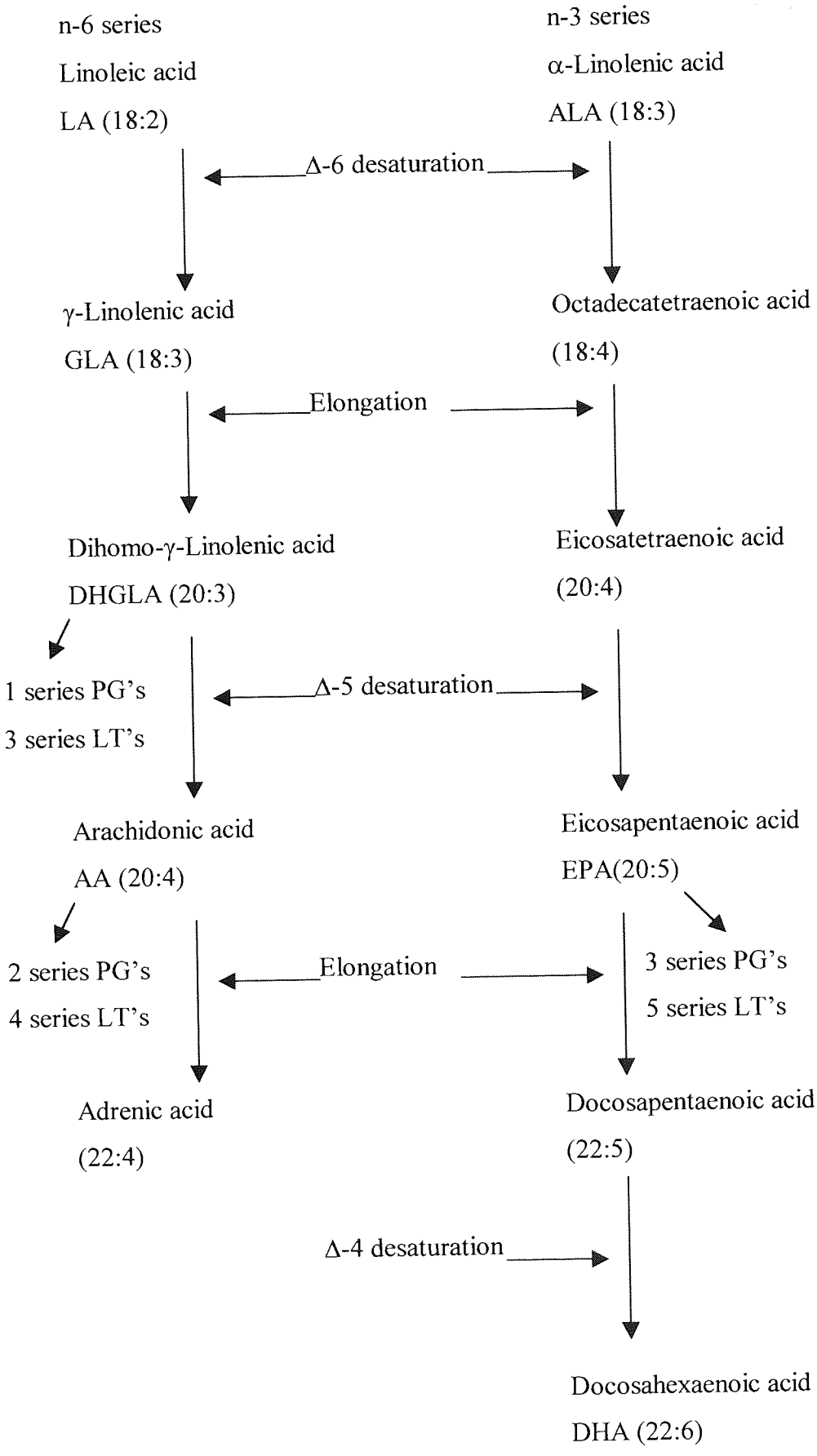



Fig 12) The Desaturation and Elongation of n-3 and n-6 Polyunsaturated Fatty Acids.

## 4.2 Polyunsaturated Fatty Acids and Cancer - Epidemiological Evidence and the Greenland Eskimo!

There is a large body of evidence directly implicating dietary fat in the development of many human cancers. Initial observations demonstrated a proportional relationship between the degree of saturation and risk of various carcinomas, including ovarian (Risch HA 1994), breast (Palmer S 1994), prostate (Giovannucci 1993 and Gann 1994, Bosland et al 1999), colorectal (Nicholson et al 1988), oesophageal (Brown et al 1995) and pancreatic (Howe 1990).

More recent observations (again initially derived from epidemiological data) have now shown that unsaturated fat can influence risk also. The role of the n-6 series of polyunsaturated fatty acids (PUFAs) has been particularly implicated, whilst an inverse relationship appears to exist with consumption of fish derived fatty acids belonging to the n-3 series particularly the n-3 eicosapentaenoic acid (EPA).

As early as 1976 Band et al, showed that the age adjusted risk of cancer amongst native Greenland Eskimos, whose dietary fat is derived almost exclusively from fish and aquatic mammals, is very low. The results of Kaiser et al (1989) demonstrated an inverse association between percent calories from fish and breast cancer rates and that when 23 dietary variables were assessed, only this one significantly affected the association with disease frequency. Carroll and Braden (1985) showed that n-6 fatty acids enhance mammary tumourigenesis, but that n-3 fatty acids have inhibitory effects at higher levels. One study (Godley 1995), measured the fatty acid composition of adipose tissue (to reflect the dietary consumption of fatty acids over a number of years) and unusually found that there was no relationship between essential fatty acid intake and breast cancer. However the authors argue that this may well be due to laboratory error associated with measuring extremely small levels of fatty acids and that small intra- and inter-individual variations in storage, transportation and mobilization of fatty acids may well have obscured real differences associated with diet. Caygill

et al (1996) on the other hand showed a very clear positive association between animal fat intake and breast and colorectal cancers and a strong negative association between fish oil consumption and these carcinomas.

Some population studies have shown that the anti-cancer effects of n-3 fatty acids are only seen in those countries in which there is also a high animal fat intake. It has been suggested that it is not fish oil consumption per se which is important, but the ratio of fish oil to animal fat. This suggests that fish oil consumption is associated with protection against the promotional effects of animal fat. The data of Caygill et al (1996) showed that this is indeed the case for both breast and colorectal cancer.

## 4.3 Polyunsaturated Fatty Acids and Cancer - Experimental Evidence

### 4.3.1 Effects on Tumour Growth

It is well established that the development of cancer occurs in two principle stages. Initiation, a permanent and irreversible change in the DNA of the transformed cell, and promotion, a continual modulation of cell growth by a variety of environmental factors. Typically animal and in vitro studies show that n-6 PUFAs tend to stimulate tumour promotion and also metastasis, neovascularisation and the progression of cachexia whilst n-3 fatty acids frequently inhibit these processes.

Hillyard and Abraham (1979) showed that a diet containing as little as 0.1% linoleate was sufficient to significantly enhance the growth rate of mammary adenocarcinoma in BALB/c mice and that AA accumulates in the membrane lipids in the human gastric cancer cell line HGT (Denizot et al 1992). Similarly Karmali et al (1984) found that AA levels were increased in neoplastic tissues compared to normal human mammary tissues. However the authors comment that such comparisons are only relevant at the tissue and not cellular level as normal mammary tissue consists mostly of

adipocytes whereas tumour tissue consists mostly of epithelial cells, mesenchymal tissue, histiocytes and lymphocytes.

Using the MDA-MB-231 human breast cancer cell line Rose and Connolly (1990), showed that linoleic acid (n-6) stimulated cell growth, whereas DHA and EPA caused a dose related inhibition. Using a battery of COX/LOX inhibitors it was shown that manipulation of MDA-MB-231 cell growth was in this case mediated by leukotriene metabolites. Interestingly the effects of oleic acid (n-9) depended on concentration. The fact that OA, LA and ALA, the precursor n-3 fatty acid all compete for the same Δ-6 desaturase (but with different affinities) causes competition between the three FA groups and at relatively high concentrations, one FA could inhibit the conversion of another and hence its entry into eicosanoid biosynthetic pathways.

The effects of EPA and DHA on tumour growth in F344 rats has been investigated by Karmali et al (1984), who demonstrated a significant reduction in weight and tumour volume after four weeks of treatment. Both EPA and DHA were found in the phospholipid fraction of the tumour cells and this combined with the observation that content and synthesis of 2-series prostaglandins ($PGE_2$, $PGF_2\alpha$, along with 6-keto-$PGF_1\alpha$ and $TXB_2$) were inhibited supported the theory that inhibitory effects were due to interference with arachidonic acid metabolism.

Maehle et al (1994) showed that the growth of tumourigenic (THKE) cells was inhibited 25% more than immortalised (IHKE) cells at 80μM EPA and 35% more at 40μM DHA, and that this correlated with inhibition of tumour growth in nude mice fed 55% EPA and 35% DHA. This study is interesting because by integrating v-Ha-ras into an immortalised cell line, resulting in tumourigenicity, tumour progression could be studied, and it was at this stage that the cells became sensitive to the growth inhibitory effects of n-3 fatty acids. This demonstrates that the n-3 effect was a late event during the progression to malignancy (and not due to modification of gene expression or the p21 ras protein for example).

Other workers (Nelson and Bedanier 1994) have shown that a two week culture in 100μM n-6 linoleic acid is not capable of transforming a normal primary breast cell line to a mammary cell carcinoma, suggesting that in this case (unlike the control DMBA, which was capable of transformation), linoleic acid was not acting as an initiator.

Sakaguchi et al (1990) examined the role of a normal versus a high n-3 fatty acid or a high saturated fat diet on the growth of COLO-320 and HT-29 human colon cancer cell lines in nude mice. It was discovered that the n-3 diet significantly reduced the tumour growth of both tumours after 4 weeks ($P<0.05$). This was accompanied by a significant incorporation of n-3 fatty acids in red cell membranes, adipose tissue and both neutral and phospholipid fractions of the tumour lipids and a concomitant reduction of LA and AA in these tissues. Significantly, this was most greatly evidenced in the metabolically labile phospholipid fraction. This supports the work of Kitada et al (1981) who showed that lipid mobilisation in tumour bearing mice is largely utilised for membrane synthesis by tumours rather than for energy utilisation by beta-oxidation which occurs in normal mice. Another interesting observation was that mitotic indices did not vary between treatment groups implying that the effect of n-3 FAs is to contribute to tumour cell destruction rather than to impede cell division.

This was also the case for BALB/c mice fed a diet containing 10% corn oil which has about 60% of its fatty acids as linoleate compared to a control group without linoleate, where no differences were found in the duration of the cell cycle phases and the only significantly different parameter was the rate of tumour cell loss (Gabor et al 1985). In fact tumour cell loss for adenocarcinomas from control mice was twice that of mice fed the high corn oil diet. Dietary linoleate did not influence inflammatory cell infiltration and so the increase in mass could not have been caused by non specific swelling. Furthermore the fact that the COX inhibitor indomethacin reduced the elevated tumour growth rate, again provide support for the hypothesis that tumour growth is in some models mediated by prostaglandins (Gabor et al 1985).

Conversely others have shown that an inhibitor which prevents the conversion of linoleate to arachidonate – eicosa-5, 8, 11, 14tetraynoic acid (TYA) caused a 3-5 times reduction in the size of adenocarcinomas in C3H mice fed a high linoleate diet compared to tumour bearing mice fed the same diet but in the absence of TYA. The fact that aspirin, another prostaglandin synthesis inhibitor did not affect tumour size led the authors to conclude that the growth of this mammary tumour was not related to prostaglandin synthesis but to the availability of arachidonate (Rao and Abraham 1977). Their findings further suggested that the tumour content of arachidonate, rather than linoleate is related to tumour growth.

Borgeson et al (1989) demonstrated that fish oil significantly reduces the growth rate of MX-1 human mammary carcinomas in mice from a mean 1847mg to mean 896mg, as well as increasing sensitivity to two anti-neoplastic agents – mitomycin C and doxorubicin. Enhancement of the chemotherapeutic indices of these agents may be due to an intracellular membrane target site, or to an effect on the transport of the drug to its active site. The notion that n-3 diets alter the axis of arachidonic acid metabolism such that the eicosanoids produced do not favour tumour growth is not supported by this work. In contrast, Borgeson et al showed that $PGI_3$ could not be detected in aortic endothelium, which had been incubated with radiolabelled EPA, bringing into question the role of n-3 FAs as PG precursors in all tissues.

It has also been proposed that EPA may effect apoptosis through 'anoikis' (the induction of apoptosis as a result of loss of cell contact) in the colorectal cell line HT29 (Clarke et al 1999). It was shown that EPA increased the rate at which adherent HT29 cells shed into the medium in a manner that was not associated with classical apoptosis and did not reduce mitosis. The authors postulate that anoikis may be the mechanism through which EPA affects tumour growth in vivo.

In mice bearing an azaserine induced pancreatic carcinoma and fed a high saturated fat diet, dietary supplementation of EPA yielded confusing results.

The high fat diet had a strong promoting effect, which EPA did not reverse. However EPA did cause a decrease in atypical acinar cell foci and pancreatic levels of 6-keto-PG-F1$\alpha$, $PGF_2$ and $TXB_2$ (Appel and Wouterson 1994). Interestingly it was found that EPA also, in this model, and in contrast to other findings (see previous), led to a decrease in cell proliferation indicating that its effects may be to inhibit tumour cell growth as well as to increase tumour cell destruction. A possible explanation for the overall lack of tumour growth inhibition by EPA might be the unchanging levels of n-6 in relation to n-3 in these experimental diets, it is possible that the effects of n-3 are only evident when there is a sufficient n-6 challenge. Another reason may be that the EPA preparation used (maxEPA) also contained DHA, and it has been demonstrated that this causes hypertrophy in some tumour cells (Stillwell et al 1993), which could potentially mask the anti-neoplastic effects of EPA.

Erickson and Hubbard (1996) have shown that select macrophage functions can be altered by dietary fats. These include tumouricidal activity (which may be downregulated by $PGE_2$) and the production of cytokines, dietary fish oils can alter the longevity (but not the maximal production) of soluble TNF$\alpha$ for example).

An interesting body of work has come from the laboratories of Begin and Ellis, whose findings are inconsistent with the general consensus, in that they have repeatedly demonstrated anti-tumour activity with both n-3 and n-6 fatty acids. Begin et al (1986) demonstrated that the n-6 FAs, DGLA and LA were cytotoxic to fibroblast, breast, lung and prostate tumour cells, but not to healthy cells. They also found that the n-6 fatty acids GLA and LA were cytotoxic to malignant cells. However, after twelve days, normal fibroblasts incubated with these fatty acids exhibited cytopathic signs indicating their effects were not selective. Under the same conditions, EPA was selectively toxic to lung and prostate tumour cells but damaged both healthy and malignant fibroblasts which were co-cultured, unlike the n-3 DHA which

was cytopathic to all of the transformed cells but which did not damage any of the non-malignant cells.

Begin et al (1986) showed that LA, GLA, DGLA, AA, ALA and EPA killed human prostate, breast and lung cancer cells but not healthy counterparts (although their rate of division was lowered). The n-3 DHA on the other hand could not discriminate between healthy and malignant cells and was cytotoxic to both. Based on their ability to eliminate tumour cell clones, the authors suggest that GLA, AA and EPA were the most effect agents.

The authors postulate that a possible explanation for the cytotoxicity of EPA to healthy fibroblasts may be a consequence of toxic material accumulated from disintegrated co-cultured tumour cells and that the variability of EPA might indicate that its action in vivo might depend on the type and site of the tumour. An important point to consider is that the possible disparities amongst the literature might reflect the heterogeneity of PUFA metabolism.

Sakaguchi et al (1981) suggest several reasons to explain the failure of EPA to attenuate tumourigenesis in some models, including the use of an unusual model, a fish oil diet which contains proportionally low levels of n-3 fatty acids, an n-3 diet which contains proportionally low levels of EPA or poor lipid incorporation by the tumour.

The work from this laboratory has focused on the anti-tumourigenic effects of EPA in the MAC16 model. Early work showed that a diet supplemented with fish oil (50% of total calories) caused a significant reduction in tumour growth without any effects on food intake or toxicity (Tisdale and Dhesi 1990). The specificity of this effect was demonstrated by Tisdale and Beck (1991) who showed that related n-6 and n-3 fatty acids had no effect. Whilst GLA has been shown to have some antitumour effect in vivo, it is only at a concentration of 5g/kg compared to 1.25-2.5g/kg for the maximal effect of EPA (Beck et al 1991). It appears that tumour stasis induced by EPA is mediated through an increase in the rate of cell loss from 38-71% (Hudson et al 1993) rather than a decrease in cell production. This is important from a

therapeutic point of view since most solid tumours tend to be populated by long-lived cells and expansion occurs not by a high rate of cell production but by a low rate of cell loss (Tisdale 1993). In fact Beck et al (1991) showed that and that the life span of tumour bearing mice was doubled by administration of 1.25-2.5g/kg EPA/day.

It was originally thought that because the effects of EPA upon accretion of weight were more pronounced than the anti-proliferative effects within the tumour, that the mechanisms were separate. This notion found further support in the observations of Hudson et al (1993) who showed that the anti-proliferative but not the anti-cachectic effects of EPA could be reversed by oral adminstration of LA. This increased tumour growth by reducing the rate of cell loss by 45% despite the incorporation of EPA into tumour phospholipids leading the authors to suggest that – in the case of the tumour- the effects of EPA may be mediated through its ability to interfere with the tumour promoting effects of other (possibly n-6) fatty acids. Furthermore, it is thought that the effects of EPA upon tumour growth may be indirect and a result of interference with catabolic release of n-6 fatty acids.

### 4.3.2 Effects on Angiogenesis and Metastasis

As well as effects upon tumour initiation and promotion, the effects of fatty acids upon neovascularisation and metastatic potential have been investigated. Using the angiogenesis model human omental microvascular endothelial (HOME) cells, it was shown that α-guaiaconic acid (GR12) and its derivative GS-01 inhibited arachidonic acid metabolism, migration and tubular formation of endothelial cells. Furthermore, the enhanced tube formation of HOME cells caused by co-incubation with oesophageal cancer cells was almost completely inhibited by GR12 and GS-01. GS-01 (but not GR12) also inhibited development of capillary networks in an in vivo model, leading the authors to conclude that this inhibitor of arachidonic acid metabolism modulated tumour angiogenesis (Ito et al 1993).

Fatty acid modulation of metastasis has been investigated by several groups, including Rose et al (1995) who showed that n-6 rich diets stimulate the growth and metastasis of the human breast cancer cell MDA-MB-435 in athymic nude mice, whilst n-3 fatty acids exert a suppressive effect. Diets supplemented with DHA and EPA caused a statistically significant reduction in tumour size and the occurrence and severity of lung metastases. Unsurprisingly the representation of these acids in tumour phospholipids increased, along with a significant reduction in the concentrations of arachidonic acid, 12- and 15-HETE, and $PGE_2$, suggesting that the mechanism involved suppression of tumour eicosanoid biosynthesis probably through competition for Δ-6 desaturases for the conversion of LA to AA. Similarly it has been shown that female Lewis/Wistar rats with MAC 33 subcutaneous implants exhibit a reduced tumour growth and lung metastases when fed a diet consisting of 30% fish oil (Torosian et al 1995).

Liu et al (1994) showed that the a highly metastatic melanoma cell line (HM340) produced predominantly 12-HETE from arachidonic acid, compared to a poorly metastatic cell line (LM180) which produced equal quantities of HETEs suggesting that this eicosanoid may be a determinant of metastatic potential. The same workers reported that treatment of tumour cells with exogenous 12(S)-HETE, but not other HETEs, augments the ability of tumour cells to adhere to endothelium and form lung colonies (Honn et al 1992), whilst others have suggested that 15-HETE is a positive regulator of tumour cell adhesion to endothelium (Bastida et al 1990). Thus it appears that the stereoconfiguration of HETEs may be crucial to their biological activity.

### 4.3.3 Effects on Cachexia

Human studies have shown that eighteen patients with unresectable pancreatic carcinoma receiving dietary supplementation of 1g fish oil containing 18% EPA and 12% DHA, and an initial median weight loss of 2.9kg/month, had a median weight gain of 0.3kg/month after three months.

This was accompanied by a reduction in acute phase protein production and stabilisation of resting energy expenditure (Wigmore et al 1996). There was also a concomitant increase in EPA and DHA incorporation into plasma phospholipids from 0 to a median 5.3% and 3.5% respectively.

Barber et al (2000) showed that after 3 weeks of 2g pure EPA/day enriched diet, patients with unresectable pancreatic carcinoma had an average increased weight gain of 1kg/month as opposed to an average weight loss of 2.9kg/month prior to treatment. Furthermore the energy expenditure in response to feeding had risen and the fasting oxidation levels fell to control. In short the metabolic cost of feeding was normalised, possibly through lowering the production of pro-inflammatory cytokines, although the report also showed that the energy cost of feeding was not elevated in these patients and that it therefore can not contribute to the apparent block to the accretion of lean tissue in cachexia.

Likwise, Barber and Fearon (2001) have shown that up to 18g/day has been tolerated in adult cachectic pancreatic cancer patients refractory to chemotherapy and radiotherapy with some patients taking 27g for at least a month. That this was accompanied, by few adverse side effects (and that other studies by this group have demonstrated a preservation of weight, increased quality of life and survival time) demonstrates the huge potential benefit of EPA in the treatment of cachexia.

Falconer et al (1994) reported the results of a phase I clinical trial in unresectable pancreatic carcinoma patients using a 10 day intravenous infusion followed by oral administration of the lithium salt of GLA. After one month, the red cell phospholipid composition altered to reflect the modified diet. T cell function was improved and pro-inflammatory cytokine production reduced. However the median survival time was increased by only 2-5 months, suggesting that this parameter would not be markedly improved by GLA therapy.

In a similar study, Wigmore et al (2000), this time using a 95% pure free acid preparation of EPA, undertook a comprehensive analysis of 26 cachectic patients with advanced pancreatic cancer. Prior to treatment patients had a median weight loss of 2kg/month, but after four weeks of 1-6g/day, patients had a median weight gain of 0.5kg, a stabilisation of weight which remained throughout the 12 weeks of the study. Wigmore and colleagues suggested that one mechanism through which EPA might exert its effects is through a reduction in proinflammatory cytokine production, as a downregulation of pro-inflammatory cytokine release by EPA has been previously demonstrated (Wigmore et al 1997).

Similar results have been found in animal studies performed in this laboratory.

As mentioned, it has been shown that the anti-cachectic and anti-tumour effects of EPA can be dissociated. Hudson and Tisdale (1994) demonstrated that linoleic acid could suppress the anti-proliferative but not the anti-cachectic effect of EPA suggesting two distinct pathways.

Using pure strain NMRI mice transplanted with the MAC16 tumour, Tisdale and Dhesi (1990) showed that diets which derived 50% calories from fish oil, caused a significant reduction in host weight loss, which was greater than would be predicted from shrinkage of the tumour. Body composition analysis showed that there was an increase in total carcass fat and muscle dry weight without an alteration in total body water. This showed that the weight gain was due to a reduction in the loss of essential body compartments, (which occurs in cancer cachexia) and was not the result of non-specific water retention. The related GLA had no effect showing the specificity of EPA and unlike the antitumour agents cyclophosphamide and 5-fluorouracil, which were toxic, EPA achieved similar anti-tumour effects with no host toxicity.

Although skeletal muscle catabolism comprises elements of decreased synthesis and increased degradation it is the latter which is the most

significant. Beck et al (1991) demonstrated that EPA in vivo, had the ability to inhibit protein degradation without any effect upon protein synthesis.

In terms of effects upon protein catabolism, elevation of the activity of the ubiquitin-proteasome pathway is most causative of skeletal muscle proteolysis in this and other models, and EPA prevents upregulated 'chymotrypsin-like' activity and expression of proteasome subunits and also the related ubiquitin conjugating enzyme $E2_{14k}$. This demonstrates that EPA antagonizes loss of skeletal muscle proteins in cancer cachexia by down regulation of proteasome expression (These findings are discussed in chapters 7 and 8 and published in part in Whitehouse et al 2001).

It has also been demonstrated that EPA reverses PIF induced alterations in carbohydrate metabolism. Hypoglycaemia, increased glucose utilisation by brain, heart, brown fat, along with decreased utilisation by kidney, white fat, diaphragm and gastrocnemius muscle resulting from administration of PIF, is attenuated by EPA. This suggests that PIF as well as affecting tumour growth and cachexia also has a direct effect on glucose metabolism (Hussey and Tisdale 1999).

It has been shown that EPA also prevents another significant aspect seen in the MAC16 and other models. Lipolysis in isolated murine adipocytes induced by LMF, salbutamol and ACTH was prevented by EPA suggesting the effect is exerted at a central step. LMF results in an elevation of cAMP levels which was also prevented by EPA (Tisdale and Beck 1991). Tisdale (1993a) demonstrated that attenuative effects of EPA upon cAMP were due to an inhibition of adenylate cyclase and that the process was GTP dependent, non-competitive with isoprenaline, and could be eliminated by pre-treatment with pertussis toxin (which ADP-ribosylates the $\alpha$-subunit of an inhibitory G protein, thus inactivating it). Taken together these observations meant that EPA inhibited cAMP formation , at least in part, due to a Gi mediated inhibition of adenylate cyclase. This was confirmed in 1993, when Tisdale demonstrated that the effects of EPA upon lipolysis were

exerted through Gi (Tisdale 1993b). However it has also been shown that direct stimulation of adenylate cyclase by forskolin (which does not involve a receptor) can be decreased by EPA, suggesting a direct interaction of EPA and adenylate cyclase (Price and Tisdale 1998)

Tisdale and Beck (1991) again demonstrated that in vivo adminstration of EPA preserved fat mass in NMRI mice bearing the MAC16 tumour in a specific manner, as related n-6 and n-3 fatty acids had no effect. Whilst GLA has been shown to have some effect at high concentrations in vivo, it has no effect upon LMF induced lipolysis in vitro and was highly toxic (Beck et al 1991). That smaller doses of $PGE_1$ (the metabolite of GLA), could inhibit lipolysis in vitro probably suggests the activity of GLA in vivo arises from its metabolism to $PGE_1$ (Beck et al 1991). Similarly whilst DHA has been shown to be modestly effective in attenuating lipolysis in vivo, it is possible that the effects are due to the retro-conversion of DHA to EPA (Price and Tisdale 1998).

#### 4.3.3.1 The involvement of $PGE_2$

Smith and Tisdale showed that it was possible to inhibit cachexia with the cyclooxygenase inhibitor indomethacin and the lipoxygenase inhibitor BWA4C. These observations combined with the significantly elevated levels of $PGE_2$ observed in cachexia and in gastrocnemius muscles incubated in serum from MAC16 cachectic mice (Smith and Tisdale 1993b) led to the notion that this prostaglandin might be an intermediate in cachectic proteolysis which might or might not be causative.

Rodeman and Goldberg (1982) were the first to demonstrate that $PGE_2$ was capable of inducing skeletal muscle catabolism in the rat. This has been demonstrated in the Yoshida hepatoma model (Tessitore et al 1994), whilst other investigators have shown that $PGE_2$ and arachidonate do not affect total or myofibrillar protein degradation in many conditions and that the cyclo-oxygenase inhibitor indomethacin does not affect proteolysis in sepsis (Hasselgren et al 1990). Furthermore $PGE_2$ (and also $PGE_1$) have also been

implicated as angiogenic factors (Reviewed in Ito et al 1993), whilst Rose et al (1995) showed that $PGE_2$ had no relationship to the occurrence of severity of metastases. Thus whilst some in vitro studies have implicated $PGE_2$ in the process of cachexia, its role is controversial.

As discussed in chapter 4.1.1, the first step in the production of $PGE_2$ is the release of arachidonic acid from membrane phospholipids, a reaction catalysed by phospholipase $A_2$. Free arachidonic acid can then be metabolised via cyclooxygenase enzymes to form prostaglandins. This suggests that the activation of $PLA_2$ may be the first step in the induction of proteolysis by PIF. However metabolism of arachidonic acid can also occur through lipoxygenase enzymes, resulting in the formation of HETEs. Smith et al (1999) showed that PIF caused a rise in both prostaglandins and HETEs but when these eicosanoids were added to C2C12 myotubes only 15-HETE was capable of stimulating protein degradation, suggesting that this metabolite rather than $PGE_2$ is responsible for the protein degradation induced by PIF.

## 4.4 The potential mechanisms through which fatty acids can affect tumour growth and cachexia.

How can fatty acids and their metabolites influence tumour growth and cachexia? Current thinking suggests several options:-

1. Alteration of the eicosanoid axis to augment/inhibit a catabolic and tumour-growth promoting environment.
2. Incorporation into membrane phospholipids leading to altered signal transduction capabilities.
3. Oxidative stress
4. Post-translational modification of signal transduction proteins and effects on transcription.

PUFAs all compete for the same desaturases (but with different affinities). This causes competition between the FA groups and at relatively high concentrations, one FA could inhibit the conversion of another and hence its entry into eicosanoid biosynthetic pathways. EPA is incorporated into phospholipids at the expense of AA, thus suppressing prostanoid synthesis. The incorporation of EPA into phospholipids at the expense of AA can generate a set of eicosanoids, which (unlike the strong immunosuppressive and platelet aggregatory activities of AA metabolites) tend to suppress tumour growth rather than favour it.

Another suggestion is that of Gabor et al (1985) who proposed that linoleate might induce an increase in tumour mass either by promoting an invasion of host IC into the neoplasm, accelerating tumour cell proliferation either by reducing the intermitotic time or by increasing the fraction of proliferating cells or decreasing the tumour K1.

### 4.4.1Modification of signal transduction

Fatty acids (FAs) can act as both modulators and messengers and they are commonly involved in feedback mechanisms, since phospholipases themselves are modulated by fatty acids. FAs can act directly on the cell membrane as first messengers or become incorporated into membrane phospholipids, and when liberated in response to other signals, act as a second messenger. They can also modulate signals coming from other pathways such as steroid hormones. In general the action of FAs can be exerted by free (non-esterified) FAs, bound or unbound to fatty acid binding proteins, before and after metabolism or incorporation into lipids.

FAs are good candidates for the role of modulator, in that they exist for a short period and are limited spatially within the cell. The effect of a free fatty acid (FFA) is brief since the pool is very small, and multiple enzymes exist, either to rapidly catabolize or re-incorporate them into structural lipids. The fact that FA binding proteins exist to modulate concentrations adds support to the idea.

Many of the enzymes involved in signal transduction pathways such as cAMP or PKC can be positively or negatively regulated by fatty acids or their metabolites. FAs can regulate ion fluxes and $Ca^{2+}$ mobilisation. When Jurkat cells for example, are exposed to FAs they block $Ca^{2+}$ influx through $Ca^{2+}$ channels, but activate $K^{+}$ channels and appear to interact directly with channel proteins themselves, or with some other component of the membrane (reviewed in Sumida et al 1993).

#### 4.4.1.1 Lipid Peroxidation and Free Radical Production

It has long been established that the by-products of oxidative metabolism and certain physiological signalling agents give rise to various forms of reactive

oxygen species (ROS) such as superoxide anion ($O_2^-$), hydroxyl radicals (OH), singlet oxygen ($^1O_2$) and nitric oxide (NO) radicals.

As well as the electron transport chain other sources of ROS include the nuclear membrane which contains an NADPH dependent electron transport chain resulting in the production of $O_2^-$; the decomposition of oxyhaemaglobin, the endothelium and photo-irradiation of tryptophan (Mates and Sanchez-Jimenez 2000).

Significantly ROS can also be formed during the peroxidation of fatty acids and during their subsequent metabolism to biologically active eicosanoids. Also the desaturase system generates superoxide anion during the introduction of double bonds into unsaturated fatty acids (see figure 12 pp62) (Mates and Sanchez-Jimenez 2000).

Additionally certain metabolic products such as hydrogen peroxide, haeme and free iron can act as strong pro-oxidants because of their ability to generate extremely reactive hydroxyl radical through nonenzymatic reactions. Under normal circumstances, all of these oxidants are detoxified by interaction with various reducing and sequestering agents such as thioredoxin, glutathione, tocopherol, ferritin, biliverdin, and bilirubin or by enzymes such as superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase and haeme oxygenase (reviewed in Lavrovsky et al 2000). Oxidative stress results when there is an imbalance between free radical generation and these various antioxidant defence mechanisms.

The reactive oxygen species (ROS) produced are thought to act as subcellular messengers which influence a number of genes and signal transduction pathways. Redox changes that alter gene expression can be due to both increases and *decreases* in oxidation. Furthermore it has been observed that anti-oxidation can stimulate the expression of certain genes.

The question that arises is 'how could redox changes affect transcription factors and signal transduction?' The molecular mechanisms are not fully

understood but it seems that oxidation or reduction of protein sulphydrils leads to conformational changes that could inhibit/augment DNA binding activity, release inhibitory subunits or promote protein complex formations necessary for signal transduction to proceed (Allan and Tresini 1999).

Van den Berg et al (1993) for example, showed that the molecular surface areas of oxidised fatty acids (1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)-phosphatidylcholine and 1-palmitoyl-2(9/13-hydroxylinoleoyl)phosphatidyl-choline) were increased by as much as 50% compared to the non-oxidised parent molecule.

Free radicals may also induce several DNA sequence changes: point mutations, deletions, gene amplification/rearrangements that result in the activation of apoptosis, proto-oncogenes and/or the inhibition of tumour suppressor proteins

Whilst the varied effects of PUFAs upon signal transduction in cancer versus normal cells could be due to differential uptake, differential distribution of the fatty acid, to derived eicosanoids/unknown metabolites or to the generation of peroxides, the fact that LOX/COX inhibitors indomethacin, nordihydroguaiaretic acid and caffeic acid fail to block the destruction of certain cancer cells, but vitamin E and butylated hydroxyanisole inhibit cytotoxicity, suggests that oxidation products may be involved (Begin et al 1986b).

Sakaguchi et al (1981) also demonstrated that the effectiveness of a given PUFA in killing tumour cells correlated with its ability to generate superoxide anions and related oxygen radicals as determined by nitrotetrazolium blue reduction and to undergo lipid peroxidation (Begin et al 1986b). Moreover, many types of tumour cells exhibit a decreased rate of lipoperoxidation compared to normal cells as a result of lower content of PUFAs (Cheeseman et al 1984). This raises an interesting question, whether or not lipid peroxidation represents a coincidental outcome of radical-

induced damage or if lipid peroxidation products are in some cases, directly deleterious to the cells.

It has also been demonstrated that LA and AA are capable of inducing specific genotoxic damage in isolated DNA, in a mechanism related to the degree of unsaturation since the monounsaturated fatty acid – oleate had no effect. It is thought that it was the enzymatic peroxidation of LA and AA which was responsible for the DNA damage in this case, since superoxide anion was generated during the peroxidation steps and this was subsequently converted into singlet oxygen (De Kok 1994).

The work of Borgeson et al (1989) discussed earlier in this chapter, demonstrated that the therapeutic index of the anti-neoplastic agents doxorubicin and mitomycin C against the human mammary adenocarcinoma MX-1 is increased in animals fed a diet high in n-3 fatty acids. This is relevant here because of the fact that the activity of these drugs is due to the generation of toxic oxygen radicals, suggesting that feeding fish oil predisposes these carcinoma cells to lipid peroxidation and increases their susceptibility to pro-oxidant anti-tumour agents.

Conjugated fatty acids have been shown to inhibit the growth of many cancers. Igarishi and Miyazawa (2000) suggested that conjugating EPA (CEPA) and DHA (CDHA) might prove doubly cytotoxic. They found that CEPA and CDHA showed extensive cytotoxicity with LD50 at 13 and 16μM respectively in the colorectal adenocarcinoma cell line DLD1 whilst having no effect on normal human fibroblast cell line (MRC5, TIG-103 and KMS6). It is thought that the mechanism was mediated by membrane phospholipid peroxidation because phospholipid hydroperoxide levels were elevated. It seems that these conjugated forms and particularly trienoic structures play an important role in this cytotoxicity. Possibly the trienoic structure may be particularly susceptible to oxidative stress; the fact that tumours frequently have deficient antioxidant defence mechanisms and that the hydrophobic radical scavenger α-tocopherol prevented the cytotoxic effects suggests that

the selective cytoxicity toward tumour cell lines results from an increased sensitivity to oxidative damage. The DNA condensation and fragmentation evidenced, indicate the involvement of apoptosis in this mechanism.

A point to consider is the levels of vitamin E in the diet. The requirement for vitamin E (and other anti-oxidants) is dependent upon the dietary levels of PUFAs. A ratio of 0.6(mg vitamin E/g PUFA) is required to prevent the development of vitamin E deficiency, whilst an excess of vitamin E may itself influence tumour growth. Vitamin E has been shown to inhibit PUFA cytotoxicity toward breast carcinomas and inhibit chemically induced breast carcinogenesis (Sakaguchi et al 1981).

Beckman et al (1994) investigated lipid peroxidation in the skin of SD1 mice following application of a tumour promoter (TPA 12-0-tetradecanoylphorbol-13-acetate). A substantial accumulation of hydroxyphospholipids (particularly derivatives of linoleic acids but also HETEs ) was seen in test animals which was associated with the development of tumours

Similarly Lynette et al (1994) measured radical adduct formation in cultured endothelial cells supplemented with DHA and EPA which had been subjected to oxidative stress, (-the addition of $FeSO_4$ to induce lipid peroxidation). They found that the presence of lipid derived free radicals was greatly enhanced in cells which had been supplemented with these fatty acids during growth. Other fatty acids (including AA, GLA and ALA) also increased radical adduct formation but to a lesser extent, while mono-unsaturated oleic acid actually decreased formation by 35%-45%. These findings suggested that endothelial cells become more susceptible to oxidative injury when exposed to PUFAs and particularly the n-3 fatty acids DHA and EPA.

On the other hand Begin et al (1986) - using the loss of fatty acid from the membrane and the generation of hydroperoxide breakdown products as indicators of lipid peroxidation – found that GLA and AA were the most

cytotoxic whereas DHA was the least effective. They argue that the effectiveness of a given fatty acid in killing cancer cells is due to the extent of its lipid peroxidation (with GLA, AA and EPA possessing 3, 4 and 5 double bonds respectively and having the highest toxicity) and not necessarily related to the omega group.

Gonzalez et al (1991) have also shown an inverse relationship between fish oil diets, lipid peroxidation and tumour volume. Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were transplanted into athymic nude mice which were then fed diets containing different amounts and types of fat. After 6-8 weeks tumour volume was largest in those animals fed 20% corn oil and lowest in those animals given 19% fish oil. Tumour lipid peroxidation levels were significantly increased only in fish fed animals and this effect was attenuated by co-administration of antioxidant, and augmented by ferric citrate. Thus the growth inhibition of these tumours appeared to be due to tumour lipid peroxidation products of fish derived oils.

Interestingly, one group has shown a correlation between the rate of superoxide anion production in breast cancer compared to controls and an inverse relationship between the antioxidant enzyme catalase, supporting the oxidative stress hypothesis in breast carcinogenesis (Ray et al 2000).

It has also been demonstrated that the PUFAs AA, DGLA and EPA suppress human T-cell growth in vitro by a mechanism which was clearly independent of $PGE_2$. These fatty acids induced free radical generation and lipid peroxidation. The anti-oxidant vitamin E and the superoxide anion quencher superoxide dismutase blocked the effect suggesting that these PUFAs induced growth suppression through a mechanism which is free radical dependent (Madhavi et al 1994).

n-3 Fatty acids have also been shown to inhibit oxidative damage in human erythrocytes induced by a free radical generator. There was an age dependent increase in membrane anion transport which was significantly reduced in the old erythrocytes of 18 subjects who had been fed 0.5ml fish

oil per day, but not in individuals who had been given safflower oil (Mills et al 1995).

In cell free chemical systems the rate of peroxidation is proportional to the degree of unsaturation. The studies discussed here have often shown a relative cytotoxic effectiveness or ineffectiveness, which cannot be explained purely by the number of double bonds. Although this discrepancy might be explained by differential uptake or incorporation into phospholipids, it suggests a high degree of fatty acid specificity

The role of oxidants in tumour cells is well documented. However, little is known about the effects of oxidants upon skeletal muscle. Gecha et al (1991) found that the oxidant phenylhydrazine increased protein breakdown in skeletal muscle, whilst H2O2 and glucose oxidase decreased proteolysis suggesting that the effects may differ depending on the oxidant. Thus there is a body of evidence which clearly provides a role for specific fatty acids as signal modifiers acting at the cell membrane level. There are however, relatively fewer studies implicating an effect of FAs upon transcription.

### 4.4.2 Fatty Acid Control of Transcription

An interesting study providing evidence to support the concept that certain fatty acids can regulate gene expression and generate second messengers was provided by Tiwari et al (1991) who, using LA and EPA investigated MCF-7 cells, immortalised, and transfected with ras to render them oestrogen independent. Surprisingly both fatty acids were capable of inducing the gene 1-8 in the parental line but not in the ras transfected line, whilst the gene 2-5A was unaffected by either fatty acid in either cell line. Expression of the Her2/Neu oncogene was also investigated and found to be enhanced by both LA and EPA. The authors postulate that the disparity between cell lines suggest that the generation of a specific second messenger may be involved.

One body of work, on the transcriptional control of adipose cell differentiation has come from the laboratory of Ailhaud and Amri. Amri et

al (1991) demonstrated that the AP2 gene (adipocyte lipid binding protein gene) was activated at the transcriptional level by duodecameric long chain fatty acids. It is hypothesised that FAs rapidly and reversibly trigger the synthesis of trans-acting factors (either at a transcriptional or translational level), which in turn regulate the transcription of the AP2 gene. Similarly chronic exposure of cells to palmitate leads, in a dose dependent manner, to terminal differentiation events through enhancing post confluent mitoses and overexpression of terminal differentiation-related genes. (Ailhaud et al 1995). The effects of FAs in adipose cells in culture are similar to those of fibrates (both fibrates and FAs are amphipathic carboxylates and affect genes involved in lipid metabolism). Also it has been demonstrated that a substitution analogue of arachidonic acid, 5, 8, 11, 14-eicosatetraynoic acid was found to fully activate PPARα. Taken together the authors postulate that a receptor of this type is responsible for the transcriptional effects of FAs in pre-adipose cells and in adipose differentiation (Ailhaud et al 1995). Arachidonic acid can also indirectly control adipose cell differentiation and the expression of late marker genes through its metabolites $PGI_2$ and $PGF_2\alpha$ (Ailhaud 1993). Ailhaud et al (1995) identified a member of the steroid/thyroid hormone receptor superfamily by cDNA cloning from a mouse ob1771 preadipose cell library as the likely fatty acid activated receptor implicated in the transcriptional effects of fatty acids in adipose cells. Ntambi (1995) demonstrated that the SCD 1 (stearoyl Co-A desaturase) gene was stimulated by a fat free diet in 3T3-L1 adipocytes and decreased by a diet high in PUFAs, where triglycerides and monounsaturated fatty acids had no effect. Thus it seems that FAs or their metabolic products regulate the expression of several genes involved in lipid metabolism. An interesting possibility is that these genes all share common cis/regulatory elements and/or trans factors that mediate gene repression/transcription

PPAR receptors have also been implicated in the effects of FAs upon Mo LPL (macrophage lipoprotein lipase) gene expression. Michaud and Renier (2001) used DNA binding assays to show an enhanced binding of nuclear proteins to the peroxisome proliferator response element (PPRE) consensus

sequence of the LPL promoter, from macrophage cells treated with a range of saturated and PUFAs. MoLPL mRNA expression was increased by LA and PA but interestingly not with EPA or AA. Overall their results provided the first evidence for a direct regulatory effect of FAs upon expression of this gene, and also suggested a potential role for PPARs in the regulation of gene expression by FAs.

Liu et al (2001) demonstrated that the n-3 fatty acids EPA and particularly DHA inhibit phorbol 12-tetradecanoate 13-acetate induced transactivation of the transcription factor activator protein a (AP-1), and subsequent transformation in mouse epidermal JB6 cells, whilst AA abrogated the inhibitory effects mediated by DHA. Although it has been shown that AP-1 can be up and down regulated by the MAP kinases and that blocking MAP-kinases leads to the inhibition of AP-1 transactivation and subsequent cell transformation, the authors found no effect of DHA or EPA upon activation of members of the MAP kinase family suggesting that the inhibitory effects of n-3 FAs upon AP-1 is mediated by some other mechanism than the MAP kinases.

The role of AA has also been examined in prostate cancer PC3 cells. It has been shown that expression of the early gene c-fos and the immediate early gene COX-2 is increased, minutes after addition of AA and that after three hours, the synthesis of $PGE_2$, via COX-2 was elevated. Previous studies have demonstrated that AA is primarily delivered by low density lipoprotein (LDL) via its receptor LDLr. This work showed that normal cholesterol feedback was lost in prostate cancer, suggesting that unregulated overexpression of LDLr in tumour cells would permit increased availability of AA which induces immediate early genes c-fos and COX-2 (Hughes-Fulford 2001). On the other hand Siddiqui et al (2001) have shown that AA has no effect upon Jurkat cells whereas similar concentrations (60μM and 90μM) induced apoptosis and an associated proteolysis of caspase 3, possibly mediated through serine/threonine kinases.

Hirano et al (2001) showed that CETP (cholesterol ester transfer protein) expression is modulated by fatty acids in Hep G2 cells. Specifically AA, EPA and DHA all lowered CETP mRNA levels to less than 50% controls. Their results demonstrate that fatty acids regulate CETP expression, although they argue that the effect is most likely dependent upon the degree of unsaturation of the acyl carbon chain and not the position of the omega group.

Badawi et al (1998) examined whether n-6 and n-3 FAs might exert their effects in breast cancer, through altering the expression of genes. They found that COX1 expression was increased by 30% in Sprague Dawley rats fed diets containing high levels of n-6 PUFAs compared to n-3 pair fed rats. Similarly COX2 expression and activity was increased, as was expression of the p21-ras protein and Ha-ras mRNA. These observations indicate that n-6 PUFAs are able to increase the expression of these genes in rat mammary glands, whereas n-3 PUFAs do not possess this ability. An interesting suggestion is that in mammary carcinogenesis, the tumour promoting activity of n-6 PUFAs may be related to their ability to increase levels of p21-ras.

DeWille et al (1993) showed that MMTV/v-Ha-ras transgenic mice fed a 25% corn oil diet (high in n-6 FAs), had not only increased incidence of mammary tumours but also increased levels of ras mRNA providing further evidence that these PUFAs influence genetic expression itself.

Similarly Etkind et al (1995) considered transcription of the mtv-1 locus of the mouse mammary tumour virus in C3Hf mice fed high and low fat diets, and found that the former but not the latter increased its transcription. The MMTV LTR contained signals for the initiation and termination as well as binding sites for steroid hormones. That certain PUFAs were capable of accelerating hormonally controlled MMTV RNA transcription, demonstrates how these fatty acids can act at the molecular/genetic level.

Fatty acids can also modify peptide signals by directly binding to them and altering conformation, as is the case with α–fetoprotein which competitively binds oestradiol (Nunez 1993).

How is it that fatty acids might attach to signal transduction or other proteins to influence transcription? It seems that they can be linked to an amino acid residue directly or indirectly as a component of a phosphatidyl moiety attached to a COOH- terminal amino acid through an intervening glycan structure. Direct linkage can occur co- or post-translationally. The former involves the N-myristoyl transferase catalysed modification of an N-terminal glycine to which the FA (myristoyl CoA in this case) is bound via an amide bond (reviewed in Muszbek and Laposata 1993). However post translational FA modification appears to involve thioester linkages which have a more relaxed fatty acid specificity than cotranslational acylation.

A novel study by Muszbek and Laposata (1993) examined the involvement of PUFAs in posttranslational fatty acid acylation. Using platelets as a model protein, Muszbek and Laposata demonstrated covalent linkage of two PUFAs - arachidonate and eicosapentaenoate, through ester linkages, demonstrating that direct binding of PUFAs to proteins occurs in vivo. They postulated that the linkages could either be thioester involving a cysteine residue, or *O*-ester linkages that might involve hydroxyl groups of serine or threonine residues.

Finding a single explanation for the effects of PUFAs upon tumour growth and cachexia is impossible, there are a myriad possibilities in which PUFAs could exert their effects, the situation is further complicated in that the many possibilities are interrelated and that the metabolism of PUFAs is heterogenous. To summarise, current thinking suggests that PUFAs can affect tumour growth directly or through their metabolites, by incorporation into the membrane, thereby potentially altering receptor binding capacity, enzyme function or permeability to pharmacological agents. Alteration of membrane structure in this way also changes the substrate lipid availability

which could favourably or unfavourably affect the pool of eicosanoid precursors. Eicosanoids regulate many cell functions important for tumour growth, by altering eicosanoid axis, the production of pro- or anti-inflammatory cytokines and lysosomal enzymes (that initiate tumour invasion and metastasis) could be altered, affecting functions such as chemotactic migration and anchorage of tumour cells to relevant substrata. Furthermore by changing the eicosanoid environment, immune functions important in tumour surveillance and most significantly signal transduction capabilities could be altered, leading to changes in gene expression which could promote or inhibit tumour growth and cachexia. It seems that PUFAs can also modify these functions directly without incorporation into the bilayer and/or further metabolism. This could occur by direct uptake into the cell, through carrier mediated transport, and then either direct binding to transcription factors or through second messenger intermediates. Moreover, the incorporation of PUFAs into the membrane can lead to lipid peroxidation products which can themselves act as second messengers or cause radical induced cell death. The effects upon cachexia can arise indirectly as a proportional result of the effects upon tumour growth or directly, through alteration in the environment to favour catabolism or by the expression of genes responsible for proteolysis.

The aim of this work therefore, is to examine the mechanisms of the fatty acid eicosanoids 15-HETE and eicosapentaenoate in skeletal muscle catabolism and its attenuation, and to further characterise those signal transduction pathways involved in their action.

## 5. Materials and Reagents

### 5.1 Materials

Affiniti Research Products Ltd. Exeter UK

Mouse Monoclonal Anti 20s α1, 2, 3, 5, 6 and 7

Amersham Pharmacia Biotech UK Ltd, Bucks, UK.

[γ-$^{33}$P] Adenosine Triphosphate

Enhanced Chemiluminescence Detection system

Hybond ECL Nitrocellulose Membrane

Hyperfilm ECL

Hyperfil MP

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

pd(N)6 Random Hexamer 5' Phosphate

Autogen-Bioclear UK Ltd, Wilstshire, UK.

Goat Polyclonal Anti NFκB-p65

Rabbit Anti-Human IκBα (FL):sc-847

Biorad Laboratories GmBH, Munich, Germany.

Biorad Protein Assay

30% Acrylamide/Bis-Acrylamide Solution

Ammonium Persulphate

Calbiochem Novabiochem, CA, USA

SN50-NFκB Cell Permeable Inhibitor Peptide

Coulter Electronics BMDH, Beds, UK

Coulter Counter ZM

'Isoton II' Electrolyte Solution

DAKO inc. Glostrup Denmark

Peroxidase Conjugated Goat Anti Rabbit Immunoglobulins

Peroxidase Conjugated Rabbit Anti Mouse Immunoglobulins

Fisher Chemicals, Loughborough, Leicester, UK

'Hi safe 3' Scintillator Fluid

Gibco BRL Life technologies, Paisley, Scotland

Dulbeccos Minimum Essential Medium with Phenol Red (DMEM)

Dulbeccos Minimum Essential Medium without Phenol Red

Foetal Calf Serum (FCS)

Horse Serum (HS)

Multiwell Tissue Culture Plates

Penicillin/Streptomycin Solution

Tissue Culture Flasks

Trypsin

MWG AG Biotech, Germany

Primus 25/96 Thermocycler

NovoCastra Laboratories, Newcastle, UK

Anti Myosin Heavy Chain (Fast) Antisera

Oxoid, Hampshire, UK.

Phosphate Buffered Saline Tablets

Packard Instrument Company, Meriden CT, USA).

'Tri-carb 2000CA' Liquid Scinitillation Analyzer

Promega, WI, USA

100bpDNA Ladder

Blue/orange 6X Loading Dye

Deoxynucleotide Triphosphates

Ethidium Bromide

Gel Shift 5X Binding Buffer

T4 Polynucleotide Kinase 10x Buffer

TBE 10x Buffer (1L)

TE Buffer

$MgCl_2$ (3.5mM)

M-MLV Reverse Transcriptase

NFκB Oligonucleotide

5x Reverse Transcription Buffer

rRnasin RNase Inhibitor

Taq Polymerase

PCR Buffer (10x)

Agarose

Scotia Pharmaceuticals, Stirling, UK

Eicosapentaenoic Acid for in vivo experiments

Sigma-Aldrich Company Ltd., Dorset, UK.

1-nitroso-2-naphthol

15(s)-Hydroxyeicosatetraenoic Acid

Boric Acid

Bovine Serum Albumin

Chloroform

Cycloheximide

(3-[2-(3,5-dimethyl-2-oxocyclohexyl0-2-hydroxyethyl]glutarimide) (Triton x100)

Dichloroethane

Diethylpyrocarbonate

Dithiothreitol (DTT)

EDTA

Eicosapentaenoic Acid (for in vitro experiments)

EGTA

Ethanol

Glucose

Glycerol

HEPES

Hydrochloric Acid

Isopropanol

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

Leupeptin

Magnesium Chloride

Nitric acid

Phenylalanine

Phenylmethylsulfonylfluoride (PMSF)

Potassium Chloride

'Rainbow' Molecular Weight Markers

Sodium Hydrogen Carbonate

Sodium Orthovanadate

TEMED

Trichloroacetic Acid (TCA)

Tri-Reagent

Tris

Rabbit anti mouse $E2_{14k}$ antisera was kindly donated by Dr S Wing (see Rajopurohitam et al 1999). Mouse anti mouse P42 and mouse anti mouse MSS1 antisera were kindly supplied by Dr J Arnold.

## 5.2 Buffers and Solutions

### General Reagents

Krebs Buffer

| | |
|---|---|
| Sodium chloride | 17.24g |
| Potassium chloride | 0.932g |
| Potassium dihydrogen orthophosphate | 0.405g |
| Magnesium sulphate | 0.735g |
| Sodium hydrogen carbonate | 5.250g |
| Calcium chloride | 0.671g |
| | In 2,500ml |

Krebs-Heinseleit Bicarbonate Buffer

As above but the following added just prior to use

6mM D-Glucose

0.12% BSA

0.5mM Cycloheximide

Q Sepharose (QS) Buffer 1

| | |
|---|---|
| Tris | 3.03g |
| EGTA | 0.4755g |
| DTT | 0.386g |
| PMSF | 0.1M |
| in isopropanolol | 12.5ml |
| | In 2,500ml |

### Tissue Culture Reagents

Dulbecco's Media without Phenol Red

10% FCS

1% Penicillin/streptomycin

Dulbecco's Media with Phenol Red

10% Foetal calf serum

1% Glutamine

1% Penicillin / streptomycin

Phenol red indicator

Sterile Filtered Tritiated Phenylalanine Solution

60mg (0.06g) Phenylalanine in 4.5ml sterile water

0.5ml L - [2, $6^3$ - H] Phenylalanine

**Western Analysis Reagents**

Blocking Buffer

10mM Tris pH 7.5

100mM NaCl

0.1% Tween 20

5% Marvel

Homogenising Buffer:

500 mM Tris pH7.5

100 mM ATP

50 mM $MgCl_2$

50mM DTT

Polyacrylamide Gel Formulations

| | 12% Separating Gel (ml) | Stacking Gel (ml) |
|---|---|---|
| Deionised water | 26.4 | 27.2 |
| 30% Acrylamide solution | 32 | 6.8 |
| Tris (1.5MpH8.8(run)/1.0M6.8(Stack) | 20 | 5 |
| 10%Ammonium persulphate | 0.8 | 0.4 |
| 10% Sodium dodecyl sulphate | 0.8 | 0.4 |
| TEMED | 0.048 | 0.04 |

Sample Buffer

125mM Tris pH 6.8

4% SDS

10% Glycerol

0.006% Bromophenol blue

2% β Mercaptoethanol

Transfer Buffer

25mM Tris

190mM Glycine

20% Methanol

Wash Buffer

10mM Tris pH 7.5

100mM NaCl

0.1% Tween 20

**Molecular Biology Reagents**

EMSA High Salt Buffer

50mM HEPES (pH 7.8)

50mM KCl

300mM NaCl

0.1mM EDTA

1mM DTT

0.4mM PMSF

0.2mM NaF

0.2mM Orthovanadate

10% Glycerol

EMSA Native PAGE Buffer x10 (1L)

Tris 30g

Glycine 144g

EMSA Non-Denaturing 8% Polyacrylamide Separating Gel

9.5ml Distilled $H_20$

5ml 1.5M Tris pH8.8

200µL 10% APS solution

5.3ml 30% Acrylamide solution

16µL TEMED

EMSA Non-Denaturing 5% Polyacrylamide Stacking Gel

6.93ml Distilled $H_20$

1.3ml 1.5M Tris pH8.8

100µL 10% APS solution

1.67ml 30% Acrylamide solution

10µL TEMED

EMSA Wash Buffer

10mM HEPES/KOH pH7.5

10mM KCl

2mM $MgCl_2$

1mM DTT

0.1mM EDTA

0.4mM PMSF

0.2mM NaF

0.2mM Sodium orthovanadate

0.3mg/ml Leupeptin

Gel Loading 10x Buffer

250mM Tris-HCl (pH7.5)

0.2% Bromophenol blue

40% Glycerol

Gel Shift 5X Binding Buffer

20% Glycerol

5mM $MgCl_2$

2.5mM DTT

250mM NaCl

50mM Tris-HCl )pH7.5)

0.25mg/ml poly (dI-dC).poly(dI-dC)

RNAse Free Water

0.1% Diethylpyrocarbonate in de-ionised water, (prepared in autoclaved glassware and incubated at 37°C overnight before use)

RNA Storage Solution

10ml Deionised formamide

3.5ml 37% Formaldehyde

2ml MOPS 5X buffer

T4 Polynucleotide Kinase 10x Buffer

700mM Tris-HCl (pH7.6)

100mM $MgCl_2$

50mM DTT

TBE 10x Buffer

107.8g Tris

~55g Boric acid

7.44g EDTA

The components are added to 800ml water in the order listed above, except that the final few grams of boric acid are used to adjust to pH 8.3 at this stage. The volume is finally adjusted to 1L

TE Buffer

10mM Tris/HCl (pH8.0)

1mM EDTA

## 6.1 In vitro Methods

### 6.1.1 Purification of PIF

Tumours were removed (see section 6.2.3 also) from NMRI mice with established weight loss (20-25%), flash frozen and stored at –20°C until use. They were thawed and homogenised in 5ml/g in 'Q-Sepharose 1' buffer. After centrifugation (4,000 rpm. 15 min.), 38% w/v ammonium sulphate was slowly added to the supernatant whilst stirring on ice. The solution was then left stirring overnight at 4 ° C. Centrifugation at 4,500rpm for 20min was followed by ultracentrifugation at 30,000 rpm for 35 min to remove any fat. After which time the homogenate was dialysed several times in a 10,000 mw cut off ultrafiltration cell (Amicon) against 2-3 changes of 300ml PBS. The retentate was centrifuged at 4,500 rpm for 20 min and the sample circulated on an affinity column overnight. The column itself contains antibodies raised against murine PIF (Todorov et al 1996; see chapter 2.2), which are 'Affi-Gel' coupled to protein A, immobilized on an agarose support. PIF was eluted from the column the next day with 0.1M glycine, pH 2.5, into tubes containing 500µl Tris pH 8.0. It was then re-dialysed in equal volume PBS and concentrated to 0.5-1.0ml.

### 6.1.2 Subculturing and Myotube Formation

For all cell culture techniques, a murine, C311 strain, myoblast subclone - C2C12- was used and all procedures were carried out aseptically. Cells were stored under liquid nitrogen, at -276°f in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 20% foetal calf serum (FCS) and 10% dimethyl allyl sulphoxide (DMSO). Cells were resurrected in 20% FCS DMEM with 5% penicillin/streptomycin (P/S) and discarded after 18 passages.

Myoblasts were passaged prior to confluency every 3-4 days in 10% FCS, 5%P/S DMEM at $2x10^4$/ml. Briefly, the cells were washed in sterile PBS and incubated for a few minutes in 2-5ml sterile 1% Trypsin in PBS to

disrupt the monolayer. The appropriate amount of DMEM was then added and the cells subcultured into sterile multiwell plates or flasks, as required, and incubated at 37°C in 95% $O_2$ and 5 % $CO_2$. Cells were counted where necessary using a standard Coulter Counter ZM and Isoton II electrolyte solution.

When the cells reached 90% confluency (as observed microscopically), they were incubated in DMEM containing 1% P/S and 2% horse serum, which was changed every 48h. This provides a growth factor deficient environment leading to the activation of the transcription factors Myo D and Myf 5 to initiate differentiation, resulting in fusion of myoblasts into multinucleated myotubes (Black et al 1998), which were used for experimentation.

### 6.1.3 The Effects of EPA on PIF Induced Proteolytic Degradation in C2C12 Myotubes

C2C12 cells were plated and differentiated into myotubes according to the standard method. Before the cells were totally differentiated 20µl of [$^3$H]-Phe corresponding to 2µCi was added to each well and the cells were incubated at 37°C in 5%$CO_2$ overnight. ([$^3$H]-Phe was pre-prepared by adding 60mg 'cold' phenylalanine to 500µL [$^3$H]-Phe in 4500µL PBS). The following day (during which time myotubes would have fully formed), the media was discarded and the cells rinsed twice in PBS. DMEM with phenol red (supplemented with 10% foetal calf serum and 1% penicillin / streptomycin) was added along with 50µM EPA (complexed to an equal mass of Bovine Serum Albumin and neutralized with equimolar $NaHCO_3$). Following a two hour pre-incubation, the media was removed and replaced with DMEM without phenol red (supplemented as previous) along with a range of concentrations of PIF (0-0.4µg/ml), 2mM 'cold' phenylalanine and cycloheximide. Cells were then incubated for a further 24hours as before. Finally, 1ml of supernatant was removed and added to 6ml optiphase 'hi safe 3' scintillator fluid. The [$^3$H] disintigrations/minute were analysed using a 'Tri-carb 2000CA' liquid scinitillation analyser.

### 6.1.4 The Effects of EPA on 15-HETE Induced Proteolytic Degradation in C2C12 Myotubes

As above except 15-HETE added in a range of concentrations (0-0.5μg/ml) instead of PIF.

### 6.1.5 The Effects of EPA on Proteasomal 'Chymotrypsin-Like' Activity in PIF Treated C2C12 Myotubes.

Cells were pre-treated with EPA (complexed to BSA and neutralised in $NaHCO_3$) for two hours and then PIF added at a range of concentrations (0-0.4μg/ml) for 24hours. Myotubes were then washed twice in ice cold PBS and scraped in approximately 1ml homogenising buffer. Samples were then sonicated for three pulses of 15sec, with 10sec intervals and centrifuged at 15,000 rpm for 10min to pellet insoluble material.

A stock solution of subtrate (10mg N-succinyl-leu-leu-val-tyr-7-amido 4 methyl coumarin in 600μl Dimethyl Sulphoxide) was diluted 1:1000 in 100mM Tris/HCl pH8.0 for use. 100μl was added to 50-100μl of prepared sample. A duplicate set of samples was included to which 10μl of lactacystin had been added to give a final concentration of 10μM per well. Samples were then incubated for 1h on ice.

Fluorescence of the substrate was measured using an LS50 Luminescence Spectrometer (Perkin-Elmer) at excitation 360nm and emission 460nm and values were adjusted for equal protein concentrations and minus a reaction blank.

### 6.1.6 The Effects of EPA on Proteasomal 'Chymotrypsin-Like' Activity in 15-HETE Treated C2C12 Myotubes.

As above except 15-HETE added in a range of concentrations (0-0.5μg/ml) instead of PIF

### 6.1.7 The Effects of the NFkB Inhibitor SN50 Peptide on PIF and 15(s)HETE induced Upregulation of the 'Chymotrypsin-Like' Activity of the Proteasome.

C2C12 myotubes were pretreated with 18μM of the cell permeable NFkB inhibitor peptide SN-50 for 20min. A vehicle control (as well as a no treatment control) was included, to differentiate any effects caused by the solvent. After 20mins at $37^0$C in 5%$CO_2$, 0μg/ml, 0.01μg/ml and 0.05μg/ml 15(s)-HETE or 0.1μg/ml and 0.4μg/ml PIF was added, and the cells incubated for 24 hours. The cells were then scraped into homogenising buffer and assayed for chymotrypsin activity in the presence and absence of 10μM lactacystin as described previously.

### 6.1.8 Determination of Protein Concentration

Protein concentrations were determined using a standard commercially available colourimetric protein assay (Biorad UK) according to the manufacturers instructions. To ensure accuracy of the measurements, 1-10μg protein was electrophoresed on a polyacrylamide gel (see methods 6.1.9) which was then stained in 1% 'Coomassie blue' (in 40% methanol. 10% acetic acid) for one hour. Gels were destained in 3-4 changes of 40%methanol/10% acetic acid until bands were visible.

### 6.1.9 Western Blotting Protocol

The media was rinsed from myotubes and they were homogenised and sonicated in 500-2000μl homogenising buffer (as described above). After

centrifugation to pellet insoluble material, the supernatant was assayed for protein concentration. Homogenates were denatured in electrophoresis sample buffer by heating to 95°C for 5 minutes and electrophoresed on a 12% SDS-polyacrylamide gel (10cm x 10cm) along with 'Rainbow' molecular weight markers. Parallel gels were also electrophoresed and then stained in 1% coomassie blue to ensure equal loading.

Proteins were electrotransferred to a nitrocellulose membrane using an enclosed system for 2h at a constant voltage of 80v. After transfer, the membranes were rinsed in wash buffer and transferred to blocking buffer for 1 hour at room temperature.

Primary antisera were diluted in blocking buffer (see table below) and added to membranes for 1h at room temperature. This was followed by washing in 0.1%PBS-T for one hour at room temperature with agitation, changing the wash buffer every 15-20min. Anti mouse or anti-rabbit IgG:HRP (horse radish peroxidase), diluted 1:2000 in wash buffer, was added and incubated for 1hour at room temperature. After incubation with secondary antibody the membranes were washed for a further 90min with agitation, again changing buffer every 15-20min.

| Antisera | Origin | Dilution | Source |
|---|---|---|---|
| $E2_{14k}$ | Rabbit polyclonal | 1:1000 | Dr S Wing |
| IκBα | Rabbit polyclonal | 1:400 | Autogen Bioclear UK |
| Myosin | Mouse monoclonal | 1:250 | Novocastra UK |
| P42 | Mouse monoclonal | 1:120 | Dr J Arnold |
| MSS1 | Mouse monoclonal | 1:100 | Dr J Arnold |
| 20S | Mouse monoclonal | 1:1500 | Affiniti Research UK |

Proteins were detected using an 'Enhanced Chemiluminescence (ECL)' system, which is based upon the oxidation of luminol by HRP, resulting in light emission, detected by a blue light sensitive autoradiography film.

## 6.2 Molecular Biology Methods

### 6.2.1 The Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay (EMSA) or gel retardation assay determines the binding interaction between DNA and DNA binding proteins, and is based upon the observation that complexed protein and DNA will migrate more slowly through a non-denaturing gel than unbound DNA or oligonucleotides. DNA or a corresponding oligonucleotide can be labelled with [$\gamma$-$^{33}$P]ATP and the protein bound and 'free' DNA forms can be quantitated using autoradiograohy.

#### 6.2.1.1 Labelling of Consensus Oligonucleotides

2µl NFκB (1.75pmol/µl), the sequence of which is shown below; 1µl T4 Polynucleotide Kinase 10x Buffer; 2µCi [$\gamma$-$^{33}$P]ATP; 5µl nuclease free water and 1µl T4 polynucleotide kinase was assembled in a sterile microcentrifuge tube and incubated at 37°C for 10min. The reaction was stopped by the addition of 1µl 0.5M EDTA followed by 89µl of TE buffer.

NFκB Oligonucleotide Sequence

5' – AGT TGA GGG GAC TTT CCC AGG C – 3'
3' – TCA ACT CCC CTG AAA GGG TCC G – 5'

#### 6.2.1.2 Dose Response

C2C12 cells, differentiated into myotubes according to the standard method, were pre-incubated in 50µM EPA for 2h. PIF (0.1µg/ml or 0.4µg/ml) and 15-HETE (0.01µg/ml or 0.05µg/ml) was then added for 20-30minutes.

For further investigations, the myotubes were pre-incubated in 10μM lactacystin or 18μM SN50 (NFκB inhibitor peptide) for 20mins followed by a 1h incubation in 0.1μg/ml and 0.4μg/ml. PIF.

### 6.2.1.3 Preparation of Nuclear Proteins

The cells were rinsed,scraped and pelleted in wash buffer. The pellets were resuspended in 300μl of the same wash buffer and incubated on ice for 15mins. 30μl of 1% 'Triton x-100' (octyl phenoxy polyethoxyethanol) was then added and the cells lysed by vortexing. A 30sec centrifugation at 14,000rpm pelleted the nuclei and the supernatant was removed. The nuclear pellet was resuspended in 50μl of ice cold high salt buffer to solubilise nuclear proteins and the suspension was kept on ice for 20min, with a 30sec vortex every 3-5 mins. A centrifugation at 14,000 rpm for 5min yielded the supernatant containing the protein extract. The concentration of nuclear extracts was measured by protein assay (as described previously). Measurements were repeated three times (where possible) to ensure the accuracy of the assay.

### 6.2.1.4 DNA Binding Reaction

2μl gel shift binding buffer and 10μg nuclear extract (for each test) were added in a sterile microfuge tube, along with 2μl unlabelled NFkB (competitor control) or 2μl of a different, unlabelled oligonucleotide (non-competitor control). A negative control reaction was also included which contained gel shift binding buffer and no sample. The volumes of the tests and controls were equalised with nuclease free water and the reactions were incubated at room temperature for 10min. 2μl of $\gamma$-$^{33}$P-NFkB was then added and the samples were incubated for a further 20min. Finally 1μl of gel loading 10X buffer was added to the negative control and the reaction products were analysed via electrophoresis.

#### 6.2.1.5 Gel Preparation and Electrophoresis

The EMSA separation was performed on an 8% non-denaturing polyacrylamide gel, with a native 5% polyacrylamide stacking gel which had been allowed to polymerise for at least 2h. The gels were pre-electrophoresed for 10min at 150mV and then electrophoresed (after the addition of samples) at 150mV for approximately 30min, or until the bromophenol blue dye front reached three-fourths down the gel. The gel was then dried between Whatman 3MM filter paper and plastic wrap and exposed to 'Hyperfilm MP' for 48hours at –70°C.

### 6.2.2. Competitive Quantitative Reverse Transcription Polymerase Chain Reaction (cQRT-PCR)

#### 6.2.2.1. RNA Extraction

RNA was extracted at 4°C, under RNase free conditions, using a 'Tri-Reagent' guanidine thiocyanate phenol method based on that of Chomczynski and Sacchi (1987). Each muscle was homogenized in 1ml 'Tri-Reagent'. C2C12 myotubes were rinsed in PBS, and either lysed directly by the addition of 1ml/10cm$^2$ area of 'Tri-Reagent', or scraped into PBS and frozen at –70°C until use. In the latter case the cells were lysed by resuspension in 'Tri-Reagent' once they were thawed.

A 10min, 4°C, 12,000rpm centrifugation removed insoluble material to leave a protein and RNA containing supernatant. A 5min room temperature incubation allowed complete dissociation of nucleoprotein complexes. 200µl chloroform/ml 'Tri-Reagent' was added and the samples incubated for 5 minutes at room temperature. A 12,000g centrifugation for 15 min separated the mixture into a red protein containing organic phase, a DNA interphase and an RNA containing upper phase.

This RNA containing phase was transferred to a fresh tube and 0.5ml isopropanol was added per ml 'Tri-reagent' used. A 10 minute room temperature incubation was followed by centrifugation at 12,000g for 10min at 4°C, which precipitated the RNA at the bottom of the tube.

The RNA was washed in 1ml of 75% ethanol, vortexed and pelleted at 7,500g for 5 minutes at 4°C. The ethanol was removed and the RNA allowed to partially air dry for a few minutes. The RNA was stored in RNA storage solution at -20°C until use and quantified by OD at 260nm, assuming that an OD of 1=40µg RNA. Total concentrations were calculated thus:-

$$\frac{\text{A260nm x 40}}{\text{Dilution factor}} = \text{RNA (µg/ml)}$$

### 6.2.2.2 Competitor Titration and Reverse Transcription

The competitor used for the titration was a 76bp deletion mutant cloned by Miss J Khal of this laboratory. A serial dilution of the competitor RNA (generally ranging from 0.078 to 2.5ng was added to an unchanging amount of target RNA for each sample.

2µg sample RNA and a range of concentrations of competitor RNAs was added to tests and to a negative tube and heated at 95°C for 5minutes. 1µg pd(N)6 random hexamer 5' phosphate was then added to each tube on ice, and the samples were heated at 70°C for 5min.

To each reaction vessel, the following was then added; 5µl 5x reverse transcription buffer, 6µl each of 10mM dNTP's (2 deoxyadenosine 5' triphosphate , 2 deoxyguanosine 5' triphosphate, 2 deoxycytosine 5' triphosphate, 2 deoxythymidine 5' triphosphate, pre-diluted in 1% diethylpyrocarbonate treated water), 1µl rRnasin RNase inhibitor and 1µl M-MLV Reverse Transcriptase.

Except in the case of the negative controls in which the Reverse Transcriptase was omitted. The volume was adjusted to 25µl with nuclease free water and the reactions incubated at 37°C for 1h.

### 6.2.2.3 PCR

Following this incubation the PCR amplification mix was prepared by adding 12.5µl (equaling 1µg RNA) to 50µl PCR 'master-mix'. This contained 5µl PCR buffer (10x), 3.7µl C2 forward primer (1pmol), 3.8µl C2 reverse primer (1pmol), 6µl 3.5mM $MgCl_2$, 2.5µl Taq polymerase, added in order, and adjusted to 50µl with nuclease free water. The reactions were then incubated at 95°C and subjected to a 30 cycle PCR using a 'Primus 25/96 Thermocycler'. The denaturing step was at 95°C for 1min, annealing at 58°C for 1 minute and elongation for 72°C for 2min.

The C2 forward and reverse primers was also designed by Miss Jwan Khal in this laboratory and the sequences are shown below.

Forward 5' - CGC ACG CAG TGC TGG TTG CAC – 3'

Reverse 3' - GTA CGA GCT GAT TGA GAA CGG – 5'

Competitor 5' - GTA CGA GCT GAT TGA GAA CGG **CAT AAC CAG CAA TGA GCA GCC** – 3'

### 6.2.2.4 Analysis of PCR Products

PCR products were analysed by electrophoresis on a 2% agarose/TBE gel to which 1µl/10ml ethidium bromide was added, prior to setting. A 100b.p. DNA ladder marker lane was included and the gels were electrophoresed at 65mV for 1h in TBE buffer. RNA integrity was assessed using a UV transilluminator and densitometrically analyzed using Windows 'Grabbit', 'Gelworks 1d' and 'Phoretix 1d' software.

### 6.2.3 In Vivo Methods

#### 6.2.3.1 Tumour Transplantation

Pure strain female NMRI mice were obtained from our own breeding colony and transplanted with fragments of the MAC 16 tumour (see Double et al 1975) into the flank by means of a trocar. (as described previously – Bibby et al 1982). MAC16 tumours were originally derived from colon tumours induced by dimethylhydrazine in NMRI mice (Double, Ball and Cowen 1975) and were originally provided for transplantation by Drs. Double and Bibby of Bradford University, UK.

Transplantation of tumours was performed by Mr M Wynter and Mr W Fleary. The animals were fed a standard rat and mouse breeding diet and (with the exception of starvation experiments) had unlimited access to food and water throughout the experiment.

#### 6.2.3.2 The Effects of Dietary EPA

10-14 days after transplantation when tumours were palpable and weight loss had reached 5% of total body weight, treatment was initiated. This point was chosen to allow for weight loss and complete tumour take to occur. At this point the animals were randomized according to tumour volume to receive either EPA, vehicle only or no treatment.

EPA treated animals received a single daily 100µl dose by gavage, equivalent to 0.5g EPA/kg and 2.5g/kg body weight and delivered in olive oil. Control animals received a 100µl daily gavage of olive oil only. A second control group were kept in identical conditions, but received neither treatment.

Body weights were measured daily and at the same time each day. Tumour volumes were also estimated daily by the use of calipers, using the formula:-

$$\frac{\text{length (cm) x (width (cm))}^2}{2} = \text{Approximate Tumour volume } (cm^3).$$

The end point for the study was decided beforehand or taken to be when there was tumour ulceration, weight loss reached 6-7g or 20% body weight or if the animals became moribund according to the United Kingdom Coordinating Committee for the welfare of animals with neoplasms.

At the end of the study the animals were sacrificed by cervical dislocation and the tumours, muscles and other organs excised as appropriate. Animals were sacrificed at 9am in all experiments, to minimise diurnal variation.

### 6.2.3.3 Measurement of Proteasome 'Chymotrypsin-like' Activity in NMRI Mice Bearing the MAC16 Tumour

Muscles were excised and flash frozen in liquid nitrogen, where they were stored at –70°C until use. When thawed they were homogenised in 1:5 volume of homogensising buffer and then sonicated for three pulses of 15sec, with 10sec intervals. A centrifugation at 15,000 rpm for 10min pelleted insoluble material.

The rest of this assay is as described previously (chapter 6.1.5 pp101) except that 10-20µl of prepared sample was used

### 6.2.3.4 Measurement of Total Protein Breakdown in Skeletal Muscle Using an In Vitro Tyrosine Release Assay

Soleus muscle were excised and quickly ligated at resting length, by the tendons to steel clips, where they were placed in ice cold isotonic PBS. The muscles were incubated for 2hours with agitation in a 37°C water bath in 3ml Krebs-Heinseleit bicarbonate buffer and in a constant 5%$CO_2$ environment.

After this incubation the muscles were blotted and weighed and 2ml of the buffer removed and deproteinised with 200µl ice cold 30% trichloroacetic acid. Insoluble material was pelleted by centrifugation at 2800g for 10min. 1ml each of nitric acid and of 0.1% 1-nitroso-2-naphthol (prepared in 95% ethanol) was added to thee supernatant, which was mixed and incubated in glass tubes at 55°C for 30min. After cooling for 10mins, 5ml dichloroethane was added and the tubes mixed and centrifuged at 2800g for 10min. Tyrosine is neither synthesized nor degraded in the muscle and as it rapidly equilibrates between intracellular pools and the external medium, its release can be used as a marker of proteolysis. Tyrosine fluorescence was measured using a Perkin Elmer LS50 Fluorimeter at an excitation wavelength of 460nm and an emission wavelength of 570nm.

#### 6.2.3.5 The Effects of EPA in Acute Starvation in NMRI Mice

Pure strain female NMRI mice were treated with 0.5g/kg, 2.5g/kg EPA or the equivalent volume of olive oil vehicle. A weight matched control group were kept in identical conditions but received neither EPA nor vehicle (fasted control) and a fourth non-fasted control group were included for comparison. Animals were dosed daily by oral gavage for 48 hours after which time the animals were fasted for 24h, though free access to water was maintained. At the end of the experiment animals were sacrificed by cervical dislocation as described previously (chapter 6.2.3.2 pp110).

#### 6.2.3.6 An Investigation of the Specificity of EPA on the Proteasome Pathway in Starvation. The Effects of Docosahexaenoic Acid, Linoleic Acid and the Lipoxygenase Inhibitor - CV6504 in Fasted NMRI Mice and the Effects of EPA in Non Fasted Mice.

To determine the specificity of EPA on the proteasome pathway in acute starvation, the experiment was repeated using docosahexaenoic acid (DHA), linoleic acid (LA) and the lipoxygenase inhibitor CV6504 in NMRI mice,

which had been fasted for 24hours. Non treated, non fasted, non fasted EPA treated, and fasted vehicle controls were included.

NMRI mice were assorted into 7 weight matched groups (5 animals per group with an average weight of 20g), to receive either no treatment, EPA, DHA, LA or olive oil vehicle (all 2.5g/kg), or CV6504 (10mg/kg). All of the groups with the exception of EPA treated and no treatment were then fasted for 24hours although free access to water was maintained.

After fasting the mice were sacrificed by cervical dislocation, the soleus and gastrocnemius muscles removed and prepared for tyrosine release assay (soleus) and Western analyses (gastrocnemius), as described previously.

# 7 The Effects of EPA in an In Vivo model of Cachexia –NMRI mice Bearing the MAC16 Colon Adenocarcinoma.

## 7.1 Introduction

EPA has been shown to be an effective inhibitor of the induction and progression of cachexia in clinical studies with human patients and in in vivo animal studies. It has been shown to attenuate proteolysis in skeletal muscle and lipolysis of adipose tissue, which are the main indicators of cachexia, both in vivo and in vitro. (See chapters 4.2 and 4.3). Whilst there is some evidence that DHA can attenuate cachexia, in the MAC16 model and many others the effect is specific for EPA and all other structurally related fatty acids are ineffective (Hudson et al 1993).

In this set of experiments, EPA was administered to NMRI mice bearing the cachexia inducing MAC16 tumour, and the effects upon weight loss, tumour growth and expression and activity of members of the ubiquitin proteasome pathway was examined.

## 7.2 Results and Discussion

### 7.2.1 Effect on Body Mass

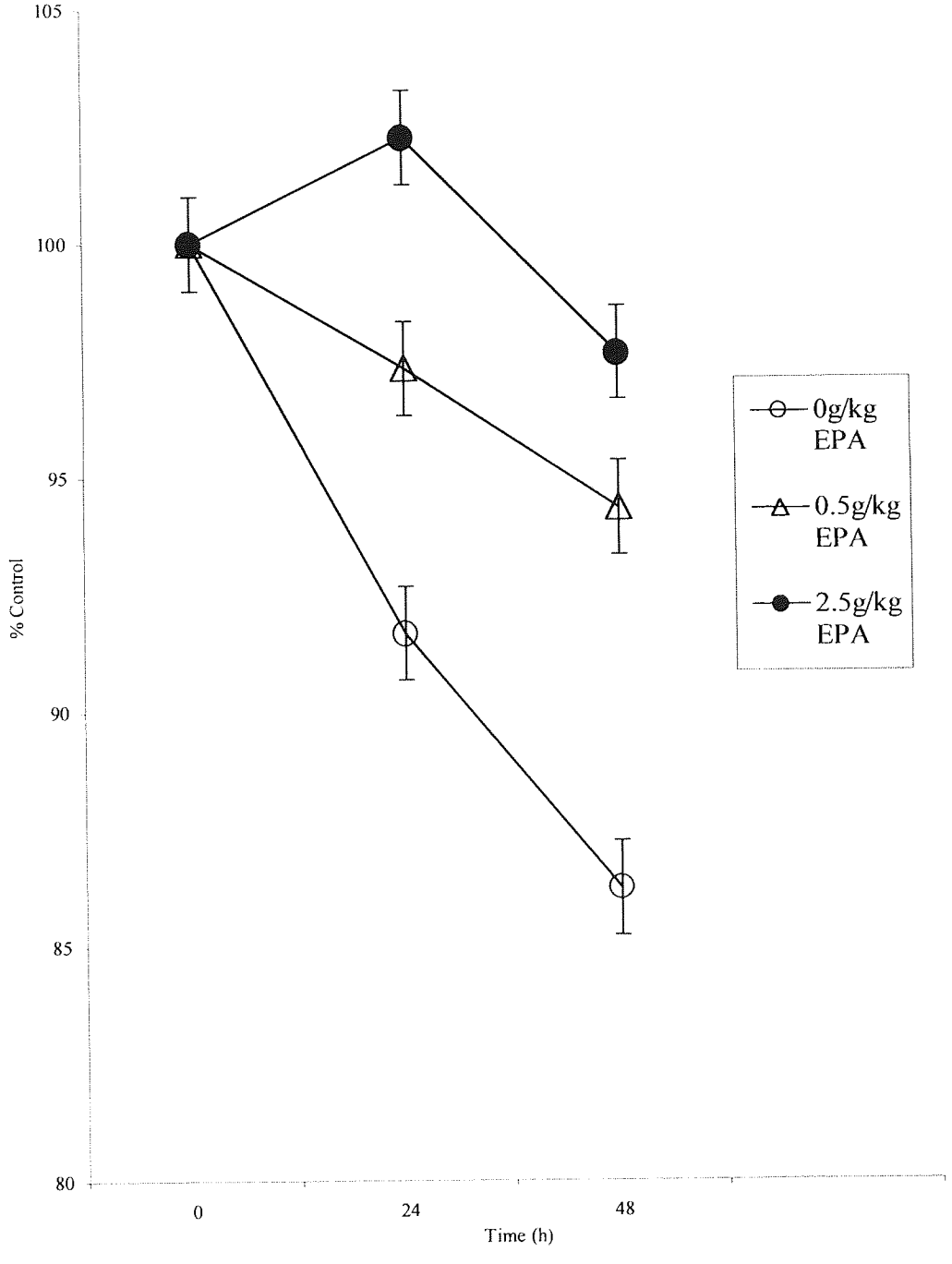
NMRI mice were transplanted with the cachexia-inducing tumour MAC16 by means of a trocar into the flank. Once weight loss was established (an average of 8.1%), the animals were sorted into weight matched groups to receive either EPA or an equal volume of olive oil vehicle control, EPA was administered p.o. at 0.5g/kg and 2.5g/kg daily by oral gavage. Figure 7.2.1.1 (overleaf) shows the effect of EPA on body mass. There is a dose dependent reduction in the amount of weight lost. Statistical significance (as determined by one way ANOVA, with Tukey's post-test, unless otherwise stated) was reached after 24hours where $p<0.05$ for 0.5g/kg EPA and $p<0.01$ for 2.5g/kg. After 48hours $p<0.01$ for 0.5g/kg EPA and $p<0.001$ for 2.5g/kg.

It has been previously demonstrated that the attenuation of proteolysis by EPA in the MAC16 model is accomplished through pathways which are not lysosomal or calpain dependent. Both Lorite et al (1998) and Whitehouse et al (2001; see appendices) have showed that blocking the lysosomal pathway with methylamine (an inhibitor of lysosomal acidification) does not prevent the EPA induced attenuation of muscle proteolysis in MAC 16 animals, indicating that EPA exerts its effects on a proteolytic pathway which is not lysosomal. Similarly whilst E64 an inhibitor of cysteine proteases (Barret et al 1982) including calpains, can, (in this model) attenuate muscle loss in its own right, (suggesting that the $Ca^{2+}$ dependent pathway is involved), EPA does not inhibit the attenuation of proteolysis in the presence of E64 and E64 reduces proteolysis identically, irrespective of the presence of EPA (Whitehouse et al 2001). This suggests that this is not the main target pathway for the attenuation of protein degradation by EPA.

It should not be forgotten that ATP-dependent proteolysis is elevated in animals bearing the MAC16 tumour (Lorite et al 1998), EPA causes a reduction in proteolysis in tumour bearing mice which is not further reduced

by ATP depletion suggesting that inhibition of this pathway is the primary mechanism through which EPA exerts its anti-proteolytic effects.

**Fig 7.2.1.1) The effects of p.o. dosing of EPA on Body Mass of female NMRI mice bearing the MAC16 tumour with established weight loss (8.1%)**

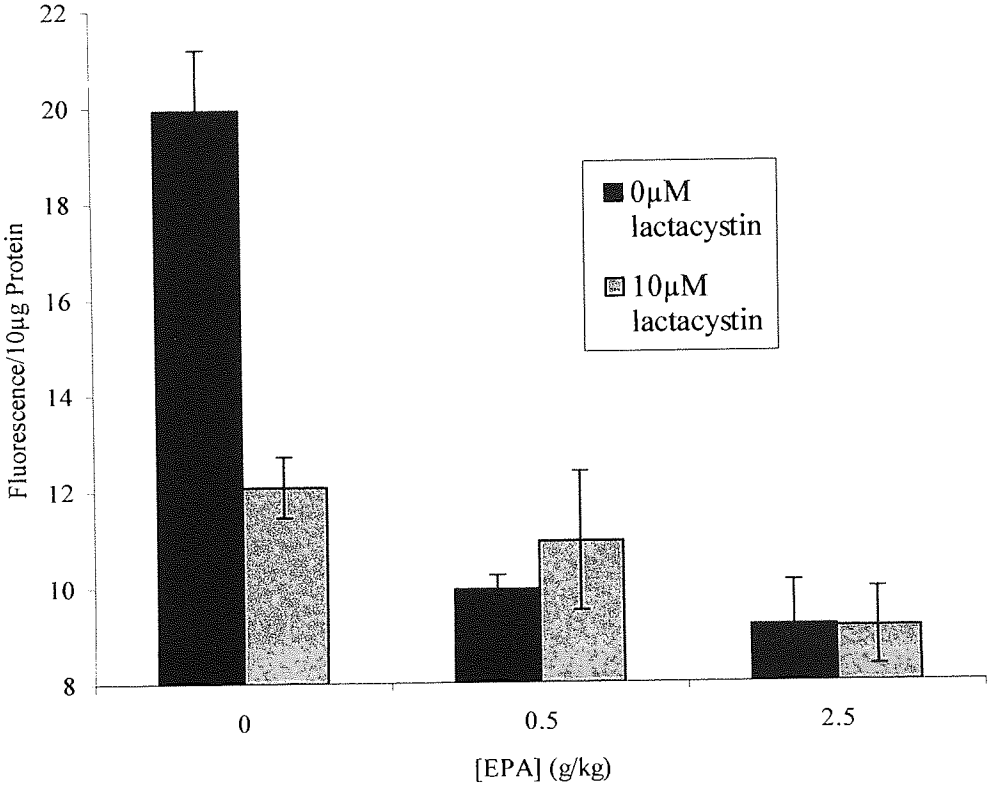


### 7.2.2 Effects on Skeletal Muscle

To investigate this further, the effects of EPA on proteasome activity were examined. Suc-LLVY-AMC is a peptide substrate of the 'chymotrypsin-like' enzyme activity of the proteasome. (This is the most dominant catalytic activity and the one most widely used in the literature as an indicator of proteasome function). Once cleaved the fluorescent AMC (amino methyl coumarin) is released and is proportional to the level of proteasome activity.

Figure 7.2.2.1 shows the effects of EPA on the 'chymotrypsin-like' enzyme activity of the proteasome in gastrocnemius muscles excised from mice bearing the MAC16 tumour. Lactacystin is a specific proteasome inhibitor which was included to differentiate any fluorescence from non-proteasomal sources. One way ANOVA (with Tukey's post test) demonstrated extreme statistical significance ($P<0.0005$) when comparing 0g/kg and 2.5g/kg EPA (in the absence of lactacystin) and no significance comparing observations with and without lactacystin ($P>0.5$). Thus, there was a complete loss of lactacystin suppressible activity suggesting that EPA completely inhibited the increased activity seen in cachectic muscle.

**Fig 7.2.2.1) The effects of EPA on the 'chymotrypsin-like' enzyme activity of the proteasome in gastrocnemius muscles from mice bearing the MAC16 tumour, in the presence and absence of 10μM lactacystin and EPA .**

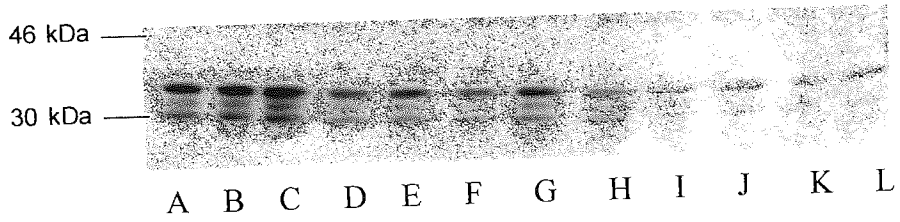


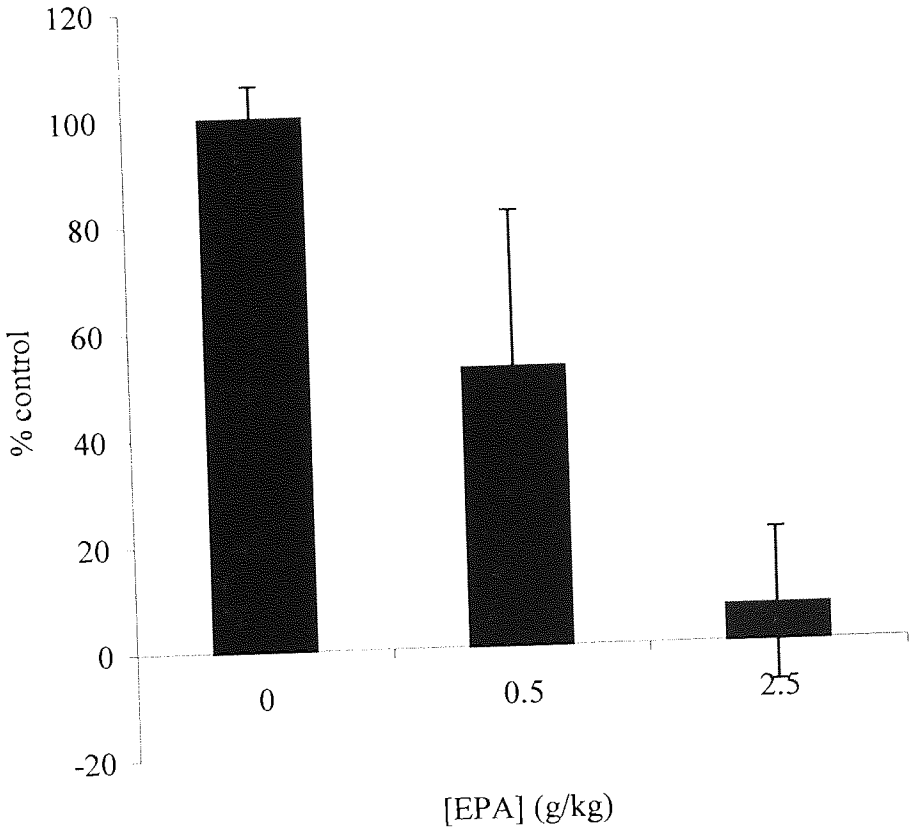

To determine levels of proteasome subunits in the cell, soluble extracts were Western blotted using an MCP231 antibody, reactive against six different α-subunits. Figure 7.2.2.2a shows a Western blot and densitometric analysis of 20S proteasome expression in the gastrocnemius muscle. There was a dose dependent decrease of 58% ($P<0.001$) and 95% ($P<0.001$) for 0.5g/kg and 2.5g/kg respectively, in cellular levels of 20S in the three bands detected.

**Figs 7.2.2.2) Western and densitometric analyses demonstrating the effects of EPA on expression of proteasome subunits, myosin and $E2_{14k}$ in gastrocnemius of NMRI mice bearing the MAC16 tumour and with established weight loss (8.1%).**

Lanes A-D = vehicle control; lanes E-H = 0.5g/kg EPA; lanes I-L = 2.5g/kgEPA)

**Fig 7.2.2.2a) 20Sα subunits**



Whilst Combaret et al (1999) and Combaret et al (1991) found that the mRNA level of the ATPase subunit MSS1 of the 19S complex was the only regulatory subunit increased in cancer cachexia; Figure 7.2.2.2b shows that P42 expression is altered in this case. P42 is an ATPase subunit of the 19S regulatory particle, necessary for formation of the 26S proteasome (Tanahashi 1999) and the only regulatory subunit tested. EPA causes a respective 69% ($P<0.01$) and 78% ($P<0.01$) reduction at 0.5g/kg and 2.5g/kg in treated animals. The increased expression of P42 may facilitate the rapid proteolysis of muscle proteins in cancer cachexia. ATPase subunits provide energy for the association of 20S and 19S particles and also the unfolding of ubiquitinated substrates for entry into the core.

The functional consequences of decreased proteasome activity and expression were demonstrated by Western analyses using an anti-myosin antisera. Figure 7.2.2.2c shows that cellular levels of myosin increase in the presence of EPA by 42% (not statistically significant) and 97% ($P<0.01$) at 0.5g/kg and 2.5g/kg respectively.

An interesting finding was that, despite changes in proteasome expression, the ubiquitin conjugating enzyme $E2_{14k}$ was not affected by EPA (fig 7.2.2.2d). Wing and Banville (1994) have suggested that this may be the rate limiting step in ubiquitin conjugation. Conversely, Lorite et al (1998) used MAC16 bearing mice and NMRI mice treated with purified PIF and demonstrated a 42% elevation in the levels of ubiquitin conjugates in MAC16 mice and a significant increase in PIF treated mice. The accumulation of ubiquitin conjugates suggests an increased flux of proteins through the pathway and that hydrolysis and not ubiquitin conjugation is rate limiting.

**Fig 7.2.2.2 continued) Western and densitometric analyses demonstrating the effects of EPA on expression of proteasome subunits, myosin and $E2_{14k}$ in gastrocnemius of NMRI mice bearing the MAC16 tumour and with established weight loss (8.1%).**

Lanes A-D = vehicle control; lanes E-H = 0.5g/kg EPA; lanes I-L = 2.5g/kgEPA)

**Fig 7.2.2.2b) P42**

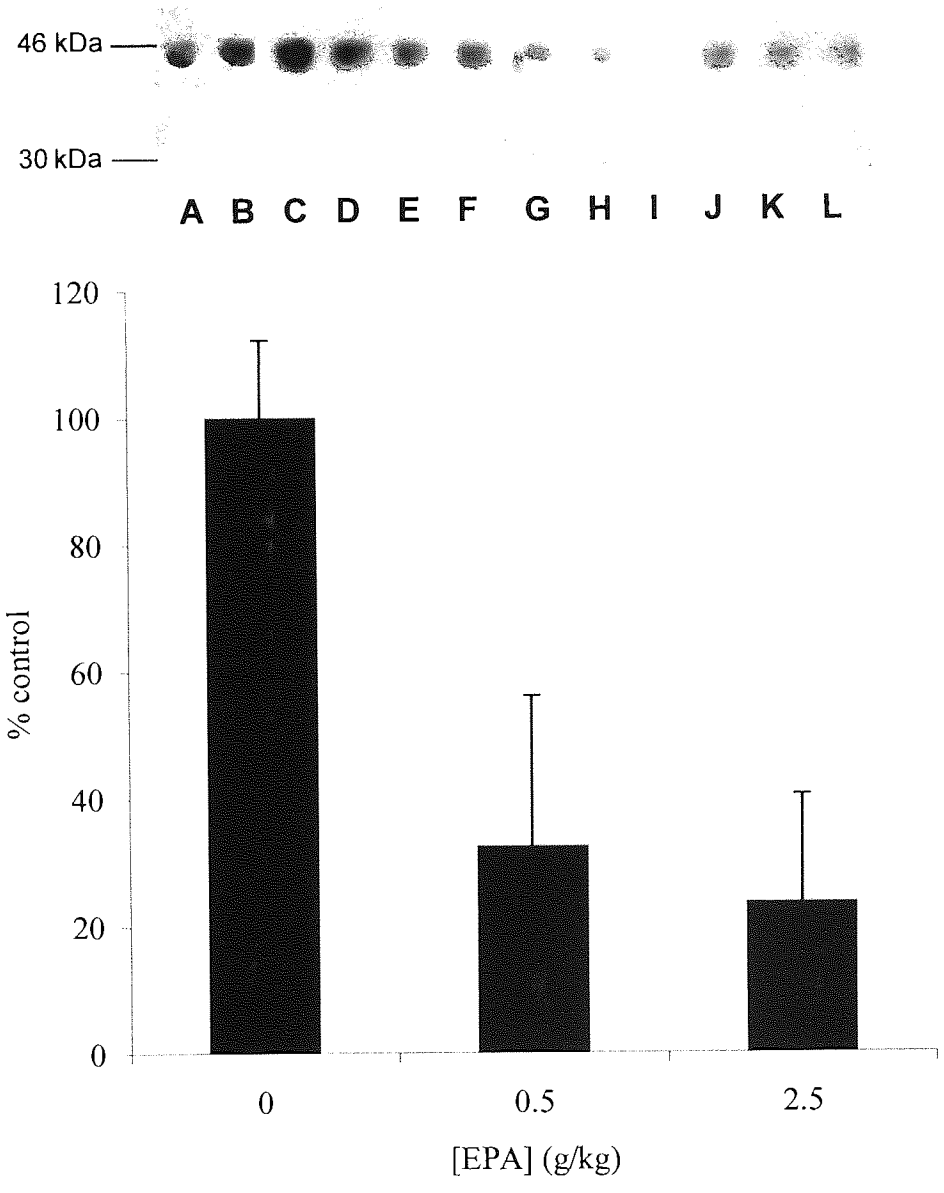
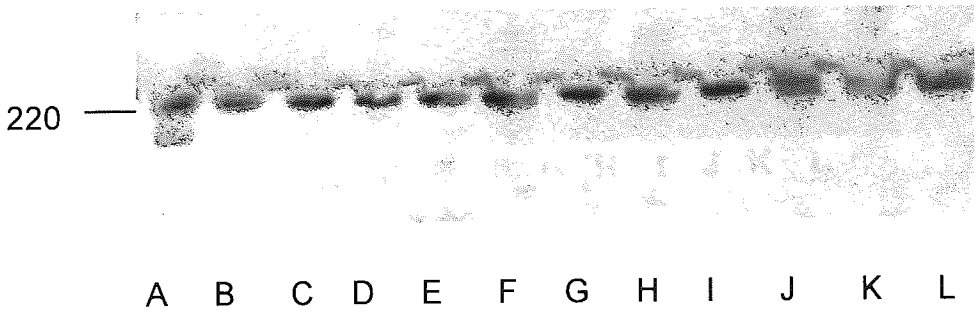


**Fig 7.2.2.2c) Myosin**



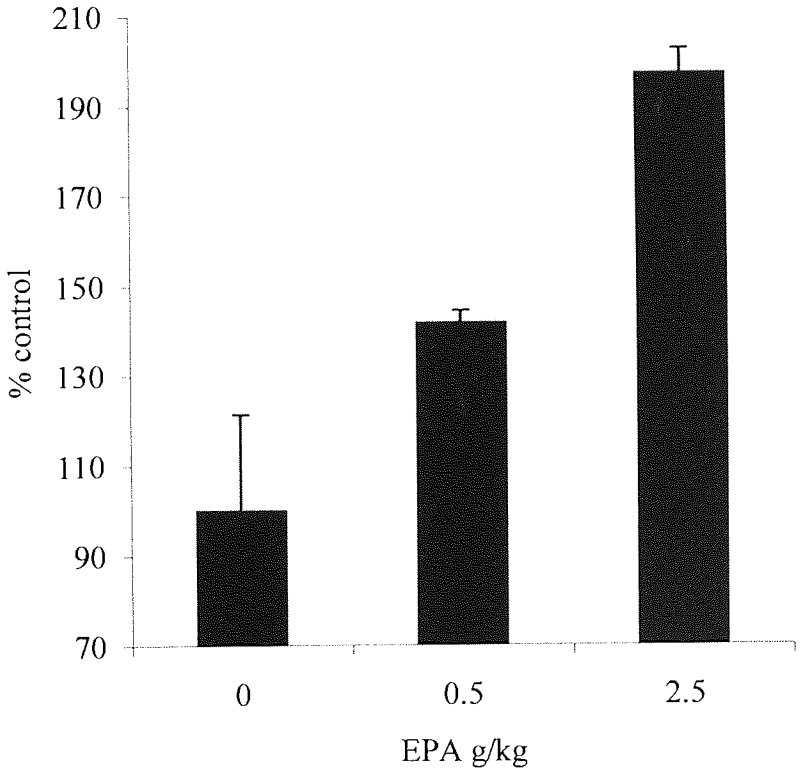
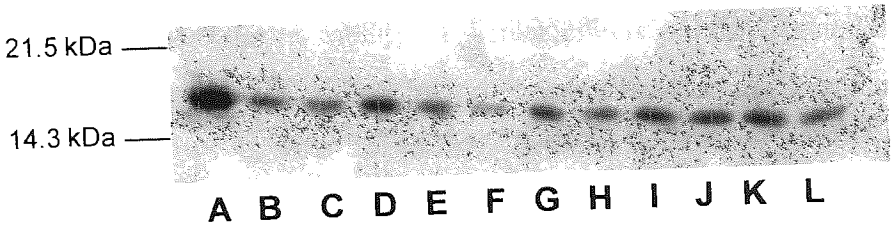
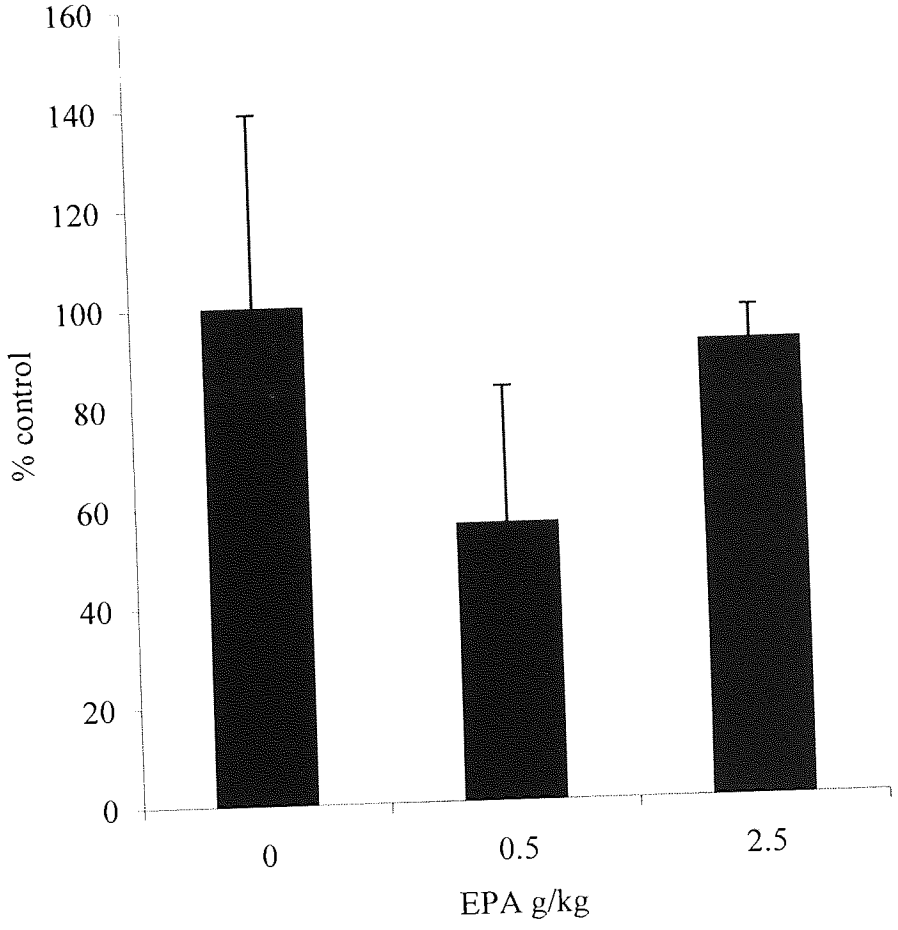



Fig 7.2.2.2d) $E2_{14k}$

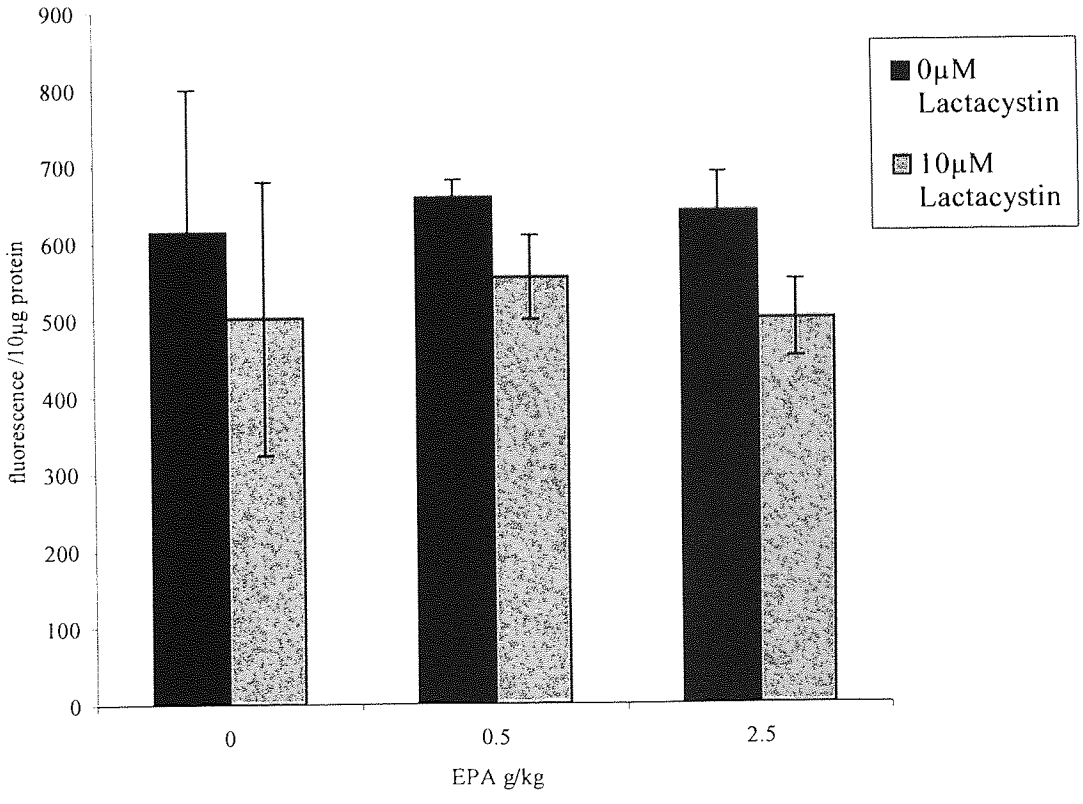


### 7.2.3 Effects on Visceral Protein Reserves

To investigate the possibility of similar effects on non-skeletal muscle, liver samples were also analysed for proteasome activity and expression. Figure 7.2.3.1 shows 'chymotrypsin-like' activity in the liver. There was no significant difference in 'chymotrypsin-like' activity in the presence of EPA, similarly no statistically significant difference in the levels of 20S, P42 or $E2_{14k}$ could be detected (data not shown), indicating that the effects of EPA on the proteasome pathway are specific to skeletal muscle and not visceral protein. This confirms the findings of Lorite et al (1998) who showed that in PIF treated mice, significant decreases were evident in soleus and gastrocnemius muscle mass, but not in the liver, kidney or heart suggesting that the action of PIF is mediated predominantly on skeletal muscle.

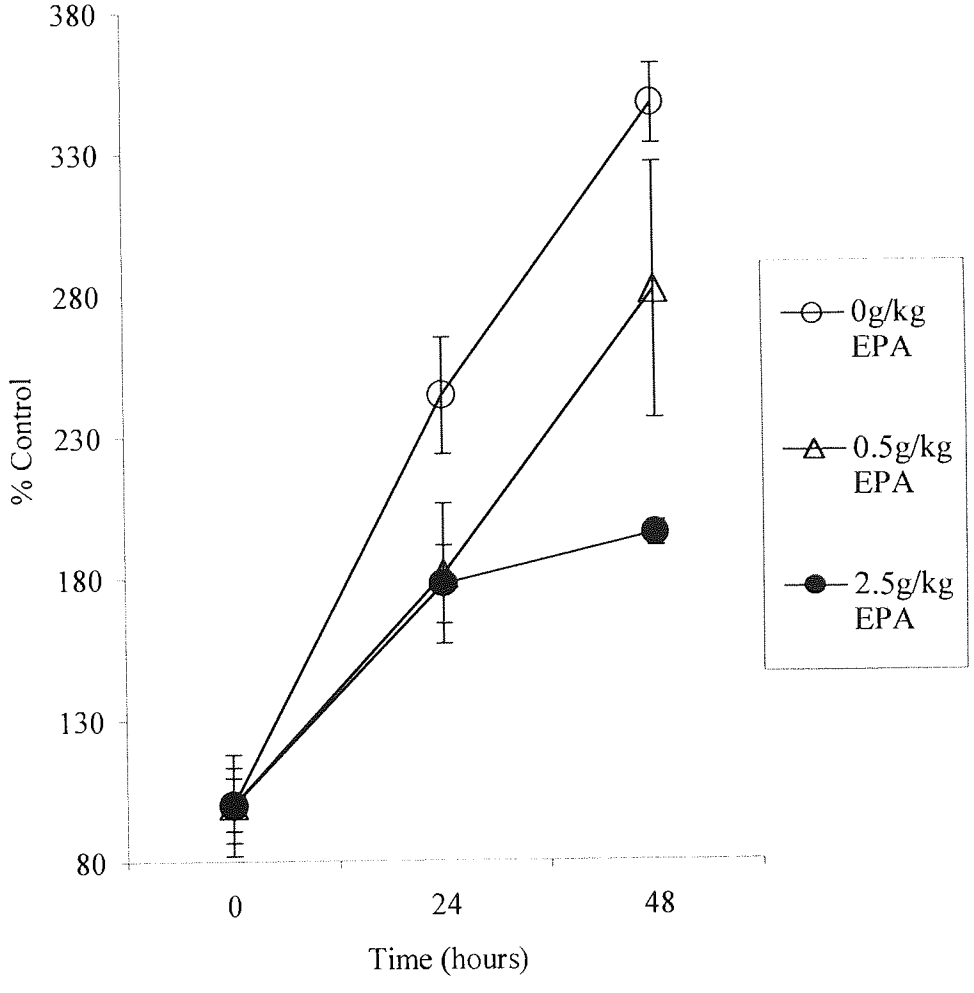
**Fig 7.2.3.1) The effects of EPA on the 'chymotrypsin-like' enzyme activity of the proteasome in the liver of NMRI mice bearing the MAC16 tumour with established weight loss (8.1%).**



### 7.2.4 Effects on Tumour Growth

EPA not only inhibits proteolysis but also tumour growth (Beck et al 1991). Figure 7.2.4.1 shows that tumour volume is decreased by an average 35% after 24 hours for 0.5g/kg, and 45 % for 2.5g/kg. After 48 hours tumour volume is reduced by 60.66% for 2.5g/kg (although 0.5g/kg showed no difference compared to control). However these results did not reach statistical significance.

**Fig 7.2.4.1) The effects of p.o. dosing of EPA on tumour volume of female NMRI mice bearing the MAC16 tumour and with established weight loss (8.1%)**



Proteasome activity and 20Sα expression in the tumour were also examined. Both concentrations of EPA reduced the 'chymotrypsin-like' activity of the proteasome by approximately 20% ($p<0.01$) (fig 7.2.4.2). EPA reduced 20S expression at a concentration of 2.5g/kg (figure 7.2.4.3) although this did not reach statistical significance. Thus, EPA may act as an antitumour agent through inhibition of the proteasome.

**Fig 7.2.4.2) The effects of EPA on the 'chymotrypsin-like' enzyme activity of the proteasome in the MAC16 tumour of NMRI mice with established weight loss (8.1%).**

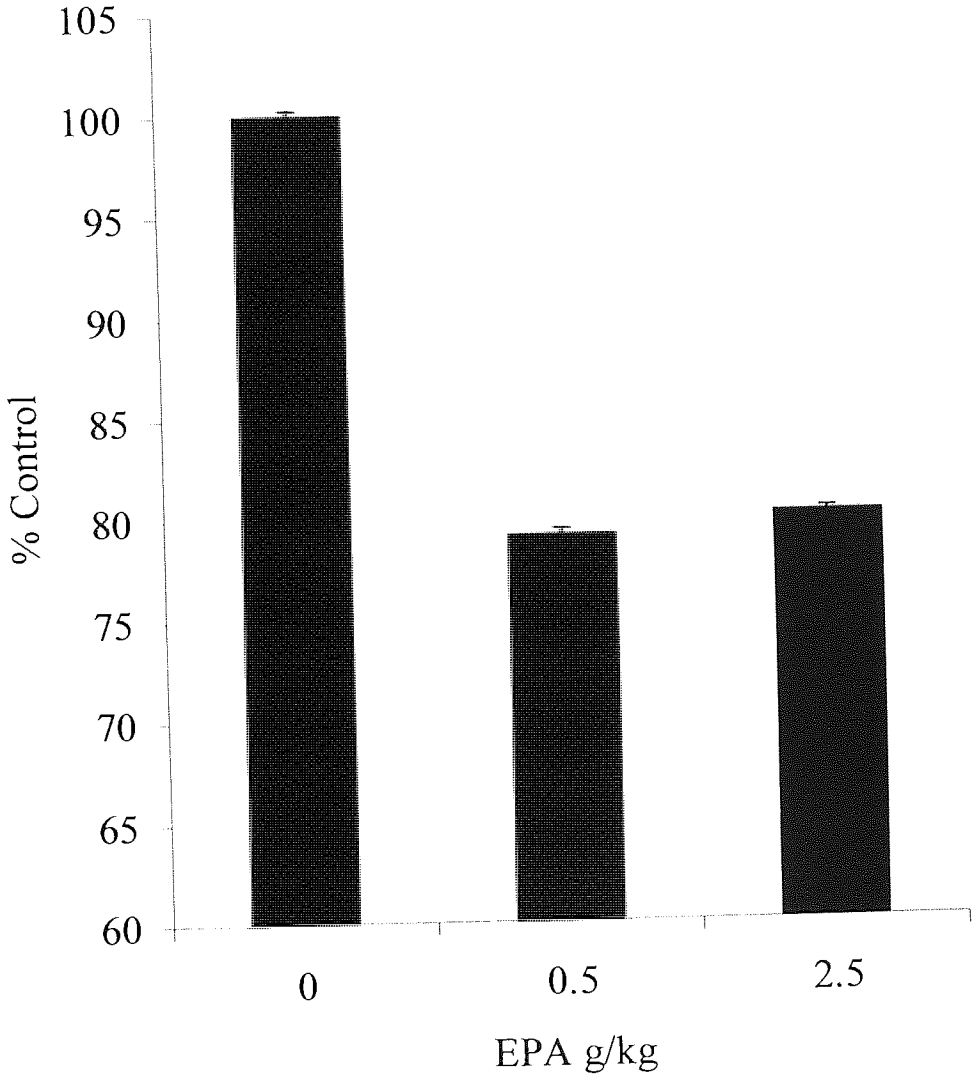
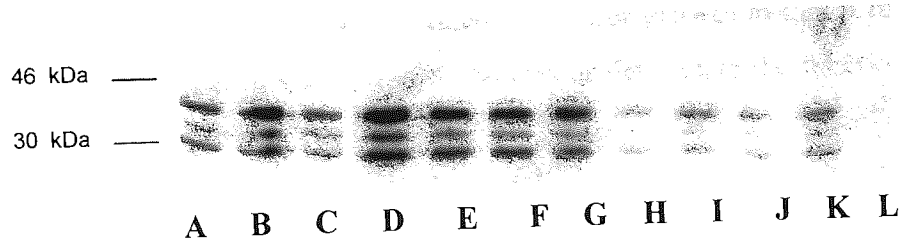
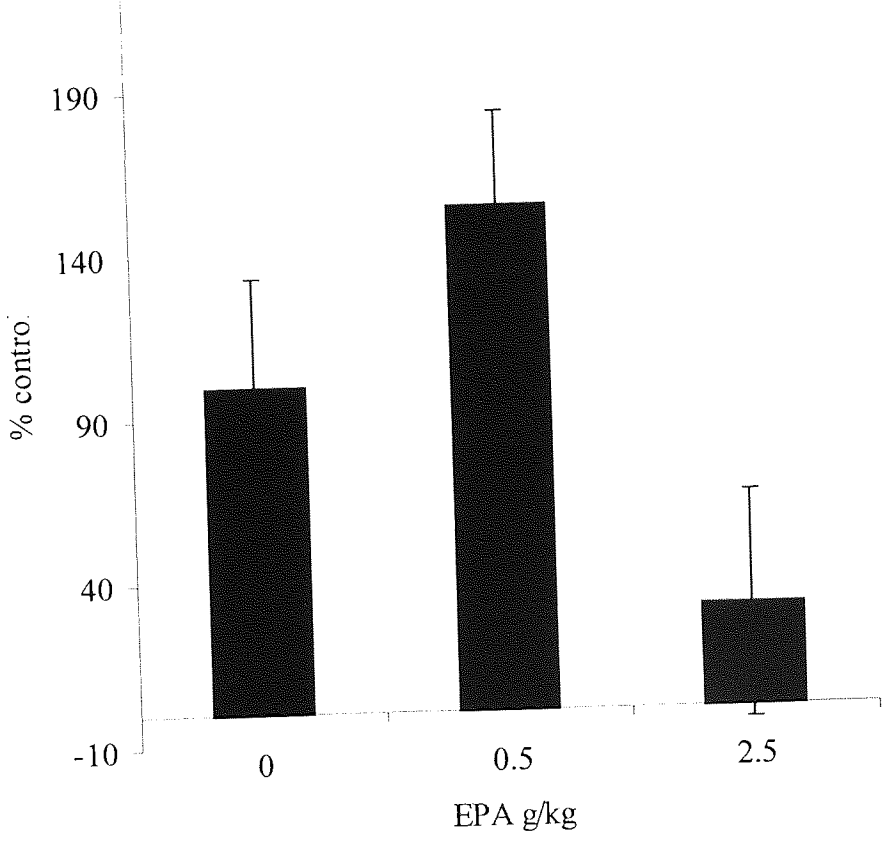


**Fig 7.2.4.3) Western and densitometric analyses to show the effects of EPA on expression of 20Sα in MAC16 tumour of NMRI mice with established weight loss (8.1%).**

Lanes A-D = vehicle control; lanes E-H = 0.5g/kg EPA; lanes I-L = 2.5g/kgEPA)



These results demonstrate an elevation of proteasome expression in both tumours and skeletal muscle of MAC16 bearing mice. Increased proteasome function in the latter is of obvious advantage to tumour growth through the mobilisation of amino acids for substrate utilisation and incorporation into the structural components of the tumour cell. However the significance of an elevated proteasome function/expression in the tumour is less clear. Proteasome upregulation may be beneficial to tumour growth in that it might mediate the destruction of anti-apoptotic factors or deregulate the function of tumour suppressor proteins.

These results show that EPA has a distinct antitumour effect which is correlated to an inhibition of proteasome expression. One possible explanation is that EPA inhibits the expression of the proteasome which is involved in the degradation of key cell cycle or signal transduction molecules. If these factors were themselves inhibitory to cell growth, inhibition of their degradation would promote apoptosis which would be reflected as a decrease in tumour size. In support of this hypothesis Blagoskonny et al (1996) and Pagano et al (1995) have shown that the *inhibitor* of cyclin dependent kinase can be regulated by the ubiquitin-proteasome pathway. Another candidate target is NFkB itself (see chapter 10) which has been shown to function as an endogenous apoptosis inhibitor (Begg and Baltimore 1996).

Whilst it seems that there is some evidence for the involvement of the proteasome in apoptosis, it is conflicting and not well investigated. It seems likely that the involvement of the proteasome is cell system specific. According to current evidence the main enzymes involved in this process are caspases, although degradation of caspase precursors is thought to be proteasome mediated, (Schutte and Ramaekers 2000). However, the role of caspases has not been investigated here and so cannot be commented on further.

The incorporation of highly polyunsaturated n-3 fatty acids from EPA into tumour membranes would be expected to increase the susceptibility of lipids to lipid peroxidation and pro-oxidant induced oxidative stress which could result in apoptosis through breaking membrane integrity, production of toxic aldehyde by-products, inhibition of thiol containing proteins and DNA degradation.

Oxidative stress (discussed in detail in chapters 4.4.1.1 (pp78) and 10.1.2 (pp193) is important in many pathologies due to the generation of reactive oxygen species. These can promote partial unfolding of proteins resulting in exposure of previously buried hydrophobic domains to ubiquitin conjugating enzymes and to proteolytic enzymes including the proteasome. Oxidatively damaged proteins often lack their normal functional properties.

The fact that tumours frequently have deficient antioxidant defence mechanisms and that the hydrophobic radical scavenger α-tocopherol often prevents cytotoxic effects, suggests that the selective cytoxicity toward tumour cell lines results from an increased sensitivity to oxidative damage.

Supporting this notion, Borgeson et al (1989) demonstrated that the therapeutic index of the anti-neoplastic agents doxorubicin and mitomycin C against the human mammary adenocarcinoma MX-1 is increased in animals fed a diet high in n-3 fatty acids. The fact that activity of these drugs is due to the generation of toxic oxygen radicals suggests that feeding fish oil predisposes these carcinoma cells to lipid peroxidation and increases their susceptibility to pro-oxidant anti-tumour agents.

In a similar way Igarishi and Miyazawa (2000) suggested that conjugating EPA (CEPA) and DHA (CDHA) into a triene structure may make it particularly susceptible to oxidative stress and thus might prove doubly cytotoxic. They found that conjugated EPA and DHA showed extensive cytotoxicity in the colorectal adenocarcinoma cell line DLD1, whilst having

no effect on normal human fibroblast cell lines (MRC5, TIG-103 and KMS6).

It is thought that the molecule is easily peroxidized, stored in the membrane phospholipids of tumours, where it generates toxic oxygen radicals causing cell death. In a demonstration of the susceptibility of the proteasome itself to direct oxidative damage, Reinheckel et al (2000) showed that the ATP stimulated degradation of the fluorogenic substrate suc –LLVY-MCA by the 26S proteasome was abolished by 1mM $H_2O_2$ in K562 human leukaemic cells.

Rashba-Step et al (1997) have suggested that an increase in membrane phospholipid peroxides induced cytosolic phospholipase $A_2$ activation, an interesting observation considering that PIF induces an increase in the levels of $PLA_2$ within the cell as an early event. It might be possible that as well as affecting proteasome subunit expression, PIF might affect $PLA_2$ expression possibly by a feed back mechanism.

A possible explanation for the effects of EPA in preventing muscle catabolism is suggested by Rotman et al (1992). The literature suggests that intracellular calcium sequestered in the ER is required for the maintenance of post-transcriptional protein synthesis in many cell types and AA and various metabolites are putative effectors of calcium mediated processes. Rotman et al, showed that exogenous AA and other fatty acids (the potencies of which generally rose as a function of increasing unsaturation) mobilised intracellular calcium to the extracellular space and inhibited protein synthesis in GH3 pituitary, CG glial tumour and HeLa cells, properties which are consistent with those expected for a physiological regulator that inhibits protein synthesis through mobilising calcium sequestered in the ER. However whilst protein synthesis has been shown to be lowered in the MAC16 model, it is thought that the effects on muscle wasting are primarily mediated by a massive increased protein degradation (Smith and Tisdale 1993b)

Other workers (Combaret et al 1999 and Llovera et al 1994) have commented upon the possibility that elevated TNFα levels seen in some cases of cachexia, corresponding to elevated proteasome subunit expression, might suggest TNFα as mediating the upregulation. This is interesting because TNFα is a known inducer of NFκB and implies a role for this transcription factor.

## 8. The Effects of EPA in an In Vitro Model of Cachexia – PIF and 15-HETE treated C2C12 Myotubes.

### 8.1 Introduction

There is a wide body of evidence demonstrating that PIF is directly responsible for the increased protein catabolism seen in cancer cachexia and that this condition can be manipulated favourably by n-3 fatty acid intake and particularly by EPA. However the mechanisms underlying these observations are not well understood. There is evidence that the ubiquitin proteasome pathway is largely responsible for skeletal muscle wasting in cachexia in this and other models. To investigate this further, a series of experiments were carried out examining the effects of EPA and purified PIF upon proteasome expression in an in vitro muscle model. The mus musculus C311myoblast cell line – C2C12 was differentiated into myotubes representative of mature differentiated muscle and used for all in vitro experiments.

In a seminal study, Smith et al (1999) considered the significance of the competitive effects of EPA upon arachidonic acid metabolism in this cell line. Using cells which contained pre-labelled arachidonate and in the presence of PIF, an increase in the levels of released arachidonate and a decrease in the levels of cellular arachidonate at a maximum of 4nM PIF demonstrated that PIF was indeed causing a release of cell bound arachidonate through interactions with PLA-2 (Smith et al 1999). The fate of the freed arachidonate was mapped using chloroform:methanol extraction of the radiolabelled eicosanoid products which were monitored via UV absorbance compared to authentic standards. $PGE_2$, $PGF_2\alpha$ and 5-, 12- and 15-HETE were produced. When the effects of these metabolites on protein degradation were investigated, it was found that only 15-HETE was capable of inducing protein degradation. In fact PIF and 15-HETE produced significant increases in degradation and all with the typical bell shaped dose-

response curve thought to represent receptor down-regulation (Smith et al 1999).

This study was significant because it demonstrated a mechanism that explained why $PGE_2$ might be elevated, but was not a causative factor, and also because it was the initial suggestion that the intracellular signal for protein degradation induced by PIF might be 15-HETE.

Whilst HETEs have long been indirectly implicated in the induction of cachexia (in that some of the observed biological actions of n-6 fatty acids in cachexia may be mediated through HETEs) there is a paucity of research which directly addresses their involvement. Falconer et al (1994) have shown that 5-HETE at μM concentrations, potentiated the effects of arachidonate and served as an inhibitor of protein synthesis whilst 5-HETE and LT B4 have been shown to inhibit cell proliferation and DNA synthesis (Reviewed in Ito et al 1993).

Rose et al (1995) have shown that n-6 rich diets stimulate the growth and metastasis of the human breast cancer cell line MDA-MB-435 in athymic nude mice whilst n-3 fatty acids exert a suppressive effect. Diets supplemented with DHA and EPA caused a statistically significant reduction in tumour size, and the occurrence and severity of lung metastases. A significant reduction in the concentrations of arachidonic acid, 12- and 15-HETE and $PGE_2$ was observed in tumour cell phospholipids suggesting that the mechanism involved suppression of tumour eicosanoid biosynthesis, probably through competition for Δ-6 desaturases for the conversion of LA to AA.

Rose et al (1995b) showed that 12- and 15-HETE were elevated in MBA-MD-231 tumours which had an increased metastatic potential, although they could not distinguish between the effects of the two. They also showed that elevated levels of these eicosanoids were present in tumours from animals

fed a high n-6 diet, and that these levels could be reduced by feeding a high n-3 diet.

Liu et al (1994) have shown that exogenous treatment of B16 amelanotic melanoma cells with 12-HETE increases their metastatic potential and that endogenous 12-HETE levels could be correlated to metastatic potential in vivo. This could also be correlated to a translocation of protein kinase C from the cytosol to the membrane and to increases in the expression of integrin αIIbβ3, which has been shown to enhance tumour cell adhesion, tumour cell lung colony formation and to facilitate tumour cell spreading on matrix proteins.

Exogenous 12-HETE mediated activation of protein kinase C has been shown to increase cell surface expression of integrins, enhance adhesion and increase experimental metastasis in tumour cells. Chen et al (1994) demonstrated that endogenous 12-HETE is preferentially produced by some human, rat and mouse melanoma cell lines and plays a role in tumour cell adhesion to matrix in vitro and lung colonisation in vivo.

Further evidence for 12-HETE in metastasis was provided by Honn et al (1994b) who showed that it was capable of inducing endothelial cell retraction in a manner similar to that induced by co-cultured highly colonising B16a cells but not by healthy cells. 12-HETE is also capable of inducing the phosphorylation and upregulating the expression of gp78, a cell surface protein involved in the binding of motility factors (Timar et al 1993).

An interesting study by Honn et al (1994a) showed that 12-HETE increased cathepsin B activity in a melanoma cell line with high colonising potential (B16a), but not in a model with low colonising potential (B16 F1). It is thought that cathepsin B is important in focal degradation of basement membranes during tumour cell invasion. Furthermore 12-HETE upregulated the surface expression of proteins able to mediate invasion, adhesion, degradation and migration.

Hussey and Tisdale (1996b) showed that specific lipoxygenase inhibitors (including BWA4C and BWB70C), which inhibited growth of MAC series tumours both in vivo and vitro causing a decrease in production of 5- and 12-HETE. Similarly it has been shown that CV-6504 when added to sensitive (MAC13, MAC16, MAC26 and CaCo2) but not resistant (A549 and DU-145) cell lines inhibits the production of HETEs from arachidonic acid. As some CV6504 sensitive cell line (MDA-MB-231 and PC-3) are known to require linoleic acid for growth these results further suggested that some tumours are indeed dependent upon arachidonic acid metabolites (i.e. HETEs) for growth and that interference with the production of such metabolites might produce a specific tumour growth inhibition. (Hussey and Tisdale 1997).

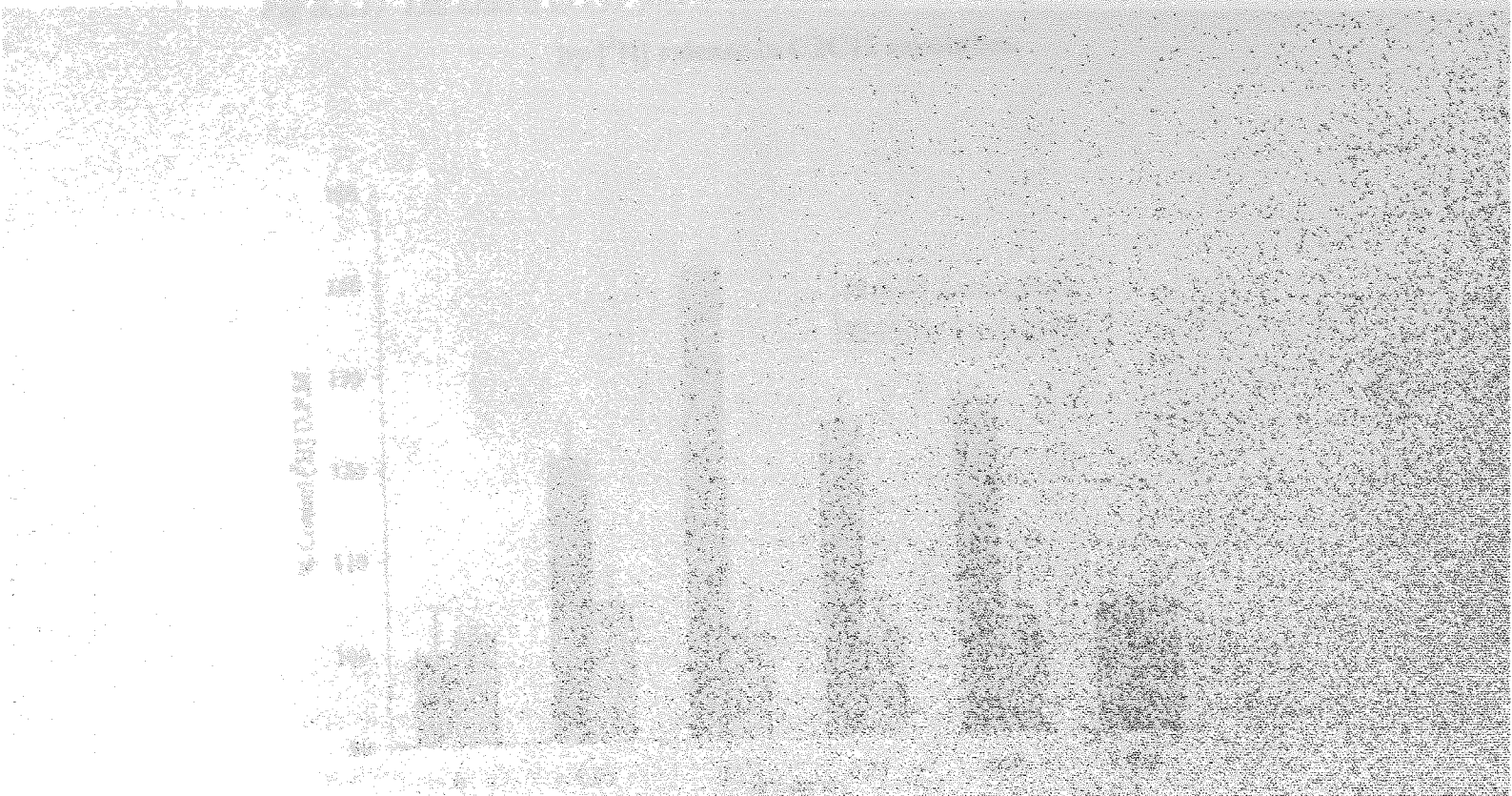
If 15-HETE is indeed the intracellular mediator of proteasome induced protein catabolism in this model, interference with its generation from precursors would predictably result in an attenuation of proteasome upregulation and of proteolysis.

On the other hand 15-HETE may well be important in the structural lipids of the tumour also, or in creating the correct eicosanoid environment, and EPA might interfere with 15-HETE generation, not directly, but by inhibiting lipolysis, thus preventing mobilisation of those fatty acids which the tumour is unable to synthesise and are required from the host. Alternatively 15-HETE or its metabolites may be preferentially mobilised and incorporated into the tumour and EPA might function to prevent this.

It is feasible that 15-HETE, could exert a cachectic effect through incorporation into the membrane phospholipids of muscle cells, where, due to its easily peroxidizable nature, it could result in the generation of toxic oxygen radicals causing cell death in the muscle, or in altering the oxidative environment to affect redox sensitive genes. For example Reinheckel et al (2000) showed that the ATP stimulated degradation of the fluorogenic proteasome substrate suc –LLVY-MCA by the 26S proteasome was

abolished by 1mM $H_2O_2$ in K562 human haematopoietic cells, demonstrating high susceptibility to oxidative stress.

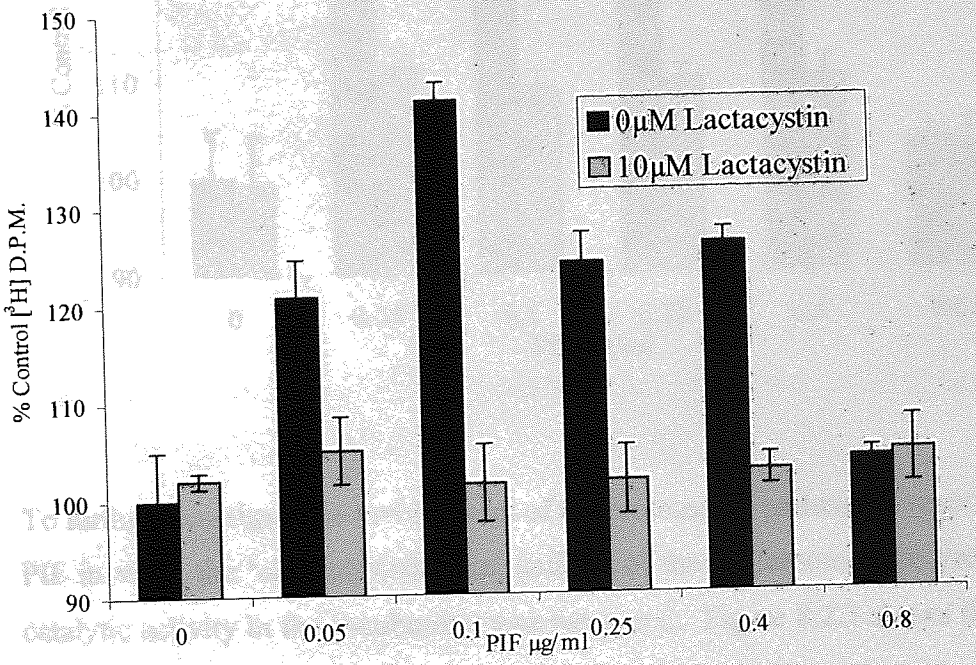
Another possible explanation is that EPA might affect signal transduction pathways which are activated by 15-HETE, and which in turn serve to increase proteasome function and expression. This could be accomplished through an effect upon translation or transcription. That fatty acids can modulate translational events has already been demonstrated. It is plausible that 15-HETE could upregulate translational events in the muscle resulting in a rapid elevation of proteasome expression and that EPA exerts a competitive effect upon this translational modification. Rotman et al (1992) for example showed that various fatty acids could inhibit protein synthesis in a translation dependent event, and in a large number of cell lines demonstrating the potential feasibility of the effect of an eicosanoid upon translational processes.



## 8.2 Results and Discussion

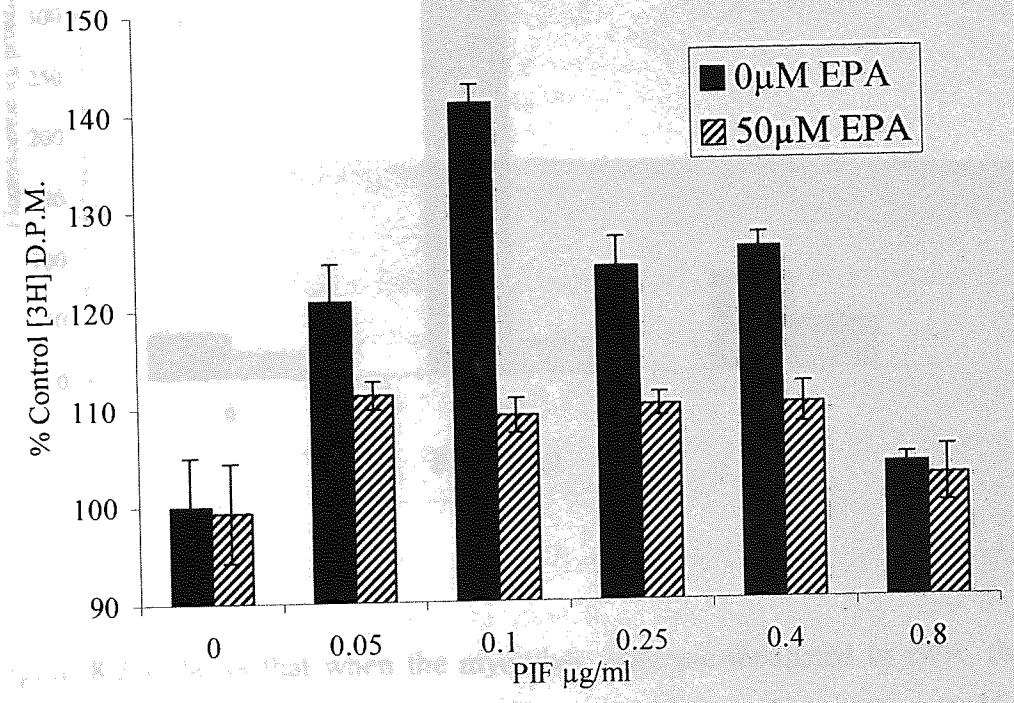
It has been previously demonstrated that the proteasome proteolytic pathway is responsible for the bulk of skeletal muscle degradation seen in the MAC16 model. To determine the pathway's involvement in response to PIF in vitro, C2C12 myotubes where pre-treated with PIF and release of tritiated phenylalanine was measured as a marker of protein degradation. A specific proteasome inhibitor – lactacystin, was included to differentiate any effects arising from non-proteasomal sources. Figure 8.2.1 shows that PIF concentrations ranging from 0.05μg/ml to 0.4μg/ml stimulated protein degradation with a maximal peak of stimulatory activity (approx. 41% untreated control) at 0.1μg/ml PIF ($p<0.01$ as determined by one way ANOVA with Tukey's post test). The lack of stimulation of activity at higher concentrations is typical of a receptor mediated response and probably reflects receptor desensitisation at higher concentrations

**Fig 8.2.1) The effects of PIF and lactacystin on protein degradation as measured by [$^3$H] release in C2C12 myotubes.**



When 10μM lactacystin was included, the increased degradation seen in the presence of PIF, was reduced to control levels indicating that the elevated catabolism was due to increased proteasome activity. Fig 8.2.2 shows that the addition of EPA also resulted in an attenuation of protein degradation at all concentrations of PIF. This was most noticeable at the maximal stimulatory concentration of PIF (0.1μg/ml), when the 41% increase in degradation was reduced by 32% ($p<0.01$).

**Fig 8.2.2) The effects of PIF and EPA on protein degradation as measured by [$^3$H] release in C2C12 myotubes in the presence and absence of EPA**




To further investigate the involvement of the proteasome pathway in response to PIF in vitro, the 'chymotrypsin-like' activity of the proteasome (the dominant catalytic activity in the β-subunits) was measured. Figure 8.2.3 shows that the

activity of the proteasome is elevated by more than 10fold in the presence of 0.1μg/ml PIF (previously determined active concentration) where $p<0.001$. In order to determine the specificity of the response, lactacystin was also included. This completely abolished the rise in proteasome activity clearly demonstrating that the proteasome proteolytic pathway is elevated in response to PIF.

**Fig 8.2.3) The effects of PIF upon the proteasome pathway.**

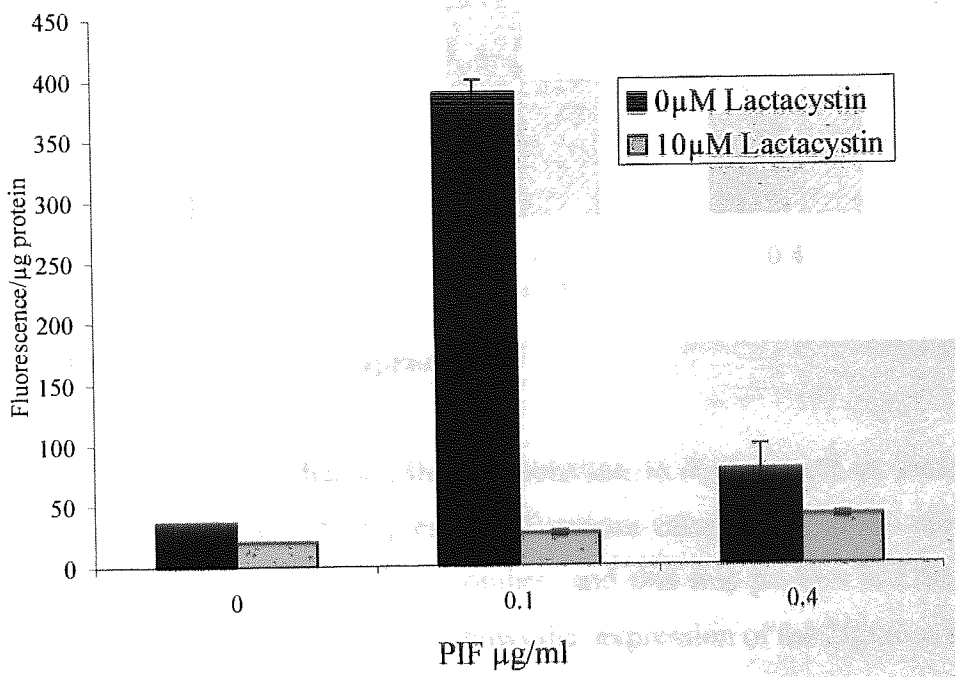
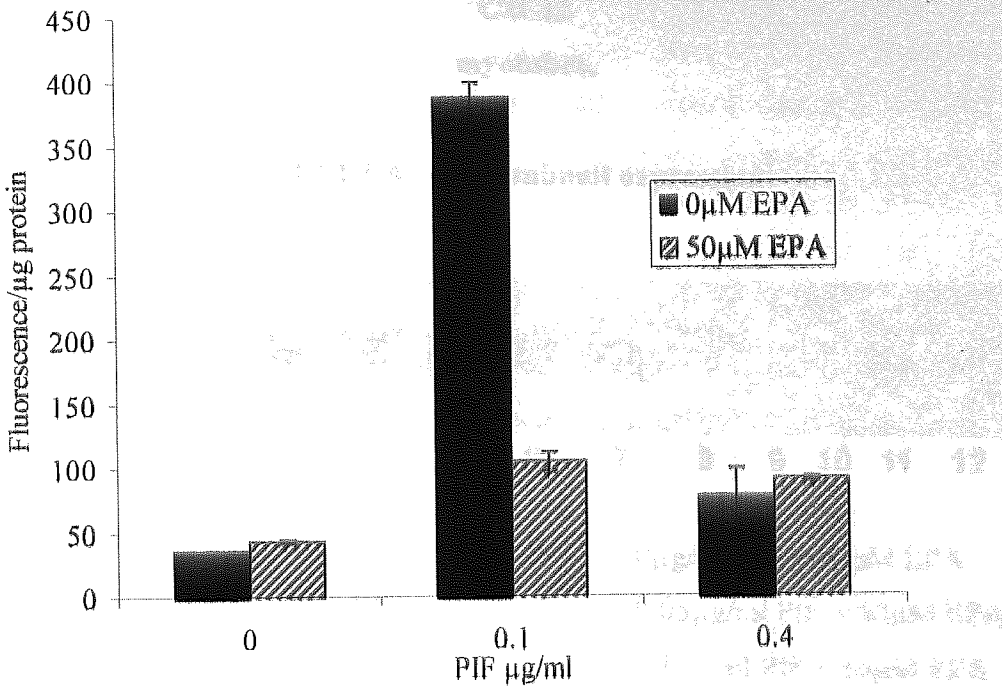



Figure 8.2.4 shows that when the myotubes were pre-incubated in EPA, the elevation in proteasome activity of 1079% untreated control, was attenuated by 787% to 292% control ($p<0.001$)

**Fig 8.2.4) The effects of EPA and PIF upon the proteasome pathway.**

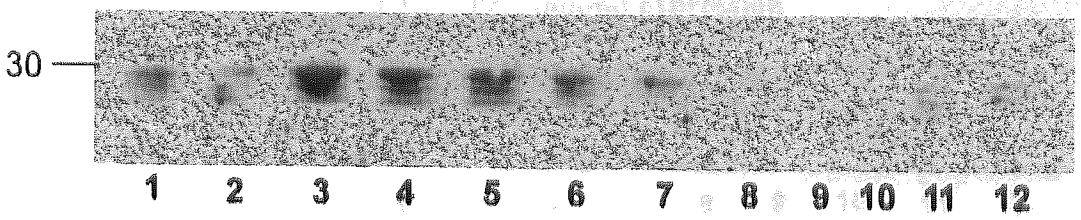



### 8.2.1 Proteasome subunit expression

Figure 8.2.1.1 shows that a 24hour incubation in PIF as well as increasing activity, also increases the expression of various components of the ubiquitin proteasome pathway in C2C12 myotubes, and that this process is effectively inhibited by EPA. Figure 8.2.1.1a shows that expression of the 20S α-subunits, detected by Western blotting with an MCP-231 antisera, increases over the concentration range of 0.1-1µg/ml PIF, with a maximum stimulation of 25% at 0.1µg/ml PIF. This increased expression is attenuated by 46% when the myotubes were pre-incubated for 2hours in 50µM EPA. (The concentration of EPA was decided by preliminary experiments which demonstrated the greatest effect upon proteasome activity at 50µM – data not shown).

**Figs 8.2.1.1 A-C) Western and densitometric analyses demonstrating the effects of PIF and EPA on expression of proteasome subunits and $E2_{14k}$ in C2C12 myotubes.**

**8.2.1.1 A– 20Sα subunit expression**




| | | | |
|---|---|---|---|
| 1 | 0μg/ml PIF + 0μM EPA | 7 | 0μg/ml PIF + 50μM EPA |
| 2 | 0.05μg/ml PIF + 0μM EPA | 8 | 0.05μg/ml PIF + 50μM EPA |
| 3 | 0.1μg/ml PIF + 0μM EPA | 9 | 0.1μg/ml PIF + 50μM EPA |
| 4 | 0.2μg/ml PIF + 0μM EPA | 10 | 0.2μg/ml PIF + 50μM EPA |
| 5 | 0.4μg/ml PIF + 0μM EPA | 11 | 0.4μg/ml PIF + 50μM EPA |
| 6 | 1μg/ml PIF + 0μM EPA | 12 | 1μg/ml PIF + 50μM EPA |

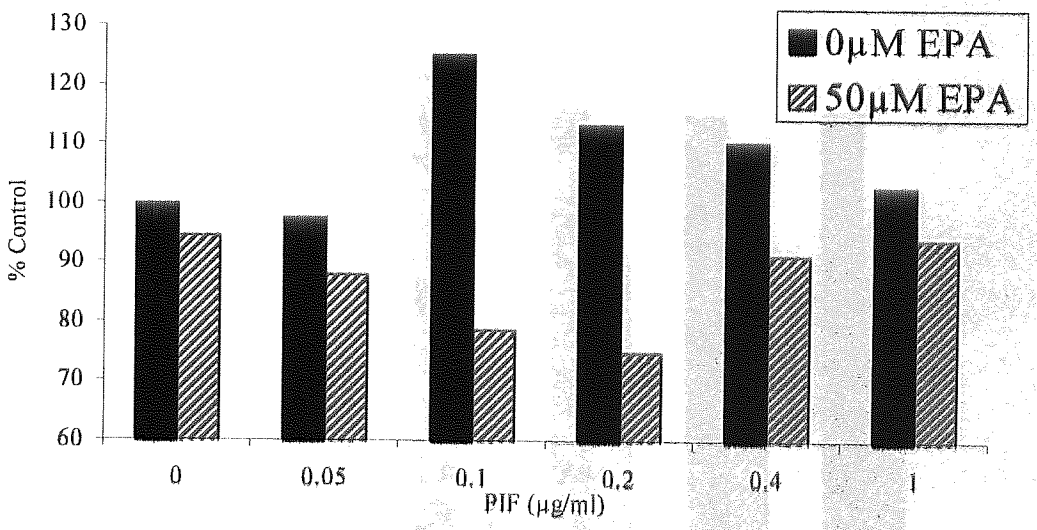
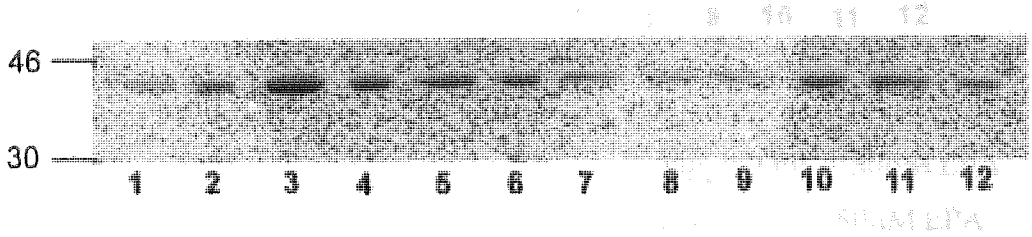


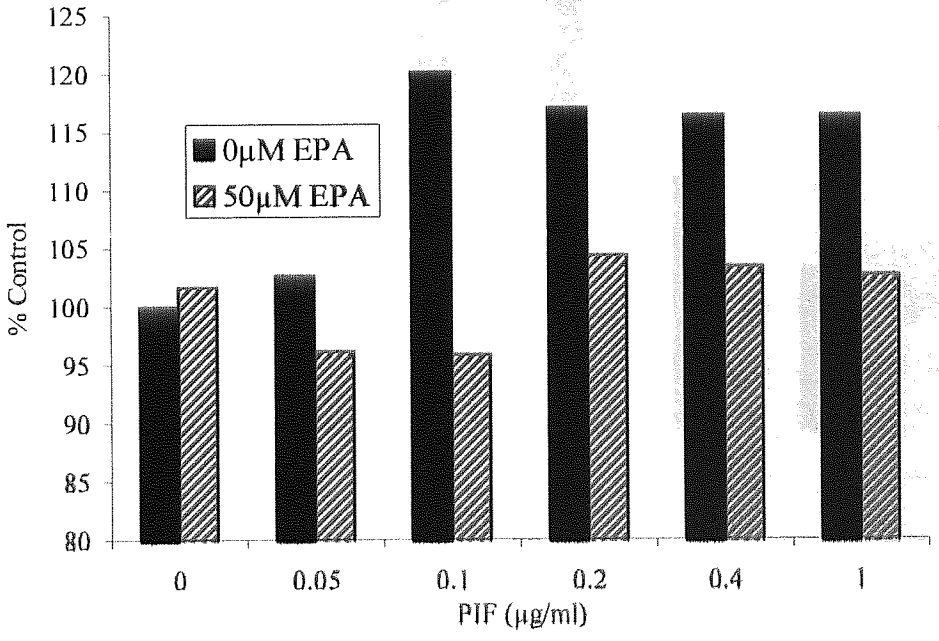

Figure 8.2.1.1b shows a similar effect for the P42 proteasome subunit. This is an ATPase dependent subunit of the 19S regulator that promotes ATP dependent association of the 20S and 19S complexes to form the 26S proteasome

(Tanahashi et al 1999). P42 expression in increased by 20%, 17%, 16% and 16% by 0.1, 0.2, 0.4 and 1μg/ml PIF respectively. When the myotubes were preincubated in EPA, the levels of P42 detected were similar to controls. As with expression of 20S, the maximal stimulatory effect of PIF was observed at 0.1μg/ml (20% above controls). The maximal inhibition was also observed at this concentration, when EPA attenuated P42, such that expression was actually below that of controls.

**8.2.1.1 B –P42 subunit expression**

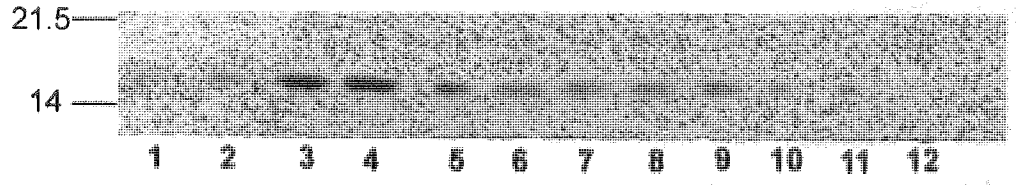



| | | | |
|---|---|---|---|
| 1 | 0μg/ml PIF + 0μM EPA | 7 | 0μg/ml PIF + 50μM EPA |
| 2 | 0.05μg/ml PIF + 0μM EPA | 8 | 0.05μg/ml PIF + 50μM EPA |
| 3 | 0.1μg/ml PIF + 0μM EPA | 9 | 0.1μg/ml PIF + 50μM EPA |
| 4 | 0.2μg/ml PIF + 0μM EPA | 10 | 0.2μg/ml PIF + 50μM EPA |
| 5 | 0.4μg/ml PIF + 0μM EPA | 11 | 0.4μg/ml PIF + 50μM EPA |
| 6 | 1μg/ml PIF + 0μM EPA | 12 | 1μg/ml PIF + 50μM EPA |

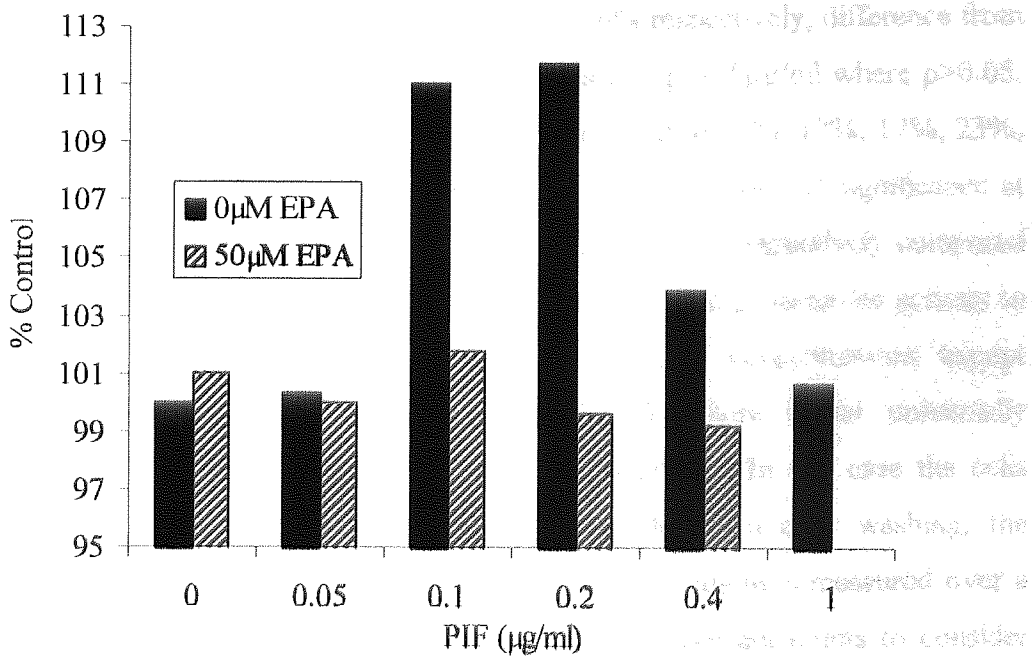


PIF also increased the expression of the ubiquitin conjugating enzyme $E2_{14k}$ (figure 8.2.1.1c) by 11% at 0.1μg/ml and this was prevented by pre-incubation in EPA, when $E2_{14k}$ expression did not rise above control levels.

**8.2.1.1 C– $E2_{14k}$ expression**




| | | | |
|---|---|---|---|
| 1 | 0μg/ml PIF + 0μM EPA | 7 | 0μg/ml PIF + 50μM EPA |
| 2 | 0.05μg/ml PIF + 0μM EPA | 8 | 0.05μg/ml PIF + 50μM EPA |
| 3 | 0.1μg/ml PIF + 0μM EPA | 9 | 0.1μg/ml PIF + 50μM EPA |
| 4 | 0.2μg/ml PIF + 0μM EPA | 10 | 0.2μg/ml PIF + 50μM EPA |
| 5 | 0.4μg/ml PIF + 0μM EPA | 11 | 0.4μg/ml PIF + 50μM EPA |
| 6 | 1μg/ml PIF + 0μM EPA | 12 | 1μg/ml PIF + 50μM EPA |



It has been previously demonstrated that PIF causes a release of cell-bound arachidonate and that of all the eicosanoid products only 15-HETE was capable of inducing protein degradation (Smith et al 1999). The observation that both PIF and 15-HETE produced changes in protein degradation with similar dynamics suggested that 15-HETE may be the intracellular mediator for PIF.

To determine whether 15-HETE was capable of inducing proteolysis in mature muscle cells in vitro, protein degradation was measured in C2C12 myotubes pretreated with 15-HETE and EPA. The proteasome inhibitor lactacystin was included to ensure the specificity of the response. Figure 8.2.1.2 demonstrates that 15-HETE can directly induce protein degradation in isolated skeletal muscle myotubes with kinetics similar to those seen in its effects upon the proteasome, and that this process can be attenuated by EPA (8.2.1.2A) and by lactacystin (figure 8.2.1.2B). [$^3$H]-phenylalanine release (a marker of protein degradation) was elevated in C2C12 myotubes treated with 0.005μg/ml, 0.01μg/ml, 0.05μg/ml, 0.1μg/ml, 0.25μg/ml 15-HETE by 37%, 41%, 50%, 43%, 36% ($p<0.001$) and 16% respectively, difference from control where $p<0.001$ for all concentrations except 0.5μg/ml where $p>0.05$. In the presence of EPA these figures were reduced to 21%, 17%, 17%, 23%, 21% and 20% of untreated controls. This reached statistical significance at 0.01μg/ml and 0.05μg/ml where $p<0.05$ and $p<0.01$, respectively compared to 15-HETE treated alone. 10μM lactacystin inhibited proteasome activity to below control levels ($p<0.001$ for all 15-HETE concentrations except untreated control which was not significant). There is no universally accepted method for measuring protein degradation. In this case the cells have been labelled with an amino acid-[$^3$H]-Phe, then after washing, the release of this isotope from cell protein into the medium is measured over a successive period. However, there are several important points to consider when interpreting this data. Firstly, it is impossible during the wash stage, to remove those labelled amino acids which are not incorporated into the cells but are in the intracellular free pools and secondly the technique can only measure short lived proteins, whereas long lived proteins like actin and

myosin (which the technique may be representative of) have a longer turnover time.

**Figure 8.2.1.2A) The effects of 15-HETE on protein degradation as measured by [$^3$H]-Phe release in C2C12 myotubes in the presence and absence of EPA**

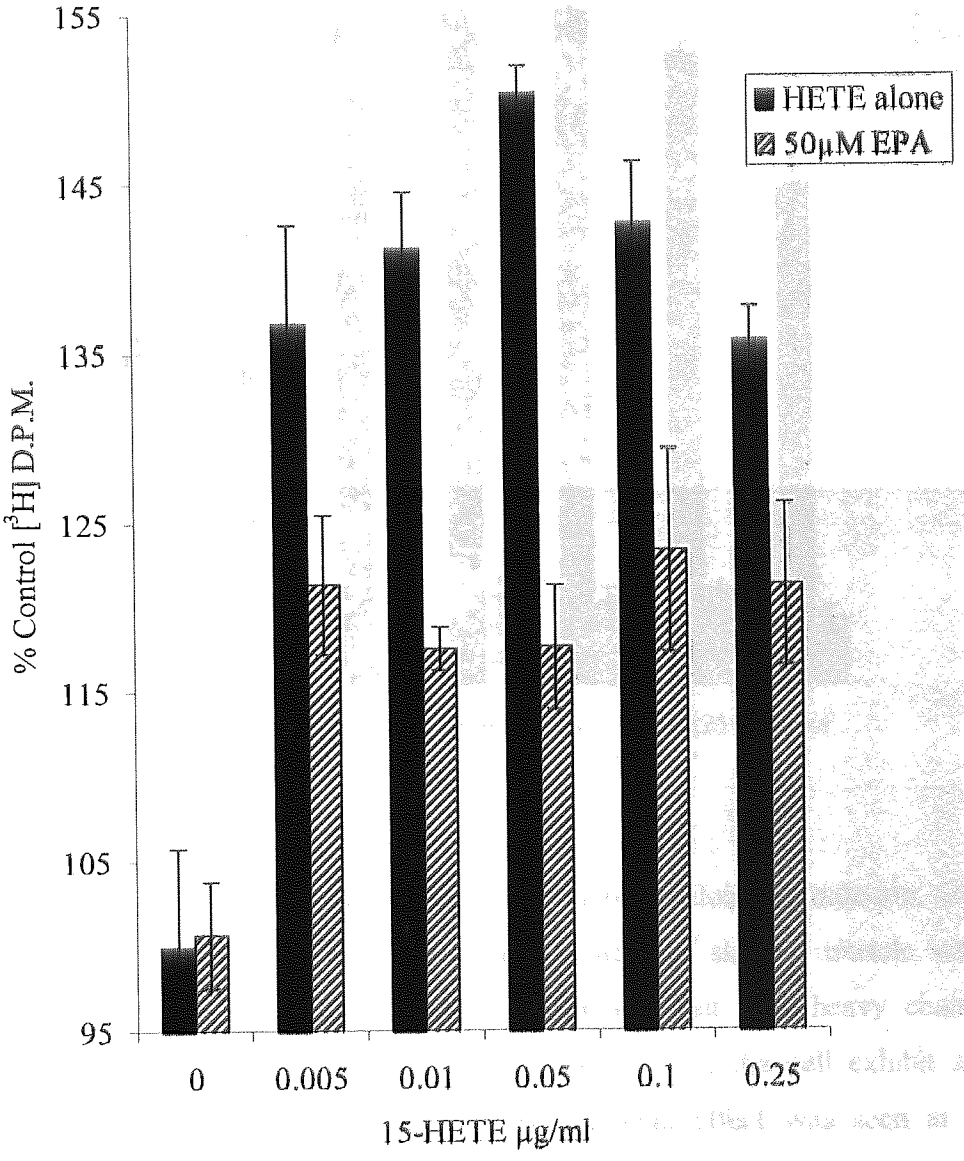
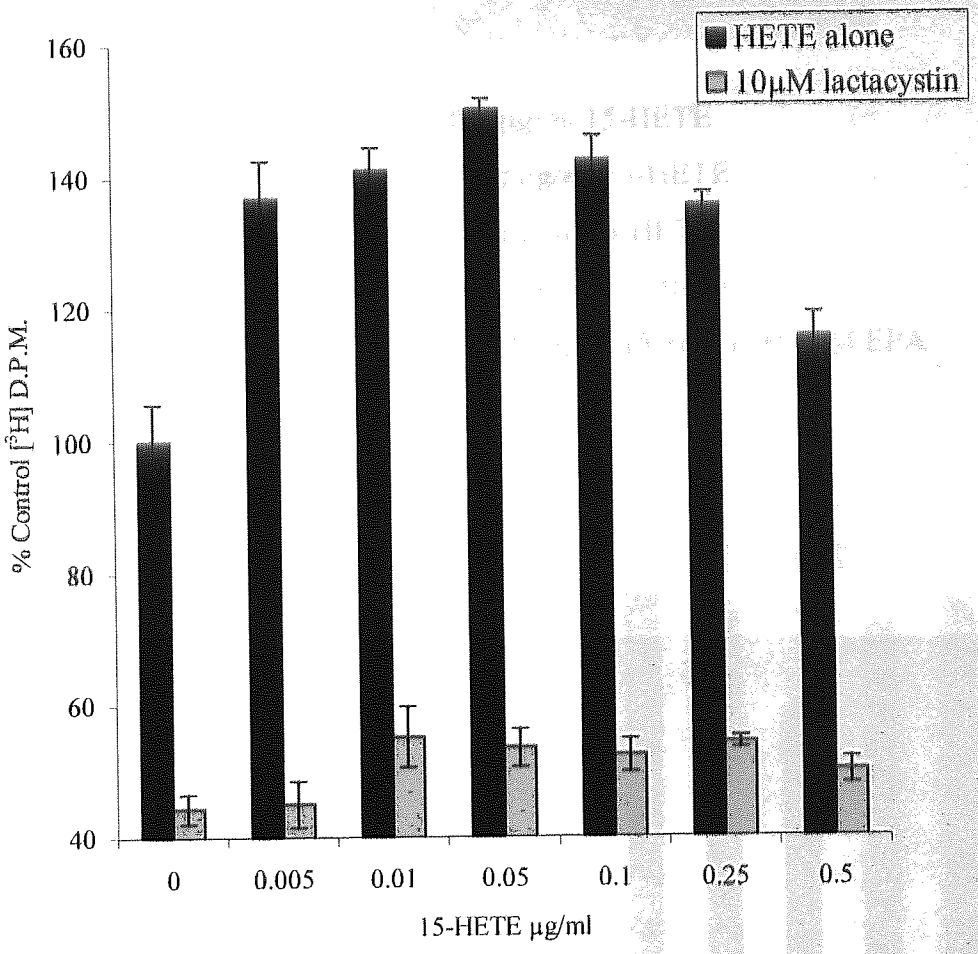
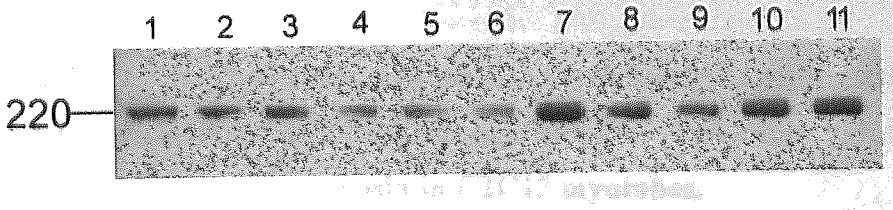


**Figure 8.2.1.2B) The effects of 15-HETE on protein degradation as measured by [$^3$H]-Phe release in C2C12 myotubes in the presence and absence of lactacystin**

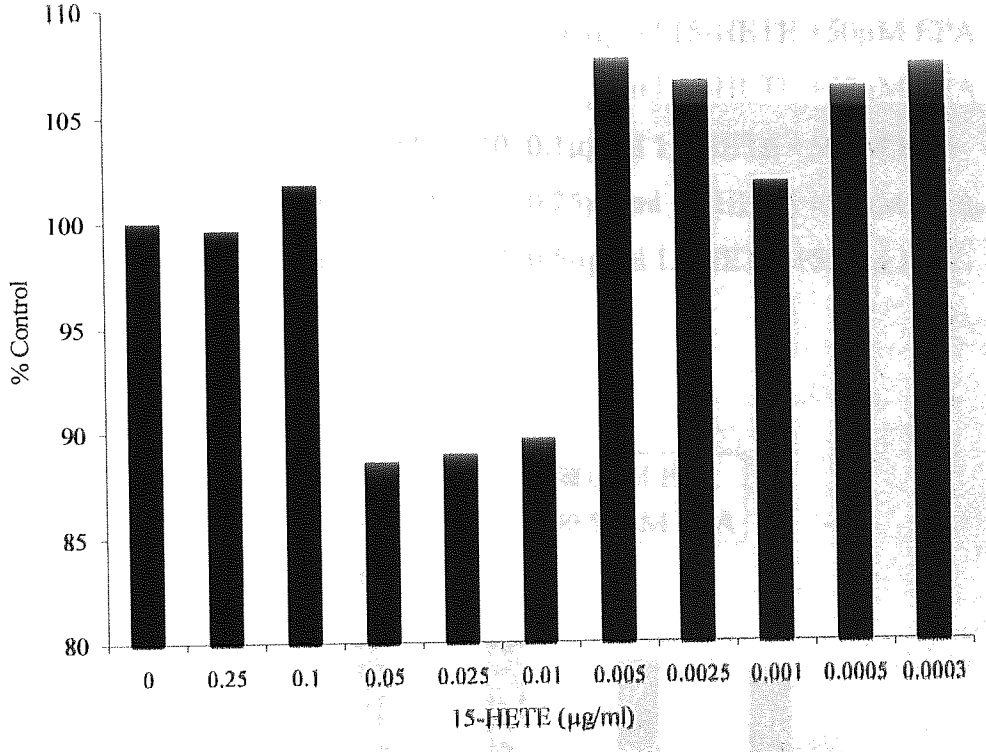



In order to verify the effects of 15-HETE upon protein degradation, the expression of myosin the major structural protein of skeletal muscle was examined. Western blots were probed for myosin fast type heavy chain. Figure 8.2.1.2c (overleaf) shows that myosin levels in the cell exhibit an inverse bell shaped response curve. The maximal effect was seen at a concentration of 0.01-0.05μg/ml 15-HETE of 10-11% control and this correlated with the effects on proteasome activity (discussed in chapter 10).

**Fig 8.2.1.2C) Western and densitometric analyses demonstrating the effects of 15-HETE on expression of myosin in C2C12 myotubes.**




1. 0μg/ml 15-HETE
2. 0.25μg/ml 15-HETE
3 0.1μg/ml 15-HETE
4 0.05μg/ml 15-HETE
5 0.025μg/ml 15-HETE
6 0.01μg/ml 15-HETE
7 0.005μg/ml 15-HETE
8 0.0025μg/ml 15-HETE
9 0.001μg/ml 15-HETE
10 0.0005μg/ml 15-HETE
11 0.00025μg/ml 15-HETE +50μM EPA

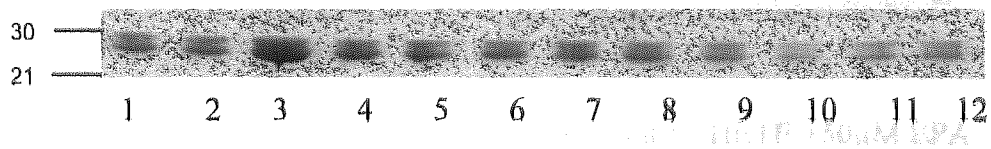



To test whether 15-HETE might affect the proteasome, parallel experiments were performed in which 15-HETE was added to C2C12 myotubes in the presence and absence of 50μM EPA and protein degradation and proteasome activity (see chapter 10) and expression were measured. Figure 8.2.1.3a shows that the expression of 20S α–subunits are stimulated by 15-HETE to a

maximum of 14% above untreated control at 0.05μg/ml, a process which was effectively inhibited by EPA, where expression of subunits fell to below controls

**Figs 8.2.1.3 A-C) Western and densitometric analyses demonstrating the effects of 15-HETE and EPA on expression of proteasome subunits and myosin in C2C12 myotubes.**

**8.2.1.3 A – 20Sα subunit expression**




1 0μg/ml 15-HETE + 0μM EPA
2. 0.01μg/ml 15-HETE + 0μM EPA
3 0.05μg/ml 15-HETE + 0μM EPA
4 0.1μg/ml 15-HETE + 0μM EPA
5 0.25μg/ml 15-HETE + 0μM EPA
6 0.5μg/ml 15-HETE + 0μM EPA
7 0μg/ml 15-HETE +50μM EPA
8 0.01μg/ml 15-HETE +50μM EPA
9 0.05μg/ml 15-HETE +50μM EPA
10 0.1μg/ml 15-HETE +50μM EPA
11 0.25μg/ml 15-HETE +50μM EPA
12 0.5μg/ml 15-HETE +50μM EPA

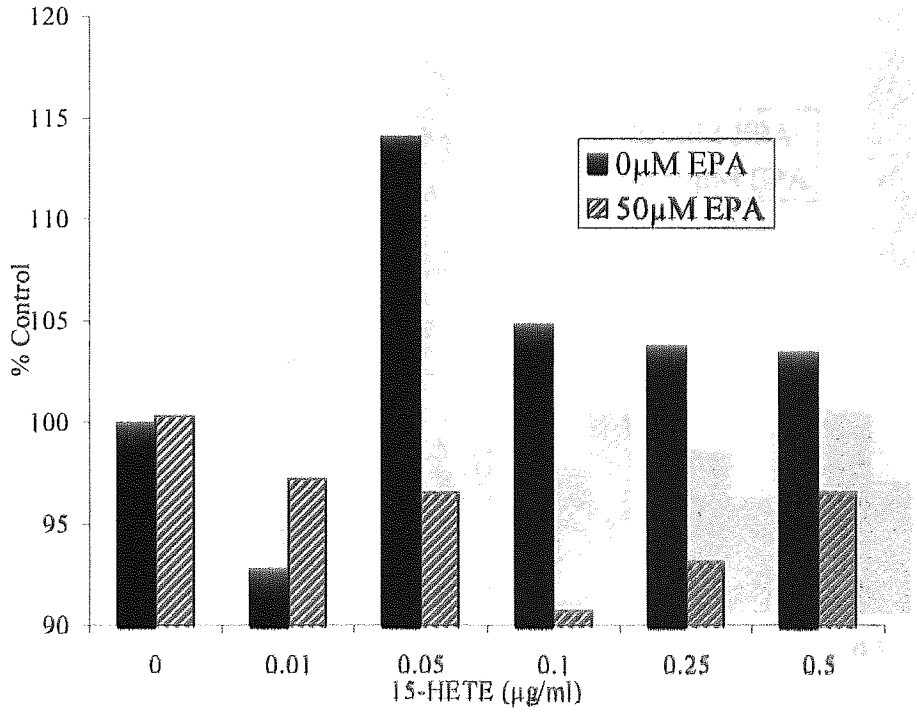
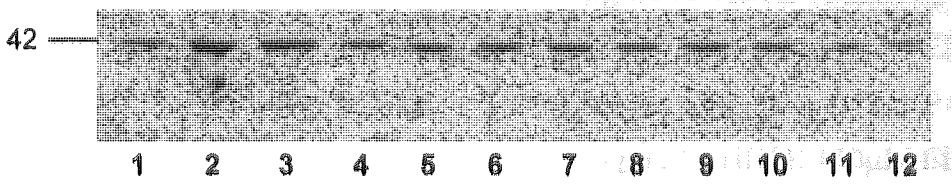
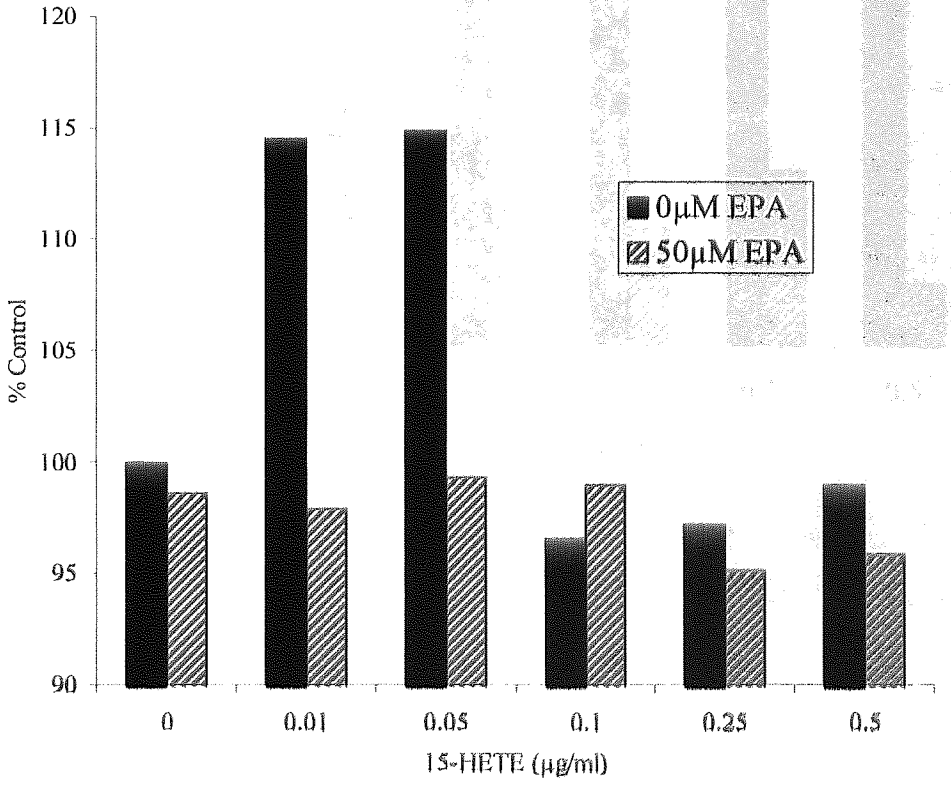


Figure 8.2.1.3b shows a similar pattern of results for MSS1, where levels of this subunit were increased by 15% at the maximal stimulatory concentration (0.01-0.05μg/ml) and decreased by 18% in the presence of EPA. The expression of $E2_{14k}$ (fig 8.2.1.3c) was not particularly increased by 15-HETE (approximately 1% at all concentrations) and reduced to approximately 97% untreated control by 50μM EPA.

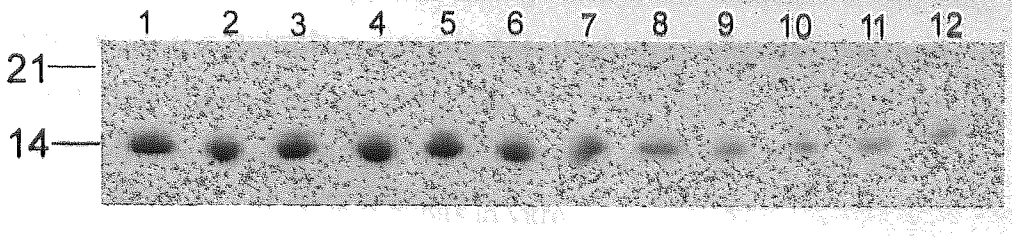
**8.2.1.3 B – MSS1 expression**



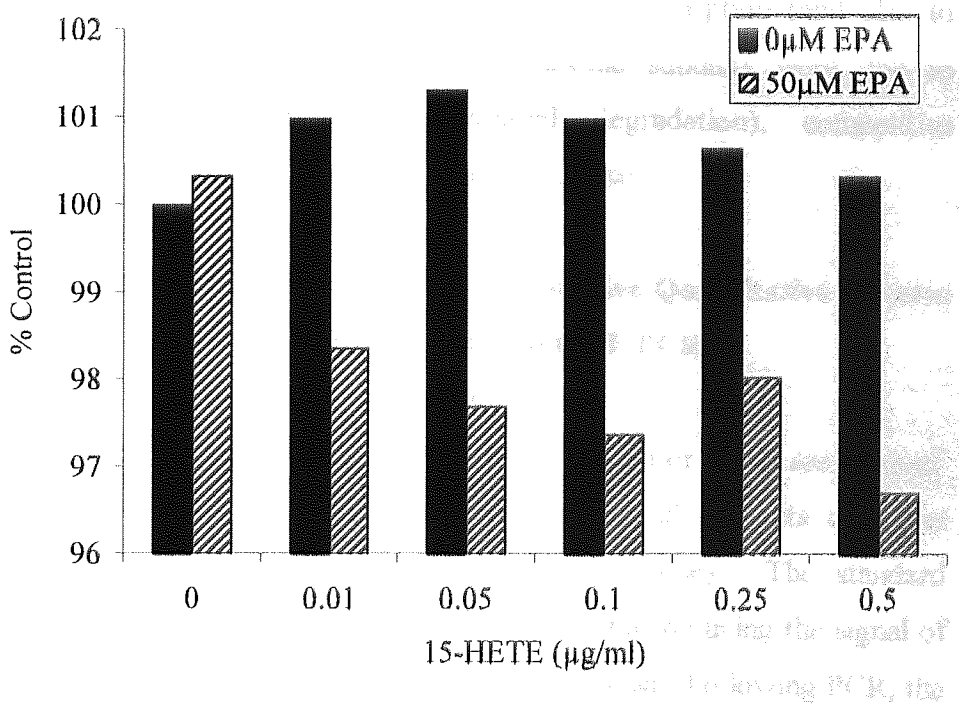

1 0μg/ml 15-HETE + 0μM EPA
2 0.01μg/ml 15-HETE + 0μM EPA
3 0.05μg/ml 15-HETE + 0μM EPA
4 0.1μg/ml 15-HETE + 0μM EPA
5 0.25μg/ml 15-HETE + 0μM EPA
6 0.5μg/ml 15-HETE + 0μM EPA
7 0μg/ml 15-HETE +50μM EPA
8 0.01μg/ml 15-HETE +50μM EPA
9 0.05μg/ml 15-HETE +50μM EPA
10 0.1μg/ml 15-HETE +50μM EPA
11 0.25μg/ml 15-HETE +50μM EPA
12 0.5μg/ml 15-HETE +50μM EPA



### 8.2.1.3 C – $E2_{14k}$ expression




1 0μg/ml 15-HETE + 0μM EPA
2 0.01μg/ml 15-HETE + 0μM EPA
3 0.05μg/ml 15-HETE + 0μM EPA
4 0.1μg/ml 15-HETE + 0μM EPA
5 0.25μg/ml 15-HETE + 0μM EPA
6 0.5μg/ml 15-HETE + 0μM EPA
7 0μg/ml 15-HETE +50μM EPA
8 0.01μg/ml 15-HETE +50μM EPA
9 0.05μg/ml 15-HETE +50μM EPA
10 0.1μg/ml 15-HETE +50μM EPA
11 0.25μg/ml 15-HETE +50μM EPA
12 0.5μg/ml 15-HETE +50μM EPA



These results demonstrate that both PIF and 15-HETE can increase expression of proteasome subunits and (possibly) the ubiquitin conjugating enzyme in a concentration dependent bell shaped response and with a maximal stimulatory peak of 0.1μg/ml (PIF) and 0.05μg/ml (15-HETE). What is also evidenced is that 50μM EPA can effectively attenuate the increased expression of these subunits in vitro.

The simplest way to interpret the effect of EPA upon 15-HETE mediated upregulation of the proteasome is that it competes for the cellular machinery responsible for the generation of 15-HETE through arachidonic acid metabolism, and as such is upstream of 15-HETE. However the fact that EPA can attenuate and sometimes completely abolish the effects of 15-HETE upon the proteasome, when 15-HETE is added directly, suggests that EPA is acting through a pathway which is downstream of 15-HETE generation.

To test whether EPA could act at the level of transcription (and also to confirm that the increased levels of proteasome subunits were due to increased production and not decreased degradation), competitive quantitative reverse transcription PCR was performed.

### 8.2.2 Transcriptional Events and The Competitive Quantitative Reverse Transcriptase Polymerase Chain Reaction (cQRT-PCR)

In competitive RT-PCR known quantities of competitor DNA are 'spiked' into a series of PCR reaction tubes containing equal amounts of target cDNA. (and therefore equal amounts of target gene). The standard competes with the native for primers and enzyme, thus reducing the signal of the native when the standard is in excess and vice versa. Following PCR, the amount of products generated by the control and target are compared. The amounts of competitor DNA yielding equal amounts of products gives the initial amount of the target gene.

The initial step in RT-PCR is the production of a single-strand complementary DNA copy (cDNA) of the RNA through the action of the

retroviral enzyme, reverse transcriptase. An oligonucleotide primer is required to initiate DNA synthesis. The primer anneals the RNA and the cDNA is extended toward the 5' end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase. The RT step is the source of most of the variability in quantitative RT-PCR.

Following PCR, the final step in QRT-PCR is detection and quantification of amplification products. The two broad classes of detection techniques are 'end-point' and 'real-time' measurements. Although real time methods offer the potential for improved quantification, the errors in sample manipulation are minimized with end-point quantification. This latter method was adopted for all investigations here and bands were visualised using the fluorescent intercalating dye –ethidium bromide.

QRT-PCR is sensitive, however due to the exponential nature of amplification, small errors can become magnified. The QRT-PCR protocol employed here makes use of stringent controls, which ensure the reliability of the assay. Whilst DNA standards have been used successfully, they are not the optimal choice, because they do not compensate for the inherent variability of the RT step, therefore homologous RNA standards were used. These can be defined as an in-vitro transcribed RNAs that share the same primer binding sites as the native RNA except for a small insertion, deletion or mutation to facilitate differentiation from the native signal during quantification. All primers were designed, and competitor cDNAs cloned by Miss J Khal of this laboratory. The competitor cDNAs were 76bp deletion mutants. This small alteration helps to ensure that the amplification characteristics of both template and competitor RNA were similar. This is important because a 5% difference in amplification efficiency between two initially equal targets can result in one product appearing to be twice the amount of the other after 26 cycles. Co-amplified standards have been used throughout the RT-PCR investigations as these control this potential variability. The use of internal controls that contain the same primer template sequences as the target makes it possible to determine the absolute amount of target cDNA by allowing known amounts of competitor DNAs to

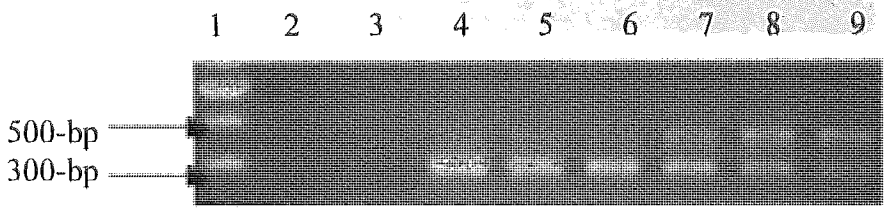
compete with the target for primer binding during the amplification. Furthermore, a control RT-PCR in which reverse transcriptase was omitted and a control PCR in which reverse transcribed RNA was omitted, was included in all experiments. The cQRT-PCR used here employs wild type and deletion mutant competitive primers for the C2 gene. This is the 263 residue α-6 subunit of the 20S proteasome (variously known as nu, Pros 30 and p30k), which studies have shown is elevated in rats bearing a cachexia inducing tumour (Temparis et al 1994), and which is recognised by the 20S MCP231 antisera used here.

Figure 8.2.2.1 shows C2 mRNA levels in C2C12 myotubes treated with 0, 0.01 and 0.05μg/ml 15-HETE, in the presence (figs 8.2.2.1D-F) and absence (figs 8.2.2.1 A-C) of 50μM EPA. Figure 8.2.2.1.A is the untreated control, the upper band represents a 384bp (base pair) target RNA whilst the lower band shows the 76bp deletion (309bp) competitor. Lane 1 represents a DNA 100bp ladder, lane 2 is the product of a PCR negative control and lane 3 represents the RT negative. These controls and the linearity of co-amplified standards ensure that the products represent a genuine change in RNA levels and that no non-specific amplification has occurred. The controls although only shown in figure 8.2.2.1A were included in every PCR reaction.

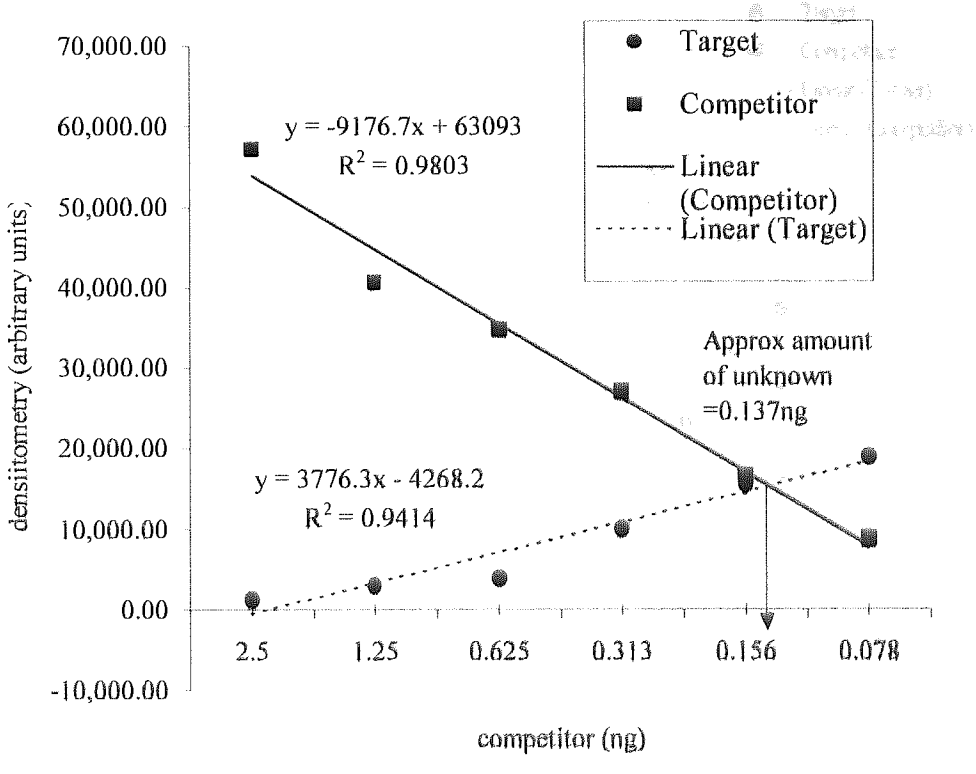
RNA is quantified by plotting the optical density of standard signal and unknown signal versus cRNA concentration (in nanograms). The intercept can then be used to quantify the unknown RNA. Figs 8.2.2.1A-F show that RNA in untreated controls is approximately 0.14ng (fig 8.2.2.1A), this does not change after the addition of 0.01μg/ml 15-HETE (fig 8.2.2.1B), however in the presence of 0.05μg/ml 15-HETE C2 RNA increased to 0.23ng (fig 8.2.2.1C) equalling 164% control. However target RNA did not differ from controls when EPA was added prior to 0.05μg/ml 15-HETE (fig 8.2.2.1F), this is represented diagrammatically in figure 8.2.2.2 (pp159) and demonstrates that 15-HETE can increase, and EPA inhibit, proteasome subunit expression at the level of transcription.

**Fig 8.2.2.1 A-F) Competitive Quantitative Reverse Transcription Polymerase Chain Reaction (cQRT-PCR) quantification of C2 proteasome subunit mRNA levels in 15-HETE treated C2C12 myotubes in the presence and absence of 50μM EPA**

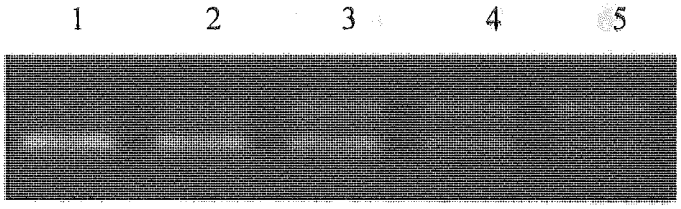
**8.2.2.1A) 0μg/ml 15-HETE + 0μM EPA**



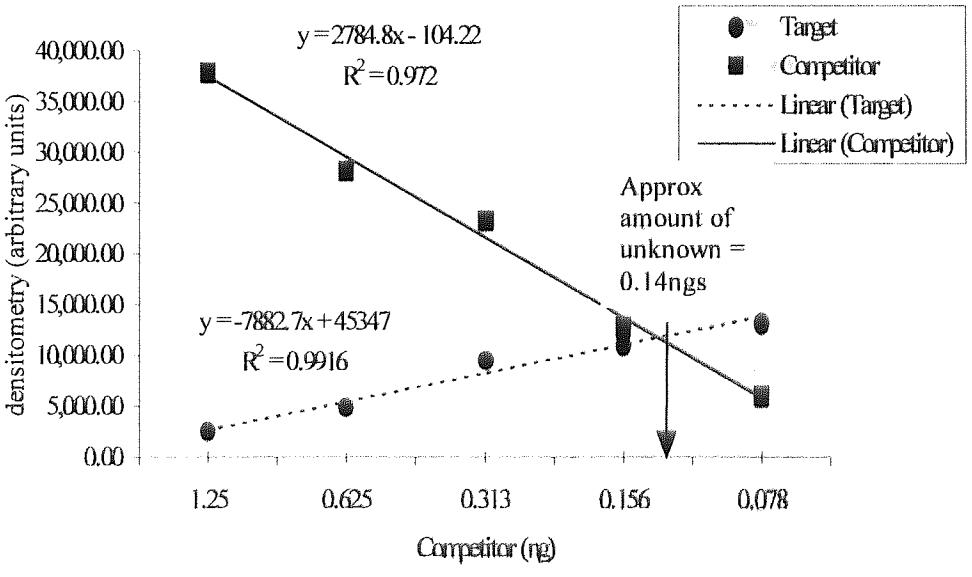

| | | | |
|---|---|---|---|
| 1 | 100base pair DNA ladder | 6 | 0.625ng cRNA |
| 2 | PCR Negative Control | 7 | 0.3125ng cRNA |
| 3 | RT Negative Control | 8 | 0.156ng cRNA |
| 4 | 2.5ng competitor RNA(cRNA) | 9 | 0.078ng cRNA |
| 5 | 1.25ng cRNA | | |



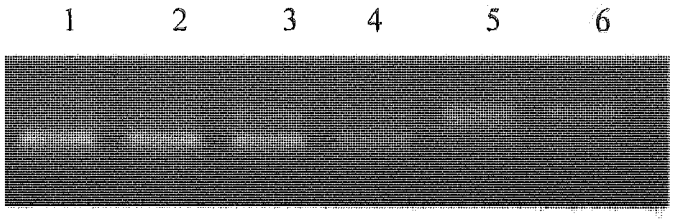
### 8.2.2.1B) 0.01µg/ml 15-HETE + 0µM EPA



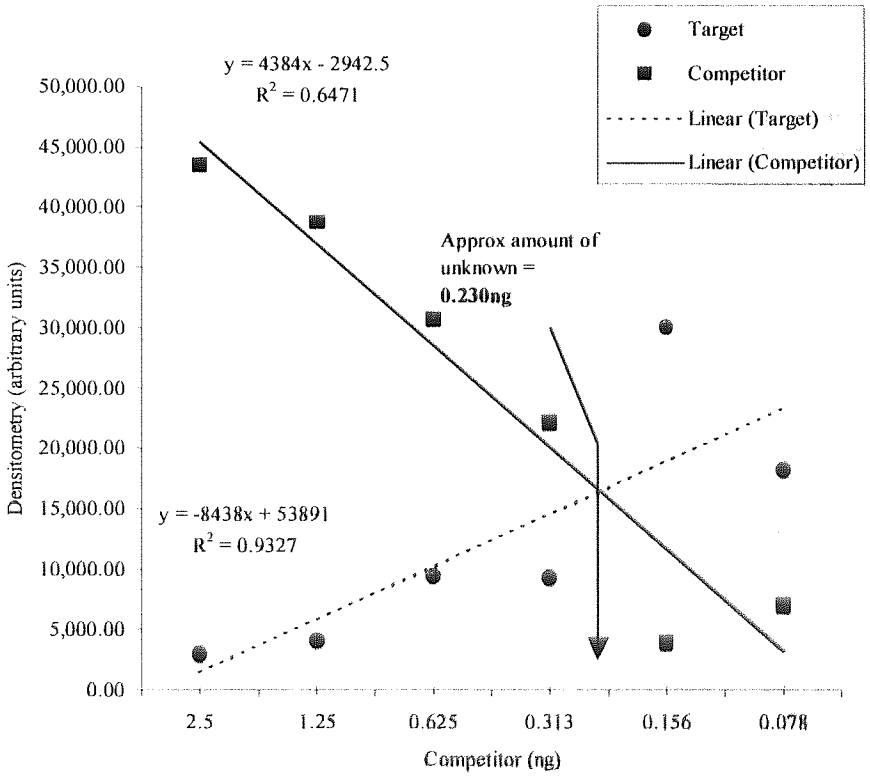

| | | | |
|---|---|---|---|
| 1 | 1.25ng cRNA | 4 | 0.156ng cRNA |
| 2 | 0.625ng cRNA | 5 | 0.078ng cRNA |
| 3 | 0.313ng cRNA | | |



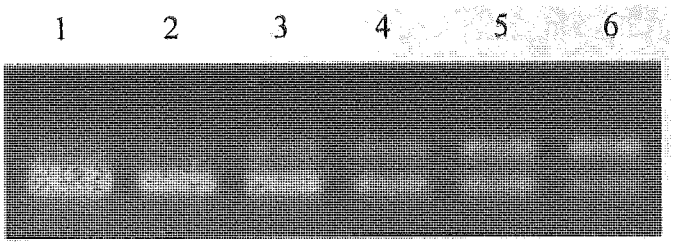
### 8.2.2.1 C) 0.05µg/ml 15-HETE + 0µM EPA



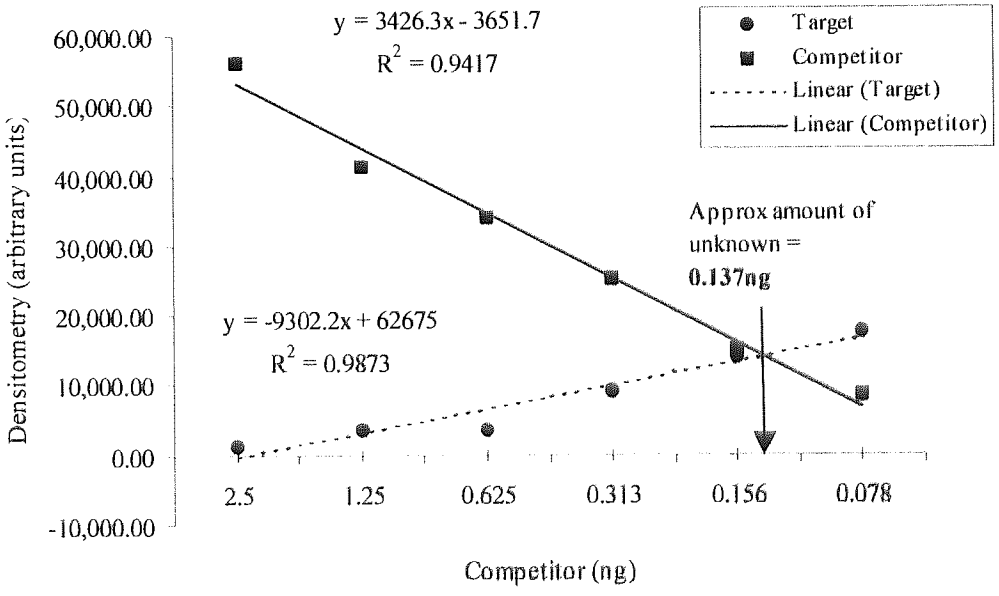

| | | | |
|---|---|---|---|
| 1 | 2.5ng cRNA | 4 | 0.313ng cRNA |
| 2 | 1.25ng cRNA | 5 | 0.156ng cRNA |
| 3 | 0.625ng cRNA | 6 | 0.078ng cRNA |



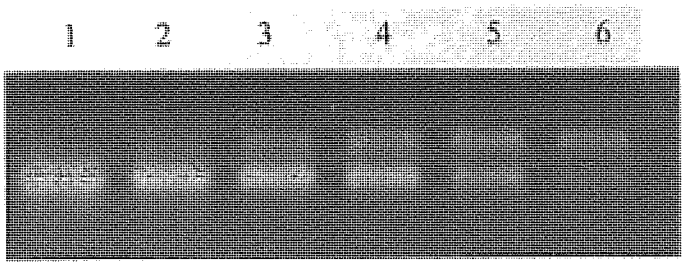
### 8.2.2.1D) 0µg/ml 15-HETE + 50µM EPA



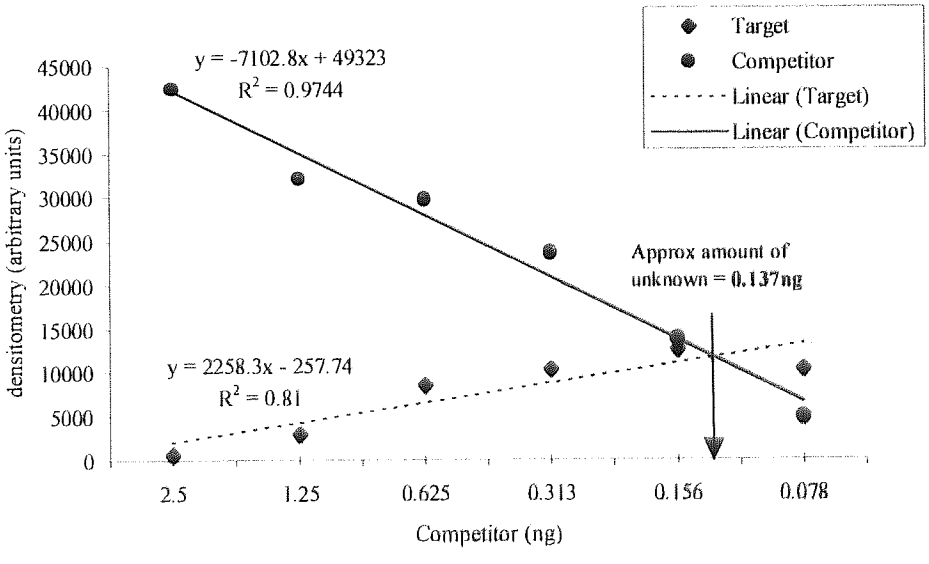

| | | | |
|---|---|---|---|
| 1 | 2.5ng cRNA | 4 | 0.313ng cRNA |
| 2 | 1.25ng cRNA | 5 | 0.156ng cRNA |
| 3 | 0.625ng cRNA | 6 | 0.078ng cRNA |



### 8.2.2.1 E) 0.01µg/ml 15-HETE + 50µM EPA

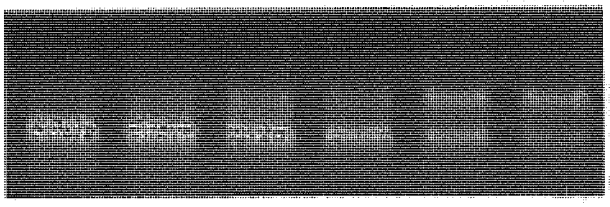



| | | | |
|---|---|---|---|
| 1 | 2.5ng cRNA | 4 | 0.313ng cRNA |
| 2 | 0.125ng cRNA | 5 | 0.156ng cRNA |
| 3 | 0.625ng cRNA | 6 | 0.078ng cRNA |



### 8.2.2.1 F) 0.05µg/ml 15-HETE + 50µM EPA

1 2 3 4 5 6



| | | | |
|---|---|---|---|
| 1 | 2.5ng cRNA | 4 | 0.313ng cRNA |
| 2 | 0.125ng cRNA | 5 | 0.156ng cRNA |
| 3 | 0.625ng cRNA | 6 | 0.078ng cRNA |

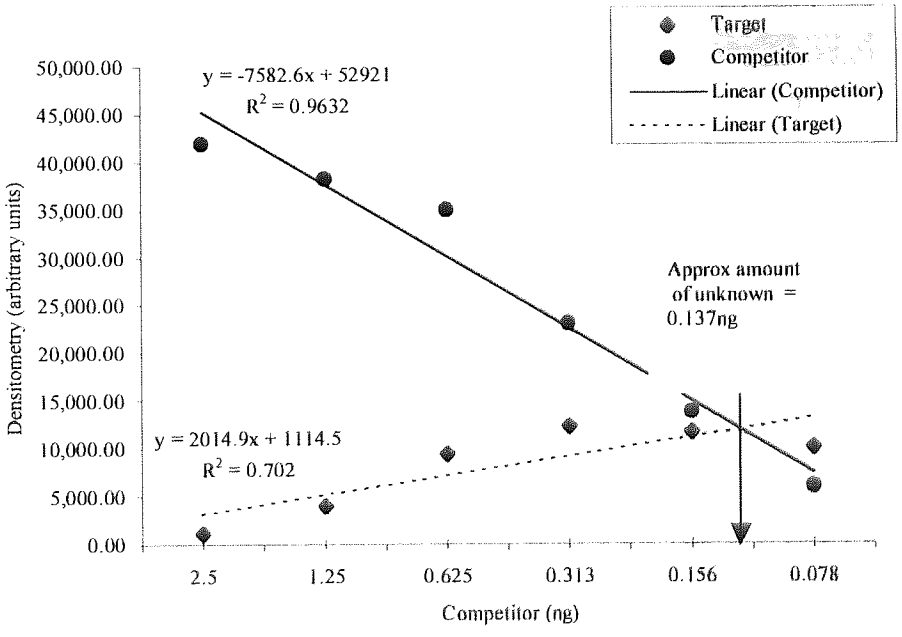
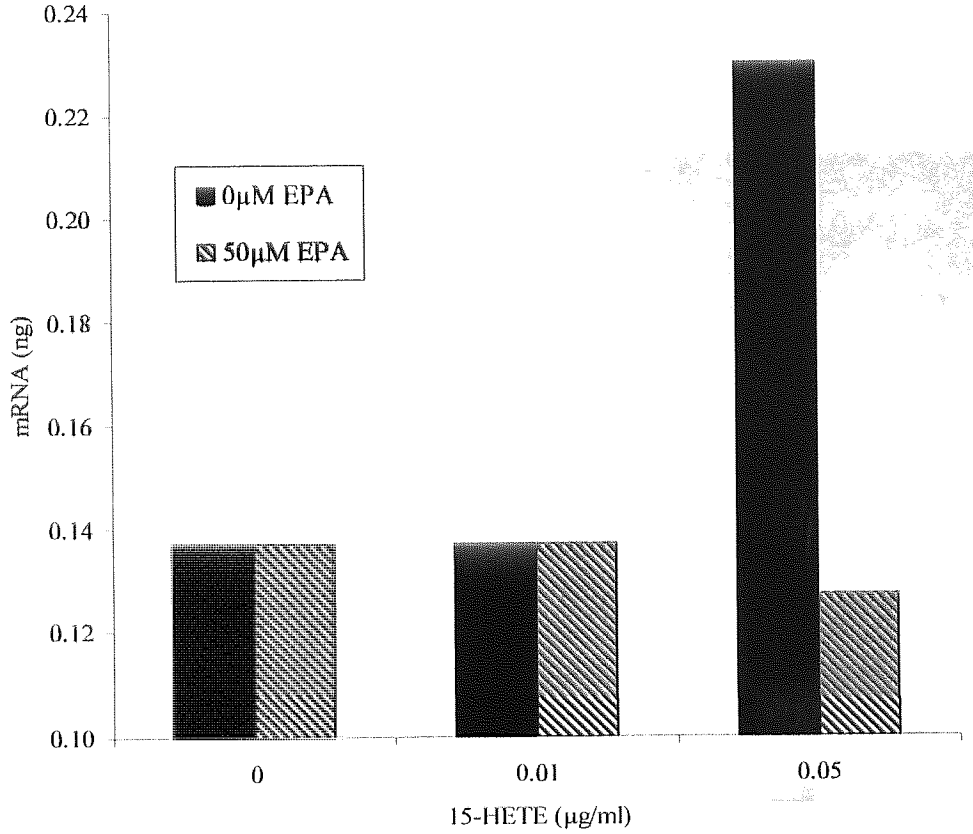


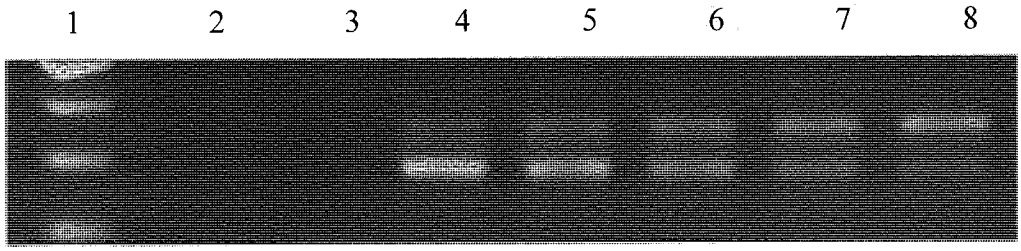
**Fig 8.2.2.2) Quantification of C2 proteasome subunit mRNA levels in 15-HETE treated C2C12 myotubes in the presence and absence of EPA**



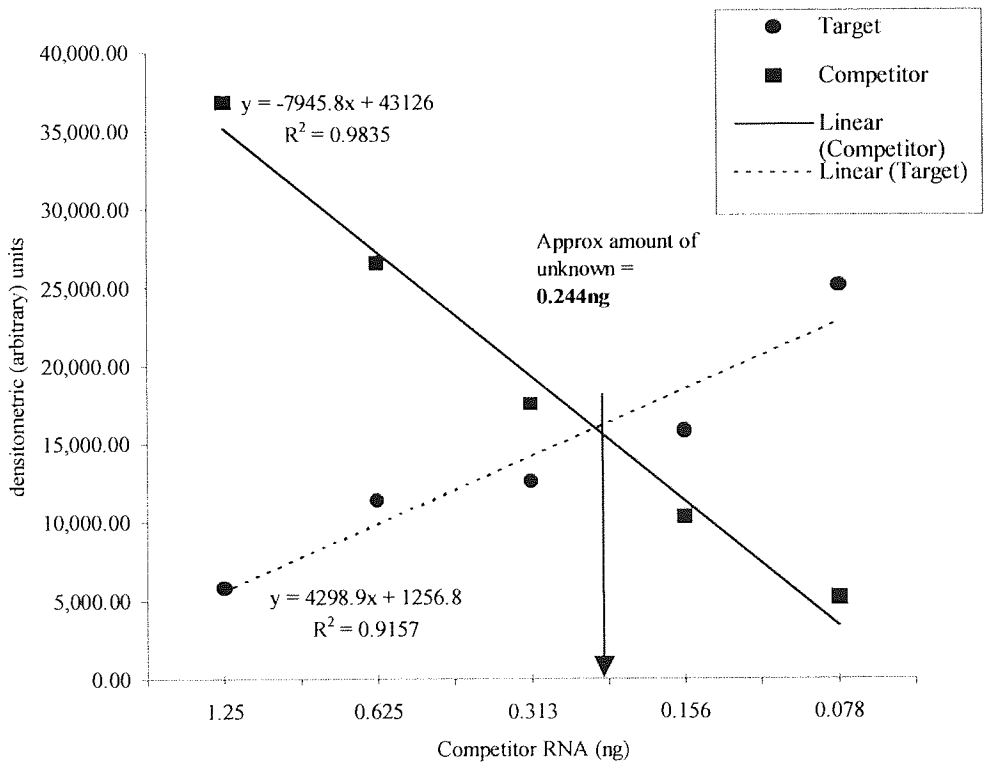

These experiments were repeated using PIF, figure 8.2.2.3 shows the effects of 0, 0.1 and 0.4μg/ml PIF in C2C12 myotubes upon C2 RNA levels in the presence (figures 8.2.2.3D-F) and absence (figs 8.2.2.3 A-C) of 50μM EPA. These figures show that 0.1μg/ml PIF (fig 8.2.2.3B) increases C2 mRNA levels by over 500% (from 0.24ng in untreated controls to 1.25ng), and that this increase was abolished in the presence of EPA (fig 8.2.2.3E) when C2 RNA levels were not significantly increased above controls (0.3ng). 0.4μg/ml on the other hand, had little effect with increases of 0.261ng in the absence (fig 8.2.2.3 C) and 0.3ng in the presence (fig 8.2.2.3F) of EPA. This is shown in figure 8.2.2.4 (pp166) and demonstrates that 0.1μg/ml is capable of stimulating expression of the C2 gene which correlates with a peak of expression of proteasome subunits in C2C12 myotubes.

**Fig 8.2.2.3 A-F) Competitive Quantitative Reverse Transcription Polymerase Chain Reaction (cQRT-PCR) quantification of C2 proteasome subunit mRNA levels in PIF treated C2C12 myotubes in the presence and absence of 50μM EPA**

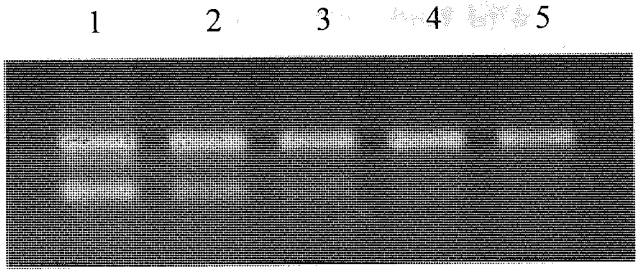
**8.2.2.3A) 0μg/ml PIF + 0μM EPA**



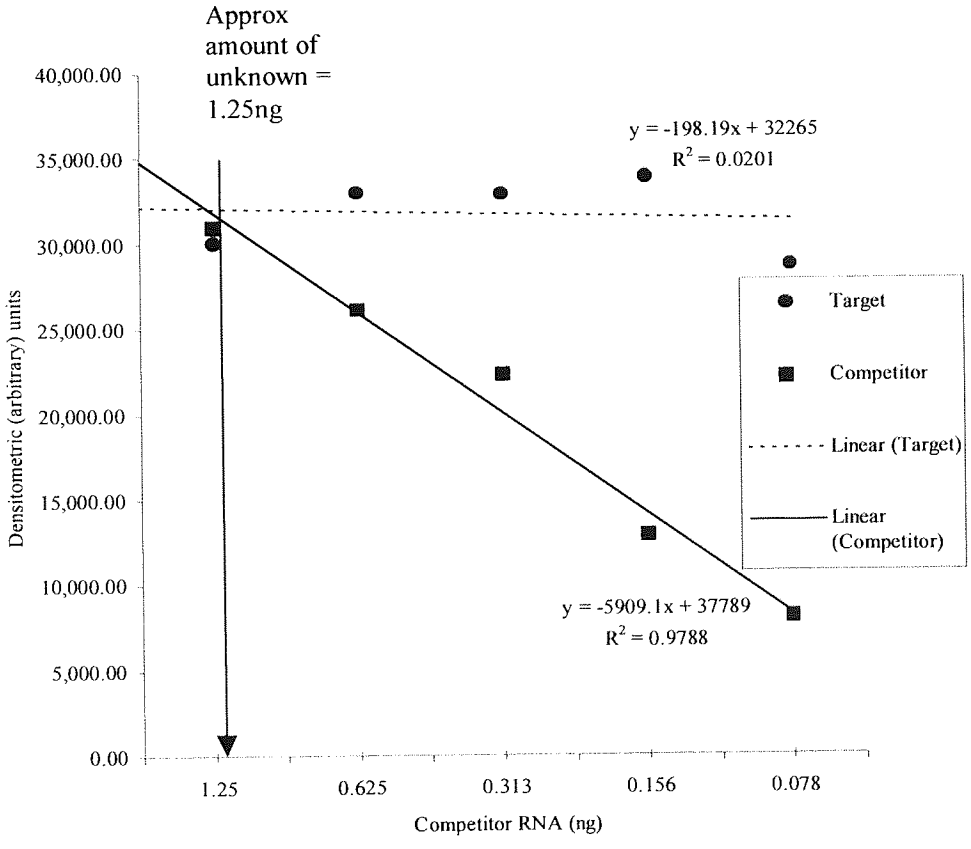

| | | | |
|---|---|---|---|
| 1 | 100 base pair DNA ladder | 5 | 0.625 ng cRNA |
| 2 | PCR Negative | 6 | 0.313ng cRNA |
| 3 | RT Negative | 7 | 0.156ng cRNA |
| 4 | 1.25ng cRNA | 8 | 0.078ng cRNA |



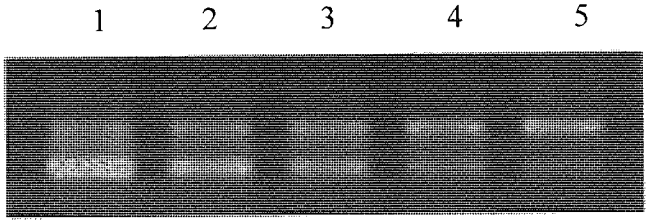
## 8.2.2.3B) 0.1µg/ml PIF + 0µM EPA



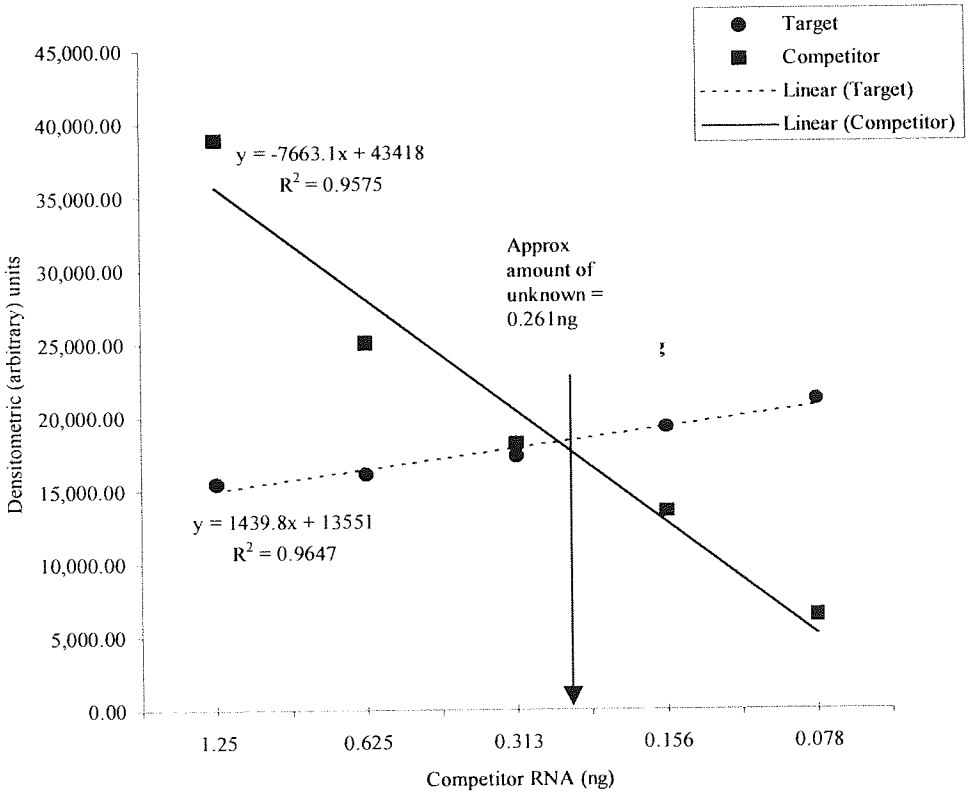

| | | | |
|---|---|---|---|
| 1 | 1.25ng cRNA | 4 | 0.156ng cRNA |
| 2 | 0.625ng cRNA | 5 | 0.078ng cRNA |
| 3 | 0.313ng cRNA | | |



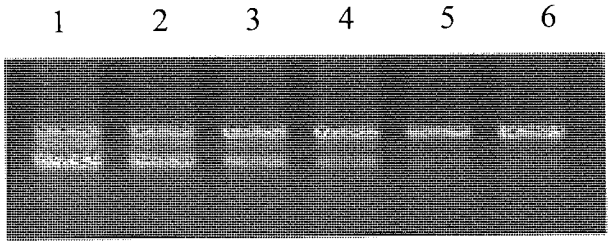
## 8.2.2.3C) 0.4µg/ml PIF + 0µM EPA



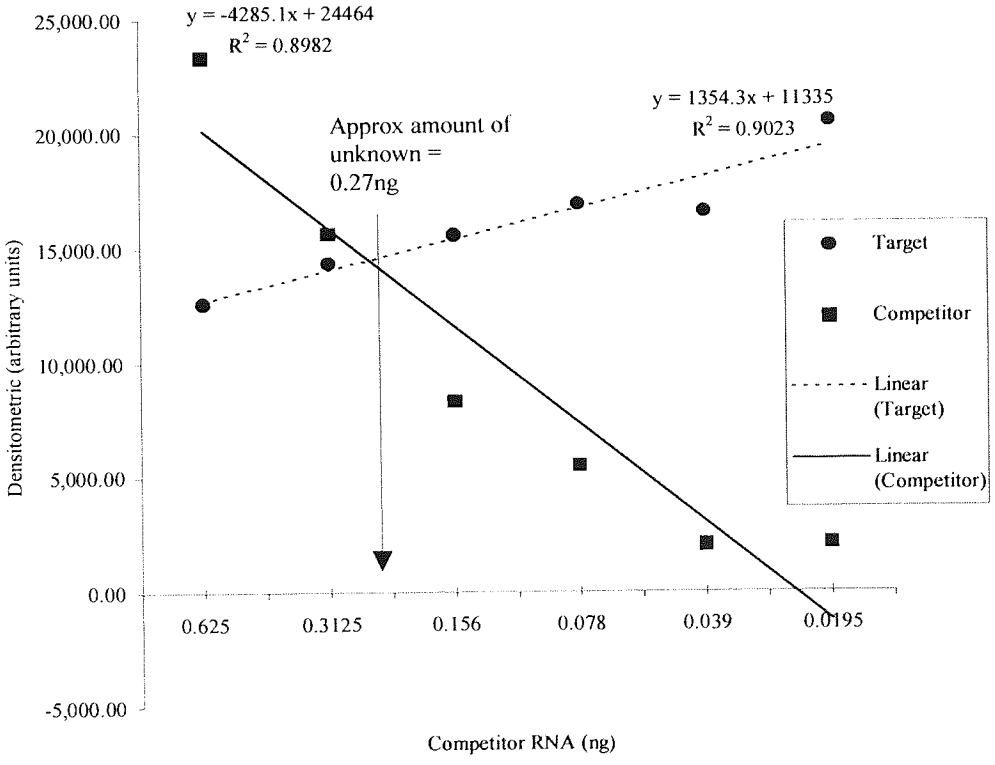

| | | | |
|---|---|---|---|
| 1 | 1.25ng cRNA | 4 | 0.156ng cRNA |
| 2 | 0.625ng cRNA | 5 | 0.078ng cRNA |
| 3 | 0.313ng cRNA | | |



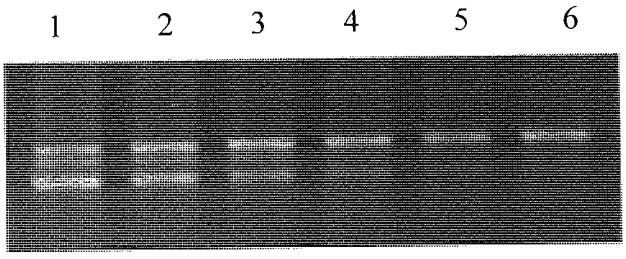
### 8.2.2.3D) 0µg/ml PIF + 50µM EPA



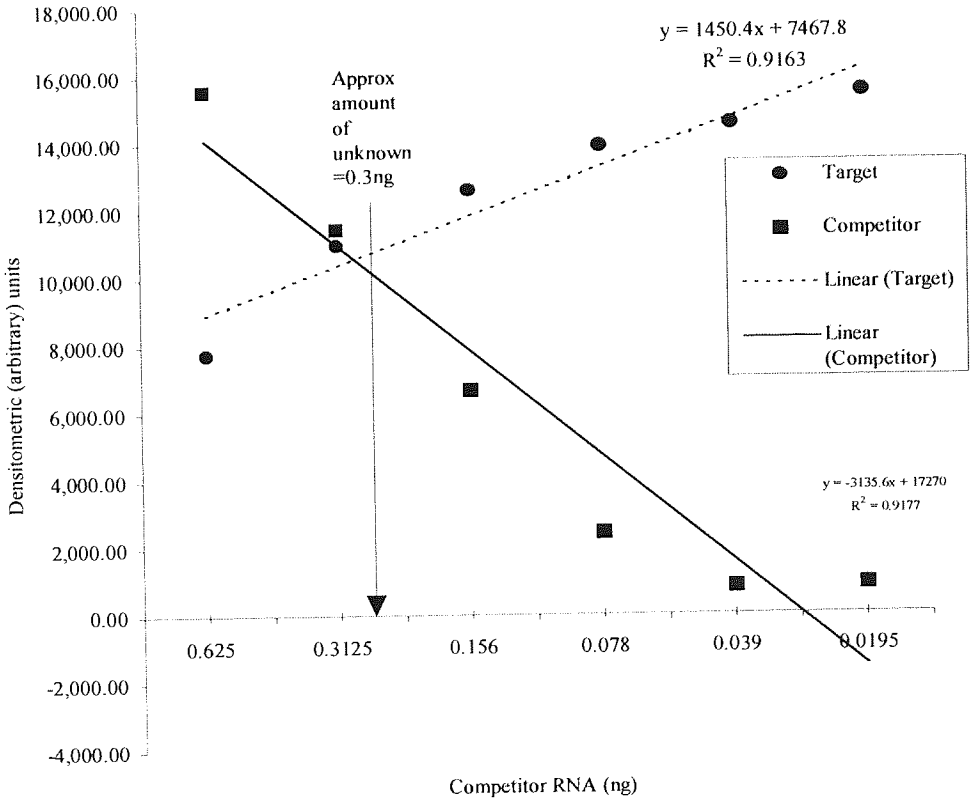

| | | | |
|---|---|---|---|
| 1 | 0.625ng cRNA | 4 | 0.078ng cRNA |
| 2 | 0.3125ng cRNA | 5 | 0.039ng cRNA |
| 3 | 0.156ng cRNA | 6 | 0.0195ng cRNA |



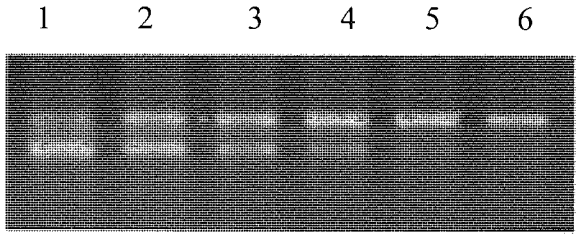
## 8.2.2.3E) 0.1µg/ml PIF + 50µM EPA




| | | | |
|---|---|---|---|
| 1 | 0.625ng cRNA | 4 | 0.078ng cRNA |
| 2 | 0.3125ng cRNA | 5 | 0.039ng cRNA |
| 3 | 0.156ng cRNA | 6 | 0.0195ng cRNA |



### 8.2.2.3F) 0.4µg/ml PIF + 50µM EPA




| | | | |
|---|---|---|---|
| 1 | 0.625ng cRNA | 4 | 0.078ng cRNA |
| 2 | 0.3125ng cRNA | 5 | 0.039ng cRNA |
| 3 | 0.156ng cRNA | 6 | 0.0195ng cRNA |

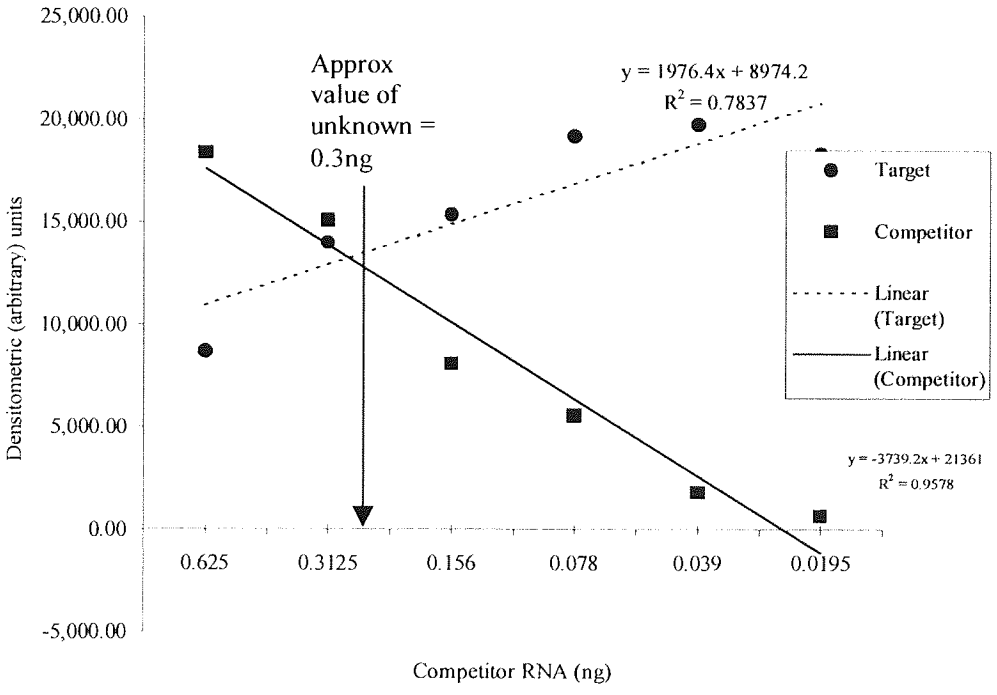
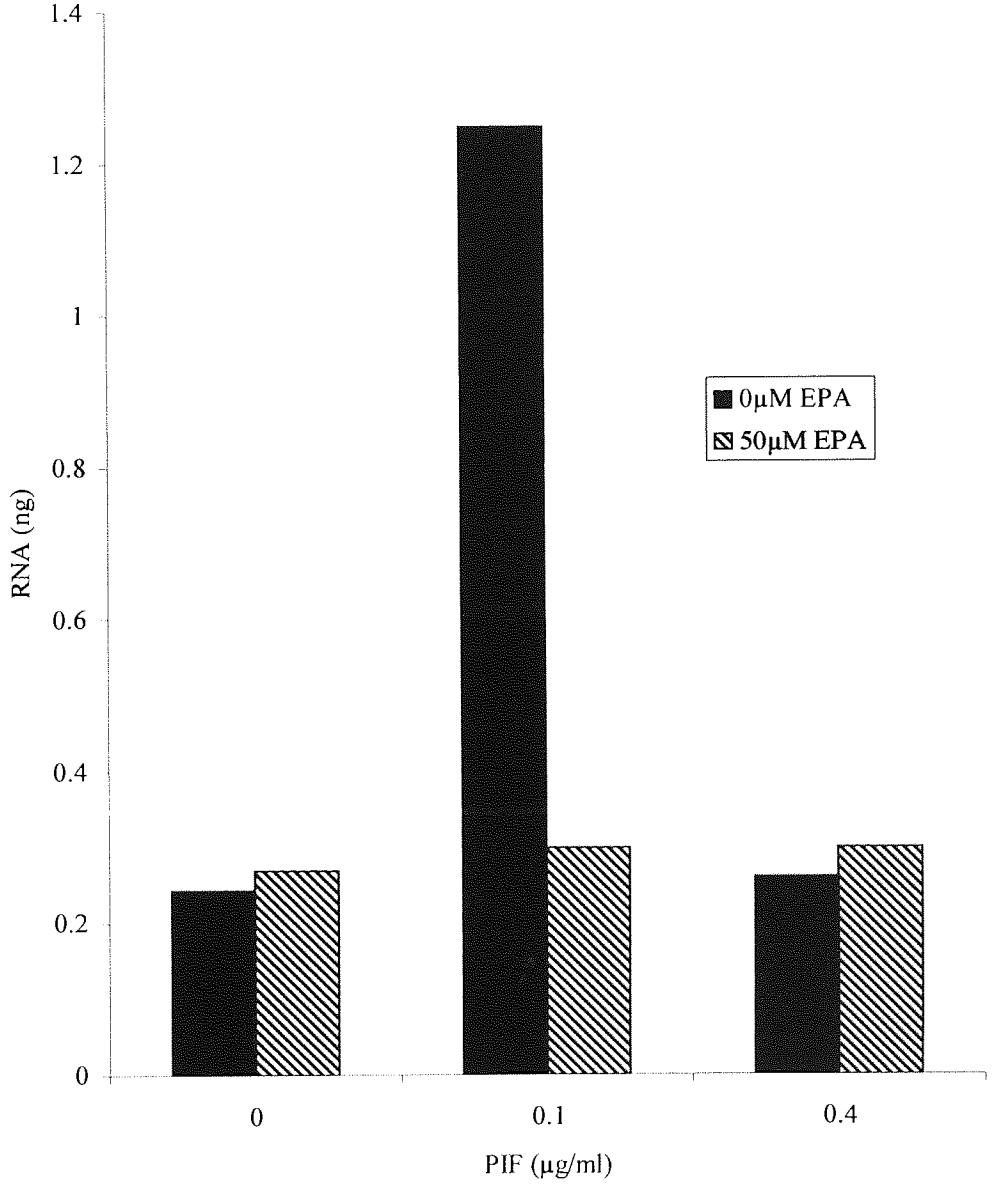


**Fig 8.2.2.4) Quantification of C2 proteasome subunit mRNA levels in PIF treated C2C12 myotubes in the presence and absence of EPA**




The results presented in this chapter demonstrate that 15-HETE is indeed capable of increasing proteasome subunit expression in C2C12 myotubes. This is a transcriptional event and suggests that 15-HETE may well be the intracellular mediator of PIF. That EPA can inhibit the expression of C2 RNA and the levels of proteasome subunits in the cell suggests that it is also

acting at the level of transcription. This effect is still a biochemical puzzle. For example, how is it that EPA is transferred to the cell/nucleus interior? It is possible that this lipophilic fatty acid penetrates cell membranes by diffusion or that specific carrier proteins are involved. The latter postulate might be supported by the discovery of specific transporters with high affinity for long chain fatty acids. FATP-1 for example, is a murine membrane protein which facilitates the uptake of FA in 3T3-L1 cells (Scaffer and Lodish 1994), which appears to have high inter-species homology (Blask et al 1999). The fact that a peroxisome proliferator activated response element was identified in the FATP1 gene and that LA (a natural PPAR$\alpha$ and $\gamma$ ligand) upregulated FATP1 expression (Trohnert et al 1999) implies a role for this receptor also. PPAR transcription factors are interesting potential targets for PUFA intervention, the $\alpha$-subtype for example is predominantly expressed in tissues with high catabolic rates of fatty acids including muscle, and its activity has been shown to be modulated by a number of PUFAs including EPA (Devchand et al 1996).

# 9 The Effects of EPA in an In Vivo Model of Acute Starvation

## 9.1 Introduction

It has been demonstrated here and elsewhere, that EPA can attenuate protein catabolism in the cancer cachexia, this is (at least in part) due to its effects on attenuating the elevated proteasome activity and expression (discussed in earlier chapters). Since there is evidence that this pathway is elevated in acute fasting also, the effect of EPA on muscle protein degradation and proteasome function was investigated in a series of experiments during acute fasting in mice, in order to determine whether protein catabolism in starvation and cancer cachexia were mediated through a common pathway which could be inhibited by EPA.

The adaptive response to starvation in the normal individual comprises two phases. The first is an initial depletion of glycogen from muscle and liver stores coupled to an increase in skeletal muscle catabolism to provide amino acids for gluconeogenesis in the liver (Kettlehut et al 1985). After a few days gluconeogenesis and skeletal muscle catabolism are suppressed and replaced by the second, long term response which involves the breakdown of adipose tissue and subsequent release of free fatty acids, these are converted into ketone bodies and utilized for energy by the peripheral tissues and brain. The most noticeable outcome of this situation is a depletion of fat reserves but a preservation of skeletal muscle and glucose.

This was demonstrated by Moley (1987) who showed that in cases of simple starvation only a very small amount of skeletal muscle is lost and that over three quarters of the weight lost is from fat depletion. Compare this to the findings in cancer cachexia where skeletal muscle : adipose tissue loss is often 50:50. Furthermore several studies have shown that it is not possible to reverse cachectic weight loss by increasing caloric intake as is the case in simple starvation (Reviewed in Tisdale 1997). This latter finding is echoed by those of Preston et al (1987) who undertook a comprehensive study in which lung cancer patients with severe weight loss were matched by age,

sex, height and pre-illness weight with controls. In this study 85% fall in total body fat was seen which clearly reflects a prolonged negative energy balance. Secondly there was a 75% fall in skeletal muscle protein mass although the non muscle compartment was preserved.

It is worth commenting also on the work of Kitada et al (1980) who used 1-$^{14}C$ labelled linoleic acid implanted to AKR mice and found that in tumour bearing animals fat appeared largely in the tumour whereas in non-tumour bearing animal controls and non-tumour bearing animals who had been fasted for 24hours fat was mobilised and appeared largely as respiratory $CO_2$. This underlies the difference between fat metabolism in starvation and cachexia.

Thus it can be appreciated that cachexia is far more complicated that simple starvation and that the switching from the early to late phase starvation response does not occur in the normal manner. However the early phase and therefore short term fasting also, still leads to mobilization of muscle protein, as does cachexia. In starvation this results from both a decrease in protein synthesis and an increase in myofibrillar and non-myofibrillar protein breakdown (Kettlehut et al 1998).

The relative contribution of the various proteolytic systems in acute fasting has been considered by several groups. Lysosomal proteolysis has been shown to be elevated (Wing and Goldberg 1993), whilst cathepsin B, H and B+L activities are unchanged or decreased (Berkhou et al 1994) and mRNA for cathepsin L and D unchanged (Medina et al 1995). Calcium dependent proteolysis in the rat was also found to be unchanged (Wing and Goldberg 1993). Lowell et al (1986) showed that the hindquarters of fasted rats perfused under conditions which use chloroquine to block lysosomal acidification or leupeptin to inhibit the calpains and cathepsins B, H and L does not affect proteolysis as measured by 3-methylhistidine release. However in a similar experiment, ATP depletion almost completely suppressed the elevated proteolysis seen in the muscles of fasted rats and the

rise in proteolysis was accompanied by increased expression of ubiquitin mRNA (Medina et al 1991).

Since then expression of other markers of the ubiquitin proteasome pathway have been shown to be elevated in acute fasting. These include $E2_{14k}$ (Wing and Banville 1994), ubiquitylated proteins (Wing et al 1994) and 20S proteasome subunits (Medina et al 1995).

Significantly it has been shown that upon refeeding, ubiquitin dependent proteolysis, ubiquitin, 20S (Medina et al 1995) and $E2_{14k}$ (Wing and Banville 1994) expression returned to normal. Medina et al (1995) also demonstrated that ubiquitin mRNA whilst elevated in skeletal muscle remained at control levels in heart, liver, kidney and fat implying that the effect is specific to striated muscle. Thus it is now thought that most of the increased myofibrillar protein breakdown in skeletal muscle during acute fasting is due to the activation of the ubiquitin proteasome pathway.

To determine whether the effects of EPA upon proteasome activity and expression in fasting, might be mediated by its ability to interfere with the generation of 15-HETE, a specific LOX inhibitor was used. CV6504 (2,3,5-trimethyl-6-(3-pyridylmethyl)1,4-benzoquinone) has been shown to inhibit the conversion of arachidonic acid to 5, 12 and 15-HETEs (Hussey and Tisdale 1996)and to effectively inhibit the growth of murine adenocarcinomas including MAC13, MAC16 and MAC26 in vivo (Hussey et al 1996), the anti-tumour activity of which could be suppressed by concurrent adminstration of linoleic acid, suggesting that linoleate metabolism was responsible for the effect.

## 9.2 Results and Discussion

Figure 9.2.1 shows the effects of p.o. dosing of 2.5g/kg EPA upon the body weight of NMRI mice which had been fasted for 24hours. There is a 5% reduction in the loss of body weight after 24 hours and 48 hours in those animals pretreated with EPA compared to untreated and vehicle control groups. However this is not statistically significant ($p<0.5$). Likewise there was no difference between wet weights of the soleus and gastrocnemius muscles (figs. 9.2.2 and 9.2.3 respectively) ($p<0.5$).

**Fig 9.2.1) The effects of p.o. dosing of EPA on bodyweight of female NMRI mice fasted for 24hours.**

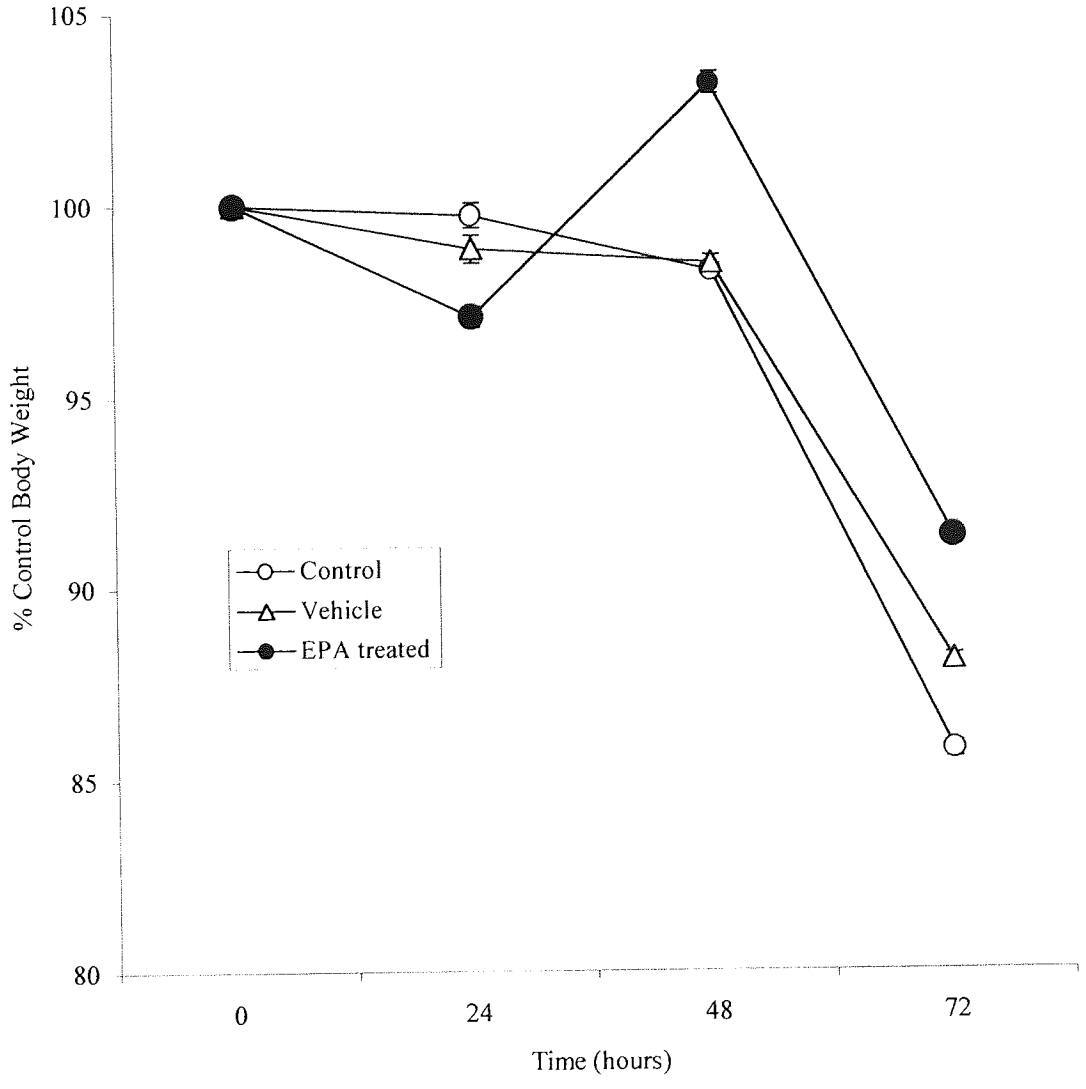


**Fig 9.2.2) The effects of p.o. dosing of EPA on soleus muscle mass of female NMRI mice fasted for 24hours**

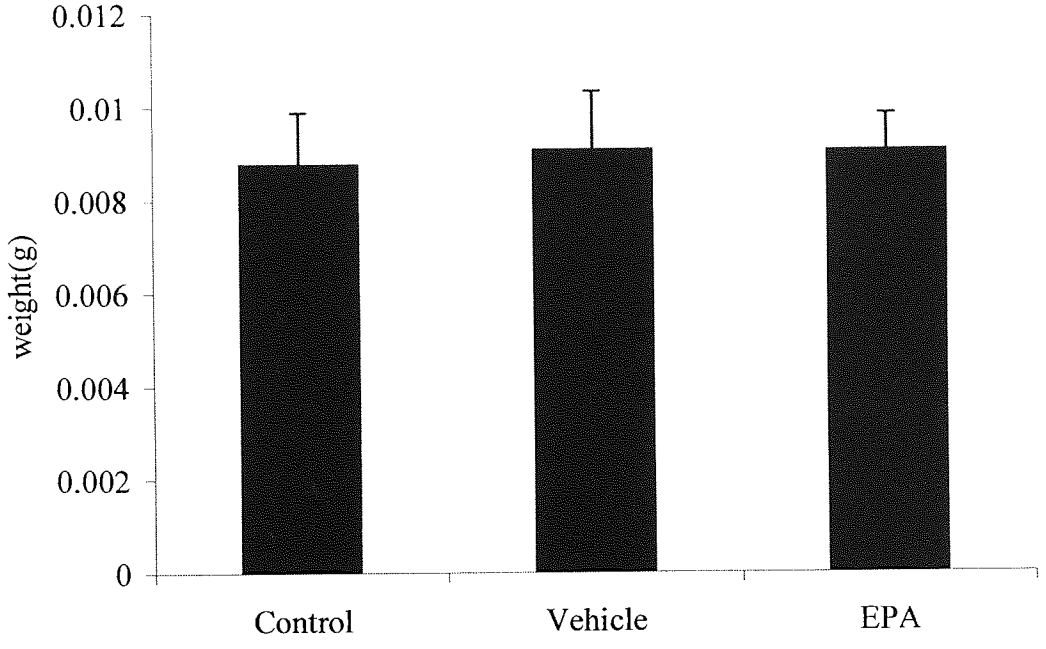
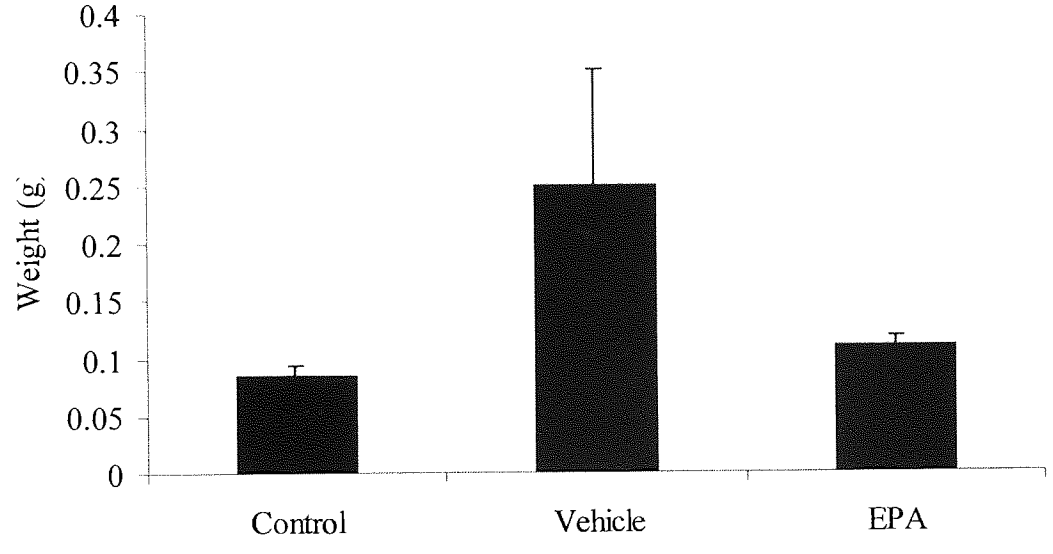
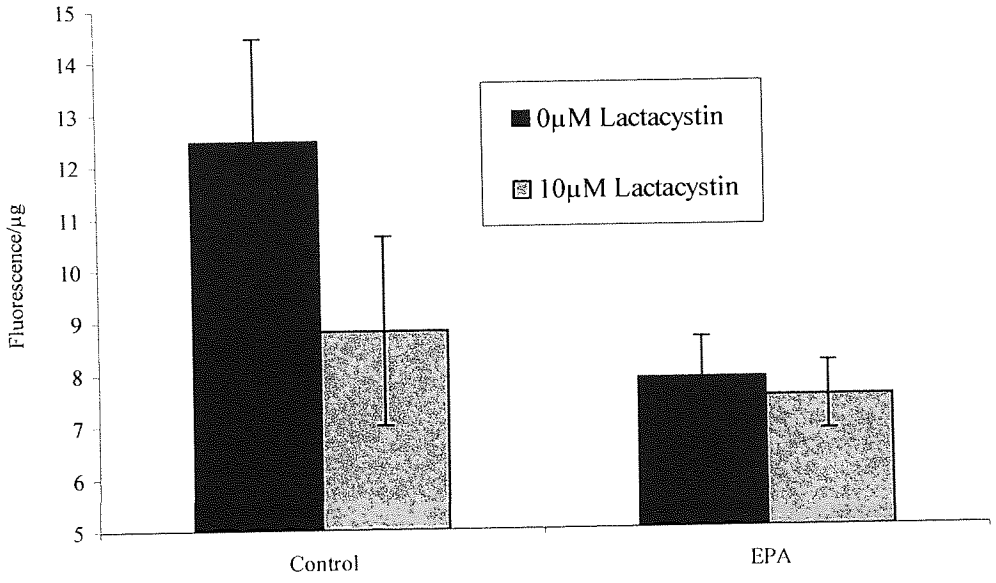



**Fig 9.2.3) The effects of p.o. dosing of EPA on gastrocnemius muscle mass of female NMRI mice fasted for 24hours**



However a complete inhibition of the elevated Chymotrypsin like enzyme activity of the proteasome was evidenced as is shown in fig 9.2.4 (control vs EPA $p<0.05$).

**Fig 9.2.4) The effects of p.o. dosing of EPA on the 'chymotrypsin-like' enzyme activity of the proteasome (in the presence and absence of lactacystin) in gastrocnemius muscles from 24hour fasted female NMRI mice**

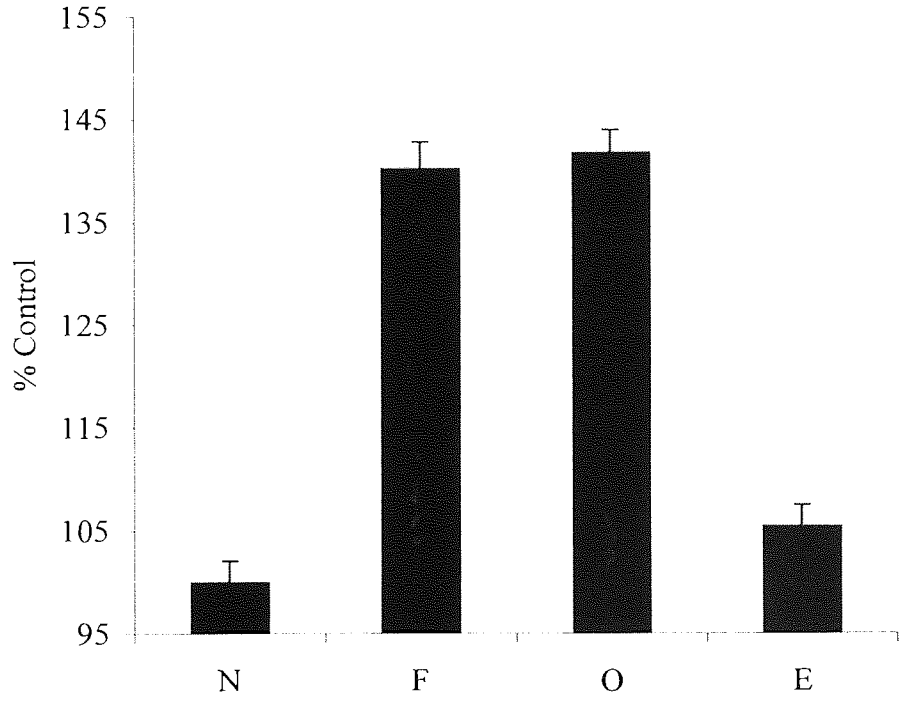



Cellular expression of proteasome subunits in the gastrocnemius muscle was measured by Western blotting of soluble fractions. Fig 9.2.5 shows the levels of various proteasome subunits and related enzymes. Similar results are found for all those members tested in that expression was elevated in untreated and vehicle treated fasted animals, but reduced to that of non starved animals in those fasted mice pre-treated with EPA. This was the case for 3 $\alpha$ subunits of the 20S proteasome as detected by an anti MCP-231 antibody (fig 9.2.5a) in which expression was elevated by approximately 40% in fasted and vehicle groups ($p<0.001$) but reduced by approximately 35% in the presence of EPA ($p<0.01$). Figure 9.2.5b demonstrates expression of P42, an ATPase subunit of 19S. Similarly elevated expression was seen, approximately 34% in both fasted and vehicle groups ($p < 0.001$) but this was reduced to control levels by EPA (101% untreated control $p < 0.001$). The ubiquitin conjugating enzyme $E2_{14k}$ (fig 9.2.5c), was also

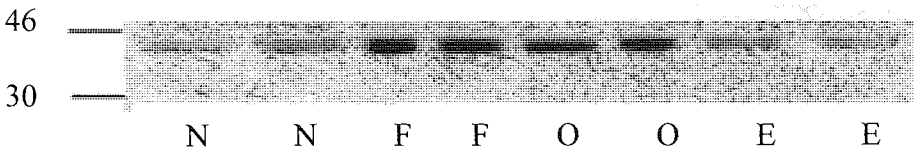
elevated by 17% and 21% in the vehicle and fasted controls respectively, whilst expression was 92% untreated control in the presence of EPA ($p<0.01$).

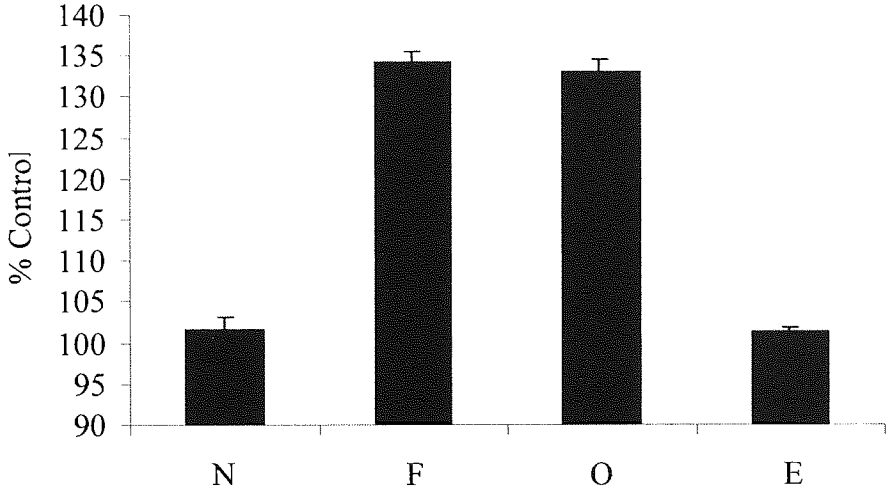
**Fig.9.2.5) Western blots (and densitometry) of soluble extracts of gastrocnemius muscles from non-fasted mice (N), fasted mice (F), olive oil pretreated fasted mice (O) and EPA pretreated fasted mice (E) probed for expression of 20S proteasome α-subunits (A), p42(B) and $E2_{14k}$ (C).**

**9.2.5 A) 20Sα Expression**

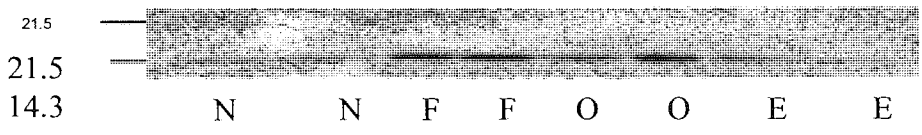



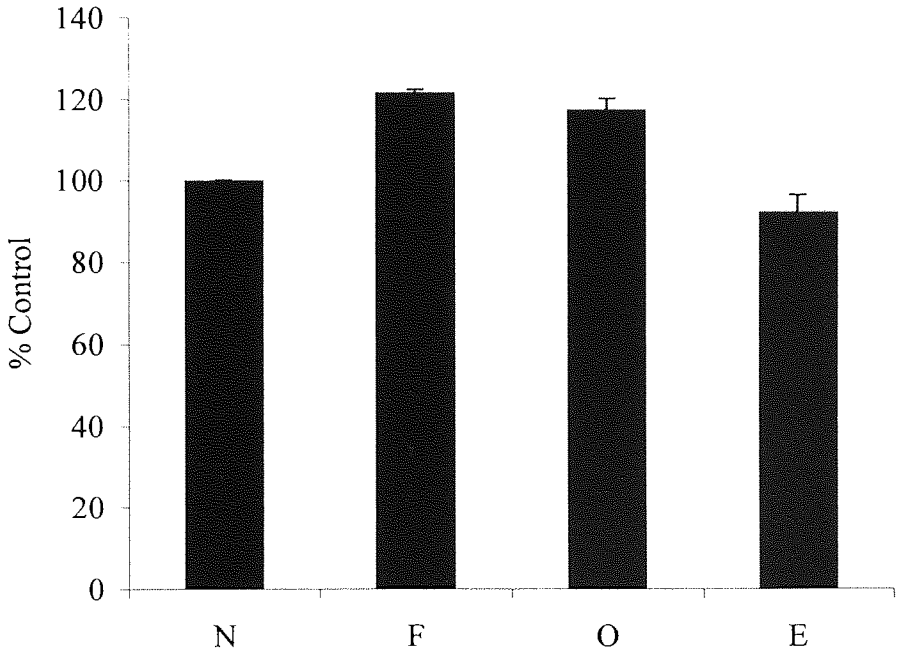
### 9.2.5 B) P42 Expression




### 9.2.5 C) $E2_{14k}$ Expression

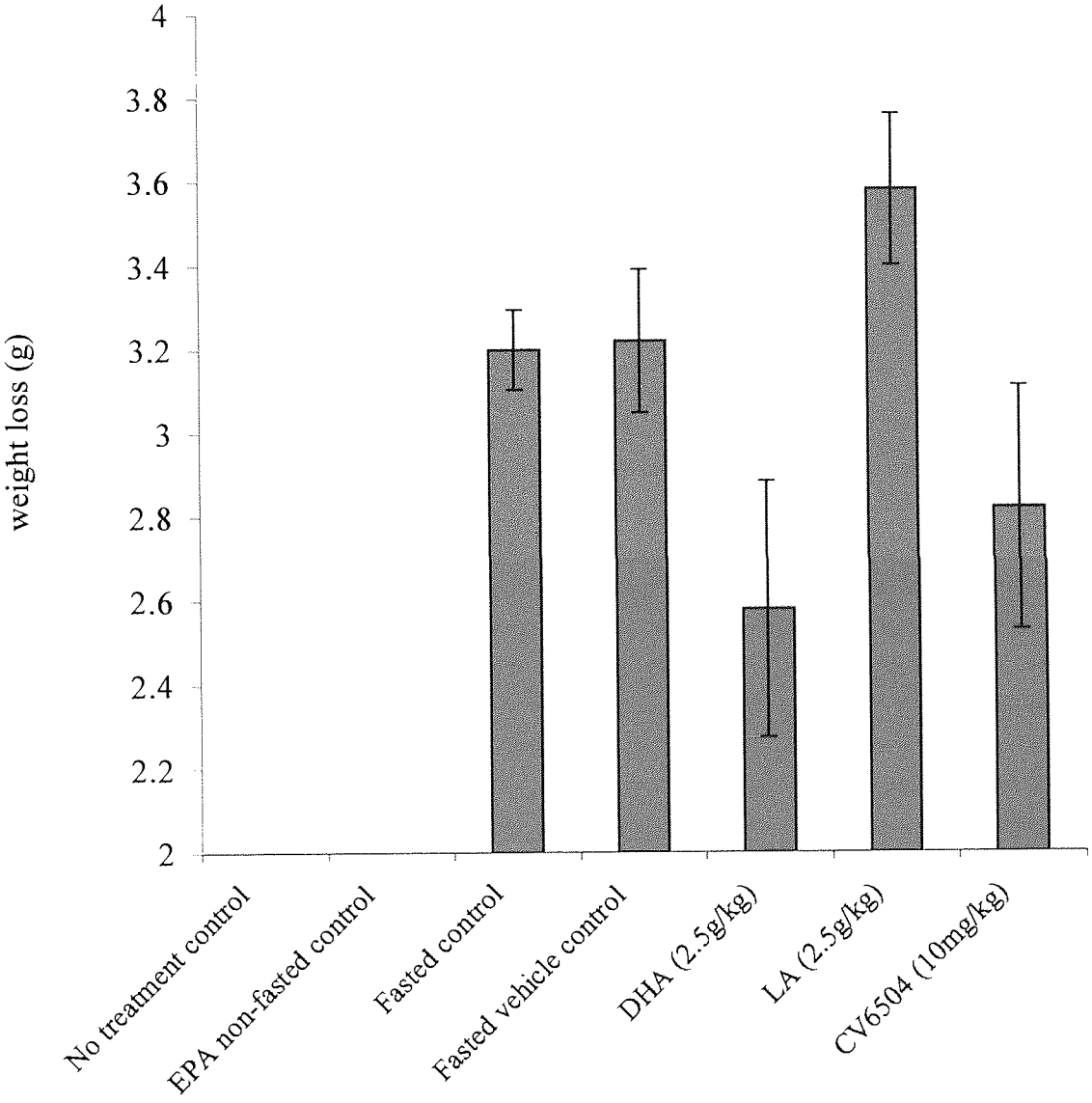


To determine if the effect was specific for EPA, further experiments were performed using the related n-3 fatty acid DHA and a typical n-6 fatty acid LA. To consider the role of the LOX pathway with regards to the involvement of the putative intermediate 15-HETE, a further test group was included, these animals were administered the LOX inhibitor CV6504.

The data in fig 9.2.6 (overleaf) shows the effects of CV6504 and these fatty acids upon body weight in fasted and non fasted animals. There was no weight loss in the untreated and EPA non-fasted control groups and an average weight loss of 3.2g± 0.095 and 3.22g± 0.17 respectively, for the fasted and fasted vehicle controls. (Compare charts 9.2.1 pp171 and 9.2.6 pp177). This was attenuated by 11.88% by CV6504 implying that the LOX pathway is indeed involved. The n-6 LA on the other hand, actually increased weight loss by a further 11%. DHA caused a 20% reduction in weight loss suggesting that it might have some activity in vivo. However, in subsequent investigations of tyrosine release (fig 9.2.7) and proteasome activity (fig 9.2.9) DHA was not effective.

**Fig 9.2.6) The effects of on body weight of EPA, DHA, LA and CV6504 in fasted mice and EPA in non-fasted mice**




Protein degradation in which tyrosine release from the gastrocnemius muscles of fasted mice was measured (fig 9.2.7), was elevated up to 227% of controls in fasted animals compared to untreated controls and this elevation was reduced by only 21% and 26% in those animals treated with DHA and

LA compared to 46% in those animals pre-treated with CV6504 ($p<0.001$) and 42% in those animals pre-treated with EPA ($p<0.02$) (fig 9.2.8)

**Fig 9.2.7) Tyrosine release from soleus muscles of non-fasted control mice, EPA pretreated non-fasted mice, fasted control mice, olive oil pretreated fasted mice, DHA pretreated fasted mice, LA pretreated fasted mice, and CV-6504 pretreated fasted mice.**

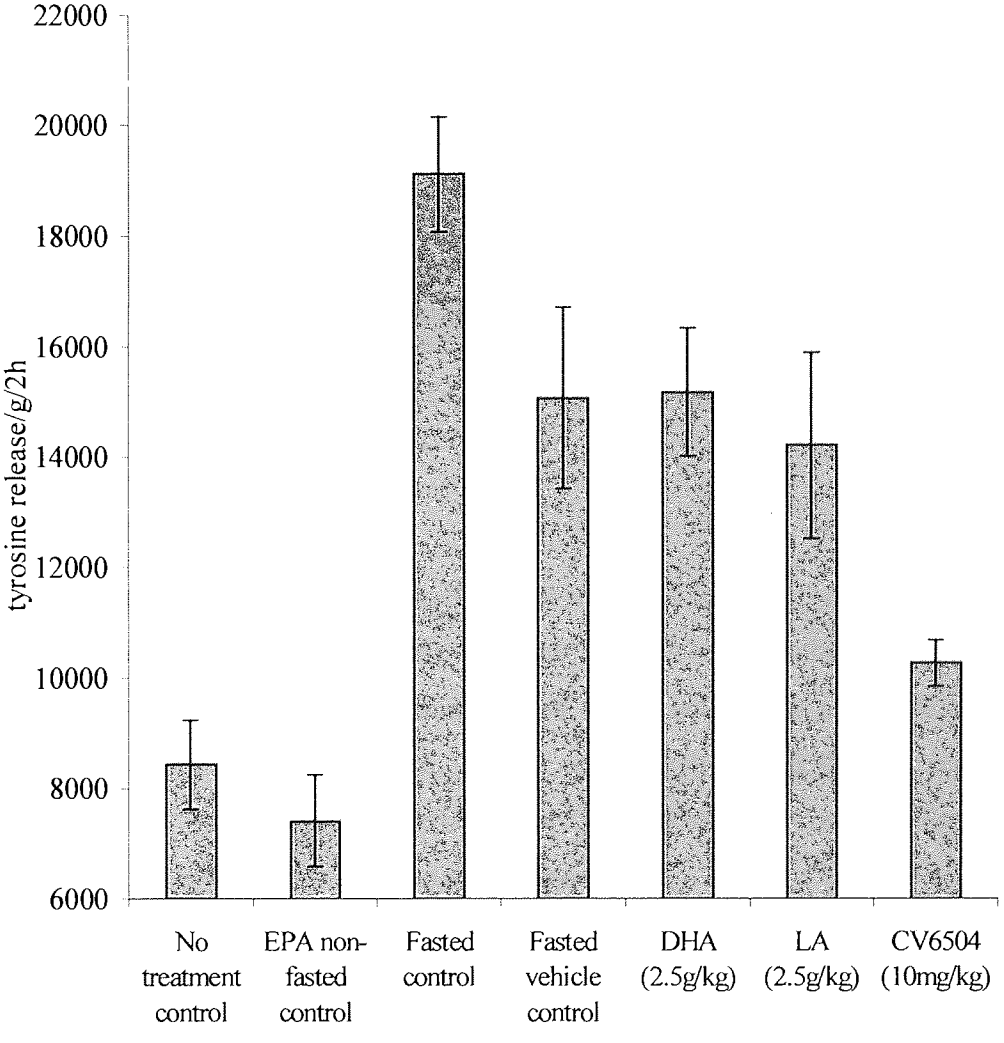
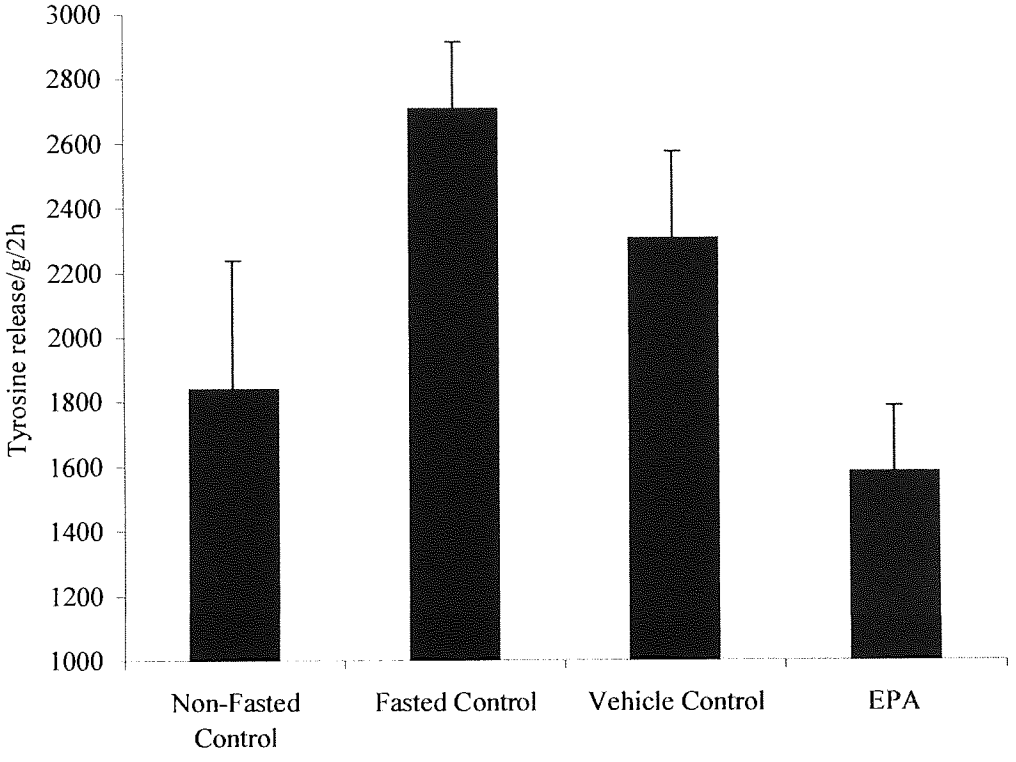
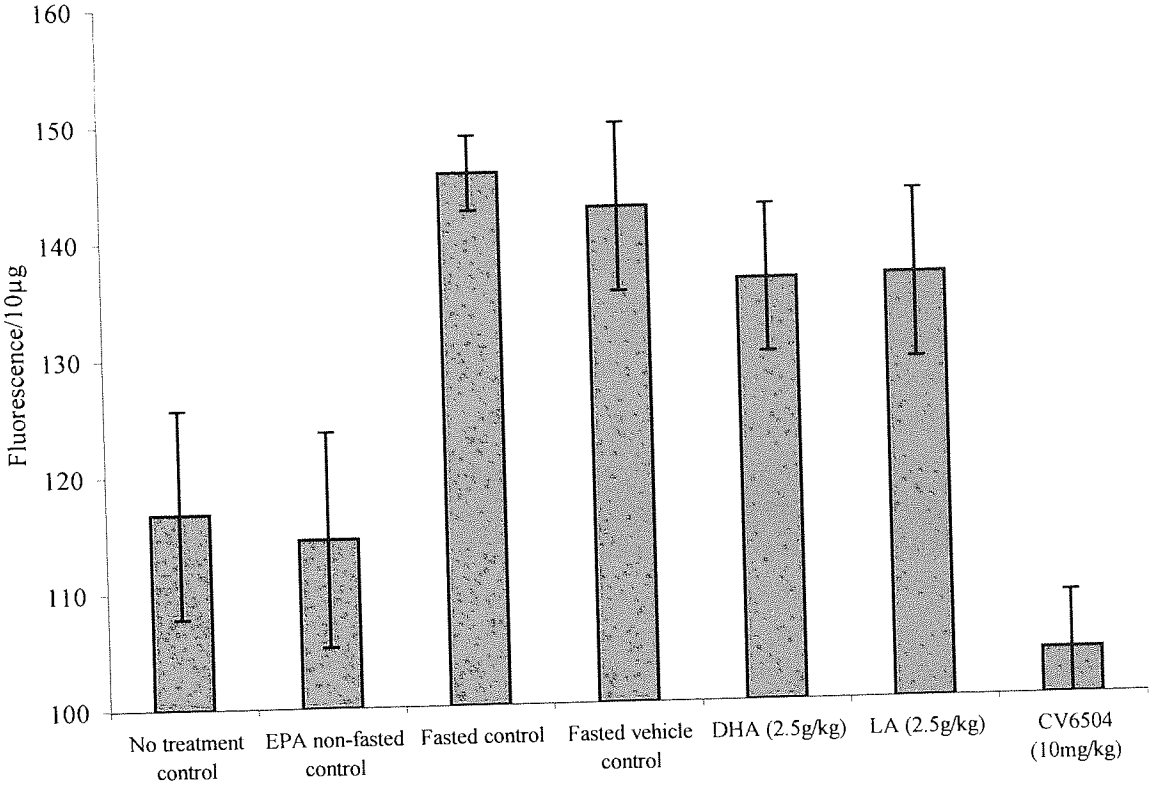


**Fig 9.2.8) Tyrosine release from soleus muscles of non-fasted control mice, EPA pretreated non-fasted mice, fasted control mice, olive oil pretreated fasted mice, and EPA treated fasted mice,**




A similar pattern was observed for proteasome activity (figure 9.2.9) as measured using the fluorogenic substrate suc-LLVY in which activity was increased by 25% in fasted animals and whilst activity in mice pre-treated with DHA and LA was reduced by only 6.5% and 6.4% respectively. Activity in those animals pretreated with CV6504 was reduced by 28% ($p<0.01$) below control non-fasted animals.

**Fig 9.2.9a and b) Chymotrypsin-like enzyme activity in gastrocnemius muscles** (for explanation of categories see previous legends)



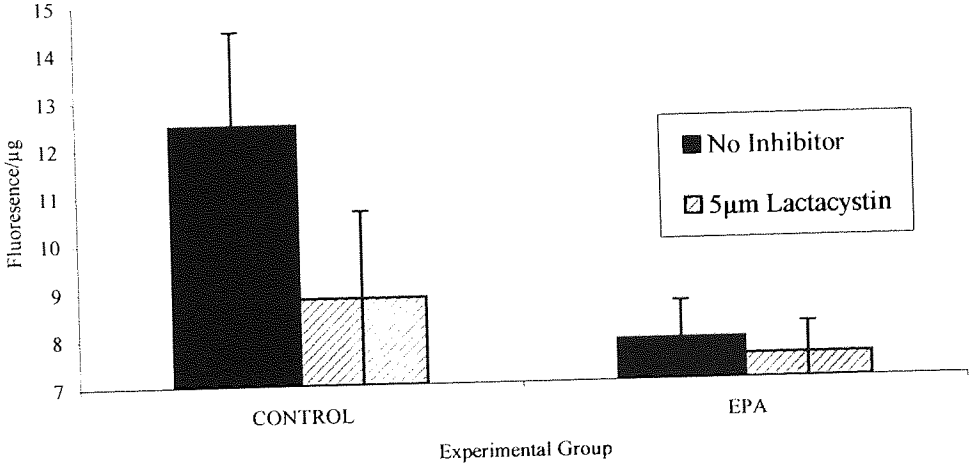
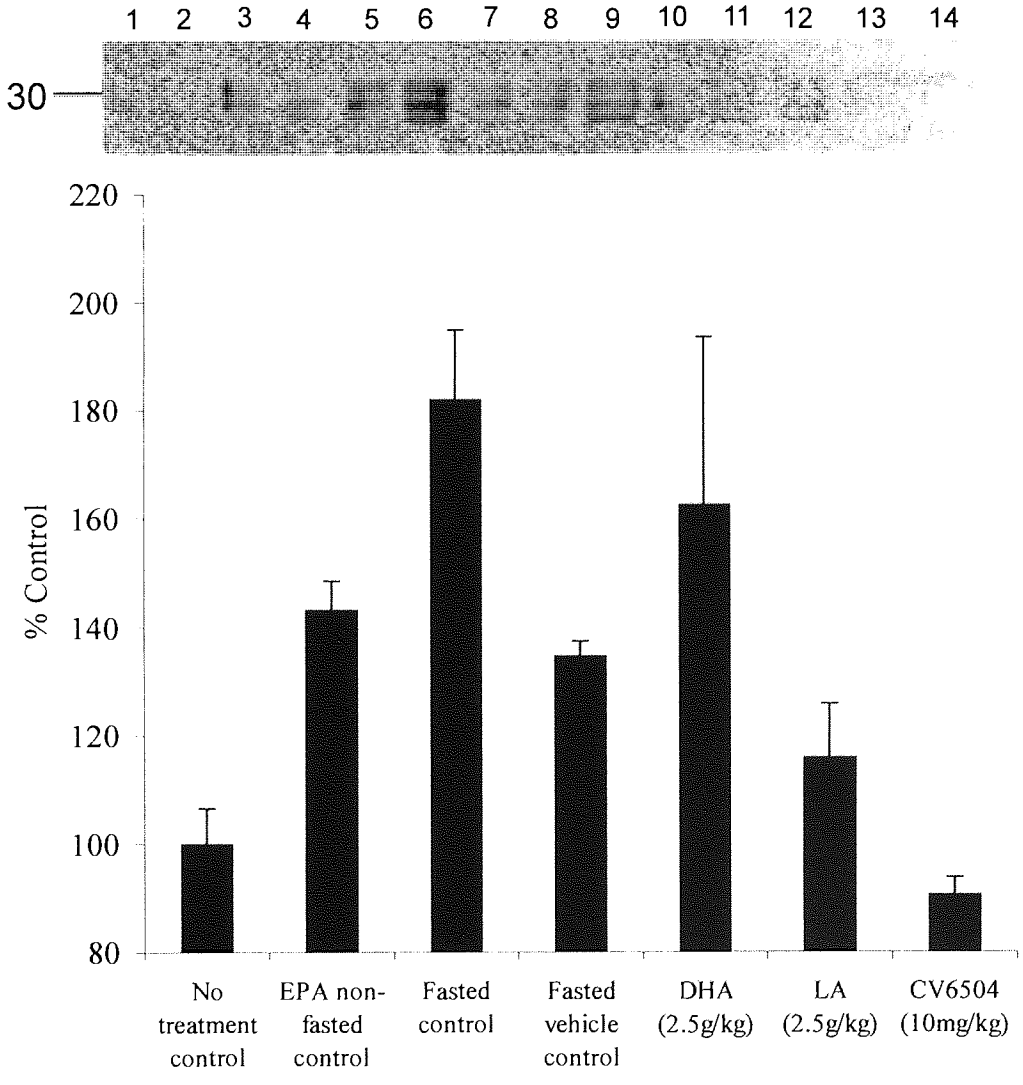




Figure 9.2.10 shows the expression of 20S proteasome α-subunits and the densitometric analysis of this Western. Expression is elevated in fasted and vehicle-fasted groups (by 82% $p<0.05$ and 34%) and this is attenuated by EPA and CV6504 ($p<0.05$) but not by DHA or LA (no significant difference from fasted control $p>0.05$).

**Fig 9.2.10) Western blots of soluble extracts of gastrocnemius muscles from non-fasted mice (lanes 1 and 2), fasted mice (lanes 3 and 4), olive oil pre-treated fasted mice (lanes 5 and 6), DHA pre-treated fasted mice (lanes 7 and 8), LA pre-treated fasted mice (lanes 9 and 10), EPA pre-treated fasted mice (lanes 11 and 12) and CV-6504 pre-treated fasted mice (lanes 13 and 14) probed for expression of 20S proteasome α-subunits.**

**Densitometric analysis**



An EPA non fasted control group was also included and several experiments demonstrated that EPA has no effect upon body weight (fig 9.2.6), muscle catabolism (fig 9.2.7) or proteasome activity (fig 9.2.9), suggesting that its effect is not to indiscriminately prevent protein degradation but more to preserve muscle in conditions of elevated catabolism. Taken together these results suggest that EPA attenuates protein catabolism in starvation by inhibiting the upregulation of members of the ubiquitin proteasome pathway.

That EPA can affect the production of 15-HETE is certain and that 15-HETE is capable of inducing protein degradation and proteasome upregulation has been shown in chapter 8. The competitivity of EPA for enzymes of the AA cascade influences the generation of 15-HETE via the LOX pathway and also its further metabolism to biologically active eicosanoids. If EPA is exerting its anti cachectic effects in part through interference with 15-HETE generation it might be expected to do so in a wide variety of catabolic conditions. The findings presented in this chapter suggest that at least in acute starvation this is indeed the case. The fact that the LOX inhibitor CV6504 attenuated proteasome function, expression and protein degradation, with dynamics comparable to EPA, further suggests that EPA is anti-cachexic partly due to its ability to prevent 15-HETE production. Furthermore that DHA and LA had no effect upon proteasome function or expression, it demonstrates that the effect is specific for EPA

Whilst the idea of metabolic competition for 15-HETE production provides an appealing explanation for the effects of EPA, it does not help to explain how PIF/HETE induces an upregulation of proteasome activity in the first place.

A key signal transduction family is NFκB. This is a plausible target for a number of reasons; it is critical for many important cell responses, it is exquisitely redox sensitive (possible allowing its manipulation by peroxidised fatty acids) and not least of all because Watchorn et al (2001) has recently demonstrated its involvement in the reponse to PIF in the liver.

The fact that CV6504, as well as possessing LOX inhibitory activity also has been shown to scavenge active oxygen species (Hussey et al 1996) might further suggest that it is this ability to manipulate a redox sensitive mechanism like NFκB, which results in its anti-tumour ability.

## 10 The Involvement of the NFκB Signal Transduction Pathway in Proteasome Upregulation

### 10.1 Introduction

NFκB is a DNA binding transcription factor which exists in the cytoplasm of most cell types as a dimer of one of a number of subunits. Each subunit belongs to a large family of proteins identified by a conserved n-terminal region called the Rel homology domain (RHD) and which are listed in table 7 below. The RHD consists of two domains of anti-parallel β sheets packed into a sandwich structure that resembles the structure of the immunoglobulin fold (Diehl and Hannink 1994, Perkins et al 1994). Within the RHD lies the DNA binding/dimerisation domains and nuclear localisation sequence (NLS) (Reviewed in Liou and Baltimore 1993).

Table 7) NFκB/Rel and IκB Nomenclature

| Chromosomal Locus | Gene | Proteins | Alternative Designations |
|---|---|---|---|
| NFκB1 | NFκB1 | NFκB 1 (p105 / p50) | KBF1, EBP1 |
| NFκB2 | NFκB2 | NFκB 2 (p100 or p52) | p49, NFκB |
| Rel | c-rel | Rel | |
| Rel A | rel a | Rel A or p65 | p65, NFκB |
| Rel B | rel b | Rel B | I-rel |
| IκB A | IκB a | IκBα | 37kDa,MAD-3 |
| IκB B | IκB b | IκBβ | 43kDa |
| NFkB1 | NFκB 1 | IκBγ | 70kDa |
| BCL3 | bcl 3 | Bcl-3 | 45-56kDa |
| IκBD | IκBd | IκBδ | |
| IκBR | IκBR | IκBR | |
| IκBδ | IκBR | IκBL | |

Class I NFκB proteins (P50 and P52 only) are unique amongst the Rel family in that they are formed as precursors (P105 and P100 respectively) and the 'classical' NFκB comprising one P50 and one P65 subunit therefore contains both a class I and a class II protein (Reviewed in May and Ghosh 1997)

Rel/NFκB proteins are regulated by interaction with a family of structurally related proteins called Inhibitory κB (IκB). In resting cells NFκB is sequestered in the cytoplasm by IκB, which is bound to the RHD.

IκB proteins themselves form a small family, with a core composed of six or more ankyrin repeats, an N-terminal regulatory domain and a PEST containing c-terminal domain, which is thought to be important in phosphorylation, inhibition of NFκB DNA binding and in regulating the stability of IκB (reviewed in Whiteside and Israel 1997). In mammals several IκBs have been identified including IκBα, IκBβ, IκBε, IκBγ, IκBδ, IκBR, IκBL and Bcl3. The different molecules show specificity for inhibiting varying NFκB complexes. This led to the hypothesis that different subsets of NFκB dependent genes are controlled by different IκBs. (Beg et al 1995)

The three classical IκBs are α, β and ε. The first is rapidly degraded in response to inducers of NFκB and is thought to represent the rapid transient response to stimuli. It has been proposed that IκBβ regulates the persistent response of a subset of NFκB inducers (Thompson et al 1995), whilst IκBε is thought to control a slow transient response (Whiteside and Israel 1997).

Some IκBs including IκBα contain a nuclear export sequence and can enter the nucleus where they combine with NFκB dimers and actively transport them to the cytoplasm (Aranzena-Seisdedos et al 1997).

Upon stimulation, the classical pathway for NFκB activation involves IκB phosphorylation. In the case of IκBα this occurs at two conserved serine residues at positions 32 and 36 (Brown et al 1995) in the N-terminal regulatory domain and is catalysed by a serine specific kinase. Both IκB-β and IκB-ε contain similarly located residues suggesting a similar regulatory mechanism for each (Perkins 1997). Phosphorylated IκBs are then further post translationally modified by the addition of polyubiquitin, the major

acceptor sites being arginines 21 and 22. (Reviewed in Karin 1999) Ubiquitination and degradation via the proteasome pathway follows the archetypal E1-E3 enzymatic cascade (this has been discussed in chapter 3.1 pp40).

It has been shown that the only regulated step in IκB degradation is its phosphorylation; and that proteolysis of IκBα also requires sequences at the COOH terminus. A mutant missing 41 residues from the COOH terminal, has been shown to be phosphorylated but is resistant to proteolysis (Brown et al 1995) indicating that phosphorylation is necessary but not sufficient for proteolysis. That the deleted sequences contained areas rich in pro, glu/asp, ser and thre residues suggests that these so called PEST sequences may be necessary for IκBα proteolysis. That the mutant inhibited transfecting ability, but not the ability to bind P65 suggests that cytoplasmic retention and inhibition of DNA binding appear to be separable functions of IκBα, with the latter requiring additional COOH sequences (Brown et al 1995). Degradation of IκB frees the NLS, allowing NFκB to migrate to the nucleus and initiate transcription. In the nucleus, NFκB recognises a specific decameric DNA binding sequence called a κB element, although there is specificity between different NFκB complexes in terms of the sequence to which they will preferentially bind. For example the sequence 5'-GGGRNNYYCC-3' (where R=any purine, Y=any pyrimidine and N any base) is preferred by p50/RelA dimers, whilst p52 displays a distinct specificity for the κB site 5'-GGGATTCCCC-3'. (Reviewed in Perkins 1997). The selectivity in DNA binding means that genes with promoters or enhancers containing various κB elements have the potential to be regulated by specific NFκB complexes. However, when NFκB is activated, it does not necessarily lead to the induction of all genes that contain a κB element within their promoter or enhancer regions. One of the ways in which transcriptional specificity is achieved is through interaction with heterologous transcription factors, for example the induction of HIV-1 LTR by NFκB is dependent upon synergistic, co-operative binding of the constitutively active Sp-1 transcription factor to upstream Sp-1 elements (Perkins et al 1997).

As mentioned, the critical regulatory step in the activation of NFκB is the phosphorylation of IκBα. This is initiated by a high molecular weight (700kDa) IκB kinase complex (IKK). Originally identified in resting cells and later found to be activated in TNFα treated cells, it was discovered that IKK consists of two catalytic subunits IKKα and IKKβ which preferentially form heterodimers in vivo. This catalytic core is coupled to a regulatory subunit referred to as the NFκB essential modulator (NEMO), also designated FIP-3, IKAPP1, or IKKγ, which it is thought functions to link the core to upstream signalling molecules and also to provide a scaffold. Phosphorylation of IKK is the signal for IκB phosphorylation. Both subunits of IKK have been shown to phosphorylate serines 32 and 36 in IκBα. (Reviewed in Peters and Maniatis 2001). The major structural and functional motifs of IKK are shown in the diagram below.

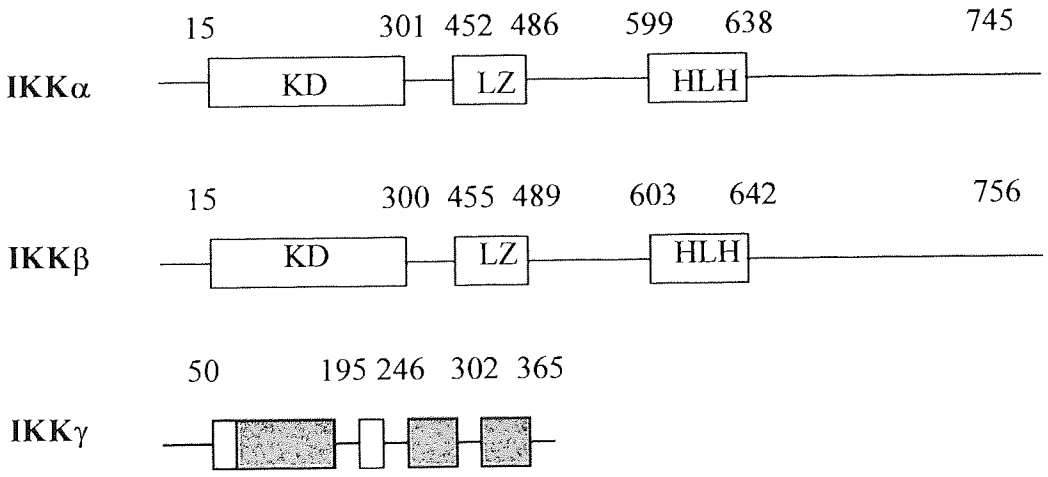



Figure 13) The Three components of IKK (adapted from Karin 1999)

*KD = Kinase domain*

*Open boxes in IKKγ indicate a helical regions and closed boxes denote leucine zipper regions.*

Delhase et al (1999) have suggested a three-step model for the regulation of IKK activity by phosphorylation. Briefly this proposes that IKK kinase (IKKKs) are recruited to IKK via its γ-subunit. This results in phosphorylation of the β-subunit, and this in turn autophosphorylates the remaining subunit, C-terminal serines and surrounding IKKs. It is thought that autophosphorylation is accomplished by intermolecular reactions such that the interaction between α and β (mediated via leucine zippers containing the activation loop in the kinase domain) is altered and the activation loop and C-terminal residues phosphorylated. Multiple phosphorylated serines (<9) results in an inhibition of kinase activity, such that autophosphorylation will eventually shut down IKK, once the upstream signal has disappeared, thus ensuring transient activation of IKK. Deficits in this mechanism prove disastrous. Indeed constitutively active IKK has been detected in Hodgkins disease (Krappmann et al 1999), and is thought to protect some tumour cells from apoptosis (Gilmore et al 1997).

There are exceptions to serine phosphorylation mediated degradation of IκB by the proteasome. Other methods by which NFκB can be activated include, phosphorylation of IκB at tyrosine 42 which causes dissociation and degradation of IκB by a proteasome independent mechanism (Imbert et al 1996). Tyr 42 phosphorylated IκBα appears to associate with the regulatory subunit of PI(phosphoinositide)-3 kinase. The exact mechanisms through which PI3 kinase regulates NFκB activation is not clear. However it is thought that an interaction with the SH2 domain of PI3 results in dissociation of IκB from NFκB and that phosphorylation of nuclear P65 (also by PI-3 kinase) may be involved (Beraud et al 1999). However Tyr 42 is not conserved in all IκBs and so the universality of the pathway is questionable (reviewed in Karin 1999).

Zhong et al (1997) demonstrated that PKA inhibitors reduced NFκB activity. In fact it seems that the catalytic subunit of PKA is actually contained within

the inactive NFκB/IκB complex, upon dissociation of IκB, PKA is activated in a cAMP independent manner to phosphorylate P65 at serine 276 and increase transcriptional activity. Similarly TNFα induces phosphorylation of P65 at Ser 529 inducing transcriptional activity not involving proteolysis of IκB (Wang et al 1998).

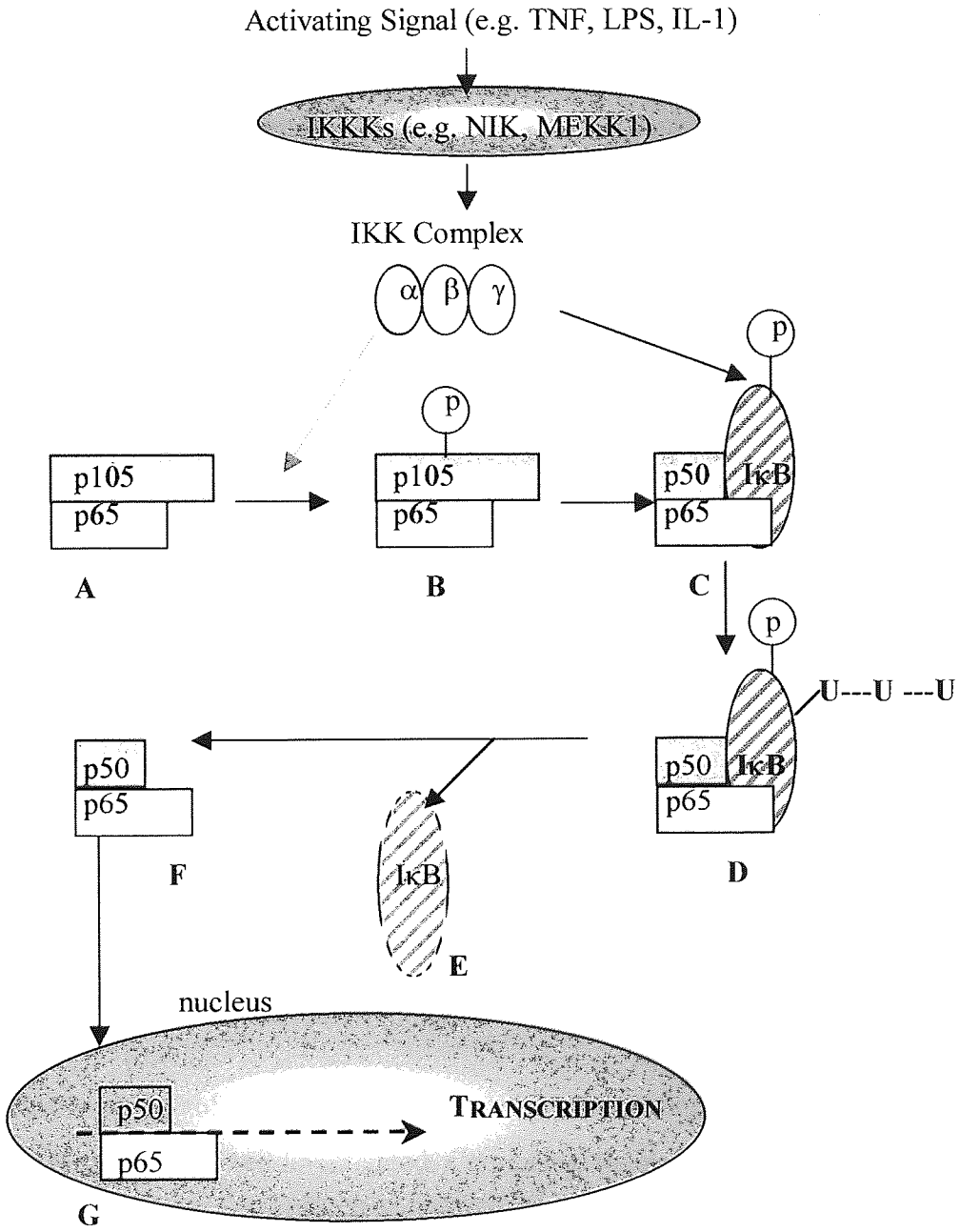
The upstream effectors of IKK are still the subject of intense investigation. An IKK related kinase - NAK (NFκB Activating Kinase) has recently been identified (Tojima et al 2000), which activates IKK through direct phosphorylation of its β activation loop. NAK it seems, is structurally related to other kinases including IKK-1, IKKε and TBK1 (reviewed in Peters and Maniatis 2001) which are known to activate NFκB, but not through phosphorylation of IκB, suggesting that they act further upstream.

Support for the notion that 'cross-talk' exists between NFκB and the MAPK pathway has come from several sources. For example, it is known that TNFα is an inducer of both MAPK and NFκB. NIK (which activates NFκB through IKK's), structurally resembles MAPK kinase kinases. MEKK1 (mitogen activated protein kinase kinase kinase) in particular, which also phosphorylates members of the NFκB pathway, namely IKK kinase and IKKα and IKKβ. However, the most convincing evidence is that the MAPK members MKK2 and MKK3 also directly activate the NFκB members IKKα and IKKβ and that using specific MAPK (P38) inhibitors, inhibits NFκB dependent gene transcription (reviewed in Janssen-Heininger et al 2000). The observation that several NFκB activators including IL-1β and TNFα converge on NIK and that MEKK2 and MEKK3 induce IKK activation and IκBα phosphorylation suggests that a core element of the combined signalling cascade may be MEKK3/IKK (reviewed in Schoonbroodt and Piette 2000).

The kinases responsible for activation of NAK, IKK-1 and IKKε (if these are indeed true IKK kinases) are not fully understood although again members of the MAPK signalling pathway including MAP/MEKK-1 and TRAF may be involved (Karin 1999). However, the fact that interaction of NIK and IKKα has only been observed in vitro at supraphysiological concentrations and not under physiological conditions, and that IKKα, the preferential target for NIK is not directly involved in IKK activation questions its involvement in the NFκB cascade (Karin 1999).

The 'classical' pathway for NFκB activation is represented in figure 14, shown overleaf.

Figure 14) The Classical Activation Pathway for NFκB




*The p50 subunit is formed from a precursor molecule p105 (A), this is phosphorylated by IKK (B) resulting in cleavage to yield p50 which associates with p65 and an IκB to form a mature, inactive NFκB (C). Upon appropriate stimulation, the IκB of the NFκB/IκB complex becomes phosphorylated by IKK (C). IκB is then ubiquitinated (D) and degraded via the proteasome (E). The free NFκB complex (F) then migrates to the nucleus and initiates transcription (G).*

After entering the nucleus and gaining access to the promoters and enhancers of the genes that it regulates, NFκB stimulates transcription by interacting with the transcriptional complex and with co-activator proteins. It has recently been shown that Rel A containing dimers interact with the p300 and CBP co-activator proteins (Perkins 1997) highlighting another area in which the transcriptional specificity of NFκB can be modified.

Of obvious importance, is the ability to appropriately inhibit or shut down the NFκB response. Predictably this appears to be a highly regulated event. Negative feedback loops exist in which κB dependent transcription of inhibitory IκBα or p100/p105 is activated (Baldwin 1996). A particularly interesting example of a negative feedback is that of the A20 protein, which is induced by NFκB resulting in NFκB resistance to TNF, LPS and IL-1 in several cell lines (reviewed in Perkins 1997).

### 10.1.1 The role of lipids

It is thought hat the genes for molecules involved in cell growth and division are regulated by NFκB / IκB, and as PUFAs such as EPA affect these activities, the pathway is potentially, a good target site for PUFA intervention. .

However the role of lipid second messengers as modulators of NFκB is not an area which is well investigated. Ceramides, the products of sphingomyelin hydrolysis, have been shown to activate the NFκB cascade. Fernandez and Dobbelaere (1999) demonstrated that in vitro ceramide treatment of TpM (803) lymphocyte cells resulted in a rapid reduction in the steady state levels of phosphorylated IκBα and IκBβ, although whether this was due to enhanced degradation of the phosphorylated form or to dephosphorylation was not clear. This report is interesting because it is an independent observation that control of the NFκB signalling pathway can involve a glycerolipid.

A recent study demonstrated that the trifluoromethyl ketone analogues of both EPA and AA are capable of suppressing TNFα induced activation of NFκB and in vitro cytosolic phospholipase A2 enzyme activity in a human keratinocyte cell line- HaCaT (Thommesen et al 1999)

### 10.1.2 The role of Redox

It has long been established that NFκB is redox responsive. This is another reason that makes it an attractive potential target for PUFA intervention. Several studies have examined the effects of an altered redox environment upon NFκB activation. It has been shown that reactive oxygen species (ROS) have increased (Tenjinbaru et al 1999; Devary et al 1993; Uckun et al 1993; Binizzi et al 1996; Li et al 1998), decreased (Jin et al 1997; Kang et al 1998; Schenk et al 1994; Flescher et al 1998) or had no effect (Suzuki et al 1995) upon various parameters of NFκB activity including translocation of various subunits to the nucleus in response to LPS, $H_20_2$ and TNFα.

It is interesting to note the work of Begin et al (1988) who investigated the relationship between tumour cell cytotoxicity by PUFAs and the level of lipid peroxidation. Formation of HPETEs (the intermediate of HETEs) results from the successive formation of PUFA radicals. The reaction with neighbouring PUFAs creates a chain reaction of free radical propagation which vitamin E stops. Begin et al found that large amounts of conjugated dienes were in fact still formed in vitamin E treated cells. Vitamin E reduced the production of HPETE radicals, suggesting that HPETEs do not themselves constitute the peroxidation products rather they appear as precursors of the toxic material. Furthermore transition metals catalyse HPETE decomposition. Iron and copper accelerated the destruction of tumour cells, further suggesting that the products of HPETE decomposition were in fact the active cytotoxic product.

Behl et al (1999), has shown that the anti-oxidant tocopherol in its synthetic and natural forms protects against oxidative stress by *increasing* the transcriptional activity of NFκB in clonal hippocampal and primary cerebellar neurones treated with the excitatory amino acid glutamate and with the hydroxyl radical precursor hydrogen peroxide. Similarly, DNA binding and transactivation of NFκB has also been induced in HeLa cells upon treatment with the *anti*oxidants pyrrolidone dithiocarbamate and N-acetyl-L-cysteine (Meyer et al 1993). On the other hand Grilli et al (1996) showed that the *suppression* of neurotoxin induced NFκB mediates neuro-protective properties, suggesting that the induction of NFκB in this case results in the production of pro-apoptotic genes.

NFκB like other transcription factors is sensitive to oxidative modification of a particular cysteine at position 62 in p50, which is crucial for DNA binding activity (Toledano et al 1991). When such residues undergo oxidation, the function of the transcription factor can be dramatically altered by preventing the DNA binding or transactivating activity by modifying contact with the basal transcription machinery.

It has recently been demonstrated that ROS cause the release of IκB from the NFκB complex (Schreck et al 1991) and that many types of anti-oxidants diminish or completely eliminate NFκB activation. For example BHA and tocopherol can decrease NFκB translocation and activity (Meyer et al 1992). The precise molecular mechanisms through which antioxidants influence NFκB is unknown, but there is some evidence that AOE372, an antioxidant (redox sensitive) thioredoxin peroxidase may function to regulate IκB phosphorylation (Jin et al 1997). Likewise several serine phosphatases are known to be redox sensitive (Reviewed in Allen and Tresini 1999). Oxidative stress could for example affect the structure of receptors such that they could no longer be phosphorylated or inactivate the dephosphorylation enzymes. Both possibilities could result in a predominance of spontaneous kinase activity (Schoonbroodt and Piette 2000)

Thus not only is NFκB itself susceptible to redox manipulation, but potentially so too are those kinases and phosphatases upstream.

It is well known that peroxidised lipids can inhibit mitosis and that even low levels can totally halt cell cycle progression (Reviewed in Allan and Tresini 1995). Furthermore the growth rate of tumour cells has been found to correlate inversely with lipid peroxidation. This is significant because taken together these observations suggest one mechanism (i.e. redox manipulation of NFκB) through which the peroxidisable eicosanoids 15-HETE and/or EPA might affect tumour cell growth and cachexia.

### 10.1.3 The role of NFκB in Cancer and Cachexia

#### 10.1.3.1) NFκB and Tumour Growth

There is a large body of evidence suggesting a role for the rel/NFκB/IκB family in oncogenesis and cell growth. For example v-rel, c-rel, rel-A (NFκB subtypes) and Bcl-3, P100, P105 and IκBα (IκB subtypes) have all been linked with malignant transformation as a result of either gene amplification, rearrangement, overexpression or translocation (Reviewed in Luque and Galinas 1997).

When injected into chickens, the v-Rel oncogene of the reticolendotheliosis virus strain T (Rev-T), rapidly induces aggressive and fatal lymphomas within 7-14days (Gilmore et al 1996). Carrasco et al (1996) demonstrated that the transforming activity of v-Rel was not confined to avian species when they induced aggressive T-cell lymphoma in transgenic mice expressing v-Rel. The transforming ability of the oncogene is due to the deletion of 118 C-terminal amino acids that which are though to be involved in the cytoplasmic sequestration of the non-transforming counterpart (Reviewed in Kamens et al 1990).

Rearrangements and amplifications of cellular rel and NFκB genes have also been implicated in lymphoid tumours (reviewed in Lucque and Galinas 1997). C-rel has been shown to be amplified in both diffuse and follicular large cell lymphoma and rearranged in primary mediastinal B-cell, diffuse large cell and in avian T-cell lymphomas. It has been demonstrated that Rel a is rearranged in B-cell non Hodgkin's lymphoma and in multiple myeloma; amplified in diffuse large cell lymphoma; point mutated in multiple myeloma and constitutively active in Hodgkin's disease. Similarly NFκB1 is rearranged in acute lymphoblastic leukaemia, and NFκB2 is rearranged in cutaneous T-cell lymphoma, B-cell non-Hodgkin's lymphoma, B-cell chronic lymphocytic leukaemia and in multiple myeloma. In solid tumours, these subunits have been overexpressed, amplified or variantly spliced in non-small cell lung, ovarian, breast and colon carcinomas (Reviewed in Luque and Galinas 1997).

Interestingly constitutive activation of upstream activators of NFκB has also been linked to certain cancers. Palayoor et al (1999) considered the NFκB status in the prostate cancer cell lines PC-3 and DU-145 and found that not only was DNA binding of NFκB constitutively activated, but so to was IKK and in particular IKKα.

The role of NFκB in apoptosis is controversial. The first indication came from studies with the avian Rev-T virus discussed above. Neiman et al (1991) have shown that lymphoma derived cell lines and those transfected with Rev-T display suppression of apoptosis induced by a variety of apoptotic agents. Conversely it has recently been shown that the inhibition of NFκB results in abrogated p53 induced apoptosis (Ryan et al 2000). This kind of disparity amongst the literature prevailed for many years and it was unclear whether activation or inhibition of gene expression by NFκB promoted cell survival.

However, accumulated evidence now suggests that NFκB generally provides more of a protective role against cell death. The observations that CD40

ligand binding in WEHI-231 B cells rescues those cells from antigen receptor mediated apoptosis in a mechanism associated with a maintenance of Rel activity, and that treatment of WEHI-231 B cells with pyrrolidinedithiocarbamate – an NFκB inhibitor – induced apoptosis suggest that NFκB is critical for cell survival in this cell line (Reviewed in Sonenshein 1997)

The molecular mechanisms involved in these processes are not yet fully understood, recent studies have suggested that the anti-apoptotic proteins TRAF(TNF receptor associated factor)-1 and TRAF-2, cIAP(cellular inhibitor of apoptosis protein)-1 and cIAP-2 which are transcriptionally regulated by NFκB function to suppress apoptosis through the inhibition of caspase-8 activity (Wang et al 1998)

Azuma et al (2001) showed that the potent chemotherapeutic agent – 5-Fluorouracil (5-FU) suppresses NFκB activation in the human salivary gland cancer cell line cl-1 by mediating upregulation of IκBα expression. This did not affect the expression of the upstream kinases IKKs, NIK or MEKK1 although the activity of IKK was suppressed in cells treated with 2μg/ml 5-FU. The expression of FLIP (Fas associated death domain-like interleukin 1-converting enzyme-inhibitory protein) was also downregulated and as apoptosis was clearly observed, the authors postulated that 5-FU exhibited its anti-tumour activity through suppression of NFκB via inhibition of IKK activity.

Cytokines play an important role in cancer cachexia. IL-1, IL-6, IFNγ and particularly TNFα may be involved in some instances of cachexia where they can cause metabolic changes affecting tumour growth. The role of TNFα induced activation of NFκB has recently been the focus of much attention.

It appears that TNFα activates two signalling pathways in cells. One leads to apoptosis and the other to survival. It is when the balance between apoptotic

and survival signals is imbalanced that cell death results. The survival pathway activates NFκB and results in the production of anti-apoptotic proteins, an important example of which is thought to be the protein GSK-3β. (Reviewed in Pomerantz and Baltimore 2000).

In support of this idea, Beg and Baltimore (1996) have shown that the fibroblasts and macrophages from relA double knockout mice when compared to $RelA^{+/+}$ have reduced viability in response to TNFα. That reintroduction of RelA into knockout mice resulted in enhanced survival demonstrates that RelA is essential for protection from TNFα. This work demonstrated that the RelA subunit has an 'active' role in protecting cells from TNFα death and not just a 'developmental' role which results in $RelA^{-/-}$ cells which are predisposed to death in the presence of TNFα. Thus it appears – at least in this model- that TNFα transmits one signal eliciting cell death and another which is dependent on RelA, that protects against cell death by the induction of gene expression. Consistently the apoptotic gene A20 for example is induced in $RelA^{+/+}$ 3T3 cells but not in $RelA^{-/-}$ cells after TNFα treatment. However the finding that transfection of A20 in RelA knockouts does not prevent cell death suggests that multiple genes are probably required.

Using the TNFα resistant human fibrosarcoma cell line HT1080 and an IκBα Ser => Ala HT1080I mutated super repressor, Wang et al (1996) showed that TNFα induced apoptosis only in the super repressor mutant and not in the control cell line. Similarly both the chemotherapeutic agent daunorubicin and ionising radiation activated NFκB in control but not HT1080I cells and this corresponded to their ability to induce cell death. Conversely the chemotherapeutic agent staurosporine did not affect NFκB activation or induce apoptosis.

Thus there is evidence that the activation of NFκB and apoptosis induced by TNFα are separate events that occur independently. An idea supported by

the observation that apoptosis occurs most rapidly in the absence of de novo RNA and protein synthesis (reviewed in Antwerp et al 1996).

### 10.1.3.2 NFκB and Cachexia

As well as involvement in tumour growth, agonist and particularly TNFα induced activation of NFκB has been linked to a modulation of two critical aspects of cachexia, the immune response and skeletal muscle proteolysis

Caamano et al (1999 and 2000) showed that when $RelB^{-/-}$ and $NF\kappa B_2^{-/-}$ mice were challenged with *toxoplasma gondii* they developed severe toxoplasmic encephalitis within a few weeks post infection due to a reduced capacity of splenocytes to produce IFN-γ, showing that these NFκB subunits are critical for a functioning immune response.

Prostaglandins, which (as discussed in earlier chapters) have long been known to affect immune responses are the product of COX2 metabolism. COX 2 has been shown to be involved in cancers of the lung (Hida 1998), epithelium (Chan 1999) and particularly colon (Reddy 1996). Lee and Ip (1992) showed that COX inhibitors suppressed arachidonic acid metabolism to $PGE_2$ in the TMT-081 rat mammary tumour cell line. This correlated with a suppression in DNA synthesis and cell growth. This is significant because it has been shown that COX-2 is an NFκB inducible gene (Rossi et al 2000) and that NFκB regulates COX-2 expression (which is constitutively expressed) in the human gastric cancer cell line AGS (Lim et al 2001).

Regarding the involvement of NFκB in skeletal muscle proteolysis, the most interesting aspect is that it seems possible that it may be a shared intermediate in both catabolic and anabolic pathways.

For example catabolic doses of glucocorticoids can antagonise NFκB induced proteasome suppression by increasing cytosolic levels of IκBα and by interfering with the binding of NFκB to its response element in the C3

promotor region. (Llovera 1997). Glucocorticoids have also been shown to inhibit NFκB activity in extracts from the cerebral cortex of rats following stimulation with various seizure inducing treatments (Unlap and Jope 1995)

Du et al (2000) demonstrated that NFκB is a repressor of C3 proteasome subunit expression in rat L6 muscle cells and that preventing the transcription of just one subunit (C2) reduces not only the number of functioning proteasomes, but also proteolytic activity and protein degradation (Grune et al 1998). Du et al (2000) also showed that glucocorticoids stimulate C3 subunit expression by opposing the suppressor action of NFκB. It is thought that glucocorticoids antagonize the interaction of the NFκB protein with an NFκB response element in the promoter region of C3. The region, between –400 and –256, did in fact contain two elements similar to a consensus c-rel/NFκB element: a downstream element ($NF\kappa B_{(d)}$) in a forward orientation and an upstream inversely orientated element ($NF\kappa B_{(u)}$), the latter of which actually functions as a negative transcriptional regulatory element. The authors also found that increasing the levels of activated NFκB with cells cotransfected with an expression plasmid encoding dominant negative forms of IκBα pC3-460 and pCMVIκB (K21R/K22R) and also 24hour incubations in TNFα, IFNγ and LPS, suppressed C3 subunit promoter activity and expression. Accordingly the NFκB inhibitor pyrrolidine dithiocarbamate, stimulated C3 subunit transcription. Surprisingly supershift analyses showed that the P65 subunit was a constitutively active negative transcriptional regulator of C3 expression. This reflects the unusual nature of these findings because NFκB is more typically inducible.

However a variety of evidence is in direct contrast to the idea of NFκB as an anabolic transcription factor, suggesting in fact that it also plays a pivotal role in muscle catabolism.

It has recently been shown that the rise in cytokine production seen in cachexia may be critical in the loss of muscle mass, in that cytokines

function to inhibit the formation of new myofibres. Guttridge et al (2000) showed that TNFα prevented the differentiation of myoblasts into myotubes which were capable of synthesising myosin. It is thought that TNFα activates NFκB which suppresses MyoD (a bHLH transcription factor family member responsible for skeletal muscle differentiation and myofibril formation) and also the late stage differentiation marker - myosin heavy chain (MHC) in differentiated myotubes. These workers also showed that Interleukin 1β, Il-6 and IFNγ in combination with TNFα (and that IFNγ alone) had no effect in vitro, on skeletal muscle specific gene expression. However TNFα in combination with IFNγ did cause a significant reduction in Myo D and MHC levels.

Similarly Kawamura et al (1999) used synthetic double stranded oligodeoxynucleotides as 'decoy' cis elements to block the binding of nuclear factors to promoter regions. They found that transfection of an NFκB decoy (compared to a 'scrambled' control decoy) resulted in attenuation of the loss of overall weight, epididymal fat and gastrocnemius muscle mass in mice bearing the cachexia inducing MAC26 tumour. That this was accompanied by a decrease in IL-6 mRNA in the tumour (although no affect on tumour growth was observed) and that IL-6 contains a κB like sequence in its promoter region, led the authors to hypothesize that it was the cytokines regulated by NFκB which played the pivotal role in the induction of cachexia in MAC26 bearing animals. Whilst, it has been shown that TNFα and IFNγ can activate NFκB and induce weight loss in animals, when added to ex vivo skeletal muscle samples they cannot induce degeneration or be linked to an increase in proteasome subunit expression, supporting the notions that these factors cannot induce muscle catabolism directly and that other factors are required for cachexia (Moldawer et al 1987).

The biology of muscle loss is complicated further by the effects of NFκB upon apoptotic pathways as discussed in chapter 10.1.3.1. It has been shown that NFκB can prevent or induce cell death and be activated under conditions of both anabolism and catabolism. This there are conflicting theories about

the relative contribution of NFκB mediated apoptosis in skeletal muscle catabolism, none of which have been investigated to date.

However despite the role of apoptosis, the main element of muscle loss is increased degradation of structural proteins by the proteasome pathway. Penner et al (2001) examined the role of glucocorticoids in NFκB activation, this time using a rat model of sepsis induced muscle cachexia. Coecal ligation and puncture resulted in an early (4h) upregulation of NFκB activity followed by an inhibition after 16hours to below control levels. There were, however, no consistent changes seen in IκBα levels in muscles from sham operated rats, although changes in IκBα are rapid and transient and could have been missed. Furthermore it is possible to activate NFκB without the degradation of IκB. The observation that the glucocorticoid receptor antagonist RU38486 increased NFκB activity and that RU38486 has been shown to prevent the increased ubiquitin expression and protein degradation in septic muscle, suggested that glucocorticoids regulated this transcription factor and that it might be important in the degradation of septic muscle.

Li et al (1998) have demonstrated that differentiated skeletal muscle cells treated with TNFα lose total protein and adult myosin heavy chain content. They also demonstrated that NFκB is activated by concentrations of TNFα in skeletal muscle, similar to those seen in cachectic cancer patients and that the system is regulated by endogenous reactive oxygen species. The response observed was similar to that seen in cachexia in that there was muscle atrophy without overt cell death. However, the investigators comment that it is possible that although NFκB is activated, it assumes a protective role and is not directly responsible for the increased catabolism. Interestingly this study –as well as being the first to demonstrate the significance of the NFκB pathway in muscle - also showed that the activity of NFκB could be correlated to an increased ubiquitin conjugation to muscle proteins and a subsequent rise in ubiquitin mRNA, suggesting that **NFκB might function to increase the expression of members of the ubiquitin-proteasome pathway in skeletal muscle.**

Watchorn et al (2001) was the first to establish a link between NFκB and PIF. The factor exhibits substantial binding to only two adult tissues - skeletal muscle and liver. Using isolated human hepatocytes and the HepG2 cell line, Watchorn and colleagues showed that PIF influences hepatic gene expression, through NFκB and STAT-3 dependent pathways after only 15-30min and after 24hours a significant increase in the production of IL-6 and ICAM (intercellular adhesion molecule)-1 in primary hepatocytes was detected. The effects of PIF on several hepatic genes were then examined and the authors found that after 48hours CRP(C reactive protein) was increased significantly and transferrin levels were decreased significantly in primary hepatocytes. This work demonstrates that PIF can increase pro-inflammatory cytokine production via NFκB and as the authors suggest, this may contribute to the role of PIF in cachexia via its ability to produce an environment which favours a chronic inflammatory state.

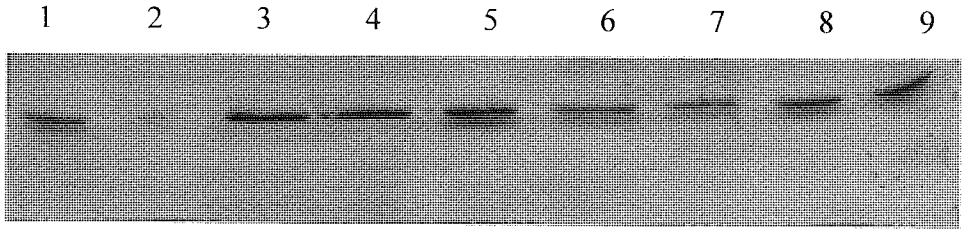
Taken together the observations of Li et al (1998) and Watchorn et al (2001) suggest a role for NFκB in the response to PIF, and that, in muscle NFκB can function to modulate expression of the proteasome pathway, the single most significant pathway responsible for the elevated skeletal muscle catabolism induced in the MAC16 model. The question that arises is, do PIF and 15-HETE mediate their effects upon the proteasome pathway in skeletal muscle through an NFκB dependent mechanism also?

## 10.2 Results and Discussion

### 10.2.1 The effects of PIF/15-HETE and EPA upon cytosolic IκBα levels

Figure 10.2.1.1 shows IκBα levels in C2C12 myotubes treated with 0.1 and 1μg/ml PIF for 30, 60 and 120 min. There was a decrease in the levels of cellular IκBα after a 30min incubation in 0.1μg/ml PIF, but not at 1μg/ml. This correlates with previous studies which have shown that the protein degrading activity and effects upon the proteasome peak at 0.1μg/ml PIF and fall by 1μg/ml. When analysed densitometrically (fig 10.2.1.2) the levels of IκBα fall by 75% (from 100% untreated control to 25%). The levels of IκBα return to normal after 60min and remain at this level after 120min indicating that the cell has recovered. IκBα was chosen for study as it is rapidly degraded (and resynthesized) by inducers of NFκB (reviewed in Whiteside and Israel 1997), a quality that facilitates its experimental manipulation and measurement. These results support the hypothesis that this IκB subtype represents an immediate and transient response of NFκB.

**Fig. 10.2.1.1) The Effects of PIF on IκBα Expression in C2C12 Myotubes at 30, 60 and 120 mins.**




| | | |
|---|---|---|
| 1 | 0μg/ml PIF | 30mins |
| 2 | 0.1μg/ml PIF | 30mins |
| 3 | 1μg/ml PIF | 30mins |
| 4 | 0μg/ml PIF | 60mins |
| 5 | 0.1μg/ml PIF | 60mins |
| 6 | 1μg/ml PIF | 60mins |
| 7 | 0μg/ml PIF | 120mins |
| 8 | 0.1μg/ml PIF | 120mins |
| 9 | 1μg/mlPIF | 120mins |

**Fig. 10.2.1.2) The effects of PIF on IkBα expression at 30, 60 and 120mins in C2C12 myotubes - Densitometric analysis**

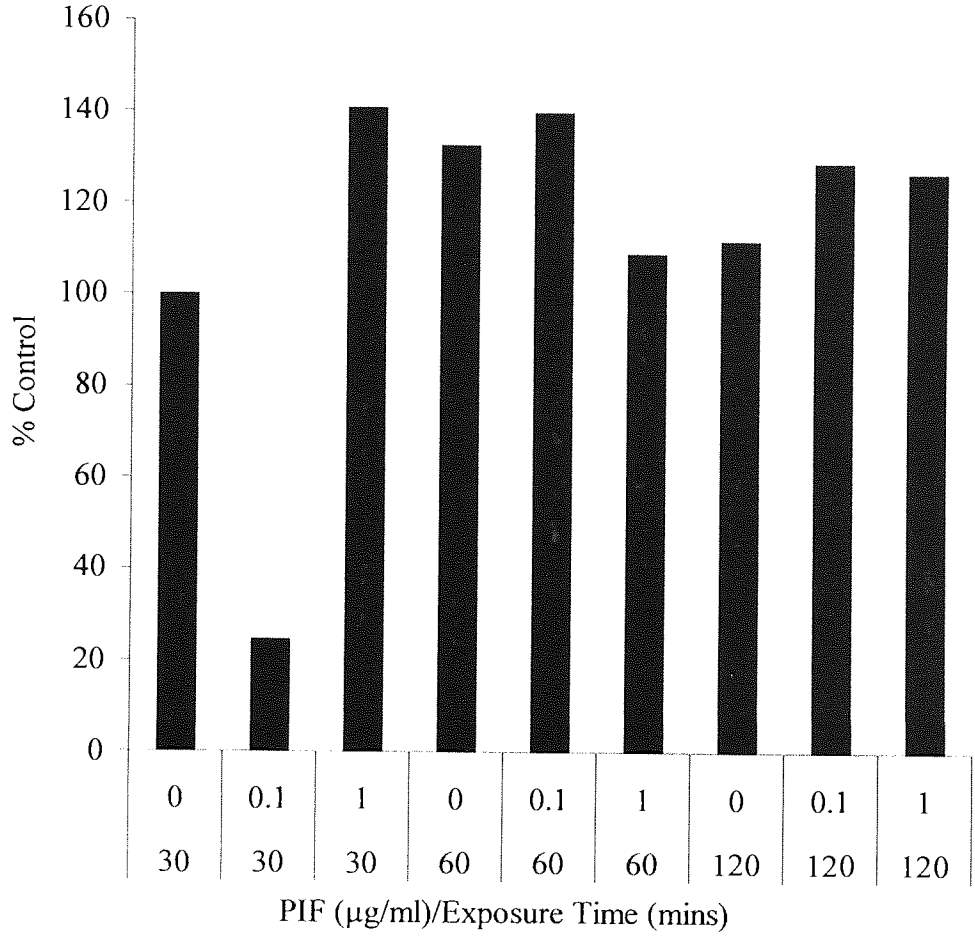
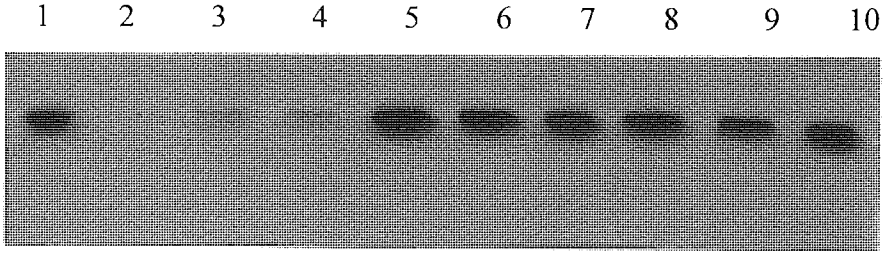



Figure 10.2.1.3 shows IκBα levels in C2C12 myotubes treated with a wider concentration range of PIF for 30min. There was a decrease in IκBα levels, at 0.1μg/ml, 0.2μg/ml and 0.4μg/ml of 99.9%, 89.7% and 87.1% control, respectively (figure 10.2.1.4). This peak of activity at the concentration of 0.1μg/ml corresponds to the effects of PIF on proteasome activity and expression. However 1μg/ml PIF and all concentrations in the presence of EPA do not fall below control levels. This indicates that EPA prevents the degradation of IκBα.

**Fig 10.2.1.3) The effects of PIF on IkBα expression in C2C12 myotubes in the presence and absence of EPA**




| | | | |
|---|---|---|---|
| 1 | 0μg/ml PIF + 0μM EPA | 6 | 0μg/ml PIF + 50μM EPA |
| 2 | 0.1μg/ml PIF +0μM EPA | 7 | 0.1μg/ml PIF +50μM EPA |
| 3 | 0.2μg/ml PIF +0μM EPA | 8 | 0.2μg/ml PIF +50μM EPA |
| 4 | 0.4μg/ml PIF +0μM EPA | 9. | 0.4μg/mlPIF +50μM EPA |
| 5 | 1μg/ml PIF + 0μM EPA | 10 | 1μg/mlPIF +50μM EPA |

**Fig 10.2.1.4) The effects of PIF on IkBα expression in C2C12 myotubes in the presence and absence of EPA - Densitometric analysis**

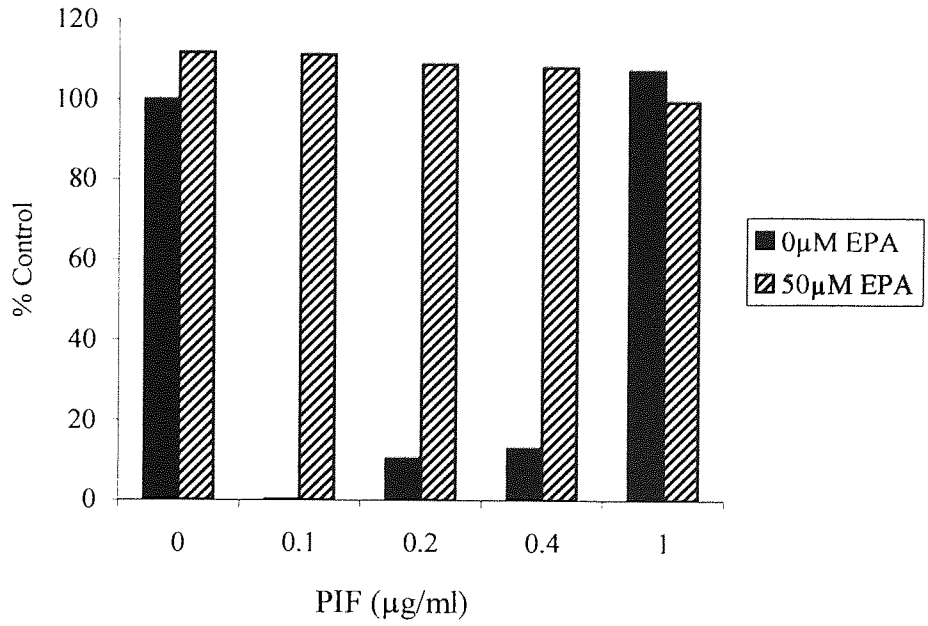
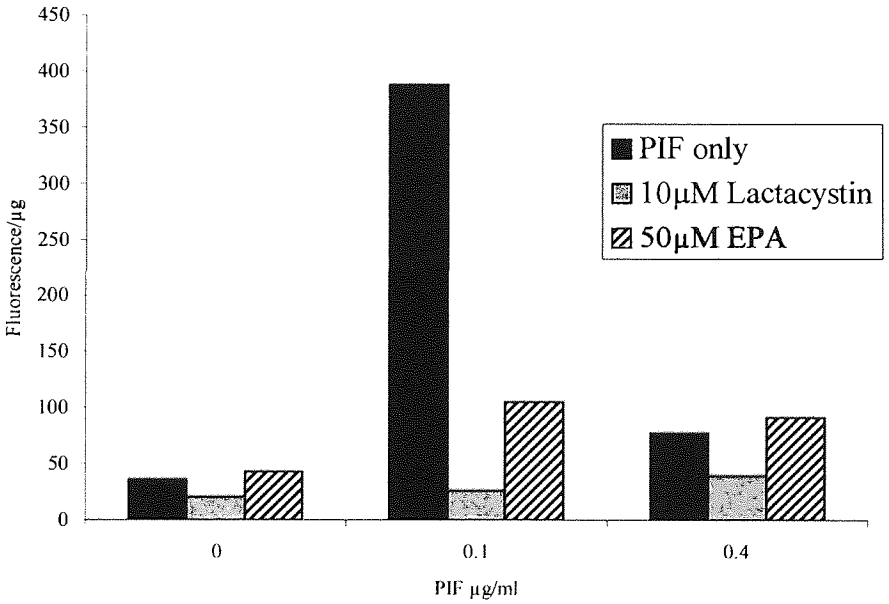


Figure 10.2.1.5 shows that 0.1μg/ml PIF produces a statistically significant ($p<0.001$) activation of the chymotrypsin-like enzyme activity of the proteasome after 24hours as determined by one way ANOVA with Tuckey-Kramer post test. This activity is reduced to control levels in the presence of EPA and lactacystin and correlates inversely with IκBα levels.

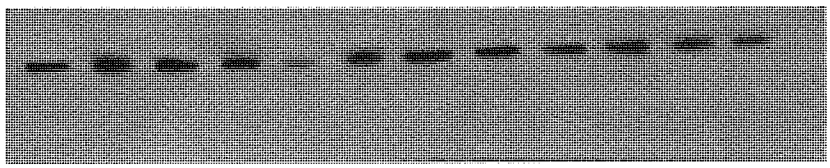
**Fig 10.2.1.5) The effects of 24hour exposure of PIF on the 'Chymotrypsin-like' activity of the proteasome in C2C12 myotubes in the presence and absence of EPA**




Figures 10.2.1.6 and 10.2.1.7 show a similar pattern of results for the effects of 15-HETE on IκBα levels. A concentration of 0.05μg/ml 15-HETE resulted in IκBα levels which were decreased by 85% as analysed by densitometry. In the presence of EPA this decrease was completely abolished and levels of IκBα did not differ from normal. Again this concentration correlated to the peak of activity seen for proteasome expression.

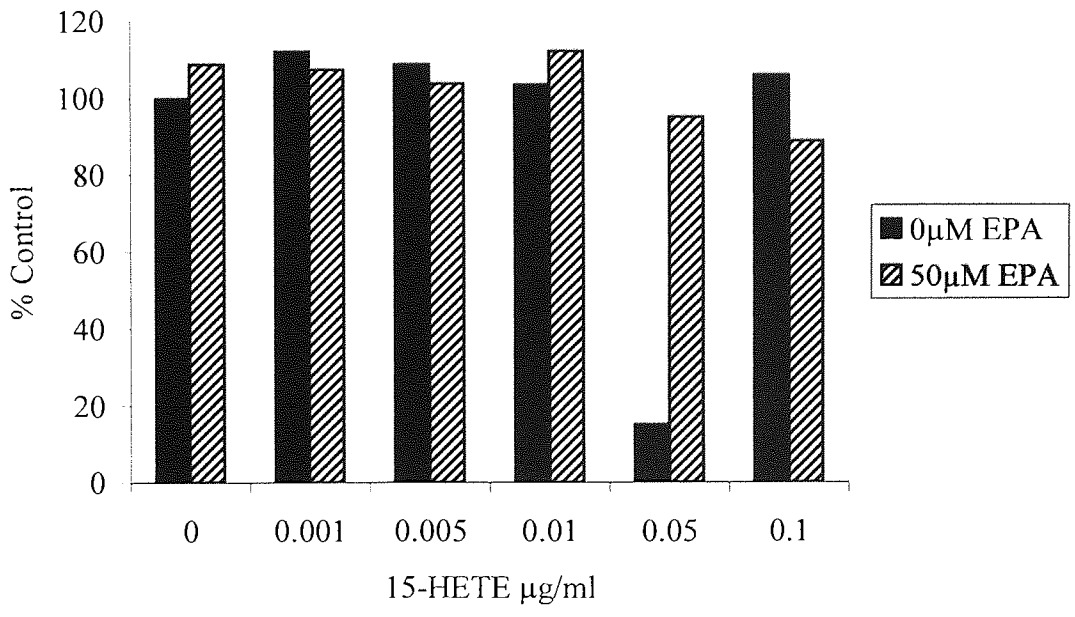
**Fig 10.2.1.6) The effects of 15-HETE on IkBα expression in C2C12 myotubes in the presence and absence of EPA**

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



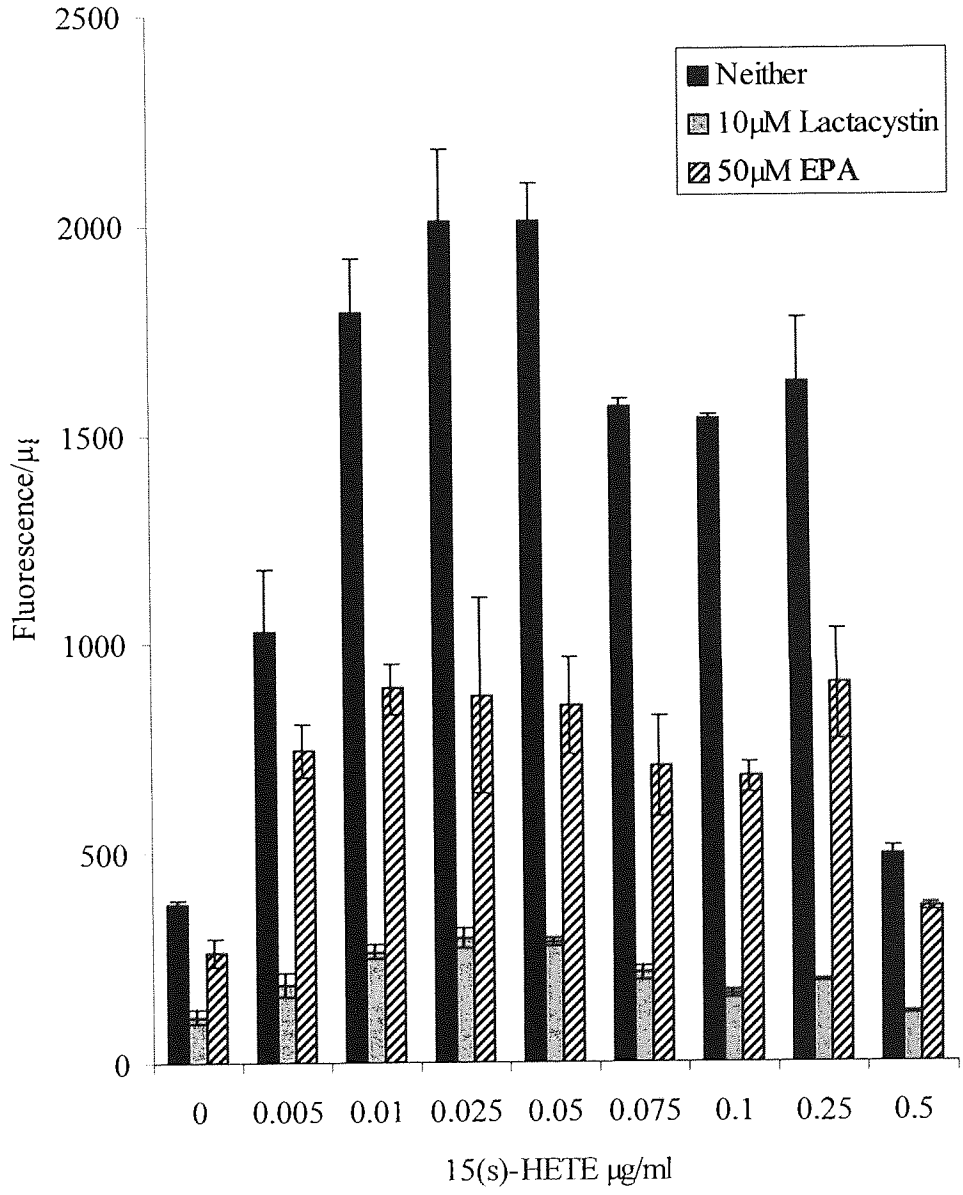
1 0μg/ml 15-HETE+0μM EPA
2 0.001μg/ml 15-HETE+0μM EPA
3 0.005μg/ml 15-HETE+0μM EPA
4 0.01μg/ml 15-HETE+0μM EPA
5 0.05μg/ml 15-HETE+0μM EPA
6 0.1μg/ml 15-HETE+0μM EPA
7 0μg/ml 15-HETE+50μM EPA
8 0.001μg/ml 15-HETE+50μM EPA
9 0.005μg/ml 15-HETE+50μM EPA
10 0.01μg/ml 15-HETE+50μM EPA
11 0.05μg/ml 15-HETE+50μM EPA
12 0.1μg/ml 15-HETE+50μM EPA

**Fig 10.2.1.7) The effects of 15-HETE on IkBα expression in C2C12 myotubes in the presence and absence of EPA - Densitometric analysis**



A similar picture has emerged of altered proteasome activity in myotubes incubated in 15-HETE (fig 10.2.1.8). One Way ANOVA with Tuckey-Kramer post-test shows that the activity is elevated at 0.005μg/ml 15-HETE where $p<0.01$, and at all other concentrations (i.e.0.01-0.25μg/ml) to $p<0.001$ (Excepting 0.5μg/ml where $p>0.05$). The peak of activity centred around 0.05μg/ml when proteasome activity increased more than five-fold (525% of untreated control). This correlated with the concentration at which IκBα levels in the cell were measurably decreased. In the presence of EPA and lactacystin these increased activities were all reduced, 0.05μg/ml showing the most significant reductions from 525% to 76% and 223% untreated control for lactacystin and EPA respectively. In the presence of 50μM EPA (and also 10μM lactacystin) there is no difference between untreated controls and any concentration of HETE ($P>0.05$), indicating that EPA has reduced the elevated proteasome activity to a level that is not significantly different from controls.

**Fig 10.2.1.8) The effects of 24hour exposure of 15-HETE on the chymotrpysin like activity of the proteasome in C2C12 myotubes in the presence and absence of EPA**

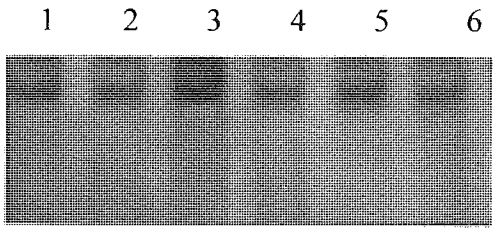


### 10.2.2 The effects of PIF/15-HETE and EPA upon nuclear NFκB levels

Fig 10.2.2.1 and 10.2.2.2 are EMSAs showing both 15-HETE (10.2.2.1) at 0.01μg/ml and 0.05μg/ml and PIF (10.2.2.2) at 0.1μg/ml and 0.4μg/ml (previously determined active range) in the presence and absence of EPA, on the electrophoretic mobility of a $\gamma^{33}$-p labelled NFκB oligonucleotide. The EMSAs presented in this chapter are representative of several experiments, each EMSA included control reactions in which samples were incubated with unlabelled NFκB and an unlabelled unrelated oligonucleotide (CREB), the competition of the κB sites on the unlabelled NFκB, with the $\gamma^{33}$-p labelled NFκB oligonucleotide, for the available NFκB in the nuclear sample, results in a weaker shifted band. Presence of an unlabelled unrelated oligonucleotide does not alter the intensity of the specific NFκB band although it alters the intensity of non-specific bands, thus by comparing competitors and non-competitors to test reactions, the specific NFκB shift can be identified. All EMSA gels were electrophoresed until the dye front almost reached the base of the gel, typically specific shifts like the ones shown here, were found in the middle of the gel. Only one sequence specific band was identified by competition assays suggesting stimulation of a single dimer pair by PIF and 15-HETE. Figures 10.2.2.3 and 10.2.2.4 are the densitometric analyses of these gel shifts and demonstrate an increase of 81% and 181% for 0.1 and 0.4μg/mlPIF, in the presence of EPA the increased activity is reduced by 25% and 144% respectively. 15-HETE increased nuclear levels of NFκB by 174% at 0.05μg/ml, and this was reduced by 190% in the presence of EPA. A repeat EMSA (figs 10.2.3.4 and 10.2.3.5pp218-219) shows that 15-HETE increases nuclear NFκB levels by 43%, 75% and 67% at 0.01μg/ml, 0.05μg/ml and 0.1μg/ml respectively and that in the presence of EPA, these levels were reduced to 5%, 29% and 37% increase above untreated control respectively. The increased levels of NFκB demonstrated in the nucleus in response to PIF and 15-HETE and the attenuation of this by EPA correspond to those concentrations, which induce both degradation of IκBα and increased proteasome activity.

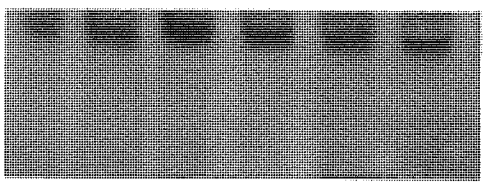
**Fig 10.2.2.1) The Effects of PIF and 15-HETE on the Electrophoretic Mobility of [$\gamma^{33}$p]-NFkB in C2C12 myotubes in the presence and absence of EPA**

**Figure 10.2.2.1**




1 0μg/ml 15-HETE+0μM EPA
2 0.01μg/ml 15-HETE+0μM EPA
3 0.05μg/ml 15-HETE+0μM EPA
4. 0μg/ml 15-HETE+50μM EPA
5 0.01μg/ml 15-HETE+50μM EPA
6 0.05μg/ml 15-HETE+50μM EPA

**Figure 10.2.2.2**




1 0μg/ml PIF+0μM EPA
2 0.1μg/ml PIF+0μM EPA
3 0.4μg/ml PIF+0μM EPA
4 0μg/ml PIF+50μM EPA
5 0.1μg/ml PIF+50μM EPA
6 0.4μg/ml PIF+50μM EPA

**Fig 10.2.2.3) The effects of PIF on the electrophoretic mobility of [$\gamma^{33}$-P]-NFkB in the presence and absence of EPA - Densitometric analysis**

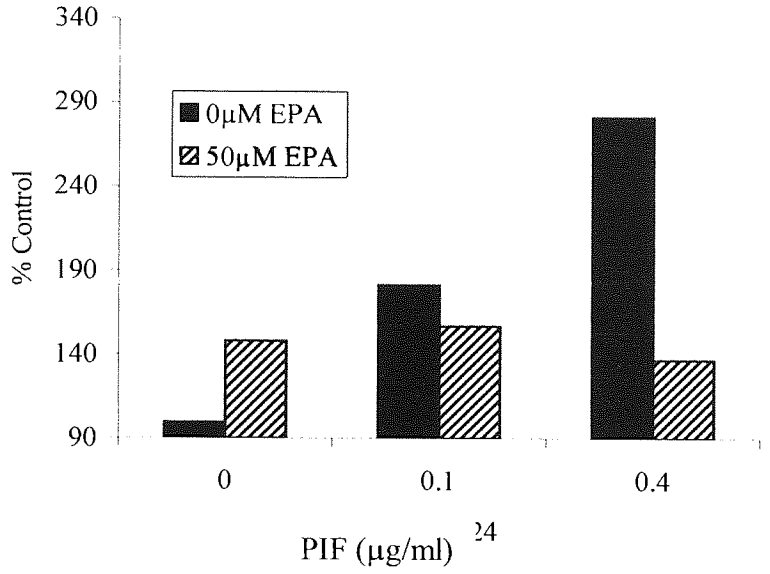
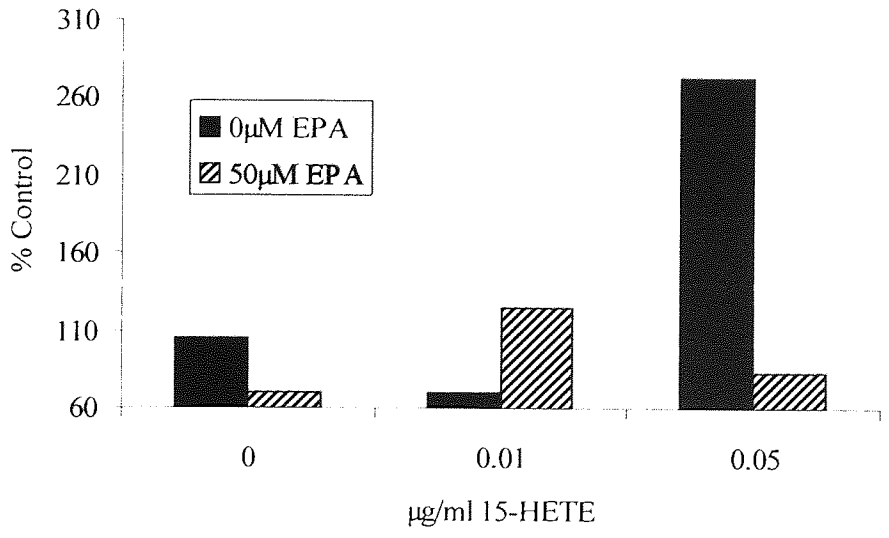



**Fig 10.2.2.4) The effects of 15-HETE on the electrophoretic mobility of [$\gamma^{33}$-P]-NFkB in the presence and absence of EPA - Densitometric analysis**



### 10.2.3 Dimer composition

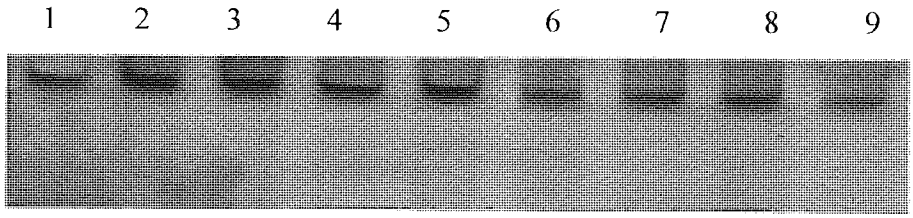
It has been established that different IκB's inhibit different NFκB subunits. It is thought that IκBα and β preferentially inhibit Rel and Rel-A (P-65) containing complexes (reviewed in May and Ghosh 1997) It is tempting to speculate that as IκBα is degraded by PIF after 30min, that either Rel or P65 might be subunits which are involved in the response to PIF. A role for P65 in muscle degeneration was provided by Guttridge et al (2000) who showed that the P65 subunit alone strongly blocked Myo D activity, and that overexpression of this subunit was enough to reduce Myo D mRNA levels. Deletion analysis showed that nucleotides 539-914 were required for this response and that the regulation of Myo D was dependent on an NFκB responsive gene.

The role of Rel was not investigated and so cannot be commented on further. However, supershift analysis with an anti-P65 antibody, did not result in any further shift, above that seen in the absence of anti-P65 (results not shown). This might indicate that the P65 subunit is not part of the dimer which translocates in response to PIF, although it may be that the assay simply failed to measure the shift.

Figures 10.2.3.1 – 10.2.3.3 show experiments performed using SN50. This is a peptide which controls agonist induced nuclear translocation of the P50 subunit of NFκB, it contains a hydrophobic region conferring cell membrane permeability and a functional cargo representing the nuclear translocation sequence of P50. It inhibits translocation of P50 containing dimers in many cell lines and the effects appear to be irrespective of the agonist used (Lin et al 1995). The observed inhibitory effects of SN50 reflect its ability to enter the cell and compete for the cellular machinery responsible for nuclear translocation and do not involve proteolysis of IκB. The maximum inhibitory effect observed in murine endothelial cell lines was at 18μM (Lin et al 1998). This concentration in C2C12 myotubes inhibited nuclear migration of NFκB without inducing toxicity (as observed microscopically).

PIF (0.1 and 0.4μg/ml) increased the levels of NFκB found in the nucleus of C2C12 myotubes by 109 and 72% above control, respectively. SN50 reduced nuclear NFκB (as evidenced by figures 10.2.3.1 and 10.2.3.2), such that in cells treated with 0.1μg/ml PIF and SN50 , nuclear NFκB was 21% of that in untreated controls whilst in cells treated with 0.4μg/ml PIF, in the presence of SN50, nuclear NFκB levels were lower than controls. This correlated with an increased proteasome activity of 158% ($p<0.01$) and 119% ($p<0.05$) respectively. The activity was reduced to 109% and 104% of untreated control in the presence of SN50 although this did not quite reach statistical significance (figure 10.2.3.3).

**Fig 10.2.3.1) The effects of SN50 and lactacystin on a PIF induced shift in the Electrophoretic Mobility of [$\gamma^{33}$p]-NFkB in C2C12 myotubes**




1 0μg/ml PIF
2 0.1μg/ml PIF
3 0.4μg/ml PIF
4 0μg/ml PIF+18μM SN50
5 0.1μg/ml PIF+18μM SN50
6 0.4μg/ml PIF+18μM SN50
7 0μg/ml PIF +10μM lactacystin
8 0.1μg/ml PIF +10μM lactacystin
9 0.4μg/ml PIF +10μM lactacystin

**Fig10.2.3.2) The effects of SN50 and lactacystin on a PIF induced shift in the Electrophoretic Mobility of [$\gamma^{33}$p]-NFkB in C2C12 myotubes – Densitometric analysis**

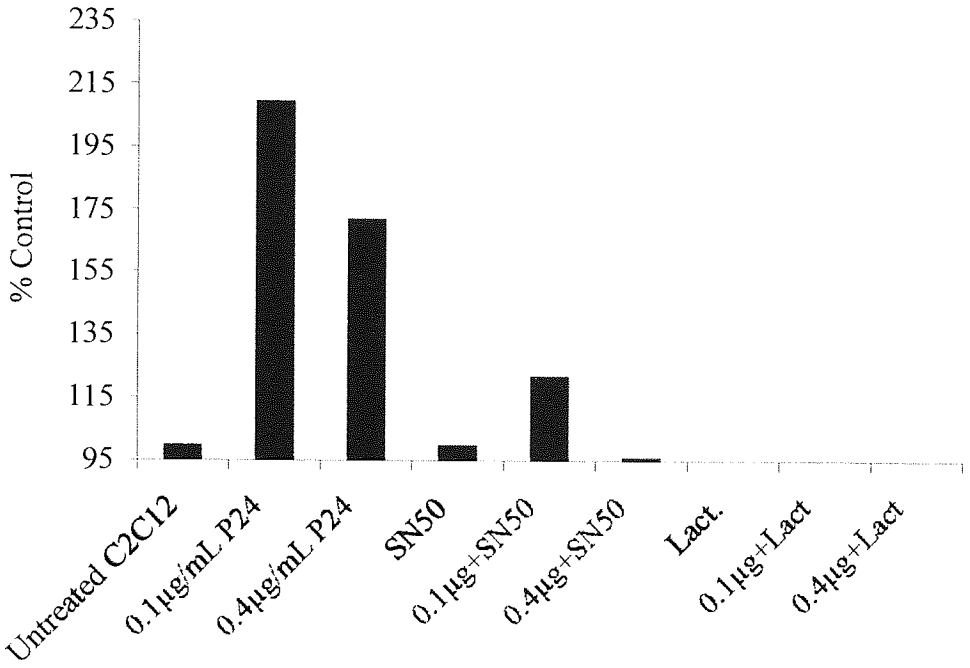
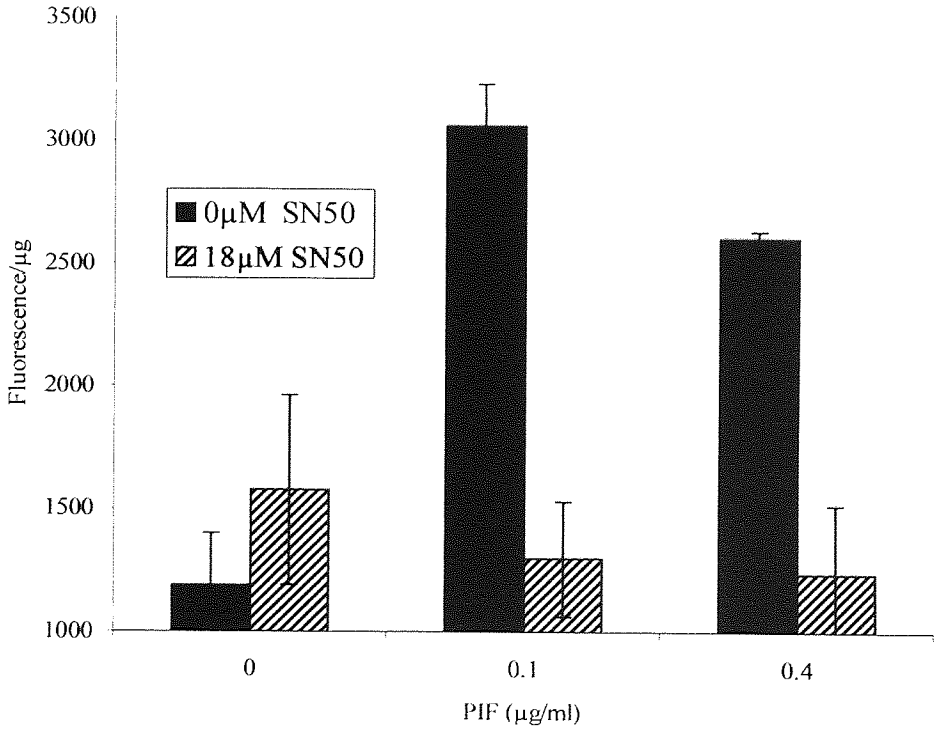
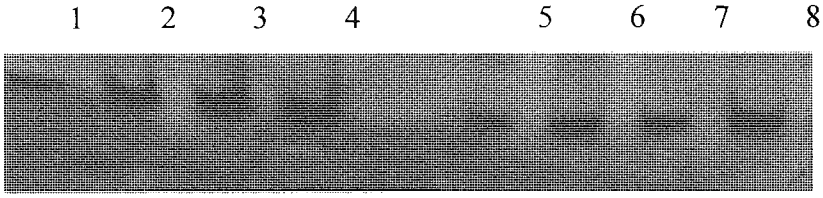



**Fig 10.2.3.3) The effects the SN50 on PIF induced upregulation of the 'chymotrypsin-like' activity of the proteasome.**



Similarly in myotubes which had been incubated in 15-HETE, nuclear expression of NFκB (figures 10.2.3.4 and 10.2.3.5 discussed earlier) was increased by an average 62%, a figure which fell to an average 38% in the presence of EPA. Figure 10.2.3.6 shows that, this correlated with substantial increases in proteasome activity of 172% and 227% ($p<0.05$) for 0.01μg/ml and 0.05μg/ml respectively, that fell to 83% and 128% in the presence of SN50.

**Fig 10.2.3.4) The effects of 15-HETE on the electrophoretic mobility of [$\gamma^{33}$p]-NFkB in C2C12 myotubes in the presence and absence of EPA**




| | | |
|---|---|---|
| 1 | 0μg/ml | 15(s)HETE+0μM EPA |
| 2 | 0.01μg/ml | 15(s)HETE+0μM EPA |
| 3 | 0.05μg/ml | 15(s)HETE+0μM EPA |
| 4 | 0.1μg/ml | 15(s)HETE+0μM EPA |
| 5 | 0μg/ml | 15(s)HETE+50μM EPA |
| 6 | 0.01μg/ml | 15(s)HETE+50μM EPA |
| 7 | 0.05μg/ml | 15(s)HETE+50μM EPA |
| 8 | 0.1μg/ml | 15(s)HETE+50μM EPA |

**Fig 10.2.3.5) The effects of 15-HETE on the electrophoretic mobility of [$\gamma^{33}$p]-NFkB in C2C12 myotubes in the presence and absence of EPA – Densitometric analysis.**

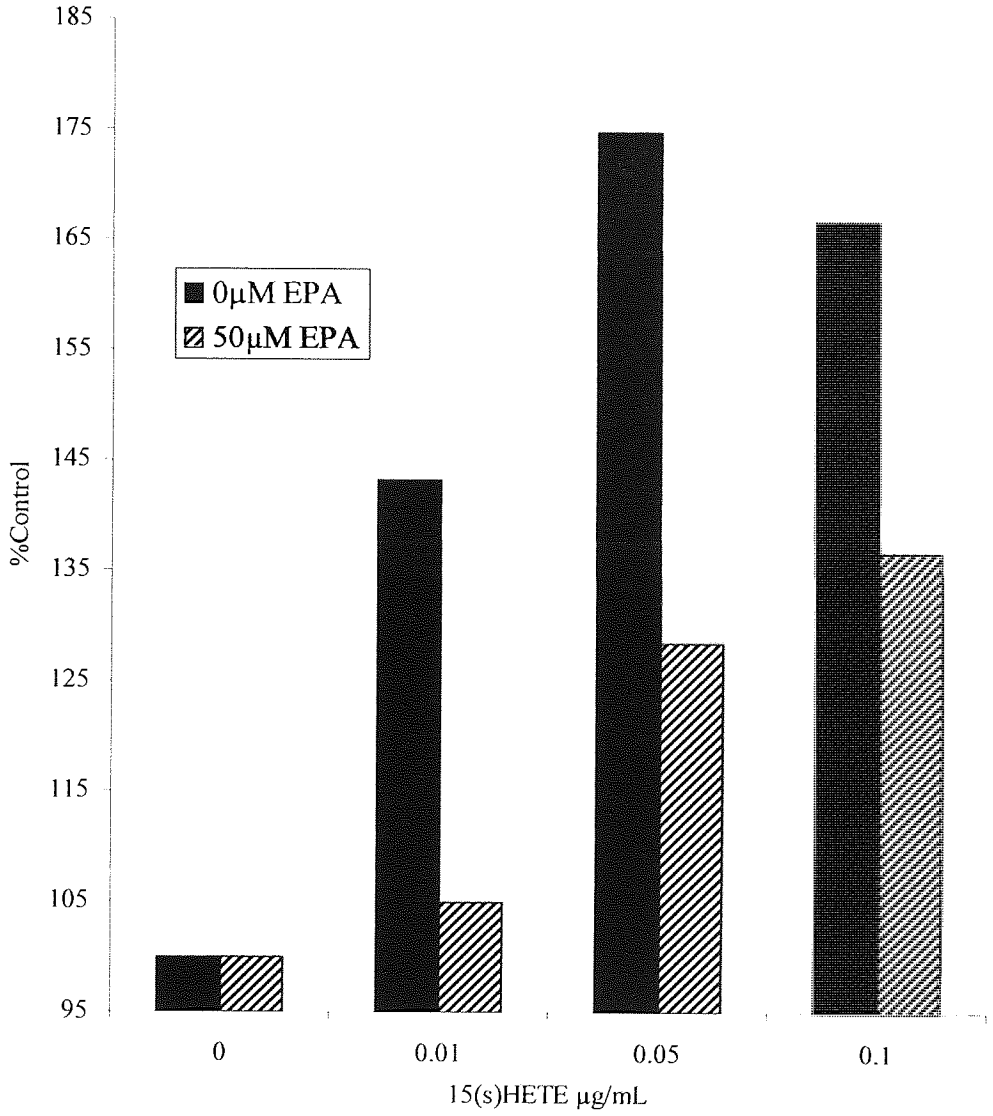
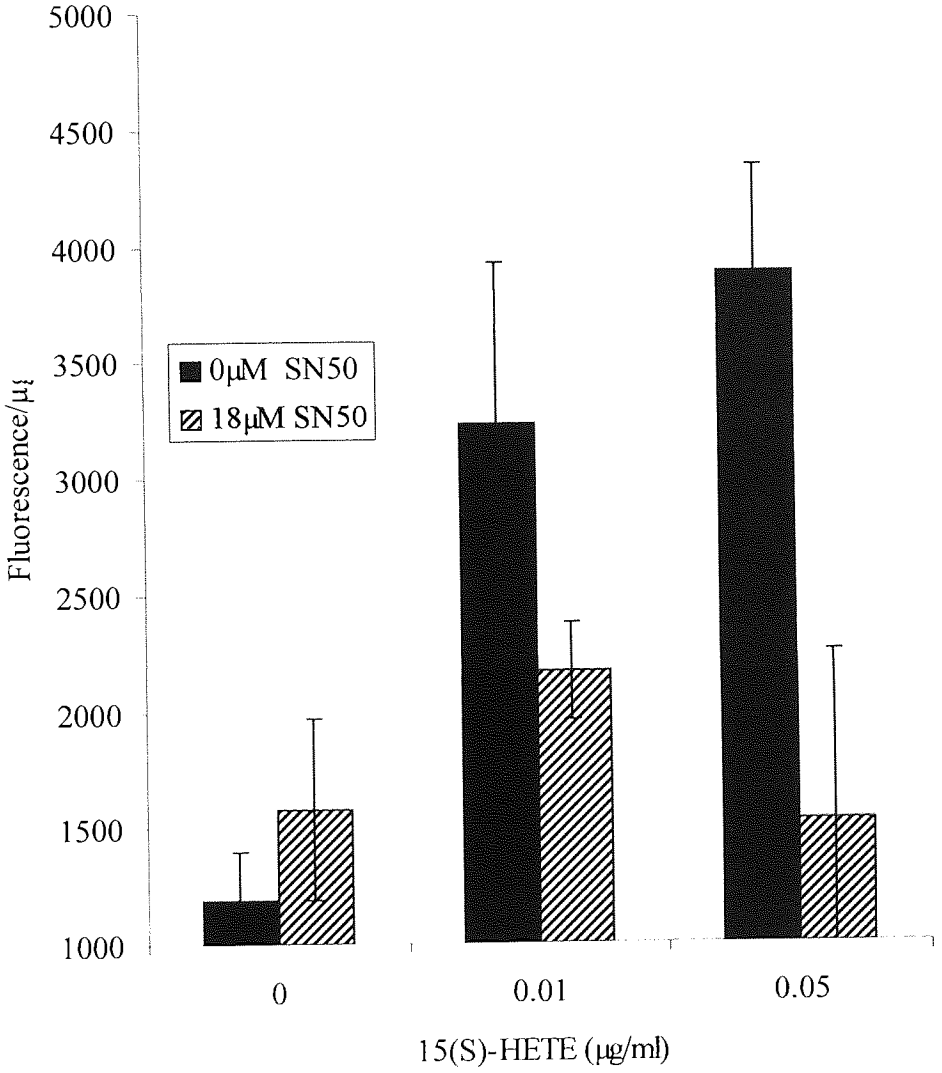


**Fig 10.2.3.6) The effects of the SN50 on 15-HETE upregulation of the 'chymotrypsin-like' activity of the proteasome**




SN50 is purportedly a specific NFκB inhibitor. Taken together the most logical explanation is that PIF through 15-HETE is effecting an upregulation in proteasome activity through an NFκB dependent mechanism which can be inhibited by EPA.

Similar results have been observed in the presence of EPA (figures 10.2.2.1, 10.2.2.2 and 10.2.2.3pp213-214). However it seems that EPA prevents nuclear translocation of NFκB by preventing the degradation of IκB (figure 10.2.1.3pp207). SN50 on the other hand is inhibitory in that it competes for the active sites on NFκB, and has no effect on IκB proteolysis, so it is likely that the effects of EPA on proteasome activity are mechanistically unrelated to those of SN50.

Another interesting observation is that SN50 is purportedly a specific P50 inhibitor. If those effects upon proteasome activity are specific, it implies a role for the P50 subunit in the response to PIF.

### 10.2.4 Methods of activation and inhibition of NFκB in this model

Figures 10.2.3.1 (pp216) and 10.2.3.2 (pp217) show that lactacystin (a specific proteasome inhibitor) reduced levels of nuclear NFκB to below control levels, this may suggest that activation of NFκB by PIF/15-HETE involves the most common and well studied pathway of NFκB activation - phosphorylation of IκB, followed by degradation via the ubiquitin-proteasome pathway (discussed in the introduction to this section), or it may reflect inhibition of the proteasome mediated activation of an upstream kinase.

Several experiments were performed to determine if those effects of PIF/15-HETE upon the proteasome could be duplicated by a documented inducer of NFκB. E.Coli lipopolysaccharide was added to C2C12 myotubes at a range of concentrations, however no conclusive differences in proteasome activity or expression were observed (data not shown). It is well established that NFκB exhibits subunit specificity and these results probably reflect recruitment of an NFκB dimer in response to lipopolysaccharide different to that involved in the response to PIF/HETE.

Those observations of PIF upon protein degradation combined with the effects of SN50 upon proteasome activity suggest that PIF/15-HETE upregulates proteasome activity/expression through an NFκB dependent mechanism that can be attenuated by EPA.

How could 15-HETE activate NFκB ? There are multiple possibilities. One explanation (discussed in the introduction) is that 15-HETE is oxidized (and possibly subject to further oxidation). It could result in the generation of ROS which might regulate the redox sensitive NFκB, possibly through oxidation of constituent proteins which could augment DNA binding activity or promote the release from or degradation of IκB. Alternatively, the 'membrane perturbation hypothesis' (Spector et al 1988) proposes that various forms of HETE produce their effects through incoporation into

membrane phospholipids. The presence of acyl chains containing a polar hydroxy group may possibly perturb the normal structural relations within the lipid bilayer. Among the possibilities are disruption of tight packing within the hydrophobic core of the bilayer, orientation of the HETE acyl chain such that it extends into the hydrophilic, cytoplasmic or extracellular environment, or the clustering of phospholipids containing HETE chains such that their polar groups could interact and disrupt the usual hydrocarbon environment surrounding transduction proteins, possibly upstream of NFκB.

The upstream effects of the PIF/HETE on the induction of the proteasome are still unknown. The interaction of PIF with the membrane is thought to be a receptor mediated event, resulting in elevation of $PLA_2$. This is the primary mediator responsible for AA release. Once released it may be that AA is preferentially metabolised through 15-LOX pathways to result in the production of the cachectic mediator 15-HETE.

Another possibility is that NFκB is the first port of call for PIF. It activates nuclear translocation and switches on transcription of proteasome genes directly. Alternatively the initial (or one of the many) κB dependent genes might be for example, another transcription/growth control factor. C-myc and ras for example (reviewed in May and Ghosh 1997) are κB inducible transcription factors which might ultimately switch on proteasome transcription 'second' generation. It is possible that some of the target genes for NFκB could be hormones such as insulin, glucagon, thyroid hormone, glucocorticoids and catecholamines, or cytokines such as $PGE_2$ or IL-1 which favour a catabolic environment or regulate muscle growth.

It is well established that the biological effects of NFκB dependent transcription are wide and far reaching. Whilst it is highly plausible (and these results support the hypothesis) that the ultimate target genes for PIF/NFκB are members of the ubiquitin proteasome pathway, there are a few other candidates which might promote proteolysis and which might also be transcribed in response to NFκB, which are worthy of mention. One such

possible target for PIF/NFκB is the Myo D gene. Myo D is a member of the bHLH transcription factor family responsible for skeletal muscle differentiation, although expressed at low levels in adult skeletal muscle, in response to injury, Myo D expression is upregulated. Guttridge et al (2000) demonstrate that TNFα and IFNγ function through NFkB to suppress Myo D synthesis by repressing the accumulation of Myo D mRNA. The authors propose that in this cytokine induced cachexia, Myo D expression could be suppressed which would inhibit the formation of new myofibres and cause the degeneration of newly formed myotubes. These combined effects would result in an impaired ability to both produce and repair muscle cells.

Kawamura et al (1999) used a synthetic oligonucleotide as a 'decoy' cis element to block the binding of NFκB to the promotor regions of its target genes (possibly pro-inflammatory cytokines), thus preventing NFκB mediated gene transactivation. When injected directly into the tumours this had no effect on tumour growth itself in the MAC26 in vivo model. However it drastically attenuated the symptoms of cachexia including loss of body weight, epidiymal fat and gastrocnemius muscle. This provides a clear link between NFκB inducible genes and the state of cachexia in the related MAC26 model.

Myosin is a major functional protein of adult skeletal muscle and loss of skeletal muscle myosin is found in MAC16 animals and in prolonged exposure to PIF in vitro. However as Solomon and Goldberg (1996) discuss, the ubiquitin-proteasome pathway does not disassemble myofibrils directly, so other proteases must first break down these complexes before myosin can be degraded by the proteasome. It is possible that these are also κB dependent genes.

The role of EPA in preventing NFκB degradation seems more clear cut. EPA prevents degradation of IκB, possibly by stabilising the NFκB/IκB complex directly, thus preventing nuclear translocation and transcription of NFkB dependent proteolytic genes. That EPA can bind to proteins in vivo

was demonstrated by Muszbek and Laposata (1993) who examined the involvement of PUFAs in posttranslational fatty acid acylation. Using platelets as a model protein, Muszbek and Laposata demonstrated covalent linkage to two PUFAs - arachidonate and eicosapentaenoate demonstrating that direct binding of PUFAs to proteins occurs in vivo. They postulated that the linkages could either be thioester involving a cysteine residue or *O*-ester linkages that might involve hydroxyl groups of serine or threonine residues.

Alternatively, because phosphorylation of IκB is one of the latter stages in the NFκB cascade, one or more of the many upstream effectors could be the site of intervention by EPA. It has been shown that the related eicosanoid epoxyeicosatrienoic acid (EET) products of the cytochrome P450 pathway inhibit the degradation of IκB and NFκB mediated gene transcription also (Node et al 1999). 11,12-EET inhibited TNFα activated IKK activity by more than 90% in endothelial cells but did not affect IKK directly in cell free kinase assays, suggesting that 11,12-EET affected IKK and IκB through an upstream mechanism.

It has also been shown that direct binding to IKK (of sodium salicylate and aspirin) can inhibit NFκB mediated gene expression in cells activated with TNFα, NIK, HTLV-1 TAX and MEKK1. Direct binding to IKK was observed, but not to other kinases including CREB, SAPK, p38 or ERK2, suggesting that the effect was specific for NFκB. In contrast dexamethasone and the COX inhibitor indomethacin prevented NFκB activation by these agents showing that the effects were not due to inhibition of prostaglandin synthesis. Further studies showed that aspirin bound directly to the β subunit of IKK. This was a non-covalent and irreversible/slowly reversible interaction, which resulted in competition for its binding to ATP (Yin et al 1998). It is possible that EPA exerts its effects upon the inhibition of IκBα degradation through interference with upstream effectors like IKK, possibly also by direct binding.

There are several other possible sites at which EPA could intervene. Binding of this eicosanoid could either directly block or induce changes to the stereochemical configuration of phosphorylation or ubiquitination sites of IκB or upstream kinases such that their activation was inhibited. For example Yaron et al (1997) using a cell free system, showed that ubiquitination of N-terminally phosphorylated IκBα still occurred, demonstrating that the only regulated step in IκBα degradation was the phosphorylation reaction, in contrast to the ubiquitinating activity which is constitutive. Whether the IκBα levels - which were preserved in the presence of EPA - were phosphorylated was not investigated, but it is possible that EPA might act to prevent phosphorylation. Alternatively, Yaron et al identified the recognition component of the phospho-IκB specific E3 activity, a protein named $E3RS^{IκB}$. A docking site for $E3RS^{IκB}$ has been identified in the N-terminal phosphoacceptor sites for IκB (Yaron et al 1998), and this provides yet another region in which EPA mediated configurational change or obstruction would result in preservation of IκB. Didonata et al (1996) and Scherer et al (1995) showed that the major acceptor sites for ubiquitin in IκBα are arginines 21 and 22, whose substitution with lysines considerably retards IκBα degradation. An alteration of this site might be expected to prevent the binding of ubiquitin enzymes, or the generation of a sufficiently large polyubiquitin chain to act as a signal for degradation. Similarly the kinase activities of IKKα and IKKβ, and their abilities to be phosphorylated depend upon leucine zipper mediated dimerisation. Leucine zipper mutations abolish kinase activity (Zande et al 1997). Other work (Karin 1999) has suggested that C-terminal regions of IKKγ are necessary for recruitment of upstream kinases. An alteration in the sterochemical configuration of any of these sites by binding of EPA might result in the presevation of IκB.

It is not known whether EPA affects the NFκB/IκB complex directly, or whether the IKK complex or the cellular machinery that regulates IKK is the target. It is possible that EPA affects the assembly or conformation of the IKK enzyme complex, or it may inhibit a single IKK subunit. It also remains

to be determined whether EPA can exert similar inhibitory effects upon other NFκB dependent genes. The observation that EPA inhibits proteolysis in acutely starved NMRI mice, as well as those bearing the MAC16 tumour might suggest the inhibitory mechanism is general to proteolysis arising from multiple causes. Furthermore, that animals have demonstrated improved survival with n-3 PUFAs to endotoxin challenge (a known NFκB inducer) and in burn injury is further evidence that fatty acid control of this transcription factor across multiple disease states is feasible.

NFκB is required in part to maintain cell viability, through the transcription of inhibitors of apoptosis, in response to environmental stress or cytotoxic agents. Stabilisation of IκB and blockade of NFκB activity has been demonstrated to make cells more susceptible to apoptosis and that chemotherapeutic agents such as 5-fluoruracil exert their apoptotic effects on tumour cells by suppressing the antiapoptotic activities of NFκB by suppressing IKK function (Azuma et al 2001). Furthermore NFκB has been implicated in controlling the cell surface expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule-1, and the intercellular adhesion molecule-1. These cell adhesion molecules are involved in tumour metastasis and angiogenesis in vivo. During metastasis, these molecules direct the adhesion and extravasation of tumour cells to and from the vasculature to distant tissues. Inhibition of IκB degradation may also limit metastasis via the attenuation of NFκB dependent cell adhesion molecule expression and make dividing cancer cells more sensitive to apoptosis. Thus agents which inhibit NFκB function could act through multiple mechanisms to arrest tumour growth, tumour spread and angiogenesis.

Alternatively, as discussed earlier, ROS can be formed during the peroxidation of fatty acids and during the subsequent metabolism of these to biologically active products. Combine this with the observation that oxidant changes can both negatively and positively affect NFκB. Thus, hypothetically, EPA could generate free radicals during peroxidation which

could act to 'switch off' upstream NFκB effectors, whilst 15-HETE could function to 'switch on' NFκB, both in a redox dependent fashion.

The findings presented here demonstrate that a PIF/15-HETE signalling pathway exists in differentiated muscle which functions to increase expression and activity of the ubiquitin proteasome pathway via an NFκB dependent mechanism, and that EPA directly modulates this signalling mechanism by preventing the degradation of IκB and subsequent activation of NFκB. This results in an attenuation of the elevated muscle catabolism seen in cancer cachexia.

## 11. Conclusions and Closing Comments

Both in vivo studies in muscle from NMRI mice bearing the cachexia inducing MAC16 tumour and in vitro studies using the myotube C2C12 model and purified proteolyis inducing factor (PIF) have shown that activation of NFκB occurs concurrently with an increase in activity, mRNA and protein levels of several key proteasome subunits. Increased activity of the ubiquitin proteasome pathway is most commonly the main cause of muscle wasting in cancer cachexia.

EPA prevents NFκB activation by preventing the degradation of IκB. This is accompanied by a decrease in the levels of proteasome activity, subunit mRNA and protein expression.

Taken together, these observations suggest that PIF induces proteasome subunit expression in the skeletal muscle of tumour bearing mice and that this is an NFκB dependent event, which is attenuated by EPA through prevention of the nuclear translocation of NFκB.

On balance, the consensus among the literature favours the idea that proteasome inhibitors act more commonly as anti tumour agents than to promote tumour growth and that NFκB functions commonly to produce anti apoptotic proteins. It has been shown here that EPA inhibits the growth of the MAC16 tumour in vivo and in vitro and also inhibits the elevated expression of proteasome subunits in the tumour. The role of NFκB in the tumour was not investigated. This may mean that EPA affects proteasome expression through a pathway which is independent of NFκB and unrelated to its effects in muscle. However it is tempting to speculate that PIF may have a constitutive function in the tumour and that is to upregulate NFκB and thus the levels of anti-apoptotic proteins and that the effects of EPA upon tumour growth might be exerted through the inhibition of the anti-apoptotic activities of NFκB, thus creating a pro-apoptotic environment.

It may well be that the inhibition of NFκB activation is the important step and the point at which the tumour cytotoxic and anti-cachectic properties of EPA converge. Elevated NFκB could mediate the increased proteasome expression in muscle whilst conversely in the tumour it could favour the production of anti apoptotic proteins. Previous studies have in fact demonstrated that the effects of EPA arise from an increase in cell loss rather than a decrease in proliferation (Tisdale et al 1996). Elevation of the proteasome in the tumour may function to degrade key signal transduction or cell cycle molecules which are anti-apoptotic, or it may positively feedback to increase both the degradation of IκB and other upstream effectors and possibly increase the processing of newly transcribed NFκB Class 1 precursors. Numerous studies have demonstrated the importance of NFκB in the activation of pro-inflammatory cytokines, and that EPA is capable of downregulating pro-inflammatory cytokine release (Wigmore et al 1996, 1997, Watchorn et al 2001). If EPA is affecting cachexia and tumour growth through intervention at a single point (i.e. NFκB), then it is also possible that preventing the transcription of NFκB dependent pro-inflammatory cytokines would further engender an anti-inflammatory and anti-cachexic environment. Alternatively elevated proteasome levels may not be particularly beneficial to tumour cell survival at all, but merely reflect an increased NFκB functioning, possibly due to circulating PIF.

What is certain is that EPA prevents the degradation of IκB. A convincing (but speculative) theory might suggest a single point (i.e. the preservation of IκB) at which EPA could exert seemingly opposite effects, that is to say preservation of cells in the muscle and destruction of cells in the tumour.

EPA might preserve IκB by physically stabilising the NFκB/IκB structure. Whether there is a direct interaction is not known, neither is the origin of PIF or 15-HETEs interaction with the pathway. There are multiple possibilities. Logically, given the ultimate aim of PIF/15-HETE to increase proteasome expression in muscle, it may act at any point in the cascade which will increase the activity of NFκB and therefore the activity of the proteasome.

Thus 15-HETE might function to *increase* ubiquitination, phosphorylation and degradation and *decrease* transcription and expression of IκB or other upstream inhibitors. In short it could upregulate an NFκB enhancer or negatively regulate an inhibitor. EPA on the other hand, might negatively regulate an enhancer or positively regulate an inhibitor, such that expression and/or activity of NFκB kinases is increased.

The observation that no difference in the expression of IκBα in untreated and EPA treated controls might suggest that EPA functions to prevent the degradation of IκBα rather than increase its production. However, it might be that EPA increases the production of IκBα as a compensatory mechanism when its degradation is elevated (i.e. when the cell is challenged with PIF).

The results presented here also demonstrate that EPA attenuates upregulation of proteasome subunits and proteasome activity (which correlated with an inhibition of protein catabolism as measured by tyrosine release) in animals which are not tumour bearing, but have been fasted for 24hours. That EPA can affect the production of 15-HETE is certain. The competitivity of EPA for enzymes of the AA cascade influences the generation of 15-HETE via the LOX pathway. If EPA is exerting its anti cachectic effects in part through interference with 15-HETE generation it would explain its ability to do so in a wide variety of catabolic conditions. The fact that the LOX inhibitor CV6504 attenuated proteasome function, expression and protein degradation, with dynamics comparable to EPA, suggests that EPA is anti-cachexic partly through interference in the arachidonic cascade and its ability to prevent 15-HETE production. However, the observation that CV6504 can act as a scavenger of active oxygen species (Hussey et al 1996) might also suggest that it is this ability to manipulate a redox sensitive system (like NFκB) rather than the ability to interfere with the arachidonic acid cascade directly, which is responsibly for its anti-tumour activities.

Hypothetically then, given that both 15-HETE and EPA are highly unsaturated, that ROS can be generated by the peroxidation of lipids and by

their breakdown and that NFκB can be both positively and negatively regulated by oxidative stress, it is possible that both could regulate NFκB transcription through a redox dependent mechanism.

There is already a vast array of clinical, experimental and epidemiological evidence showing that EPA possesses anti-tumour properties and that it has the ability to correct metabolic abnormalities associated with cachexia resulting in the accretion of lean tissue. As the true test for any therapy is the preservation of body mass it suggests that EPA has huge potential benefit in the treatment of cachexia and might eventually lead to a nutritional therapy which could prolong the survival and quality of life for millions of cancer patients.

Therefore the explanation of those mechanisms by which EPA exerts its effects is all important, for as Costa asked as early as 1963..... "Is cachexia reversible ? This unanswered question constitutes a perpetual challenge to the researcher".

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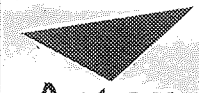

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